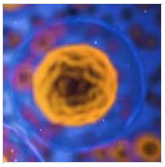

Version 3

Mar 31, 2021

Multiplexed scNOMe-seq protocol based on isolated single nuclei V.3

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2 *Works for me* dx.doi.org/10.17504/protocols.io.btutnnwn

Single_Cell_Uchicago

Sebastian Pott

SUBMIT TO PLOS ONE

ABSTRACT

What follows is the protocol for performing single-cell Nucleosome Occupancy and Methylome sequencing on single nuclei (scNOMe-seq). This plate-based method, which can be used to simultaneously assess nucleosome occupancy and DNA methylation in single nuclei, draws upon the approach laid out in Luo et al., 2018. Everything from the initial nuclei sorting step to the final library preparation that directly precedes sequencing is included in this protocol. This protocol is optimized for non-neuronal nuclei and can be applied to a range of different cell types (e.g., intestinal organoid, fibroblasts).

This is the method we are using and it is working.

Adapted from "Robust single-cell DNA methylome profiling with snmC-seq2," by C. Luo et al., 2018, *Nature Communications 9*(1), pp. 1-6. Copyright 2018 by the authors.

ATTACHMENTS

Multiplexed scNOMe-seq protocol based on isolated single nuclei references.pdf

DOI

dx.doi.org/10.17504/protocols.io.btutnnwn

PROTOCOL CITATION

Sebastian Pott, Michael Wasney, Nadia Khan 2021. Multiplexed scNOMe-seq protocol based on isolated single nuclei. **protocols.io**
https://dx.doi.org/10.17504/protocols.io.btutnnwn
Version created by Michael Wasney

KEYWORDS

single cell NOMe-seq, Bisulfite conversion, DNA methylation, Chromatin accessibility

CREATED

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LAST MODIFIED

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OWNERSHIP HISTORY

Mar 31, 2021 Sebastian Pott

PROTOCOL INTEGER ID

48755

GUIDELINES

Protocol Overview:

1. Nuclei Isolation and GpC Methylation
2. FACS sorting
3. Lysis
4. Bisulfite conversion
5. Random primed DNA Synthesis
6. Inactivation of Free Primers & dNTPs
7. Sample Cleanup
8. Adaptase Reaction
9. Library Amplification
10. Library Cleanup
11. Qubit Quantification and QC

MATERIALS TEXT

MATERIALS

S-adenosylmethionine (SAM) (32mM) - 0.5 ml **New England Biolabs Catalog #B9003S**

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

Shrimp Alkaline Phosphatase (rSAP) - 500 units **New England Biolabs Catalog #M0371S**

Magnesium Chloride **Fisher Scientific Catalog #AC223210010**

Sera-Mag SpeedBeads Carboxylate-Modified Magnetic Particles **Ge Healthcare Catalog #44152105050350**

RNase Inhibitor **Lucigen Catalog #30281-2**

Ethylenediaminetetraacetic Acid (0.5M Solution/pH 8.0), Fisher BioReagents **Fisher Scientific Catalog #BP2482-500**

Tris-HCl **Sigma Aldrich**

Elution Buffer (EB) **Qiagen Catalog #19086**

PBS - Phosphate-Buffered Saline (10X) pH 7.4 **Invitrogen - Thermo Fisher Catalog #AM9625**

NP-40 **Sigma Aldrich**

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Tris (1 M), pH 8.0, RNase-free **Thermo Fisher Catalog #AM9855G**

Poly Ethylene Glycol (PEG) 8000 **Sigma Aldrich Catalog #89510-250G-F**

Sodium Chloride (5M) **Invitrogen - Thermo Fisher Catalog #AM9760G**

10X GpC Methyltransferase Buffer **New England Biolabs Catalog #B0227S**

M-Digestion Buffer (2X) **Zymo Research Catalog #D5021-9**

Proteinase K w/ storage buffer set **Zymo Research Catalog #D3001-2**

CT Conversion Reagent **Zymo Research Catalog #D5001-1**

M-Solubilization Buffer **Zymo Research Catalog #D5021-7**

M-Dilution Buffer **Zymo Research Catalog #D5002-2**

M-Reaction Buffer **Zymo Research Catalog #D5021-8**

M-Binding Buffer **Zymo Research Catalog #D5040-3**

M-Wash Buffer **Zymo Research Catalog #D50074**

M-Desulphonation Buffer **Zymo Research Catalog #D5040-5**

M-Elution Buffer **Zymo Research Catalog #D5041-6**

10X Blue Buffer **Enzymatics Catalog #B0110L**

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

dNTP (10mM each) **Contributed by users**

Exonuclease I **Enzymatics Catalog #X8010L**

Accel-NGS® Adaptase™ **Swift Biosciences Catalog #33096**

Kapa HiFi HotStart ReadyMix (2X) **Kapa Biosystems Catalog #KM2602**

Primers:

