Feb 03, 2026

ME-seq protocol V1_a single cell trimodal assay

DOI
https://dx.doi.org/10.17504/protocols.io.5qpvowmwxl4o/v1

Bohan Zhu[1], Sai Ma[1]

[1]Icahn School of Medicine at Mount Sinai

Bohan Zhu
Icahn School of Medicine at Mount Sinai

OPEN ACCESS

DOI: https://dx.doi.org/10.17504/protocols.io.5qpvowmwxl4o/v1

Protocol Citation: Bohan Zhu, Sai Ma 2026. ME-seq protocol V1_a single cell trimodal assay. **protocols.io** https://dx.doi.org/10.17504/protocols.io.5qpvowmwxl4o/v1

Protocol status: Working
We use this protocol and it's working

Created: June 09, 2025

Last Modified: February 03, 2026

Protocol Integer ID: 219816

Keywords: single cell assay, Chromatin accessibility, Gene expression, DNA methylation, seq protocol v1-a single cell trimodal, single cell trimodal, seq protocol v1-a, seq, dna methylation, chromatin accessibility, gene expression, cell platform, assay

Abstract

ME-seq is a scalable single-cell platform for simultaneous co-profiling of DNA methylation, chromatin accessibility, and gene expression.

Guidelines

This protocol can be used to multiplex multiple samples in one run. Keep the samples in seperate tubes during fixation, trasnposition and RT, until loaded into Round 1 barcoding plate.

This protocol can be slightly modified for profiling DNA methylation or gene expression only.

Before starting

1 Based on empirical experience, we recommend the following guidelines to ensure the high-quality sequencing results from the experiment:
1. By default, perform all steps without specific instructions either on ice or at 4°C. Ensure all reagents are fully thawed and thoroughly mixed before use, and maintain most of them on ice throughout the experiment.
2. To enhance cell/nuclei recovery, we recommend using pre-lubricated tubes coated additionally with 7.5% BSA prior to centrifugation.
3. To preserve Tn5 enzyme quality, aliquot the protein immediately upon receipt. Assemble Tn5 with oligonucleotides freshly before the experiment.
4. Prepare all reagents containing RNase inhibitors freshly just before use.

Buffer preparation

2 ▪ **Prepare the following buffer and store at -80°C**

	A	B	C
	10× oxidation buffer	**Volume (µl)**	**Final conc.**
	1M HEPES, pH 8.0	500	500 mM
	0.5M alpha-KG	20	10 mM
	0.5M L-ascorbic acid	40	20 mM
	100mM DTT	100	10 mM
	5M NaCl	200	1000 mM
	H2O	140	
	Total	1000	

Note: To avoid repeated freeze-thaw cycles, we recommend aliquoting the 10x oxidation buffer into 20 µl portions and discarding each aliquot after use.

▪ **Prepare the following buffers and store at -20°C**

	A	B	C
	Tn5 dilution buffer	**Volume (µl)**	**Final conc.**
	1M Tris-HCl, pH 7.5	50	50 mM

	A	B	C
	5M NaCl	20	100 mM
	5mM EDTA	20	0.1 mM
	100mM DTT	10	1 mM
	10% NP-40	10	0.1%
	100% glycerol	500	50%
	H2O	390	
	Total	1000	

Note: The concentration of stock EDTA is 0.5M, it needs to be diluted to 5mM first and then used to prepare the Tn5 dilution buffer

	A	B	C
	5× SMART RT buffer	**Volume (µl)**	**Final conc.**
	100mM DTT	400	40 mM
	1M Tris-HCl, pH 8.0	125	125 mM
	100mM GTP	50	5 mM
	5M NaCl	30	150 mM
	1M MgCl2	12.5	12.5 mM
	H2O	382.5	
	Total	1000	

- **Prepare the following buffers and store at 4°C**

	A	B	C
	STE buffer	**Volume (ml)**	**Final conc.**
	1M Tris-HCl, pH 8.0	0.5	10 mM
	5M NaCl	0.5	50 mM
	0.5 M EDTA	0.1	1 mM

	A	B	C
	H2O	48.9	
	Total	50	

	A	B	C
	NI buffer	**Volume (µl)**	**Final conc.**
	1M Tris-HCl, pH 7.5	400	10 mM
	5M NaCl	80	10 mM
	1M MgCl2	120	3 mM
	H2O	39400	
	Total	40000	

	A	B	C
	2× reverse crosslinking buffer	**Volume (µl)**	**Final conc.**
	1M Tris-HCl, pH 8.0	1000	100 mM
	5M NaCl	200	100 mM
	20% SDS	20	0.04%
	H2O	8780	
	Total	10000	

Note: SDS may precipitate at 4°C. Before use, warm the buffer at room temperature for at least 1 hour, then vortex thoroughly to completely redissolve the SDS.

