Day One

Tissue Preparation and Shearing

In Advance: Have dry ice and a bucket of liquid nitrogen ready**.** Add protease inhibitors to your buffers.

1. Slice frozen tissue into ~ 200 mg sections, quickly weigh and record weight
2. Place weighed frozen tissue sample into the center of labeled Covaris tissueTube TT1 (cat# 520001) with a collection tube attached and loosened 1/4 turn (note: it is best to keep the size of the tissue below 300mg per bag).
3. Keep your sample on dry ice then submerge in LN2 right before using the Covaris Hammer.
4. Turn on Covaris hammer. Note settings, setting one is for softer tissue, setting 6 is for tougher tissues. We always use setting six.
5. Seat your sample in the Covaris device, select force (1-6) then activate.
6. Remove your sample quickly and submerge in LN2. Assess whether or not additional pulverization is required. Remember to keep your tissue frozen.
7. Repeat pulverization until you achieve homogenous powder. This usually requires 1 blow or activation, sometimes 2.
8. Remove the tube and cap your samples.

** At this point, you can proceed with your protocol or store samples at -80. **

9. Invert bag and tap frozen material into 5 ml Eppendorf LoBind tube (cat. # 223100034). Keep material frozen to ensure transfer.
10. Once transferred place tube on wet ice.
11. Add 1 ml room temperature (RT) PBS with 2x protease inhibitors to the tissueTube to collect remaining material by gently pipetting down the sides of the tissueTube as well as collecting material from the neck of the tissueTube.
12. Pipette this additional material into the 5ml tube on ice.
13. Repeat rinses of TissueTube but do not exceed 4mL total volume.
14. Re suspend tissue with wide orifice tip. Be careful to remove your entire sample from pipet tip. Note* if you are working with sticky samples, just invert tube to mix sample.
15. Cross-link tissue in (RT) PBS with 2x protease inhibitors to a final concentration of 1% formaldehyde. (Add 62.5λ of fresh 16% methanol free formaldehyde per **1 mL** solution)
16. Incubate at RT for 15’ with gentle agitation on a rotator or rocker.

17. Quench crosslinking by adding fresh 2.5M glycine to a final concentration of 0.125M.
(Assuming previous vol. was 1062.5λ **per mL**; add 53.125λ **per mL** of freshly prepared 2.5M Glycine)

18. Incubate at RT for 5' w/ gentle agitation, then place on ice for 5 min.

19. Centrifuge at 3000 rpm, 5', 4°C and aspirate supt. (NOTE: increase speed if material does not pellet).

20. Wash samples with 4 mL of 4°C PBS w/ 2x protease inhibitors per tube.

21. Centrifuge at 3000 rpm, 5', 4°C and aspirate supt.

22. Repeat PBS wash, incubation and centrifugation.

23. Resuspend cross-linked tissue in 4°C PBS w 2x protease inhibitors and aliquot material as evenly as possible into 1.5 mL eppendorf tubes so that each tube represents approximately 25-50mg of cross-linked material.

24. Centrifuge at 3000 rpm, 5', 4°C and aspirate supt. (adjust speed if material does not pellet).

25. If pellets are not equal in size, you can make adjustments using additional 4°C PBS

* You can snap freeze cross-linked material (store - 80°C) or proceed to tissue lysis and shearing.

Tissue Lysis

1. Re-suspend each ~25-50mg cross linked tissue in 1mL of Cell Lysis Buffer (CLB) w/ 2x protease inhibitors (20mM Tris pH 8.0, 85mM KCl, 0.5% NP40).

2. Incubate samples on ice for 10' intermittently pipetting samples with wide orifice tip to achieve homogenous suspension.

3. Spin at 2500-3000rpm, 5', 4°C to pellet nuclei then aspirate supt. (adjust speed if necessary so that material pellets)

4. Repeat steps 1-3 but only incubate on ice for 5' during the second cell lysis step.

5. Re-suspend the pellet in a final volume of 1 mL of 4°C Nuclear Lysis Buffer (NLB) w/ 2x pi (10mM Tris pH 7.5, 1% NP40, 0.5% Sodium Deoxycholate, 0.1% SDS).

