Day One

Prior to starting your ChIPs and Shearing:

1. Completely thaw frozen cross linked pellets on ice.
2. Turn on sonifiers and cooling system allow system to reach -1 °C before shearing
3. Cool bench top centrifuge to 4°C
4. Prepare all of your buffers with protease inhibitors in advance
5. **Keep all buffers on ice**.
6. Use DNA low bind tubes throughout protocol (eppendorf cat# 022431021)

For 1 Step Lysis

1. Resuspend cross linked cell pellet in 100-300ul of 1% SDS Lysis Buffer + protease inhibitors for 10' on ice (1% SDS, 10mM EDTA, 50 mM Tris-HCL pH 8.1)
2. After 10' bring the volume up to 1mL with ChIP Dilution Buffer + protease inhibitors (16.7mM Tris-HCl pH 8.1, 167mM NaCl, 0.01% SDS, 1.1% Triton X-100, 1.2mM EDTA) and begin your shearing. Following shearing protocol of choice.

For 2 Step Lysis

Cell and Nuclear Lysis:

Note: for frozen cells, completely thaw on ice.

1. Resuspend cross linked cell pellet (1e7) in 1mL of Cell Lysis Buffer (CLB)+ protease inhibitors (pi) (20mM Tris pH 8.0, 85mM KCl, 0.5% NP40).
2. Make sure that sample is well resuspended by pipetting.
3. Incubate 10' on ice, spin for 3.5', 5000xg, 4°C.
4. Remove supt., (Optional: repeat steps 3 and 4 only incubate on ice for 5')
5. Add 1mL Nuclear Lysis Buffer (NLB) + pi. (10mM Tris-HCl pH7.5, 1% NP40, 0.5% Na Deoxycholate, 0.1%SDS). **For Branson sonifier shearing, make sure that your sample is in an unlabeled (no sticker) 1.5 ml tube in order to fit into our tube holder**
6. Incubate on ice 10' and proceed to shearing. Follow shearing protocol of choice.

*At this point you can freeze material at -80°C; this is called a **nuclear pellet**; Nuclear pellets can be a little more difficult going into solution after they have been frozen and stored.

Day One (cont'd)

Setting Up ChIPs:

From previous optimization, you should know the actual cell number in your samples.

1. Add the appropriate amount of sheared chromatin to each tube. For instance, If you want 1e6 cells for a ChIP and there are 1e7 cells/mL, you would add 100λ of sheared chromatin. When diluting your chromatin with ChIP Dilution Buffer, keep in mind that you want your final concentration of SDS to be 0.1%. **Note:** If you shear in the Covaris LE, your sheared material will have a higher concentration of both cells and SDS.

1. Adjust your ChIP vol. with ChIP Dilution Buffer (CDB) plus protease inhibitors to desired volume. (16.7mM Tris-HCl pH 8.1, 167mM NaCl, 0.01% SDS, 1.1% Triton X-100, 1.2mM EDTA).

2. Add 1ug of your antibody of interest to each tube. (conc. of Ab may vary)

3. Rotate your samples end over end O/N at 4°C.

Day Two

In Advance : Prepare thermal cycler for 65°C. Have buffers ready so the beads won't dry out during washes.

****Note: Dynabeads should constitute ≤ 10% of total volume (make vol. adjustments as necessary).**

Dynabeads Preparation and Incubation with ChIP:

1. For each ChIP, aliquot 50λ of Protein A/G (50/50) magnetic beads into 2ml low bind tube.
2. Place tubes on magnet and remove supernatant.
3. Add 1mL of blocking buffer with protease inhibitors to each 2mL tube containing 50λ beads.
4. Mix by placing tubes on magnet then turning tubes 360°.
5. Repeat steps 2 and 3.
6. Aspirate blocking buffer and add 100λ of fresh blocking solution to pre washed beads.
7. Remove ChIPs from rotation at 4°C, pulse spin and transfer material to 2ml tube with prewashed beads.

8. Incubate for 1hr at 4°C, rotating end over end.

Day Two (cont'd)

Washes:

In Advance : Prepare thermal cycler for 65°C. Have buffers ready so the beads won't dry out during washes