	A	B	C
	2× B&W buffer	**Volume (µl)**	**Final conc.**
	1M Tris-HCl, pH 7.5	100	10 mM
	0.5M EDTA	20	1 mM
	5M NaCl	4000	2 M
	H2O	5880	

	A	B	C
	Total	10000	

- **Prepare the following buffers fresh on the day of the experiment and keep them on ice.**

	A	B	C
	PBSI	**Volume (µl)**	**Final conc.**
	1× PBS, pH 7.4	992.2	
	7.5 % BSA	5.3	0.04%
	Enzymatics RNase inhibitor	2.5	0.26%
	Total	1000	

	A	B	C
	NI-RI buffer	**Volume (µl)**	**Final conc.**
	1M Tris-HCl, pH 7.5	10	10 mM
	5M NaCl	2	10 mM
	1M MgCl2	3	3 mM
	Enzymatics RNase Inhibitor	2.5	0.25%
	SUPERase•In RNase inhibitor	2.5	0.25%
	H2O	980	
	Total	1000	

	A	B	C
	HCBI buffer	**Volume (µl)**	**Final conc.**
	7.5% BSA	26.7	0.04%
	5M NaCl	10	10 mM

	A	B	C
	1M MgCl2	15	3 mM
	1M HEPES, pH 7.2	50	10 mM
	Enzymatics RNase inhibitor	12.5	0.25%
	H2O	4885.8	
	Total	5000	

	A	B	C
	LAND buffer	**Volume (µl)**	**Final conc.**
	1M Tris, pH 7.5	11	10 mM
	5M NaCl	2.2	10 mM
	1M MgCl2	3.3	3 mM
	10% IGEPAL CA-630	11	0.1%
	25× cOmplete protease inhibitor cocktail, EDTA-free	44	1×
	95 mg/ml Pefabloc Protease Inhibitor	5.8	0.5 mg/ml
	SUPERase•In RNase inhibitor	2.75	0.25%
	H2O	1020	
	Total	1100	

	A	B	C
	1× B&W-T buffer	**Volume (µl)**	**Final conc.**
	2× B&W buffer	500	1×
	100% Tween-20	0.5	0.1%
	H2O	499.5	
	Total	1000	

	A	B	C
	2× TD buffer	**Volume (µl)**	**Final conc.**
	1M Tris-HCl, pH 7.5	16	20 mM
	1M MgCl2	8	10 mM
	DMF	80	10%
	H2O	696	
	Total	800	

	A	B	C
	Transposition buffer	**Volume (µl)**	**Final conc.**
	0.2M Tris-acetate	8.25	33 mM
	5M K-acetate	0.66	66 mM
	1M Mg-acetate	0.5	10 mM
	100% DMF	8	16%
	40 U/µl Enzymatic RI	0.85	0.68 U/µl
	100% PIC	0.2	0.4%
	1x PBS	15	
	H2O	9.04	
	Total	42.5	

Oligos preparation

3 ▪ **Barcoding oligo plates preparation**

1. Order the barcoding oligos in deepwell plates from IDT:
The Excel file below could be used for ordering. If 96 barcodes per round is good enough, order the oligos on the tabs labeled as 'R1_plate1', 'R2_plate1', and 'R3_plate1'; If 192 barcodes per round is desired, additionally order the oligos on the tabs labeled as 'R1_plate2', 'R2_plate2', and 'R3_plate2'. Order the oligos with formulation as 100 µM in IDTE buffer, pH 8.0 and purification as standard desalting.

three round barcoding plate oligos.... 29.5KB

2. Order the linkers and blocking oligos in tubes from IDT:
The linkers are used to hybridize with the barcoding oligos in each plate, while the blocking oligos are utilized during the cell/nuclei barcoding steps. Details of both linker and blocking oligos are provided below.

	A	B	C	D	E
	Name	Sequence	Scale	Formulation	Purification
	phos-Round 1_linker	/5Phos/GACTGGTAGGAGCGTTCGGACGATCATGGG	1 µmol	100 µM in IDTE, pH 8.0	STD
	phos-Round 2_linker	/5Phos/CAAGTATGCAGCGCGCTCAAGCACGTGGAT	1 µmol	100 µM in IDTE, pH 8.0	STD
	phos-Round 3_linker	/5Phos/AGTCGTACGCCGATGCGAAACATCGGCCAC	1 µmol	100 µM in IDTE, pH 8.0	STD
	Round 1 blocking	CCCATGATCGTCCGAACGCTCCTACCAGTC	1 µmol	100 µM in IDTE, pH 8.0	STD
	Round 2 blocking	ATCCACGTGCTTGAGCGCGCTGCATACTTG	1 µmol	100 µM in IDTE, pH 8.0	STD
	Round 3 blocking	GTGGCCGATGTTTCGCATCGGCGTACGACT	1 µmol	100 µM in IDTE, pH 8.0	STD

Table of linkers and blocking oligos for the ME-seq cell/nuceli barcoding plates

3. Anneal the barcoding oligo plates:
3.1 Thoroughly thaw the three deep-well plates containing barcoding oligos at room temperature. Firmly invert each plate five times to ensure complete mixing of the solution, then centrifuge at 300 × g for 1 minute at room temperature to prevent cross-contamination.
3.2 Dilute 1200µl of Round 1 linker oligo (100µM) with 10800µl of STE buffer. Mix 90µl of diluted Round 1 linker oligo with 10µl of Round 1 oligo (100µM) in a 96 well PCR plate.
3.3 Dilute 1200µl of Round 2 linker oligo (100µM) with 8400µl of STE buffer. Mix 88µl of diluted Round 2 linker oligo with 12µl of Round 2 oligo (100µM) in a 96 well PCR plate.
3.4 Dilute 1440µl of Round 3 linker oligo (100µM) with 8064µl of STE buffer. Mix 86µl of diluted Round 3 linker oligo with 14µl of Round 3 oligo (100µM) in a 96 well PCR plate.
3.5 Seal the plates with peelable foil heat seal (Bio-Rad, catalog # 1814045) using a Bio-Rad PX1 plate sealer by the sealling program: 180 °C for 5 seconds.

