Simultaneous measurement of DNA methylation and nucleosome occupancy in single cells using scNOMe-seq

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Abstract

Single-cell Nucleosome Occupancy and Methylome sequencing (scNOMe-seq) is a multimodal assay that simultaneously measures endogenous DNA methylation and nucleosome occupancy (i.e., chromatin accessibility) in single cells. scNOMe-seq combines the activity of a GpC methyltransferase, an enzyme which methylates cytosines in GpC dinucleotides, with bisulfite conversion, whereby unmethylated cytosines are converted into thymines. Because GpC methyltransferase acts only on cytosines present in non-nucleosomal regions of the genome, the subsequent bisulfite conversion step detects not only the endogenous DNA methylation, but also reveals the genome-wide pattern of chromatin accessibility. Implementing this technology at the single-cell level helps to capture the dynamics governing methylation and accessibility vary across individual cells and cell types. Here, we provide a scalable plate-based protocol for preparing scNOMe-seq libraries from single nucleus suspensions.

Keywords: scNOMe-seq, Single Cell, DNA methylation, nucleosome occupancy, chromatin accessibility, GpC Methyltransferase, bisulfite sequencing, epigenetic modification, fluorescence-activated cell sorting

1. Introduction

Multimodal technologies allow for the simultaneous characterization of multiple biological features in the same samples, making it possible to directly observe relationships between them [1–8]. Increasingly, these assays are being implemented at the level of single cells. Single-cell data reveals cell-type-specific features and regulatory dynamics that are not apparent in data from bulk assays.

Nucleosome Occupancy and Methylome sequencing (NOMe-seq) is a multimodal assay that quantifies both endogenous DNA methylation and nucleosome occupancy[9–11]—two genomic features associated with modulation of transcription—and has been adapted for application on single cells (scNOMe-seq) [1–3]**.** DNA methylation, which in mammalian cells almost always occurs in CpG dinucleotides, is strongly associated with transcriptional repression[12]. Nucleosomes block most DNA-dependent processes by hindering access of transcription factors and other cellular machinery to the underlying genetic sequence[13]. Conversely, nucleosome depleted regions (NDRs) identify gene regulatory elements, such as promoters and enhancers. In surveying both features at the single-cell level, scNOMe-seq can help to elucidate the functional relationship between DNA methylation and nucleosome occupancy within cell-type-specific contexts [14](**Figure 1**).

scNOMe-seq utilizes the *M.CviPi* GpC Methyltransferase to methylate all accessible cytosine residues in GpC dinucleotides. Importantly, these residues are normally unmethylated in mammalian cells [15]. By following GpC Methyltransferase treatment with bisulfite conversion, all unmodified cytosine residues are converted facilitating the detection of both accessible GpC residues and endogenous DNA methylation at CpG residues [1, 11].

To detect cytosine methylation this protocol adapts a plate-based library strategy developed for single cell bisulfite sequencing that use cellular barcoding to facilitate pooling[16, 17]. Sequenced scNOMe-seq libraries are aligned to a human genome, allowing for the methylation statuses of all captured cytosines in CpG and GpC contexts to be retrieved. Because CpG and GpC dinucleotides occur relatively frequently, a single read can simultaneously capture the accessibility and endogenous methylation of the genomic locus to which the read maps.

The protocol below starts from single cell suspensions and combines initial preparation and GpC treatment of nuclei [18] with preparation of single-cell bisulfite libraries [16]. However, in our experience this protocol can be performed following most nuclei isolations, and we successfully used nuclei isolated from frozen human heart samples (**Figure 2**).

2. Materials

While performing this protocol, use ultrapure water and analytical grade reagents to prepare solutions and follow institutional and material-related safety guidelines when disposing of waste.

