# Day One

## Prior to starting your ChIPs and Shearing:

1. Turn on sonifiers and cooling system allow system to reach -1 °C before shearing
2. Cool bench top centrifuge to 4°C
3. Prepare and pre-chill all of your buffers with protease inhibitors in advance
4. Use DNA low bind tubes throughout protocol (eppendorf cat# 022431021)

## Preparing Dynabeads:

1. Make Blocking Buffer + Protease Inhibitors (pi)
2. For each ChIP, aliquot 50λ protein A/G (50/50) Dyna beads per 2ml tube
3. Place each tube on magnetic stand and aspirate supernatant
4. Add 1mL of blocking buffer to each 2ml tube containing pre cleared beads
5. Mix by placing tubes on magnet then turning tubes 360°
6. Repeat steps 2 and 3
7. Aspirate blocking buffer and add 100 - 200λ of fresh blocking solution to pre washed beads

## Conjugating Antibody and Magnetic Beads:

**NOTE:** the percentage of A/G beads should be ≤ 10% of the total ChIP volume. Make adjustments accordingly.

1. Add 1ug of antibody to each tube containing 50/50 Protein A/G magnetic beads w/ blocking solution.
2. Rotate for 1hr at 4°C. Make sure that the rotating motion keeps the liquid moving and the beads wet.
3. Place tubes on magnet, aspirate supt. and wash the beads x2 with blocking buffer.
4. After removing supt., resuspend beads in 50- 200λ fresh blocking buffer and set aside until you are ready to add the correct volume of sheared chromatin that corresponds to your target cell number.

## Cell Lysis:

**Note:** for frozen cells, completely thaw on ice.
For low cell no.samples use “Alon's SCN v2.0.docx” protocol.

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., resuspend in additional 1mL CLB + pi for 5’ on ice.
5. Spin 5000xg, 3.5’ @ 4°C and remove supt.*
6. Add 1mL Nuclear Lysis Buffer (NLB) + pi. (10mM Tris-HCl pH7.5, 1% NP40, 0.5% Na Deoxycholate, 0.1%SDS).
7. Incubate on ice 10’ and proceed to shearing

*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.

## Chromatin Shearing using the Branson Sonifier:

Using the Branson Sonifier can be subjective. Our set up has a cooling system that uses a glycerol/water mix and is set to -1°C. In addition, a moving platform holds a metal rack that is chilled and keeps tubes in place upon sonication. Amplitude varies from one instrument to the next but the energy output in watts will not, therefore we use watts rather than amplitude as one of our parameter. The following are guidelines only.

## Settings:

**On:** 0.7 seconds, **Off:** 1.3 seconds
**Time:** 2 minutes intervals (waiting in between cycles prevents sample from over heating)
**Watts:** ~12

## Shearing:

1. Clean probes with dd$H_2O$ before and after use.
2. Place nuclear lysate in 1.5 ml eppendorf tube in pre chilled 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. If samples splash or foam, re-adjust tube and/or platform.
8. After you have completed your shearing incubate samples on ice for 10' and clean the sonifier tip.
9. After 10', spin lysate for 10' at max speed @ 4°C. Transfer supernatant to a new tube avoiding debris.
10. Pool all chromatin from the same samples; remove 10λ for reverse cross linking. This is your **input material.** Label this tube and store at 4°C or -20°C until reverse crosslinking. This will be your starting material for your "whole cell extract library" or "control" library.
11. Add 5M NaCl to your pooled chromatin samples to a final conc. of 167mM.
    (This will adjust your ChIPs to the same concentration of NaCl as the ChIP Dilution Buffer (CDB)).
12. Add the appropriate amount of sheared chromatin to your pre blocked and conjugated protein A/G magnetic beads. For instance, If you want 1e6 cells for a ChIP and there are 1e7 cells/ml, you would add 100λ of sheared chromatin.
13. Adjust vol. to 500 - 1000λ with ChIP Dilution Buffer (16.7mM Tris-HCl pH 8.1, 167mM NaCl, 0.01% SDS, 1.1% Triton X-100, 1.2mM EDTA).

14. Rotate your samples O/N at 4°C.

## Day Two

### Washes:

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

**RIPA Buffer washes x6** (0.1% DOC, 0.1%SDS, 1% Triton X-100,140mM NaCl,1mM EDTA, 20mM Tris-HCl pH8.1)

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1. Remove ChIPs from rotation and briefly spin tubes to remove any sample from caps., place on magnet and remove suRemove tubes from magnet, add 200λ of RIPA buffer to rinse beads to bottom of 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)**
2. 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.
3. Move plate on magnet advancing column of wells either left or right. This will help to mix your sample.
4. Repeat steps 3 and 4. If you are satisfied with the bead transfer, you may continue with three more RIPA washes in 96 well plate or continue to wash beads from O/N tubes transferring supt to wells.
5. 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)
6. Wash x2 with 200λ **LiCl Buffer** (0.25M LiCl,1% NP40,1% Na Deoxycholate, 1mM EDTA,10mM Tris-HCl pH 8.1)
7. Wash x2 with **TE**
8. Add 50λ **ChIP Elution Buffer** (10mM Tris-Cl pH 8.0, 5mM EDTA, 300mM NaCl, 0.1% SDS) **and** 8λ reverse x linking mix (250mM Tris-HClpH 6.5, 1.25M NaCl, 62.5mM EDTA, 5mg/ml Proteinase K, 62.5ug/ml RNAse A) to each well. Cover plate with film, pulse vortex, spin briefly and reverse x link entire sample including beads at 65°C O/N.
9. 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 x15, 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 removed ethanol. Spin briefly, return to magnet and remove all traces EtOH .
7. Allow beads to dry, RT ~2'. The pellets should appear shiny and not cracked (over dried).
8. Elute material in 22λ 10mM Tris-HCl pH 8.0, mix with pipet, incubate RT, 3' and return to magnet.
9. 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"

## Epigenomics Mag Bead Buffers:

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

**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

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

**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)