3.6 Anneal the Round 1, Round 2, and Round 3 barcoding plates using the PCR prgram (total time: ~1 hr 34 min) bellow:

	A	B
	95 °C	2 min
	-1 °C per cycle	-0.1°C/sec, slow ramp
	20 °C	2 min
	4 °C	hold

PCR cycling condidtions for the three round barcoding plates

4. Aliquot 10 ul of annealed oligo from each well of the original 96-well plate to a new 96-well plate (you will have 9 sets of the three round barcoding plates) and seal the plates with microseal 'F' foil seal (Bio-Rad, catalog #MSF1001B). Label the plates with correct name. The rounds have to be in order during split-pool to make the hybridization work.
5. Store the plates at -20°C, they are good for at least 9 months, and probably 12 months.

4

- **Ordering and annealing oligos for Tn5 assembly**

1. Order the following oligos from IDT

	A	B	C	D	E
	Name	Sequence	Scale	Purification	Formulation
	Read 1	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG	1 umol	HPLC	100 uM in IDTE, pH 8.0
	Read 2	/5Phos/ACGCTCCTACCAGTCAGATGTGTATAAGAGACAG	1 umol	HPLC	100 uM in IDTE, pH 8.0
	Blocked_ME_Comp	/5Phos/C*T*G*T*C*T*C*T*T*A*T*A*C*A*/3ddC/	1 umol	HPLC	100 uM in IDTE, pH 8.0

2. Prepare tagmentation adapter mix in PCR tube

	A	B
	Tagmentation Adapter R1	Vol.
	100 uM Read 1	20
	100 uM Blocked ME_Comp	40

	A	B
	1 M Tris pH 8.0	0.6
	5 M NaCl	0.6
	Total	61

Adapter R1

	A	B
	Tagmentation Adapter R2	Vol.
	100 uM Read 2	20
	100 uM Blocked_NE_Comp	40
	1 M Tris pH 8.0	0.6
	5 M Nacl	0.6
	Total	61

Adapter R2

3. Anneal oligos in a PCR cycle as follows.

	A	B
	95 ºC	2 min
	-1 ºC per cycle	-0.1 ºC per second, slow ramp
	20 ºC	2 min
	4 ºC	hold
	Total	1 hr 34 min

4. Heat the 100% glycerol to 65 ºC in a PCR. Make two 60 ul aliquots in two PCR tubes and place them on ice to cool them down.
5. Add 60 µl of annealed adapter R1 to one tube of ice-cold glycerol and mix by pipetting to obtain 120 µl of adapter mixture R1. Do the same with 60 µl of annealed adapter R2 and the second tube of glycerol to obtain 120 µl of adapter mixture R2. These adapter mixtures can later be used for assembly with unloaded Tn5.

5

- **Ordering the ME-seq oligos**

1. Order the two sets of index primers, Ad1. X and Ad2.X, in deepwell plates from IDT: The Excel file below could be used for ordering. Order the oligos with formulation as 100 µM in IDTE buffer, pH 8.0 and purification as standard desalting.

index primers.xlsx 16.2KB

2. All the below oligos are nessary for performing ME-seq. Most of them can be ordered from IDT, except the template switching oligo from Qiagen.

	A	B	C	D	E
	Name	Sequence	Scale	Purification	Formulation
	Poly(T) RT primer	/5Phos/ACGCTCCTACCAGTCNNNNNNNNNN/iBiodT/TTTTTTTTTTTTTVN	1 umol	HPLC	
	Random RT primer	/5Phos/ACGCTCCTACCAG/iBiodT/CNNNNNN	1 umol	HPLC	
	RNA PCR primer	AAGCAGTGGTATCAACGCAGAGT	100 nmol	STD	
	P5 primer	AATGATACGGCGACCACCGA	100 nmol	STD	
	P7 primer	CAAGCAGAAGACGGCATACGAGAT	100 nmol	STD	

DNA oligos for ME-seq from IDT

	A	B	C	D	E
	Name	Oligo type	Scale	Purifiation	Sequence
	TSO oligo	RNA oligo	1 umol	RNase-Free HPLC	AAGCAGTGGTATCAACGCAGAGTGAATrGrG+G

Template switching oligo (TSO)

Nucleosome depletion and fixtion in cells or nuclei

6 This section consists of two main parts: nucleosome depletion and fixation. The nucleosome depletion step should be performed only if whole-genome DNA methylation profiling is desired. Otherwise, proceed directly to Step 19 to fix your cells or nuclei.

7 Install the swinging bucket centrifuge rotor in the Eppendorf centrifuge and set the temperature as 4 ºC

8 Freshly prepare the LAND buffer and HCBI buffer and place them on ice. The recipe of these buffers could be found above.

