Revised 11/05/09

Experiment:

DATE:

ChIP of (cell line): Chip-Seq

Purpose: Prepare DNA Library for Solexa

Methods and Materials:
Nanosep MF Filter tube(VWR Cat.29300-642)
Pushion Hot Start High-fidelity DNA Polymerase(NEB Cat.F-540L)
Quick Ligation Kit (NEB Cat.M2200L)
End-it DNA End-repair Kit (Epicentre Technologies Cat. No. ER0720)
Klenow (3'-5' exo-) (5U/ul) Invitrigen
Dark Reader (Iso BioExpress U-2235-1)
Minelute purification (Qiagen)
Adapter Oligo mix (1:10 in H2O) (From Illumina's Kit)
PCR primer 1.1(From Illumina's Kit)
PCR primer 2.1(From Illumina's Kit)
SYBR Gold (Invitrigen S11494)

Procedure:

1. End-Repair

1. Use 1-10 ng as starting materials in 34ul of TE or 10mM Tris (EB). (Do not quantitate ChIP samles.) DNA ends are repaired to blunt ends by T4 DNA polymerase and phosphorylated at 3' ends by T4 Polynucleotide Kinase.

	ml	x 8
DNA+H2O	34	
10X End-repair Buffer	5	40
2.5mM dNTPs	5	40
10mM ATP	5	40
END-IT enzyme mix	1	8
Total vol.	50	16/per rxn

2. Incubate for 45 min at room temperature.
3. Minelute purification (Qiagen). Elute once in 20ul of EB, the second time elute in 12uL.
4. Total elute volume is 32uL.

2. Addition of an 'A' Base to the 3' End of the DNA fragments

		ml	x 8
DNA from section 1		32	
	10X NEB Buffer 2	5	40
	1mM dATP	10	80
Klenow (NEB exo-) (5U/ul)		3	24
	Total vol.	50	18/per rxn

1. Incubate for 30min. at 37C.
2. Minelute purification. Elute **twice** in 10ul of EB. Total elute volume is 20uL.
3. Speedvac for 10 min to condense sample to 4ul.

3. Ligation of Adapters to the Ends of the DNA Fragments

15ng-50ng starting DNA scale	**m**	x8	
DNA from section 2	4		
2X Ligase Buffer	5		40
Adapter Oligo mix (1:10 in H2O)	0.5		4
DNA Ligase (1U/m)	0.5		4
Total vol.	10	6ul/per rxn	

1. Incubate for 15min. at room temperature.
2. Minelute purification. Elute **twice** in 20ul of EB. Total elute volume is 40uL.
3. You can store in -80degree for days.

4. Gel Purification of the Products From the Ligation Reaction

- Prepare 8 % polyacrymide gel or buy:

	Volume (mL)
40% acryl (29:1)	15
10X TBE	7.5
ddH20	51.7
10% APS	0.750
TEMED	0.075
Total	75

Running buffer 1X TBE(1L) .

1. **Spin-vac sample for 30''-5min to get rid of ETOH residue in sample.**
2. Add 6x Bromo phenol blue/xylene cyanol loading dye to samples.
3. Load 3 µl 100bps ladder into the first lane and last lane.
4. Load all samples into every other lane.
5. Run at 220V for about 2 hours, when the blue dye reached the 2/3 way of the gel.
6. Stain gel with 100ml 1X TAE, 12uL 10,000 x SYBR Gold (Invitrigen S11494) for 15-20 min. Shake the plate every 5 min. Cover the plate with foil to avoid the light.
7. Excise bands around 200bps-400bps with a clean scalpel.
   - (Optional:Save the band around 400bp-800bp to do Chip-hyb.)
8. Put gel pieces in 0.5mL tube (with bottom of tube punctured 3 times with needle). Put 0.5mL tube into a 2mL tube, and centrifuge at 14k for 2 min.
9. Add 2X volume EB Buffer(about 500uL EB buffer), Shake for O/N in the cold room.

