**Hi-C protocols**

*Crosslinking*

1)  Grow two to five million mammalian cells under recommended culture conditions to about 80% confluence. Pellet suspension cells or detached adherent cells by centrifugation at 1000 rpm for 5 minutes.

2)  Resuspend cells in fresh medium at a concentration of 1x106 cells per 1ml media. In a fume hood, add freshly made formaldehyde solution (sigma, F1635) to a final concentration of 1%, v/v. Incubate at room temperature for 10 minutes with mixing.

3)  Add 2.5M glycine solution to a final concentration of 0.2M to quench the reaction. Incubate at room temperature for 5 minutes on rocker.

4)  Centrifuge for 5 minutes at 1500 rpm at 4°C. Discard supernatant into an appropriate collection container.

5)  Resuspend cells in 1x106 cells per 1ml cold 1X PBS and spin for 5 minutes at 1500 rpm at 4°C. Discard supernatant and freeze cell pellets in dry ice.

6)  Either proceed to the rest of the protocol or store cell pellets at -80°C.

*Lysis and Restriction Digest*

7)  Combine 300 μl of ice-cold Hi-C lysis buffer (10mM Tris-HCl pH8.0, 10mM NaCl, 0.2% Igepal CA630, protease inhibitor (Sigma, 4693159001)). Add to one crosslinked pellet of cells.

8)  Incubate cell suspension on ice for >15 minutes. Centrifuge at 2500xG for 5 minutes. Discard the supernatant.

9)  Wash pelleted nuclei once with 500μl of ice-cold Hi-C lysis buffer.

10)  Gently resuspend pellet in 50μl of 0.5% sodium dodecyl sulfate (SDS) and incubate at 62°C for 5-10 minutes.

11)  After heating is over, add 145μl of water and 25μl of 10% Triton X-100 (Sigma, 93443) to quench the SDS. Mix well, avoiding excessive foaming. Incubate at 37°C for 15 minutes.

12)  Add 25μl of 10X NEBuffer2 and 100U of MboI restriction enzyme (NEB, R0147) and digest chromatin overnight or for at least 2 hours at 37°C with rotation.

*Marking of DNA Ends, Proximity Ligation, and Crosslink Reversal*

13)  Incubate at 62°C for 20 minutes to inactivate MboI, then cool to room temperature.

14)  To fill in the restriction fragment overhangs and mark the DNA ends with biotin, add 50μl of fill-in master mix:

37.5μl of 0.4mM biotin-14-dATP (Life Technologies, 19524-016)

1.5μl of 10mM dCTP
1.5μl of 10mM dGTP
1.5μl of 10mM dTTP

8μl of 5U/μl DNA Polymerase I, Large (Klenow) Fragment (NEB, M0210)

15)  Mix by pipetting and incubate at 37°C for 1 hour with rotation.

16)  Add 900μl of ligation master mix:

663μl of water
120μl of 10X NEB T4 DNA ligase buffer (NEB, B0202)

100μl of 10% Triton X-100
12μl of 10mg/ml Bovine Serum Albumin (100X BSA)

5μl of 400 U/ μl T4 DNA Ligase (NEB, M0202)

17)  Mix by inverting and incubate at room temperature for 4 hours with slow rotation.

18)  Spin for 5 minutes at 2500 rpm. Resuspend nuclei in 500 μl TE (pH8.0), SDS 0.5%. Degrade protein by adding 5μl of 20mg/ml proteinase K (NEB, P8102) and incubate

at 55°C for 30 minutes.

19) Add 50μl of 5M sodium chloride and incubate on thermomixer (900 rpm) at 65°C overnight.

*DNA Shearing and Size Selection*

20)  Cool tubes at room temperature.

21)  Add 0.1X volumes of 3M sodium acetate, pH 5.2 and 1ml of pure ethanol. Mix by inverting and incubate at -80°C for 15 minutes.

22)  Centrifuge at max speed, 4°C for 15 minutes. Carefully remove the supernatant by pipetting.

23)  Wash 800μl of 70% ethanol. Centrifuge at max speed for 5 minutes.

24)  Remove all supernatant and wash the pellet once more with 800μl of 70% ethanol.

25)  Dissolve the pellet in 130μl of 1X Tris buffer (10 mM Tris-HCl, pH 8) and incubate at 37°C for 15 minutes to fully dissolve the DNA.

26)  To make the biotinylated DNA suitable for high-throughput sequencing using Illumina sequencers, shear to a size of 200-500bp using the following parameters: Instrument: Covaris ME220 (Covaris, Woburn, MA)

Volume of Library: 130μl in a Covaris microTUBE
Durations: 53
Peak Power: 70
Duty Factor: 20%
Cycle/Burst: 1000

27)  Transfer sheared DNA to a fresh 1.5ml tube. Wash the Covaris vial with 70μl of water and add to the sample, bringing the total reaction volume to 200μl. Run 1μl DNA on a 2% agarose gel to verify successful shearing. For libraries containing fewer than 2x106 cells, the size selection using AMPure XP beads described in the next steps could be performed on final amplicons rather than before biotin pull-down.

28)  Warm a bottle of AMPure XP beads (Beckman Coulter, A63881) to room temperature.

29)  Add exactly 120μl of beads to the reaction. Mix well by pipetting and incubate at room temperature for 5 minutes.

30)  Separate on a magnet. Transfer the clear solution to a fresh tube, avoiding any beads. The supernatant will contain fragments shorter than 500bp.

31)  Add exactly 54μl of fresh AMPure XP beads to the solution. Mix by pipetting and incubate at room temperature for 5 minutes.

32)  Separate on a magnet and keep the beads. Fragments in the range of 200-400bp will be retained on the beads. Discard the supernatant containing degraded RNA and short DNA fragments.

