Experiment: _____

Date: _____ Tissue: _____

Purpose: **ChIP-Seq**

Antibodies: _____

_____

11x cross-link buffer:

| *Regent Stock Solution* | *Final concentration* | *Vol for 10 mL of 11xstock* |
|---|---|---|
| 5 M NaCl | 0.1M | 0.2 ml |
| 0.5 M EDTA | 1 mM | 20 uL |
| 0.5 M EGTA, pH8.0 | 0.5 mM | 10 uL |
| 1 M Hepes pH8.0 | 50 mM | 0.5 mL |
| $dH_2O$ | | 6.29 mL |
| | | 2.98 ml |

| *LB1 buffer* | For 50 ml | For 10ml | For 30 ml | *Final* |
|---|---|---|---|---|
| 1 M HEPES, pH 7.5 | 2.5 ml | 0.5 ml | 1.5 ml | 50mM |
| 5 M NaCl | 1.4 ml | 280 µl | 0.84 ml | 140 mM |
| 0.5M EDTA, pH 8.0 | 100 µl | 20 µl | 60 µl | 1 mM |
| 50% glycerol | 10 ml | 2 ml | 6 ml | 10% |
| NP-40 10% | 2.5 ml | 0.5 ml | 1.5 ml | 0.5% |
| Triton X-100 10% | 1.25 ml | 250 µl | 750 µl | 0.25% |
| DDW | 32.25 ml | 6.45 ml | 19.35 ml | |
| Immediately before use, add EDTA-free complete protease inhibitors (Roche). 1 tablet for each 50ml buffer. | | | | |

| *LB2 buffer* | *For 50 ml:* | For 10 ml | *Final* |
|---|---|---|---|
| 5 M NaCl | 2ml | 0.4 ml | 200mM |
| 0.5 M EDTA, pH 8.0 | 100 µl | 20 ul | 1 mM |
| 0.5 M EGTA, pH 8.0 | 50 µl | 10 ul | 0.5 mM |
| 1 M Tris·Cl, pH 8.0 | 500 µl | 100 ul | 10 mM |
| DDW | 47.35ml | 9.47 ml | |
| Immediately before use, add EDTA-free complete protease inhibitors (Roche). 1 tablet/50ml buffer. | | | |

Day 1: _____

**A. Crushing the tissues with Covaris CPO2 and Cut to small pieces**

Weight of bags contained organs: _____ gram
Weight of bags: (no sample) _____gram
Organ Net weight: _____grams

1. Crush frozen tissue using Covaris machine two times.
2. Sep up Petri-dish with 500 ul pre-cooled PBS on ice.
3. Place crushed tissue immediately in cold PBS, keep on ice. Immediately cut the tissue in dish with a razor blade (average size of tissue pieces after cutting should be about 1mm).
4. Add 2-3ml PBS to dish to resuspend tissue, transfer buffer with tissue into 15ml Falcon tube, fill the 15 ml tube with PBS to 10 ml.

**B. Cross-Linking**

5. Add 1ml 11x cross-link buffer (add formaldehyde freshly prior to use).
6. Rotate tube 15 min. at room tempreture.
7. Add 579 ul 2.5M glycine to stop crosslinking, rotate 5 min. at room tempreture.
8. Centrifuge sample at 2000g for 3 min at 4°C. Wash pellet once in 10 ml ice-cold PBS and resuspend in 7mL ice-cold PBS and transfer to 15ml glass douncer.
9. Dounce tissue first with loose (10 times) and later with the tight pestle (15 times). Transfer the tissue to new 15ml tube, centrifuge at 2500g for 3 min. at 4°C.

**C. Cell Lysis and Sonication**

***All lysis buffers should be supplemented with protease inhibitor-EDTA free.** ***Dissolve 1 Protease Inhibitor tablet in 50 mL buffer (need time to dissolve, do it half hour before the cells are ready for next step.) Just before use, add 250 μL of 100 mM PMSF and 50 μL of 1M DTT to RIPA buffer.***

