In nucleus Hi-C protocol for *C. elegans* embryos

Compiled by Erika Anderson, July 2016.

Crosslinking, isolating nuclei, and digestion

1. Bleach gravid hermaphrodites to obtain at least 0.5g of embryos. Freeze down in an equal volume of 1x M9 in 1ml aliquots and store at −80 °C
2. Thaw embryos on ice and supplement with 1 mM PMSF and 5 mM DTT.
3. Wash once in 30 ml formaldehyde solution (1× M9 solution with 2% (v/v) formaldehyde, Polysciences 18814-20) by spinning for 1 min at 2000rpm.
4. Crosslink in 50 ml of formaldehyde solution for 30 min at room temperature while shaking.
5. Wash once with 50 ml of 100 mM Tris-HCl, pH 7.5 to quench the reaction.
6. Wash twice with 50 ml of 1× M9.
7. Transfer to a 1.5ml tube and wash once in 1ml of lysis buffer (10 mM Tris-HCl, pH 8.0, 10 mM NaCl and 0.2% (v/v) Igepal CA-630 (Sigma I8896)) supplemented with 5 mM DTT, 1 mM PMSF, 0.1% (v/v) protease inhibitors (EMD 539134) and 0.5 mM EGTA. Spin at 2000g for 1min.
8. To obtain extract, resuspend the pellet in 750ul lysis buffer and dounce embryos 10 times using the large pestle A (Kontes 2 ml glass dounce, Spectrum 985-44182; clearance 0.076–0.127 mm), and then 10 times using the small pestle B (clearance 0.01–0.069 mm) on ice.
9. Spin extract for 5 min at 100*g* at 4 °C, and save the supernatant.
10. Resuspend the pellet in 750µl of supplemented lysis buffer and dounce again. Repeat steps 8-10 until the supernatant becomes clear (5-7 times).
11. Spin again at 100g for 5min, and remove supernatant to avoid broken carcasses.
12. Combine all supernatants. Mix 9ul of nuclei with 1ul of 10ug/ml DAPI and count the nuclei using a haemocytometer. I dilute the nuclei 1:100 and count the nuclei in the center square. Nuclei/ml = (nuclei in the center square) x (dilution factor) x 10^4. Typical yield is ~$3x10^8$ nuclei from 0.5g of embryos.
13. Spin down 1.5 x 10^8 nuclei for 5 min at 2,000*g* at 4°C in a low retention tube. Unused nuclei can be resuspended in Nuclei storage buffer (50mM Tris-Cl pH 8.0, 25% glycerol, 5mM MgAc2, 0.1mM EDTA, 5mM DTT), frozen in liquid nitrogen, and stored at -80°.
14. Remove the supernatant, wash once with 500ul lysis buffer, and repeat the spin.
15. Gently resuspend pellet in 50ul of 0.5% SDS and incubate at 62°C for 5-10 min.
16. 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.
17. Add 25µl of 10X DpnII Buffer and 2ul (100U) of DpnII restriction enzyme and digest chromatin overnight at 37°C with rotation. A significant amount of digestion takes place within 2hr.

Biotin incorporation, ligation, and crosslink reversal

18. Incubate at 62°C for 20 minutes to inactivate restriction enzyme, then cool to room temperature.
19. 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 exo minus

20. Mix by pipetting and incubate at 37°C for 45 minutes-1.5 hours with rotation.
21. Add 900µl of ligation master mix:
    535µl of water
    240µl of 5X T4 DNA ligase buffer (Invitrogen)
    100µl of 10% Triton X-100
    12µl of 10mg/ml Bovine Serum Albumin (100X BSA)
    13µl of 1 U/ µl T4 DNA Ligase (Invitrogen)
22. Mix by inverting and incubate at room temperature for 4 hours with slow rotation.
23. Degrade protein by adding 50µl of 20mg/ml proteinase K (NEB, P8102) and 120µl of 10% SDS and incubate at 55°C for 30 minutes.
24. Add 130µl of 5M sodium chloride and incubate at 68°C overnight or for at least 1.5 hours.

