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scNMT-seq v2

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We use this protocol and it's working

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Abstract

scNMT-seq (single cell Nucleosome, Methylome, and Transcriptome sequencing) allows the parallel study of a single cell chromatin status, methylation profile, and transcriptome.

Here, we are developing and testing modifications of the scNMT-seq pipeline. The protocol is carried out in 96w plates and typically takes 4-5 days to complete.
The number of pre-amplification cycles is adjusted to tackle the problem of poor recovery after BS conversion.Primers are optimized for first-strand and second-strand synthesis to solve the problem of unmapped reads and poor amplification. Both are testified as compatible with theoriginal the original scNMTseq.

Materials

- GpC Methyltransferase (M.CviPI) - 1,000 units **New England Biolabs Catalog #**M0227L
- IGEPAL-CA630 **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**I3021 SIGMA-ALDRICH
- RNase Inhibitor **Thermo Fisher Catalog #**N8080119
- RLT Plus Buffer **Qiagen**
- Dynabeads MyOne Streptavidin T1 **Thermo Fisher Scientific Catalog #**65601
- Superscript II **Invitrogen - Thermo Fisher Catalog #**18064014
- ERCC RNA Spike-In Mix or order custom-made synthetic sequences **Thermo Fisher Scientific Catalog #**4456740
- dNTP Mix (10 mM ea) **Thermo Fisher Catalog #**18427013
- Nextera XT DNA Sample Preparation Kit, 96 samples **Illumina, Inc. Catalog #**FC-131-1096
- Nextera XT Index Kit v2 Set A (96 indexes 384 samples) **Illumina, Inc. Catalog #**FC-131-2001
- Betaine 5M **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**B0300
- Magnesium Chloride (MgCl2) Solution - 6.0 ml **New England Biolabs Catalog #**B9021S
- DTT **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**D0632
- Kapa HiFi Hotstart ReadyMix (2x) **Kapa Biosystems Catalog #**KK2612
- Ampure XP beads **Beckman Coulter Catalog #**A63881
- Lambda DNA **Thermo Fisher Catalog #**SD0011
- EZ-96 DNA Methylation-Direct MagPrep **Zymo Research Catalog #**D5044
- Klenow (3′→ 5′ exo-) (High Concentration) **Enzymatics Catalog #**P7010-HC-L
- QuantiFluor® dsDNA System **Promega Catalog #**E2670

	A	B
	Name	**Sequence (5′ to 3′)**
	Oligo-dT primer	Biotin-TEG-AAGCAGTGGTATCAACGCAGAGTACTTTTTTTTTTTTTTTTTTTTTTTTTTTTVN
	TSO	AAGCAGTGGTATCAACGCAGAGTACATrGrG+G
	IS PCR	AAGCAGTGGTATCAACGCAGAGT
	Pre-amplification primer	Biotin-TGACTGGAGTTCAGACGTGTGCTCTTCCGATCT**HHHHHHH*H**
	Adapter2 primer	ACACTCTTTCCCTACACGACGCTCTTCCGATCT**DDDDDDD*D**

	A	B
	PE 1.0	AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGATC*T
	iPCRTag primer	CAAGCAGAAGACGGCATACGAGATXXXXXXXXGTGACTGGAGTTCAGACGTGTGCTCTTCCGATC*T

All oligos should be ordered with HPLC purification

Cell isolation and GpC methylation

1

Prepare plates containing **2.5µl** GpC methylase reaction mixture in each well:

	A	B	C	D
	Component (initial)	**Component(final)**	**Volume (µl)**	**Mastermix (110 samples)**
	M.CviPI reaction buffer (10x)	1x	0.25	27.5
	M.CviPI (4U/µl)	2U	0.5	55
	SAM (**320µM**)	160µM	1.25	137.5
	IGEPAL (10%)	0.1%	0.025	2.75
	RNasein (20U/µl)	1U/µl	0.125	13.75
	Nuclease-free water		0.35	38.5

2

Isolate cells manually or using FACS in 2.5 µL of GpC methylase reaction buffer

3

After cell isolation, spin down plates at **≥1000g** for **≥10s** 4 °C

4

Incubate the samples at 37 °C for 00:15:00 15m

5 Stop reaction by adding 5 µL **RLT plus buffer**

6 Stoe the plates at -80 °C until processed

Oligo-dT$_{30}$VN bead preparation 30m

7 Add 55 µL Dynabeads into a new Eppendorf tube. Place the tube on a magnet for 00:02:00 and discard supernatant 2m

8 Resuspend beads in 200 µL **Dynabead solution A (** [M] 0.1 Mass Percent **NaOH,** [M] 0.05 Mass Percent **NaCl)**. Place the tube on a magnet for 00:02:00 and discard supernatant 2m

