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Multiplexed scNOME-seq protocol based on isolated single nuclei V.1

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Protocol status: Working

We use this protocol and it's working

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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. Adapted with permission.

Nuclei isolation and GpC methylation step adapted from "Methylation-Sensitive Single-Molecule Analysis of Chromatin Structure," by T. Miranda et al., 2010, pp. 21.17.1-21.17.16. Copyright 2010 by John Wiley & Sons, Inc. Adapted with permission.

Attachments



Multiplexed scNOME-

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Guidelines

Protocol Overview:

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



Materials

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 **Merck MilliporeSigma (Sigma-Aldrich)**
- ✕ Elution Buffer (EB) **Qiagen Catalog #19086**
- ✕ PBS - Phosphate-Buffered Saline (10X) pH 7.4 **Invitrogen - Thermo Fisher Catalog #AM9625**
- ✕ NP-40 **Merck MilliporeSigma (Sigma-Aldrich)**
- ✕ Tris (1 M), pH 8.0, RNase-free **Thermo Fisher Catalog #AM9855G**
- ✕ Poly Ethylene Glycol (PEG) 8000 **Merck MilliporeSigma (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) Exonuclease I **Enzymatics Catalog #X8010L** Accel-NGS® Adaptase™ **Swift Biosciences Catalog #33096** Kapa HiFi HotStart ReadyMix (2X) **Kapa Biosystems Catalog #KM2602****Primers:**

Primer	Sequence (5' to 3')
P5L_AD001_H	/5SpC3/TTCCCTAC ACGACGCTCTTCC GATCTATCACG(H1: 33340033)(H1)(H1) (H1)(H1)(H1)(H1)(H1) (H1)
P5L_AD002_H	/5SpC3/TTCCCTAC ACGACGCTCTTCC GATCTCGATGT(H1: 33340033)(H1)(H1) (H1)(H1)(H1)(H1)(H1) (H1)
P5L_AD004_H	/5SpC3/TTCCCTAC ACGACGCTCTTCC GATCTTGACCA(H1: 33340033)(H1)(H1) (H1)(H1)(H1)(H1)(H1) (H1)
P5L_AD006_H	/5SpC3/TTCCCTAC ACGACGCTCTTCC GATCTGCCAAT(H1: 33340033)(H1)(H1) (H1)(H1)(H1)(H1)(H1) (H1)
P5L_AD007_H	/5SpC3/TTCCCTAC ACGACGCTCTTCC GATCTCAGATC(H1: 33340033)(H1)(H1) (H1)(H1)(H1)(H1)(H1) (H1)
P5L_AD008_H	/5SpC3/TTCCCTAC ACGACGCTCTTCC GATCTACTTGA(H1: 33340033)(H1)(H1) (H1)(H1)(H1)(H1)(H1) (H1)
P5L_AD010_H	/5SpC3/TTCCCTAC ACGACGCTCTTCC GATCTTAGCTT(H1: 33340033)(H1)(H1) (H1)(H1)(H1)(H1)(H1) (H1)
P5L_AD012_H	/5SpC3/TTCCCTAC ACGACGCTCTTCC GATCTCTTGTA(H1:



	33340033)(H1)(H1) (H1)(H1)(H1)(H1)(H1) (H1)
P5ind_501	AATGATACGGCGA CCACCGAGATCTA CACACGATCAGAC ACTCTTTCCCTACA CGACGCTCT
P5ind_502	AATGATACGGCGA CCACCGAGATCTA CACTCGAGAGTAC ACTCTTTCCCTACA CGACGCTCT
P5ind_503	AATGATACGGCGA CCACCGAGATCTA CACCTAGCTCAAC ACTCTTTCCCTACA CGACGCTCT
P5ind_504	AATGATACGGCGA CCACCGAGATCTA CACATCGTCTCACA CTCTTTCCCTACAC GACGCTCT
P5ind_505	AATGATACGGCGA CCACCGAGATCTA CACTCGACAAGAC ACTCTTTCCCTACA CGACGCTCT
P5ind_506	AATGATACGGCGA CCACCGAGATCTA CACCCTTGGAAC ACTCTTTCCCTACA CGACGCTCT
P5ind_507	AATGATACGGCGA CCACCGAGATCTA CACATCATGCGACA CTCTTTCCCTACAC GACGCTCT
P5ind_508	AATGATACGGCGA CCACCGAGATCTA CACTGTTCCGTACA CTCTTTCCCTACAC GACGCTCT
P5ind_509	AATGATACGGCGA CCACCGAGATCTA CACATTAGCCGAC ACTCTTTCCCTACA CGACGCTCT
P5ind_510	AATGATACGGCGA CCACCGAGATCTA CACCGATCGATACA CTCTTTCCCTACAC GACGCTCT
P5ind_511	AATGATACGGCGA CCACCGAGATCTA CACGATCTTGCAC

	ACTCTTTCCCTACA CGACGCTCT
P5ind_512	AATGATACGGCGA CCACCGAGATCTA CACAGGATAGCAC ACTCTTTCCCTACA CGACGCTCT
P7ind_701	CAAGCAGAAGACG GCATACGAGATAGG CAATGGTGA CTGG AGTTCAGACGTGT GCTCTT
P7ind_702	CAAGCAGAAGACG GCATACGAGATTCA CCTAGGTGA CTGG AGTTCAGACGTGT GCTCTT
P7ind_703	CAAGCAGAAGACG GCATACGAGATCAT ACGGAGTGA CTGG AGTTCAGACGTGT GCTCTT
P7ind_704	CAAGCAGAAGACG GCATACGAGATGTC ATCGTGTGA CTGG AGTTCAGACGTGT GCTCTT
P7ind_705	CAAGCAGAAGACG GCATACGAGATTTA CCGACGTGA CTGG AGTTCAGACGTGT GCTCTT
P7ind_706	CAAGCAGAAGACG GCATACGAGATACC TTCGAGTGA CTGG AGTTCAGACGTGT GCTCTT
P7ind_707	CAAGCAGAAGACG GCATACGAGATACG CTTCTGTGA CTGG AGTTCAGACGTGT GCTCTT
P7ind_708	CAAGCAGAAGACG GCATACGAGATACG CTTCTGTGA CTGG AGTTCAGACGTGT GCTCTT

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)
- 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 (SSlbio 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 start

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 in the fridge.

Reagent	Reaction concentration (based on reaction volume)	Volumes for 2 384-well plates (+ 15%) (μL)	Volu mes for singl e reacti on (μL)
M-Digestion Buffer (2X)	1X	883.2	1
Proteinase K (1.9%)	0.095%	88.32	0.1
Distilled H ₂ O		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 quantity 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.

CITATION

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.

LINK

<https://doi.org/10.1002/0471142727.mb2117s89>

- 2.1 **RSB buffer (10X)** recipe:
 - 100mM Tris-Cl, pH 7.4



- 100mM NaCl
- 30mM MgCl₂


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

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%)

Note

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.



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 methylase buffer such that there are 1M cells per 75 µL.

Note

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

17 Prepare two 1.5 mL eppendorf tubes with the following mixture for incubation:

Reagent	Reaction concentration (based on reaction volume)	Amount (µL)
GpC methylase buffer (10X) (NEB)	0.5X	7.5
SAM (32mM) (NEB)	320µM	1.5
GpC Methyltransferase (4U/µL) (NEB)	1.33U/µL	50
Distilled H ₂ O		16
Nuclei		75
total		150

**Reaction volume: 150 μ L**

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

- 18 Incubate at 37°C for 7.5 minutes.

37 °C 7.5 minutes

- 19 Add a boost of 25 μ L GpC Methyltransferase (100U) and 0.75 μ L 32mM SAM to the nuclei.