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Primer	Sequence (5' to 3')
P5L_AD001_H	/5SpC3/TTCCCTACACGACGCTCTTCCGATCTATCACG(H1:33340033)(H1)(H1)(H1)(H1)(H1)(H1)(H1)(H1)
P5L_AD002_H	/5SpC3/TTCCCTACACGACGCTCTTCCGATCTCGATGT(H1:33340033)(H1)(H1)(H1)(H1)(H1)(H1)(H1)(H1)
P5L_AD004_H	/5SpC3/TTCCCTACACGACGCTCTTCCGATCTTGACCA(H1:33340033)(H1)(H1)(H1)(H1)(H1)(H1)(H1)(H1)
P5L_AD006_H	/5SpC3/TTCCCTACACGACGCTCTTCCGATCTGCCAAT(H1:33340033)(H1)(H1)(H1)(H1)(H1)(H1)(H1)(H1)
P5L_AD007_H	/5SpC3/TTCCCTACACGACGCTCTTCCGATCTCAGATC(H1:33340033)(H1)(H1)(H1)(H1)(H1)(H1)(H1)(H1)
P5L_AD008_H	/5SpC3/TTCCCTACACGACGCTCTTCCGATCTACTTGA(H1:33340033)(H1)(H1)(H1)(H1)(H1)(H1)(H1)(H1)
P5L_AD010_H	/5SpC3/TTCCCTACACGACGCTCTTCCGATCTTAGCTT(H1:33340033)(H1)(H1)(H1)(H1)(H1)(H1)(H1)(H1)
P5L_AD012_H	/5SpC3/TTCCCTACACGACGCTCTTCCGATCTCTTGTA(H1:33340033)(H1)(H1)(H1)(H1)(H1)(H1)(H1)(H1)
P5ind_501	AATGATACGGCGACCACCGAGATCTACACACGATCAGACACTCTTTCCCTACACGACGCTCT
P5ind_502	AATGATACGGCGACCACCGAGATCTACACTCGAGAGTACACTCTTTCCCTACACGACGCTCT
P5ind_503	AATGATACGGCGACCACCGAGATCTACACCTAGCTCAACACTCTTTCCCTACACGACGCTCT
P5ind_504	AATGATACGGCGACCACCGAGATCTACACATCGTCTCACACTCTTTCCCTACACGACGCTCT
P5ind_505	AATGATACGGCGACCACCGAGATCTACACTCGACAAGACACTCTTTCCCTACACGACGCTCT
P5ind_506	AATGATACGGCGACCACCGAGATCTACACCCTTGGAAACACTCTTTCCCTACACGACGCTCT
P5ind_507	AATGATACGGCGACCACCGAGATCTACACATCATGCGACACTCTTTCCCTACACGACGCTCT
P5ind_508	AATGATACGGCGACCACCGAGATCTACACTGTTCCGTACACTCTTTCCCTACACGACGCTCT
P5ind_509	AATGATACGGCGACCACCGAGATCTACACATTAGCCGACACTCTTTCCCTACACGACGCTCT
P5ind_510	AATGATACGGCGACCACCGAGATCTACACCGATCGATACACTCTTTCCCTACACGACGCTCT
P5ind_511	AATGATACGGCGACCACCGAGATCTACACGATCTTGCACACTCTTTCCCTACACGACGCTCT
P5ind_512	AATGATACGGCGACCACCGAGATCTACACAGGATAGCACACTCTTTCCCTACACGACGCTCT
P7ind_701	CAAGCAGAAGACGGCATACGAGATAGGCAATGGTGACTGGAGTTCAGACGTGTGCTCTT
P7ind_702	CAAGCAGAAGACGGCATACGAGATTCACCTAGGTGACTGGAGTTCAGACGTGTGCTCTT
P7ind_703	CAAGCAGAAGACGGCATACGAGATCATACGGAGTGACTGGAGTTCAGACGTGTGCTCTT
P7ind_704	CAAGCAGAAGACGGCATACGAGATGTCATCGTGTGACTGGAGTTCAGACGTGTGCTCTT
P7ind_705	CAAGCAGAAGACGGCATACGAGATTTACCGACGTGACTGGAGTTCAGACGTGTGCTCTT
P7ind_706	CAAGCAGAAGACGGCATACGAGATACCTTCGAGTGACTGGAGTTCAGACGTGTGCTCTT
P7ind_707	CAAGCAGAAGACGGCATACGAGATACGCTTCTGTGACTGGAGTTCAGACGTGTGCTCTT
P7ind_708	CAAGCAGAAGACGGCATACGAGATACGCTTCTGTGACTGGAGTTCAGACGTGTGCTCTT

