Supplementary Protocol for Methyl-HiC in bulk and single cells

Reagents:

- TC Media (Gibco)
- 37% Formaldehyde (J.T. Baker)
- 2.5M Glycine
- PBS (Gibco, 10010-023)
- 1M Tris-HCl pH = 8 (Life Tech)
- 5M NaCl (Teknova)
- NP-40 (Sigma)
- Protease Inhibitor (Sigma, P8340)
- 10% SDS (Life Tech)
- Triton X-100 (Sigma)
- 1M Tris-HCl pH = 7.5 (Life Tech)
- 1X Tris Buffer (10mM Tris-HCl, pH = 8)
- 0.4nM biotin-14-dATP (Life Tech, 19524-016)
- 3M Sodium Acetate pH = 5.2 (Sigma)
- SPRI Beads (Beckman or Home-made)
- Tween-20 (Sigma)
- 0.5M EDTA (Life Tech)
- Dynabeads My One T1 Streptavidin beads (Life Tech)
- 100mM ATP (Thermo, R0441)
- 100mM dNTPs (Thermo, R0181)
- TruSeq PCR Primers
- EZ-96 DNA Methylation-Direct™ Kit (Deep Well Format) (Zymo D5023)
- Accel-NGS Adaptase Module (96 rxns) (Swift 33096)
- unmethylated lambda DNA

Enzymes and Buffers:

- NEB Buffer2 (NEB, B7002)
- DpnII (NEB, R0176)
- Proteinase K (NEB, P8102)
- 5U/ul Klenow (NEB, M0210)
- 10X T4 DNA ligase buffer (NEB, B0202)
- BSA (10mg/mL stock) (NEB)
- 400U/ul T4 DNA Ligase (NEB, M0202)
- 10U/ul NEB T4 PNK (NEB, M0201)
- 3U/ul NEB T4 DNA Polymerase (NEB, M0203)
- 5U/ul Klenow (NEB, M0210)
- 5U/ul Klenow Exo- (NEB, M0212)
- 2X NEB Quick Ligation Reaction Buffer (NEB, B2200S)
- DNA Quick Ligase (NEB, M2200)
- Klenow Exo-DNA Polymerase (high concentration 50U/μl) supplied with Blue Buffer; Enzymatics Cat. No. P7010-HC-L)
- Exonuclease I (20U/μl), (Enzymatics Cat. No. X8010F)
- Shrimp Alkaline Phosphatase (New England BioLabs Cat. No. M0371)
- KAPA HiFi HotStart Uracil+ ReadyMix PCR Kit (KK2801)

Important:

1. Use barrier, low-bind tips for all steps with direct contacts to DNA.
2. Perform all the steps below in clean room or hood and be extremely careful for contamination.

Oligos:

Random Primer(s) with a P5 adapter 5' tail sequence

P5L_AD002 /5SpC3/TTCCCTACACGACGCTCTTCCGATCTCGATGT(N1:25252525)(N1)(N1) (N1)(N1)(N1) (N1)(N1)(N1)
P5L_AD006 /5SpC3/TTCCCTACACGACGCTCTTCCGATCTGCCAAT(N1:25252525)(N1)(N1) (N1)(N1)(N1) (N1)(N1)(N1)
P5L_AD008 /5SpC3/TTCCCTACACGACGCTCTTCCGATCTACTTGA(N1:25252525)(N1)(N1) (N1)(N1)(N1) (N1)(N1)(N1)
P5L_AD010 /5SpC3/TTCCCTACACGACGCTCTTCCGATCTTAGCTT(N1:25252525)(N1)(N1) (N1)(N1)(N1) (N1)(N1)(N1)

