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in situ Hi-C

Florian Noack[1], Jeisimhan Diwakar[1], Boyan Bonev[1]

[1]Helmholtz Pioneer Campus

Boyan Bonev
Helmholtz Pioneer Campus

Protocol was adapted from the HiC 2.0 protocol (Belaghzal et al.; 2018).

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Required Kits

NextFlex index adapters (PerkinElmer, Cat. N.: NOVA-514101)
Qubit dsDNA High Sensitivity Assay (ThermoFisher, Cat. N.: Q32851)

Buffers and Mastermixes

2% Formaldehyde solution

Dilute 1ml of 16% Formaldehyde solution (ThermoFisher, Cat. N.: 28908) with 7ml PBS.

2M Glycine solution

Mix 30.024g of Ultrapure Glycine (Invitrogen, Cat. N.: 15527013) with 200ml of PBS.

Lysis buffer (1ml)

10mM Tris-HCl, pH 8.0 (ThermoFisher, Cat. N.: 15567027)
10mM NaCl (ThermoFisher, Cat. N.: AM9760G)
0.2% Igepal-CA630 (Sigma-Aldrich, Cat. N.: I3021)
1x cOmplete™, EDTA-free Protease Inhibitor Cocktail (Roche, Cat. N.: 11873580001)
0.05% RNAsin plus RNase inhibitor (Promega, Cat. N.: N261A)

For 1ml

- 10µl Tris-HCl, pH 8.0 (1M)

- 20µl Igepal-CA630 (10% stock solution, freshly prepared)
- 2µl NaCl (5M)
- 20µl cOmplete™, EDTA-free Protease Inhibitor Cocktail (50x stock)
- 0.5µl RNAsin plus RNase inhibitor
- 947.5 µl nuclease free water

Wash buffer (1ml)
10mM Tris-HCl, pH 8.0 (ThermoFisher, Cat. N.: 15567027)
10mM NaCl (ThermoFisher, Cat. N.: AM9760G)
0.05% RNAsin plus RNase inhibitor (Promega, Cat. N.: N261A)
0.5% Bovine Serum Albumin (ThermoFisher, Cat. N.: AM2618)

For 1ml
- 10µl Tris-HCl, pH 8.0 (1M)
- 2µl NaCl (5M)
- 0.5µl RNAsin plus RNase inhibitor
- 100µl Bovine Serum Albumin (5% stock solution)
- 887.5 µl nuclease free water

Biotin fill-in master mix (20µl)
- 2µl DpnII Buffer (New England Biolabs, Cat. N.: R0543)
- 12.5µl 0.4mM biotin-14-dATP (Life Technologies, Cat. N.: 195245016)
- 0.5µl 10mM dCTP (Promega, Cat. N.: U1330)
- 0.5µl 10mM dGTP (Promega, Cat. N.: U1330)
- 0.5µl 10mM dTTP (Promega, Cat. N.: U1330)
- 3.33µl 5U/µl DNA Polymerase I, Large(Klenow) Fragment(New England Biolabs, Cat. N.: M0210)
- 0.67µl nuclease free water

Ligation master mix (360µl)
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- 48µl 10X NEB T4 DNA ligase buffer (New England Biolabs,Cat. N.: B0202)
- 40µl 10% Triton X-100 (Sigma Aldrich, Cat. N.: X100)
- 1µl 5% Bovine Serum Albumin (ThermoFisher, Cat. N.: AM2618)
- 2.5µl 400 U/ µl T4 DNA Ligase (New England Biolabs,Cat. N.: M0202)
- 268.5µl nuclease free water

Tween washing buffer (10ml)
5mM Tris-HCl, pH7.5 (ThermoFisher, Cat. N.: 15567027)
0.5mM EDTA (Invitrogen,Cat. N.: AM9260G)
1M NaCl (ThermoFisher, Cat. N.: AM9760G)
0.05% Tween-20 (Sigma-Aldrich, Cat. N.: P9416)