Equipment List:

- MicroAmp™ EnduraPlate™ Optical , 384-Well Clear Reaction Plates with Barcode (Thermo Fisher cat. no. 4483273)
- Olympus 96-Well PCR Plate, Full-Skirted (Genesee Scientific cat. no. 24-302)
- Zymo-Spin 384 Well Plate, 2 pack (Zymo cat. no. C2012)
- PlateOne® Deep 96-Well 2 mL Polypropylene Plate (USA-SCI. cat. no. 1896-2000)
- 15 mL Centrifuge Tubes (Olympus cat. no. 28-103)

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- 50 mL Centrifuge Tubes (Olympus cat. no. 28-106)
- 1.7 mL Microtube (Genesee Scientific cat. no. 24-282LR)
- 0.2 mL SnapStrip® II PCR Tubes (SSIbio cat. no. 3245-00)
- Microseal® B Adhesive Sealer (Bio-Rad cat. no. MSB-1001)
- 37°C Incubator
- 384-well and 96-well Compatible Thermocycler
- DynaMag™-96 Side Magnet (Thermo Fisher cat. no. 12331D)
- DynaMag™-2 Magnet (Thermo Fisher cat. no. 12321D)
- Sorvall ST40R with Swinging Bucket Rotor that can spin at 5,000xg

BEFORE STARTING

Prepare plates with digestion mix for FACS sorting.

Nuclei Isolation and GpC Methylation

1 Before commencing with nuclei isolation and GpC Methyltransferase step, prepare 384- or 96-well collection plates with **digestion mix**. This can be prepared the day before and kept at 4ºC.

Reagent	Reaction concentration (based on reaction volume)	Volumes for 2 384-well plates (+ 15%) (µL)	Volumes for single reaction (µL)
M-Digestion Buffer (2X)	1X	883.2	1
Proteinase K (1.9%)	0.095%	88.32	0.1
Distilled H2O		794.88	0.9
Total		**1766.4**	**2**

Reaction volume: 2 µL

1.1 To prepare **Proteinase K**, add 1.04 mL of Proteinase K Storage Buffer to one tube of Proteinase K (as per Zymo Kit instructions).

2 Also prepare a large quanity of **RSB buffer (10X**) before beginning nuclei isolation and treatment. From that, make a 1:10 dilution (i.e., RSB buffer 1X) for use in the experiment.

Miranda, T. B., Kelly, T. K., Bouazoune, K., Jones, P.A. (2010). Methylation-sensitive single-molecule analysis of chromatin structure. Current protocols in molecular biology. https://doi.org/10.1002/0471142727.mb2117s89

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2.1 **RSB buffer (10X)** recipe:
- 100mM Tris-HCl, pH 7.4
- 100mM NaCl
- 30mM $MgCl_2$

This stock can be stored for up to one year at 4ºC.

2.2 Mix enough RSB buffer (1X) for the rest of the nuclei isolation and GpC methylation step (make 5 mL to be safe).

3 Start with a suspension of single cells. Count cells and use ~5-10M cells for this protocol.

4 Transfer cells to 15 mL Falcon tube.

5 Spin for 5 minutes at 500xg at 4°C.
500 x g, 4°C, 00:05:00

6 Discard supernatant and wash once with ice cold PBS. Spin for 5 minutes at 500xg at 4°C.
500 x g, 4°C, 00:05:00

7 Discard supernatant and resuspend cells in 1 mL ice-cold RSB buffer (1X).

8 Incubate for 10 minutes at room temperature.
00:10:00 at room temperature

9 Add 15 µL 1% NP-40 solution (0.015% final concentration) to the cell suspension.
15 µl NP-40 (1%)

> The NP-40 concentration might need to be adjusted depending on cell type

10 Transfer cell suspension to a dounce tissue grinder (2 mL volume) and burst the cells with 15 strokes of the pestle (both A and B work).

