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scNMT-seq V.1

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We use this protocol and it's working

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Abstract

Here we describe the full wetlab protocol for scNMT-seq (single-cell nucleosome position, methylome and transcriptome sequencing), a plate-based single-cell multi-omic method.

In scNMT-seq, a GpC methyltransferase is used to label non-nucleosome bound DNA thus encoding chromatin accessibility information within the genomic DNA. Bisulfite conversion is then used to discriminate the two chromatin states which can be read from GpC dinucleotide positions. Endogenous DNA methylation is read from the CpG positions of the same molecules and gene expression data is obtained by capturing poly-A RNA on magnetic beads and physically separating before the gDNA is bisulfite converted. This mRNA on beads is processed using Smart-seq2. The protocol is carried out in 96w plates and typically takes 3-4 days to complete.

Guidelines

scNMT-seq is essentially a combination of the following methods:

1. NOME-seq:

Genome-wide mapping of nucleosome positioning and DNA methylation within individual DNA molecules

Kelly T., Liu Y. *et al* 2012

doi: 10.1101/gr.143008.112

2. G&T-seq:

G&T-seq: parallel sequencing of single-cell genomes and transcriptomes

Macaulay I. *et al* 2015

doi: 10.1038/nmeth.3370

3. Smartseq2:

Smart-seq2 for sensitive full-length transcriptome profiling in single cells

Picelli S. *et al* 2013

doi: 10.1038/nmeth.2639

4. scBS-seq:

Single-cell genome-wide bisulfite sequencing for assessing epigenetic heterogeneity

Smallwood S., Lee H. *et al* 2014

doi: 10.1038/nmeth.3035

Protocols for each of these individual methods have been published elsewhere, however the full step-by-step protocol for scNMT-seq had not previously been available as a single document.

General guidelines

As with other single-cell sequencing methods it is important to minimise sources of contamination. We perform all pre-PCR pipetting steps in a dedicated pre-PCR room. Prior to starting, all surfaces and pipettes are wiped down with a dilute bleach solution (e.g. RNase away or similar) to mitigate RNase and DNA contamination and all tubes and PCR plates are subjected to UV irradiation. We also recommend aliquoting reagents such as oligos and dNTPs so that each tube is used once then discarded.

We use an automated pipetting robot (Agilent Bravo Workstation) for each of the pre-PCR magnetic bead portions of the protocol. This reduces hands-on time and increases reproducibility of the method. However, it is also possible to perform the whole protocol by hand with an appropriate magnet and multichannel pipettes.

Sequencing of libraries should be possible on any Illumina instrument unless certain restrictions are programmed in such as with the X10. We routinely use HiSeq 2000/2500 v4 and NextSeq500. RNA-seq libraries should be sequenced on a separate lane or flowcell to the BS-seq libraries. We have had success with read-depths of 0.5-2M per cell for the RNA-seq and 2-10M per cell for the BS-seq. We recommend paired-end with mid-long read lengths (75-125bp) for the BS-seq to maximise cytosine coverage. However, it is possible that much lower read-depths could yield informative data, especially in studies with large numbers of cells.



Indexes are incorporated into the BS-seq libraries during the final amplification step. We use the iPCRTag indexing system, which means that a custom index read primer needs to be spiked-in with the other i7 index primers for sequencing. However, another indexing system (e.g. Truseq) could be used by re-designing the second strand synthesis oligo (SSO) appropriately.