2.1 Reagents/consumables

2.1.1 Nuclei isolation and GpC Methyltransferase treatment

1. Digestion mix: Prepare 1.9% Proteinase K solution by adding 1,040 µl of Proteinase K Storage buffer to one tube with 20 mg of proteinase K (Zymo D3001-2-20) and allow Proteinase K to dissolve completely. Store Proteinase K solution at -20ºC between uses. To isolate nuclei, mix 883 µl of M-Digestion Buffer (Zymo D5020-9), 88 µl Proteinase K

solution, and 795 µl water. Digestion mix can be prepared the day before an experiment and stored at 4ºC. **(See note 2)**

2. 1X RSB Buffer [18]: Prepare a stock of 10X RSB with 100 mM Tris-HCL, pH 7.4, 100 mM NaCl, and 30 mM $MgCl_2$. Make at least 3 mL of 1X RSB (1:10 dilution of 10X RSB) per sample being processed.
3. 1X PBS
4. 1% NP-40: Make 1 mL of 1% NP-40 from 10% NP-40 by combining 10 µL of 10% NP-40 with 90 µL of water.
5. GpC Methylase reaction mix: Combine 7.5 µL 10X GpC Methyltransferase buffer, 1.5 µL 32 mM SAM, 50 µL 4U/µL GpC Methyltransferase (NEB M0277L). Nuclei will be added directly to this mix for the methylase reaction. Reserve 25 µL 4U/µL GpC Methyltransferase and 0.75 µL 32 mM SAM to boost the reaction.

2.1.2 Fluorescence-activated cell sorting

1. NucBlue™ Live Cell Stain ReadyProbes (Invitrogen R37605)

2.1.3 Bisulfite conversion

1. CT conversion reagent: Add 7.9 mL M-Solubilization Buffer and 3 mL M-Dilution Buffer to one bottle of CT Conversion reagent. Vortex vigorously for at least 10 minutes to fully dissolve the reagent, and then add 1.6 mL M-Reaction buffer (Zymo D5022). Prepared CT Conversion reagent can be stored overnight at room temperature, for a week at 4ºC, or for up to one month at -20ºC. If stored at 4ºC or -20ºC, warm solution at 37ºC prior to use.
2. EZ-96 DNA Methylation-Direct Kit (shallow-well) (Zymo D5022)

- M-Binding buffer
- M-Wash buffer: To prepare one bottle of M-wash buffer, add 144 mL of 100% ethanol to the 36 mL of concentrate in the bottle provided by the kit
- M-Desulphonation buffer
- M-Elution buffer

3. Random Primer Solution: Prepare Random Primer Solution prior to the purification by adding 64 µL 100 µM random primer stock to 728 µL M-Elution buffer. Keep on ice.

2.1.4 Random-primed DNA synthesis

1. Random Priming Master Mix: Pior to denaturing the samples as part of the Random-primed DNA synthesis step, combine 922 µL 10X Blue Buffer, 231 µL of 50 U/µL Klenow fragment (Qiagen Beverly P7010HCL), 461 µL of dNTP solution with each nucleotide at a concentration 10 mM (Thermo Fisher R0191), and 2995 µL of water. Keep on ice.

2.1.5 Inactivation of free primers and dNTPs

1. Exo/rSAP Master Mix: Prior to beginning inactivation step combine 922 µL of 20 U/µL Exonuclease I and 461 µL of 1 U/µL rSAP (Qiagen Beverly X8010L). Keep on ice.

2.1.6 Sample clean-up

1. SPRI Beads: Apportion 280 µL of Sera-Mag SpeedBeads into an Eppendorf tube and place on a magnetic stand. Allow the solution to clear of beads before carefully removing supernatant. Wash the beads—now sequestered to one side of the tube due to the magnetic stand—twice with 1 mL TE. Between washes, remove the tube from the magnet and mix by inversion before replaces tube on the stand and allowing the beads to clear. After the second wash, resuspend beads in 280 µL of TE. Meanwhile, transfer 2.52 g PEG 8000 to a 50 mL conical tube. Add 2.8 mL of 5M NaCl, 140 µL 1M Tris-HCl, pH 8, and 28 µL of 0.5M

EDTA, pH 8. Add 7 to 8 mL of water and vortex the solution until the PEG 8000 has dissolved. Add the washed Sera-Mag SpeedBeads and bring the solution up to 14 mL with water. Store at 4ºC. **(See note 3)**

2. 80% Ethanol: to make 50 mL of 80% ethanol, combine 40 mL 200 proof ethanol and 10 mL water. Vortex before use.