1. Remove ChIPs from rotation and briefly spin tubes to remove any sample from caps, place tubes on magnet and remove supt.
2. Remove tubes from magnet, add 200λ of **RIPA/140mM NaCl Buffer** (0.1% DOC, 0.1%SDS,1% Triton X-100,140mM NaCl,1mM EDTA, 20mM Tris-HCl pH8.1) to each tube. Transfer beads to 96 well plate on magnetic station. **(Save pipet tips from this step in respective tubes in order to transfer remaining beads to 96 well plate)**
3. Remove supt in wells and add another 200λ of RIPA buffer to tubes with a clean pipet tip but continue to use "saved" tip to collect remaining beads and add them to their respective wells in the 96 well plate.
4. Move plate on magnet advancing column of wells either left or right. This will help to mix your sample.
5. Repeat steps 3 and 4 until you are satisfied with the bead transfer.
6. Wash x2 with 200λ **RIPA/500mM NaCl Buffer** (0.1% DOC, 0.1%SDS ,1% Triton X-100, 500mM NaCl,1mM EDTA, 20mM Tris-HCl pH8.1)
7. Wash x2 with 200λ **LiCl Buffer** (0.25M LiCl,1% NP40,1% Na Deoxycholate, 1mM EDTA,10mM Tris-HCl pH 8.1)
8. Wash x2 with **TE**
9. Add 50λ **ChIP Elution Buffer** and 8λ reverse x linking mix to each well.
   ChIP Elution Buffer (10mM Tris-Cl pH 8.0, 5mM EDTA, 300mM NaCl, 0.1% SDS) Reverse Cross Linking Buffer (250mM Tris-HClpH 6.5, 1.25M NaCl, 62.5mM EDTA, 5mg/ml Proteinase K, 62.5ug/ml RNAse A)
10. Cover plate with film, pulse vortex, spin briefly and reverse x link entire sample including beads at 65°C for 5 hours.
11. Include 10λ input, 40λ ddH2O and 8λ reverse cross linking mix to additional well on plate (if you don't already have it).

Day Three

SPRI Clean Up After Reverse Cross Linking:

1. Place overnight reverse cross linking plate on magnet. Transfer supernatants to new well.
2. To each reversed cross linked sample, add 1.8x to 2.2x SPRI beads equilibrated to room temperature (RT).
3. Mix x10, incubate 2' RT followed by 4' on magnet (or until supt is clear). Remove supt.
4. Wash x2 with 200λ of freshly made 70% EtOH for 30s on magnet.
5. Move plate containing samples from left to right on the magnet so beads move through ethanol.
6. After 2^{cd} wash, completely remove ethanol.
7. Spin briefly, return to magnet and remove all traces EtOH .
8. Allow beads to dry, RT ~2'. The pellets should appear shiny and not cracked (over dried).
9. Elute material in 22λ 10mM Tris-HCl pH 8.0, mix with pipet, incubate RT, 3' and return to magnet.
10. Move cleared material to clean wells. This is your ChIP material that is ready for quantification.

Qubit measurement:

1. Use Qubit dsDNA HS setup
2. Make Qubit master mix
   a. (n+2)*200 λ – (n+2) of Qubit dsDNA High Sensitivity Buffer.
   b. (n+2) λ Qubit "Reagent" (pinkish red, in drawer b/c sensitive to light).
3. Aliquot 190λ of master mix to two Qubit Assay tubes and then add 10λ of standard 1 and 2 respectively.
4. Aliquot 198λ of master mix to the remaining tubes.
5. Add 2λ of ChIP material per tube.
6. Mix well via quick vortex, quick spin, let stand 1 – 5 minutes in dark drawer.
7. Measure using Qubit Spectrometer and report values in "ng/λ"

Buffers for ChIP

Blocking buffer (4°C); add PI, PBS+0.5% TWEEN, 0.5% BSA

Chip Dilution Buffer: 0.01% SDS, 1.1% Triton X-100, 1.2mM EDTA, 16.7mM Tris-HCl pH 8.1, and 167mM NaCl

RIPA Low Salt Buffer: 0.1% SDS, 1% Triton x-100, 1mM EDTA, 20mM Tris-HCl pH 8.1, 140mM NaCl, 0.1% DOC

RIPA High Salt Buffer: 0.1% SDS, 1% Triton x-100, 1mM EDTA, 20mM Tris-HCl pH 8.1, 500mM NaCl, 0.1% DOC

LiCl Wash Buffer: 250mM LiCl, 0.5% NP40, 0.5% Na Deoxycholate, 1mM EDTA,10mM Tris-HCl pH 8.1

TE Buffer pH 8.0:10mM Tris-HCl pH8.0, 1mM EDTA pH 8.0

Direct ChIP Elution buffer (RT):10mM Tris-HCl pH 8.0, 5mM EDTA, 300mM NaCl, 0.1% SDS

Reverse x-linking Buffer: 250mM Tris-HCl pH 6.5, 62.5mM EDTA pH 8.0, 1.25M NaCl, 5mg /ml Proteinase K, 62.5ug/ml RNAse A (store in small aliquots @-20 °C)

For 1 Step Lysis:

1% SDS Cell Lysis Buffer 1% SDS, 10mM EDTA, 50 mM Tris-HCL pH 8.1

For 2 Step Lysis:

Cell Lysis Buffer: 20mM Tris pH 8.0, 85mM KCl, 0.5% NP40

Nuclear Lysis Buffer: 10mM Tris-HCl pH7.5 ml, 1% NP40, 0.5% Na Deoxycholate, 0.1% SDS

The dynabeads bind to the Fc region of the antibody and will concentrate your sample on the beads