Materials

MATERIALS

✕ GpC Methyltransferase (M.CviPI) - 1,000 units **New England Biolabs Catalog #M0227L**

✕ RLT Plus Buffer **Qiagen**

✕ IGEPAL-CA630 **Merck MilliporeSigma (Sigma-Aldrich) Catalog #I3021 SIGMA-ALDRICH**

✕ Kapa HiFi Hotstart ReadyMix (2x) **Kapa Biosystems Catalog #KK2612**

✕ Recombinant RNasin(R) RNase Inhibitor, 10,000u **Promega Catalog #N2515**

✕ Agencourt AMPure XP SPRI beads **Beckman Coulter Catalog #A63881**

✕ Nextera XT DNA Sample Preparation Kit, 96 samples **Illumina, Inc. Catalog #FC-131-1096**

✕ Nextera XT Index Kit, 96 indices, 384 samples **Illumina, Inc. Catalog #FC-131-1002**

✕ Dynabeads MyOne Streptavidin C1 **Invitrogen - Thermo Fisher Catalog #65001**

✕ Superscript II **Invitrogen - Thermo Fisher Catalog #18064014**

✕ EZ-96 DNA Methylation-Direct MagPrep **Zymo Research Catalog #D5044**

✕ Klenow (3'→ 5' exo-) (High Concentration) **Enzymatics Catalog #P7010-HC-L**

	Name	Sequence (5' to 3')
	bio-Smartseq2-dT	/5BiotinTEG/AAGCAGTGGT ATCAACGCAGAGTACTTTT TTTTTTTTTTTTTTTTTTTT TTTTTTVN
	Smartseq2 TSO	AAGCAGTGGTATCAACGCA GAGTACATrGrG+G
	ISPCR	AAGCAGTGGTATCAACGCA GAGT
	First strand oligo	/5SpC3/CTACACGACGCTC TTCCGATCTNNNNNN
	Second strand oligo	TGCTGAACCGCTCTTCCG ATCTNNNNNN
	PE1.0	AATGATACGGCGACCACC GAGATCTACACTCTTTCCC TACACGACGCTCTTCCGAT C*T
	iPCRTag	CAAGCAGAAGACGGCATA CGAGATXXXXXXXXXGAGAT CGGTCTCGGCATTCTGC TGAACCGCTCTTCCGATC* T



iTag sequencing primer	AAGAGCGGTTTCAGCAGGA ATGCCGAGACCGATCTC
------------------------	--

Oligo sequences. All oligos should be ordered with HPLC purification.


Troubleshooting

Single-cell collection and methylase reaction

1 repare GpC methylase reaction buffer:

	Rea gent	amo unt per sam ple	96 well plat e (x12 0)
	Nucl ease free water	1.42 5	171
	GpC met hyltr ansf eras e buff er (10x)	0.25	30
	SAM	0.01 25	1.5
	IGEP AL CA- 630 (1%)	0.25	30
	RNa se- in (40 U/ul)	0.06 25	7.5
	M.C viPI (4U/ ul)	0.5	60
	Tota l	2.5	300

2 Collect single cells in


 2.5 µL of freshly prepared GpC methylase reaction mix (keep chilled on ice)

Centrifuge at $\geq 1000g$ for $\geq 10s$.

Note

We have successfully used both flow sorting and manual pipetting for single-cell collections.


3 Incubate on a thermocycler:

 00:15:00


 37 °C


4 Add  5 μL RLT plus


Centrifuge at $\geq 1000g$ for $\geq 10s$.


5 Store at  -80 °C

Prepare oligo-dT on beads

6 Pipette  55 μL Dynabeads (MyOne Streptavidin C1) into a microcentrifuge tube.
Place on a magnet and remove supernatant.


7 Resuspend beads in  55 μL solution A (0.1M NaOH, 0.05M NaCl) by pipetting. Place on a magnet and remove supernatant.

8  [go to step #7](#) : Repeat solution A wash one more time



9 Resuspend beads in  55 μL solution B (0.1M NaCl) . Place on magnet and remove supernatant.

10  [go to step #8](#) : Repeat solution B wash once.

11 Resuspend beads in  55 μL 2x B&W (2 M NaCl, 10 mM Tris-HCl, 1 mM EDTA) . Place on magnet and remove supernatant.

12  [go to step #11](#)




- 13 Resuspend beads in  55 μ L 2x B&W and  55 μ L bio-Smartseq2-dT (100uM) .


