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scNMT-seq V.3

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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 accessiblity 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 pysically separating before the gDNA is bisulfite converted. This mRNA on beads is pocessed 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 iradiation. We also reccomend 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 multichanel 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 reccommend 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 yeild 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-desigining 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/AAGCAGTGGTATCAACGCAGAGTACTTTTTTTTTTTTTTTTTTTTTTTTTTTVN
	Smartseq2 TSO	AAGCAGTGGTATCAACGCAGAGTACATrGrG+G
	ISPCR	AAGCAGTGGTATCAACGCAGAGT
	First strand oligo	/5SpC3/CTACACGACGCTCTTCCGATCTNNNNNN
	Second strand oligo	TGCTGAACCGCTCTTCCGATCTNNNNNN
	PE1.0	AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGATC*T
	iPCRTag	CAAGCAGAAGACGGCATACGAGATXXXXXXXXGAGATCGGTCTCGGCATTCCTGCTGAACCGCTCTTCCGATC*T

	iTag sequencing primer	AAGAGCGGTTCAGCAGGA ATGCCGAGACCGATCTC

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

Troubleshooting

Single-cell collection and methylase reaction

1 Prepare GpC methylase reaction buffer:

	Reagent	amount per sample	96 well plate (x120)
	Nuclease free water	1.425	171
	GpC methyltransferase buffer (10x)	0.25	30
	SAM	0.0125	1.5
	IGEPAL CA-630 (1%)	0.25	30
	RNase-in (40 U/ul)	0.0625	7.5
	M.CviPI (4U/ul)	0.5	60
	Total	**2.5**	**300**

2 Collect single cells in

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

Centrifuge at ≥1000g for ≥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 ≥1000g for ≥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 supernatent.

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

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 supernatent.

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 supernatent.

12 go to step #11

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

Note

bio-Smartseq2-dT: 5'-Biotin-TEG-AAGCAGTGGTATCAACGCAGAGTACTTTTTTTTTTTTTTTTTTTTTTTTTTTTVN-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 supernatent. Resuspend beads in 110 µL 1x B&W buffer (1M NaCl, 5mM Tris, 0.5mM EDTA) . Place on magnet and remove supernatent.

16 go to step #15

17 Place on magnet and remove supernatent. Resuspend beads in:
830 µL Nuclease free water ,
110 µL 5x first strand buffer (Invitrogen, Superscript II) ,
43 µL RNasin (Promega) ,
10 µL 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	**4355**

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
	Betaine (5M)	2	220
	DTT (100 mM, Superscript II)	0.5	55
	dNTPs (10mM each)	1	110
	MgCl2 (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 pelette. Aspirate 17.5 µL and transfer to empty lobind 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 pelette. Aspirate 15 µL and transfer to empty lobind 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 µL 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 µL 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 µL AMPure XP beads. Mix thoroughly and incubate for 5 minutes.

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

34 Elute cDNA in 25 µL water

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

Expected result

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

Nextera XT library prepation

36

Note

This section is performed outside of pre-PCR room.

In a 96 well or a 384 well plate, add 2.25ul 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 µL of each indexed cDNA library. Purify using 0.7x AMPure XP beads with 2× 80% ethanol washes.

43 QC using Bioanalyzer.

Genomic DNA purification

44

Add 50 µL AMPure XP beads to gDNA samples. Vortex thoroughly and incubate for 00:30:00

45 Place on a magnet until beads pellet. Remove supernatent, wash twice with 100 µL 80% ethanol . Remove supernatent. Immediatley resuspend in 10 µL 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 disolving 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 bisuflite converted DNA. Mix for 00:05:00

52 Plate on magnet until beads pellet. Remove supernatent.

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

54 Place on magnet until beads pellet. Remove supernatent.

55 Add 100 µL 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 µL 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:

	Reagent	amount per sample	96 well plate (x120)
	Nuclease free water	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 µL first strand synthesis mix . incubate at 60 °C 00:05:00

64 Place on magnet until beads pellet. Transfer 20 µL supernatent 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 thermocyler 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

	(10uM)		
	Klenow exo- (50U/ul)	0.5	55
	Total	**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 thermocylcer as follows:

4 °C 00:05:00

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

37 °C 00:30:00

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

73 For the fifth and final round, incubate for an additional 1 hour:

37 °C 01:00:00

4 °C hold.

Exonuclease treatment

74 50 µL Exonuclease mix

	Reagent	**amount per sample**	**96 well plate(x120)**
	Nuclease free	48	5760

	water		
	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 magent and wait until beads pellet. Remove supernatent.

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

79 Place plate on magent and wait until beads pellet. Remove supernatent.

80 Add 180 µL 80% ethanol on the magnet.

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

82 Resuspend beads in 49 µL second strand master mix

	Reagent	**amount per sample**	**96 well plate (x120)**

	Nuclease free water	40	4800
	10x Blue buffer	5	600
	dNTP mix (10mM each)	2	240
	Second strand oligo (10uM)	2	240
	Total	**49**	**5880**

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

85 Add 0.5 µL Klenow exo- (50U/ul, 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 supernatent.

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 supernatent.

91 Wash twice with 80% ethanol.

92 Remove supernatent. 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 readymix (2x)	25	3000

	PE1.0 (10uM)	1	120
	Total	**48**	**5760**

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: CAAGCAGAAGACGGCATACGAGATXXXXXXXXGAGATCGGTCTCGGCATT 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.