Broad ChIP Protocol for Full REMC (6 marks)

DAY 1

1) Sonicate cells to fragment sizes of 200-800bp:
   i) Fresh cell pellets can be used directly, while frozen cell pellets are taken from -80°C freezer, placed in an ice bucket on the diagonal so that the pellet is up. Thaw this way for 30 minutes.
   ii) Dissolve 1 mini Protease Inhibitor tablet in 10 ml SDS lysis Buffer. Re-suspend each $3x10^6$ cell pellet in 0.27 ml SDS lysis buffer containing the protease inhibitor cocktail. Be sure to thoroughly re-suspended cell pellet in SDS-Lysis buffer.
   iii) Combine all aliquots of lysed cells and mix well by pipetting. Aliquot 300µl into each of 6x1.5ml tubes. Incubate on ice for 10 min.
   iv) Sonication:
      Bioruptor setting: Power on high, 30s on, 30s off
      (a) Put the 1.5 ml tube in the bioruptor adaptor.
      (b) All slots must be filled and balanced. Make sure water is up to the required water line.
      (c) Put the adaptor inside the reservoir
      (d) Turn on bioruptor. Make sure the sonication is running at 30s on / 30s off
      (e) Check on samples after every 15 minutes of sonication and restart the bioruptor.
      (f) Generally, the time course is for 20, 30, 40 and 50min intervals.

2) After sonication, spin samples at max speed for 10 min at 4º C. A pellet of insoluble material and SDS should form.

3) After centrifugation, collect supernatant from all tubes and pool into a 15 ml conical tube. There should be a total of 0.8mL supernatant. Increase the volume to 21mL with 19.2mL ChIP Dilution Buffer (CDB) with protease inhibitors and mix well. Remove 50µl aliquot for WCE and store at 4º C until ready for reverse crosslinks. Remember to stay on ice at all times.

Table 1

	Mark	Cell #	Supernatant (mL)	IP rxn volume (mL)	Antibody (ug/ChIP)
1	K27me3	4.50E+06	4.5	4.5	4.5
2	K36me3	1.50E+06	1.5	1.5	1.5
3	K4me1	3.00E+06	3	3	0.6
4	K4me3	3.00E+06	3	3	1.5
5	K9ace	4.50E+06	4.5	4.5	4.5
6	K9me3	4.50E+06	4.5	4.5	10 or 1.5?

4) Reference table 1, add appropriate antibody to each individual IP reaction and incubate overnight at 4º C in a rotator at setting 20-24.

DAY 2

5) Pre-equilibrate beads by aliquoting slurry of protein- A sepharose-4B beads into eppendurf tube. Spin 3000g, 30s- check bed volume.
   a) Follow Table 2 as to how much bed volume to add to each reaction

Table 2

		cell #	bed volume (uL)
REMC	K27me3	4.5×10^6	35
REMC	K36me3	1.5×10^6	15
REMC	K4me1	3×10^6	21
REMC	K4me3	3×10^6	21
REMC	K9ace	4.5×10^6	35
REMC	K9me3	4.5×10^6	35

   b) When you have the correct bed volume, remove and save sup by adding it to stock tube. Wash beads 30 sec each time with 2 vol. cold CDB w/ protease inhibitors. Wash as many times as necessary in order to achieve the 2 vol. washes.
   c) After 2 vol washes, resuspend in CDB so that beads are dilute enough to manipulate easily. For instance, if you have 770µl bed vol for 11 samples (70µl bed vol /sample), add ~880µl CDB (in 2ml tube) = final vol. of 1650µl or 150µl total/sample. Start by aliquoting 140µl to each sample. If there is sample left over, try to evenly distribute it to samples. Incubate beads w/ sample for 1 hr at 4ºC in rotator setting 22-24.

6) After 1 hr incubation, spin samples in Beckman centrifuge at ~3000g (845 rpm) for 1 min. at 4º C. Aspirate sup being sure not to disturb the bead pellet. You can leave a small amount of buffer and remove it with pipet tip. *also, beads can stay on ice for 1-2 hrs at this point

7) Re-suspend the beads w/ the DNA-His-Ab complex in 500µl of Low Salt Immune Complex Wash Buffer. Pipet into eppendorf tube and save this tip. With clean tip, add another 500µl Low Salt Buffer to 15ml falcon. This will ensure that remaining beads are successfully removed. Incubate ea sample for 5 min on its side in ice bucket. Mix by hand ~ 2x during 5 min. incubation. After incubation, spin samples 3000 g or 6.6 rpm for 30 sec. Aspirate sup. Taking care not to disturb the bead pellet.

8) Repeat previous step.

9) Re-suspend the beads in 1 ml of LiCl Immune Complex Wash Buffer to wash.

