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NOMe-seq of fixed cells

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protocol .

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Protocol is based on the NOMe-seq protocol (Kelly et al.; 2012) with major modifications in the incubation times to work with fixed cells (Nordström et al.; 2019) and at the library preparation step.

Florian Noack, Boyan Bonev 2021. NOMe-seq of fixed cells. **protocols.io** https://protocols.io/view/nome-seq-of-fixed-cells-brdwm27e

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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

GpC methylation mix I (for control DNA)

- 5µl 10x GpC buffer (New England Biolabs, Cat. N.: M0227S)
- 1µl 32mM SAM (New England Biolabs, Cat. N.: M0227S)
- 5µl M.CviPI (New England Biolabs, Cat. N.: M0227S)
- 19µl nuclease free water

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

GpC wash buffer (250µl)
- 25µl GpC buffer (New England Biolabs, Cat. N.: M0227S)
- 50µl 5% Bovine Serum Albumin (ThermoFisher, Cat. N.: AM2618)
- 175µl nuclease free water

GpC reaction buffer (50µl)
- 7.5µl M.CviPI (New England Biolabs, Cat. N.: M0227S)
- 5µl 10x GpC buffer (New England Biolabs, Cat. N.: M0227S)
- 1µl 32mM SAM (New England Biolabs, Cat. N.: M0227S)
- 36.5µl nuclease free water

Library amplification mix (75µl)
- 2.5µl indexing primers of the Methyl-Seq Set A Indexing Kit (Swift Bioscience, Cat. N.: 36024)
- 25µl 5x EpiMark Hot Start Taq Reaction Buffer (New England Biolabs, Cat. N.: M0490)
- 2.5µl 10mM dNTPs (New England Biolabs, Cat. N.: N0447L)
- 0.65µl EpiMark Hot Start Taq (New England Biolabs, Cat. N.: M0490)
- 44.35µl nuclease free water

Prepare control DNA

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

To prepare GpC methylated control DNA, mix 10µl of CpG methylated pUC19 DNA (Zymo Research, Cat. N.: D5017) with 10µl of unmethylated lambda DNA (Promega, Cat. N.: D1521).

2 Perform GpC methylation by mixing 20µl of the pUC19/lambda DNA mix with 30µl of the GpC methylation mix I and incubate for 1h @ 37 ˚C followed by 20 minutes at 65 ˚C.

3 Perform a 1x AmpureXP (Agencourt, Cat. N.: A63881) purification by adding 50 µl of room temperature AmpureXP beads to the samples followed by a incubation of 10 minutes at room temperature under slow rotation.

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

5 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.

6 Resuspend the beads with 16µl 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 Covaris microTUBE-15 AFA Beads Screw-Cap tubes (Covaris, Cat. N.: 520145).

7 Shear the 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

8 Perform another 1x AmpureXP purification as described above und elute the purified DNA in 30µl nuclease-free water.

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

Fixation of cells

10 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.

11 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.

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

13 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 FAC-sorted, directly used for NOME-seq or pelleted (2500xg for 5 minutes at 4˚C) and then snap frozen in liquid nitrogen for storage at -80˚C.

NOMe-seq: Cell Lysis and GpC methylation

14 **NOTE: this protocol was optimized for 1x10^4 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.

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

16 Wash the nuclei pellet by adding 250µl GpC wash buffer followed by a centrifugation at 2500xg for 5 minutes at 4°C and the removal of the supernatant.

17 Carefully resuspend nuclei with 50µl GpC reaction buffer.

18 Incubate for 3 hours at 37 ˚C with slight shaking (500 RPM) and add to the reaction every hour 0.5µl 32mM SAM (New England Biolabs, Cat. N.: M0227S) and 1µl of M.CviPI (New England Biolabs, Cat. N.: M0227S).

NOMe-seq: Decrosslinking and shearing

19 Add 478µl freshly prepared Proteinase-K digestion mix and incubate at 55°C for 30 minutes.

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

21 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.

22 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 10-15 minutes.

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

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

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

26 Remove as much as possible of the ethanol and air-dry 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.

27 Transfer 16µl of the sample into a Covaris microTUBE-15 AFA Beads Screw-Cap tube (Covaris, Cat. N.: 520145) and sheare 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

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

NOMe-seq: Bisulfite Conversion and Lib. preparation

29 **NOTE: for the highest yield, the libraries should be prepared directly after the bisulfite conversion and not more than 100ng of sheared DNA should be used.**

Prior the bisulfite conversion add roughly 0.05% M.CviP-treated 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.

30 Elute the bisulfite converted DNA in 16µl low EDTA TE water provided with the Accel-NGS® Methyl-Seq DNA Library Kit (Swift Bioscience, Cat. N.: 30024).

31 Use 15µl of the bisulfite converted DNA for the library preparation using the Accel-NGS® Methyl-Seq DNA Library Kit according to the manual instruction until the final amplification step. Elute the DNA after the last purification step in 50µl low EDTA TE water.

32 Final amplification is performed with the with the EpiMark Hot Start Taq (New England Biolabs, Cat. N.: M0490S) in 5 separate reactions in order to achieve a higher complexity. To do so, pipette 10µl of the purified and adapter ligated DNA in 5 separate PCR tubes and add 15µl of the Library amplification mix containing sample specific Methyl-Seq Set A Indexing Primers (Swift Bioscience, Cat. N.: 36024).

33 Amplify library using the following PCR program: 95˚C 30s; {95˚C 15s, 61˚C 30s, 68 ˚C 60s} x10-14; 68˚C 5min; Hold at 10˚C.

34 After the PCR is finished pool the different reactions of a sample together (~125µl total volume) and perform a 0.8x AmpureXP (Agencourt, Cat. N.: A63881) purification by adding 100µl of room temperature AmpureXP beads to the samples followed by a incubation of 10 minutes at room temperature under slow rotation.

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

36 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.

37 Resuspend the beads with 15µl 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)