Note

bio-Smartseq2-dT: 5'-Biotin-TEG-AAGCAGTGGTATCAACGCAGAGTACTTTVN-3' (IDT, purified with HPLC and resuspended in nuclease free water)


- 14 Rotate tube containing beads such that beads remain in solution for a minimum of


 00:15:00 .

Note


Beads can be prepared in advance and stored at  4 °C for up to 1 month

Wash oligo dT beads and prepare for dispensing


- 15 Place on magnet and remove supernatant. Resuspend beads in  110 μ L 1x B&W buffer (1M NaCl, 5mM Tris, 0.5mM EDTA) . Place on magnet and remove supernatant.

- 16  [go to step #15](#)

- 17 Place on magnet and remove supernatant. Resuspend beads in:

 830 μ L Nuclease free water ,

 110 μ L 5x first strand buffer (Invitrogen, Superscript II) ,

 43 μ L RNasin (Promega) ,

 10 μ L Diluted ERCC spike-in

Note

This assumes 2.5ul GpC reaction plus 5ul RLTplus. If using bigger volumes, adjust upward in proportion.

**Note**

ERCC spike-in is optional. The concentration used will depend on cell type (RNA content).

mRNA separation

18 Prepare G&T-seq wash buffer:

Reagent	96 well plate
Nuclease free water	3430
5x First strand buffer (Invitrogen Superscript II)	430
DTT (100 mM, Invitrogen Superscript II)	430
Tween-20 (100 %)	22





Recombinant RNasin (40 U/ul)	43
Total	435

19 Prepare RT mastermix:

Reagent	amount per sample	96 well plate (x110)
Nuclease free water	3.715	408.65
5x First strand buffer (Superscript II)	2	220
Beta ine (5M)	2	220
DTT (100 mM, Superscript II)	0.5	55
dNTPs (10mM each)	1	110
MgCl ₂ (1M)	0.06	6.6


Smartseq2 TSO (100 uM)	0.1	11
Recombinant RNasin (40 U/ul)	0.125	13.75
Superscript II (200 U/ul)	0.5	55
Total	10	1100


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


21 Mix at maximum speed for  00:10:00

Note

We perform mRNA separation steps on an Agilent Bravo liquid handling robot. If performing this step manually, intermittent vortexing for 10 minutes achieves the same result.

22 Place on magnet until beads pellet. Aspirate  17.5 μL and transfer to empty lobe plate for gDNA collection.


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


24 Mix at maximum speed for  00:01:00

Note

If performing manually, a thorough vortexing here suffices.



25 Place on magnet until beads pellet. Aspirate  15 μ L and transfer to empty lobe plate for gDNA collection.

26  go to step #22 Repeat steps 22-25 twice more.


Note

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

Note



Pause point. gDNA can be frozen at -20C for later processing.

27 Add  10 μ L RT mastermix to beads.



28 Mix at maximum speed for  00:01:00

Reverse transcription

29 Incubate on a thermocycler as follows:

 01:00:00  42 °C

 00:30:00  50 °C


 00:10:00  60 °C

Note

Vortex plate at 30 and 60 minute time points to resuspend beads.

cDNA amplification



30 Add  12 μ L PCR mastermix by hand using a multichannel pipette.



	Reagent	amount per cell	96 well plate
	KAPA Hifi 2x ReadyMix	11	1210
	ISPCR primer (10uM)	0.25	27.5
	Water	0.75	82.5
	Total	12	1320

31 Cycle as follows:

 00:03:00  98 °C

18 Cycles of:



 00:00:20  98 °C

 00:00:15  67 °C

 00:06:00  72 °C

Followed by:

 00:05:00  72 °C

 00:00:00 pause - usually overnight  4 °C

Note

Cycle number depends on cell type. 18 is sufficient for mouse ES cells and embryo cells.

Note


Pause point. cDNA can be frozen at -20C for later processing.

cDNA purification using 1x AMPure XP


32

Note

This section is performed outside of pre-PCR room.

Add  18 μ L AMPure XP beads. Mix thoroughly and incubate for 5 minutes.