10) Repeat previous step **but** during this incubation, remove tubes to bench top to equilibrate to RT.

11) Re-suspend beads in 1 ml of RT TE pH 8.0 (10mM Tris-HCl, 1mM EDTA). Incubate 5', RT.

12) Repeat previous step use pipet to remove any residual TE going into the next step.

13) Re-suspend bead pellet in 125 µl of **ChIP Elution Buffer** with DTT (1X TE, pH 8.0, 1% SDS, 150mM NaCl, 5mM DTT). Add DTT just before use. Inc. beads w/ elution buffer at 65º C, 10 min. Spin 6.6k, 1 min. Remove sup and pipette into PCR dish- save tip in well next to sample. Add another 125 µl of elution buffer to the beads and repeat incubation and spin. Remove eluate with the saved tip and add to previous 125 µl of elution buffer (total vol = 250 µl). Mix sample and aliquot 100 µl into the well to the right of the original and then an additional 100µl to the right of the second well.

14) To the WCE **only,** add 200µl ddH2O, 10 µl of 5M NaCl, 12.5µl 20% SDS and 1.25µl 1M DTT. The enriched samples DO NOT require the addition of these things due to the salt concentration in the elution buffer.

15) Reverse crosslink samples in DNA amplifier by incubating at 65º C for at least 5 hours. (Optional: Can use PCR machine placing 300µl mineral oil over the all samples including WCE.)

DAY 3

16) After incubation at 65º C remove the samples and bring to room temperature. Add equal volume of Proteinase K (approx 250 µl, 1mg/ml, 0.2 mg glycogen) to each sample and incubate at 37º C for 2 hr.

17) After Proteinase K treatment, perform the following phenol/chloroform extractions:

a) Add 500µl of 4° phenol to each sample, vortex 30s, spin HS, 5 min. Remove top aqueous layer, transfer to new tube. Set pipet to >500µl to ensure removal of entire sample.

b) Add 500µl of 4° phenol:chloroform:isoamyl-OH and proceed as above.

c) Add 500µl of 4° chloroform: isoamyl alcohol 25:1, vortex 30 sec., spin HS, 5 min. and transfer aqueous layer to new tube having set pipet to < 450µl to ensure no phenol is transferred w/ sample.

d) Precipitate DNA by adding 1/10 vol of 3M sodium acetate (pH 5.0) and 2 vol. abs ethanol. Mix well, inc. O/N @ -20°C or 2hr at -80°C.

DAY 3/4

18) After overnight incubation, spin samples at max speed for 40 min at 4°C.

19) Aspirate ethanol taking care w/ pellet. Add 1 ml 70% ethanol and spin at max speed for 15 min. Aspirate ethanol and pulse spin to concentrate remaining ethanol. Remove remaining ethanol by pipet. Allow pellets to air dry for ~ 10-15 min. Re-suspend in 1x TE pH, 8.0 with RNase at a ratio of 35 µl TE: 1µl RNase. (The final volume will depend on the number of cells you started

with, i.e.: the amount of DNA you expect per tube and the volume required for your next step.) Incubate at 37°C for 30 min. then pulse spin.

20) You should have enriched samples and one WCE sample each suspended in 1x TE.

21) Use 2µl of each sample to quantify the yields with Invitrogen Qubit.

Buffers and Solutions for ChIP

SDS lysis buffer (with protease inhibitor)
1% SDS,
10mM EDTA,
50mM Tris-HCl, pH 8.1

Proteinase K/ Glycogen

Proteinase K 20mg/ml	500µl
Glycogen 1 mg/ml	100µl
ddH_2O	9.4ml

Chip Dilution Buffer
0.01% SDS
1.1% Triton X-100
1.2mM EDTA
16.7mM Tris-HCl pH 8.1
167mM NaCl

Low Salt Immune Complex Wash Buffer
0.1% SDS
1% Triton X-100
2mM EDTA
20mM Tris-HCl pH 8.1
150mM NaCl

LiCl Immune Complex Wash Buffer
0.25M LiCl
1% NP40
1% deoxycholate
1mM EDTA
10mM Tris-HCl pH 8.1

TE Buffer pH 8.0
10mM Tris-HCl, pH 8.0
1mM EDTA pH 8.0

Elution buffer with DTT
10mM Tris-HCl pH8.0
1mM EDTA pH 8.0
1% SDS
150mM NaCl
5mM DTT

4 X loading buffer
50mM Tris pH8.0
40mM EDTA
40% (w/v) sucrose
0.25% orange G

Format for 96 well PCR dishes:

Whole Cell Extract (input)
Place 50 µl of sample in first well. Add 200 µl of salts. Mix by pipeting. Remove 100µl to well B from A. Remove 100 µl of sample to well C from A.