2.1.7 Adaptase reaction

1. Adaptase Master Mix: combine 450.5 µL of Elution Buffer (Qiagen 19086), 212 µL Buffer G1, 212 µL Reagent G2, 132 µL Reagent G3, 53 µL Enzyme G4, and 53 µL Enzyme G5. Pipette to mix and keep on ice.

2.1.8 Library amplification

1. P5L PCR Primer Mix: 1.2 µM P5L primer (working concentration of 600 nM when combined with P7L primer). Mix 1.2 µL of 100 µM P5L stock with 98.8 water. Keep on ice before use.

2. P7L PCR Primer Mix: 2 µM P7L primer (working concentration of 1 µM after being combined with P5L primer). Mix 2 µL 100 µM P7L primer with 98 µL water. Keep on ice before use.

3. 2X Kapa Hifi Mix (Roche 07958935001)

2.1.9 Library clean-up

1. SPRI Beads
2. 80% Ethanol
3. Elution Buffer (Qiagen 19086)
4. Qubit 4 Fluorometer (Invitrogen or Qubit Flex Fluorometer (Invitrogen Q33327)

2.1.10 Primers and Barcodes

1. HPLC purified random primers (added after bisulfite conversion): H: A, G, or T

   Barcode 1:

   /5SpC3/TTCCCTACACGACGCTCTTCCGATCTATCACG(H1:33340033)(H1)(H1)(H1)(H1)(H1)(H1)(H1)(H1)

   Barcode 2:

   /5SpC3/TTCCCTACACGACGCTCTTCCGATCTCGATGT(H1:33340033)(H1)(H1)(H1)(H1)(H1)(H1)(H1)(H1)

   Barcode 3:

   /5SpC3/TTCCCTACACGACGCTCTTCCGATCTTGACCA(H1:33340033)(H1)(H1)(H1)(H1)(H1)(H1)(H1)(H1)

   Barcode 4:

   /5SpC3/TTCCCTACACGACGCTCTTCCGATCTGCCAAT(H1:33340033)(H1)(H1)(H1)(H1)(H1)(H1)(H1)(H1)

   Barcode 5:

   /5SpC3/TTCCCTACACGACGCTCTTCCGATCTCAGATC(H1:33340033)(H1)(H1)(H1)(H1)(H1)(H1)(H1)(H1)

   Barcode 6:

   /5SpC3/TTCCCTACACGACGCTCTTCCGATCTACTTGA(H1:33340033)(H1)(H1)(H1)(H1)(H1)(H1)(H1)(H1)

   Barcode 7:

   /5SpC3/TTCCCTACACGACGCTCTTCCGATCTTAGCTT(H1:33340033)(H1)(H1)(H1)(H1)(H1)(H1)(H1)(H1)

Barcode 8:

/5SpC3/TTCCCTACACGACGCTCTTCCGATCTCTTGTA(H1:33340033)(H1)(H1)(H1)(H1)(H1)(H1)(H1)(H1)

These random priming oligos differ from the ones provided by Swift Biosciences and were provided in Luo et al. [17].