Custom P5 Indexing PCR Primers

P5L_D501	AATGATACGGCGACCACCGAGATCTACACTATAGCCTACACTCTTTCCCTACACGACGCTCT
P5L_D502	AATGATACGGCGACCACCGAGATCTACACATAGAGGCACACTCTTTCCCTACACGACGCTCT
P5L_D503	AATGATACGGCGACCACCGAGATCTACACCCTATCCTACACTCTTTCCCTACACGACGCTCT
P5L_D504	AATGATACGGCGACCACCGAGATCTACACGGCTCTGAACACTCTTTCCCTACACGACGCTCT
P5L_D505	AATGATACGGCGACCACCGAGATCTACACAGGCGAAGACACTCTTTCCCTACACGACGCTCT
P5L_D506	AATGATACGGCGACCACCGAGATCTACACTAATCTTAACACTCTTTCCCTACACGACGCTCT
P5L_D507	AATGATACGGCGACCACCGAGATCTACACCAGGACGTACACTCTTTCCCTACACGACGCTCT
P5L_D508	AATGATACGGCGACCACCGAGATCTACACGTACTGACACACTCTTTCCCTACACGACGCTCT

Custom P7 Indexing PCR Primers

P7L_D701	CAAGCAGAAGACGGCATACGAGATCGAGTAATGTGACTGGAGTTCAGACGTGTGCTCTT
P7L_D702	CAAGCAGAAGACGGCATACGAGATTCTCCGGAGTGACTGGAGTTCAGACGTGTGCTCTT
P7L_D703	CAAGCAGAAGACGGCATACGAGATAATGAGCGGTGACTGGAGTTCAGACGTGTGCTCTT
P7L_D704	CAAGCAGAAGACGGCATACGAGATGGAATCTCGTGACTGGAGTTCAGACGTGTGCTCTT
P7L_D705	CAAGCAGAAGACGGCATACGAGATTTCTGAATGTGACTGGAGTTCAGACGTGTGCTCTT
P7L_D706	CAAGCAGAAGACGGCATACGAGATACGAATTCGTGACTGGAGTTCAGACGTGTGCTCTT
P7L_D707	CAAGCAGAAGACGGCATACGAGATAGCTTCAGGTGACTGGAGTTCAGACGTGTGCTCTT
P7L_D708	CAAGCAGAAGACGGCATACGAGATGCGCATTAGTGACTGGAGTTCAGACGTGTGCTCTT
P7L_D709	CAAGCAGAAGACGGCATACGAGATCATAGCCGGTGACTGGAGTTCAGACGTGTGCTCTT
P7L_D710	CAAGCAGAAGACGGCATACGAGATTTCGCGGAGTGACTGGAGTTCAGACGTGTGCTCTT
P7L_D711	CAAGCAGAAGACGGCATACGAGATGCGCGAGAGTGACTGGAGTTCAGACGTGTGCTCTT
P7L_D712	CAAGCAGAAGACGGCATACGAGATCTATCGCTGTGACTGGAGTTCAGACGTGTGCTCTT

in situ HiC (Adapted from Rao et.al, 2014)

Crosslinking

1. Harvest adherent or suspension cells and pellet by centrifugation at 300xG for 5min.
2. Suspend in fresh media at 1M cells/ml.
3. Add formaldehyde to a final concentration of 1%. Incubate at RT for 10min with mixing.
4. Add 2.5M Glycine to a final concentration of 0.2M to quench reaction. Incubate at RT for 5min with mixing.
5. Centrifuge at 300xG for 5min at 4 °C. Remove supernatant.
6. Wash by adding ice cold PBS and split to several tubes with each 2M cells. Spin at 300xG for 5min at 4 °C. Discard supernatant.
7. Flash freeze pellet and either proceed to lysis or store at -80.