For 10ml
- 50µl Tris-Hcl pH7.5 (1M)
- 10µl EDTA (0.5M)
- 2ml NaCl (5M)
- 50µl 10% Tween-20 (10% stock solution, freshly prepared)
- 7890 µl nuclease free water

2x Binding buffer (1ml)
10mM Tris-HCl, pH 7.5 (ThermoFisher, Cat. N.: 15567027)
1mM EDTA (Invitrogen, Cat. N.: AM9260G)
2M NaCl (ThermoFisher, Cat. N.: AM9760G)

For 1ml
- 10µl Tris-HCl, pH 7.5 (1M)
- 2µl EDTA (0.5M)
- 400µl NaCl (5M)
- 588 µl nuclease free water

Biotin Removal Master Mix (100µl):
- 10µl of 10X NEB T4 DNA ligase buffer (New England Biolabs, Cat. N.: B0202)
- 2µl of 25mM dNTP mix (New England Biolabs, Cat. N.: N0447L)
- 5µl of 10U/ul T4 PNK (New England Biolabs, Cat. N.: M0201)
- 4µl of 3U/ul T4 DNA polymerase I (New England Biolabs, Cat. N.: M0203)
- 1µl of 5U/ul DNA polymerase I, Large (Klenow) Fragment (New England Biolabs, Cat. N.: M0210)
- 78µl nuclease free water

dATP Attachment Master Mix (100µl):
90µl of 1x NEBuffer 2 (New England Biolabs, Cat. N.: B7002S)
5µl of 10mM dATP (Promega, Cat. N.: U1330)
5µl of 5U/ul NEB Klenow exo minus (New England Biolabs, Cat. N.: M0212)

Cell fixation 30m

1 Resuspend dissociated cells in PBS to reach a maximal cell concentration of $2x10^6$ cells/ml and add freshly prepared 2% Formaldehyde solution to reach a final concentration of 1%. Incubate for 10 minutes at RT with slow rotation.

2 Add 2.0M glycine solution (Invitrogen, Cat. N.: 15527013) to a final concentration of 0.2M to quench the reaction. Incubate at room temperature for 5 minutes with slow rotation.

3 Centrifuge for 5 minutes at 500xg at 4°C. Discard supernatant into an appropriate collection container.

4 Resuspend cells in 1ml of cold 1x PBS with 0.5% BSA (ThermoFisher, Cat. N.: AM2618) and count the cell number.

Fixed cells can be either stained for ImmunoFACS, directly used for in-situ HiC or pelleted (2500xg for 5 minutes at 4˚C) and then snap frozen in liquid nitrogen for storage at -80˚C.

in situ Hi-C: Cell Lysis 15m

5 **NOTE: this protocol was optimized for ~3x10^4 cells.**

Pellet fixed cells for 5 minutes at 2500xg (4˚C) and carefully resuspend them with 100µl of ice-cold Lysis Buffer.

If cells were frozen, thaw the cell pellet first on ice.

6 Incubate on ice for exactly 10 minutes and centrifuge at 2500xg for 5 minutes at 4°C. and remove the supernatant.

7 Wash nuclei once with 190µl wash buffer. Centrifuge at 2500xg for 5 minutes and remove as much of the supernatant as possible without disturbing the nuclei pellet.

in situ Hi-C: DpnII Digestion and biotin fill-in

8 Gently resuspend nuclei pellet in 20µl of 0.5% SDS (Invitrogen, Cat. N.: AM9823) and incubate at 62°C for 10 minutes. Afterwards place on room temperature.

9 Add first 56µl of water and then 10µl of freshly made 10% Triton X-100 solution (Sigma Aldrich, Cat. N.: X100) to quench the SDS. Mix well by carefully pipetting up-down (avoiding excessive foaming). Incubate at 37°C for 15 minutes on a thermomixer with a rotation speed of 600 rpm.

