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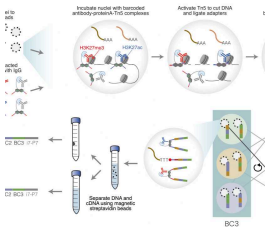
scMTR-seq



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Human Developmental B...

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We use this protocol and it's working

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Abstract

Here, we describe a protocol for scMTR-seq (single cell multi-targets and mRNA sequencing), which is a multi-omics sequencing technology that can simultaneously profile multiple histone modifications or other chromatin-bound proteins and transcriptome in the same single cells. scMTR-seq has high sensitivity, high cell recovery and is highly scalable in terms of starting material requirement. The protocol has the following steps: i) pre-assemble antibodies specific for each target histone modification or protein with indexed proteinA-Tn5-adapters; ii) perform in situ Tn5-mediated tagmentation with indexed complexes; iii) capture nuclear mRNA with a barcoded poly-T primer followed by in situ reverse transcription; iv) label single cells using split-pool combinatorial barcoding. We have applied scMTR-seq to profile histone modifications and transcriptomes in single cells from a broad range of heterogeneous samples including pluripotent stem cell differentiation and mouse pre-implantation embryos.

Attachments

scMTR-seq_primers.xl...

17KB

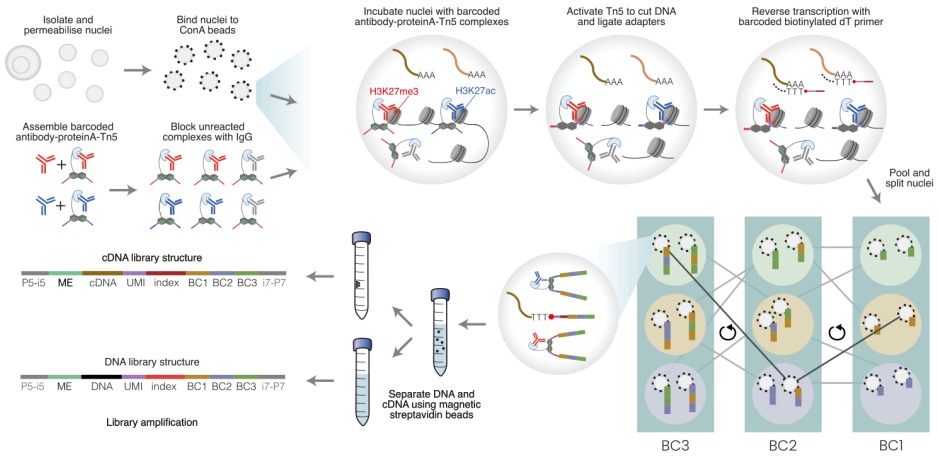
Protocol materials

- Nuclease-free water, not DEPC-treated **Life Technologies Catalog #**AM9932
- Roche Complete Protease Inhibitor EDTA-Free tablets **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**5056489001
- DPBS (no Ca, no Mg) **Thermofisher Catalog #**14190144
- Bovine Albumin Fraction V (7.5% solution) **Thermo Fisher Catalog #**15260037
- Trypan Blue Solution, 0.4% **Thermo Fisher Catalog #**15250061
- 16% Formaldehyde (w/v) Methanol-free **Thermo Fisher Scientific Catalog #**28906
- Concanavalin-coated magnetic beads **Bangs Laboratories Catalog #**BP531
- EDTA (0.5 M), pH 8.0, RNase-free **Thermo Fisher Catalog #**AM9260G
- Sodium Chloride (5M) **Invitrogen - Thermo Fisher Catalog #**AM9760G
- 1M MgCl2 **Invitrogen - Thermo Fisher Catalog #**AM9530G
- Anti-Histone H3 (acetyl K27) antibody - ChIP Grade **Abcam Catalog #**ab4729
- Anti-Histone H3 (mono methyl K4) antibody - ChIP Grade **Abcam Catalog #**ab8895
- Anti-Histone H3 (tri methyl K36) antibody - ChIP Grade **Abcam Catalog #**ab9050
- Tri-Methyl-Histone H3 (Lys27) (C36B11) Rabbit mAb **Cell Signaling Technology Catalog #**9733
- Anti-Histone H3 (tri methyl K4) antibody - ChIP Grade **Abcam Catalog #**ab8580
- Anti-Histone H3 (tri methyl K9) antibody - ChIP Grade **Abcam Catalog #**ab8898
- Normal Rabbit IgG Control **R&D Systems Catalog #**AB-105-C
- PEG 6000 (Poly(ethylene glycol)) **Bio Basic Inc. Catalog #**PB0432.SIZE.500g
- dNTP Set 100 mM Solutions **Thermo Fisher Scientific Catalog #**R0182
- Protector RNase Inhibitor **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**3335399001
- SUPERaseIN RNase Inhibitor **Thermo Fisher Scientific Catalog #**AM2696
- Maxima H Minus Reverse Transcriptase **Thermo Fisher Scientific Catalog ##**EP0741
- T4 DNA Ligase - 100,000 units **New England Biolabs Catalog #**M0202L
- Protector RNase Inhibitor **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**3335402001
- 10% Triton X-100 **Merck MilliporeSigma (Sigma-Aldrich)**
- SDS, 10% Solution, RNase-free **Thermo Fisher Catalog #**AM9822
- 1M Tris-HCl (pH 8.0) **Thermo Fisher Scientific Catalog #**15568025
- Proteinase K **Thermo Fisher Scientific Catalog #**EO0491
- Dynabeads MyOne Streptavidin C1 **Invitrogen - Thermo Fisher Catalog #**65001

- PMSF **Merck MilliporeSigma (Sigma-Aldrich)** **Catalog #**P7626
- Ficoll PM-400 **Merck MilliporeSigma (Sigma-Aldrich)** **Catalog #**F5415-50ML
- HotStart ReadyMix (KAPA HiFi PCR kit) **Kapa Biosystems** **Catalog #**KK2601
- Ampure XP beads **Beckman Coulter** **Catalog #**A63881
- NEBNext® High-Fidelity 2X PCR Master Mix **New England Biolabs** **Catalog #**M0541
- Glycerol **Merck MilliporeSigma (Sigma-Aldrich)** **Catalog #**G5516
- 1 M Hydroxyethyl piperazineethanesulfonic acid pH 7.9 (HEPES (K)) **Merck MilliporeSigma (Sigma-Aldrich)** **Catalog #**H3375
- 1 M Potassium Chloride (KCl) **Merck MilliporeSigma (Sigma-Aldrich)** **Catalog #**P3911
- 2 M Spermidine **Merck MilliporeSigma (Sigma-Aldrich)** **Catalog #**S2501
- PBS **Thermo Fisher Scientific** **Catalog #**28374
- Glycine
- 1 M Hydroxyethyl piperazineethanesulfonic acid pH 7.5 (HEPES (Na)) **Merck MilliporeSigma (Sigma-Aldrich)** **Catalog #**H3375
- 5M NaCl **Ambion** **Catalog #**AM9760G
- 1 M Calcium Chloride (CaCl2) **Fisher Scientific** **Catalog #**BP510
- 1 M Manganese Chloride (MnCl2) **Merck MilliporeSigma (Sigma-Aldrich)** **Catalog #**203734
- Trizma® hydrochloride solution **Merck MilliporeSigma (Sigma-Aldrich)** **Catalog #**T2319
- NP-40 10% **Merck MilliporeSigma (Sigma-Aldrich)** **Catalog #**11332473001
- 10% Tween 20 **Bio-Rad Laboratories** **Catalog #**1662404
- Tris HCl Buffer 1M Solution, Sterile pH 7.5 **Bio Basic Inc.** **Catalog #**SD8124.SIZE.450ml
- Pierce™ Dimethylformamide (DMF), Sequencing grade **Thermo Fisher Scientific** **Catalog #**20672
- 5M NaCl solution **Thermo Fisher Scientific** **Catalog #**AM9759
- DTT **Merck MilliporeSigma (Sigma-Aldrich)** **Catalog #**D0632

Solution Preparation

1



Overview of scMTR-seq

Solution Preparation

1.1 **Protease Inhibitor Cocktail (PIC)** (100x):

Dissolve 1 tablet of

Roche Complete Protease Inhibitor EDTA-Free tablets **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**5056489001

in 500 µL

Nuclease-free water, not DEPC-treated **Life Technologies Catalog #**AM9932 ,

aliquot and store at -20 °C .