2. P5 primers (added during library amplification)

P501:

AATGATACGGCGACCACCGAGATCTACACACGATCAGA**C**ACTCTTTCCCTACACGACGCTCT

P502:

AATGATACGGCGACCACCGAGATCTACACTCGAGAGTACACTCTTTCCCTACACGACGCTCT

P503:

AATGATACGGCGACCACCGAGATCTACACCTAGCTCAACACTCTTTCCCTACACGACGCTCT

P504:

AATGATACGGCGACCACCGAGATCTACACATCGTCTCACACTCTTTCCCTACACGACGCTCT

P505:

AATGATACGGCGACCACCGAGATCTACACTCGACAAGACACTCTTTCCCTACACGACGCTCT

P506:

AATGATACGGCGACCACCGAGATCTACACCCTTGGAAACACTCTTTCCCTAC

ACGACGCTCT

P507:

AATGATACGGCGACCACCGAGATCTACACATCATGCGACACTCTTTCCCTAC

ACGACGCTCT

P508:

AATGATACGGCGACCACCGAGATCTACACTGTTCCGTACACTCTTTCCCTACA

CGACGCTCT

P509:

AATGATACGGCGACCACCGAGATCTACACATTAGCCGACACTCTTTCCCTAC

ACGACGCTCT

P510:

AATGATACGGCGACCACCGAGATCTACACCGATCGATACACTCTTTCCCTAC

ACGACGCTCT

P511:

AATGATACGGCGACCACCGAGATCTACACGATCTTGCACACTCTTTCCCTACA

CGACGCTCT

P512:

AATGATACGGCGACCACCGAGATCTACACAGGATAGCACACTCTTTCCCTAC

ACGACGCTCT

3. P7 primers (added during library amplification)

P701

CAAGCAGAAGACGGCATACGAGATAGGCAATGGTGACTGGAGTTCAGACGT

GTGCTCTT

P702

CAAGCAGAAGACGGCATACGAGATTCACCTAGGTGACTGGAGTTCAGACGTG

TGCTCTT

P703

CAAGCAGAAGACGGCATACGAGATCATACGGAGTGACTGGAGTTCAGACGT

GTGCTCTT

P704

CAAGCAGAAGACGGCATACGAGATGTCATCGTGTGACTGGAGTTCAGACGTG

TGCTCTT

P705

CAAGCAGAAGACGGCATACGAGATTTACCGACGTGACTGGAGTTCAGACGTG

TGCTCTT

P706

CAAGCAGAAGACGGCATACGAGATACCTTCGAGTGACTGGAGTTCAGACGTG

TGCTCTT

P707

CAAGCAGAAGACGGCATACGAGATACGCTTCTGTGACTGGAGTTCAGACGTG

TGCTCTT

P708

CAAGCAGAAGACGGCATACGAGATGAGTAGAGGTGACTGGAGTTCAGACGT GTGCTCTT

2.2 Equipment

2.2.1 Assay

1. KIMBLE® Dounce tissue grinder set (DWK 885300-0001)
2. 384-well clear reaction plates (Applied Biosystems 4483285)
3. Adhesive PCR sealing foil sheets (Thermo Fisher 00139148)
4. Zymo-Spin 384-Well DNA Binding Plates (Zymo C2012)
5. Thermo Scientific™ Nunc™ 96-Well Polypropylene DeepWell™ Storage Plates (Thermo Fisher Scientific 95040462) (**See note 5)**
6. DynaMag™-96 Side Skirted Magnet (Thermo Fisher Scientific 12027)
7. 96-well PCR plate (Genesee Scientific 24-302)
8. DynaMag™-2 Magnet (Thermo Fisher Scientific 12321D)
9. 1.7 ml microtube, clear (Genesee Scientific 24-282LR)
10. 0.2 ml 8-well PCR strip tubes (Genesee Scientific 21-125)
11. Flow cytometer (e.g., BD FACSAria™ Fusion)
12. Thermocycler(s) with 96 and 384-well blocks
13. Centrifuge outfitted with a swinging bucket rotor capable of spinning at 5,000xg, maintaining a temperature of 4 ºC, and accommodating both plates and microtubes
14. 12-channel pipette set capable of handling volumes ranging from 0.5 µl – 300 µl
15. Standard wet laboratory equipment (e.g., pipette set, serological pipette, 4ºC refrigerator and -20ºC Freezer, etc.)