33)  Keeping the beads on the magnet, wash twice with 700μl of 70% ethanol without mixing.

34)  Leave the beads on the magnet for 5 minutes to allow remaining ethanol to evaporate.

35)  To elute DNA, add 300μl of 1X Tris buffer, gently mix by pipetting, incubate at room temperature for 5 minutes, separate on a magnet, and transfer the solution to a fresh 1.5ml tube.

36)  Quantify DNA by Qubit dsDNA High Sensitivity Assay (Life Technologies, Q32854) and run undiluted DNA on a 2% agarose gel to verify successful size selection.

*Biotin Pull-Down and Preparation for Illumina Sequencing*

37)  Prepare for biotin pull-down by washing 75μl of 10mg/ml Dynabeads MyOne Streptavidin T1 beads (Life technologies, 65602) with 400μl of 1X Tween Washing Buffer (1X TWB: 5mM Tris-HCl (pH 7.5); 0.5mM EDTA; 1M NaCl; 0.05% Tween 20). Separate on a magnet and discard the solution.

38)  Resuspend the beads in 300μl of 2X Binding Buffer (2X BB: 10mM Tris-HCl (pH 7.5); 1mM EDTA; 2M NaCl) and add to the reaction. Incubate at room temperature for 15 minutes with rotation to bind biotinylated DNA to the streptavidin beads.

39)  Separate on a magnet and discard the solution.

40)  Wash the beads by adding 600μl of 1X TWB. Heat the tubes on a Thermomixer at 55°C for 2 min with mixing. Reclaim the beads using a magnet. Discard supernatant.

41)  Repeat wash.

42)  Resuspend beads in 100ul 1X NEB T4 DNA ligase buffer (NEB, B0202) and transfer to a new tube. Reclaim beads and discard the buffer.

43)  To repair ends of sheared DNA and remove biotin from unligated ends, resuspend beads in 100μl of master mix:
 88μl of 1X NEB T4 DNA ligase buffer

2μl of 25mM dNTP mix
5μl of 10U/μl NEB T4 PNK (NEB, M0201)
4μl of 3U/μl NEB T4 DNA polymerase I (NEB, M0203)
1μl of 5U/μl NEB DNA polymerase I, Large (Klenow) Fragment (NEB, M0210)

44)  Incubate at room temperature for 30 minutes. Separate on a magnet and discard the solution.

45)  Wash the beads by adding 600μl of 1X TWB Heat the tubes on a Thermomixer at 55°C for 2 min with mixing. Reclaim the beads using a magnet. Discard supernatant.

46)  Repeat wash.

47)  Resuspend beads in 100μl 1X NEBuffer 2 and transfer to a new tube. Reclaim beads and discard the buffer.

48)  Resuspend beads in 100μl of dATP attachment master mix:

90μl of 1X NEBuffer 2
5μl of 10mM dATP
5μl of 5U/μl NEB Klenow exo minus (NEB, M0212)

49)  Incubate at 37°C for 30 minutes. Separate on a magnet and discard the solution.

50)  Wash the beads by adding 600μl of 1X TWB Heat the tubes on a Thermomixer at 55°C for 2 min with mixing. Reclaim the beads using a magnet. Discard supernatant.

51)  Repeat wash.

52)  Resuspend beads in 100μl 1X Quick ligation reaction buffer (NEB, B6058) and transfer to a new tube. Reclaim beads and discard the buffer.

53)  Resuspend in 50μl of 1X NEB Quick ligation reaction buffer.

54)  Add 2μl of NEB DNA Quick ligase (NEB, M2200). Add 1.8μl of an Illumina indexed adapter (25 μM, Bioo Scientific). Record the sample-index combination. Mix thoroughly.

55)  Incubate at room temperature for 15 minutes. Separate on a magnet and discard the solution.

56)  Wash the beads by adding 600μl of 1X TWB and transferring the mixture to a new tube. Heat the tubes on a Thermomixer at 55°C for 2 min with mixing. Reclaim the beads using a magnet. Remove supernatant.

57)  Repeat wash.

58)  Resuspend beads in 100μl 1X Tris buffer and transfer to a new tube. Reclaim beads and discard the buffer.

59)  Resuspend in 50μl of 1X Tris buffer.

*Final Amplification and Purification*

60)  Amplify libraries by 5x 50μl PCR reactions directly off of the T1 beads with 4-6 cycles, using Illumina primers (12.5 μM, Bioo Scientific) and Q5 Hi-Fidelity DNA polymerase (NEB).

61)  After amplification is complete, bring the total library volume to 250μl.

62)  Separate on a magnet. Transfer the solution to a fresh tube and discard the beads.

63)  Warm a bottle of AMPure XP beads to room temperature. Gently shake to resuspend the magnetic beads. Add 175μl of beads to the PCR reaction (0.7X volumes). Mix by pipetting and incubate at room temperature for 5 minutes.

64)  Separate on a magnet and remove the clear solution.

65)  Keeping the beads on the magnet, wash once with 700μl of 70% ethanol without mixing.

66)  Remove ethanol completely. To remove traces of short products, resuspend in 100μl of 1X Tris buffer and add another 70μl of AMPure XP beads. Mix by pipetting and incubate at room temperature for 5 minutes.

67)  Separate on a magnet and remove the clear solution.

68)  Keeping the beads on the magnet, wash twice with 700μl of 70% ethanol without mixing.

69)  Leave the beads on the magnet for 5 minutes to allow the remaining ethanol to evaporate.

70)  Add 30-50μl of 1X Tris buffer to elute DNA. Mix by pipetting, incubate at room temperature for 5 minutes, separate on a magnet, and transfer the solution to a fresh labeled tube. The result is a final *in situ* Hi-C library ready to be quantified and sequenced using an Illumina sequencing platform.