33 Place on magnet until beads precipitate. Remove supernatant. Wash twice with 80% ethanol. Remove supernatant and air-dry beads for 5 minutes at room temperature.

34 Elute cDNA in  25 μ L water

35 Check quality of a subset (e.g. 11 samples) of cDNA samples using a Bioanalyzer high sensitivity DNA chip.

Expected result

Fragment size range of 1kb to 5kb. Concentration of 100 to 500 pg/ μ L. If concentration is higher then dilute samples with water prior to Nextera XT library prep.

Nextera XT library preparation

36

Note

This section is performed outside of pre-PCR room.

In a 96 well or a 384 well plate, add 2.25 μ L Tagmentation mastermix to each well:


	Reagent	amount per sample	96 well plate (x120)	384 well plate
	Tagment DNA buffer	1.5	180	650
	Amplicon tagment mix	0.75	90	325
	Total	2.25	270	975

37 Using a multi-channel pipette, add 0.75ul of cDNA sample (100-500pg/ul) to the mastermix. Vortex the plate and spin down.


38 In a thermocycler incubate as follows:

 55 °C  00:05:00 ,

 4 °C pause

39 Add  0.75 µL NT buffer using multichannel pipette. Centrifuge then vortex to mix and centrifuge again.

40 Add  2.25 µL NPM (from Nextera XT kit)

Add  1.5 µL pre-mixed and diluted i7 and i5 primers

Using a multichannel pipette. Centrifuge and vortex to mix.

Note

We prepare pre-mixed indexing primers in 96w plates for this step. Using multichannel pipettes, transfer each of 8 i5 primers to 12 columns and each of 12 i7 primers to 8 rows, then dilute 5-fold with water.

41 Cycle as follows:

 72 °C  00:05:00



🔥 95 °C ⌚ 00:02:00

10 cycles of:

🔥 95 °C

🔥 65 °C

🔥 72 °C

Followed by:

🔥 72 °C ⌚ 00:05:00

🔥 4 °C hold.

- 42 Pool 🧪 1 μ L of each indexed cDNA library. Purify using 0.7x AMPure XP beads with 2 \times 80% ethanol washes.
- 43 QC using Bioanalyzer.

Genomic DNA purification

44

Add 🧪 50 μ L AMPure XP beads to gDNA samples. Vortex thoroughly and incubate for ⌚ 00:30:00

45

Place on a magnet until beads pellet. Remove supernatant, wash twice with 🧪 100 μ L 80% ethanol . Remove supernatant. Immediately resuspend in 🧪 10 μ L of nuclease free water.

Note

We use an Agilent Bravo for these wash steps.

Prepare bisulfite conversion reagent

46

Bisulfite reagent is prepared according to manufacturers instructions (Zymo EZ-methylation direct):


Add 🧪 7.9 mL M-Solubilization Buffer and 🧪 3 mL M-Dilution Buffer to a bottle of CT Conversion Reagent. Mix at room temperature with frequent vortexing or shaking for at



least 10 minutes. Add  1.6 mL M-Reaction Buffer and vortex thoroughly.

Bisulfite conversion

47 Place gDNA sample plate on magnet to pellet beads.

48 Add  65 μL of prepared CT Conversion Reagent solution to each sample on the magnet.

Note

Do not mix sample and beads with CT reagent – beads should remain pelleted during conversion to avoid excessive dissolving of magnetic beads.




49 Incubate on a thermocycler as follows:



 98 °C  00:08:00

 64 °C  03:00:00


 4 °C pause until purification (maximum duration of overnight)

Desulphonation and purification

50 Add  533 μL Zymo Magbinding beads to  32 mL M-Binding buffer, mix and dispense  305 μL per well of deepwell plate using multichannel and reservoir.