2.2.2 Sequencing

1. Access to next Generation Sequencing platform: Illumina-based sequencing platform (e. g., NovaSeq 6000)

3. Methods

3.1 Assay

3.1.1 Nuclei isolation and GpC Methyltransferase treatment

1. Prepare digestion mix on ice. Deliver 2 µL of mix to every well of two 384-well plates. Plates with digestion mix can be prepared the day before the experiment and stored at 4ºC. **(See note 2)**
2. Obtain a suspension of single cells. This protocol was optimized for use with a total of 5-10 million cells. Centrifuge single cell suspension at 500xg for 5 minutes at 4ºC, remove supernatant, suspend in 1 mL ice-cold PBS, and centrifuge the sample again at the same settings. Discard supernatant and suspend in 1 mL 1X RSB buffer. Incubate for 10 minutes at room temperature.
3. Add 15 µL of 1% NP-40 to the cell suspension (NP-40 concentration may need to be adjusted depending on the cell type). Transfer cell suspension to a 2 mL dounce tissue grinder and add 1 mL of 1X RSB. Homogenize cell suspension using 15 strokes of both pestle A and B (number of strokes may be adjusted to accommodate the particular cell-/tissue-type being handled). Transfer lysed cells to a new 1.5 mL Eppendorf tube and centrifuge at 800xg for 5 minutes at 4ºC. Discard supernatant and wash with 1 mL 1X RSB. Incubate for 30 seconds to 1 minute at room temperature. Centrifuge at 800xg for 5 minutes at 4ºC.

4. Resuspend nuclei in 1X GpC methyltransferase buffer such that there are 1 million nuclei per 75 µL of buffer. If there are less than 1 million nuclei, suspend in 75 µL of buffer. Meanwhile, prepare GpC Methylase Reaction Mix. Add the 75 µL of nuclei to the reaction mix and incubate at 37ºC for 7.5 minutes. Add a boost of 25 µL GpC Methyltransferase and 0.75 µL 32 mM SAM and incubate for another 7.5 minutes at 37ºC. (**See note 4**)
5. Quench the reaction by adding 500 µL 1X PBS and spin at 800xg for 5 minutes at 4ºC. Resuspend in 0.5 – 1 mL of 1X PBS and add 2 drops of Hoechst per mL of sample (1 drop for 0.5 mL, two drops for 1 mL). Keep sample on ice for 15 minutes before commencing with Fluorescence-activated cell sorting (FACS).

3.1.2 Fluorescence-activated cell sorting

1. We use the BD FACSAria™ Fusion system to sort individual nuclei into 384-well plates. However, any system with this capability should work equally well.
2. Our gating strategy is focused on recovering intact single nuclei and excluding cellular debris (**Figure 3**). This needs to be adjusted based on the input material.
3. Sort a single nucleus into each well of the 384-well plates being processed. Place plates on ice when sorting is complete.

3.1.3 Bisulfite conversion

1. Prepare CT Conversion Reagent and add 15 µL of it to each well of two 384-well reaction plates. Pipette up and down eight times to mix the sample. Seal the plates and quick spin at 2,000xg for 10 seconds at room temperature. Place plates into a thermocycler able to accommodate 384-well plates, and run the following program:
    a. 98ºC for 8 minutes

b. 64ºC for 3.5 hours

c. Hold at 4C

(See note 5)

2. Prior to purifying bisulfite-converted samples, prepare Random Primer Solutions (eight separate solutions for primers 1 through 8). Keep primer solutions on ice until use.
3. Place two Zymo-Spin 384-Well DNA Binding Plates on two 96-Well Polypropylene DeepWell™ Storage Plates and add 80 µL of M-Binding buffer to each well. Transfer bisulfite-converted samples to the 384-Well DNA Binding Plates and pipette up and down eight times to mix the samples. Centrifuge at 5,000xg for 5 minutes at room temperature. **(See note 6)**
4. Discard flow through in the 96-Well Storage Plates and add 100 µL M-Wash buffer to each well of the 384-well DNA binding plates. Centrifuge at 5,000xg for 5 minutes at room temperature.
5. Discard flow through in the 96-Well Storage Plates and add 50 µL M-Desulphonation buffer to each well of the 384-well DNA binding plates. Incubate at room temperature for 15 minutes, and then centrifuge at 5,000xg for 5 minutes at room temperature.
6. Discard flow through in the 96-Well Storage Plates. Add 100 µL M-Wash buffer and centrifuge at 5,000xg for 5 minutes at room temperature. Repeat this wash step once more.
7. Place 384-well DNA binding plates on 384-well reactions Plates (the two 96-Well Storage Plates can be disposed). Add 7 µL of one of the eight random primer solutions to each well of each plate, such that half of the primers are delivered to one plate and the one half are delivered to the other plate, and such that every other well—along both ranks

