

in situ Hi-C Experimental Standards of the ENCODE Consortium

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 300xG for 5 minutes.
- 2) Resuspend cells in fresh medium at a concentration of 1×10^6 cells per 1ml media. In a fume hood, add freshly made formaldehyde solution 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 300xG at 4°C. Discard supernatant into an appropriate collection container.
- 5) Resuspend cells in 1ml of cold 1X PBS and spin for 5 minutes at 300xG at 4°C. Discard supernatant and flash-freeze cell pellets in liquid nitrogen or dry ice/ ethanol.
- 6) Either proceed to the rest of the protocol or store cell pellets at -80°C.

Lysis and Restriction Digest

- 7) Combine 250µl of ice-cold Hi-C lysis buffer (10mM Tris-HCl pH8.0, 10mM NaCl, 0.2% Igepal CA630) with 50µl of protease inhibitors (Sigma, P8340). 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 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 NEBuffer 2 and 100U of MboI restriction enzyme (NEB, R0147) or 25µl of 10X NEBuffer 3.1 and 100U of DpnII enzyme (NEB, R0543) 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 45 minutes 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) Pellet nuclei by centrifuge at 2500xG for 5 minutes. Discard the supernatant.
- 19) Resuspend pellet in 300µl of 10mM Tris-HCl, pH8; 0.5M NaCl; 1% SDS solution. Add 50µl of 20mg/ml proteinase K (NEB, P8102) and incubate at 55°C for 30 minutes.
- 20) Increase temperature to 68°C and incubate overnight to reverse cross-links.

DNA Shearing and Size Selection

- 21) Cool tube at room temperature.
- 22) Add 2X volumes of pure ethanol, mix by inverting and incubate at -80°C for 15 minutes.
- 23) Centrifuge at max speed, 2°C for 15 minutes. Keep the tubes on ice after spinning and carefully remove the supernatant by pipetting.
- 24) Resuspend in 800µl of 70% ethanol. Centrifuge at max speed for 5 minutes.
- 25) Remove all supernatant and wash the pellet once more with 800µl of 70% ethanol.
- 26) 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.
- 27) To make the biotinylated DNA suitable for high-throughput sequencing using Illumina sequencers, shear to a size of 300-500bp using the following parameters:
 - Instrument: Covaris LE220 (Covaris, Woburn, MA)
 - Volume of Library: 130µl in a Covaris microTUBE
 - Fill Level: 10

Duty Cycle: 15
 PIP: 500
 Cycles/Burst: 200
 Time: 58 seconds

- 28) 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 a 1:5 dilution of 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.
- 29) Warm a bottle of AMPure XP beads (Beckman Coulter, A63881) to room temperature. To increase yield, AMPure XP beads can be concentrated by removing some of the clear solution before the beads are mixed for use in the next steps.
- 30) Add exactly 110µl (0.55X volumes) of beads to the reaction. Mix well by pipetting and incubate at room temperature for 5 minutes.
- 31) Separate on a magnet. Transfer the clear solution to a fresh tube, avoiding any beads. The supernatant will contain fragments shorter than 500bp.
- 32) Add exactly 30µl of fresh AMPure XP beads to the solution. Mix by pipetting and incubate at room temperature for 5 minutes.
- 33) 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.
- 34) Keeping the beads on the magnet, wash twice with 700µl of 70% ethanol without mixing.
- 35) Leave the beads on the magnet for 5 minutes to allow remaining ethanol to evaporate.
- 36) 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.
- 37) 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

Perform all the following steps in low-bind tubes.

- 38) Prepare for biotin pull-down by washing 150µl 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.
- 39) 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.
- 40) Separate on a magnet and discard the solution.
- 41) 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.
- 42) Repeat wash.
- 43) Resuspend beads in 100µl 1X NEB T4 DNA ligase buffer (NEB, B0202) and transfer to a new tube. Reclaim beads and discard the buffer.
- 44) 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 with 10mM ATP
 - 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)
- 45) Incubate at room temperature for 30 minutes. Separate on a magnet and discard the solution.
- 46) 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.
- 47) Repeat wash.
- 48) Resuspend beads in 100µl 1X NEBuffer 2 and transfer to a new tube. Reclaim beads and discard the buffer.
- 49) 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)
- 50) Incubate at 37°C for 30 minutes. Separate on a magnet and discard the solution.
- 51) 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.
- 52) Repeat wash.
- 53) Resuspend beads in 100µl 1X Quick ligation reaction buffer (NEB, B6058) and transfer to a new tube. Reclaim beads and discard the buffer.
- 54) Resuspend in 50µl of 1X NEB Quick ligation reaction buffer.

- 55) Add 2µl of NEB DNA Quick ligase (NEB, M2200). Add 3µl of an Illumina indexed adapter. Record the sample-index combination. Mix thoroughly.
- 56) Incubate at room temperature for 15 minutes. Separate on a magnet and discard the solution.
- 57) 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.
- 58) Repeat wash.
- 59) Resuspend beads in 100µl 1X Tris buffer and transfer to a new tube. Reclaim beads and discard the buffer.
- 60) Resuspend in 50µl of 1X Tris buffer.

Final Amplification and Purification

- 61) Amplify the Hi-C library directly off of the T1 beads with 4-12 cycles of PCR, using Illumina primers and protocol (Illumina, 2007). (Note that recent lots of some streptavidin beads may interfere with PCR; to avoid this, one can remove the DNA from the streptavidin beads by heating at 98C for 10 minutes after step 59 and then removing the beads with a magnet.)
- 62) After amplification is complete, bring the total library volume to 250µl.
- 63) Separate on a magnet. Transfer the solution to a fresh tube and discard the beads.
- 64) 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.
- 65) Separate on a magnet and remove the clear solution.
- 66) Keeping the beads on the magnet, wash once with 700µl of 70% ethanol without mixing.
- 67) 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.
- 68) Separate on a magnet and remove the clear solution.
- 69) Keeping the beads on the magnet, wash twice with 700µl of 70% ethanol without mixing.
- 70) Leave the beads on the magnet for 5 minutes to allow the remaining ethanol to evaporate.
- 71) 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 using an Illumina sequencing platform.