11 Transfer lysed cells to a 1.5 mL eppendorf tube.

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12 Centrifuge cells for 5 minutes at 800xg at 4°C.
800 x g, 4°C, 00:05:00

13 Discard the supernatant **without disturbing the pellet.** Wash with 1 mL 1X RSB buffer (without NP-40).
1 mL 1X RSB (without NP-40)

14 Incubate in RSB buffer for 30 seconds – 1 minute
00:00:30 - **00:01:00**

15 Centrifuge for 5 minutes at 800xg at 4°C.
800 x g, 4°C, 00:05:00

16 Discard supernatant and resuspend in 1X GpC methyltransferase buffer such that there are 1M cells per 75 µL.

If there are <1M cells, resuspend the pellet in 75 µL

17 Prepare a 1.5 mL eppendorf tube with **GpC Methylase Reaction Mix** for incubation:

A	B	C
Reagent	**Reaction concentration (based on reaction volume)**	**Amount (µL)**
GpC methyltransferase buffer (10X) (NEB)	0.5X	7.5
SAM (32mM) (NEB)	320µM	1.5
GpC Methytransferase (4U/µL) (NEB)	1.33U/µL	50
Distilled H2O		16
Nuclei		75
total		**150**

Reaction volume: 150 µL

After adding 75 µL of nuclei to the final mixtures of the above ingredients, pipette to mix.

18 Incubate at 37ºC for 7.5 minutes.
37 °C 7.5 minutes

19 Add a boost of 25 µL GpC Methyltransferase (100U) and 0.75 µL 32mM SAM to the nuclei.
25 µl GpC methyltransferase **0.75 µl 32mM SAM**

20 Incubate at 37°C for 7.5 minutes.

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37 °C 7.5 minutes

21 Add 500 µL 1X PBS and spin for 5 minutes at 800 g at 4°C.

500 µl of 1X PBS

800 x g, 4°C, 00:05:00

22 Remove supernatant and resuspend in **0.5-1 mL** 1X PBS.

500 µl – **1 mL 1X PBS**

23 Add 2 drops of Hoechst.

If the cells are resuspended in <500 µL of 1X PBS, use 1 drop of Hoechst

24 Keep on ice for ~15 minutes before FACS sorting.

On ice ~15 minutes

25 After FACS, heat plates to 50ºC for 20 minutes in a thermocycler to facilitate nuclei lysis.

50 °C 20 minutes

Store plates at -20ºC or move on to the next step.

Bisulfite Conversion

26 Add 15 µL **CT conversion reagent** to each well of 2 384-well plate. Pipette up and down 8 times to mix the sample.

15 µl CT conversion reagent

26.1 To prepare **CT Conversion Reagent** add the following buffers to one bottle of CT Conversion Reagent:

- 7.9 mL M-Solubilization Buffer
- 3 mL M-Dilution Buffer

Once the reagent is fully dissolved through shaking and vortexing vigorously, add:

- 1.6 mL M-Reaction Buffer

27 Seal the plates with adhesive film and quick spin for 10 seconds at 2,000xg at room temperature. 10s

2000 x g, Room temperature , 00:00:10

28 Place the plate in a thermocycler and run the following program:

98°C 8 minutes
64°C 3.5hrs
4°C Hold

29 Prior to cleaning up bisulfite conversion reactions, make **Random Primer Solution** for each of the 8 primers being

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used such that each is at a final molarity of 500nM. Keep on ice.

Reagent	Reaction concentration (based on reaction volume)	Volumes for 2 384-well plates (+ 8 extra reactions) (µL)	Volumes for single reaction (µL)
Random primer stock (100µM)	500nM	3.64	0.035
M-Elution Buffer		728	7
Total		**731.64**	**7**

Reaction volume: 7 µL

30 Place two Zymo-Spin 384-Well DNA Binding Plate on two 2.0 mL 96-Well Deep Well Plates.

31 Load 80 µL M-Binding Buffer to each well of the Zymo-Spin 384-Well DNA Binding Plates.

80 µl M-Binding Buffer

32 Transfer bisulfite conversion reactions to the Zymo-Spin 384-Well DNA Binding Plates. Pipette up and down 8 times to mix the sample.

