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Multiplexed scNOME-seq protocol based on isolated single nuclei

Forked from [Multiplexed scNOME-seq protocol based on isolated single nuclei](#)

Sebastian Pott¹, Michael Wasney¹, Nadia Khan¹

¹Department of Medicine, University of Chicago

1 Works for me dx.doi.org/10.17504/protocols.io.brhum36w

Michael Wasney

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.brhum36w

PROTOCOL CITATION

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FORK NOTE

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KEYWORDS

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

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46356

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](#)

☒ [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:

Primer	Sequence (5' to 3')
P5L_AD001_H	/5SpC3/TTCCCTACACGACGCTCTCCGATCTATCACG(H1:33340033)(H1)(H1)(H1)(H1)(H1)(H1)(H1)(H1)
P5L_AD002_H	/5SpC3/TTCCCTACACGACGCTCTCCGATCTCGATGT(H1:33340033)(H1)(H1)(H1)(H1)(H1)(H1)(H1)(H1)
P5L_AD004_H	/5SpC3/TTCCCTACACGACGCTCTCCGATCTTGACCA(H1:33340033)(H1)(H1)(H1)(H1)(H1)(H1)(H1)(H1)
P5L_AD006_H	/5SpC3/TTCCCTACACGACGCTCTCCGATCTGCCAAT(H1:33340033)(H1)(H1)(H1)(H1)(H1)(H1)(H1)(H1)
P5L_AD007_H	/5SpC3/TTCCCTACACGACGCTCTCCGATCTCAGATC(H1:33340033)(H1)(H1)(H1)(H1)(H1)(H1)(H1)(H1)
P5L_AD008_H	/5SpC3/TTCCCTACACGACGCTCTCCGATCTACTTGA(H1:33340033)(H1)(H1)(H1)(H1)(H1)(H1)(H1)(H1)
P5L_AD010_H	/5SpC3/TTCCCTACACGACGCTCTCCGATCTTAGCTT(H1:33340033)(H1)(H1)(H1)(H1)(H1)(H1)(H1)(H1)
P5L_AD012_H	/5SpC3/TTCCCTACACGACGCTCTCCGATCTTTGTA(H1:33340033)(H1)(H1)(H1)(H1)(H1)(H1)(H1)(H1)
P5ind_501	AATGATACGGCGACCACCGAGATCTACACGATCAGACACTCTTCCCTACACGACGCTCT
P5ind_502	AATGATACGGCGACCACCGAGATCTACACTCGAGGTACACTCTTCCCTACACGACGCTCT
P5ind_503	AATGATACGGCGACCACCGAGATCTACACCTAGCTCAACACTCTTCCCTACACGACGCTCT
P5ind_504	AATGATACGGCGACCACCGAGATCTACACATCGTCTCACACTCTTCCCTACACGACGCTCT
P5ind_505	AATGATACGGCGACCACCGAGATCTACACTCGACAAGACACTCTTCCCTACACGACGCTCT
P5ind_506	AATGATACGGCGACCACCGAGATCTACACCTTGAAACACTCTTCCCTACACGACGCTCT
P5ind_507	AATGATACGGCGACCACCGAGATCTACACATCATGCGACACTCTTCCCTACACGACGCTCT
P5ind_508	AATGATACGGCGACCACCGAGATCTACACTGTTCCGTACACTCTTCCCTACACGACGCTCT
P5ind_509	AATGATACGGCGACCACCGAGATCTACACATTAGCCGACACTCTTCCCTACACGACGCTCT
P5ind_510	AATGATACGGCGACCACCGAGATCTACACCGATCGATACACTCTTCCCTACACGACGCTCT
P5ind_511	AATGATACGGCGACCACCGAGATCTACACGATCTTGACACTCTTCCCTACACGACGCTCT
P5ind_512	AATGATACGGCGACCACCGAGATCTACACAGGATAGCACACTCTTCCCTACACGACGCTCT
P7ind_701	CAAGCAGAAGACGGCATACGAGATAGGCAATGGTGACTGGAGTTCAGACGTGTGCTCTT
P7ind_702	CAAGCAGAAGACGGCATACGAGATTACCTAGGTGACTGGAGTTCAGACGTGTGCTCTT
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)

- 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 Methylation step, prepare 384- or 96-well collection plates with **digestion mix**. These can be prepared the day before and kept at 4°C.