---

10. The next day, Quick spin the tubes.
11. 50°C for 15 min on thermomixer. Spin 2 min @ 14k.
12. Transfer the **supernatant** to two Nanoseq column per sample, spin 2 min @ 14k.
13. Collect supernatant into new 1.5ml eppendorf tube.
14. Add EB Buffer to bring to a total volume of 500ul.
15. Add 1/10 volume(50ul) 3M NaOAC(ph 5.2) and vortex to mix.
16. Add 4ul glycogen(20mg/ml) and 1ml cold 100% ETOH(2.5volumn), vortex.
17. Freeze at -80°C for 30 min (O/N),spin 13.8K for 15min in cold room. Remove supernatant.
18. Wash with 1mL 70% EtOH, vortex, spin in cold room for 5min @13.8k rpm, remove all traces of EtOH, air dry 5 minutes (pellet turns clear).
19. Resuspend precipitated DNA in 20ul EB. (on thermomixer 37°C for 2-5 min)

5. Enrichment of adapter-modified DNA fragments by PCR

	Volume (µl)
DNA from section 4	20
5x Phusion HF Buffer (NEB)	10
10mM dNTPs	1
H2O	13.5
10µM Solexa_PCR_up	2.5
10µM Solexa_PCR-lo	2.5
HotStart Phusion (NEB)	0.5
Total	50

- Amplify using the following PCR protocol,
  Step 1: 98°C for 30sec;
  Step 2: 98°C for 10sec;
  Step 3: 65°C for 30sec;
  Step 4: 72°C for 30sec;
  Step 5: go to step 2, 17 cycles
  Step 6: 72°C for 5min;
  Step 7: 4°C forever;

- PCR purify. Elute twice in 20ul of EB.Total elute volume is 40uL.
- You can store in -80degree for days.

6. Gel Purification of the Products From the Ligation Reaction

1. Prepare 8 % polyacrymide gel or buy:

	Volume (mL)
40% acryl (29:1)	15
10X TBE	7.5
ddH20	51.7
10% APS	0.750
TEMED	0.075
Total	75

2. Running buffer 1X TBE(1L) .

3. Spin-vac sample for 30''-5min to get rid of ETOH residue in sample.
4. Add 6x Bromo phenol blue/xylene cyanol loading dye to samples.
5. Load 3 µl 100bps ladder into the first lane and last lane.
6. Load all samples into every other lane.
7. Run at 220V for about 2 hours, when the blue dye reached the 2/3 way of the gel.
8. Stain gel with 100ml 1X TAE, 12uL 10,000 x SYBR Gold (Invitrigen S11494) for 15-20 min. Shake the plate every 5 min. Cover the plate with foil to avoid the light.
9. Excise bands around 200bps-400bps with a clean scalpel.
   - (Optional:Save the band around 400bp-800bp to do Chip-hyb.)
10. Put gel pieces in 0.5mL tube (with bottom of tube punctured 3 times with needle). Put 0.5mL tube into a 2mL tube, and centrifuge at 14k for 2 min.
11. Add 2X volume EB Buffer(about 500uL EB buffer), Shake for O/N in the cold room.

12. The next day, Quick spin the tubes.

13. Incubate at 50°C for 15 min on thermomixer. Spin 2 min @ 14k.
14. Transfer the **supernatant** to two Nanoseq column per sample, spin 2 min @ 14k.
15. Collect supernatant into new 1.5ml eppendorf tube.
16. Add EB Buffer to bring to a total volume of 500ul.
17. Add 1/10 volume(50ul) 3M NaOAC(ph 5.2) and vortex to mix.
18. Add 4ul glycogen(20mg/ml) and 1ml cold 100% ETOH(2.5volumn), vortex.
19. Freeze at -80°C for 30 min (O/N),spin 13.8K for 15min in cold room. Remove supernatant.
20. Wash with 1mL 70% EtOH, vortex, spin in cold room for 5min @13.8k rpm, remove all traces of EtOH, air dry 5 minutes (pellet turns clear).
21. Resuspend precipitated DNA in 15ul EB. (on thermomixer 37°C for 2-5 min)
22. OD the samples by using Qubit.
23. Dilute the samples down to 10nM (if the conc is greater than 2ng/ul).

The website for calculation is listed below:
http://www.idtdna.com/analyzer/Applications/DilutionCalc/Default.asp

Stock Concentration: sample conc(ng/ul) must be greater than 2ng/ul
Stock Volume: 10ul
Final Concentration: 10nM
Final Volume: 10ul
Molecular Weight: average size which you selected x 650(g/mol)