51 Add sample to deepwell plate containing beads and binding buffer. Mix thoroughly then transfer  100 μL back to sample plate, mix thoroughly and transfer back to deepwell plate to maximise recovery of bisulfite converted DNA. Mix for  00:05:00


52 Plate on magnet until beads pellet. Remove supernatant.

53 Add  180 μL freshly prepared 80% ethanol. Mix thoroughly.

54 Place on magnet until beads pellet. Remove supernatant.




55 Add  100 μ L M-Desulphonation buffer (Zymo) . Mix thoroughly.

56 Incubate  00:15:00

Note

Incubate for ≥ 15 minutes and ≤ 25 minutes

57 Place on magnet and remove supernatent.

58 Add  180 μ L freshly prepared 80% ethanol . Mix thoroughly.

59 Place on magnet until beads pellet. Remove supernatent.

60  go to step #58 : Repeat once more.

61 Dry beads  60 °C  00:10:00

62 Prepare first strand master mix for elution:






	Rea gent	amo unt per sam ple	96 well plat e (x12 0)
	Nucl ease free wate r	31.8	3816



10x Blue buffer (Enzymatics)	4	480
dNTP mix (10mM each)	1.6	192
First strand oligo (10uM)	1.6	192
Total	39	4680

Note

First strand oligo: /5SpC3/CTACACGACGCTCTTCCGATCTNNNNNN (IDT, HPLC purified). This primer contains the Illumina PE read 1 sequence.

- 63 Resuspend beads in  20 μ L first strand synthesis mix . incubate at  60 °C
 00:05:00
- 64 Place on magnet until beads pellet. Transfer  20 μ L supernatant to fresh lobind PCR plate.
- 65  go to step #63 : Repeat elution once more.

First strand synthesis



66 Place sample plate on thermocycler at 65 °C 00:03:00 then immediately cool on ice.

67 Add 1 µL Klenow exo- (50 U/ul, Enzymatics) using multichannel and PCR strip.

68 Incubate on a thermocycler as follows:

4 °C 00:05:00

Slow ramp from 4 °C to 37 °C at 30s per 1C

37 °C 00:30:00

4 °C hold.

69 95 °C 00:00:45 95 °C for 45 seconds then immediately cool 4 °C using two ice blocks.

70 Add 2.5 µL first-strand extra cycles mix

	Reagent	amount per sample	96 well plate (x110)
	Nuclease free water	0.65	71.5
	10x Blue buffer	0.25	27.5
	dNTP mix (10mM each)	0.1	11
	First strand oligo	1	110



	(10u M)		
	Klen ow exo- (50U /ul)	0.5	55
	Tota l	2.5	275

Note

First strand oligo: /5SpC3/CTACACGACGCTCTTCCGATCTNNNNNN (IDT, HPLC purified).
This primer contains the Illumina PE read 1 sequence.

71 Incubate on a thermocycler as follows:

🌡️ 4 °C ⌚ 00:05:00

Slow ramp from 🌡️ 4 °C to 🌡️ 37 °C at 30s per 1C

🌡️ 37 °C ⌚ 00:30:00

🌡️ 4 °C hold.

72 ➡️ go to step #69 Repeat first strand synthesis an additional 3 times

73 Incubate the 5th and final round of first strand synthesis as follows:

🌡️ 4 °C ⌚ 00:05:00

Slow ramp from 🌡️ 4 °C to 🌡️ 37 °C at 30s per 1C

🌡️ 37 °C ⌚ 01:30:00

🌡️ 4 °C hold.

Exonuclease treatment

74 🧴 50 µL Exonuclease mix

	Rea gent	amo unt per	96 well plat
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		sample	e(x120)
	Nuclease free water	48	5760
	Exonuclease I (NEB)	2	240
	Total	50	600

75 37 °C for 1 hour. 37 °C 01:00:00

1st Strand Purification

76 Add 70 µL AMPureXP beads per well of a deepwell plate. Transfer 100 µL sample to deepwell plate. Mix thoroughly and incubate at room temperature for 10 minutes.

