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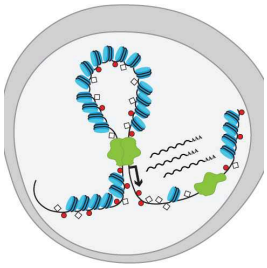
3DRAM-seq enables joint epigenome profiling of spatial genome organization, chromatin accessibility and DNA methylation at high resolution V.1



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We use this protocol and it's working

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Abstract

3DRAM-seq (3D genome, RNA, Accessibility and Methylation sequencing) is a novel multiomic method that simultaneously interrogates spatial genome organization, chromatin accessibility, DNA methylation and gene expression at high resolution. It utilized enzymatic treatment of bulk fixed nuclei with the GpC methyltransferase M.CviPI prior to restriction digestion, biotin fill-in and proximity ligation as in Hi-C. The resulting 3C DNA is then sheared to ~550bp, followed by bisulfite conversion and library preparation based on the Accel-NGS® Methyl-Seq DNA but using streptavidin beads after the adaptor ligation step to enrich for fragments containing informative contacts. 3DRAM-seq can be performed with as few as 200 000 cells and can be combined with immunoFACS to profile the epigenome landscape in a cell-type-specific manner.

Materials

Required Kits

Quick-RNA FFPE Miniprep (Zymo Research, Cat. N: R1008) with Zymo-Spin IC Columns (Zymo Research, Cat. N: C1004-250)
Accel-NGS Methyl-Seq DNA Library Kit (Swift Bioscience, Cat. N: 30024, now xGen Methyl-Seq DNA Library Prep IDT, Cat. N.: 10009860)
Methyl-Seq Set A Indexing Kit (12 indices, 24 rxns) (Swift Bioscience, Cat. N: 36024, now IDT, Cat. N. 10009965 or 10005975)
EZ DNA Methylation-Gold Kit (Zymo Research, Cat. N: D5005)
Qubit dsDNA High Sensitivity Assay (ThermoFisher, Cat. N. Q32851)
Qubit RNA HS Assay Kit (ThermoFisher, Cat. N.: Q32852)
Bioanalyzer High Sensitivity RNA 6000 Pico Kit (Agilent, Cat. N.: 5067-1513)

Buffers and Mastermixes

2% Formaldehyde solution

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

2M Glycin solution

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

GpC methylation mix I (30µl)

- 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

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 Bovine Serum Albumin (ThermoFisher, Cat. N: AM2618)
- 175µl nuclease free water

GpC reaction buffer (100µl)
- 15µl M.CviPI (New England Biolabs, Cat. N: M0227S)
- 10µl 10x GpC buffer (New England Biolabs, Cat. N: M0227S)
- 2µl 32mM SAM (New England Biolabs, Cat. N: M0227S)
- 73µl 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% 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 Bovin Serum Albumin (5% stock solution)
- 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 (NEB, 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, M0202)
- 537µl nuclease free water

Tween washing buffer (10ml)
5mM Tris-HCl, pH7.5 (ThermoFisher, Cat. N: 15567027)
0.5mM EDTA (Invitrogen, 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, 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 (75µl)
- 2.5µl indexing primers of the Methyl-Seq Set A Indexing Kit (Swift Bioscience, Cat. N: 36024; now IDT, Cat. N. 10009965 or 10005975)
- 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

Troubleshooting

3DRAM-seq: Methylation controls

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 min. at 65 degree.

3 Add 40ul (0.8x) of room temperature Ampure XP beads and mix well by pipetting up and down 10 times. Incubate at RT for 5min.

4 Separate on a magnet and remove the clear solution.

5 Keeping the beads on the magnet, wash twice with 200µl of 80% ethanol without mixing.

6 Leave the beads on the magnet for 5 minutes to allow the remaining ethanol to evaporate.

7 Resuspend the beads with 16µl nuclease-free water, incubate for 10 minutes at RT, 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).

8 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

9 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 min.).

10 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 RT under slow rotation.

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

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

13 Resuspend the beads with 50µl nuclease-free water, incubate for 10 minutes at RT, pellet the beads on a magnetic-rack and transfer the DNA containing supernatant into a new tube.

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

Cell fixation

30m

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

16 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 at rotating wheel.

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

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

19 Fixed cells are pelleted (2500xg for 5min. at 4˚C) and can be either used directly for 3DRAM-seq, ImmunoFAC-sorted or snap frozen in liquid nitrogen for storage at -80˚C.