33 Centrifuge for 5 minutes at 5,000xg.

5000 x g, Room temperature , 00:05:00

34 Discard the flow through by decanting and add 100 µL M-Wash Buffer to each well of the 384-Well DNA Binding Plates.

100 µl of M-Wash buffer

35 Centrifuge for 5 minutes at 5,000xg.

5000 x g, Room temperature , 00:05:00

36 Discard the flow through by decanting and add 50 µL M-Desulphonation Buffer to each well of the 384-Well DNA Binding Plates.

50 µl M-Desulphonation Buffer

37 Incubate at room temperature for 15 minutes.

Room temperature 15 minutes

38 Centrifuge for 5 minutes at 5,000xg.

5000 x g, Room temperature , 00:05:00

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39 Discard the flow through by decanting and add 100 µL M-Wash Buffer to each well of the 384-Well DNA Binding Plates.

100 µl M-Wash Buffer

40 Centrifuge for 5 minutes at 5,000xg.

5000 x g, Room temperature , 00:05:00

41 Repeat wash steps (39 and 40) once more.

42 Place the 384-Well DNA Binding Plates on 2 new 384-well PCR plates. Add 7 µL Random Primer Solution to each well of the 384-Well DNA Binding Plates (fig. 1).

7 µl Random Primer Solution

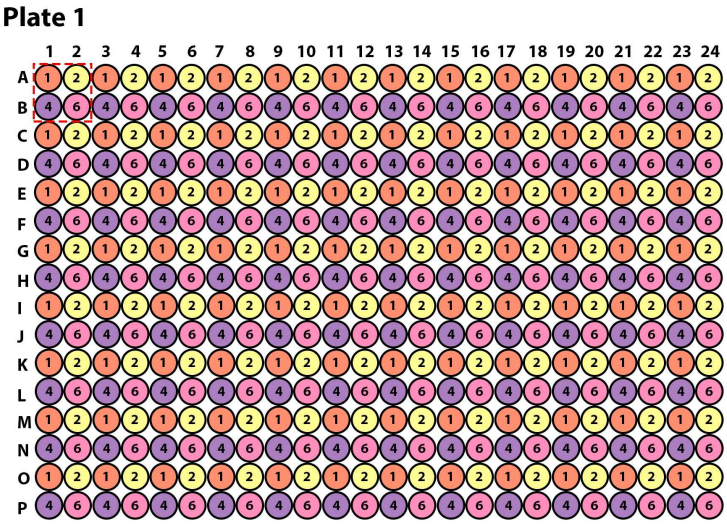

Figure 1a. Primer scheme for plate 1 (the 8 wells inside of the red squares will be pooled into a single well of a 96-well plate in the sample cleanup step).

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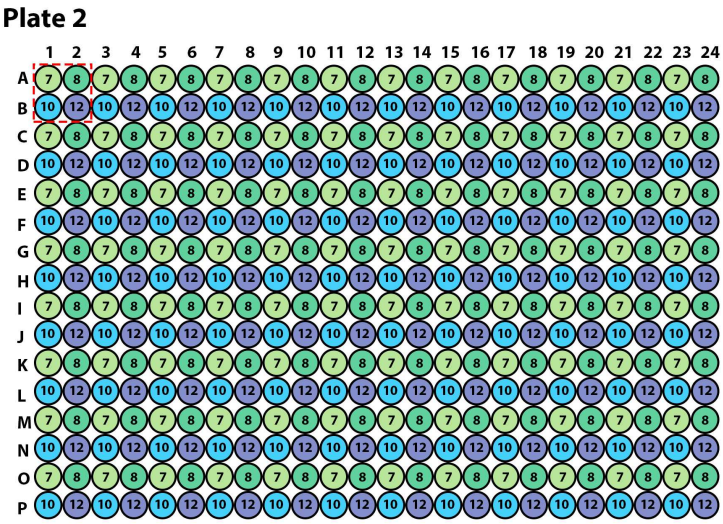

Figure 1b. Primer scheme for plate 2 (the 8 wells inside of the red squares will be pooled into a single well of a 96-well plate in the sample cleanup step).

