ChIP-seq Protocol

Day 1

Note: *Needed to add protease inhibitor, PMSF, and DTT to the Hypotonic buffer. Besides protease inhibitor, PMSF, DTT, also add the phophatase inhibitors to 1X RIPA throughout the protocol if there is no specific instruction.

1. Take out one 15 mL Falcon tubes, each containing 2 X 10 ^7 of frozen cross-linked cells. Add 1 mL of cold PBS into each tube. Thaw cells on ice.

2. Spin the cells at 1,500 rpm for 3 minutes. Remove PBS and add 2 mL of hypotonic buffer. Resuspend cells and leave the tubes on ice for 10 minutes to swell the cells. *Tap to bottom of the tube to dislodge the cells before adding the buffer which makes the task easier.

3. Dounce homogenize on ice, 30 strokes, to break open the cells. Keep the total time to 15 minutes for hypotonic lysis/homogenization steps. * Use the 7 mL dounce homogenizer

4. Transfer lysate to 2 mL microfuge tubes. Centrifuge at 600 g (rcf) at 4 0 C for 5 min, Discard supernatant.

5. Resuspend nuclear pellets in 1 mL hypotonic buffer. Centrifuge at 600 g (rcf) at 4 0 C for 5 min, Discard supernatant.

6. Resuspend nuclear pellets in 2 mL 1X RIPA buffer, transfer nuclear lysate into 15ml Bioruptor tube with 300ul sonication beads (Diagenode C01020031), (Beads washed with 1ml 1X PBS once and 1X RIPA buffer once) Incubate for 30 min on ice.

7. Sonication cycles 30sec on / 30sec off, 16 rounds. Votexed every 4 cycles.
*Optimal sonication condition needed to be determined for any new cell line.
8. Centrifuge tubes at 3000 rpm for 5 minutes at 4 0 C.

9. Transfer lysate to 2 ml microfuge tubes and clarify by centrifugation at 14,000 rpm at 4 0 C for 15 minutes. Pool supernatants back together in one 15 ml Falcon tubes. 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.

10. Add 5ug GFP antibody. Incubate at 4 0 C on a neutator for 12-16 hours (overnight).

Day 2:

1. Remove 80 μL of Protein A/G-agarose for each sample (2X10^7 cells) to 1.5 ml microfuge tubes and wash twice with 1 ml of ice cold 1X RIPA buffer (containing

protease inhibitor, DTT, PMSF, and phosphatase inhibitors). Spin at 5,000 rpm for 1 minute at 4 ^{0}C.

2. Resuspend the Protein A/ G-agarose in 80 µL 1XRIPA. Add the beads (use another 100 µL 1XRIPA to insure you get all the beads) to tubes containing Ag-Ab complex and incubate for 1 hour at 4 ^{0}C on neutator rocker.

3. Centrifuge the tubes at 1,500 rpm for 3 minutes, wash the protein G-agarose beads 3 times with 10 ml of **fresh, ice cold** 1X RIPA buffer and once with ice-cold PBS. Wash each time for 15 minutes.

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 the beads in 15 ml tube with **400 µL** ice-cold 1X PBS (to be sure all the beads are collected) and transfer to the 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 (Elute 1). 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.

9. Reverse cross-linked ChIP DNA and input DNA overnight at 65 ^{0}C.

Day 3: _____

1. Add **250 µL** 1X TE containing 100 µg RNase to **each 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 samples to 15 ml tube. Purify ChIP DNA using Qiagen PCR purification column. ChIP (sample size **500 µL**; add 2.5 ml of elution buffer). Elute ChIP DNA in **35 µL** EB.

Step 1: End Repair:

Use ChIP DNA prepared from 2 x 10^7 cells or gel purified ChIP DNA of 0.15-0.35 kb in size.
a) Combine and mix the following components in a microfuge tube.

ChIP DNA to be end-repaired	1-34 μL (however much DNA you isolated from the band)
10X End-Repair Buffer	5 μL
2.5 mM dNTP Mix	5 μL
10 mM ATP	5 μL
Sterile water	*X* μL to bring reaction volume to 49 μL
End-Repair Enzyme Mix	1 μL
Total reaction volume	50 μL

b) Incubate at room temperature for 45 minutes.

c) Purify on one QIAquick column using the QIAquick Purification Kit and protocol. Elute in 34 μL EB.

*Follow QIAquick PCR purification protocol.
*Use the column stored at room temperature.

Step 2: Addition of 'A' base to 3` Ends

a) Combine and mix the following components in a microfuge tube.

DNA from step 1	1-34 μL
Klenow buffer (NEB2)	5 μL
1 mM dATP	10 μL
Klenow (3' to 5' exo minus)	1 μL
Total reaction volume	50 μL

b) Incubate at 37 ^{0}C for 30 minutes.

c) Purify on one QIAquik **MinElute** column, using the MinElute PCR purification Kit and protocol. Elute in **12 μL** EB.

*Follow QIAquick PCR purification protocol.
*Use the column stored at 4 ^{0}C.

Step 3: Adapter Ligation

a) Dilute the Illumina adapters 1:10 with water for gel purified DNA. **DO NOT** reuse diluted adapters.

b) Combine and mix the following components in a microfuge tube.

DNA purified from Step 2	12 μL
2X DNA ligase buffer	15 μL
TruSeq Adapter oligo mix (1:10)	1 μL
DNA ligase	2 μL
Total reaction volume	30 μL

c) Incubate for 15 minutes at room temperature.

d) Purify on one QIAquik **MinElute** column, using the MinElute PCR purification Kit and protocol. Elute in **19** μL EB.

*Follow QIAquick PCR purification protocol.
*Use the column stored at 4 ^{0}C.

e) Run adapter ligated DNA on a 2% Agarose EX-Gel.

f) Excise gel in the range of 450-650 bp with a clean scalpel. Be sure to take photos of the gel before and after the gel slices are excised.

g) Purify the DNA from the agarose slices using Qiagen Gel Extraction Kit. Elute in **24 μL** EB.

Day 4:_____

Step 4: PCR Amplification and Size Selection

a) Dilute PCR primers (1.1 and 2.1) 1:1 with water and use 1 μL of each primer in a 50 μL reaction.

b) Combine and mix the following components in a PCR tubes (or plate).

DNA from Step 3	24 μL
Phusion DNA polymerase	25 μL
Diluted PCR primer 1.1	0.5 μL

Diluted PCR primer 2.1	0.5 μL
Total reaction volume	50 μL

c) 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): 14 more cycles

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

Step 4: Hold at 4 ^{0}C

d) Purify on one QIAquik **MinElute** column, using the MinElute PCR purification Kit and protocol. Elute in **19 μL** EB.

e) Run adapter ligated DNA on a 2% Agarose EX-Gel.

f) Excise gel in the range of 150-350 bp with a clean scalpel. Be sure to take photos of the gel before and after the gel slices are excised.

g) Purify the DNA from the agarose slices using Qiagen Gel Extraction Kit. Elute in **12 μL** EB

h) Measure the DNA concentration (ng/μL) using Nanodrop spec. or Qubit.