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Version 2

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Multiplexed scNOMe-seq protocol based on isolated single nuclei V.2

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1 Works for me dx.doi.org/10.17504/protocols.io.bgfvjtn6

Single_Cell_Uchicago

Sebastian Pott

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

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

NAME	CATALOG #	VENDOR
S-adenosylmethionine (SAM) (32mM) - 0.5 ml	B9003S	New England Biolabs
GpC Methyltransferase (M.CviPI) - 1,000 units	M0227L	New England Biolabs
Shrimp Alkaline Phosphatase (rSAP) - 500 units	M0371S	New England Biolabs
Magnesium Chloride	AC223210010	Fisher Scientific
Sera-Mag SpeedBeads Carboxylate-Modified Magnetic Particles	44152105050350	Ge Healthcare
RNase Inhibitor	30281-2	Lucigen
Ethylenediaminetetraacetic Acid (0.5M Solution/pH 8.0), Fisher BioReagents	BP2482-500	Fisher Scientific
Tris-HCl		Sigma Aldrich

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NAME	CATALOG #	VENDOR
Elution Buffer (EB)	19086	Qiagen
PBS - Phosphate-Buffered Saline (10X) pH 7.4	AM9625	Invitrogen - Thermo Fisher
NP-40		Sigma Aldrich
Tris (1 M), pH 8.0, RNase-free	AM9855G	Thermo Fisher
Poly Ethylene Glycol (PEG) 8000	89510-250G-F	Sigma Aldrich
Sodium Chloride (5M)	AM9760G	Invitrogen - Thermo Fisher
10X GpC Methyltransferase Buffer	B0227S	New England Biolabs
M-Digestion Buffer (2X)	D5021-9	Zymo Research
Proteinase K w/ storage buffer set	D3001-2	Zymo Research
CT Conversion Reagent	D5001-1	Zymo Research
M-Solubilization Buffer	D5021-7	Zymo Research
M-Dilution Buffer	D5002-2	Zymo Research
M-Reaction Buffer	D5021-8	Zymo Research
M-Binding Buffer	D5040-3	Zymo Research
M-Wash Buffer	D50074	Zymo Research
M-Desulphonation Buffer	D5040-5	Zymo Research
M-Elution Buffer	D5041-6	Zymo Research
10X Blue Buffer	B0110L	Enzymatics
Klenow (3'→5' exo-)	P7010-HC-L	Enzymatics
dNTP (10mM each)		
Exonuclease I	X8010L	Enzymatics
Accel-NGS® Adaptase™	33096	Swift Biosciences
Kapa HiFi HotStart ReadyMix (2X)	KM2602	Kapa Biosystems

MATERIALS TEXT

Primers:

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)