Please note that snap freezing leads to degradation of RNA, therefore cells for RNA isolation should be directly processed after fixation or ImmunoFACS.

RNA-seq library construction

20 **NOTE: this protocol was optimized for ~2×10^4 cells.**

Pelleted cells are resuspended with 95µl nuclease free water and RNA is isolated using the Quick-RNA FFPE Miniprep (Zymo Research, Cat. N: R1008) according the manual instructions starting from the tissue digestion step.

To minimize the elution volumne Zymo-Spin IC Columns (Zymo Research, Cat. N: C1004-250) can be used.

21 Purified RNA is quantified using Qubit RNA HS Assay Kit (ThermoFisher, Cat. N.: Q32852) and quality of the RNA is measured using the Bioanalyzer High Sensitivity RNA 6000 Pico Kit (Agilent, Cat. N.: 5067-1513).

22 100ng of high quality RNA was used for RNA library generation using the NEBNext® Single Cell/Low Input RNA Library Prep Kit (New England Biolabs, Cat. N.: E6420) according to the manual instruction.

3DRAM-seq: Cell lysis and GpC methylation

15m

23 **NOTE: this protocol was optimized for ~2×10^5 cells.**

Pellet fixed cells for 5min. at 2500xg (4˚C) and carefully resuspend them with 190µl of ice-cold Lysis Buffer.

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

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

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

26 Carefully resuspend nuclei with 100µl GpC reaction buffer.

27 Incubate for 3 hours at 37 ˚C with slight shaking of the tubes (500 RPM) and subsidize the reaction every hour with 1µl 32mM SAM (New England Biolabs, Cat. N: M0227S) and 2µl of M.CviPI (New England Biolabs, Cat. N: M0227S).

3DRAM-seq: DpnII Digestion and biotin fill-in

28 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.

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

30 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 rotationspeed of 600 rpm.

31 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 rotationspeed of 600 rpm.

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

33 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 min).

MethylHiC: Proximity ligation and decrosslinking

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

35 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.

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

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

38 Distribute the upper aqueous phase into two 2ml tubes and add to each tube 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.

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

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

41 Wash each tube with 200µl freshly prepared 70% cold ethanol and pool the samples together in one 0.5ml tube (total volume ~ 400µl, make sure the glycogen pellets are transfered). Centrifuge at maximal speed for 5 minutes at 4°C.

42 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.

3DRAM-seq: DNA shearing (Covaris)

43 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

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

3DRAM-seq: Biotin removal

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

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

3DRAM-seq: Bisulfite Conversion

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

Prior the bisulfite conversion add roughly 0.01% GpC methylated-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.

48 Elute the bisulfite converted DNA in 31µl low EDTA TE water provided with the Accel-NGS® Methyl-Seq DNA Library Kit (Swift Bioscience, Cat. N: 30024; now xGen Methyl-Seq DNA Library Prep IDT, Cat. N.: 10009860).

3DRAM-seq: Library construction and Biotin pulldown

49 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.

50 Use 30µl of the bisulfite converted DNA for the library preparation using 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).**

Please note that one reaction volume can be used if less than 100ng of DNA was used for the bisulfite conversion.

51 After the ligation resuspend samples with 30 µl steril water 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.

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

53 After the last wash remove as much as possible from the Tween washing buffer and wash beads twice with 400 µl sterile water. Thereafter resuspend beads in 50µl nuclease free water.

54 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 archive a higher complexity. To do so pipette 10µl of streptavidin bead solution 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; now IDT, Cat. N. 10009965 or 10005975).

55 Amplify library using the following PCR program: 95˚C 30s; {95˚C 15s, 61˚C 30s, 68 ˚C 80s} x10-11; 68˚C 5min; Hold at 10˚C.

56 After the PCR is finished, pellet the beads and pool the library containing supernatant of different reactions of a sample together (~125µl total volume).

57 Perform a 0.65x AmpureXP (Agencourt, Cat. N: A63881) purification by adding 81.25µl of room temperature AmpureXP beads to the samples followed by a incubation of 10 minutes at RT under slow rotation.

Please note that a <0.6x AmpureXP beads can lead to the loss of the whole library !

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

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

60 Resuspend the beads with 15µl nuclease-free water, incubate for 10 minutes at RT, 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)