Prior to starting your ChIPs and Shearing

Turn on sonifiers and cooling system allow system to reach -2°C before shearing
Cool bench top centrifuge to 4°C
Prepare all of your buffers in advance with EDTA free protease inhibitors (pi) (Roche # 11836170001)
Keep all buffers on ice
Use DNA low bind tubes throughout protocol (eppendorf cat# 022431021)

For Fresh or Frozen Cells

After you have done your optimization, you can use fresh cross linked pellets directly or you can thaw cross linked pellets that have been stored at -80°C.

1. Thaw frozen cross linked cells on ice at an angle so that the pellet is facing upward.
2. As the pellet thaws, you can invert the tube – make sure that the pellet has thawed completely.

Cell Lysis

1. For every 1E7 cells, resuspend material in 3 mL of Cell Lysis Buffer (CLB): (20mM Tris pH 8.0, 85mM KCl, 0.5% NP40) + protease inhibitors (pi)
2. Make sure that sample is well resuspended
3. Incubate 10' on ice with intermittent pipetting
4. Spin ~3000 x g for 3.5' at 4°C
5. Remove supernatant taking care not to disturb the pellet

   *Can freeze at this point at -80°C; this is called a **nuclear pellet**

Nuclear Lysis

1. For every 1e7 cells, add 300λ of Nuclear Lysis Buffer (NLB): (10mM Tris-HCl pH7.5, 1% NP40, 0.5% Na Deoxycholate, 0.1% SDS) + pi
2. Slowly resuspend the material with a pipet to avoid bubbles. You may have to use wider then narrower tips to accomplish this.
3. Incubate on ice 10' then add ~ 650 – 700λ of NLB + pi to a final vol 1mL
4. Proceed to shearing.

Chromatin Shearing using the Branson Sonifier

DAY 1

Shearing with Branson Sonifier can be somewhat subjective. Our set up has a cooling system that uses a glycerol/water mix and is set to -2°C. In addition, a moving platform holds a metal rack that is chilled and keeps tubes in place upon sonication. The following are guidelines only and reflect our current methods

Settings:

On: 0.7 seconds
Off: 1.3 seconds
Time: 2 minutes
Watts: 10-12
Wait in between each 2min. cycle for ~ 1-2 min to allow sample to stay cool otherwise, the probe may heat up the chromatin.

Shearing:

1. Clean probes with ddH2O before and after use
2. Place nuclear lysate in 1.5 ml eppendorf tube in rack that is part of our Branson set up
3. Raise the platform so that the sonifier tip is centered in the tube.
4. Allow the tip to touch the bottom of the tube and then back off the tip so that there is approximately 1-1.5mm of space between the tip and the bottom of the tube.
5. Begin shearing using the settings above and stop in between cycles to allow sample to cool.
6. Watch the tube for a few seconds to make sure sample is not splashing or foaming.
7. After you have completed your shearing cycles (determined by optimizing), remove samples
8. Clean Branson probes again w/ ddH2O
9. Place aliquots on ice for at least 10' in pre-chilled and pre labeled tubes
10. After 10' spin lysate for 10' at max speed @ 4°C. Transfer supernatant to a new tube leaving any residual debris behind.
11. Pool all cleared lysates from the same cell lines; remove 10ul for reverse cross linking. This is your input material
12. Add 5M NaCl to your pooled chromatin samples to a final conc. of 167mM
    a. This will adjust your ChIPs to the same NaCl concentration as the ChIP Dilution Buffer (CDB): (0.01% SDS, 1.1% Triton X-100, 1.2mM EDTA, 16.7mM Tris-HCl pH 8.1, and 167mM NaCl).

Setting up ChIPs

From previous optimization, you should know the actual cell number in your samples. Once you pool your chromatin, aliquot the appropriate amount of cells to each 1.5mL tube for your subsequent ChIPs raising the vol. to 1mL with CDB + pi

1. For each IP, record the cell # and vol. of antibody per IP, concentrations of Ab may vary. If an antibody is not robust, you can either adjust the cell number or antibody vol.

2. Make sure that tubes are closed tightly and/or put parafilm over the caps so that the ChIPs won't leak and/or contaminate nearby ChIPs.

3. Rotate 4°C overnight. ChIPs can either rotate end over end or rocking, speed will vary with equipment. Make sure rotation is slow enough so that bubbles are not created.

Washing ChIPs and Preparing Protein A Sepharose

1. Use 15λ of Protein-A Sepharose beads (bed vol.) for each ChIP.
2. Remove twice the required bed volume of beads from the stock tube and aliquot into an eppendorf tube.
    a. Spin 3,000g, 30s at 4°C. Remove ½ of the vol. and put back into stock tube of Protein-A Sepharose.
    b. Wash beads x2 in 2 volumes of cold CDB + pi.
    c. Resuspend beads in CDB + pi to make 1:4 slurry.
    d. Quickly spin your IPs so that samples settle to bottom of tube (nothing in caps)
    e. Add the appropriate volume of beads to each IP (in this case, it would be 60λ)
    f. Incubate for 1 hour rotating at 4°C ~10-17rpm depending on mixer in cold room.
3. After 1 hour incubation, spin samples at ~3,000 x g for 1' at 4º C.