Avoid repeated freeze-thaw cycles

1.2 **RNase Inhibitor (RI) mix** (80x)

Mix:

- SUPERaseIN RNase Inhibitor **Thermo Fisher Scientific Catalog #**AM2696
- Protector RNase Inhibitor **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**3335399001

at a 1:1 ratio, and store at -20 °C

1.3 PBS with inhibitors (PBSI)

- 1x **PIC**
- 0.4x **RI** mix
- 0.04% BSA
  Bovine Albumin Fraction V (7.5% solution) **Thermo Fisher Catalog #**15260037
  diluted in DPBS (no Ca, no Mg) **Thermofisher Catalog #**14190144

eg: 2 µL **PIC +** 1 µL **RI +** 1.06 µL BSA + 196 µL DPBS

1.4 2x Nuclei Extraction (NE) buffer

	A	B	C	D
	Reagents	Stocking	Working 1x	2x /µL
	HEPES(K+) pH 7.9	1M	20 mM	1000
	KCl	1M	10 mM	500
	Spermidine	2M	0.5 mM	12.5
	Triton-X100	10% (vol/vol)	0.1%	500
	Glycerol	100%	20%	10000
	PIC*	100x	2x	500
	RI*	80x	2x	625
	Nuclease-free water			11862.5
			total	25000

**** Only add 2x PIC and 2x RI mix just before use.***
Prior to these additions, the buffer can be stored at 4 °C for up to 3 months.
NB To achieve 40% glycerol in the 2x NE buffer, we typically add 12.5g of 100% glycerol.

- 1 M Hydroxyethyl piperazineethanesulfonic acid pH 7.9 (HEPES (K)) **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**H3375
- 1 M Potassium Chloride (KCl) **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**P3911
- 2 M Spermidine **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**S2501
- 10% Triton X-100 **Merck MilliporeSigma (Sigma-Aldrich)**

- Glycerol **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**G5516
- **PIC** (100x)
- **RI** (80x)
- Nuclease-free water, not DEPC-treated **Life Technologies Catalog #**AM9932

1.5 **5x Fixation quench buffer**

	A	B	C	D
	Reagents	Stocking	Working 1x	/µL
	Glycine	2.5M	125 mM	50
	Tris-HCl pH 8.0	1M	50 mM	50
	Triton-X100	10%	0.1% (in buffer)	2
	BSA*	7.5%	0.1%	13.4
	PBS			84.6
			total	200

**** Only add BSA just before use.*** Store 4 °C .

- Glycine
- 1M Tris-HCl (pH 8.0) **Thermo Fisher Scientific Catalog #**15568025
- 10% Triton X-100 **Merck MilliporeSigma (Sigma-Aldrich)**
- Bovine Albumin Fraction V (7.5% solution) **Thermo Fisher Catalog #**15260037
- PBS **Thermo Fisher Scientific Catalog #**28374

1.6 **Wash Buffer**

	A	B	C	D
	Reagents	Stocking	Working 1x	/µL
	HEPES(Na+) pH 7.5	1 M	20 mM	1000
	NaCl	5 M	150 mM	1500

	A	B	C	D
	Spermidine	2 M	0.5 mM	12.5
	BSA*	7.5%	1%	6666
	PIC*	100x	1x	500
	RI*	80x	1x	625
	Nuclease-free water			39696
			total	50000

**** Only add 1x PIC, 1x RI mix, and 1% BSA just before use.***
Prior to these additions, buffer can be stored at 4 °C for up to 3 months.

- 1 M Hydroxyethyl piperazineethanesulfonic acid pH 7.5 (HEPES (Na)) **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**H3375
- 5M NaCl **Ambion Catalog #**AM9760G
- 2 M Spermidine **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**S2501
- Bovine Albumin Fraction V (7.5% solution) **Thermo Fisher Catalog #**15260037
- **PIC** (100x)
- **RI mix** (80x)
- Nuclease-free water, not DEPC-treated **Life Technologies Catalog #**AM9932

1.7 **Binding Buffer**

	A	B	C	D
	Reagents	Stocking	Working 1x	/µL
	HEPES (Na+) pH 7.5	1 M	20 mM	200
	KCl	1 M	10 mM	100
	CaCl2	1 M	1 mM	10
	MnCl2	1 M	1 mM	10
	Nuclease-free water			9680
			total	10000

Store the buffer at 4 °C for up to 6 months

- 1 M Hydroxyethyl piperazineethanesulfonic acid pH 7.5 (HEPES (Na)) **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**H3375
- 1 M Potassium Chloride (KCl) **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**P3911
- 1 M Calcium Chloride (CaCl2) **Fisher Scientific Catalog #**BP510
- 1 M Manganese Chloride (MnCl2) **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**203734
- Nuclease-free water, not DEPC-treated **Life Technologies Catalog #**AM9932

1.8 **Nuclei Isolation Buffer (NIB)**

	A	B	C	D	E
	Reagents	Stocking	Working 1x	/µL	
	Trizma buffer pH 7.5	1 M	10 mM	500	
	NaCl	5 M	10 mM	100	
	MgCl2	1 M	3 mM	150	
	NP-40	10%	0.1%	500	
	Nuclease-free water			48750	
			total	50000	

Add PIC and RI mix just before use

- Trizma® hydrochloride solution **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**T2319
- Sodium Chloride (5M) **Invitrogen - Thermo Fisher Catalog #**AM9760G
- 1M MgCl2 **Invitrogen - Thermo Fisher Catalog #**AM9530G
- NP-40 10% **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**11332473001
- Nuclease-free water, not DEPC-treated **Life Technologies Catalog #**AM9932

1.9 **STE buffer**

	A	B	C	D
	Reagents	Stocking	Working 1x	/µL
	Tris pH 8.0	1 M	10 mM	500
	NaCl	5 M	50 mM	500
	EDTA	0.5 M	1 mM	100
	Nuclease-free water			48900
			total	50000

- 1M Tris-HCl (pH 8.0) **Thermo Fisher Scientific Catalog #**15568025
- Sodium Chloride (5M) **Invitrogen - Thermo Fisher Catalog #**AM9760G
- EDTA (0.5 M), pH 8.0, RNase-free **Thermo Fisher Catalog #**AM9260G
- Nuclease-free water, not DEPC-treated **Life Technologies Catalog #**AM9932

1.10 **1x B&W-T buffer**

	A	B	C	D
	Reagents	Stocking	In buffer	/µL
	Tris pH 8.0	1M	5 mM	50
	NaCl	5M	1 M	2000
	EDTA	0.5 M	0.5 mM	10
	Tween 20	10%	0.05%	50
	Nuclease-free water			7890
			total	10000

- 1M Tris-HCl (pH 8.0) **Thermo Fisher Scientific Catalog #**15568025
- Sodium Chloride (5M) **Invitrogen - Thermo Fisher Catalog #**AM9760G
- EDTA (0.5 M), pH 8.0, RNase-free **Thermo Fisher Catalog #**AM9260G
- 10% Tween 20 **Bio-Rad Laboratories Catalog #**1662404
- Nuclease-free water, not DEPC-treated **Life Technologies Catalog #**AM9932

1.11 **2x Binding & Washing buffer**

	A	B	C	D
	Reagents	Stocking	In buffer	/µL
	Tris pH 8.0	1M	10 mM	10
	NaCl	5M	2 M	400
	EDTA	0.5 M	1 mM	2
	Nuclease-free water			588
			total	1000

Add Superase RI before use (1µL RI to 45µL 2x Binding & Washing buffer)

- 1M Tris-HCl (pH 8.0) **Thermo Fisher Scientific Catalog #**15568025
- Sodium Chloride (5M) **Invitrogen - Thermo Fisher Catalog #**AM9760G
- EDTA (0.5 M), pH 8.0, RNase-free **Thermo Fisher Catalog #**AM9260G
- Nuclease-free water, not DEPC-treated **Life Technologies Catalog #**AM9932

1.12 **4x Tag Buffer for cDNA tagmentation**

	A	B	C	D
	Reagents	Stocking	In Buffer	/µL
	Tris-HCl pH 7.5	1M	40mM	40
	MgCl2	1M	20mM	20
	Dimethylformamide (DMF)	100%	20%	200
	Nuclease-free water			740
			total	1000