9 Transfer a certian number of cells to a new 1.5 ml eppendorf centrifuge tube. The number of the cells should be larger than 500,000 to recovery enough nucleosome depleted cells for fixation.

10 Spin at 300xg for 4 min at 4 ºC. Carefully remove and discard the supernatant.

11 Gently resuspend the cells in 195 µl of ice-cold LAND buffer using pipetting.

12 Add 5 µl of 0.5 M lithium diiodosalicylate solution to the cell suspension and mix thoroughly by gentle pipetting. Incubate the mixture on ice for 5 minutes. The suspension should appear cloudy after the addition of the lithium solution.

13 Immediately add 1 ml of LAND buffer to the tube after incubation and mix thoroughly by gentle pipetting until the solution is no longer cloudy.

14 Spin at 500xg for 10 minutes at 4 ºC. Carefully remove and discard the supernatant.

15 Wash with 200 µl of HCBI buffer without disturbing pellet.

16 Spin at 500xg for 5 minutes at 4 ºC. Carefully remove and discard the supernatant.

17 Resuspend the cells in 100 µl of HCBI buffer and determine the cell density using a hemocytometer. Adjust the suspension by adding additional HCBI buffer as needed to obtain a final density of 1,000 cells per µl.

18 Add a certain volume of 1.6% formaldehyde (diluted from commercial 16% formaldehyde using H2O) to get a final concentration of formaldehyde to fix the cells (0.2% for cells from primary tissue; 1% for fragile immune cells, like primary PBMCs).
For example: For 500,000 non-immune cells in 500 µl of HCBI, add 71.4 µl of 1.6% formaldehyde to the cell suspension and mix well.

19 Incubate at room terperature for 5 minutes.

20 Add 28.1 µl of 2.5 M glycine, 25 µl of ice-cold 1 M Tris (pH 8.0), and 7 µl of 7.5% BSA solution to the tube. Mix thoroughly to quench the fixation. The volumes of these three reagents should be adjusted proportionally based on the initial volume of the cell suspension.

21 Incubate on ice for 10 minutes.

22 Spin at 750xg for 5 minutes at 4 ºC. Carefully remove and discard the supernatant.

23 Wash the pellet carefully without disturbing it using 500 µl of ice-cold HCBI.

24 Spin at 750xg for 5 minutes at 4 ºC. Carefully remove and discard the supernatant.

25 Wash the pellet carefully without disturbing it using 500 µl of ice-cold HCBI.

26 Spin at 750xg for 5 minutes at 4 ºC. Carefully remove and discard the supernatant.

27 If working with nucleosome depleted cells or nuclei, resuspend the cells in 50 µl of NI buffer. Proceed directly to step 34 to count the cell density. Otherwise, resuspend the cells in 100 µl of NI-RI buffer with 0.1% NP-40 (mix 99 µl of NI-RI buffer with 1 µl of 10% NP-40).

28 Incubate the cells on ice for 5 minutes for cultured cells or 3 minutes for cells from primary tissue.

29 Immediately add 900 µl of NI-RI buffer to the tube and mix well.

30 Spin at 900xg for 5 minutes at 4 ºC. Carefully remove and discard the supernatant.

31 Wash the pellet carefully without disturbing it using 500 µl of ice-cold NI-RI.

32 Spin at 900xg for 5 minutes at 4 ºC. Carefully remove and discard the supernatant.

33 Resuspend the cells in 50 µl of NI buffer and determine the cell density using a hemocytometer. Adjust the cell density to 2000 cells per µl by adding more NI buffer to the tube.

Transposition

34 Assemble the unloaded Tn5 with two annealed adapters, R1 and R2, using the recipe below. Incubate at 30 ºC for 1 hour. (**Tn5 should be assembled freshly, no overnight storage!!!**)

	A	B
	Name	**Volume (µl)**
	Annealed R1	4
	Annealed R2	4
	unloaded Tn5	0.5
	Tn5 dilution buffer	7.5
	Total	16

Recipe for assembling the Tn5 for 60,000 cells (6 reactions, 2.5 µl of assembled Tn5 per reaction, 10,000 cells per reaction)

The volume of each componant in the recipe should be changed proportionally according to the number of transposition reaction.

35 Mix 30 µl of cells (60,000 fixed cells) with 255 µl of transposition buffer by pipetting. Add 15 µl of assembled Tn5, mix well by pipetting.

36 Aliquot the reaction into PCR tubes, 50 µL per tube. Place the tubes on a thermomixer and incubate at 37 ºC for 30 minutes at 500 rpm.

37 Pool all the cell suspension in the PCR tubes together in a new 1.5 ml eppendorf tube. Add 500 µL of NI-RI buffer with 0.01% digitonin into it and mix well.

38 Spin at 1000xg for 5 minutes at 4 ºC. Carefully remove and discard the supernatant.

39 Wash the pellet with 500 µL of NI-RI buffer. Spin at 1000xg for 5 minutes at 4 ºC. Carefully remove and discard the supernatant.

40 Resuspend the cells in 10 µL of NI-RI buffer.

Poly(A) tailing

41 Performing Poly(A) tailing can substantially improve single-cell RNA library quality when working with immune system samples (e.g., PBMCs, HSCs). If you are not using such samples, you may skip this step and proceed directly to reverse transcription.

