ATAC-Seq on frozen tissue **5/20/15**

Materials and reagents:

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.				

2ml glass tissue grinder and pestle (Kimble chase, 885301-0002, 885300-0002)
40 µm cell strainer (Fisher, 352340)
Illumina Tagment DNA buffer (Cat#: 15027866) (TD buffer)
Illumina Nextera Tagment DNA enzyme (Cat#: 15027916) (TDE)
NEBNext High-Fidelty 2xPCR Master Mix (NEB M0514S)
10,000 x SYBR Green I (Invitrogen Cat# -7563)
Protocol:
1. Crush frozen tissue (5-10mg) into fine powder while cold (dry ice and LN2).
2. Suspend pulverized tissue in 1 ml ice-cold 1x PBS (1.5 ml tube) by tapping, then centrifuge at 2000g for 3 min. at 4°C.
3. Remove supernatant and resuspend pellet in 1 ml LB1 (1.5 ml tube).
4. Rock tubes at 4°C for 10 min. (Prepare 3 times sample number of 1.5 ml tube, 2 ml tube and 6 well plate for later use)
5. Transfer sample to 2 ml glass douncer, loose stroke (A) 15 times, and then transfer to 1.5 ml tube.
6. Centrifuge 2000g for 5 min at 4°C.
7. Aspirate supernatant and resuspend in 1 ml 1 x cold PBS.
8. Filter with 40 um cell strainer (in 6 well plate), use 1 ml 1 x PBS to wash plate.
9. Count nuclei with cell counter (ideally want 10^5 nuclei; tryptan blue stains nuclei; typically 4-9 um)
10. Transfer 50,000 nuclei (mix before transfer) to 1.5ml tube, do replicates or triplicates for each sample here. If volume is lower than 50ul, add PBS to 1.5 ml tube to make the volume 50 ul. Centrifuge at 500 x g for 10 min at 4 degree C (be careful of the tube direction). Aspirate supernatant with pipet (should be very careful).
11. Add 12.5 ul TD buffer, 10 ul ddH_2O, 2.5 ul TDE to each tube and then transfer to PCR tubes. Usually master mix could be made, then add 25 ul master mix to each nuclei sample.
12. Incubate at 37°Cfor 1hr on PCR machine (preferably with shaking)
13. Use Qiagen MinElute to isolate transposed DNA in 10 ul EB.
    Product can be stored at -20°C, or proceed with library amplification
14. Library amplification:
    a. To amplify transfosed DNA fragments, combine the following in a PCR tube:
    10 ul Transposed DNA
    9.7 ul Nuclease Free H_2O
    2.5 ul 25uM customized Nextera PCR primer 1
    2.5 ul 25uM customized Nextera PCR primer 2 (Barcode)
    0.3 ul 100 x SYBR Green I
    25 ul NEBNext High-Fidelity 2x PCR Master
    50 ul Total

    b. PCR Cycles:
    (1) 72°C 5 min
    (2) 98°C 30 sec
    (3) 98°C 10 sec
    (4) 63°C 30 sec
    (5) 72°C 1 min
    (6) Repeat steps 3-5, 4x
    (7) Hold at 4°C

15. When doing PCR, prepare qPCR master mix (in order to reduce GC and size bias in PCR, the first PCR reaction do 5 cycles as above, qPCR was used here to monitor how many more PCR cycles needed to avoid saturation)

5 ul 5 cycles PCR amplified DNA
4.44 ul Nuclease Free H_2O
0.25 ul 25 uM Customized Nextera PCR primer 1
0.25 ul 25uM customized Nextera PCR primer 2 (choose any barcoded one, use same one for each sample)
0.06 ul 100 x SYBR Green I
5 ul NEBNext High-Fidelity 2x PCR Master
15 ul Total

Transfer each 15 ul reaction sample to the well of qPCR 384 well plate.

qPCR cycle as follows: (or use saved protocol: New experiment from template—JDL-atac Run Protocol)

(1) 98 °C 30 sec 4.8°C/s 1 cycle
(2) 98°C 10 sec 4.8°C/s
63°C 30 sec 2.5°C/s 20 cycles Quantification
72°C single 1 min 4.8°C/s
(3) 64°C 1 sec 2.5°C/s

16. The additional number of cycles needed for the remaining 45 ul PCR reaction is determined as following:
(1) Plot linear fluorescent intensity vs. Cycle
(2) maximum fluorescent minus minimum fluorescent, get the fluorescent intensity difference
(3) ¼ of fluorescent intensity plus minimum fluorescent =lower flurescent
1/3 of fluorescent intensity plus minimum fluorescent =upper flurescent
(4) Calculate the number of cycle that is corresponded to the plot of lower fluorescent and upper fluorescent

17. Put back remaining 45 ul PCR reaction to PCR machine:

(1) 98°C 30 sec
(2) 98°C 10 sec
(3) 63°C 30 sec
(4) 72°C 1 min
(5) Repeat steps 2-4, x times (use the calculation cycles, usually 2-5 cycles)
(6) Hold at 4°C

18. Purify amplified library using Qiagen MinElute PCR purification kit. Elute in 20 ul EB.
19. Gel size selection and purification:
Load purified PCR product on 2% EX-gel, no loading dye, run 6 min, label the gel, collect everything except 60bp primer dimer strong band, do gel extraction, elute 20 ul with EB buffer.
20. Determine DNA concentration using Qubit.
21. Bioanalyzer
Gate 150-1000bp, record average size, concentration (pg/ul) and molarity (nmol/l). Give a score for each sample's peak shape for later sequencing result matching (check if peak shape correlate with sequencing data).