43 Incubate for 5 minutes at room temperature.

Room temperature 5 minutes

44 Centrifuge for 5 minutes at 5,000xg and discard the 384-Well DNA Binding Plate.

5000 x g, Room temperature , 00:05:00

45 Seal the 384-well PCR plate with adhesive film and store at -20°C for up to 1 week.

-20 °C for up to 1 week

Random-primed DNA synthesis

46 Prior to denaturing the samples, make **Random Priming Master Mix** and keep sealed on ice:

A	B	C	D
Reagent	**Reaction concentration (based on reaction volume)**	**Volumes for 2 384-well plates (+ 20%) (µL)**	**Volumes for single reaction (µL)**
Blue Buffer (10X)	1X	922	1
Klenow exo (50U/µL)	1.25U/µL	231	0.25
dNTP (10mM each)	500uM each	461	0.5
Distilled H2O		2995	3.25
Total		**4609**	**5**

Reaction volume: 10 µL

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Reaction volume is 10 µL because it's assumed that 2 µL is lost during the centrifugation in step 44 (Luo et al., 2018)

47 Denature the samples by placing the 384-well PCR plates on a thermocycler and run the following program:

95°C 3 minutes

48 Immediately place the plate on ice for 2 minutes.

On ice 2 minutes

49 Add 5 µL Random Priming Master Mix to each well of the 384-well PCR plates.

5 µl Random Priming Master Mix

50 Vortex to mix the samples and quick spin for 10 seconds at 2,000xg.

2000 x g, 00:00:10

51 Place the plates in a thermocycler and run the following program:

4°C 5 minutes
25°C 5 minutes
37°C 60 minutes
4°C Hold

Inactivation of Free Primers & dNTPs

52 Add 1.5 µL **Exo/rSAP Master Mix** to each well of the 384-well PCR plates.

1.5 µl Exo/rSAP Mix

A	B	C	D
Reagent	**Reaction concentration (based on reaction volume)**	**Volumes for 2 384-well plates (+20%) (µL)**	**Volumes for single reaction (µL)**
Exonuclease I (20U/µL)	1.74U/µL	922	1
rSAP (1U/µL)	0.043U/µL	461	0.5
Total		**1383**	**1.5**

Reaction volume: 11.5 µL

53 Vortex and quick spin for 10 seconds at 2,000xg.

2000 x g, 00:00:10

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54 Place the plate in a thermocycler and run the following program:

37°C 30 min
4°C Hold

Sample clean-up

55 Before proceeding with sample clean-up, prepare enough **SPRI beads** (14 mL) for the whole experiment. Store beads at 4°C and always take out 30 minutes prior to use.

55.1 Mix Sera-Mag SpeedBeads and transfer 280 µL to a 1.5 mL tube.

280 µl Sera-Mag SpeedBeads

55.2 Place beads on a magnetic stand until the solution is clear of beads. Carefully remove the supernatant.

55.3 Wash twice with 1 mL TE. For each wash, remove the tube from the magnet and mix by inversion.

1 mL TE

55.4 Resuspend washed beads in 280 µL TE.

280 µl TE

55.5 Add 2.52 g PEG 8000 to a new 50 mL sterile conical tube.

2.52 g PEG 8000

55.6 Add 2.8 mL of 5M NaCl to the 50 mL tube.

2.8 mL 5M NaCl

55.7 Add 140 µL 1M Tris-HCl pH=8.0 and 28 µL of 0.5M EDTA pH=8.0 to the 50 mL tube.

140 µl 1M Tris-HCl pH=8.0

28 µl 0.5M EDTA pH=8.0

55.8 Add 7-8 mL distilled H_2O and mix until PEG 8000 dissolves into solution.

7 mL distilled H2O – **8 mL distilled H2O**

55.9 Add the washed Sera-Mag SpeedBeads to the 50 mL conical tube.

55.10 Add enough distilled H_2O to bring the total volume up to 14 mL. Vortex before each use.

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56 Add 73.6 µL (0.8x) SPRI beads to each well of a clean 96-well PCR plate.