- Tris HCl Buffer 1M Solution, Sterile pH 7.5 **Bio Basic Inc. Catalog #**SD8124.SIZE.450ml
- 1M MgCl2 **Invitrogen - Thermo Fisher Catalog #**AM9530G
- Pierce™ Dimethylformamide (DMF), Sequencing grade **Thermo Fisher Scientific Catalog #**20672
- Nuclease-free water, not DEPC-treated **Life Technologies Catalog #**AM9932

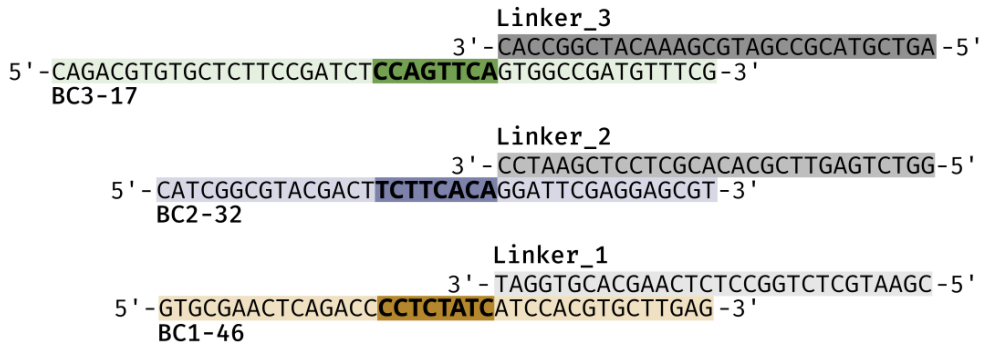
1.13 **Tn5 dilution buffer**

	A	B	C	D
	Reagents	Stocking	In Buffer	/uL
	Tris-HCl pH7.5	1M	10mM	10
	NaCL	5M	100mM	20
	Glycerol	100%	50% (0.625g)	500
	DTT	1mM	0.1M	10
	Nuclease-free water			460
			total	1000

- Tris HCl Buffer 1M Solution, Sterile pH 7.5 **Bio Basic Inc. Catalog #**SD8124.SIZE.450ml
- 5M NaCl solution **Thermo Fisher Scientific Catalog #**AM9759
- Glycerol **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**G5516
- DTT **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**D0632
- Nuclease-free water, not DEPC-treated **Life Technologies Catalog #**AM9932

Prepare oligonucleotides for split pool barcoding

2 This section prepares the oligonucleotides that will add the sequence used to identify individual cells.



Example Annealed Barcodes & Linkers

Equipment:

- PCR machine (thermocycler)
- RNAse-free 96-well plates (Twin-Tec PRC plate, 96 Lobind, Semi-skirted)

Equipment

Eppendorf Twin tec PCR plates, 96 wells	NAME
96 well PCR plate	TYPE
Eppendorf	BRAND
EP0030128770	SKU
https://www.sigmaaldrich.com/GB/en/product/sigma/ep0030128770	LINK

3 **Primers**

Linker Primers

	A	B
	Name	Sequence
	Linker_1	CGAATGCTCTGGCCTCTCAAGCACGTGGAT
	Linker_2	GGTCTGAGTTCGCACACGCTCCTCGAATCC
	Linker_3	AGTCGTACGCCGATGCGAAACATCGGCCAC

Barcode Primers

Split pool barcoding for cell identification, example primer sequence and index position:

```
BC1_#01
GTGCGAACTCAGACC CTGTAGCC ATCCACGTGCTTGAG
                                  |     Index     |
```

BC1

	A	B
	Name	Sequence
	BC1_#01	/5Phos/GTGCGAACTCAGACCAACGTGATATCCACGTGCTTGAG
	BC1_#02	/5Phos/GTGCGAACTCAGACCAAACATCGATCCACGTGCTTGAG
	BC1_#03	/5Phos/GTGCGAACTCAGACCACCACTGTATCCACGTGCTTGAG
	BC1_#04	/5Phos/GTGCGAACTCAGACCCATCAAGTATCCACGTGCTTGAG
	BC1_#05	/5Phos/GTGCGAACTCAGACCCTGTAGCCATCCACGTGCTTGAG
	BC1_#06	/5Phos/GTGCGAACTCAGACCAACCGAGAATCCACGTGCTTGAG
	BC1_#07	/5Phos/GTGCGAACTCAGACCAAGGTACAATCCACGTGCTTGAG
	BC1_#08	/5Phos/GTGCGAACTCAGACCACAGCAGAATCCACGTGCTTGAG
	BC1_#09	/5Phos/GTGCGAACTCAGACCACCTCCAAATCCACGTGCTTGAG
	BC1_#10	/5Phos/GTGCGAACTCAGACCACGCTCGAATCCACGTGCTTGAG
	BC1_#11	/5Phos/GTGCGAACTCAGACCACTATGCAATCCACGTGCTTGAG
	BC1_#12	/5Phos/GTGCGAACTCAGACCAGAGTCAAATCCACGTGCTTGAG
	BC1_#13	/5Phos/GTGCGAACTCAGACCAGATCGCAATCCACGTGCTTGAG
	BC1_#14	/5Phos/GTGCGAACTCAGACCCAATGGAAATCCACGTGCTTGAG
	BC1_#15	/5Phos/GTGCGAACTCAGACCCAGCGTTAATCCACGTGCTTGAG
	BC1_#16	/5Phos/GTGCGAACTCAGACCCATACCAAATCCACGTGCTTGAG
	BC1_#17	/5Phos/GTGCGAACTCAGACCCCAGTTCAATCCACGTGCTTGAG
	BC1_#18	/5Phos/GTGCGAACTCAGACCCGAACTTAATCCACGTGCTTGAG
	BC1_#19	/5Phos/GTGCGAACTCAGACCCGACTGGAATCCACGTGCTTGAG
	BC1_#20	/5Phos/GTGCGAACTCAGACCCTCAATGAATCCACGTGCTTGAG
	BC1_#21	/5Phos/GTGCGAACTCAGACCCTGAGCCAATCCACGTGCTTGAG
	BC1_#22	/5Phos/GTGCGAACTCAGACCGAATCTGAATCCACGTGCTTGAG
	BC1_#23	/5Phos/GTGCGAACTCAGACCGAGCTGAAATCCACGTGCTTGAG
	BC1_#24	/5Phos/GTGCGAACTCAGACCGATAGACAATCCACGTGCTTGAG
	BC1_#25	/5Phos/GTGCGAACTCAGACCGCTAACGAATCCACGTGCTTGAG
	BC1_#26	/5Phos/GTGCGAACTCAGACCGCTCGGTAATCCACGTGCTTGAG

	A	B
	BC1_#27	/5Phos/GTGCGAACTCAGACCGGAGAACAATCCACGTGCTTGAG
	BC1_#28	/5Phos/GTGCGAACTCAGACCGGTGCGAAATCCACGTGCTTGAG
	BC1_#29	/5Phos/GTGCGAACTCAGACCGTACGCAAATCCACGTGCTTGAG
	BC1_#30	/5Phos/GTGCGAACTCAGACCGTCGTAGAATCCACGTGCTTGAG
	BC1_#31	/5Phos/GTGCGAACTCAGACCGTGTTCTAATCCACGTGCTTGAG
	BC1_#32	/5Phos/GTGCGAACTCAGACCTCTTCACAATCCACGTGCTTGAG
	BC1_#33	/5Phos/GTGCGAACTCAGACCTGGAACAAATCCACGTGCTTGAG
	BC1_#34	/5Phos/GTGCGAACTCAGACCTGGCTTCAATCCACGTGCTTGAG
	BC1_#35	/5Phos/GTGCGAACTCAGACCTGGTGGTAATCCACGTGCTTGAG
	BC1_#36	/5Phos/GTGCGAACTCAGACCTTCACGCAATCCACGTGCTTGAG
	BC1_#37	/5Phos/GTGCGAACTCAGACCAACTCACCATCCACGTGCTTGAG
	BC1_#38	/5Phos/GTGCGAACTCAGACCAAGAGATCATCCACGTGCTTGAG
	BC1_#39	/5Phos/GTGCGAACTCAGACCAAGGACACATCCACGTGCTTGAG
	BC1_#40	/5Phos/GTGCGAACTCAGACCAATCCGTCATCCACGTGCTTGAG
	BC1_#41	/5Phos/GTGCGAACTCAGACCACACGACCATCCACGTGCTTGAG
	BC1_#42	/5Phos/GTGCGAACTCAGACCAGCCATGCATCCACGTGCTTGAG
	BC1_#43	/5Phos/GTGCGAACTCAGACCATCATTCCATCCACGTGCTTGAG
	BC1_#44	/5Phos/GTGCGAACTCAGACCCACCTTACATCCACGTGCTTGAG
	BC1_#45	/5Phos/GTGCGAACTCAGACCCCTAATCCATCCACGTGCTTGAG
	BC1_#46	/5Phos/GTGCGAACTCAGACCCCTCTATCATCCACGTGCTTGAG
	BC1_#47	/5Phos/GTGCGAACTCAGACCGAACAGGCATCCACGTGCTTGAG
	BC1_#48	/5Phos/GTGCGAACTCAGACCGATGAATCATCCACGTGCTTGAG