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P5L_AD012_H	/5SpC3/TTCCCTACACGACGCTCTTCCGATCTCTT GTA(H1:33340033)(H1)(H1)(H1)(H1)(H1)(H1)(H1)(H1)
P5ind_501	AATGATACGGCGACCACCGAGATCTACACACGAT CAGACACTCTTTCCCTACACGACGCTCT
P5ind_502	AATGATACGGCGACCACCGAGATCTACACTCGAG AGTACACTCTTTCCCTACACGACGCTCT
P5ind_503	AATGATACGGCGACCACCGAGATCTACACCTAGC TCAACACTCTTTCCCTACACGACGCTCT
P5ind_504	AATGATACGGCGACCACCGAGATCTACACATCGT CTCACACTCTTTCCCTACACGACGCTCT
P5ind_505	AATGATACGGCGACCACCGAGATCTACACTCGAC AAGACACTCTTTCCCTACACGACGCTCT
P5ind_506	AATGATACGGCGACCACCGAGATCTACACCCTTG GAAACACTCTTTCCCTACACGACGCTCT
P5ind_507	AATGATACGGCGACCACCGAGATCTACACATCAT GCGACACTCTTTCCCTACACGACGCTCT
P5ind_508	AATGATACGGCGACCACCGAGATCTACACTGTTC CGTACACTCTTTCCCTACACGACGCTCT
P5ind_509	AATGATACGGCGACCACCGAGATCTACACATTAG CCGACACTCTTTCCCTACACGACGCTCT
P5ind_510	AATGATACGGCGACCACCGAGATCTACACCGATC GATACACTCTTTCCCTACACGACGCTCT
P5ind_511	AATGATACGGCGACCACCGAGATCTACACGATCT TGCACACTCTTTCCCTACACGACGCTCT
P5ind_512	AATGATACGGCGACCACCGAGATCTACACAGGAT AGCACACTCTTTCCCTACACGACGCTCT
P7ind_701	CAAGCAGAAGACGGCATACGAGATAGGCAATGG TGACTGGAGTTCAGACGTGTGCTCTT
P7ind_702	CAAGCAGAAGACGGCATACGAGATTCACCTAGGT GACTGGAGTTCAGACGTGTGCTCTT
P7ind_703	CAAGCAGAAGACGGCATACGAGATCATACGGAGT GACTGGAGTTCAGACGTGTGCTCTT
P7ind_704	CAAGCAGAAGACGGCATACGAGATGTCATCGTGT GACTGGAGTTCAGACGTGTGCTCTT
P7ind_705	CAAGCAGAAGACGGCATACGAGATTTACCGACGT GACTGGAGTTCAGACGTGTGCTCTT
P7ind_706	CAAGCAGAAGACGGCATACGAGATACCTTCGAGT GACTGGAGTTCAGACGTGTGCTCTT
P7ind_707	CAAGCAGAAGACGGCATACGAGATACGCTTCTGT GACTGGAGTTCAGACGTGTGCTCTT
P7ind_708	CAAGCAGAAGACGGCATACGAGATACGCTTCTGT GACTGGAGTTCAGACGTGTGCTCTT

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)

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- 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 (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 in the fridge.

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.

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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

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

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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.

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 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

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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.

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

Bisulfite Conversion

25 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

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

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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 µL M-Wash Buffer to each well of the 384-Well DNA Binding Plates.

100 µl 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 µL M-Desulphonation Buffer to each well of the 384-Well DNA Binding Plates.

50 µl 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

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38 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

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 µL Random Primer Solution to each well of the 384-Well DNA Binding Plates.

7 µl Random Primer Solution

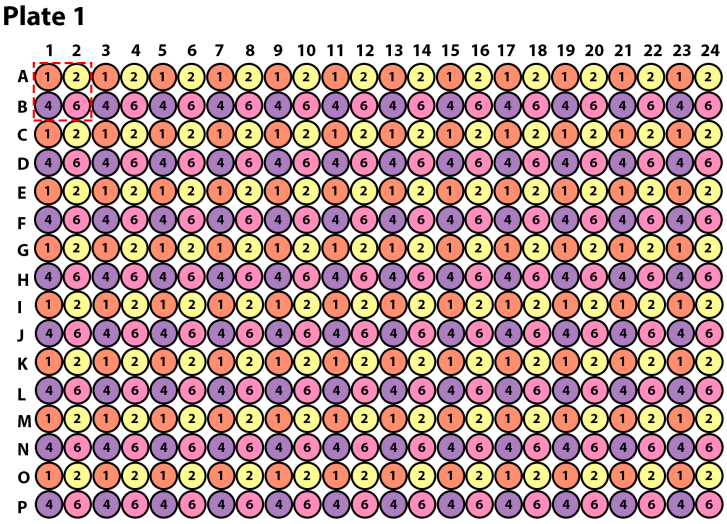

Figure 1a. Primer scheme for plate 1 (the 8 wells inside of the red squares will be pooled into a single well of a 96-well plate in the sample cleanup step).