DNA Purification, Shearing, and Size Selection

25. Cool tubes at room temperature. Split into two 750µl aliquots in 2ml tubes and add 1.6X volumes of pure ethanol and 0.1X volumes of 3M sodium acetate, pH 5.2, to each tube. Mix by inverting and incubate on ice for 15 minutes.
26. Centrifuge at max speed at 4°C for 15 minutes. Keep the tubes on ice after spinning and carefully remove the supernatant by pipetting.
27. Resuspend, combining the two aliquots, in 800µl of 70% ethanol. Centrifuge at max speed for 5 minutes.
28. Remove all supernatant and wash the pellet once more with 800µl of 70% ethanol.
29. After removing all the ethanol, resuspend 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.
30. Transfer to a 130ul Covaris tube and shear on the S2 Covaris using this program:
    duty cycle, 10%
    intensity, 4
    cycles/burst, 200
    time, 55 s
31. 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. Save 3ul to run on a gel to check shearing.
32. Warm a bottle of AMPure XP beads (Beckman Coulter, A63881) to room temperature. Add exactly 110µl (0.55X volumes) of beads to the reaction. Mix thoroughly by pipetting at least 8 times and incubate at room temperature for 5 minutes.
33. Separate on a magnet for 5min. Transfer the clear supernatant (which contains fragments under 500bp) to a fresh tube, avoiding any beads.

34. Add exactly 40µl of fresh AMPure XP beads to the solution. Mix by pipetting and incubate at room temperature for 5 minutes.
35. Separate on a magnet and keep the beads. Fragments in the range of 300-500bp will be retained on the beads. Discard the supernatant containing degraded RNA and short DNA fragments.
36. Keeping the beads on the magnet, wash twice with 700µl of freshly made 70% ethanol without mixing.
37. Spin for 10 seconds at 1000rpm, replace on the magnet, and remove the remaining ethanol. Leave the beads on the magnet for 3 minutes to allow residual ethanol to evaporate.
38. 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.
39. Quantify DNA by Qubit dsDNA High Sensitivity Assay (Life Technologies, Q32854) and run 4ul on an agarose gel to verify successful size selection.

Biotin Pull-Down and Library Preparation

40. Resuspend Dynabeads MyOne Streptavidin T1 beads by vortexting (Life technologies, 65602) Wash 150µl of beads 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 for 1 min and discard the solution. Wash the beads twice more in 150ul of 1X Tween Washing Buffer.
41. 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.
42. Separate on a magnet for 2-3 min and remove the solution. To calculate how much DNA was pulled down by the beads, measure the concentration in the solution by Qubit.
43. 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. Discard supernatant.
44. Repeat wash.
45. Resuspend beads in 100ul 1X End-Repair buffer and transfer to a new tube. Reclaim beads and discard the buffer.
46. Resuspend beads in 100ul End Repair mix:
    10ul 10X End-Repair buffer
    10ul 2.4mM dNTP mix
    10ul 10mM ATP
    2ul End-Repair enzyme mix
    68ul water
47. Incubate at room temperature for 45 minutes with gentle rotation.
48. 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. Discard supernatant.
49. Repeat wash.

50. Resuspend beads in 100µl 1X NEBuffer 2 and transfer to a new tube. Reclaim beads and discard the buffer.
51. Resuspend beads in 100µl of dATP attachment master mix:
    10µl 10X NEBuffer 2
    50µl 1mM dATP
    5µl 5U/µl NEB Klenow exo minus (NEB, M0212)
    35ul water
52. Incubate at 37°C for 30 minutes with gentle rotation. Separate on a magnet and discard the solution.
53. 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. Discard supernatant.
54. Repeat wash.
55. Resuspend beads in 100µl 1X Quick ligation reaction buffer (NEB, B6058) and transfer to a new tube.
56. Reclaim beads and discard the buffer.
57. Resuspend in 50ul of ligation master mix and record the sample-index combination:
    25ul 2x Quick ligase ligation buffer
    2ul NEXTflex DNA Barcode (Bioo Scientific, 514101)
    2ul quick ligase
    21ul water
58. Incubate at room temperature for 15 minutes. Separate on a magnet and discard the solution.
59. 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.
60. Repeat wash.
61. Resuspend beads in 100µl 1X Tris buffer and transfer to a new tube. Reclaim beads and discard the buffer.
62. Resuspend in 50µl of 1X Tris buffer.