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 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 (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.

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-HCl, 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 a 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.

- 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 two 1.5 mL eppendorf tubes 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 Methyltransferase (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 37°C 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.

🔥 **37 °C 7.5 minutes**

- 21 Add 500 µL 1X PBS and spin for 5 minutes at 800xg 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 50°C for 20 minutes in a thermocycler to facilitate nuclei lysis.

🔥 **50 °C 20 minutes**

Store plates at -20°C 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 10s at 2,000xg at room temperature.

🌀 **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 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 2 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**

39 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**

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 μ L Random Primer Solution to each well of the 384-Well DNA Binding Plates (Fig. 1).

 **7 μ L Random Primer Solution**

Plate 1

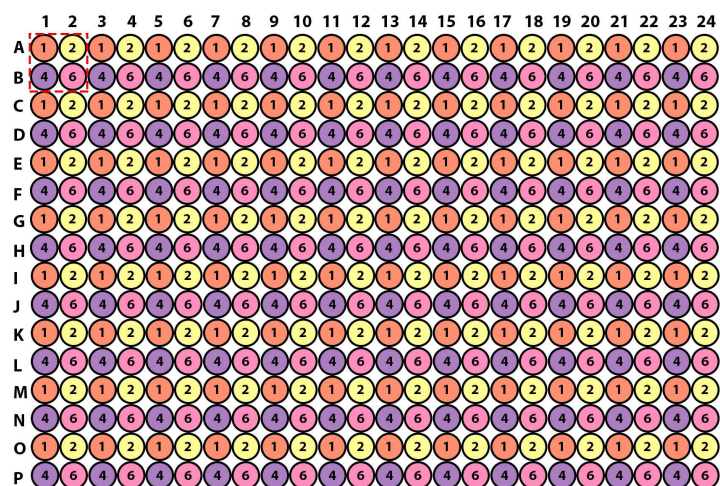


Figure 1a. Primer scheme for plate 1 (the 8 wells inside of the red squares will be pooled into a single well of a 96-well plate in the sample cleanup step).

Plate 2

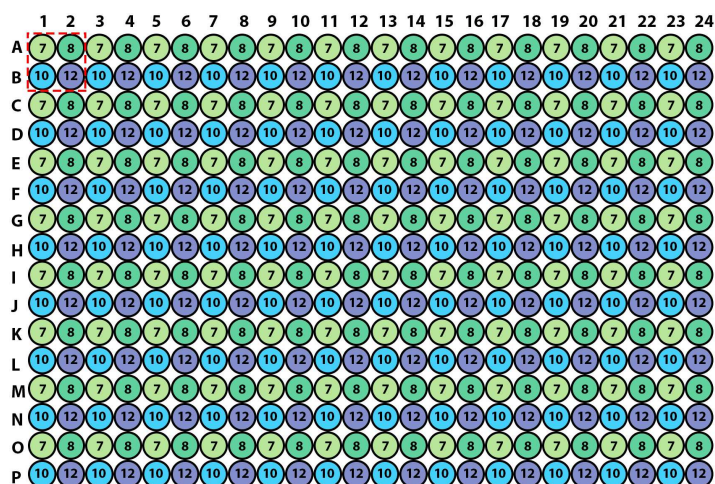


Figure 1b. Primer scheme for plate 2 (the 8 wells inside of the red squares will be pooled into a single well of a 96-well plate in the sample cleanup step).

- 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

Reaction volume is 10 μ L because it's assumed that 2 μ L 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 μ L Random Priming Master Mix to each well of the 384-well PCR plates.

 **5 μ L Random Priming Master Mix**

50 Vortex 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 μ L **Exo/rSAP Master Mix** to each well of the 384-well PCR plates.