BC2

	A	B
	Name	Sequence
	BC2_#01	/5Phos/CATCGGCGTACGACTAACGTGATGGATTCGAGGAGCGT

	A	B
	BC2_#02	/5Phos/CATCGGCGTACGACTAAACATCGGGATTCGAGGAGCGT
	BC2_#03	/5Phos/CATCGGCGTACGACTACCACTGTGGATTCGAGGAGCGT
	BC2_#04	/5Phos/CATCGGCGTACGACTCATCAAGTGGATTCGAGGAGCGT
	BC2_#05	/5Phos/CATCGGCGTACGACTCTGTAGCCGGATTCGAGGAGCGT
	BC2_#06	/5Phos/CATCGGCGTACGACTAACCGAGAGGATTCGAGGAGCGT
	BC2_#07	/5Phos/CATCGGCGTACGACTAAGGTACAGGATTCGAGGAGCGT
	BC2_#08	/5Phos/CATCGGCGTACGACTACAGCAGAGGATTCGAGGAGCGT
	BC2_#09	/5Phos/CATCGGCGTACGACTACCTCCAAGGATTCGAGGAGCGT
	BC2_#10	/5Phos/CATCGGCGTACGACTACGCTCGAGGATTCGAGGAGCGT
	BC2_#11	/5Phos/CATCGGCGTACGACTACTATGCAGGATTCGAGGAGCGT
	BC2_#12	/5Phos/CATCGGCGTACGACTAGAGTCAAGGATTCGAGGAGCGT
	BC2_#13	/5Phos/CATCGGCGTACGACTAGATCGCAGGATTCGAGGAGCGT
	BC2_#14	/5Phos/CATCGGCGTACGACTCAATGGAAGGATTCGAGGAGCGT
	BC2_#15	/5Phos/CATCGGCGTACGACTCAGCGTTAGGATTCGAGGAGCGT
	BC2_#16	/5Phos/CATCGGCGTACGACTCATACCAAGGATTCGAGGAGCGT
	BC2_#17	/5Phos/CATCGGCGTACGACTCCAGTTCAGGATTCGAGGAGCGT
	BC2_#18	/5Phos/CATCGGCGTACGACTCGAACTTAGGATTCGAGGAGCGT
	BC2_#19	/5Phos/CATCGGCGTACGACTCGACTGGAGGATTCGAGGAGCGT
	BC2_#20	/5Phos/CATCGGCGTACGACTCTCAATGAGGATTCGAGGAGCGT
	BC2_#21	/5Phos/CATCGGCGTACGACTCTGAGCCAGGATTCGAGGAGCGT
	BC2_#22	/5Phos/CATCGGCGTACGACTGAATCTGAGGATTCGAGGAGCGT
	BC2_#23	/5Phos/CATCGGCGTACGACTGAGCTGAAGGATTCGAGGAGCGT
	BC2_#24	/5Phos/CATCGGCGTACGACTGATAGACAGGATTCGAGGAGCGT
	BC2_#25	/5Phos/CATCGGCGTACGACTGCTAACGAGGATTCGAGGAGCGT
	BC2_#26	/5Phos/CATCGGCGTACGACTGCTCGGTAGGATTCGAGGAGCGT
	BC2_#27	/5Phos/CATCGGCGTACGACTGGAGAACAGGATTCGAGGAGCGT
	BC2_#28	/5Phos/CATCGGCGTACGACTGGTGCGAAGGATTCGAGGAGCGT

	A	B
	BC2_#29	/5Phos/CATCGGCGTACGACTGTACGCAAGGATTCGAGGAGCGT
	BC2_#30	/5Phos/CATCGGCGTACGACTGTCGTAGAGGATTCGAGGAGCGT
	BC2_#31	/5Phos/CATCGGCGTACGACTGTGTTCTAGGATTCGAGGAGCGT
	BC2_#32	/5Phos/CATCGGCGTACGACTTCTTCACAGGATTCGAGGAGCGT
	BC2_#33	/5Phos/CATCGGCGTACGACTTGGAACAAGGATTCGAGGAGCGT
	BC2_#34	/5Phos/CATCGGCGTACGACTTGGCTTCAGGATTCGAGGAGCGT
	BC2_#35	/5Phos/CATCGGCGTACGACTTGGTGGTAGGATTCGAGGAGCGT
	BC2_#36	/5Phos/CATCGGCGTACGACTTTCACGCAGGATTCGAGGAGCGT
	BC2_#37	/5Phos/CATCGGCGTACGACTAACTCACCGGATTCGAGGAGCGT
	BC2_#38	/5Phos/CATCGGCGTACGACTAAGAGATCGGATTCGAGGAGCGT
	BC2_#39	/5Phos/CATCGGCGTACGACTAAGGACACGGATTCGAGGAGCGT
	BC2_#40	/5Phos/CATCGGCGTACGACTAATCCGTCGGATTCGAGGAGCGT
	BC2_#41	/5Phos/CATCGGCGTACGACTACACGACCGGATTCGAGGAGCGT
	BC2_#42	/5Phos/CATCGGCGTACGACTAGCCATGCGGATTCGAGGAGCGT
	BC2_#43	/5Phos/CATCGGCGTACGACTATCATTCCGGATTCGAGGAGCGT
	BC2_#44	/5Phos/CATCGGCGTACGACTCACCTTACGGATTCGAGGAGCGT
	BC2_#45	/5Phos/CATCGGCGTACGACTCCTAATCCGGATTCGAGGAGCGT
	BC2_#46	/5Phos/CATCGGCGTACGACTCCTCTATCGGATTCGAGGAGCGT
	BC2_#47	/5Phos/CATCGGCGTACGACTGAACAGGCGGATTCGAGGAGCGT
	BC2_#48	/5Phos/CATCGGCGTACGACTGATGAATCGGATTCGAGGAGCGT

BC3

	A	B
	Name	Sequence
	BC3_#01	CAGACGTGTGCTCTTCCGATCTAACGTGATGTGGCCGATGTTTCG
	BC3_#02	CAGACGTGTGCTCTTCCGATCTAAACATCGGTGGCCGATGTTTCG
	BC3_#03	CAGACGTGTGCTCTTCCGATCTACCACTGTGTGGCCGATGTTTCG