9 Repeat step 8 once

10 Resuspend beads in 200 µL **Dynabead solution B (** [M] 0.1 Mass Percent **NaCl)**. Place on a magnet for 00:02:00 and discard supernatant 2m

11 Resuspend the beads in 55 µL of **2x B&W (** [M] 2 Mass Percent **NaCl,** [M] 10 millimolar (mM) **Tris-HCl,** [M] 1 millimolar (mM) **EDTA)** and 55 µL Biotinylated Oligo-dT30VN ([M] 100 micromolar (µM)).
Incubate 00:20:00 on a thermomixer while shaking at **2000rpm** at Room temperature 20m

*In the meantime, prepare the **bead resuspension buffer***

	A	B
	Superscript FS buffer (5x)	220µl
	Nuclease-free Water	825µl
	RNase inhibitor (20U/µl)	**55µl**

After adding RNase inhibitor, use beads within 30min

*In the meantime, prepare **1x B&W** buffer by mixing* 440 µL *Nuclease-free water with* 440 µL ***2x B&W** buffer*

12 2m

Place beads on a magnet for 00:02:00 and discard supernatant

13 2m

Resuspend the beads in 200 µL **1x B&W** buffer. Place beads on a magnet for 00:02:00 and discard supernatant

14

Repeat step 13 **three** more times

15

Resuspend the beads in the **bead resuspension buffer**

Physical separation of mRNA and gDNA

16

Thaw the 96-well plate containing the single cell lysates on ice

17

Add 1 µL **ERCC spike-ins** at 1:1Million - 1:128Million dilution to each sample using a multi-dispensing pipette. Run the pulse centrifugation program to spin ERCCs down to the bottom

18

Take 4 tubes(1069 µL per tube) of **G&T wash buffer(** [M] 50 millimolar (mM) **Tris–HCl** pH 8.3 **,** [M] 75 millimolar (mM) **KCl,** [M] 3 millimolar (mM) **MgCl2, 0.5% Tween**

20 Solution) and add to each tube 137.5 µL **DTT** and 25 µL **RNaseIn**

19

Add 50 µL **G&T-Seqwash buffer** per well to the"G&T-Seq wash plate"

20

Add 10 µL **Oligo-dT beads** per well to the"bead plate"

21

Add an empty **non-skirted** 96 well plate labeled "gDNA collection"

22

Spin all plates and run the adapted G&T-separation program robotically or manually.

*While the separation program is running, prepare the **RT master mix***

	A	B	C	D
	Component (C_initial)	**C_final**	**Volume(µl)**	**Mastermix (110 samples)**
	dNTP (10mM)	1mM	0.5	55
	TSO (100µM)	1µM	0.05	5.5
	MgCl2 (1M)	6mM	0.03	3.3
	Betain (5M)	1M	1	110
	S II First strand buffer (5x)	1x	1	110
	DTT (100mM)	5mM	0.25	27.5
	Nuclease-free water		1.8	198
	RNase inh (20U/µl)	**0.5U/ µl**	**0.125**	**13.75**
	Superscript reverse transcriptase II (200U/µl)	**10U/µl**	**0.25**	**27.5**

Adding enzyme within less than 30 min before running the Reverse Transcription program

Note

Separation is performed robotically on the Hamilton platform in this protocol. If performed manually, steps should be as follows

22.1 Manually pipette 10 µL of prepared **oligo-dT beads** to each well of the **sample plate** using a multichannel pipette

22.2 Mix at maximum speed for 00:20:00 20m

22.3 Place on magnet for 00:05:00 . Aspirate 17.5 µL and transfer to the empty low-bind plate for gDNA collection 5m

22.4 Add 15 µL of G&T-seq wash buffer off magnet.

22.5 Mix at maximum speed for 00:10:00 10m

22.6 Place on magnet for 00:02:00 . Aspirate 15 µL and transfer to the empty low-bind plate for gDNA collection 2m

22.7 Repeat steps 22.3-22.6 twice more

Note

Lysate (17.5ul) combined with 3 washes (15ul each) should now have been collected into the gDNA plate

Reverse transcription 1h 45m

23

Collect the polyA(+) mRNA plate and using the multi-dispenser dispense 5 µL **RT master mix** into each well of the bead-containing 96-well plate

24

Seal the mRNA and gDNA plates and spin.