73.6 µl SPRI beads

57 Pool the samples from the two 384-well PCR plates to one 96-well PCR plate such that each well of the 96-well PCR plate holds a pool of 8 samples, with each of those samples having been indexed with *a different* distinct random primer during step 42.
(two rows of each 384-well plate combine in one row of the 96-well plate.)

58 Vortex and incubate for 5 minutes at room temperature.

Room temperature for 5 minutes

59 Quick spin for 10 seconds at 2,000xg at room temperature. 10s

2000 x g, Room temperature , 00:00:10

60 Place the 96-well PCR plate on the DynaMag™-96 Side Magnet and let stand until the solution in each well is clear of beads (~5 minutes).

61 Wash beads 3 times with 150 µL fresh 80% EtOH.

150 µl 80% EtOH

62 Remove all EtOH and let beads dry at room temperature. Do not overdry the beads.

63 Remove the plate from the magnet and add 10 µL Elution Buffer (Qiagen) to each well. Resuspend beads by pipette.

10 µl Elution Buffer (Qiagen)

64 Vortex and incubate for 5 minutes at room temperature.

Room temperature for 5 minutes

65 Quick spin for 10 seconds at 2,000xg at room temperature. 10s

2000 x g, Room temperature , 00:00:10

66 Place back on magnet and let stand until solution is clear (~5 minutes).

67 Transfer 10 µL of the supernatant from each well to a clean 96-well PCR plate. The plate can be stored at -20ºC.

10 µl of supernatant

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Adaptase Reaction

68 Prior to denaturing the samples, make **Adaptase Master Mix** and keep sealed on ice:

A	B	C
Reagent	Volumes for 1 96-well plate (+10 extra reactions) (µL)	Volumes for single reaction (µL)
Elution Buffer (Qiagen)	450.5	4.25
Buffer G1	212	2
Reagent G2	212	2
Reagent G3	132.5	1.25
Enzyme G4	53	0.5
Enzyme G5	53	0.5
Total	**1113**	**10.5**

Reaction volume: 20.5 µL

69 Denature the samples by placing 96-well plates on a thermocycler and run the following program:

95°C 3 min

70 Immediately place the plate on ice for 2 minutes.

On ice 2 minutes

71 Add 10.5 µL Adaptase Master Mix to each well of the 96-well PCR plate.

10.5 µl Adaptase Master Mix

72 Vortex and quick spin for 10 seconds at 2,000xg. 10s

2000 x g, 00:00:10

73 Place the plate in a thermocycler and run the following program:

37°C 30 min
95°C 2 min
4°C Hold

Library Amplification

74 Add 5 µL **PCR Primer Mix** to every well.

5 µl PCR Primer Mix

74.1 Dilute each **P5L primer** such that the final concentration is 600nM (0.6µM) after the two primers corresponding to each well are combined

Citation: Sebastian Pott, Michael Wasney, Nadia Khan (03/31/2021). Multiplexed scNOMe-seq protocol based on isolated single nuclei. https://dx.doi.org/10.17504/protocols.io.btutnnwn

Reagent	Mix concentration (based on reaction volume)	Volume (µL)
P5L stock (100µM)	1.2µM	1.2
Distilled H2O		98.8
Total		**100**

74.2 Dilute each **P7L primer** such that the final concentration is 1µM after the two primers corresponding to each well are combined.

Reagent	Mix concentration (based on reaction volume)	Volume (µL)
P7L stock (100µM)	2µM	2
Distilled H2O		98
Total		**100**

74.3 To a new 96-well PCR plate, add 3 µL of each P5L primer to individual columns and 3 µL of each P7L primer to individual rows (one P5L primer per column, one P7L primer per row). Each well contains the PCR Primer Mix for the corresponding well in the sample plate. (Fig. 2)