and files of the plates—contain the same primer (**Figure 4**) Once primers have been added to every well, incubate plates for 5 minutes and then centrifuge at 5,000xg for 5 minutes at room temperature. Seal the 384-well reaction plates and store at -20ºC for up to 1 week.

3.1.4 Random-primed DNA synthesis

1. Prepare Random Priming Master Mix prior to denaturing samples and keep mix on ice. (**See note 7**)
2. Denature samples by placing 384-well plates in a thermocycler and run the following program:
    a. 95ºC for 3 minutes
3. Place the plates on ice for 2 minutes.
4. Add 5 µL Random Priming Master Mix to each well of the 384-well reaction plates. Vortex to mix and quick spin at 2,000xg for 10 seconds at room temperature.
5. Place plates into a thermocycler and run the following program:
    a. 4ºC for 5 minutes
    b. 25ºC for 5 minutes
    c. 37ºC for 60 minutes
    d. Hold at 4ºC

3.1.5 Inactivation of free primers and dNTPs

1. Prepare Exo/rSAP Master Mix and keep on ice. Add 1.5 µL to each well of the 384-well reaction plates. Vortex to mix and quick spin at 2,000xg for 10 seconds at room temperature. **(See note 8)**
2. Place plates into a thermocycler and run the following program:

a. 37ºC for 30 minutes

b. Hold at 4C

3.1.6 Sample clean-up

1. Prepare 14 mL of SPRI beads.
2. Add 73.6 µL (0.8x) of SPRI beads to each well of a clean 96-well plates. Pool the samples from the two 384-well plates in the wells of the 96-well plate such that each well of the 96-well plates holds a pool of 8 samples, each with a distinct random barcode. Vortex the plates briefly and incubate for 5 minutes at room temperature.
3. Quick spin at 2,000xg for 10 seconds at room temperature and then place the 96-well plate on a DynaMag™-96 Side Skirted Magnet and let stand until the solution is clear of beads.
4. Remove supernatant and wash beads three times with 150 µL fresh 80% ethanol. After the third wash, remove the ethanol and allow the beads to dry at room temperature. Take care to not over dry beads.
5. Remove the plate from the magnet, add 10 µL of Elution Buffer (Qiagen), and suspend beads by pipetting. Vortex the plates briefly and incubate for 5 minutes at room temperature.
6. Quick spin at 2,000xg for 10 seconds at room temperature and then place the 96-well plate on a DynaMag™-96 Side Skirted Magnet and let stand until the solution is clear of beads.
7. Transfer 10 µL of the supernatant from each well to a clean 96-well plate. Store at -20ºC or move on to the next step of the protocol.

3.1.7 Adaptase reaction

1. Prepare Adaptase Master Mix prior to denaturing sample and keep mix on ice.
2. Denature the samples by placing the 96-well plate in a thermocycler and running the following program:
    a. 95ºC for 3 minutes
3. Place plate on ice for 2 minutes.
4. Add 10.5 µL Adaptase Master Mix to each well of the 96-well plate. Vortex to mix and quick spin at 2,000xg for 10 seconds at room temperature.
5. Place the plate in the thermocycler and run the following program:
    a. 37ºC for 30 minutes
    b. 95ºC for 2 Minutes
    c. Hold at 4ºC