Store gDNA at -80 °C until processed

25

1h 45m

Incubate the polyA(+) mRNA 96-well plate on a **thermomixer C** using the program below (approx. duration 01:45:00)

	A	B	C	D
	Cycle	**Temp (°C)**	**Time**	**Mixing (rpm)**
	1	42	2 min	2000
	2	42	60 min	1500
	3	50	30 min	1500
	4	60	10 min	1500

26

*In the meantime prepare **PCR mastermix***

	A	B	C
	Component	**Volume(µl)**	**Mastermix (110 samples)**
	KAPA HiFi HotStart ReadyMix (2x)	6.25	687.5
	IS PCR primer (10µM)	0.124	13.64
	Nuclease-free water	1.13	124.3

PCR amplification of cDNA

30s

27

Add 7.5 µL **PCR reaction mastermix**, seal the plate and centrifuge

28

30s

Resuspend the beads for 00:00:30 at **2000rpm** using the Thermomixer

29

Perform cDNA amplification as follows

	A	B	C
	Cycles	**Temperature (°C)**	**Time**
	1	98	3 min
	18-25	98	20 s
		67	15 s
		72	6 min
	1	72	5 min
	1	4	Hold

Amplification cycles differ

PCR cleanup of amplified cDNA

22m 10s

30

Add 12.5 µL **Agencourt AMPure beads** (1:1 ratio), mix thoroughly by pipetting up and down

31

5m

Incubate 00:05:00 at Room temperature

32 5m

Pellet the beads on a Low-elution magnet for 00:05:00

33

Remove the supernatant without disturbing the beads

34 10s

Wash the beads twice with 150 µL of freshly prepared 80% ethanol for 00:00:10

35 7m

Allow the beads to dry for approximately 00:05:00 . Resuspend in 25 µL nuclease-free water. Incubate for 00:02:00 Room temperature

36 5m

Return the 96-well plate to the magnet and allow the Agencourt AMPure beads to settle for 00:05:00

37

Carefully transfer the supernatant to a new 96-well plate

Note

Quality control: QUBIT+BIOANALYZER
expected cDNA concentration: >= 1ng/µl
expected cDNA length: 500-2000bp, peaking at 1-1.5kb

Library preparation of cDNA (Nextera XT) 9m

38

Dilute the **cDNA** of each sample to **0.2ng/µl** with nuclease-free water

39

Add 2.5 µL **Tagment DNA(TD) buffer** to a new Hard-Shell skirted 96-well plate

40 Add 1.25 µL **diluted cDNA** and 1.25 µL **amplicon tagment mix** (ATM) to TD buffer and mix

41 Centrifuge the plate at 280 x g, 20°C, 00:01:00 1m

42 Incubate on a thermal cycler

	A	B	C
	Segment	**Temp(°C)**	**Duration(min)**
	1	55	10
	2	10	Hold

43 Add 1.25 µL Neutralize Tagment Buffer (NT)

44 Vortex & spin down at 800 x g, 20°C, 00:01:00 1m

45 Incubate at Room temperature 00:05:00 5m

46 Add 1.25 µL **Index (i7) adapter** to each column and 1.25 µL **Index 2 (i5) adapter** to each row

47 Add 3.75 µL **Nextera PCR mastermix** and mix

48 Centrifuge the samples at 280 x g, 20°C, 00:01:00 and amplify as follows: 1m

	A	B	C
	Cycle	**Temp (°C)**	**Duration**
	1	72	3min
	2	95	30s
	3-14	95	10s
		55	30s
		72	30s
	15	72	5min
	16	4	Hold

49

Centrifuge the plate at 280 x g, 20°C, 00:01:00

1m

50

Purify libraries at a **0.66:1 ratio** and elute in 12.5 µL **EB buffer**

Note

Libraries can be stored for at least a year at -20°C

51

Pool libraries and quantify using qPCR

Note

expected pool concentration: 4nM
expected pool size: 250-1500bp

scBS-seq library preparation (gDNA)

52

Bisulfite conversion

39m

53

19m

Prepare the CT conversion reagent by mixing 7.9 mL **M-Solubilisation buffer** and 3 mL **M-Dilution buffer** and 00:15:00 vortexing at Room temperature

Finally,add 1.6 mL **M-Reaction buffer** and vortex 00:04:00 at Room temperature

54

Add 32.5 µL **AMPure XP beads** to the gDNA plate (0.65:1 ratio)

55

20m

Incubate 00:20:00 Room temperature

56

20m

Place the plate on the magnet for 00:20:00 and discard the supernatant

57

Wash the beads twice with 200 µL 80% ethanol

58

Resuspend the beads in 10 µL elution buffer, optionally containing 60fg unmethylated lambda DNA