6. Incubate on ice 10' intermittently pipetting samples with wide orifice tip to achieve homogenous suspension.

7. Proceed to shearing following Epigenomics Branson Shearing Protocol.

8. After shearing, incubate samples 10' on ice, spin max speed for 10', 4°C and save supt. in a clean tube. This is your chromatin prep.

9. Remove 5 -10λ chromatin from each sample for reverse cross linking

10. Once you have confirmed optimal shearing using Agilent BioAnalyzer, you can go directly to setting up your ChIPs. You can also snap freeze remaining material and store at - 80°C.

Reverse Cross Linking to check shearing and concentration of your chromatin

1. Add 10mM Tris to each 5-10λ sample aliquot (above) so that the final volume is 50ul. Add 8λ of reverse cross-linking buffer to each sample. Mix, spin and reverse cross link at 65°C for 2 hours.
2. After reverse cross-linking, perform a 2.2x SPRI clean up and check shearing on the Agilent BioAnalyzer.
3. Check concentration of prep and estimate cell number via back counting.

Day Two

Setting Up ChIPs

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

1. Add the appropriate amount of sheared chromatin to each tube. For instance, if you want 1e6 cells per ChIP and there are 1e7 cells/mL, you would add 100λ of sheared chromatin.
2. Adjust your ChIP vol. with ChIP Dilution Buffer (CDB) plus 2x 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).
3. Add 1ug of your antibody of interest to each tube. (conc. of Ab may vary)
4. Slowly rotate your samples end over end O/N at 4°C.

Day Three

In Advance: Have buffers prepared with 2x protease inhibitors so that the beads do not dry out during washes.

Dynabeads Preparation and Incubation with ChIP:

1. Aliquot 50λ of Protein A, Protein G or Protein A/G magnetic beads per ChIP into 5mL low bind tube.
2. Place tube on magnet and remove supernatant.
3. Add 3 volumes of blocking buffer with 2x protease inhibitors to the 5mL tube containing beads.
4. Mix by placing tube on magnet then turning tube 360°.
5. Repeat steps 2 and 3.
6. Aspirate blocking buffer and resuspend beads in 50λ of ChIP Dilution Buffer with 2x protease inhibitors per ChIP.
7. Remove ChIPs from rotation, pulse spin and aliquot 50ul pre-blocked beads to each ChIP.
8. Incubate for 1hr at 4°C, rotating end over end.

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

Washes:

1. Remove ChIPs from rotation and briefly spin tubes to remove any sample from caps, place tubes on magnet, remove supernatant (supt) and save supt in clean tube then remove tubes from magnet and pulse spin.

2. Add 150λ of RIPA Low Salt Buffer to each tube (0.1% DOC, 0.1%SDS, 1% Triton X-100,140 mM NaCl, 1mM EDTA, 20mM Tris-HCl pH8.1).

3. 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)

4. Add another 150λ of RIPA Low Salt 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.

5. Remove supt. from the wells of the 96 well plate but only do this for 2 to 3 samples at a time so that the beads do not dry out.

6. Continue washing tubes with RIPA Low Salt Wash Buffer and transferring the beads to their respective wells until you are confident that you have transferred all of the beads from your sample.

7. When the beads have settled in the plate, remove the low salt wash buffer from each well using a multichannel pipet.

8. Use a multichannel pipet and reservoir to add 150λ RIPA High Salt Buffer to each well (0.1% DOC, 0.1%SDS ,1% Triton X-100, 500mM NaCl,1mM EDTA, 20mM Tris-HCl pH8.1)

9. Remove RIPA High Salt Buffer with a multichannel pipet.

10. Wash x2 with 150λ LiCl Buffer – during the second wash, advance the plate on the magnet from left to right to wash beads (0.25M LiCl,1% NP40,1% Na Deoxycholate, 1mM EDTA,10mM Tris-HCl pH 8.1).

