**Day 0: Harvesting Cells**

1. Trypsinize/Harvest cells and count them. Resuspend cells in fresh media so that you have 100,000,000 (1x10e8) cells in 45 mLs of media in a 50 mL conical tube. If there is not exactly 1x10e8 cells, adjust the volume of media (and subsequent steps) by multiplying by the correction factor: Correction factor is = (X # of cells)/(1x10e8)
2. Add 1.25 mLs of 37% formaldehyde to each 45 mL tube and incubate at room temperature for 10 minutes (of, if less than 1x10e8 cells, multiply 1.25 mLs by the correction factor calculated in step 1).
3. Add 2.5mLs of 2.5M Glycine and incubate for 5 minutes at room temperature and then for 15 minutes on ice. (Or if less than 1x10e8 cells, multiply 2.5mLs of 2.5M Glycine by the correction factor calculated in step 1.
4. Split each 50mL tube (with 10e8 cells) into 4 aliquots (~12 mLs per tube in a 15 mL conical) giving 25,000,000 cells/tube. Spin these tubes down at 3,500xG for 15 minutes. Dump off supernatant, and remove last few drops with a pipette. Freeze the cells on dry ice, and store at -80˚C. (If less than 1x10e8 cells are used, still use 12mL aliquots, giving 25,000,000 cells per 15 mL conical. If there are extra, aliquot those into a 15 mL conical and mark the approximate amount of cells).

**Day 1: Cell Lysis, Restriction Enzyme Digestion**

1. Add 550µL of Lysis Buffer to one pellet and resuspend (in 1.5mL Eppendorf)

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| **Lysis Buffer** | **Stock** | **For 5 mLs** |
| 10mM Tris-HCl pH 8.0 | 1M | 50µL |
| 10mM NaCl | 5M | 10µL |
| 0.2% NP-40 | 10% | 100µL |
| 1X Complete | 50X | 100µL |
| H2O |  | 4.74 mLs |

1. Incubate on ice for 15 minutes
2. Dounce 10X with Loose Pestle (Pestle A)
3. Incubate 1 minute on ice
4. Dounce 10X with Loose Pestle (Pestle A)
5. Spin down in a table top centrifuge 5000 rpm for 5 minutes at 4°C
6. Wash the pellet twice in 500µL of ice cold 1X NEB Buffer 2.
7. Resuspend the pellet in 250µL of 1X NEB Buffer 2, split into 5 50µL aliquots
8. Add 312 µL of 1X NEB Buffer 2 to each tube
9. Add 38µL of 1% SDS to each tube and incubate at 65˚C for 10 minutes on thermomixer (800 rpm)
10. Immediately after, put on ice
11. Add 44µL of 10% Triton X-100 and be careful to avoid bubbles, let sit for 5 minutes
12. Add 400U of HindIII (20µL of 20U/µL, NEB)
13. Incubate at 37˚C overnight on thermomixer (750 rpm)

**Day 2: Biotin End-Repair, Ligation, Proteinase K digestion**

1. Place tubes on ice. Mark one as the “3C control”
2. For the 4 HiC tubes, add:

1.5µL 10mM dTTP

1.5µL 10mM dATP

1.5µL 10mM dGTP

37.5µL of 0.4mM Biotin-14-dCTP (Invitrogen)

10µL of 5U/µL Klenow (NEB)

1. Incubate at 37˚C for 45 minutes
2. Add 86µL of 10% SDS and incubate at 65˚C for 30 minutes (to all tubes, including “3C”)
3. Immediately place tubes on ice
4. Add 7.61 mLs of Ligation Mix to five 15mL conical tubes, and add the

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| **Ligation Mix** |
| 745µL 10% Triton X-100 |
| 745µL 10X Ligation Buffer |
| 80µL of 10mg/mL BSA |
| 80µL 100mM ATP |
| 5.96 mLs of H2O |

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| **10X Ligation Buffer** | **Stock** | **For 5mLs** |
| 0.5M Tris-HCl pH 7.5 | 1M | 2.5mLs |
| 100mM MgCl2 | 1.5M | 333µL |
| 100mM DTT | 1M | 0.5mL |
| H2O |  | 1.666mLs |

1. Add 50µL of 1U/µL T4 DNA Ligase (Invitrogen, this is important) to each HiC sample, and 10µL of 1U/µL T4 DNA Ligase to the 3C Control
2. Incubate at 16˚C for 4 hrs (water bath in cold room)
3. After 4 hrs, add 25µL of 20mg/mL Proteinase K
4. Incubate overnight at 65˚C