 **1.5 μ L Exo/rSAP Mix**

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

Reaction volume: 11.5 μ L

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

 **2000 x g, 00:00:10**

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 H₂O 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 H₂O to bring the total volume up to 14 mL. Vortex before each use.

- 56 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**
- 57 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 42.
(2 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.
 **2000 x g, 00:00:10**
- 60 Place the 96-well PCR plate on the DynaMagTM-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 μ L fresh 80% EtOH.
 **150 μ L 80% EtOH**
- 62 Remove all EtOH and let beads dry at room temperature. Do not overdry the beads.
- 63 Remove the plate and add 10 μ L Elution Buffer (Qiagen) to each well and resuspend beads by pipette.
 **10 μ L 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.
 **2000 x g, 00:00:10**
- 66 Place back on magnet and let stand until solution is clear (~5 minutes).
- 67 Transfer 10 μ L of the supernatant from each well to a clean 96-well PCR plate. The plate can be stored at -20°C.
 **10 μ L of supernatant**

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.

- 71 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**

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

 **10.5 μl Adaptase Master Mix**

- 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

A	B	C
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 1 μM after the two primers corresponding to each well are combined.

A	B	C
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 5 μL of each P5L primer to individual columns and 5 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)

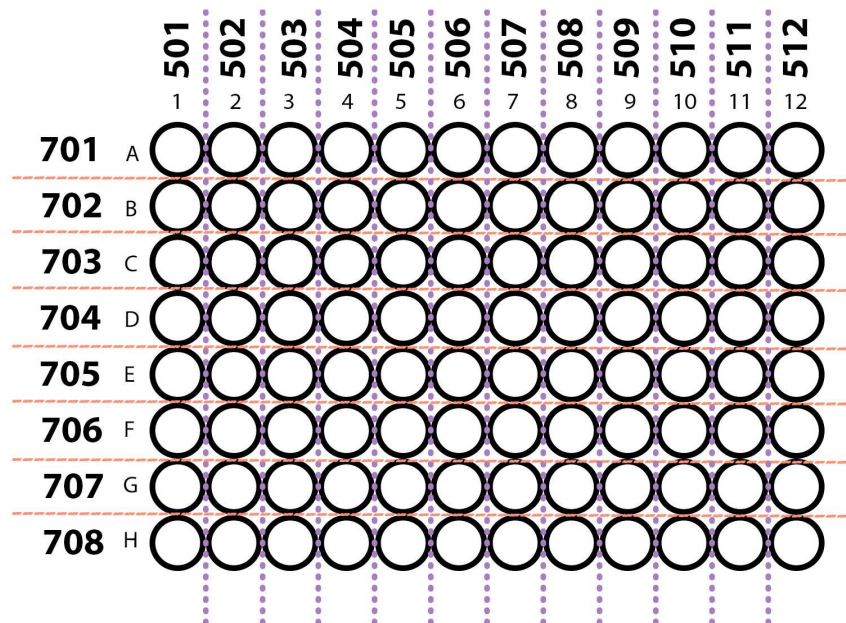




Figure 2. Each well receives a unique P5L-P7L primer combination.






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








 **5 μL PCR Primer Mix**

- 75 Add 25 μ L 2X KAPA HiFi Mix to each well.
 **25 μ L 2X KAPA HiFi Mix**
- 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

- 78 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**
- 79 Vortex and incubate for 5 minutes at room temperature.
 **Room temperature for 5 minutes**
- 80 Quick spin for 10 seconds at 2,000xg.
 **2000 x g, 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 μ L freshly made 80% EtOH.
 **150 μ L 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 μ L Elution Buffer (Qiagen) and resuspend beads by pipette.
 **25 μ L Elution Buffer (Qiagen)**

- 85 Vortex and incubate for 5 minutes at room temperature.
 **Room temperature for 5 minutes**
- 86 Quick spin for 10 seconds at 2,000xg.
 **2000 x g, 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 is 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 Remove 40 µL supernatant to 12 clean 1.5 mL Eppendorf tubes.
 **40 µl eluent**
- 96 Measure concentration of each 1.5 mL Eppendorf tube with Qubit dsDNA BR Assay Kit.