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MethylHiC

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protocol .

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Protocol was adapted from current protocols (Lee et al.; 2019; Li et al.; 2019) with major modifications mainly at the library preparation step.

Florian Noack, Boyan Bonev 2021. MethylHiC . **protocols.io**
https://protocols.io/view/methylhic-bif2kbqe

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Jul 28, 2020 Boyan Bonev Helmholtz Pioneer Campus

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Required Kits

Accel-NGS Methyl-Seq DNA Library Kit (Swift Bioscience, Cat. N.: 30024)
Methyl-Seq Set A Indexing Kit (12 indices, 24 rxns) (Swift Bioscience, Cat. N.: 36024)
EZ DNA Methylation-Gold Kit (Zymo Research, Cat. N.: D5005)
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.

MethylHiC 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

MethylHiC 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% Bovin 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 5% Bovin Serum Albumin
- 887.5 µl nuclease free water

Biotin fill-in master mix (40µl)

- 4µl DpnII Buffer (New England Biolabs, Cat. N.: R0543)
- 25µl 0.4mM biotin-14-dATP (Life Technologies, Cat. N.: 195245016)
- 1µl 10mM dCTP (Promega, Cat. N.: U1330)
- 1µl 10mM dGTP (Promega, Cat. N.: U1330)
- 1µl 10mM dTTP (Promega, Cat. N.: U1330)
- 6.66µl 5U/µl DNA Polymerase I, Large(Klenow) Fragment(New England Biolabs, Cat. N.: M0210)
- 1.34µl nuclease free water

Ligation master mix (720µl)

- 96µl 10X NEB T4 DNA ligase buffer (New England Biolabs, Cat. N.: B0202)
- 80µl 10% Triton X-100 (Sigma Aldrich, Cat. N.: X100)
- 2µl 5% Bovin Serum Albumin (ThermoFisher, Cat. N.: AM2618)
- 5µl 400 U/ µl T4 DNA Ligase (New England Biolabs, Cat. N.: M0202)
- 537µl 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

Endrepair master mix (5µl)
- 2µl 10x NEBuffer 2.1(New England Biolabs, Cat. N.: B7202S)
- 2µl 3,000 U/ml T4 DNA polymerase (New England Biolabs, Cat. N.: M0203S)
- 0.5µl 1mM dATP (Promega, Cat. N.: U1330)
- 0.5µl 1mM dGTP (Promega, Cat. N.: U1330)

Library amplification mix (30µl)
- 5µl indexing primers of the Methyl-Seq Set A Indexing Kit (Swift Bioscience, Cat. N.: 36024)
- 10µl 5x EpiMark Hot Start Taq Reaction Buffer (New England Biolabs, Cat. N.: M0490)
- 1µl 10mM dNTPs (New England Biolabs, Cat. N.: N0447L)
- 0.25µl EpiMark Hot Start Taq (New England Biolabs, Cat. N.: M0490)
- 13.75µl of nuclease free water

Prepare bisulfite conversion control

1 **NOTE: Control DNA has to prepared only once and can be reused.**

To prepare methylation controls, mix 8µl of CpG methylated pUC19 DNA (Zymo Research, Cat. N.: D5017) with 8µl of unmethylated lambda DNA (Promega, Cat. N.: D1521) in Covaris microTUBE-15 AFA Beads Screw-Cap tubes (Covaris, Cat. N.: 520145).

2 Shear DNA to a targeted 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

3 Perform biotin fill-in reaction by mixing:
15µl sheared DNA
75µl steril water

10µl 10x DpnII buffer (New England Biolabs, Cat. N.: R0543)
20µl Biotin fill-in master mix (see Materials)

Incubate at 23°C for 4 hours (900 RPM mixing; 10 sec. every 5 minutes).

4 Perform a 1x AmpureXP (Agencourt, Cat. N.: A63881) purification by adding 120 µl of room temperature AmpureXP beads to the samples followed by a incubation of 10 minutes at room temperature under slow rotation.

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

6 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.

7 Resuspend the beads with 50µl 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.

8 Quantify the yield using the Qubit dsDNA High Sensitivity Assay (ThermoFisher, Cat. N.: Q32851). Low DNA amounts expected. Control DNA can be stored at -20˚C.

Cell fixation 30m

9 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 room temperature with slow rotation.

10 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.

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

12 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 pelleted (2500xg for 5 minutes at 4˚C) and either snap frozen in liquid nitrogen for storage at -80˚C or directly used for MethylHiC.

MethylHiC: Cell Lysis 15m

13 **NOTE: this protocol was optimized for 2x10^5 cells.**

If cells were frozen, thaw the cell pellet on ice. Carefully resuspend cells with 200µl of ice-cold MethylHiC Lysis Buffer.

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

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

MethylHiC: DpnII Digestion and biotin fill-in

16 Gently resuspend nuclei pellet in 40µl of 0.5% SDS (Invitrogen, Cat. N.: AM9823) and incubate at 62°C for 10 minutes. Afterwards place at room temperature.