10 Add 10µl of 10X DpnII buffer (New England Biolabs, Cat. N.: R0543) and 4µl (400U) of DpnII restriction enzyme (New England Biolabs, Cat. N.: R0543) and digest chromatin overnight at 37°C with a rotation speed of 600 rpm.

11 Next day incubate samples at 62°C for 20 minutes, then cool the samples to room temperature.

12 To fill in the restriction overhangs and mark the DNA ends with biotin, add 20µl of Biotin fill-in master mix . Mix by pipetting carefully and incubate for 4 hours at 23°C (900 rpm mixing; 10 sec every 5 min).

in situ Hi-C: Proximity ligation and decrosslinking

13 Add 360µl of the Ligation master mix. Mix by inverting and incubate at least for 6h at 16°C ^{6h} (900 rpm mixing, 10 sec every 30 min). Alternativly the ligation reaction can be also performed overnight.

14 Degrade proteins by adding 5µl of 20mg/ml Proteinase K (New England Biolabs, Cat. N.:

P8107) and 48µl of 10% SDS (Invitrogen, Cat. N.: AM9823) and incubate at 55°C for 30 minutes.

15 Add 53µl of 5M NaCl (ThermoFisher, Cat. N.: AM9760G) and incubate at 68°C overnight (900 rpm 10sec, every 5 min.).

16 Cool tubes at room temperature, add 1:1 volume Phenol/Chloroform/Isoamylalcohol (Invitrogen, Cat. N.: 15593031), vortex for at least 30 seconds and spin with maximal speed for 10 minutes at 4˚C.

17 Take the upper aqueous phase into a 2ml tube and resuspend with 2x volumes of ice cold 100% ethanol, 1/10th of the volume 3M NaAc (Ambion, Cat. N.: AM9740) and 1µl Glycogen (ThermoFisher, Cat. N.: 10814010). Store the mix at -20˚C at least for 1 hour or -80˚C for 15 minutes.

18 Centrifuge at maximal speed for 20 minutes at 4°C. Carefully remove the supernatant by pipetting.

19 Wash with 800µl freshly prepared 70% cold ethanol followed by centrifuge at maximal speed for 5 minutes at 4°C.

20 Wash with 400µl freshly prepared 70% cold ethanol and transfer everything to a 0.5 ml tube (make sure the glycogen pellet is transfered). Centrifuge at maximal speed for 5 minutes at 4°C.

21 Remove as much as possible of the ethanol and air-dry the pellet. Dissolve pellet in 17µl of 10mM Tris-buffer (pH 7.5) (ThermoFisher, Cat. N.: 15567027) and incubate at 37°C for 15 minutes to fully dissolve DNA.

in situ Hi-C: DNA shearing (Covaris)

22 Transfer 17µl of the sample into a Covaris microTUBE-15 AFA Beads Screw-Cap tube (Covaris, Cat. N.: 520145) and shear DNA to a target size of 550bp using following parameter on a Covaris S220 sonicator:

- Peak Incident Power (W) 18
- Duty Factor 20%
- Cycles per Burst 50
- Treatment Time (s) 22

23 Quantify the sheared DNA using the Qubit dsDNA High Sensitivity Assay.

in situ Hi-C: Biotin pulldown, on-bead Biotin removal and End-repair

24 Prepare streptavidin beads by washing 100µl of 10mg/ml MyOne Streptavidin T1 beads (ThermoFisher, Cat. N.: 65602) with 400µl 1x Tween washing buffer. Afterwards resuspend beads in 200µl of 2x Binding buffer.

25 Resuspend 15µl of the sheared sample DNA with 185µl nuclease free water and 200µl of the washed MyOne Streptavidin T1 beads in binding buffer. Incubate at room temperature for 15 - 30 minutes with rotation to bind biotinylated DNA to the streptavidin beads.