Note

Do not transfer the samples from the beads
Do not dry the beads after the second wash, a dry step when purifying gDNA lowers recovery

59

Add 65 µL CT conversion reagent **without mixing**

Note

Watch out for bubbles, centrifuge shortly if necessary

60

Incubate the mixture as follows:

	A	B	C
	Segment	**Temperature(°C)**	**Duration(min)**
	1	98	8
	2	65	180
	3	4	Hold

Note

BS converted DNA is stable for 3 days at -20°C or 20h at 4°C

Purification of the bisulfite converted DNA

33m

61

Mix 300 µL M-binding buffer and 5 µL MagBinding beads

Note

Tip: to minimize loss of sample due to pipetting use a thermomixer to mix instead of pipetting
Use a deep-well plate

62 5m

Transfer the samples to the M-binding buffer - MagBinding beads mix and incubate 00:05:00 Room temperature

63 3m

Pellet the beads on a magnet for 00:03:00 and discard the supernatant

64

Resuspend the beads in 200 µL M-Wash buffer

65 15m

Pellet beads on the magnet and discard the supernatant. Resuspend the beads in 100 µL M-Desulphonation buffer and incubate 00:15:00 Room temperature

Note

The beads sink quite fast to the bottom, during these 15 mins you can slowly mix on regular basis with the thermomixer

66

Pellet beads on the magnet and discard the supernatant. Wash the beads twice with 200 µL M-Wash buffer

67 10m

Dry the beads on a heating element at 55 °C for 00:10:00

*In the meantime, prepare the **pre-amplification mix** as follows*

	A	B	C	D
	Component	**Amount (µl)**	**Final concentration**	**Mastermix (110 samples)**
	Blue buffer (10×)	4	1x	440
	dNTP mix (10mM)	1.6	0.4mM	176
	Preamp Oligo (**10 µM**)	1.6	O.4 µM	176
	H2O	32.8		3608
	Total volume	40		4400

Pre-amplification

8m 5s

68

Resuspend the beads in a 40 µL **pre-amplification mix**

69

4m

Incubate the mixture at 55 °C for 00:04:00 and place it on the magnet

70

After the beads are pelleted transfer 39 µL to a new plate

71

3m 10s

Incubate the samples 00:03:00 at 65 °C and immediately cool on a pre-cooled aluminum rack

Centrifuge the plate at 500 x g, Room temperature, 00:00:10

72

Add 1 µL **klenow exo- polymerase** (50U/µl)

Vortex the samples and amplify as follows:

	A	B	C	D
	Segment	**Temp (°C)**	**Duration (min)**	**Ramp speed (°C/min)**
	1	4	5	-
	2	4-37	8.25	4
	3	37	30	-
	4	4	Hold	

73

In the meantime, prepare 6 tubes of ***pre-amplification mix***

Note

Only add klenow exo to the mix before use

	A	B	C	D
	Component	**Amount (µl)**	**Final concentration**	**Mastermix (samples)**
	Blue buffer (10×)	0.25	1x	
	dNTP mix (10mM)	0.1	0.4mM	
	Preamp Oligo (**10 µM**)	1	4 µM	
	Klenow exo- (50 U/µl)	0.5	10 U/µl	
	H2O	0.65		
	Total volume	2.5		

74

45s

Heat the plate to 95 °C for 00:00:45 and transfer it to an aluminum rack pre-cooled on ice

75 10s

Centrifuge the plate at 500g for 00:00:10 at 15-25°C

76

Add 2.5 µL of the **pre-amplification mix**

77

Repeat steps 72-76 **five more times**

78

Incubate as follows:

	A	B	C	D
	Segment	**Temp (ºC)**	**Duration (min)**	**Ramp speed (ºC/min)**
	1	4	5	-
	2	4-37	8.25	4
	3	37	90	-
	4	4	Hold	

Note

The first-strand product can be stored ON at 4°C or for at least a month at -20°C

Exonuclease I treatment 1h

79

Dilute the samples to a volume of 98 µL with **nuclease-free water**

80 1h

Add 2 µL **exonuclease I** (20U/µl) to the pre-amplified product and incubate 01:00:00 at 37 °C with the **heated lid set to** 50 °C

Purification 18m

81

Add 75 µL **AMPure XP beads** (0.75:1 ratio) and mix thoroughly by pipetting up and down

Note

Tip check the volume of some samples first and adjust volumes of beads to add accordingly