42 Mix 40 µL of the poly(A) mix with the 10 µL of cell suspension from step 41, and incubate at 37 ºC for 15 minutes in a PCR thermocycler.

	A	B
	Name	**Volume (µL)**
	5x Maxima H Minus RT buffer	10
	Enzymatics RI	0.31
	SUPERase RI	0.63
	E. coli poly(A) enzyme	3
	rATP	5
	H2O	21.06

The recipe for poly(A) mix

43 Add 300 µL of NI-RI buffer with 0.01% digitonin to the 50 µL reaction from the last step and mix well.

44 Spin at 1000xg for 5 minutes at 4 ºC. Carefully remove and discard the supernatant.

45 Wash the cells with 400 µL of NI-RI buffer without disturbing cell pellet.

46 Spin at 1000xg for 3 minutes at 4 ºC. Carefully remove and discard the supernatant.

47 Resuspend the cells in 5 µL of NI-RI buffer.

Reverse Transcription

48 Mix 45 µL of the RT mix with the 5 µL of cell suspension from step 48, and run the reverse transcription PCR program in a PCR thermocycler.

	A	B
	Name	**Volume (µL)**
	5X SMART RT buffer	10
	Enzymatics RI	0.3
	SUPERase RI	0.6
	10 mM dNTP	2.5
	100 µM Poly(T) RT primer	5
	100 µM Random RT primer	5
	50% PEG 8000	15
	200 U/µL Maxima H Minus RT	5
	H2O	1.6

The recipe for RT mix

	A	B	C
	1	50 ºC	10 min
	3 cycles	8 ºC	12 sec
		15 ºC	45 sec
		20 ºC	45 sec
		30 ºC	30 sec

	A	B	C
		42 ºC	120 sec
		50 ºC	180 sec
	3	50 ºC	5 min

PCR program, set the lid temp as 60 ºC

49 Add 500 µL of NI-RI buffer with 0.01% digitonin to the 50 µL reaction from the last step and mix well.

50 Spin at 1000xg for 3 minutes at 4 ºC. Carefully remove and discard the supernatant.

51 Wash the cells with 400 µL of NI-RI buffer without disturbing cell pellet.

52 Spin at 1000xg for 3 minutes at 4 ºC. Carefully remove and discard the supernatant.

53 Resuspend the cell in a certain volume of NI-RI buffer. The volume of NI-RI depends on the number of samples per experiment. For example, if there are **N** samples in a single run, resuspend the the cells from each sample in 96/**N***12 µL of NI-RI buffer.

Hybridization and ligation

54 Prepare the hybridization mix according to the number of samples in the experiment. We recommend preparing **N** individual aliquots rather than a single master mix to ensure better accuracy.
Note: Completely thaw the T4 ligase buffer to room temperature before use. Vortex the tube as needed to fully dissolve any white precipitate in the solution.

	A	B
	Name	**Volume (µL)**
	10x T4 ligase buffer	6*N
	SUPERase RI	0.2*N
	Enzymatics RI	0.5*N
	7.5% BSA	1.3*N

	A	B
	T4 ligase	1.2*N
	H2O	26.6*N

Recipe for an individual aliquot of hybridization mix

55 Mix the hybridization mix with cell suspension well and then transfer the solution to a new reservoir. If you have multiple samples, use a separate reservoir for each sample.

56 Use an 8 or 12 channel multi-channel pipette to split the mixture to each of the well in a Round 1 barcoding plate. **40 µL** of the mixture per well.
Note: Make sure to thaw all the three round barcoding plate to RT before hybridization. Spin down the plates before using (1000xg for 1 minute).

57 Cover the plate with a microseal 'F' foil seal and incubate the plate on a thermomixer at 20 ºC for 1 hour at 300rpm.

58 Prepare the Round 1 blocking mix and transfer it to a new reservoir.

	A	B
	Name	**Volume (µL)**
	100 µM Round 1 blocking oligo	253
	10x T4 ligase buffer	211
	T4 ligase	48
	H2O	640

Recipe for Round 1 blocking mix

59 Add **10 µL** of Round 1 blocking mix to each of the well in the plate by multi-channel pipette. Mix the reaction well by pipetting up and down 5 times

60 Cover the plate with a microseal 'F' foil seal and incubate the plate on a thermomixer at 20 ºC for 15 minutes at 300rpm.

61 Pool the sampels into a 7.5% BSA coated reservoir by using a multi-channel pipette. After reaction in all wells have been pooled, pipette up and down 5 times to mix the solution

well in the reservoir.

62 Use an 8 or 12 channel multi-channel pipette to split the mixture to each of the well in a Round 2 barcoding plate. **50 µL** of the mixture per well.

63 Cover the plate with a microseal 'F' foil seal and incubate the plate on a thermomixer at 20 ºC for 1 hour at 300rpm.

64 Prepare the Round 2 blocking mix and transfer it to a new reservoir.

	A	B
	Name	**Volume (µL)**
	100 µM Round 2 blocking oligo	304
	10x T4 ligase buffer	211
	T4 ligase	48
	H2O	589

Recipe for Round 2 blocking mix

65 Add **10 µL** of Round 2 blocking mix to each of the well in the plate by multi-channel pipette. Mix the reaction well by pipetting up and down 5 times

66 Cover the plate with a microseal 'F' foil seal and incubate the plate on a thermomixer at 20 ºC for 15 minutes at 300rpm.