Lysis and RE Digestion

1. Make Lysis Buffer on ice (10mM Tris-HCl (pH 8.0), 10mM NaCl, 0.2% NP40 (Sigma))
    a. For 6.0mL (Note: you will need 850ul per sample)
    b. 50ul 1M Tris-HCl (pH 8.0)
    c. 10ul 5M NaCl
    d. 100ul 10% NP40
    e. 1mL Protease Inhibitor (Sigma, P8340)
    f. 4.86mL H20

Final	Stock	1ml	2ml	3ml	4ml	5ml	6ml
10mM Tris-HCl (pH 8.0)	1M Tris-HCl (pH 8.0)	10	20	30	40	50	60
10mM NaCl	5M NaCl	2	4	6	8	10	12
0.2% NP40	10% NP40	20	40	60	80	100	120
1xComplete	25x Complete	40	80	120	160	200	240
ddW	ddW	950	1900	2850	3800	4750	5700

2. Add 300ul of lysis buffer to crosslinked pellet. Suspend pellet.
3. Incubate on ice for 1hr. Centrifuge at 2500xG for 5min at 4 °C. Discard supernatant.
4. Wash pelleted nuclei by adding 500ul ice-cold lysis buffer, spin 2500xG for 5 min at 4 °C.
5. Gently suspend pellet in 50ul of 0.5% SDS and incubate for 10min at 62 °C**.**
6. After heating, add 145ul water and 25ul of 10% TritonX-100. Mix well and avoid bubbles. Incubate for 15min at 37 °C.
7. Add 26.5ul of 10X NEBuffer 3.1 and 100U (20ul) DpnII and digest chromatin for at least O/N at 37 °C at 700rpm rotation.

Biotin Fill-in, Ligation, sorting, and Bisulfite conversion

1. Inactivate MboI by incubating sample at 65 °C for 20min. Then cool to RT for >10min.
2. To biotin label fragmented ends add the following to the sample:
    a. 37.5ul of 0.4nM biotin-14-dATP (Life Tech, 19524-016)
    b. 1.5ul of 10mM dCTP
    c. 1.5ul of 10mM dTTP
    d. 1.5ul of 10mM dGTP
    e. 8ul of 5U/ul Klenow (NEB, M0210)
    f. Total = 50ul
3. Mix by pipetting and incubate for 90min at 37 °C with rotation (500rpm).
4. To ligate, add the following to the sample:
    a. 669ul of H2O
    b. 120ul of 10X T4 DNA ligase buffer (NEB, B0202)
    c. 100ul of 10% TritionX-100
    d. 6ul of 20mg/mL BSA
    e. 5ul of 400U/ul T4 DNA Ligase (NEB, M0202)
5. Mix by inversion and incubate at RT for 4hr. with slow rotation (300rpm).

The following steps will be separated to Methyl-HiC in bulk populations and Methyl-HiC in single cells. Please refer to each part accordingly.

To continue with Methyl-HiC in bulk populations:

6. To reverse crosslink, add the following to the sample:
    a. 50ul of 20mg/mL Proteinase K (NEB, P8102)
    b. 120ul of 10% SDS
7. Mix by inversion and incubate for 30min at 55 °C.
8. Add 130ul of 5M NaCl and incubate for at least O/N at 68 °C.

DNA Precipitation (~1.5hr)

1. Cool tubes to RT (~15min).
2. Split sample into two 700ul aliquots in 2mL microfuge tubes.
3. Add the following to ethanol precipitate the DNA:
    a. 1.12mL of 100% ethanol (i.e. 1.6X volumes)
    b. 70ul of 3M Sodium Acetate, pH 5.2 (i.e. 0.1X volumes)
4. Mix by inversion and incubate for at least 15min at -80 °C.
5. Centrifuge at max speed for 15min at 4 °C. Remove supernatant and keep on ice.
6. Suspend each pellet in 400ul of 70% ethanol, pool tubes into one tube of 800ul, and spin at max speed for 5min at 4 °C. Remove supernatant.
7. Wash pellet once more with 800ul of 70% Ethanol. Remove supernatant.
8. Dissolve pellet in 130ul of 1X Tris Buffer (10mM Tris-HCl, pH 8) to dissolve DNA.
9. Qubit BR

DNA sonication

1. Shear DNA using Convaris 130ul 400bp DNA sonication condition (For PE 2x100).
2. Warm a bottle of SPRI beads to RT.

Fragment Size Selection

Do a 1X SPRI clean-up. Elute in 300ul of 1X Tris Buffer.