4. Aspirate supernatant carefully, do not to disturb the bead pellet. It is fine to leave a small amount of buffer and later remove it with pipette tip. * Beads can stay on ice for 1-2 hours.

5. Re-suspend the beads in 500λ of Low Salt Wash Buffer (LSWB)**: (**0.1% SDS, 1% Triton X-100, and 2mM EDTA, 20mM Tris-HCl pH 8.1, 150mM NaCl).

    a. Pipette into new eppendorf tube and save this tip in the original tube.

    b. Add another 500λ LSWB with a clean tip to the first tube. Use the "used" tip to remove any remaining beads from the original tube to the new tube. This also helps remove any beads left in the first tip. Presumably, transferring to clean tube reduces background

6. Rotate 4°C for 5'.
7. Repeat LSWB step with without changing tubes.
8. Re-suspend the beads in 1mL of High Salt Wash Buffer (HSWB): (0.1% SDS, 1% Triton X-100, 2mM EDTA, 20mM Tris-HCl pH 8.1, 500mM NaCl)
9. Incubate 5' on ice, spin, and aspirate supernatant.
10. Repeat previous step.
11. Re-suspend the beads in 1mL of LiCl Immune Complex Wash Buffer (LICWB): (0.25M LiCl,1% NP40,1% Na Deoxycholate, 1mM EDTA,10mM Tris-HCl pH 8.1)
12. Incubate 5' on ice, aspirate supernatant.
13. Repeat previous step **but** during this incubation, rotate tubes at RT. Incubate 5', spin, and aspirate supernatant.
14. Re-suspend beads in 1mL of RT TE pH 8.0 (10mM Tris-HCl, 1mM EDTA).
15. Incubate 5' RT and aspirate supernatant.
16. Repeat previous step. Use pipette to remove any residual TE going into the next step.
17. Re-suspend bead pellet in 50µL of freshly prepared ChIP Elution Buffer: (100mM $NaHCO_3$, 1% SDS), put the tube on vortex shaker setting 6, RT for 10'. Spin 8,000 x g for 1'. Remove supernatant and pipette into a clean eppendorf tube. Add another 50µL of ChIP Elution Buffer to the beads, repeat incubation and spin. Remove supernatant and add to previous 50µL of ChIP Elution Buffer (total volume = 100µL).
18. To the WCE only**,** add water to final volume 100 µL.
19. Add 16µL Reverse Crosslinking Salt Mixture (250mM Tris-HCl pH 6.5, 62.5mM EDTA pH 8.0, 1.25M NaCl, 5mg/ Proteinase K, 62.5ug/ RNAse A) to ChIP and WCE samples.
20. Mix well, seal with parafilm and incubate at 65º C for at least 5 hours. Samples can be stored at 4°C after reverse cross linking.

AFTER REVERSE CROSS LINKING

DNA Extraction using SPRI beads

1. Remove samples reverse cross linked samples.
2. Spin plate or tubes and allow samples to sit at 37°C so that all of the material is in solution.
3. Make sure that your SPRI beads have been pre equilibrated to room temp.
4. Recover DNA by adding 1.8-2.2x vol. of SPRI beads to each sample. (max vol. plate is 300µL/well)
5. Mix by pipetting 10x and allow to sit at RT for 5'
6. Place sample on magnet for 5'
7. Remove liquid in tube/plate
8. Begin series of 2 x 70% ethanol washes (see longer version of SPRI protocol accompanying this protocol.
9. After final wash, spin tube/plate and return to magnet. Use a pipette to remove any residual ethanol.
10. Place tubes/plate at 37°C with lids open for ~5' until SPRI pellet is dry but not cracked.
11. Add 36λ of Elution Buffer (EB): (10mM Tris) to each ChIP and 50λ to input material and mix by pipetting ≥ 10x
12. Allow sample to sit for 5' then place on magnet.
13. Remove sample to clean tube and assay concentration with Qubit.
14. Proceed directly to Library Construction or store ChIPs at -20°C for short term storage or -80°C for longer term storage.

Buffers

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

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

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

Low Salt Wash Buffer: 0.1% SDS, 1% Triton X-100, 2mM EDTA, 20mM Tris-HCl pH 8.1,150mM NaCl

High Salt Wash Buffer: 0.1% SDS, 1% Triton X-100, 2mM EDTA, 20mM Tris-HCl pH 8.1, 500mM NaCl

LiCl Wash Buffer: 0.25M LiCl,1% NP40,1% Na Deoxycholate, 1mM EDTA,10mM Tris-HCl pH 8.1

Elution Buffer: 100mM $NaHCO_3$, and 1% SDS

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

Reverse x-linking Buffer: 250mM Tris-HCl pH 6.5, 62.5mM EDTA pH 8.0, 1.25M NaCl, 5mg/ Proteinase K, 62.5ug/ RNAse A