10. Resuspend each pellet of crosslinked tissue in 5 ml LB1(0.1g tissue). Rock at 4 $^0$C for 10 min (nuclear extraction).
11. Transfer sample to 7ml glass douncer, tight stroke 20 times, and then transfer to new 15 ml tube, centrifuge at 2000g for 5 min at 4 $^0$C in a table top centrifuge (If the sample volume is more than 6ml, douce the sample twice, put in the same 15 ml tube).
12. Remove supernatant, resuspend each pellet in 5 ml of LB2 buffer. Rock gently for 5 min at 4 $^0$C and 5 min at room tempreture. Spin at 2000g for 5 min at 4°C in a table top centrifuge.
13. Remove supernatant, resuspend nuclear pellets in 2 mL 1X RIPA buffer (0.1 or 0.2 g tissue) in 15ml Bioruptor tube with sonication beads (Diagenode C01020031). Put samples on ice for 30 min, centrifuge tubes at 3000 rpm for 5 minutes at 4 $^0$C.

14. Set Diagenode pico, sonication cycles 30sec on / 30sec off , 16 rounds. Votexed every 4 cycles.

***Optimal sonication condition needed to be determined for any new cell line**.

15. Centrifuge tubes at 3000 rpm for 5 minutes at 4 $^0$C.

16. Transfer lysate to 2 ml microfuge tubes and clarify by centrifugation at 14,000 rpm at 4 $^0$C for 15 minutes. Save 100 uL of lysate, label "input". Store overnight at 4 $^0$C, then carry through with rest of samples from Day 2 to Day 3, i.e. reverse crosslink at 65 $^0$C, RNase, porteinase K, and spin column steps.
*Snap freeze unused sheared chromatin in liquid nitrogen (2ml lysate containing 0.1 g tissue per tube). Store @-80 $^0$C for later use.

17. Add 5ug TF antibody or 5ug normal IgG (control sample) to each 2 ml lysates (0.1g tissue). Incubate at 4 $^0$C on a neutator for 12-16 hours (overnight).

**Day 2**:_____

1. Remove 80 μL of 50 % Protein A/G-agarose for **each sample (0.2g tissue)** to 1.5 mL microfuge tubes, and wash the beads **twice** with 1 mL of ice cold 1X RIPA buffer. Spin at 5,000 rpm, 4 $^0$C for 1 minute.

   *Use the 1000 μL wide orifice pipet tips (Rainin HR-1000W) to resuspend the Protein A/G-agarose.
2. Resuspend the beads in 80 μL in 1X RIPA. Add Protein A/G-agarose to tubes containing Ag-Ab complex (use another 100 μL 1X RIPA to wash out the beads) and incubate for 1 hour at 4 $^0$C with revolver/rotator rocking.

3. Centrifuge the tubes at 1,500 rpm for 3 minutes, wash the protein A/G-agarose beads 3 times with 10 mL of **fresh, ice cold** 1X RIPA buffer (prepare 3 tubes of RIPA with protease inhibitor, add PMSF and DTT just before use), and once with ice-cold PBS. 15 minutes each wash.

   * Protease Inhibitor, Protease Inhibitor_EDTA free could be used from this step on.
   *For washing, only use ONE tablet of protease inhibitor per 50 mL 1X RIPA buffer.

4. Resuspend Protein A/G-agarose in 800 μL ice-cold 1X PBS and transfer the beads from 15 mL tube to a 1.5 mL eppendorf tube. Wash out the beads in 15 mL tube again with 400 μL ice-cold 1X PBS (to be sure all the bead are collected) and transfer them to Eppendorf tube.

5. Centrifuge Eppendorf tube at 5,000 rpm for 1 minute. Remove PBS completely, add **100 μL of (1% SDS, 1X TE)** to each tube, resuspend Protein A/G-agarose. Incubate at 65 $^0$C for 10 minutes gentle mixing every two minutes.

6. Spin at 5,000 rpm for 1 minute (room temperature), and remove supernatant to an Eppendorf tube (Elute1). Add **150 µL of (0.67% SDS, 1X TE)** to Protein A/G-agarose pellet in each tube. Incubate again at 65 $^0$C for 10 minutes with occasional gentle vortexing. Spin at 5,000 rpm for 1 minutes (Elute 2). Combine the second elute with the first one.

7. To remove any residual Protein A/G-agarose, spin tubes at 14,000 rpm for 2 minutes at room temperature. Transfer the supernatants to new 1.5 mL tube (avoid transferring any beads.)