	A	B
	BC3_#04	CAGACGTGTGCTCTTCCGATCTCATCAAGTGTGGCCGATGTTTCG
	BC3_#05	CAGACGTGTGCTCTTCCGATCTCTGTAGCCGTGGCCGATGTTTCG
	BC3_#06	CAGACGTGTGCTCTTCCGATCTAACCGAGAGTGGCCGATGTTTCG
	BC3_#07	CAGACGTGTGCTCTTCCGATCTAAGGTACAGTGGCCGATGTTTCG
	BC3_#08	CAGACGTGTGCTCTTCCGATCTACAGCAGAGTGGCCGATGTTTCG
	BC3_#09	CAGACGTGTGCTCTTCCGATCTACCTCCAAGTGGCCGATGTTTCG
	BC3_#10	CAGACGTGTGCTCTTCCGATCTACGCTCGAGTGGCCGATGTTTCG
	BC3_#11	CAGACGTGTGCTCTTCCGATCTACTATGCAGTGGCCGATGTTTCG
	BC3_#12	CAGACGTGTGCTCTTCCGATCTAGAGTCAAGTGGCCGATGTTTCG
	BC3_#13	CAGACGTGTGCTCTTCCGATCTAGATCGCAGTGGCCGATGTTTCG
	BC3_#14	CAGACGTGTGCTCTTCCGATCTCAATGGAAGTGGCCGATGTTTCG
	BC3_#15	CAGACGTGTGCTCTTCCGATCTCAGCGTTAGTGGCCGATGTTTCG
	BC3_#16	CAGACGTGTGCTCTTCCGATCTCATACCAAGTGGCCGATGTTTCG
	BC3_#17	CAGACGTGTGCTCTTCCGATCTCCAGTTCAGTGGCCGATGTTTCG
	BC3_#18	CAGACGTGTGCTCTTCCGATCTCGAACTTAGTGGCCGATGTTTCG
	BC3_#19	CAGACGTGTGCTCTTCCGATCTCGACTGGAGTGGCCGATGTTTCG
	BC3_#20	CAGACGTGTGCTCTTCCGATCTCTCAATGAGTGGCCGATGTTTCG
	BC3_#21	CAGACGTGTGCTCTTCCGATCTCTGAGCCAGTGGCCGATGTTTCG
	BC3_#22	CAGACGTGTGCTCTTCCGATCTGAATCTGAGTGGCCGATGTTTCG
	BC3_#23	CAGACGTGTGCTCTTCCGATCTGAGCTGAAGTGGCCGATGTTTCG
	BC3_#24	CAGACGTGTGCTCTTCCGATCTGATAGACAGTGGCCGATGTTTCG
	BC3_#25	CAGACGTGTGCTCTTCCGATCTGCTAACGAGTGGCCGATGTTTCG
	BC3_#26	CAGACGTGTGCTCTTCCGATCTGCTCGGTAGTGGCCGATGTTTCG
	BC3_#27	CAGACGTGTGCTCTTCCGATCTGGAGAACAGTGGCCGATGTTTCG
	BC3_#28	CAGACGTGTGCTCTTCCGATCTGGTGCGAAGTGGCCGATGTTTCG
	BC3_#29	CAGACGTGTGCTCTTCCGATCTGTACGCAAGTGGCCGATGTTTCG
	BC3_#30	CAGACGTGTGCTCTTCCGATCTGTCGTAGAGTGGCCGATGTTTCG

	A	B
	BC3_#31	CAGACGTGTGCTCTTCCGATCTGTGTTCTAGTGGCCGATGTTTCG
	BC3_#32	CAGACGTGTGCTCTTCCGATCTTCTTCACAGTGGCCGATGTTTCG
	BC3_#33	CAGACGTGTGCTCTTCCGATCTTGGAACAAGTGGCCGATGTTTCG
	BC3_#34	CAGACGTGTGCTCTTCCGATCTTGGCTTCAGTGGCCGATGTTTCG
	BC3_#35	CAGACGTGTGCTCTTCCGATCTTGGTGGTAGTGGCCGATGTTTCG
	BC3_#36	CAGACGTGTGCTCTTCCGATCTTTCACGCAGTGGCCGATGTTTCG
	BC3_#37	CAGACGTGTGCTCTTCCGATCTAACTCACCGTGGCCGATGTTTCG
	BC3_#38	CAGACGTGTGCTCTTCCGATCTAAGAGATCGTGGCCGATGTTTCG
	BC3_#39	CAGACGTGTGCTCTTCCGATCTAAGGACACGTGGCCGATGTTTCG
	BC3_#40	CAGACGTGTGCTCTTCCGATCTAATCCGTCGTGGCCGATGTTTCG
	BC3_#41	CAGACGTGTGCTCTTCCGATCTACACGACCGTGGCCGATGTTTCG
	BC3_#42	CAGACGTGTGCTCTTCCGATCTAGCCATGCGTGGCCGATGTTTCG
	BC3_#43	CAGACGTGTGCTCTTCCGATCTATCATTCCGTGGCCGATGTTTCG
	BC3_#44	CAGACGTGTGCTCTTCCGATCTCACCTTACGTGGCCGATGTTTCG
	BC3_#45	CAGACGTGTGCTCTTCCGATCTCCTAATCCGTGGCCGATGTTTCG
	BC3_#46	CAGACGTGTGCTCTTCCGATCTCCTCTATCGTGGCCGATGTTTCG
	BC3_#47	CAGACGTGTGCTCTTCCGATCTGAACAGGCGTGGCCGATGTTTCG
	BC3_#48	CAGACGTGTGCTCTTCCGATCTGATGAATCGTGGCCGATGTTTCG

3.1 The following sub-steps describe annealing linker primers with barcode primers in RNAse-free 96-well plates (Twin-Tec PRC plate, 96 Lobind, Semi-skirted) to a total volume of 10 µL per well. At the end of go to step #3.5 the resulting plate will contain oligos with the following concentrations:

BC1-Linker_1: in round 1 plates, containing:
- 9 µL *Linker_1* and
- 10 µL *BC1* barcodes

BC2-Linker_2: in round 2 plates, containing:
- 11 µL *Linker_2* and

- 12 µL *BC2* barcodes

BC3-Linker_3: in round 3 plates, containing:

- 13 µL *Linker_3* and
- 14 µL *BC3* barcodes

3.2 To prepare stock of 9 plates of 48 barcodes:

For each linker-barcode, prepare 10 µL /well x 9 × 1.1 = 100 µL ;

- **BC1-Linker1**:
  - *Linker_1* ([M] 1 millimolar (mM) stock) 0.9 µL
  - *BC1* ([M] 100 micromolar (µM) stock) 10 µL
  - **STE buffer** 89.1 µL (go to step #1.9 STE)
- **BC2-Linker2**:
  - *Linker_2* ([M] 1 millimolar (mM) stock) 1.1 µL
  - *BC2* ([M] 100 micromolar (µM) stock) 12 µL
  - **STE buffer** 86.9 µL
- **BC3-Linker3**:
  - *Linker_3* ([M] 1 millimolar (mM) stock) 1.3 µL
  - *BC3* ([M] 100 micromolar (µM) stock) 14 µL
  - **STE buffer** 84.7 µL

3.3 Prepare linker stocks (x 48 × 1.1 = x 53; in each round, linker is the same one)

- *Linker_1* 47.7 µL + **STE buffer** 4722.3 µL
- *Linker_2* 58.3 µL + **STE buffer** 4605.7 µL
- *Linker_3* 68.9 µL + **STE buffer** 4489.1 µL

3.4 Aliquot diluted linker into 48 wells: *Linker_1* 90 µL ; *Linker_2* 88 µL ; *Linker_3* 86 µL .

- Add barcodes of *BC1* (1-48) 10 µL to *Linker_1*
- Add barcodes of *BC2* (1-48) 12 µL to *Linker_2*
- Add barcodes of *BC3* (1-48) 14 µL to *Linker_3*

3.5 Place sealed plates with annealing linker and barcodes in PCR machine with the programme:

95 °C 00:02:00, cooling down to 20 °C at -1 °C /min

Aliquot mixture 10 µL to each well into 96-well plates

Seal plates and store at -20 °C.

Solution Preparation

4 **Equipment:**

- PCR machine (thermocycler)

Primers

Barcoding for antibody Identification, with example primer sequence and index position:

	A	B
	Name	Sequence
	MEB_dU_#01	/5Phos/AGGCCAGAGCATTCGAATGGNNNNNNNN/ideoxyU/AGATGTGTATAAGAGACAG
	MEB_dU_#02	/5Phos/AGGCCAGAGCATTCGTGAGANNNNNNNN/ideoxyU/AGATGTGTATAAGAGACAG
	MEB_dU_#03	/5Phos/AGGCCAGAGCATTCGCCATANNNNNNNN/ideoxyU/AGATGTGTATAAGAGACAG
	MEB_dU_#04	/5Phos/AGGCCAGAGCATTCGGGTGTNNNNNNNN/ideoxyU/AGATGTGTATAAGAGACAG
	ME_R	/5Phos/C*T*G*T*C*T*C*T*T*A*T*A*C*A*/3ddC/
	ME_A	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG

*N indicates a phosphorothioate bond (primer sequences are in the IDT DNA format).