11. Wash x2 with low TE advancing the plate on the magnet from left to right to mix beads.

12. Cover and spin plate for 30 seconds at 1000 rpm to collect remaining low TE.

13. Place plate back on magnet to remove residual low TE.

14. Remove plate from magnet.

15. Prepare a master mix of Direct ChIP Elution Buffer (10mM Tris-Cl pH 8.0, 5mM EDTA, 300mM NaCl, 0.1% SDS and 5mM DTT directly before use) and reverse cross-linking buffer (250mM Tris-HCl pH 6.5, 1.25M NaCl, 62.5mM EDTA, 5mg/ml Proteinase K, and 62.5ug/ml RNAse A).

16. Add 50λ of master mix to each well.

17. To ensure complete dispensing of the beads, mix slowly by pipette.

18. Cover plate with foil, spin briefly, and reverse cross-link the entire sample including beads at 65°C for at least 3 hours.

19. Include 10λ input; 32λ ddH2O and 8λ reverse cross-linking mix to additional well on plate (if you have not already done so).

Addendum to Prior versions of this protocol

We have introduced an additional step to our protocol for tissues that have a limited supply. If the original ChIP did not work for some reason, we have gone back to saved and cleared supernatant to perform another round of ChIP with the same antibody.

After Reverse Cross Linking

1. Remove plate from thermocycler and spin briefly.
2. Transfer plate to magnet.
3. Transfer supernatant to clean wells leaving behind all of the Protein A/G beads.
4. Perform a 1.8 to 2x SPRI clean up on ChIPs and then quantify with Qubit. This is your ChIP material.

Saved Supernatants from ChIPs for Tissues of limited supply

1. Add 40ul of prewashed Protein A/G beads to each ChIP.
2. Incubate while rotating end over end at 4C for 1 hour.
3. Briefly spin tubes and place on magnet.
4. Transfer supernatant to fresh tube.
5. Repeat incubation with beads and transfer of supernatant to a clean tube.
6. Snap freeze this “cleared” chromatin for later use if necessary.

Buffers for Tissue ChIP

Blocking Buffer (4°C); PBS+0.5% TWEEN, 0.5% BSA add PI (protease inhibitor cocktail)

ChIP Dilution Buffer (4°C); 16.7mM Tris-HCl pH8.1, 1.1% Triton X-100, and 167mM NaCl, 1.2mM EDTA, 0.01% SDS

Direct ChIP Elution Buffer (RT); 10mM Tris-HCl pH 8.0, 5mM EDTA, 300mM NaCl, **0.1% SDS.** Complete buffer by adding 5mM DTT before adding elution buffer.

RIPA High Buffer (4°C); 0.1% SDS, 1% Triton x-100, 1mM EDTA, 20mM Tris-HCl pH 8.1, 500mM NaCl, 0.1% DOC

RIPA Low Buffer (4°C); 0.1% SDS, 1% Triton x-100, 1mM EDTA, 20mM Tris-HCl pH 8.1, 140mM NaCl, 0.1% DOC

LiCl Wash Buffer (4°C); 250mM LiCl, 0.5% NP40, 0.5% Na Deoxycholate, 1mM EDTA,10mM Tris-HCl pH 8.1

Low TE Buffer pH 8.0 (4°C); 10mM Tris-HCl pH8.0, 0.1mM EDTA pH 8.0

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

Cell Lysis Buffer for 1 Step Lysis (RT); 1% SDS, 10mM EDTA, 50 mM Tris-HCL pH 8.1

Cell Lysis Buffer for 2 Step Lysis (4°C); 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

2.5 M Glycine Solution
9.38 g glycine to 50ml water
Cover and use stir bar while applying low heat until glycine goes into solution.

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