8. Remove input DNA tube stored at 4 $^0$C and add 150 µL of 1% SDS, 1X TE.
*Hint: Add 1.5X of 1% SDS, 1X TE to sample.

9. Reverse cross-linked **ChIP DNA** and **Input DNA** overnight at 65 $^0$C.

| | |
|---|---|
| *IgG ChIP: | 250 µL |
| Antibody ChIP: | 250 µL |
| Input DNA: | 250 µL |

Day 3: _____

1. Add 250 µL 1X TE containing 100 µg RNase to each of the sample. Incubate for 30 minutes at 37 $^0$C.

2. Add 5.0 µL of 20 mg/mL Proteinase K to each sample. Incubate at 45 $^0$C for 30 minutes.

3. Transfer the samples to 15 mL tube. Purify ChIP and Input DNA using Qiagen PCR purification column (adjust the volume of elution buffer accordingly). Elute DNA in 35 µL EB (when working with Pol II antibody; elute DNA in 50 µL EB).

4. Determine the concentration of "Input DNA" (Qubit).
*Use 1 µL to read!

| Antibody | Concentration (ng/ul) | 260/280 | 260/230 |
|---|---|---|---|
| | | | |
| | | | |
| | | | |

**Bravo automatic library preparation**
Step 1: End Repair (for 24 reactions) TruSeq-End-Repair protocol
10X buffer: 140 ul
ATP: 140 ul
dNTP: 140 ul
enzyme: 28 ul

Mix the above reagents in one tube and transfer 53 ul of the mixture to the A3-H3 wells of a 1ml Nunc plate.

Transfer 34 ul of ChIP DNA to Twin-Tec Eppendorf 96-well plate A1-H1, A2-H2,A3H3. Put plates on Bravo.

Step 2: Purification--SPRISelect reagent (Beckman Coulter B23318) –TruSeq 04 AmPure Purification
90 ul beads was used for each well reaction
Step 3: Addition of 'A' base to 3` Ends—TruSeq 07 A-Tailing
NEB buffer 2: 140 ul
1mM dATP: 280 ul
Klenow: 28 ul

Mix the above reagents in one tube and transfer 53 ul of the mixture to the A4-H4 wells of a 1ml Nunc plate.

Step 4: Purification--SPRISelect reagent (Beckman Coulter B23318) –TruSeq 06 AmPure Purification
90 ul beads was used for each well reaction

Step 5: Adapter Ligation—TruSeq 08 Adapter Ligation

2 x buffer: 420 ul
Enzyme: 56 ul

Mix the above reagents in one tube and transfer 56 ul of the mixture to the A5-H5 wells of a 1ml Nunc plate.

Load 2 ul H2O and 1 ul of adapter 1, 2, 3, 4, 7, 9 to the A1-H1, A2-H2, A3-H3 well of Twin-Tec Eppendorf 96-well plate.

Step 6: Size selection--SPRISelect reagent (Beckman Coulter B23318) Nugen Chip-Seq 03 purification

50 ul beads was loaded to A1-H1, A2-H2, A3-H3 wells of 1ml Nunc plate.
14 ul beads was loaded to A1-H1, A2-H2, A3-H3 wells of Twin-Tec Eppendorf 96-well plate.

Step 7: PCR Amplification

2 x phusion: 1 ml
TruSeq primer 1.0: 20 ul
TruSeq primer 2.0: 20 ul
H2O: 35 ul

Mix the above reagents in one tube and transfer 43 ul of the mixture to 8-strip tubes and add 37 ul DNA to each well (total 80 ul).

Amplify using the following PCR protocol:

Step 1: 98 $^{0}$C---30 seconds

Step 2: [98 $^{0}$C---10 seconds] --> [65 $^{0}$C---30 seconds] -->[72 $^{0}$C---30 seconds]

(Go to Step 2): 17 more cycles

Step 3: 72 $^{0}$C---5 minutes

Step 4: Hold at 4 $^{0}$C

Step 8: Purification--SPRISelect reagent (Beckman Coulter B23318) – Nugen Chip-Seq 05 purification

40 ul beads was loaded to A1-H1, A2-H2, A3-H3 wells of 1ml Nunc plate.
11.2 ul beads was loaded to A1-H1, A2-H2, A3-H3 wells of Twin-Tec Eppendorf 96-well plate.

Step 9: Measure the DNA concentration (ng/μL) using Qubit.