4.1 Dilute oligonucleotides to [M] 200 micromolar (µM) in **STE buffer** (go to step #1.9 STE)

4.2 **pA-Tn5-MeBdU-MeR**
For each of the four MEB_dU oligonucleotides, in separate tubes mix:
- 2 µL [M] 200 micromolar (µM) phosphorylated Mosaic end adapter B dU (*MEB_dU_#N*)
- 2 µL [M] 200 micromolar (µM) blocked Mosaic end-reverse (*ME_R*) oligonucleotides

to generate [M] 100 micromolar (µM) **pre-annealed MEBdU-MER**

4.3 **pA-Tn5-MeA-MeR**
Mix:
- 2 µL [M] 200 micromolar (µM) Mosaic end adapter A (*ME_A*)
- 2 µL [M] 200 micromolar (µM) blocked Mosaic end-reverse (*ME-R*) oligonucleotides

to generate [M] 100 micromolar (µM) **pre-annealed MEA-MER**

4.4 Place the tubes in a thermocycler and run 95 °C 00:02:00 , slowly cool to 20 °C with ramp rate of -1 °C /min 2m

4.5 Mix 4 µL [M] 100 micromolar (µM) **pre-annealed MEBdU-MER** or **MEA-MER** with 40 µL of [M] 5.5 micromolar (µM) **pA-Tn5 fusion protein** and incubate on a rotating platform for 01:00:00 at room temperature. Store at -20 °C for up to 1 year. 1h

Note

We use home-made **pA-Tn5 fusion protein** generated from plasmid #124601 from Addgene **https://www.addgene.org/124601/** following this protocol:

Citation

Terri Bryson, Steven Henikoff
. 3XFlag-pATn5 Protein Purification and MEDS-loading (5x scale, 2L volume). protocols.io.

https://protocols.io/view/3xflag-patn5-protein-purification-and-meds-loading-8yrhxv6
LINK

Commercially available alternatives are:

- pA-Tn5 Transposase (10 µg) **Active Motif Catalog #**53161
- CUTANA™ Uncharged pAG-Tn5 for CUT&Tag **EpiCypher Catalog #**15-1025

Single-cell dissociation

5 **Equipment:**

- Centrifuge with swing-bucket rotor
- 1.5 mL protein lobind tube

Dissociate samples into single-cell suspension, and transfer cells to 1.5 mL protein lobind tube

5.1 Wash cells twice with **PBSI** (go to step #1.3 PBSI) by centrifuging cells in a swing-bucket rotor at 300 x g, 00:03:00 , and remove the supernatant.

Nuclei Preparation

6 To prepare nuclei:

Measure the the volume of the the single cell suspension and add **PBSI** (go to step #1.3 PBSI) to cells to a final volume of 80 µL

7 Add 80 µL **2x NE buffer** (go to step #1.4 2x NE Nuffer) (with 2x **PIC** and 2x **RI**) briefly vortex to mix and put cells on ice for 00:10:00 (adjust incubation time if needed; Trypan Blue Solution, 0.4% **Thermo Fisher Catalog #**15250061 , trypan blue positive > 95%).

Light Fixation and Quench

8 To help maintain intact nuclei for split-pool barcoding, we use a light fixation with 0.2% formaldehyde.

Equipment:

- Centrifuge with swing bucket rotor
- (optional) Mr. Frosty container

Fixation wash buffer

	A	B	C
	Reagent	Stocking	/µL
	Base Wash Buffer		867
	BSA	7.5%	133
	PIC	100x	10
	RI	80x	12.5

Quickly spin (100 x g, 00:00:05) to collect solution into the bottom, add 2 µL 16% Formaldehyde (w/v) Methanol-free **Thermo Fisher Scientific Catalog #**28906 to final 0.2%, immediately tap to mix and start stopwatch. Incubate for 00:05:00 exactly .

9 Quickly spin (100 x g, 00:00:05) to collect solution into the bottom, add 40 µL **5x Fixation Quench Buffer** (go to step #1.5 5x Fixation Quench Buffer) to quench nuclei, invert to mix, place on ice for 00:05:00 - 00:10:00 .

10 Centrifuge nuclei in a **swing-bucket rotor (pre-cooled)** at 600 x g, 4°C, 00:03:00 , carefully take out tubes and remove 180 µL from the top.
Leave ~ 20 µL in the tube without disturbing nuclei at the bottom.
Add 100 µL **Fixation wash buffer** to re-suspend cells, invert to mix.

11

Note

Optional Stopping Point

Quickly spin 100 x g, 00:00:05 , add 15 µL 100% DMSO to each sample (10-15% in final), invert to mix. Quickly spin 100 x g, 00:00:05 , place samples in a Mr. Frosty container and store at -80 °C overnight to freeze samples. The next day, samples are ready for shipment on dry ice or can be stored at -80 °C freezer for a longer term.

Binding nuclei to beads

15m

12 To immobilise nuclei, we bind the lightly-fixed nuclei with Concanavalin A (ConA)-coated magnetic beads.

Equipment:
- Magnet stand

Beads Preparation

12.1 Transfer (5 x N* x 1.1) µL
Concanavalin-coated magnetic beads **Bangs Laboratories Catalog #**BP531 bead slurry into a 1.5 mL tube , wash twice with 1 mL binding buffer, mix by pipetting, and place the tube on a magnetic stand to allow the mixture to clear, and drain liquid completely.

*where N is the number of samples

Note

Using more bead slurry could alleviate the formation of clumps. We typically use around 1 µL per 1,000 cells.

12.2 Re-suspend beads in (5 x N x 1.1) µL binding buffer, and hold on ice until nuclei are ready.

12.3 **Preparing**

Thawing Wash Buffer:

	A	B	C	D
	Reagents	Stocking	Working	/µL
	Base Wash Buffer			867
	BSA	7.5%	1%	133
	PIC	100x	1x	10
	RI	80x	0.4x	5

- 867 µL **wash buffer** (go to step #1.6 wash buffer)
- 133 µL
  Bovine Albumin Fraction V (7.5% solution) **Thermo Fisher Catalog #**15260037
- 5 µL **RI** mix
- 10 µL **PIC**

Thawing Wash buffer + EDTA:

	A	B	C	D
	Reagents	Stocking	Working	/µL
	Thawing Wash Buffer			500

	A	B	C	D
	EDTA	0.5 M	2 µM	2

- 500 µL **Thawing Wash Buffer**
- 2 µL EDTA (0.5 M), pH 8.0, RNase-free **Thermo Fisher Catalog #**AM9260G

12.4 Thaw nuclei on ice, add 500 µL **Thawing Wash Buffer**

12.5 Slowly add prepared ConA beads from step 12.2 into cell suspension while vortexing gently 1100 rpm Vortex Gently , place tubes on an end-over-end rotator for 00:05:00 - 00:10:00 Room temperature 15m

12.6 Place the tubes on a magnetic stand to allow the mixtures to clear. Carefully discard the liquid (taking care due to surface tension).

12.7 Wash once with 100 µL **Thawing Wash Buffer + EDTA** while tubes are on the magnetic stand.

DNA tagmentation

13 The steps below describe how to simultaneously tagment and profile multiple indexed antibodies using scMTR-seq. We use indexed MEBdU to multiplex each primary antibody of choice with different pA-Tn5-MEBdU-MER. To minimise cross-contamination between antibodies, we then block any unreacted pA-Tn5-MEBdu_MER by incubation with normal IgG. We have successfully profiled five different histone modifications simaltaenously with only minor off-target signals detected. Including an additional IgG profile helps to identify and further reduce off-target signals using computational methods.

As an alternative approach, we have also performed tagmentation in multiple sequential steps, rather than simultaneously (see step 13.12). Sequential profiling increases hands-on time and may lead to greater loss of nuclei, although the advantages of this alternative approach include reduced off-target signals and the opportunity to profile more targets in different rounds.