26 Separate on a magnet and discard the solution. Wash the beads twice by adding 600µl of tween washing buffer, incubating for 2 minutes at 55˚C with mixing (1000 RPM) followed by reclaim the beads using a magnet and discarding the supernatant.

27 Wash the beads once with 100µl **1x (!!!)** NEB T4 DNA ligase buffer (New England Biolabs, Cat. N.: B0202S).

28 To repair ends of the sheared DNA and to remove the biotin from unligated ends, resuspend beads in 100µl of Biotin Removal Master Mix and incubate for 30 minutes at room temperature.

29 Separate on a magnet and discard the solution. Wash the beads twice by adding 600µl of tween washing buffer, incubating for 2 minutes at 55˚C with mixing (1000 RPM) followed by reclaim the beads using a magnet and discarding the supernatant.

30 Wash the beads once with 100µl **1x (!!!)** NEB2 Buffer (New England Biolabs, Cat. N.: B7002S).

31 Resuspend beads in 100µl of dATP attachment master mix and incubate for 30 minutes at 37°C.

32 Separate on a magnet and discard the solution. Wash the beads twice by adding 600µl of tween washing buffer, incubating for 2 minutes at 55˚C with mixing (1000 RPM) followed by reclaim the beads using a magnet and discarding the supernatant.

in situ Hi-C: adapter ligation and library amplification

33 Wash the beads once with 100µl **1x (!!!)** Quick ligation reaction buffer (New England Biolabs, Cat. N.: B6058).

34 Resuspend in 50µl of **1x (!!!)** Quick ligation reaction buffer (New England Biolabs, Cat. N.: B6058), add 2µl of sample specific NextFlex index adapters (PerkinElmer, Cat. N.: NOVA-514101) and 2µl of NEB DNA Quick ligase (New England Biolabs, Cat. N.: M2200). Mix thoroughly and incubate for 15 minutes at room temperature.

35 Separate on a magnet and discard the solution. Wash the beads twice by adding 600µl of tween washing buffer, incubating for 2 minutes at 55˚C with mixing (1000 RPM) followed by reclaim the beads using a magnet and discarding the supernatant.

36 Wash the beads once with 100µl 10mM Tris -uffer pH 7.5 (ThermoFisher, Cat. N.: 15567027).

37 Resuspend beads in 42µl 10mM Tris-buffer pH 7.5 (ThermoFisher, Cat. N.: 15567027) to reach a total volume of ~45µl.

38 Amplification of the HiC library is performed directly on the beads.

Mix 45µl of the end-repaired DNA with 5µl of NEXTFlex Primer mix (PerkinElmer, Cat. N.: NOVA-514101) and 50µl NEBNext Ultra Q5 II Master Mix (New England Biolabs, Cat. N.: M0544S) to reach a total volume of 100µl. Split the reaction into 5 PCR tubes of 20µl, in order to increase the complexity of the library.

PCR is performed using following program: 98˚C 30s; {98˚C 10s, 65˚C 75s} x8-10; 65˚C 5 min.; Hold at 10˚C

39 After amplification, beads are separated using a magentic rack and the supernatant containing the final library is pooled together for each sample (volume ~100µl).

40 Final library is purified by adding 70µl of AmpureXP beads (0.7X, Agencourt, Cat. N.: A63881) to the samples followed by a incubation of 10 minutes at room temperature under slow rotation.

41 Pellet the beads on a magnatic-rack, remove the supernatant and wash the beads twice with freshly prepared 80% Ethanol.

42 After the last wash remove as much as possible of the 80% Ethanol and air-dry the beads for approximately 5 minutes at room temperature.

43 Resuspend the beads with 12µl nuclease-free water, incubate for 10 minutes at room temperature, pellet the beads on a magnetic-rack and transfer the DNA containing supernatant into a new tube. Quantify the yield using the Qubit dsDNA High Sensitivity Assay (ThermoFisher, Cat. N.. Q32851).