3.1.8 Library amplification

1. Prepare P5L and P7L primer mixes. Add 5 µL of the appropriate primers to the each well of a clean 96-well plate, such that each well has a unique P5L-P7L combination (keep note of each combination's location in the plate). Transfer 5 µL of each P5L-P7L combination to the corresponding well in the 96-well plate containing the pooled samples.
2. Add 25 µL 2X Kapa Hifi Mix to each well of the 96-well plate containing the samples. Vortex to mix and quick spin at 2,000xg for 10 seconds at room temperature.
3. Place the plate in a thermocycler and run the following program:
    a. 95ºC for 2 minutes
    b. 98ºC for 30 seconds
    c. 98ºC for 15 seconds

d. 64ºC for 30 seconds

e. 72ºC for 2 minutes

Return to step **c.** 14 times for a total of 15 cycles.

f. 72ºC for 5 minutes

g. Hold at 4C

3.1.9 Library clean-up

1. Add 40 µL SPRI beads to each well of the 96-well plate. Vortex the plate briefly and incubate for 5 minutes at room temperature and then quick spin at 2,000xg for 10 seconds at room temperature. Place the plate on a DynaMag™-96 Side Skirted Magnet and allow the solution to clear of beads.
2. Remove supernatant and wash beads twice with 150 µL of freshly made 80% ethanol. After the final wash, remove the plate from the magnet and allow beads to dry at room temperature. Take care to not over dry beads.
3. Add 25 µL Elution Buffer (Qiagen) and suspend beads by pipette. Place back on the DynaMag™-96 Side Skirted Magnet and allow solution to clear of beads. Combine supernatant from each column into 12 Eppendorf tubes, such that there is one Eppendorf tube per 96-well plate column. Add 160 µL (0.8x) SPRI beads to each of the 12 Eppendorf tubes. Pipette to mix and incubate for 5 minutes at room temperature.
4. Place the Eppendorf tubes on a DynaMag™-2 Magnet and allow solution to clear of beads. Discard supernatant and wash beads 2 times with 500 µL of 80% ethanol. After the second wash, remove all ethanol and allow beads to dry at room. Take care to not overdry beads.

5. Add 40 µL Elution Buffer (Qiagen) and suspend beads by pipette. Incubate for 5 minutes at room temperature. After incubaton, transfer 40 µL of supernatant to 12 new Eppendorf tubes.
6. Measure concentration of the libraries using a Qubit Fluorometer and assess fragment size distribution with an Agilent 2100 Bioanalyzer Fragment sizes should fall between 300 and 1,500bp (**Figure 5**). On the fluorometer, libraries with a concentration of 2-15 ng/µl are to be expected. If concentration and size distributions are as expected, proceed with sequencing of the libraries.

3.2 Sequencing

1. Using an illumina-based next generation sequencing platform (e.g., NovaSeq 6000), sequence the libraries using pair end sequencing with a 200bp cassette. We generally aim to obtain 500,000 – 1 million reads per cell. **(See note 9)**

3.3 Analysis

A full description of the analysis is outside of the scope of this protocol describing the steps top generate scNOMe-seq libraries for sequencing. We provide an example of a processing pipeline for raw scNOMe-seq data at **[github link].**

4 Notes

4.1 General

1. All steps involving 96- and 384-well plates should be performed with a 12-tip multichannel pipette. Reagents can be split between 12-tube rows of strip tubes using a

single-channel and then transferred to their final destinations in the 96- and 384-well plates using a multichannel pipette.