Biotin Pulldown and Library Prep

- Prepare 1X Tween Wash Buffer (TWB) (5mM Tris-HCl pH 7.5, 0.5mM EDTA, 1M NaCl, 0.05% Tween)
- **5ml per sample**

Final	Stock	1x10ml	2x	3x	4x	5x	6x
5mM Tris-HCl pH 7.5	1M	50ul					
0.5mM EDTA	0.5M	10ul					
1M NaCl	5M	2ml					
0.05% Tween	10%	50ul					
ddW		7.89ml					

- Prepare 2X Binding Buffer (2X "BB") (10mM Tris-HCl pH 7.5, 1mM EDTA, 2M NaCl)
- **350ul per sample**

Final	Stock	1x1ml	2x	3x	4x	5x	6x
10mM Tris-HCl pH 7.5	1M	10ul					
1mM EDTA	0.5M	2ul					
2M NaCl	5M	400ul					
ddW		588ul					

1. Add 150ul of 10mg/mL Dynabeads My One T1 Streptavidin beads ("T1 beads") to a clean 1.5mL microfuge tube and wash the beads by adding 400ul of 1X Tween Wash Buffer. Separate on magnet and discard the supernatant.
2. Suspend the beads in 300ul of 2X BB, and then transfer the beads to the sample tube. Incubate the biotin pull-down for 15min at RT.
3. Separate on a magnet and collect supernatant in a new tube (For Methyl-HiC, you can keep this supernatant as DNA methylation background ctrl).
4. Ethonal precipitation the supernatant.
5. Wash beads by adding 600ul of 1X TWB and transfer to a fresh tube. Heat on thermomixer for 2min at 55 °C with mixing. Then bind beads to magnet and remove supernatant.
6. Repeat wash in Step 6.
7. Resuspend beads in 100ul 1X NEB T4 DNA ligase buffer (NEB, B0202) and transfer to a new tube. Bind beads to magnet and discard the supernatant.
8. To repair fragmented ends and remove biotin from unligated ends, resuspend the beads in 100ul of the following:

Final	Stock	1x100ul	2x	3x	4x	5x	6x
1xNEB T4 ligase buffer	10x	10	20	30	40	50	60
0.5mM dNTP	10mM	5	10	15	20	25	30
0.5U T4 PNK (M0201)	10U/ul	5	10	15	20	25	30
0.12U T4 Polymerase (M0203)	3U/ul	4	8	12	16	20	24
0.05U Klenow (M0210)	5U/ul	1	2	3	4	5	6
ddW		75	150	225	300	375	450

9. Incubate for 30min at RT. Separate on a magnet and discard the solution.
10. Wash by adding 600ul 1X TWB and transfer to a new tube. Heat tube for 2min at 55 °C with mixing. Place sample on magnet to reclaim beads and discard supernatant.
11. Repeat previous step. Discard supernatant.
12. Resuspend beads in 100ul 1X NEB Buffer2 and transfer to a new tube. Place on magnet and discard supernatant.
13. To add dA-tail, resuspend the bedas in 100ul of the following:

Final	Stock	1x100ul	2x	3x	4x
1x NEB buffer2	10x	10	20	30	40
0.5mM dATP	10mM	5	10	15	20
0.25U Klenow(exo-) (M0212)	5U/ul	5	10	15	20
ddW		80	160	240	320