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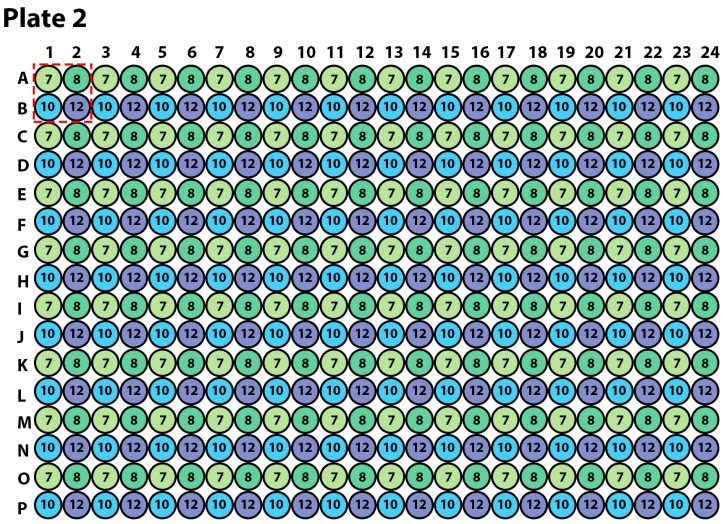

Figure 1b. Primer scheme for plate 2 (the 8 wells inside of the red squares will be pooled into a single well of a 96-well plate in the sample cleanup step).

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 H2O		2746.25	3.25
Total		**4225**	**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 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)	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

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

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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 µL TE.

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

140 µl 10mM Tris-HCl pH=8.0

28 µl 0.5M EDTA pH=8.0

54.8 Add 7-8 mL distilled H_2O 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 H_2O to bring the total volume up to 14 mL. Mix before each use.

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

73.6 µl SPRI beads

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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

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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 H2O		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.

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

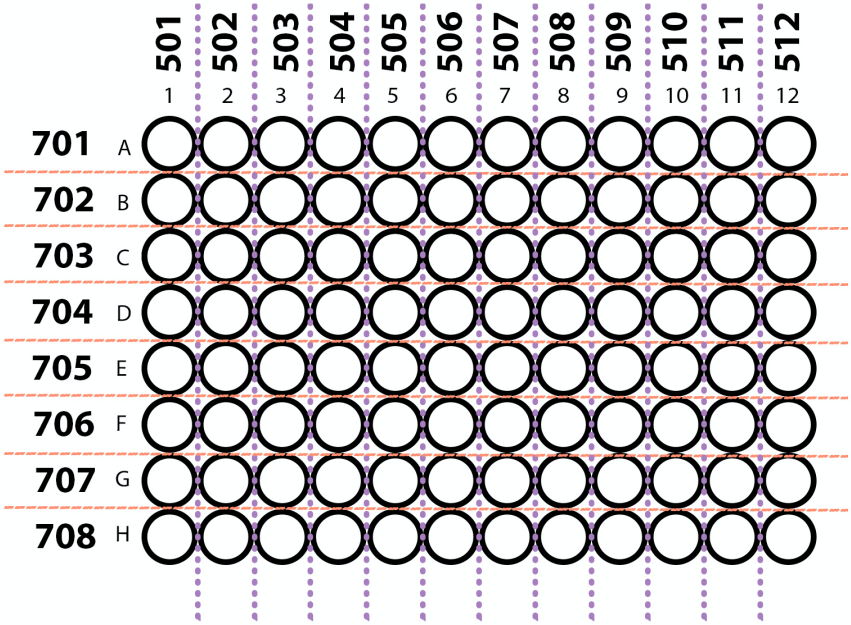

Figure 2. Each well recieves a unique P5L-P7L primer combination.

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

5 µl PCR Primer Mix

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

25 µl 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

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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

Number of cycles can be adjusted

Library clean-up

75 Add 40 µL (0.8x) SPRI Beads to each well of the 96-well PCR plate containing the sample.
40 µl 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 µL freshly made 80% EtOH.
150 µl 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 µL Elution Buffer (Qiagen) and resuspend beads by pipette.
25 µl 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).

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85 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

86 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

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

88 Remove supernatant and wash beads 2 times with 200 µL fresh 80% EtOH.

200 µl 80% EtOH

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

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

40 µl Elution Buffer (Qiagen)

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

92 Remove 40 µL supernatant to 12 clean 1.5 mL Eppendorf tubes.

40 µl eluent

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

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