13.1 Solution Preparation

Equipment:

- end-over-end rotator
- cold room
- PCR machine (thermocycler)

Preparing

13.2 **High RI wash 300 buffer**

	A	B	C
	Reagents	Stocking	/µL
	Base Wash Buffer		867
	NaCl	5M	30
	BSA	7.5%	133
	PIC	100x	10
	RI	80x	12.5

- 867 µL **wash buffer** (go to step #1.6 wash buffer)
- 30 µL [M] 5 Mass Percent
  Sodium Chloride (5M) **Invitrogen - Thermo Fisher Catalog #**AM9760G
- 133 µL
  Bovine Albumin Fraction V (7.5% solution) **Thermo Fisher Catalog #**15260037
- 12.5 µL **RI** mix
- 10 µL **PIC**

(make 100 µL per sample)

13.3 **Low RI Wash 300 buffer**

	A	B	C
	Reagents	Stocking	/µL

	A	B	C
	Base Wash Buffer		867
	NaCl	5 M	30
	BSA	7.5%	133
	PIC	100x	10
	RI	80x	5

- 867 µL **wash buffer** (go to step #1.6 wash buffer)
- 30 µL [M] 5 Mass Percent
  Sodium Chloride (5M) **Invitrogen - Thermo Fisher Catalog #**AM9760G
- 133 µL
  Bovine Albumin Fraction V (7.5% solution) **Thermo Fisher Catalog #**15260037
- 10 µL **PIC**
- 5 µL **RI** mix

(make 100 µL per sample)

13.4 **Tag buffer**

	A	B	C
	Reagent	Stocking	/µL
	Base Wash Buffer		867
	NaCl	5 M	30
	BSA	7.5%	13.3
	PIC	100x	10
	RI	80x	25
	Nuclease-free water		119.7
	MgCl2	1M	10

- 867 µL **wash buffer** (go to step #1.6 wash buffer)
- 30 µL [M] 5 Mass Percent
  Sodium Chloride (5M) **Invitrogen - Thermo Fisher Catalog #**AM9760G
- 13.3 µL
  Bovine Albumin Fraction V (7.5% solution) **Thermo Fisher Catalog #**15260037
- 25 µL **RI** mix
- 10 µL **PIC**
- 119.7 µL
  Nuclease-free water, not DEPC-treated **Life Technologies Catalog #**AM9932
- 10 µL [M] 1 Mass Percent
  1M MgCl2 **Invitrogen - Thermo Fisher Catalog #**AM9530G

(make 100 µL per sample)

13.5 Pre-assemble selected primary antibody (Ab) and indexed **pA-Tn5-MeBdU-MeR** (0.5-1h). We list below several antibodies that we have used successfully in scMTR-seq. Alternative antibodies can be tested first using bulk CUT&Tag to check for signal specificity. 1h

Example Antibodies:

- Anti-Histone H3 (tri methyl K4) antibody - ChIP Grade **Abcam Catalog #**ab8580
- Anti-Histone H3 (tri methyl K9) antibody - ChIP Grade **Abcam Catalog #**ab8898
- Anti-Histone H3 (acetyl K27) antibody - ChIP Grade **Abcam Catalog #**ab4729
- Anti-Histone H3 (mono methyl K4) antibody - ChIP Grade **Abcam Catalog #**ab8895
- Tri-Methyl-Histone H3 (Lys27) (C36B11) Rabbit mAb **Cell Signaling Technology Catalog #**9733
- Anti-Histone H3 (tri methyl K36) antibody - ChIP Grade **Abcam Catalog #**ab9050

Mix:

- 0.5 µL [M] 1 µg/µL antibody

- 0.5 µL **pA-Tn5-MeBdU-MeR**
- 4.25 µL **High RI wash 300 buffer**

place tubes on an end-over-end rotator
10 rpm, Room temperature, 01:00:00 , end-over-end rotator

13.6 Block any remaining unreacted proteinA-Tn5: 30m

Add 1 µL [M] 1 µg/µL
Normal Rabbit IgG Control **R&D Systems Catalog #**AB-105-C and incubate
Room temperature for 00:30:00

13.7 Mix pre-assembled Ab-pA-Tn5-MeBdU-MeR (6 µL x n of indexed antibodies) with ConA-bound nuclei, add **High RI wash 300** to 99.6 µL, and then add 0.4 µL [M] 0.5 Mass Percent EDTA. 2h

Gently mix, briefly spin and incubate in cold room (4 °C) Overnight at 25-30 rpm on rotator or 1-2 hrs at Room temperature

13.8 Wash three times with **Low RI Wash 300 buffer**, using the magnetic stand, and invert to mix between washes

13.9 Re-suspend with 50 µL **Tag buffer**, gently mix and place in PCR machine, incubate 37 °C for 01:00:00 1h

13.10 Directly place tube on pre-cooled rack, and add 1.67 µL [M] 0.5 Mass Percent EDTA each sample to stop tagmentation, gently tap to mix

13.11 Add 8 µL
Bovine Albumin Fraction V (7.5% solution) **Thermo Fisher Catalog #**15260037 to each sample to avoid clumps forming. Mix by pipetting or gently tapping, place on ice for 00:05:00 5m

13.12 **(Optional)**
For sequential tagmentation, wash twice with **Low RI wash 300 buffer** after adding stop buffer, and repeat from go to step #13.7 to step 13.11.

In situ Reverse Transcription

17m 12s

14 In situ reverse transcribe polyA mRNA with biotinylated dT RT primers in the nuclei.

Equipment:

- PCR machine

Primers

RT_#02

```
5'-AGGCCAGAGCATTCGCCTAANNNNNNNNTTTTTTTTTTTTTTVN-3'
Linker_1 Annealing index   UMI
```

	A	B
	Name	Sequence
	RT_#01	/5Phos/AGGCCAGAGCATTCGAGCAANNNNNNNN /iBiodT/TTTTTTTTTTTTTTVN
	RT_#02	/5Phos/AGGCCAGAGCATTCGCCTAANNNNNNNN /iBiodT/TTTTTTTTTTTTTTVN
	RT_#03	/5Phos/AGGCCAGAGCATTCGGTCGANNNNNNNN /iBiodT/TTTTTTTTTTTTTTVN
	RT_#04	/5Phos/AGGCCAGAGCATTCGTGTGANNNNNNNN /iBiodT/TTTTTTTTTTTTTTVN

Preparing

14.1 **NIB-RI** buffer

- 867 µL **NIB** buffer (go to step #1.8 Nuclei Isolation Buffer)
- 133 µL
  Bovine Albumin Fraction V (7.5% solution) **Thermo Fisher Catalog #**15260037
- 5 µL **RI** mix

- 10 µL **PIC**

(make 350 µL per sample)

14.2 **RT mix**:

	A	B	C	D
	Reagent	Stocking	Working 1x	1x volume / µL
	RT buffer	5x	1x	10
	PEG 6000	50 %	15 %	15
	RT_#N primer	100 µM	10 µM	5
	dNTPs	25 mM/each	0.5 mM	1
	RNAse Protector	40 U/µL	0.5 U/µL	0.625
	Superase RI	20 U/µL	0.5 U/µL	1.25
	Maxima H Minus RT	200 U/µL	20 U/µl	5
	Nuclease-free water			12.125
			total	50

- PEG 6000 (Poly(ethylene glycol)) **Bio Basic Inc. Catalog #**PB0432.SIZE.500g
- *RT_#N*
- dNTP Set 100 mM Solutions **Thermo Fisher Scientific Catalog #**R0182
- Protector RNase Inhibitor **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**3335399001
- SUPERaseIN RNase Inhibitor **Thermo Fisher Scientific Catalog #**AM2696
- Maxima H Minus Reverse Transcriptase **Thermo Fisher Scientific Catalog ##**EP0741
- Nuclease-free water, not DEPC-treated **Life Technologies Catalog #**AM9932

14.3 Wash in situ-tagged nuclei twice with 100 µL **NIB-RI** buffer.

14.4 Add 10 µL of **RT mix** per 10,000 nuclei to each tube, gently mix and briefly centrifuge 17m 12s

- Run following programme to synthesise the first strand of cDNA:
  50 °C 00:10:00
- 3 cycles of annealing:
  8 °C 00:00:12
  15 °C 00:00:45
  20 °C 00:00:45
  30 °C 00:00:30
  42 °C 00:02:00
  50 °C 00:03:00
  End cycles
- 50 °C 5 min
- 4 °C Hold

14.5 Add 50 µL **NIB**-**RI** to dilute sample, place tubes on PCR magnet and remove liquid

14.6 Wash once with 100 µL **NIB**-**RI** and remove liquid

Split-pool barcoding 3h 14m

15 Three rounds of split-pool barcoding were used for barcoding single cells. As there is 48 different barcodes for each round in our assay, this will generate over 110,000 barcodes combinations (# of BC1 x # of BC2 x # of BC3) after three rounds of the split-pool barcoding procedure. Both cDNA and tagged gDNA molecules will get the same combination of barcodes within the same single nuclei.