25 μ L GpC methyltransferase

0.75 μ L 32mM SAM

- 20 Incubate at 37°C for 7.5 minutes.

37 °C 7.5 minutes

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

500 μ L of 1X PBS

800 x g, 4°C, 00:05:00

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

500 μ L – 1 mL 1X PBS

- 23 Add 2 drops of Hoechst.

Note

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

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

On ice ~15 minutes

Bisulfite Conversion

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

15 μ L CT conversion reagent


25.1 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

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

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

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

98°C 8 minutes

64°C 3.5hrs

4°C Hold

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

29 Place 2 Zymo-Spin 384-Well DNA Binding Plate on two 2.0 mL 96-Well Deep Well Plates.

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

 80 μL M-Binding Buffer

- 31 Transfer bisulfite conversion reactions to the Zymo-Spin 384-Well DNA Binding Plates. Pipette up and down 8 times to mix the sample.
- 32 Centrifuge for 5 minutes at 5,000xg.
 5000 x g, Room temperature, 00:05:00
- 33 Discard the flow through by decanting and add 100 μ L M-Wash Buffer to each well of the 384-Well DNA Binding Plates.
 100 μ L of M-Wash buffer
- 34 Centrifuge for 5 minutes at 5,000xg.
 5000 x g, Room temperature, 00:05:00
- 35 Discard the flow through by decanting and add 50 μ L M-Desulphonation Buffer to each well of the 384-Well DNA Binding Plates.
 50 μ L M-Desulphonation Buffer
- 36 Incubate at room temperature for 15 minutes.
 Room temperature 15 minutes
- 37 Centrifuge for 5 minutes at 5,000xg.
 5000 x g, Room temperature, 00:05:00
- 38 Discard the flow through by decanting and add 100 μ L M-Wash Buffer to each well of the 384-Well DNA Binding Plates.
 100 μ L M-Wash Buffer
- 39 Centrifuge for 5 minutes at 5,000xg.
 5000 x g, Room temperature, 00:05:00
- 40 Repeat wash steps (38 and 39) once more.

- 41 Place the 384-Well DNA Binding Plates on 2 new 384-well PCR plates. Add 7 μ L Random Primer Solution to each well of the 384-Well DNA Binding Plates.


 7 μ L Random Primer Solution

Plate 1

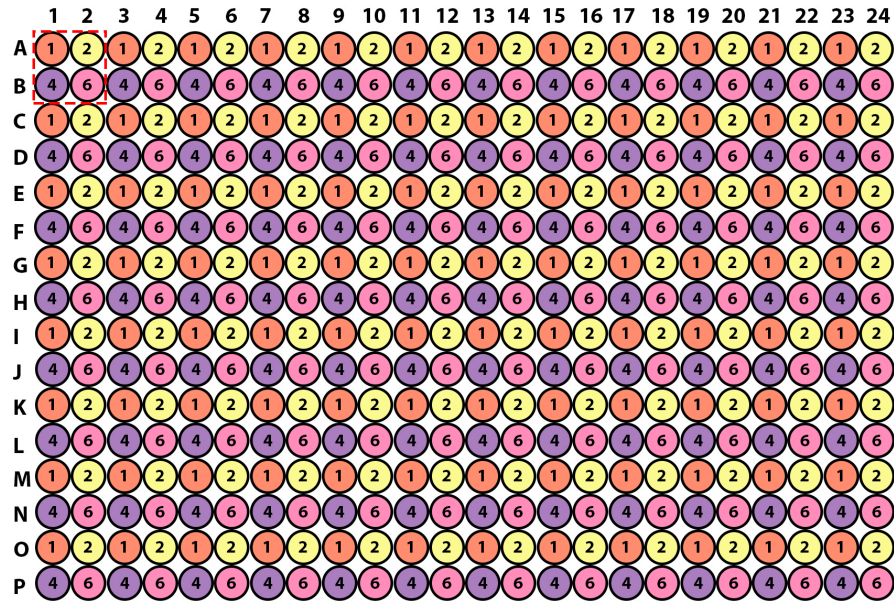


Figure 1a. Primer scheme for plate 1 (the 8 wells inside of the red squares will be pooled into a single well of a 96-well plate in the sample cleanup step).