**Day 3: Reverse Cross-Links, Purify DNA**

1. Add another 25µL of 20mg/mL Proteinase K to each tube and incubate at 65˚C for another 2 hrs
2. Cool the tubes to room temperature (don’t use ice, just let them sit for 10 minutes)
3. Transfer each tube to a 50mL conical tube.
4. Add 10mLs Phenol pH 8.0 to each tube, and vortex for 2 minutes
5. Spin the tubes for 10 minutes at 3,500 rpm
6. Transfer the supernatant to a new 50mL conical
7. Add 10mLs of Phenol:Chloroform:Isoamyl Alcohol pH 8.0 to each tube, and vortex for 2 minutes
8. Spin the tubes for 10 minutes at 3,500 rpm.
9. Transfer supernatant to a 35mL centrifuge tube. Bring the volume to 10mLs with 1X TE
10. Add 1mL of 3M Na-Acetate and mix
11. Add 25mLs of ice cold 100% Ethanol to each tube and mix by inversion
12. Incubate the tubes at -80˚C for at least 1 hour (can be overnight)
13. Spin the tubes at 4˚C for 20 minutes at 10,000g.
14. Remove the supernatant very carefully (the pellet can be very loose).
15. Remove as much residual ethanol as possible with a pipette.
16. Add 450µL of 1X TE to dissolve the pellet and transfer to a 1.5mL eppendorf.
17. Speed vac sample for 5 minutes to remove residual ethanol
18. Perform two Phenol:Chloroform:Isoamyl Alcohol extractions by adding 500µL of phenol:chloroform:isoamyl alcohol to each tube, vortex for 30 seconds, and transfer the contents to a phase lock tube, and spin at max speed for 4 minutes. Transfer the supernatant to a new 1.5mL eppendorf and repeat.
19. Transfer the final supernatant (400µL) to a new 1.5mL eppendorf tube
20. Add 40µL of 3M Na-Acetate and mix by vortexing
21. Add 1mL of 100% ice cold ethanol to each tube and invert to mix.
22. Incubate the tubes for at least 30 minutes at -80˚C.
23. Spin the tubes at max speed for 20 minutes at 4˚C.
24. Wash the pellets with 500µL of ice cold 70% Ethanol
25. Spin the samples at max speed for 5 minutes at 4˚C.
26. Discard the supernatant, and remove residual ethanol with a pipette.
27. Air dry the sample for 10 minutes
28. Resuspend in 25µL of 1X TE (can incubate at 37˚C for ~10 minutes)
29. Add 1µL of 20mg/mL RNase A to each sample and incubate for 15 minutes at 37˚C
30. Pool tubes marked for HiC, leave the 3C tube aside as a control (these samples can be frozen at -20˚C for overnight/a few days)

**Day 4: Gel to Test Library Quality, T4 Clean Up**

1. To test the quality of the HiC library, we will run it on an 0.8% agarose gel (I don’t usually do this, I have seen libraries turn out poorly that have a tight band, and I’ve seen good libraries result from a non so tight band).
2. Quantify the amount of DNA using Qubit (usually requires a 1:20 or 1:40 dilution to be within the range of the standards). Using the nanodrop gives wildly inaccurate concentrations. Alternatively, you can run the library out on a gel with a dilution series of digested genomic DNA to estimate the concentration.
3. Take 5µg of HiC library (if >5µg, set up multiple in parallel) and set up the following:

1µL 10mg/ml BSA

10µL NEB Buffer 2 10X

1µL 10mM dATP

1µL 10mM dGTP

1.67 µL T4 DNA Polymerase (3U/µL, NEB)

Water to 100µL

1. Incubate at 12˚C for 2 hrs.
2. Add 2µL of 0.5M EDTA to each sample
3. Add 1X TE to 400 µL, and 400µL of Phenol:Chloroform:Isoamyl Alcohol and phenol chloroform extract the DNA.
4. Take aqueous phase (~400µL) and add 40µL 3M NaAcetate and 1mL 100% Ethanol.
5. Keep at -80˚C for 30 minutes, spin at maximum speed for 15 minutes.
6. Wash pellet 1X in 1mL of ice cold 70% Ethanol.
7. Air Dry Pellet for 10 minutes.
8. Add water to each pellet and pool samples together to reach a final volume of 100µL of water (incubate at ~37˚C for 10 minutes to dissolve).

**Day 5:**

1. Shear DNA using a Covaris S2 sonicator using the following parameters: Duty Cycle 5, Intensity 5, Cycles/Burst 200, Time 60 seconds, for 4 cycles.
2. Repair the ends of DNA by adding the following:

100µL starting material

14µL 10X Ligation Buffer (NEB)

14µL 2.5mM dNTPs

5µL T4 DNA Polymerase (NEB)

5µL T4 Polynucleotide Kinase (NEB)

1µL Klenow (NEB)

1µL H2O

1. Incubate sample at room temperature for 30 minutes.
2. Purify with a minute column. Elute twice in 15µL 1X TE
3. Add an “A” base by adding the following reagents

30µL starting material

5µL NEB Buffer 2 10X

10µL 1mM dATP

2µL H2O

3µL Klenow Exo-

1. Incubate at 37˚C for 30 minutes
2. Inactivate by incubating at 65˚C for 20 minutes.
3. Reduce volume to 20µL by speed vac for 10 minutes.
4. Run sample on a 1.5% agarose gel in 1X TAE at 80 volts for 3.5 hours. Load 10µL of 100 bp ladder.
5. Stain with Syber Gold for 30 minutes
6. Select samples in the 300-500 bp range
7. Use QiaQuick Gel Extraction Kit to extract DNA
8. Elute twice in 50µL of 1X TE
9. Add 200µL of 1X TE to bring the total volume to 300µL.
10. Quantify the sample using the Qubit, use 2µL for Qubit.