67 Pool the sampels into a 7.5% BSA coated reservoir by using a multi-channel pipette. After reaction in all wells have been pooled, pipette up and down 5 times to mix the solution well in the reservoir.

68 Use an 8 or 12 channel multi-channel pipette to split the mixture to each of the well in a Round 3 barcoding plate. **60 µL** of the mixture per well.

69 Cover the plate with a microseal 'F' foil seal and incubate the plate on a thermomixer at 20 ºC for 1 hour at 300rpm.

70 Prepare the Round 3 blocking mix and transfer it to a new reservoir.

	A	B
	Name	**Volume (µL)**
	100 µM Round 3 blocking oligo	265
	5% digitonin	2.3
	H2O	884.7

Recipe for Round 3 blocking mix

71 Add **10 µL** of Round 3 blocking mix to each of the well in the plate by multi-channel pipette. Mix the reaction well by pipetting up and down 5 times

72 Cover the plate with a microseal 'F' foil seal and incubate the plate on a thermomixer at 20 ºC for 15 minutes at 300rpm.

73 Pool the sampels into a 7.5% BSA coated reservoir by using a multi-channel pipette. Transfer the mix to a 7.5% BSA coated 15 ml tube.

74 Spin at 1000xg for 3 minutes at 4 ºC. Carefully remove and discard the supernatant.

75 Resuspend the cells in 500 µL of NI-RI buffer and transfer to a new 7.5% BSA coated 1.5 ml eppendorf tube.

76 Spin at 1000xg for 3 minutes at 4 ºC. Carefully remove and discard the supernatant.

77 Resuspend the cells in 800 µL of NI-RI buffer and filter out the potential clumping cells through a 40 µm Flowmi cell strainer.

78 Collect the flow through cell suspension in a new 7.5% BSA coated 1.5 ml eppendorf tube.

79 Spin at 1000xg for 3 minutes at 4 ºC. Carefully remove and discard the supernatant.

80 Resuspend the cells in 50 µL of NI-RI buffer and determine the cell density using a hemocytometer.

Note: To avoid wasting too many cells in counting, we recommand to take 2 µL of cells, mix them with 8 µL of trypan blue and count the cells in hemocytometer.

81 Aliquot the retained cells into sub-pools in PCR tubes. Each sub-pool can contain between 1,000 and 20,000 cells, with a low risk of barcode collisions (less than 2.5%).

82 Bring the volume of each sub-pool up to 20 µL using NI-RI buffer.

83 Prepare the proteinase K digestion mix and add 28 µL of it to each sub-pool, mix them well.

	A	B
	Name	**Volume (µL)**
	2x reverse crosslinking buffer	25*N
	20 mg/ml proteinase K	2*N
	SUPERase RI	1*N
	total	28*N

Recipe for proteinase K digestion mix

84 Incubate the sub-pools at 55ºC for at least one hour on the thermal cycler with lid is set to 65ºC.
Note: The 55ºC incubation can be extended to overnight if needed.
STOPING POINT: The sub-pool after 55ºC incubation can be perserved at -80ºC for up to one month.

cDNA pull down and cDNA amplification

85 Retrieve one of the sub-pools from the −80ºC freezer and thaw it on ice. Add 2.5 µL of 100 mM PMSF in IPA to the sub-pool. Mix thoroughly by vortexing, then briefly centrifuge on a benchtop centrifuge.

86 Incubate the tubes for 10 minutes at room temperature.

87 Take 10 µL of MyOne Streptavidin C1 beads and transfer them into an new Eppendorf centrifuge tube.

88 Wash the beads with 100 µL of 1x B&W-T buffer twice.
Note: To wash the beads, first mix the desired buffer with the beads by pipetting. Place the tube on a magnetic rack and wait for ~30 seconds until the beads form a pellet. Carefully remove and discard the supernatant.

89 Wash the beads with 100 µL of 1x B&W-T buffer with 1 µL of SUPERase RI once.

90 Resuspend the beads in 50 µL of 2x B&W buffer with 1 µL of SUPERase RI and place the beads on ice.

91 Add the 50 µL of beads from last step to the sub-pool from step 87. Mix thoroughly by vortexing, then briefly centrifuge on a benchtop centrifuge.

92 Fix the tube on an end-to-end rotator and incubate the tube at room temperature for 60 mins at 10 rpm.

93 After the 60 mins incubation, place the tube on a magnetic rack. Transfer the supernatant to a new tube for future library preparation.
Note: The supernatant contains the transposed chromatin fragments, don't discard it!!! It could be stable on ice for several hours.

94 Wash the beads capturing the cDNA-RNA hybrids three times with 100 µL of 1× B&W-T buffer supplemented with 0.5 µL SUPERase RI.