82 10m

Incubate 00:10:00 Room temperature

*In the meantime, prepare **Adaptor 2 mix***

	A	B	C	D
	Component	**Amount (µl)**	**Final concentration**	**Mastermix (samples)**
	Blue buffer (10×)	4.7	1x	
	dNTP mix (10mM)	1.9	0.4mM	
	Adapter 2 Oligo **(10 µM)**	1.9	0.4µM	
	H2O	38		
	Total volume	46.5		

83 3m

Place on the magnet for 00:03:00 and discard the supernatant

84

Add 200 µL of 80% (vol/vol) ethanol while keeping the plate on the magnet then discard ethanol after ±10sec

85 5m

Repeat 84 once. Dry the AMPure XP beads for 00:05:00 Room temperature

Adapter 2 tagging 12m

86

Resuspend the beads in 46.5 µL **Adapter 2 mix**

87 10m

Incubate for 00:10:00 Room temperature

88

Transfer samples to a new plate

89 45s

Heat mixture to 95 °C for 00:00:45 then immediately cool on ice using an aluminum rack

90 10s

Spin down at 500g for 00:00:10 at 15–25°C

91 10s

Add 1 µL **Klenow exo-** (50 U/µl), vortex gently, and spin down at 500g for 00:00:10 at 15–25°C

92

incubate as follows:

	A	B	C	D
	Segment	**Temp (ºC)**	**Duration (min)**	**Ramp speed (ºC/min)**
	1	4	5	-

	A	B	C	D
	2	4-37	8.25	4
	3	37	30	-
	4	4	Hold	

93

In the meantime, prepare 1 tube of ***Adapter 2 mix***

	A	B	C	D
	Component	**Amount (µl)**	**Final concentration**	**Mastermix (samples)**
	Blue buffer (10×)	0.25	1x	
	dNTP mix (10mM)	0.1	0.4mM	
	Preamp Oligo (**10 µM**)	1	4 µM	
	Klenow exo- (50 U/µl)	0.5	10 U/µl	
	H2O	0.65		
	Total volume	2.5		

94

Heat the plate to 95 °C for 00:00:45 and transfer it to an aluminum rack pre-cooled on ice

45s

95

Centrifuge the plate at 500g for 00:00:10 at 15-25°C

10s

96

Add 2.5 µL of the **Adapter 2 mix**

97

Incubate as follows:

	A	B	C	D
	Segment	**Temp (°C)**	**Duration (min)**	**Ramp speed (°C/min)**
	1	4	5	-
	2	4-37	8.25	4
	3	37	90	-
	4	4	Hold	

Purification

21m 10s

98

Add 37.5 µL AMPure XP beads (**0.75:1 ratio**)

99

10m

Incubate 00:10:00 at room temperature

*In the meantime, prepare the **library amplification mix***

	A	B	C	D
	Component	**Amount (µl)**	**Final Concentration**	**Mastermix (samples)**
	KAPA HIFI HotStart ReadyMix (2x)	25	1x	
	PE1.0 **(10µM)**	1	0.2µM	
	Nuclease-free water	23		
	Total volume	49		

100

3m

Place on a magnet for 00:03:00

101

Place on a magnet for 00:03:00 and discard the supernatant 3m

102 10s

Add 200 µL ethanol (70%) without disturbing the beads. After 00:00:10 remove ethanol

103 5m

Repeat step 102 once then dry beads 00:05:00 at room temperature

Library amplification 10m

104

Resuspend the beads in 49 µL **library amplification mix**

105 10m

Incubate the mixture 00:10:00 Room temperature

106

Place on a magnet and transfer supernatant to a new plate

107

Add 1 µL **10µM reverse iPCRTag primer**(containing a sample-specific index)

Amplify as follows:

	A	B	C
	Cycles	**Temperature (°C)**	**Time**
	1	95	3 min
	17-20	98	80 s
		65	30 s
		72	30 s
	1	72	3 min
	1	4	Hold

Note

The PCR product can be stored ON at 4°C or for at least a month at -20°C

Purification of amplified libraries

28m

108 **Add 37.5µl AMPure XP beads** (0.75:1 ratio) and mix well

109 Incubate 00:10:00 Room temperature 10m

110 Place on the magnet for 00:03:00 and discard supernatant 3m

111 Add 200 µL ethanol (70%) without removing the plate from the magnet then discard the ethanol

112 Repeat step 111 once then dry beads 00:05:00 5m

113 Resuspend the beads in 15 µL **EB**

114 Incubate 00:10:00 Room temperature 10m

115 Place on a magnet then transfer supernatant to a new plate

Note

Library quantity and quality can be checked using Qubit HS Assay and Bioanalyzer
expected gDNA concentration: >= 1ng/µl
expected fragment length: >200bp and on average 400-600bp

Libraries can be stored for at least a year at -20°C