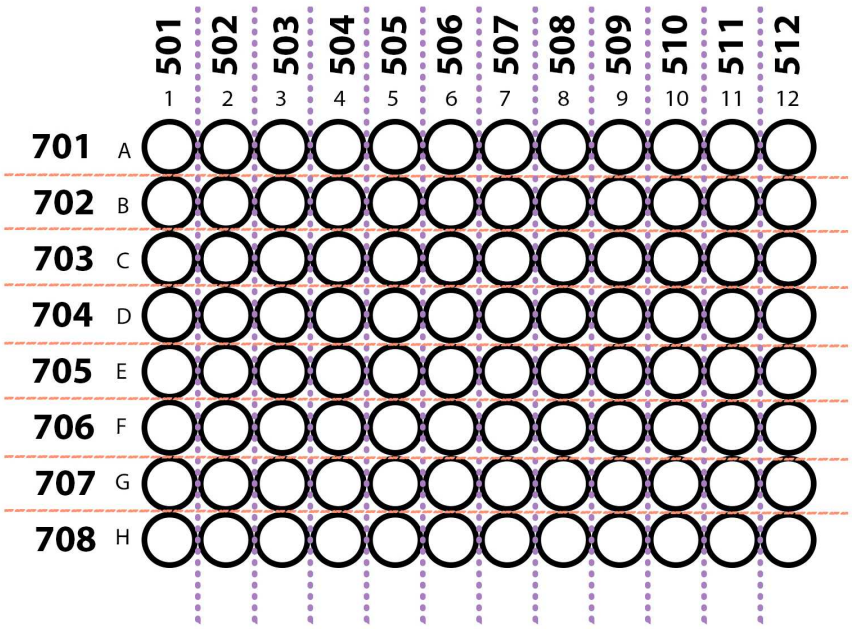

Figure 2. Each well receives a unique P5L-P7L primer combination.

74.4 Transfer 5 µL of the appropriate primer mix to the wells of the sample plate.

5 µl PCR Primer Mix

75 Add 25 µL 2X KAPA HiFi Mix to each well.

25 µl 2X KAPA HiFi Mix

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76 Vortex and quick spin for 10 seconds at 2,000xg.

2000 x g, 00:00:10

77 Place the plate in a thermocycler and run the following program:

a. 95°C 2 minutes
b. 98°C 30 seconds
c. 98°C 15 seconds
d. 64°C 30 seconds
e. 72°C 2 minutes
Return to step c 14 times (15 cycles total)
f. 72°C 5 minutes
g. 4°C Hold

Number of cycles can be adjusted

Library clean-up 20s

78 Add 40 µL (0.8x) SPRI Beads to each well of the 96-well PCR plate containing the sample.

40 µl SPRI beads

79 Vortex and incubate for 5 minutes at room temperature.

Room temperature for 5 minutes

80 Quick spin for 10 seconds at 2,000xg at room temperature. 10s

2000 x g, Room temperature , 00:00:10

81 Place 96-well plates on DynaMag™-96 Side Magnet, let stand until solution in wells is clear of beads (~5 minutes).

82 Remove supernatant and wash beads 2 times with 150 µL freshly made 80% EtOH.

150 µl 80% EtOH

83 Remove all EtOH after the last wash and remove plate from magnet. Let beads dry at room temperature. DO NOT overdry beads.

84 Add 25 µL Elution Buffer (Qiagen) and resuspend beads by pipette.

25 µl Elution Buffer (Qiagen)

85 Vortex and incubate for 5 minutes at room temperature.

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Room temperature for 5 minutes

86 Quick spin for 10 seconds at 2,000xg at room temperature. 10s

2000 x g, Room temperature , 00:00:10

87 Place back on magnet and let stand until solution is clear of beads (~5 minutes).

88 Combine 25 µL eluent from all wells in each column of the 96-well plate (8 wells per column, 12 columns) into 12 1.5 mL Eppendorf tubes.

25 µl eluent

89 Add 160 µL (0.8x) SPRI Beads to each 1.5 mL Eppendorf tube. Pipette to mix and incubate for 5 minutes at room temperature.

160 µl SPRI Beads

90 Place 1.5 mL tubes on DynaMag™-2 Magnet, let stand until solution in tubes in clear of beads (~5 minutes).

91 Remove supernatant and wash beads 2 times with 500 µL fresh 80% EtOH.

500 µl 80% EtOH

92 After the last wash, remove all EtOH and let beads dry at room temperature. DO NOT overdry beads.

93 Add 40 µL Elution Buffer (Qiagen) and resuspend beads by pipet. Incubate for 5 minutes at room temperature.

40 µl Elution Buffer (Qiagen)

94 Place tubes back on magnet and let stand until solution is clear of beads (~5 minutes).

95 Transfer 40 µL supernatant to 12 new 1.5 mL Eppendorf tubes.

40 µl eluent

96 Measure concentration of each 1.5 mL Eppendorf tube with Qubit dsDNA BR Assay Kit.

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