**Day 6: Biotin Pull Down, PCR**

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| **2X Binding Buffer** | **Stock** | **For 10 mLs** |
| 10mM Tris-HCl pH 8.0 | 1M | 100µL |
| 1mM EDTA | 0.5M | 20µL |
| 2M NaCl | 5M | 4mLs |
| H2O |  | 5.88mLs |

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| **Tween Wash Buffer** | **Stock** | **For 10 mLs** |
| 5mM Tris-HCl pH 8.0 | 1M | 50µL |
| 0.5mM EDTA | 0.5M | 10µL |
| 1M NaCl | 5M | 2mLs |
| 0.05% Tween | 10% | 50µL |
| H2O |  | 7.89mLs |

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| **10X Ligation Buffer** | **Stock** | **For 5mLs** |
| 0.5M Tris-HCl pH 7.5 | 1M | 2.5mLs |
| 100mM MgCl2 | 1.5M | 333µL |
| 100mM DTT | 1M | 0.5mL |
| H2O |  | 1.666mLs |

**Dilute the 10X Ligation buffer 1:10 (to 1X) for subsequent ligation reaction.**

1. All Washes are performed as follows: Rotate at room temperature for 3 minutes, bind to the magnetic tube for 1 minute, take the supernatant off, resuspend, and change tubes.
2. Take 60µL of C1 Streptavidin Beads (Invitrogen) and wash 2x in 400µL of Tween Wash Buffer
3. Resuspend the sample in 300µL of 2X Binding Buffer
4. Combine 300 µL of beads with 300µL of HiC DNA
5. Incubate sample at room temperature for 15 minutes with rotation
6. Wash the beads 3X in 400µL of 1X Binding Buffer
7. Wash the beads 2X in 100µL of 1X Ligation Buffer
8. Resuspend sample in 50µL of 1X Ligase Buffer.
9. Add 6pmoles of paired end adaptors per µg of HiC DNA from Qubit. Estimate is that the 1:10 adaptor oligo mix contains 2.165pmoles/µL.
10. Add 1,200 U of T4 DNA Ligase (NEB), and add 0.5µL of 100mM ATP
11. Ligate for 2hrs
12. Wash 5X with Tween Wash Buffer (400µL)
13. Wash 1X in 200µL of 1x Binding Buffer
14. Wash 1X in 200µL of NEB Buffer 2 1X
15. Wash 1X in 50µL of NEB Buffer 2 1X
16. Resuspend in 50µL of NEB Buffer 2 1X
17. Set up test PCR Reactions as follows

0.6µL of Bead bound Library

2µL of Fusion HF 5X Buffer

0.2µL 10mM dNTPs

0.15µL Primer 1.0 (10µM)

0.15µL Primer 2.0 (10µM)

0.1µL Fusion Hotstart

6.8 µL H2O

1. Run test PCR for X = 12, 15, 18, or 21 cycles with the following parameters

Step 1: 30s at 95˚C

Step 2: 10s at 95˚C

Step 3: 30s at 65˚C

Step 4: 30s at 72˚C

Go to Step 2 for X-1 cycles

Step 5: 7 minutes at 72˚C

Step 6: 4˚C forever

1. Run on an acrylamide gel for test for the ideal number of cycles

**Day 7: PCR, AMPure Clean Up, Gel for QC**

1. After visualizing gel from previous day to determine the minimum cycle number to see a decent amount of product forming, run a full scale PCR reaction using this cycle number.
2. Set PCR up as a 200µL total reaction, run it in 4x50µL PCR tubes

1x 4x

10µL Fusion HF 5X Buffer 40µL

11.5µL Beads 46µL

1µL 10mM dNTPs 4µL

0.5µL Fusion Hot Start 2µL

3.125µL Primer 1.0 (10µM) 12.5µL

3.125µL Primer 2.0 (10µM) 12.5µL

20.75µL H2O 83µL

1. Save 2µL of final PCR product to run on a gel to test for quality
2. Take remaining PCR reaction (~200µL) and add 360µL of AMPure Beads
3. Mix with a pipette 10 times, let rotate at RT for 5 minutes.
4. Place on magnet for 2 minutes
5. Remove supernatant with a pipette, add 1mL of 70% Ethanol, rotate for 1 minute, 1 minute on the beads.
6. Repeat wash 1 more time
7. Let air dry for 2 minutes on beads.
8. Elute by adding 50µL of 1X TE and pipette up and down 10 times, returning to magnet
9. Save 0.5µL and run on a gel to test quality of clean up.