17 Add first 112µl of water and then 20µl 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.

18 Add 20µl of 10X DpnII buffer (New England Biolabs, Cat. N.: R0543) and 8µl (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.

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

20 To fill in the restriction overhangs and mark the DNA ends with biotin, add 40µl 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 minutes).

MethylHiC: Proximity ligation and decrosslinking

21 Add 720µl of the Ligation master mix. Mix by inverting and incubate at least for 6 hours at 16°C (900 rpm mixing, 10 sec. every 30 min.). Alternatively, the ligation reaction can also be performed overnight. 6h

22 Degrade proteins by adding 10µl of 20mg/ml Proteinase K (New England Biolabs, Cat. N.:

P8107) and 96µl of 10% SDS (Invitrogen, Cat. N.: AM9823) and incubate at 55°C for 30 minutes.

23 Add 106µl of 5M NaCl (ThermoFisher, Cat. N.: AM9760G) and incubate at 68°C overnight (900 rpm, 10 sec., every 5 min.).

24 Cool tubes at room temperature. Split reaction into 2x 2ml tubes and add 1:1 volume Phenol/Chloroform/Isoamylalcohol (Invitrogen, Cat. N.: 15593031), vortex for at least 30seconds and spin with maximal speed for 10 minutes at 4˚C.

25 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.

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

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

28 Wash each tube with 200µl freshly prepared 70% cold Ethanol and transfer the total 400µl to a 0.5ml tube (make sure glycogen pellet is transferred). Centrifuge at maximal speed for 5 minutes at 4°C.

29 Remove as much as possible of the ethanol and airdry the pellet. Dissolve pellet in 16ul of 10mM Tris buffer pH 7.5 (ThermoFisher, Cat. N.: 15567027) and incubate at 37°C for 15 minutes to fully dissolve DNA.

MethylHiC: DNA shearing (Covaris)

30 Transfer 16µl 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

31 Quantify the yield using the Qubit dsDNA High Sensitivity Assay.

MethylHiC: Biotin removal

32 Mix 15µl of the sheared sample with 5µl of the Endrepair master mix.

33 Incubate for 4 hours at 20˚C followed by a heat inactivation for 20 minutes at 75˚C. Sample can be stored at -20˚C.

MethylHiC: Bisulfite Conversion

34 **NOTE: for the highest yield the libraries should be prepared directly after the bisulfite conversion.**

Prior the bisulfite conversion add roughly 0.05% sheared-biotinylated control DNA to the sample. Proceed with the bisulfite conversion using the EZ DNA Methylation-Gold Kit (Zymo Research) according to the manual instructions until the elution step.

35 Elute the bisulfite converted DNA in either 16µl (**<100ng DNA input**) or 31µl (**>100ng DNA input**) low EDTA TE water provided with the Accel-NGS® Methyl-Seq DNA Library Kit (Swift Bioscience, Cat. N.: 30024).

MethylHiC: Library construction and Biotin pulldown

36 For <100ng pre-bisulfite DNA input use 15µl of bisulfite converted DNA for the Accel-NGS® Methyl-Seq DNA Library Kit according to the manual instruction **until the ligation step (step 16: 15 minutes at 25˚C).**

For >100ng pre-bisulfite DNA input use 30µl of bisulfite converted DNA for the Accel-NGS® Methyl-Seq DNA Library Kit with the **double reaction volume** according to the manual instruction **until the ligation step (step 16: 15 minutes at 25˚C).**

37 Per sample prepare 25µl Dynabeads MyOne Streptavidin T1 beads (ThermoFisher, Cat. N.: 65602) by washing them with 400µl 1x Tween washing buffer and resuspend the beads in 60µl of 2x Binding buffer.

38 Add nuclease free water to the ligation reaction to reach a final volume of 60µl and subsequently add 60µl of the washed MyOne Streptavidin T1 beads in 2x Binding buffer. Incubate for 15 minutes at room temperature under slow rotation.

39 Wash beads 4 times with 400µl 1x Tween washing buffer for 2 minutes at 55˚C.

40 After the last wash remove as much as possible from the Tween washing buffer and wash beads twice with 400µl nuclease free water. Thereafter resuspend beads in 20µl nuclease free water.

41 Mix 20µl of the streptavidin bound sample with 30µl Library amplification mix. Amplify library using the following PCR program: 95˚C 30s; {95˚C 15s, 61˚C 30s, 68 ˚C 60s}x8-14; 68˚C 5min; Hold at 10˚C.

42 After final library amplification separate the streptavidin beads using a magnetic rack and transfer the supernatant into a new tube. Perform a 0.6x AmpureXP (Agencourt, Cat. N.: A63881) purification by mix 30µl of room temperature AmpureXP beads to the samples followed by an incubation of 10 minutes at room temperature under slow rotation.

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

44 After the last wash remove as much as possible of the 80% Ethanol and airdry the beads for approximatly 5 minutes at room temperature.

45 Resuspend the beads with 10µl 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.

46 Quantify the yield using the Qubit dsDNA High Sensitivity Assay (ThermoFisher, Cat. N.: Q32851).