14. Incubate for 30min at 37 °C. Separate on a magnet and discard the supernatant.
15. Wash by adding 600ul 1X TWB and transfer to a new tube. Heat tube for 2min at 55 °C with mixing. Place sample on magnet to reclaim beads and discard supernatant.
16. Repeat previous. Discard supernatant.
    - Prepare 1X NEB Quick Ligation Reaction Buffer. I dilute the 2X NEB Quick Ligation Reaction Buffer (NEB, B2200S) to 1X. This will also work fine if you use the 5X NEB Quick Ligation Reaction Buffer from the NEBNext Kit (NEB, B6058) to dilute to 1X. The only difference between these two buffers is a small difference in PEG concentration. You will need 150ul of 1X NEB Quick Ligation Reaction Buffer per sample.
17. Resuspend beads in 100ul 1X NEB Quick Ligation Reaction Buffer (NEB, B2200S) and transfer to a new tube. Place on a magnet and discard the supernatant.
18. Resuspend beads in 50ul of 1X NEB Quick Ligation Reaction Buffer. Split the mixture in two parts (10/40), one for normal HiC (not necessary to do this but it can be used as a control for the HiC part), the other for bisulfite conversion, with different adapter index (so you can multiplex them together for sequencing).
19. To ligate adapters by adding the following:

Final	Stock	1x50ul	2x	3x	4x
1x Quick ligase Buffer	2x	25	50	75	100
Qucik ligase (M2200)		2	4	6	8
Illuminar Adapter	Original	3			
ddW		20	40	60	80

20. Mix thoroughly and incubate for 15min at RT. Separate on magnet and discard supernatant.
21. Wash by adding 600ul 1X TWB and transfer to a new tube. Heat tube for 2min at 55 °C with mixing. Place sample on magnet to reclaim beads and discard supernatant.
22. Repeat previous step. Discard supernatant.
23. Resuspend beads in 100ul of 1X Tris Buffer and transfer to a new tube. Place sample on magnet and discard supernatant.
24. Resuspend beads in 20ul of 1X Tris Buffer.

Bisulfite Conversion

Do bisulfite conversion directly on the beads-bound DNA.
Add BS regents directly to the beads (130ul-20ul model);
Spike in 0.5% of same adapter-ligated lambda DNA.
After conversion, separate on a magnet and transfer the supernatant to the purification column. Then, add the binding buffer, wash buffer, dephos buffer to the beads. Finally, do PCR directly on the beads.

Final PCR Amplification and Purification (~1hr)

1. PCR with KAPA HIFI Urial+ MM. Start from 10 cycles.
2. Do a double size selection with AMPure beads. Beads ratio are 0.6-0.15 (mainly 400-700bp).
3. Do library quantification with QPCR and DNA fragments analyzer.

To continue with Methyl-HiC in single cells:

1. Centrifuge at 2500G for 5min. Discard supernatant.
2. Suspend the pallet in 1ml PBS, add 5ul DRAQ7 (1:200). (DRAQ7™ is a new far-red fluorescent dye that only stains the nuclei in dead and permeabilized cells).
3. Prior to sorting, prepare Zymo lysis buffer containing proteinase K following the Zymo EZ96 direct kit manual. Spike in 0.5% of sonicated lambda DNA (must sonicated before using, fragment size around 500bp) to the lysis buffer according to the amount of total nucleus.
4. Preload 10 µl to each well of the 96-well plate that will be used for cell sorting.
5. Perform single nuclei sorting. Make sure single nuclei is sorted to the middle of the wells. Sort single nuclei in 96 well plates with different combinations. Always include negative blank ctrl.
6. After sorting, add 65ul of the CT conversion reagent to each well. Do bisulfite conversion directly on the sorted SNs with EZ-96 DNA Methylation-Direct™ Kit (Deep Well Format) according to the manufactory manual.

Single Cell Methyl-Seq Library Prep Protocol with Adaptase Module (Adapted from Swift 33096)

1. To set up the Random Priming Step, briefly spin the plate, unseal, add P5L random primer to each well of the 96-well plate containing the single cell samples and seal the plate. For downstream 4-Plex multiplexing, use random primers that incorporate AD002, AD006, AD008 and AD010. Alternatively, if not multiplexing, add a P5L non-indexed random primer.

