ChIP Protocol for fresh or frozen cross linked cells

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 with protease inhibitors in advance
Keep all buffers on ice. Use DNA low bind tubes throughout protocol (eppendorf cat# 022431021)

For Fresh or Frozen Cells

Thaw frozen cross linked cells on ice at a 45° angle so that the pellet is facing upward. As the pellet thaws, it will fall toward the ice. At this point, you can invert the tube. Make sure that the pellet has thawed completely (we usually use 30' as our guide).

Cell Lysis

1. For every 1e7 cells, resuspend material in 3ml s of Cell Lysis Buffer (CLB) + protease inhibitors (pi)
2. Make sure that sample is well resuspended by pipetting
3. Incubate 10' on ice with intermittent pipetting
4. Aliquot evenly into micro centrifuge tubes
5. Spin 3000 x g for 3.5' at 4°C
6. Remove supernatant taking care not to disturb the pellet
7. Collect pellets into one tube and resuspend in additional 3mls of CLB + pi per 1e7 cells
8. Repeat steps 4-6
9. Collect pellets in a total volume 0.5-1.0 ml of CLB+ for every 1e7 cells and divide equally into 1.5ml tubes
10. Spin 3000 x g for 3.5' at 4°C
11. Discard supernatant and proceed to next step

    *At this point you can freeze material at -80°C; this is called a **nuclear pellet**;
    Nuclear pellets can be harder to work with later

Nuclear Lysis and Shearing

1. For every 1e7 cells, add 300λ of Nuclear Lysis Buffer (NLB) + pi . Slowly resuspend the material with a pipet to avoid bubbles. You may have to use wider then narrower tips to accomplish this.
2. Once your sample is well resuspended, add ~ 650 – 700λ of NLB + pi to a final vol 1ml
3. Iincubate 10' on ice with intermittent pipetting
4. Proceed to shearing

Chromatin Shearing using the Branson Sonifier

DAY 1

Shearing using the 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 (amplitude will vary from one instrument to the next but the energy output in watts will not) Therefore we use watts as one of our parameters rather than amplitude..
We wait 1-2' between cycles to avoid over heating the samples

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 chromatin from the same samples; remove 10λ for reverse cross linking. This is your **input material.** Clearly label this tube as input material and store at 4°C or -20°C until library preparation. This will be your starting material for your "whole cell extract library" or "control" library.
12. Add 5M NaCl to your pooled chromatin samples to a final conc. of 167mM
    a. This will adjust your ChIPs to the same concentration of NaCl as the ChIP Dilution Buffer (CDB)

Setting up ChIPs

From previous optimization, you should know the actual cell number in your samples. Once you mix your chromatin, you can aliquot the appropriate amount of cells to each tube for your subsequent ChIPs

For instance, if you know that you have 1e7 cells/ml of sheared material and you want to use 1e6 cells/IP, aliquot 100λ of sheared chromatin to each tube and complete the volume of the IP to 1ml with CDB plus pi. (Don't forget to add 5M NaCl)

1. For each IP, record the cell # and add 1λ of antibody per ml of 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.

DAY 2

Adding Beads and Washing ChIPs

Our current protocol is designed for use with Protein A Sepharose beads

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 2x 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, being sure not to disturb the bead pellet. You can leave a small amount of buffer and remove it with pipette tip. *Beads can stay on ice for 1-2 hours before proceeding to next step
5. Re-suspend the beads in 500λ of Low Salt Wash Buffer (LSWB).
   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 left over beads from the original tube to the new tube. This also helps remove any beads left in the first tip. Presumably, transfering to clean tube reduces background
6. Rotate 4°C for 5'
7. Repeat LSWB step with 1 without changing tubes
8. Re-suspend the beads in 1ml of High Salt Wash Buffer. Incubate 5' on ice, spin, and aspirate supernatant.
9. Repeat previous step.
10. Re-suspend the beads in 1ml of LiCl Immune Complex Wash Buffer. Incubate 5' on ice, spin, aspirate supernatant.
11. Repeat previous step **but** during this incubation, rotate tubes at RT.
12. Add 1 ml of RT TE pH 8.0. Incubate 5' RT, spin and aspirate supernatant.
13. Repeat previous step. Use pipette to remove any residual TE going into the next step.
14. Re-suspend bead pellet in 100λ of freshly prepared ChIP Elution Buffer
15. Incubate at 65°C for 15' (parafilm caps) then put the tubes on vortex shaker at RT for ≥ 10'on setting 6.
16. Spin 8,000 x g for 2', remove supernatant and pipette into a 96 well plate. Add 16λ of reverse cross linking buffer to each well. Cover with parafilm, vortex briefly, spin and reverse cross link at 65°C for at least 5 hours or over night.
17. To the **input material only,** add water to final volume 100λ and 16λ rev.crosslinking buffer and reverse crosslink along with your ChIPs

Day 3

After Reverse Cross Linking

1. Remove samples from 65°C. Spin plate or tubes and allow samples to sit at 37°C so that all of the material is in solution (especially if you used a thermocylcer programmed to go to 4°C after rev. cross linking)
2. Make sure that your SPRI beads have been pre equilibrated to room temp. (≥ 30')
3. Recover DNA by adding **2.2x** vol. of SPRI beads to each sample.
4. Mix by slowly pipetting 10x and allow to sit at RT for 5'
5. Place sample on magnet for 5'
6. Aspirate liquid in tube/plate
7. Begin series of 2-3 x 70% ethanol washes (see longer version of SPRI protocol accompanying this protocol).
8. After final wash, spin tube/plate and return to magnet. Use a pipette to remove any residual ethanol.
9. Place tubes/plate on bench with lids open for ~3' until SPRI pellet is dry, but not cracked
10. Add 36λ of Elution Buffer to each ChIP and 50λ to input material and mix by pipetting ≥ 10x
11. Allow sample to sit for 5' then place on magnet
12. Remove samples to clean tubes/wells and assay concentration with Qubit
13. Either 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, 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 8.1,167mM 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,}$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