Plate 2

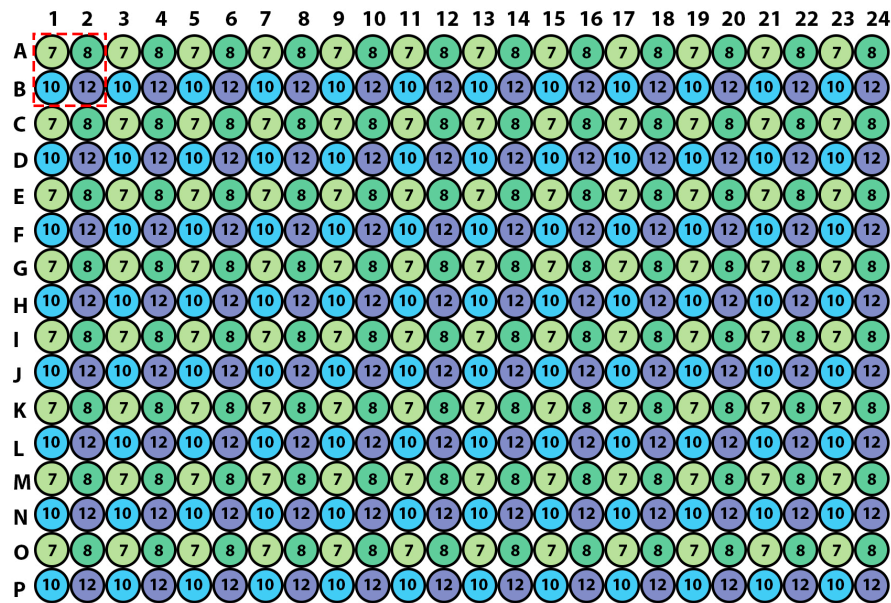


Figure 1b. Primer scheme for plate 2 (the 8 wells inside of the red squares will be pooled into a single well of a 96-well plate in the sample cleanup step).

- 42 Incubate for 5 minutes at room temperature.

Room temperature 5 minutes

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

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

- 44 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

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



Reagent	Reaction concentration (based on reaction volume)	Volumes for 2 384-well plates (+ 10%) (μL)	Volumes for single reaction (μL)
Blue Buffer (10X)	1X	845	1
Klenow exo (50U/μL)	1.25U/μL	211.25	0.25
dNTP (10mM each)	500uM each	422.5	0.5
Distilled H ₂ O		2746.25	3.25
Total		4225	5

Reaction volume: 10 μL

Note

Reaction volume is 10 μL because it's assumed that 2 μL is lost during the centrifugation in step 43 (Luo et al., 2018)

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

95°C 3 minutes

- 47 Immediately place the plate on ice for 2 minutes.

 On ice 2 minutes

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

 5 μL Random Priming Master Mix

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

 2000 x g, 00:00:10

- 50 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

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

 1.5 µL Exo/rSAP Mix

Reagent	Reaction concentration (based on reaction volume)	Volumes for 2 384- well plates (+extra) (µL)	Volu mes for singl e reacti on (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

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

 2000 x g, 00:00:10

- 53 Place the plate in a thermocycler and run the following program:


37°C 30 min

4°C Hold

Sample clean-up


- 54 Before to 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.