2. M-Digestion buffer can form a white precipitate, which can be dissolved by keeping the buffer at 37ºC for 30 minutes prior to use.
3. Make SPRI fresh for each experiment. Before each use, allow beads to warm to room temperature for 30 minutes and vortex vigorously.
4. In practice we have observed relatively small changes in global GpC levels between a single 7.5 min incubation period and double that time. However, in order to reach saturation we continue to use ~15 minutes total incubation time.
5. It takes roughly 10 minutes of vigorous vortexing for the powdered CT conversion reagent to go into solution, and per the manufacturer's instruction manual, it is normal to see trace amounts of undissolved reagent even after extensive mixing.
6. It is best to use the two 384-well plates as balances for each other for all centrifugations during the sample purification portion of the bisulfite conversion workflow. During steps that require a bench rest, begin the timer only after the reagent has been delivered to the final row the second plate.
7. Prepare all master mixes in advance of their respective steps to avoid prolonged waiting times of the sample on ice or in the thermocycler.
8. The Exo/rSAP Master Mix is very viscous, which can make it difficult to aspirate equal amounts of fluid in all channels of a multichannel pipette. During this step, it is crucial to visually check the amount of fluid in each channel prior to delivering the solution to each well of the 384-well plates.

9. Sequencing parameters may change depending on the characteristics of your libraries, and how many finals pools you choose to submit. For example, a cassette with more base pairs may be desirable to obtain additional sequencing from each fragment for libraries with longer average fragment length. Choice of flowcell should be determined by the number of pools that are being multiplexed to achieve the optimal number of reads per cell.
10. Pipette tips should be replaced whenever they come in contact with the plates or with fluid in the plates' wells (this includes most, but not all, multichannel pipetting steps).
11. If working with a thermocycler that can only handle a single plate reaction plate at once, process the plates sequentially; that is, perform a step for the first of two plates, and store that plate at 4ºC as the same step is performed on the second plate. After both plates are complete, move on to the second step.
12. SPRI beads should be prepared fresh for each experiment. Allow beads to warm to room temperature and mix vigorously before use.
13. If the concentration of your libraries is lower than expected, consider the following:
    a. Increasing the cycle number (e.g., to 16 or 17) to achieve more highly-concentrated libraries. We have not observed a huge increase in redundant reads when increasing the amplification cycle number by one or two.
    b. Ensure good pipetting technique, particularly because multichannel pipettes can be more difficult to use than normal ones. If not used properly, not all channels will aspirate the same volume of liquid. Review the directions provided by your multichannel pipette's manufacturer prior to use.

17. Manual processing of four 384-well plates works well for us. Scaling the assay up beyond that might be difficult without the right reagents and equipment, however. We routinely use 8 barcodes, which allows for two 384-well plates to be pooled together (768 cells in total) as presented above. We successfully used 16 barcodes as well, which allows for four 384-well plates to be pooled together (1,536 cells in total). Because each 384-well plate takes a significant amount of time and labor to process, some level of automation (e.g., a liquid handler and a thermocycler capable of accommodating multiple 384-well plates at once) would likely be advisable for scaling the assay beyond four plates.

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Figure legends

Figure 1. scNOMe-seq data measures chromatin accessibility and DNA methylation. Aggregated CpG (yellow) and GpC (blue) methylation data from multiple single cells at DNase Hypersensitive sites in GM12878 cells. Figure reproduced from Pott, 2017 [1], published under a Creative Commons Attribution license.

Figure 2. Multi-modal profiling of the heart captures cell-type specific epigenetic configurations in the MYH7 locus. scNOMe-seq data from an adult human heart sample comprising 1,229 cells. (A) TSNE plot with clusters corresponding to major cell types (left). (B) Pseudobulk data tracks for the corresponding clusters for both data modalities capturing chromatin accessibility (GmC, green) and DNA methylation (mCG, blue), respectively.

Figure 3. Example of a gating strategy during FACS sorting. Individual nuclei were selected based on size and DNA content. Percentages provide proportion of events within a particular gate for each scatter plot, the proportion of total events is indicated in parenthesis.

Figure 4. Loading schema for primers in the 2 384-well plates used for random priming step. Pattern shown for Wells A 1-2, B 1-2 in plates 1 and 2, respectively, is repeated across the entire plate.

Figure 5. Expected size distribution of scNOMe-seq library pools. BioAnalyzer profile shows size distribution of a representative pool of 64 individual scNOMe-seq libraries after final amplification.