<u>Amplification and Purification</u>

63. Do a test PCR and run a gel to determine how many cycles of amplification are needed. I test 8, 6, and 4 cycles by running 1/8, 1/32, and 1/128 of the sample for 11 cycles.
    Hi-C library, amplified directly off the beads
    10ul 5X Phusion HF Buffer
    0.4ul 10mM dNTPs
    2ul NEXTflex Primer Mix (12.5uM)
    0.5ul Phusion DNA Polymerase
    Water to 50ul

    Cycle:
    98° for 30sec

[98° for 10sec, 65° for 30sec, 72° for 30sec] x cycle number
72° for 5min
Hold at 12°
Aim for a final library concentration around 6ng/ul, which should be faintly visible on the gel.

64. Amplify the library using the determined number of cycles (I have found 5-6 to be sufficient). To avoid any problems with beads inhibiting the PCR, I divide the beads between seven 50ul PCRs.
65. After amplification, bring the total library volume to 350ul.
66. Separate on a magnet. Transfer the solution to a fresh tube and discard the beads.
67. Warm a bottle of AMPure XP beads to room temperature. Gently shake to resuspend the magnetic beads. Add 245µl of beads to the PCR reaction (0.7X volumes). Mix by pipetting and incubate at room temperature for 5 minutes.
68. Separate on a magnet and remove the clear solution.
69. Keeping the beads on the magnet, wash once with freshly made 700µl of 70% ethanol without mixing.
70. 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.
71. Separate on a magnet and remove the clear solution.
72. Keeping the beads on the magnet, wash twice with 700µl of freshly made 70% ethanol without mixing.
73. Leave the beads on the magnet for 5 minutes to allow the remaining ethanol to evaporate.
74. Add 25-50µl 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.
75. To check the library, clone using the Zero Blunt TOPO PCR Cloning Kit. Mix:
    1ul amplified library
    1ul salt solution
    3ul water
    1ul pCR-II-Blunt-TOPO vector

    Incubate at room temperature for 5 minutes. Transform 2ul into competent cells and plate on kanamycin plates.
76. Colony PCR using the following primers with annealing temperature 52° for Taq.
    EA235: CCA GTG AAT TGT AAT ACG ACT CAC TAT AGG G
    EA236: CGC CAA GCT ATT TAG GTG ACA CTA TAG
    For more robust colony PCR, first pick individual colonies to 20ul of water using a pipette tip. Incubate at 95° for 15 min. Use 1ul of this bacterial lysis in the PCR.
77. Submit 10ul of the final library for bioanalyzer and 100bp paired end sequencing on the HiSeq4000.

<u>Sources</u>

Crosslinking and nuclei isolation:

Crane, E., Bian, Q., McCord, R.P., Lajoie, B.R., Wheeler, B.S., Ralston, E.J., Uzawa, S., Dekker, J., and Meyer, B.J. (2015). Condensin-driven remodelling of X chromosome topology during dosage compensation. Nature.

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Rao, S.S.P., Huntley, M.H., Durand, N.C., Stamenova, E.K., Bochkov, I.D., Robinson, J.T., Sanborn, A.L., Machol, I., Omer, A.D., Lander, E.S., et al. (2014). A 3D Map of the Human Genome at Kilobase Resolution Reveals Principles of Chromatin Looping. Cell *159*, 1665–1680.

Library preparation from Meyer Lab ChIP-seq protocol