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

 280 µL Sera-Mag SpeedBeads

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

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

 1 mL TE

54.4 Resuspend washed beads in 280 μ L TE.

 280 μ L TE


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


 2.52 g PEG 8000

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

 2.8 mL 5M NaCl

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

 140 μ L 10mM Tris-HCl pH=8.0

 28 μ L 0.5M EDTA pH=8.0


54.8 Add 7-8 mL distilled H₂O and mix until PEG 8000 dissolves into solution.

 7 mL distilled H₂O –  8 mL distilled H₂O

54.9 Add the washed Sera-Mag SpeedBeads to the 50 mL conical tube and vortex before use.


54.10 Add enough distilled H₂O to bring the total volume up to 14 mL. Mix before each use.

55 Add 73.6 μ L (0.8x) SPRI beads to each well of a clean 96-well PCR plate.

 73.6 μ L SPRI beads

56 Pool the samples from the 2 384-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 40. (2 rows of each 384-well plate combine in one row of the 96-well plate.)

57 Vortex and incubate for 5 minutes at room temperature.

 Room temperature for 5 minutes

58 Quick spin for 10 seconds at 2,000xg.

 2000 x g, 00:00:10


59 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).

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


 150 µL 80% EtOH

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


62 Add 10 µL Elution Buffer (Qiagen) to each well and resuspend beads by pipette.

 10 µL Elution Buffer (Qiagen)

63 Vortex and incubate for 5 minutes at room temperature.


 Room temperature for 5 minutes

64 Quick spin for 10 seconds at 2,000xg.

 2000 x g, 00:00:10

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

66 Transfer 10 µL of the supernatant from each well to a clean 96-well PCR plate.

 10 µL of supernatant


Adaptase Reaction

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

95°C 3 min

68 Immediately place the plate on ice for 2 minutes.

69 Add 10.5 μL **Adaptase Master Mix** to each well of the 96-well PCR plate. Vortex and quick spin for 10s at 2,000xg.

 10.5 μL Adaptase Master Mix

Reagent	Volumes for 2 384-well plates (+extra) (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

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

37°C 30 min

95°C 2 min

4°C Hold

Library Amplification

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

 5 μL PCR Primer Mix

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

Reagent	Mix concentration (based on reaction volume)	Volume (μL)
P5L stock (100μM)	1.2μM	1.2
Distilled H ₂ O		98.8
Total		100

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

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

- 71.3 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. 1)