77 Place plate on magnet and wait until beads pellet. Remove supernatant.

78 Add 180 µL 80% ethanol off the magnet and mix thoroughly with pipetting.

79 Place plate on magnet and wait until beads pellet. Remove supernatant.

80 Add 180 µL 80% ethanol on the magnet.

81 Remove supernatant and air dry at 50 °C 00:05:00

82 Resuspend beads in 49 µL second strand master mix

Reagent	amount	96 well
---------	--------	---------



		per sam ple	plat e (x12 0)
	Nucl ease free water	40	480 0
	10x Blue buff er	5	600
	dNT P mix (10m M each)	2	240
	Sec ond stra nd oligo (10u M)	2	240
	Total	49	588 0

Note

Second strand oligo: TGCTGAACCGCTCTTCCGATCTNNNNNN (HPLC purified from IDT). This primer contains the Illumina PE read 2 sequence. Users wishing to use Truseq indexing primers (instead of iPCRTag) should redesign this so that it matches the Truseq read 2 sequence (TCAGACGTGTGCTCTTCCGATC).

- 83 Transfer second strand mix containing beads to a fresh 96w PCR plate.

Second strand synthesis

- 84 Incubate on a thermocycler:

98 °C 00:02:00 , then immediatly cool on ice.



85 Add  0.5 μL Klenow exo- (50U/ μL , Enzymatics) and incubate:

 4 °C  00:05:00

Slow ramp from  4 °C to  37 °C at 30s per 1C


 37 °C  01:30:00

 4 °C hold.

2nd Strand Purification

86 Prepare an aliquot of AMPure buffer by pelleting AMPure XP beads using a magnet or centrifuge and taking the supernatant.

87 Add  70 μL AMPure buffer and  50 μL water per well of a deepwell plate.

88 Transfer sample, containing beads, to the deepwell plate containing AMPure buffer and water. Mix thoroughly then incubate at room temperature  00:10:00

89 Place plate on magnet and wait until beads pellet.

90 Remove supernatant.

91 Wash twice with 80% ethanol.

92 Remove supernatant. Air dry  50 °C  00:05:00

93 Resuspend beads in  48 μL PCR master mix :

	Reagent	amount per sample	96 well plate(x120)
	Water	22	2640
	KAPA Hifi	25	3000



	read ymix (2x)		
	PE1. 0 (10u M)	1	120
	Total	48	576

Note

PE1.0:
AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGATC*T (IDT, HPLC)
This primer contains the full Illumina P5 and PE read 1 sequences.

94 Add  2 µL iTAG indexing primer (5uM)

Note

We prepare iTAG primers in 96w plates for this step.
iPCRTag: CAAGCAGAAGACGGCATACGAGATXXXXXXXXXGAGATCGGTCTCGGCATT
CCTGCTGAACCGCTCTTCCGATC*T (IDT, HPLC, XXXXXXXX = 8nt index)
This primer contains the full Illumina P7 sequence, followed by an index, followed by the Illumina PE read 2 sequence.

95 Tranfer samples to fresh 96w PCR plate.

Library amplification

96 Cycle as follows:

 95 °C  00:02:00

14 cycles of:

 94 °C  00:01:20

 65 °C  00:00:30

 72 °C  00:00:30

Followed by:



72 °C 00:05:00

4 °C hold.

Library purification

97 All subsequent steps are performed outside of pre-PCR room.

98 Make 2 pools of 48 libraries (5ul each) in 1.5ml tubes.

Note

The number of samples per pool depends on the number of indexes used. e.g. we typically use only 48 indexes and so make two pools of 48 samples each.

99 Purify each pool with 168 µL AMPure XP beads (i.e. 0.7x ratio), 2× 80% ethanol washes and elute in 100 µL water .

100 Purify each pool a second time 70 µL AMPure XP beads (i.e. 0.7x ratio), 2× 80% ethanol washes and elute in 100 µL water .

101 QC using a Bioanalyser high sensitivity chip.

Expected result

Pooled and purified scBS libraries should have a fragment length of 300-1000bp (average 450-500). Pools containing smaller fragments (especially <200bp) will result in poor alignment rates due to the presence of adapter concatemers. These should be subjected to an additional 0.7x AMPure XP purification.