95 Wash the beads once with 100 µL of STE buffer supplemented with 0.5 µL SUPERase RI.

96 Resuspend the beads in 50 µL of template switch mix.

	A	B
	Name	**Volume(µL)**
	5x SMART RT buffer	10
	20% Ficoll PM-400	10
	10 mM dNTPs	5

	A	B
	NeGen RI	5
	100 µM TSO oligo	1.25
	Maxima H Minus Reverse Transcriptase	2.5
	50% PEG8000	15
	H2O	1.25

Recipe for template switch mix

97 Fix the tube containing the beads on an end-to-end rotator and incubate the tube at room temperature for 30 mins at 10 rpm.

98 Incubate the tube containing the beads on a thermoshaker at 42 °C with shaking at 300 rpm for 1.5 h. Resuspend the beads by pipetting every 30 min.

99 Add 100 µL of ice-cold STE buffer to the tube, mix well by pipetting.

100 Place the tube on a magic rack to collect the beads, then remove and discard the supernatant.

101 Wash the beads with 200 µL of ice-cold STE buffer without distrubing the bead pellet. Remove and discard the supernatant.

102 Resuspend the beads in 50 µL of PCR mix.

	A	B
	Name	**Volume (µL)**
	2x KAPA HiFi PCR master mix	25
	25 µM RNA PCR primer	0.8

	A	B
	25 µM Ad2 primer	0.8
	H2O	23.4

Recipe for PCR mix

103 Place the tube in a PCR thermocycler and run the program as follows:

	A	B	C
	1	95 °C	3 mins
	5 cycles	98 °C	30 secs
		65 °C	45 secs
		72 °C	3 mins

cDNA initial PCR amplification program

104 Mix 2.5 µL of PCR sample from the last step with 7.5 µL of qPCR mix.

	A	B
	Name	**Volume (µL)**
	2x KAPA HiFi PCR master mix	3.75
	25 µM RNA PCR primer	0.12
	25 µM P7 primer	0.12
	20x EvAgreen	0.5
	H2O	3.01

Recipe for qPCR mix

105 Run the qPCR cycle program as follows.

	A	B	C
	1	95 °C	3 mins
	25 cycles	98 °C	30 secs
		65 °C	20 secs
		72 °C	3 mins

program for qPCR

106 Calculate the number of cycles needed to each 1/3 of the plateau fluorescence (0.33 Ct).

107 Run the continued PCR cycles as follows.

	A	B	C
	1	95 °C	3 mins
	N cycles (1/3 Ct)	98 °C	30 secs
		65 °C	45 secs
		72 °C	3 mins

Continued PCR amplification program

108 Add 2.5 µL of EB buffer back to the PCR sample and mix it with 45 µL of AMPure beads (0.9x) well by pipetting

109 Finish the beads clean-up and elute the amplified cDNA in 10 µL of EB buffer. Check the DNA concentration using Qubit and keep the record.

Gap filling, oxidation, reduction and amplification for DNA methylation library

110 Purify the supernatant from **step 94** using a Zymo DNA Clean & Concentrator column and elute the DNA sample in 12.5 µL of EB buffer.
Note: Follow the manufacturer's instructions to finish the DNA purification.

111 Transfer the 12.5 µL of DNA sample from the last step to a new PCR strip tube and mix it with 4 µL of gap filling mix well by pipetting.

	A	B
	Name	**Volume (µL)**
	10x Ampligase buffer	2
	dNTP	2

Recipe for gap filling mix

112 Place the tube with DNA sample in a PCR thermocycler and run the program as follows:

	A	B	C
	1	50 °C	1 min
	2	45 °C	10 mins
	3	ramp at 0.1 °C/sec, from 45 °C to 37°C	
	4	37 °C	hold
	5	37 °C	30 mins
	6	4 °C	hold

PCR Program for Gap Filling of Transposed DNA

Note: Add 1 µL of T4 DNA polymerase and 2.5 µL of Ampligase to the tube and mix well right before step 5, 37 °C 30-min incubation

113 Purify the gap filled DNA sample with a Zymo DNA Clean & Concentrator column and elute the DNA sample in 12 µL of H2O.

114 Prepare the oxidation reaction in a new PCR tube as follows:

	A	B
	Name	**Volume(µL)**
	DNA sample	12
	10x Oxidation buffer	2.5

	A	B
	23 uM mTET1 enzyme	6.5
	ATP	3

Recipe for the oxidation reaction

115 Add 1 µL of 2.5 mM Fe(II) solution to the oxidation reaction and mix well.
Note: The Fe(II) solution was freshly diluted from a 500 mM stock with H2O immediately before use. The 500 mM Fe(II) stock solution was stored at −80 °C.