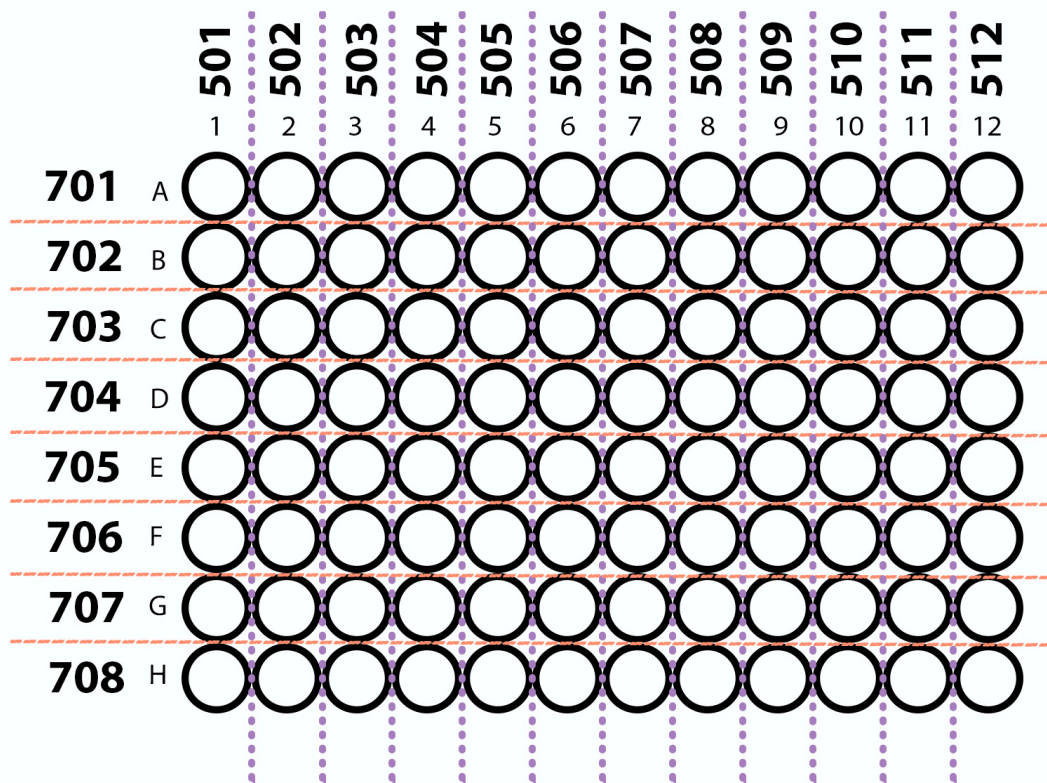


Figure 2. Each well receives a unique P5L-P7L primer combination.

71.4 Using a multichannel pipette, transfer 5 μ L of the primer mix to the sample plate.

5 μ L PCR Primer Mix

72 Add 25 μ L 2X KAPA HiFi Mix to each well.

25 μ L 2X KAPA HiFi Mix

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

2000 x g, 00:00:10

74 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
- Go to step c 14 times (15 cycles total)
- f. 72°C 5 minutes















g. 4°C Hold

Note

Number of cycles can be adjusted

Library clean-up

- 75 Add 40 μ L (0.8x) SPRI Beads to each well of the 96-well PCR plate containing the sample.
 40 μ L SPRI beads
- 76 Vortex and incubate for 5 minutes at room temperature.
 Room temperature for 5 minutes
- 77 Quick spin for 10 seconds at 2,000xg.
 2000 x g, 00:00:10
- 78 Place 96-well plates on DynaMag™-96 Side Magnet, let stand until solution in wells is clear of beads (~5 minutes).
- 79 Remove supernatant and wash beads 2 times with 150 μ L freshly made 80% EtOH.
 150 μ L 80% EtOH
- 80 Remove all EtOH after the last wash and remove plate from magnet. Let beads dry at room temperature. DO NOT overdry beads.
- 81 Add 25 μ L Elution Buffer (Qiagen) and resuspend beads by pipette.
 25 μ L Elution Buffer (Qiagen)
- 82 Vortex and incubate for 5 minutes at room temperature.
 Room temperature for 5 minutes
- 83 Quick spin for 10 seconds at 2,000xg.
 2000 x g, 00:00:10

- 84 Place back on magnet and let stand until solution is clear of beads (~5 minutes).
- 85 Combine 25 μ L 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 μ L eluent
- 86 Add 160 μ L (0.8x) SPRI Beads to each 1.5 mL Eppendorf tube. Pipette to mix and incubate for 5 minutes at room temperature.
 160 μ L SPRI Beads
- 87 Place 1.5 mL tubes on DynaMag™-2 Magnet, let stand until solution in tubes is clear of beads (~5 minutes).
- 88 Remove supernatant and wash beads 2 times with 200 μ L fresh 80% EtOH.
 200 μ L 80% EtOH
- 89 After the last wash, remove all EtOH and let beads dry at room temperature. DO NOT overdry beads.
- 90 Add 40 μ L Elution Buffer (Qiagen) and resuspend beads by pipet. Incubate for 5 minutes at room temperature.
 40 μ L Elution Buffer (Qiagen)
- 91 Place tubes back on magnet and let stand until solution is clear of beads (~5 minutes).
- 92 Remove 40 μ L supernatant to 12 clean 1.5 mL Eppendorf tubes.
 40 μ L eluent
- 93 Measure concentration of each 1.5 mL Eppendorf tube with Qubit dsDNA BR Assay Kit.



Citations

Step 2

Miranda, T. B., Kelly, T. K., Bouazoune, K., Jones, P.A.. Methylation-sensitive single-molecule analysis of chromatin structure

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