	Reagent Volume (µl)	Final Concentration
P5L random primer (5 µM)	1	250 nM

2. Prepare the Random Priming Master mix and keep on ice:

	Reagent Volume (µl)	Final Concentration
Blue Buffer (10×)	2	1x
Klenow exo- (50 U/µl)	1	50U
dNTP (10 mM each)	1	0.5 mM each
H_2O	6	
Total Volume	10	

3. Denature the samples containing random primer in the 96-well plate using a thermocycler at 95 °C for 3 minutes. Immediately chill the plate on ice for 2 minutes.
4. Briefly spin the plate, unseal, and add 10 µl of the Random Priming Master mix to each well of the 96-well plate. Seal the plate. Mix the reactions well by gently vortexing the plate, and then quickly spin the plate to collect contents.
5. Use a thermo cycler to run the Random Priming Program:
   4 °C 5 min
   0.1 °C/s to 25 °C
   25 °C 5 min
   0.1 °C/s to 37 °C
   37 °C 60 min
   4 ° C ∞
6. Briefly spin the plate, unseal, and add Exonuclease I and Shrimp Alkaline Phosphatase to each well of the 96-well plate. Seal the plate. Mix the reactions well by gently vortexing the plate, and then quickly spin the plate to collect contents.

	Reagent Volume (µl)	Units
Exonuclease 1 (20 U/µl)	2	40 U
Shrimp Alkaline Phosphatase	1	1 U

7. Use a thermo cycler to run the Enzymatic Digestion Program.
   37 °C 30 min
   4 ° C ∞
8. Briefly spin the plate, unseal, and add 0.8x SPRI beads – 18.4 µl to each well of the 96-well plate. Seal the plate. Mix the reactions well by gently vortexing the plate, and then quickly spin the plate to collect contents.
9. If multiplexing using indexed random priming, combine the solution/beads mixture from four plates into one of the plates. Wash the beads with 200 µl 80% ethanol for three times, elute in 10 µl EB. If not multiplexing, wash beads with 200 µl 80% ethanol for two times and elute in 10 µl EB.
10. Prepare the Adaptase Master mix and keep on ice.

	Reagent Volume (µl)
EB	4.25
Buffer G1	2.0
Reagent G2	2.0
Reagent G3	1.25
Enzyme G4	0.5
Enzyme G5	0.5

11. Seal the plate and denature the samples in the 96-well plate using a thermocycler Denaturation Program at 95 °C for 3 minutes. Immediately chill the tube on ice for 2 minutes.
12. Briefly spin the plate, unseal, and add 10.5 µl of the Adaptase Master mix into each well of the 96-well plate. Seal the plate. Mix the reactions well by gently vortexing the plate, and then quickly spin the plate to collect contents.
13. Use a thermocycler Adaptase Program.
    37 °C 30 min
    95 °C 2 min
14. Briefly spin the plate, unseal, and add PCR mixture below. Seal the plate. Mix the reactions well by gently vortexing the plate, and then quickly spin the plate to collect contents.

	Reagent Volume (µl)	Final Concentration
Custom P5L index primer (100 µM)	0.3	600 nM
Custom P7L index primer (10 µM)	5.0	1 µM
KAPA HiFi HotStart ReadyMix	25.0	1x

15. Use a thermo cycler Indexing PCR Program.
    95 °C 2 min
    98 °C 30 sec
    98 °C 15 sec
    64 °C 30 sec
    72 °C 2 min
    Go to step 3 for 16 times (repeat steps 3-5 for total of 17 cycles*)
    72 °C 5 min
    4 ° C ∞
16. Briefly spin the plate, unseal, and purify the amplified libraries with two rounds of 0.8x SPRI beads — 40.6 µl for the first round with the 50.8 µl sample. The elution from the first round can be any volume between 10-50 µl of Low EDTA TE. For the second round, use a corresponding 0.8x SPRI bead volume. For each round of SPRI purification, wash with 80% ethanol twice.
17. The library concentration in the eluate can be quantified for pooling and loading either by fluorometric reading or qPCR.