116 Incubate the tube with oxidation reaction in a PCR thermcycler at 37 °C for 80 mins.

117 Add 1 µL of 0.8 U/µL proteinaseK to the tube and mix well by pipetting. Incubate the tube at 50 °C for 1 hr.

118 Purify the DNA from the reaction using 45 µL of AMPure beads (1.8X) and elute the DNA in 35 µL of H2O.

119 Prepare the reduction reaction in a new PCR tube as follows:

	A	B
	Name	**Volume(µL)**
	DNA sample	35
	10M pyridine borane	5
	3M NaAc	10

Recipe for the reduction reaction

120 Incubate the reduction reaction in a thermoshaker at 37 °C for 16 hr at 850 rpm.

121 Purify the DNA sample with a Zymo DNA Clean & Concentrator column and elute the DNA sample in 20 µL of EB buffer.

122 Prepare the PCR amplification reaction in a new PCR tube as follows:

	A	B
	Name	**Volume(µL)**
	DNA sample	20
	2X KAPA HiFi HotStart Uracil+ ReadyMix	25
	25 uM Ad1 primer	1
	25 uM Ad2 primer	1
	H2O	3

Recipe for PCR mix

123 Run the PCR program as follows:

	A	B	C
	1	98 °C	45 secs
	5 cycles	98 °C	15 secs
		65 °C	30 secs
		72 °C	60 secs
	3	72 °C	60 secs

124 Mix 2.5 µL of PCR sample from the last step with 7.5 µL of qPCR mix.

	A	B
	Name	**Volume (µL)**
	2X KAPA HiFi HotStart Uracil+ ReadyMix	3.75
	25 uM Ad1 primer	0.15

	A	B
	25 uM Ad2 primer	0.15
	10X SYBRgreen	0.6
	H2O	2.85

Recipe for the qPCR mix

125 Run the qPCR cycle program as follows:

	A	B	C
	1	98 °C	45 secs
	20 cycles	98 °C	15 secs
		65 °C	30 secs
		72 °C	60 secs

qPCR program

126 Calculate the number of cycles needed to each 1/3 of the plateau fluorescence (0.33 Ct). And run the continued PCR cycles as follows.

	A	B	C
	1	95 °C	3 mins
	N cycles (1/3 Ct)	98 °C	30 secs
		65 °C	45 secs
		72 °C	3 mins

Continued PCR amplification program

127 Add 2.5 µL of EB buffer back to the PCR sample and mix it with 40 µL of AMPure beads (0.8x) well by pipetting

128 Finish the beads clean-up and elute the amplified cDNA in 20 µL of EB buffer. Check the DNA concentration using Qubit and keep the record.

small cDNA recovery

129 Take 50 ng of amplified cDNA from step 110 and transer it to a new PCR tube.

130 Add EB buffer to bring the volume to 50 µL.

131 Add 25 µL of AMPure beads (0.5x) to the tube and mix well by pipetting. Incubate at room temperature for 5 mins.

132 Transfer the entire 75 µL of supernatant to a new PCR tube and add 20 µL of AMPure beads to the tube, the total volume is 95 µL now.

133 Mix the solution well by pipetting and incubate at room temperature for 5 mins.

134 Place the tube on a magnetic rack to collect the beads and complete the bead cleanup.

135 Elute the double-selected small cDNA in 20 µL of EB buffer.

136 Prepare the PCR amplification reaction in a new PCR tube as follows:

	A	B
	Name	**Volume(µL)**
	DNA sample	20
	NEBNext ReadyMix	25
	25 uM PCR primer with index	1
	25 uM P7	1
	H2O	3

Recipe for the PCR reaction

137 Run the PCR program as follows:

	A	B	C
	1	98 °C	30 secs
	6 cycles	98 °C	10 secs
		65 °C	30 secs
		72 °C	60 secs

PCR program

138 Purify the DNA sample with 40 µL of AMPure beads (0.8x) and elute the DNA sample in 20 µL of EB buffer.

RNA library preparation

139 Assemble the unloaded Tn5 with the annealed adapter, R1, using the recipe below. Incubate at 30 ºC for 1 hour. (**Tn5 should be assembled freshly, no overnight storage!!!**)

	A	B
	Name	Volume (µl)
	Annealed R1	10
	unloaded Tn5	0.3
	Tn5 dilution buffer	9.7
	Total	20

Recipe for assembling the Tn5 for 7 reactions (7 reactions, 2.5 µl of assembled Tn5 per reaction)

140 Take 50 ng of amplified cDNA from step 110 and transer it to a new PCR tube.

141 Add EB buffer to bring the volume to 15 µL.

142 Add 7.5 µL of H2O, 25 µL of 2x TD buffer and 2.5 µL of assembled Tn5 to the tube and mix well by pipetting.

143 Incubate at 55 ºC for 5 mins.
Note: Immediately proceed to the next step to prevent over-transposition of the DNA.

144 Purify the DNA sample with a Zymo DNA Clean & Concentrator column and elute the DNA sample in 20 µL of EB buffer.

145 Prepare the PCR amplification reaction in a new PCR tube as follows:

	A	B
	Name	Volume(µL)
	DNA sample	20
	NEBNext ReadyMix	25
	25 uM Ad1 primer	1
	25 uM P7	1
	H2O	3

Recipe for the PCR reaction

146 Run the PCR program as follows:

	A	B	C
	1	98 °C	30 secs
	6 cycles	98 °C	10 secs
		65 °C	30 secs
		72 °C	60 secs

PCR program

147 Purify the DNA sample with 40 µL of AMPure beads (0.8x) and elute the DNA sample in 20 µL of EB buffer.

Library quantification and sequencing

148 Quantify using the Kapa Quant qPCR kit for Illumina, and pool libraries to 10-20 nM concentration. Expect > 30-80 nM, average library size of 450bp with minimal adapter dimers.

Library structure for both modalities