Note

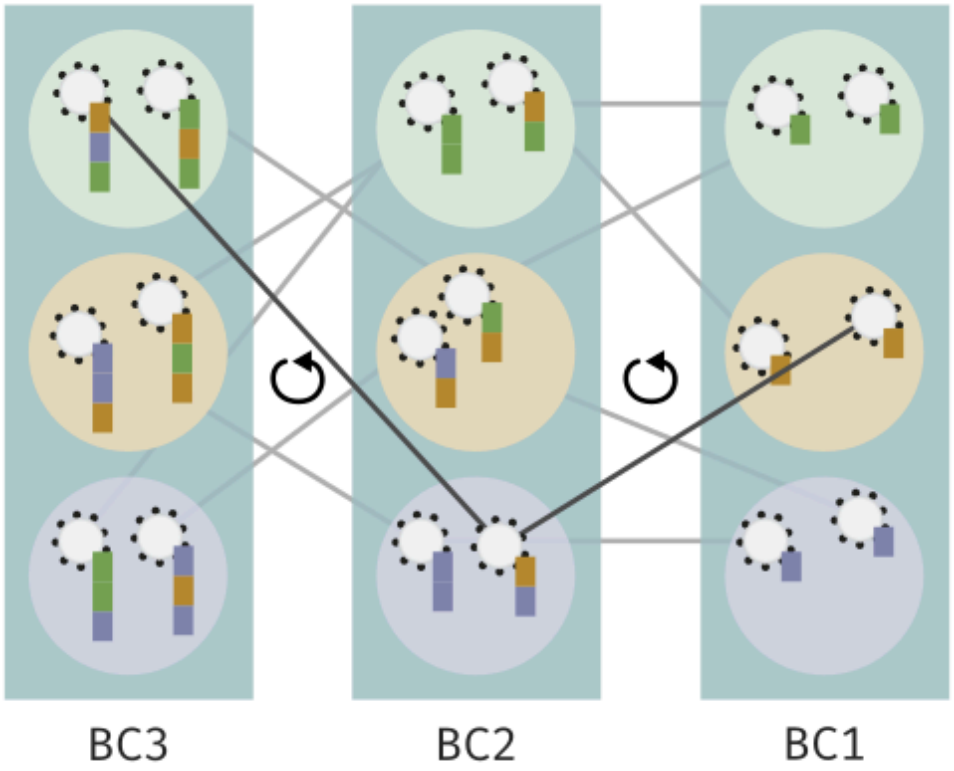
To ensure there are enough barcode combinations for barcoding each single cell/nuclei with a unique cell barcode, we adjust the conditions to keep the collision rate below 5%. The collision rate is calculated by dividing the number of cells or nuclei by the number of barcode combinations.

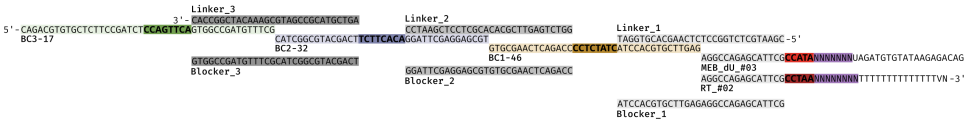
The number of cells or nuclei: as the recovery rate from the starting point is normally ~20-40%, we use the number of starting cells multiplied by the recovery rate to estimate the number of cells/nuclei.

The number of barcode combinations: in addition to the split-pool steps, we could use different RT primers and MEB adapters for different samples, and different nuclei could be aliquoted into different sub-libraries and amplified with different i5-i7 primer sets, so all these steps will contribute to the total number of barcodes combinations. Therefore, the number of barcode combinations = the number of RT primers or MEB adapters x number of BC1 x number of BC2 x number of BC3 x number of sub-libraries.

In practice, we normally adjust the number of RT primers and MEB adapters used and the number of sub-libraries to keep the collision rate below 5%.

Example calculations are included in the attached spreadsheet.





Equipment:

- Plate centrifuge
- Multichannel pipettor
- Thermomixer

Primers

	A	B
	Name	Sequence

	A	B
	Blocker_1	ATCCACGTGCTTGAGAGGCCAGAGCATTCG
	Blocker_2	GGATTCGAGGAGCGTGTGCGAACTCAGACC
	Blocker_3	GTGGCCGATGTTTCGCATCGGCGTACGACT

Preparing

15.1 **Hybridisation mix**: (2 mL per sample)

	A	B	C	D
	Reagents	Stocking	Working 1x	/µl
	T4 ligation buffer	10x	1x	200
	RNase Inhibitor	40 U/µL	0.32 U/µl	16
	SUPERase RI	20 U/µL	0.05 U/µl	5
	PIC	100x	1x	20
	Triton X-100	10% (vol/vol)	0.1%	20
	BSA	7.5%	0.1%	27
	NIB		0.25x	500
	Nuclease-free water			1212
			total	2000

- T4 DNA Ligase - 100,000 units **New England Biolabs Catalog #**M0202L
- Protector RNase Inhibitor **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**3335402001
- SUPERaseIN RNase Inhibitor **Thermo Fisher Scientific Catalog #**AM2696
- **PIC**
- 10% Triton X-100 **Merck MilliporeSigma (Sigma-Aldrich)**
- Bovine Albumin Fraction V (7.5% solution) **Thermo Fisher Catalog #**15260037
- **NIB**
- Nuclease-free water, not DEPC-treated **Life Technologies Catalog #**AM9932

15.2 **block1 mix**:

	A	B	C	D
	Blocker_1	1 mM	22 µM	12.1 µL
	T4 Ligation buffer	10x	2x	110 µL
	Nuclease-free water			427.9 µL
				550 µL

- T4 DNA Ligase - 100,000 units **New England Biolabs Catalog #**M0202L
- Nuclease-free water, not DEPC-treated **Life Technologies Catalog #**AM9932

15.3 **block2 mix**:

	A	B	C	D
	Blocker_2	1mM	26.4µM	14.52 µL
	T4 Ligation buffer	10x	2x	110 µL
	Nuclease-free water			425.48 µL
				550 µL

- T4 DNA Ligase - 100,000 units **New England Biolabs Catalog #**M0202L
- Nuclease-free water, not DEPC-treated **Life Technologies Catalog #**AM9932

15.4 **block3 mix**:

	A	B	C	D
	Blocker_3	1mM	23 µM	12.65µL
	Triton X100	10%	0.1%	5.5µL
	Nuclease-free water			531.85µL
				550µL

- 10% Triton X-100 **Merck MilliporeSigma (Sigma-Aldrich)**

- Nuclease-free water, not DEPC-treated **Life Technologies Catalog #**AM9932

Ligation mix: (0.5 mL per sample)

	A	B	C	D
	Reagents	Stocking	Working 1x	/µL
	T4 ligation buffer	10x	1x	50
	RNase Inhibitor	40 U/µL	0.32 U/µl	4
	Superase RI	20 U/µL	0.05 U/µL	1.25
	PIC	100x	1x	5
	T4 DNA ligase	2000 U/µL	20 U/µl	5
	Triton X-100	10% (vol/vol)	0.1%	5
	BSA	7.5%	0.1%	6.7
	NIB		0.2x	100
	Nuclease-free water			323.05
			total	500

- T4 ligation buffer
- Protector RNase Inhibitor **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**3335402001
- SUPERaseIN RNase Inhibitor **Thermo Fisher Scientific Catalog #**AM2696
- **PIC**
- T4 DNA Ligase - 100,000 units **New England Biolabs Catalog #**M0202L
- 10% Triton X-100 **Merck MilliporeSigma (Sigma-Aldrich)**
- Bovine Albumin Fraction V (7.5% solution) **Thermo Fisher Catalog #**15260037
- **NIB**
- Nuclease-free water, not DEPC-treated **Life Technologies Catalog #**AM9932

16 **Split-pool barcoding**

Prepare 1% BSA in DPBS for rinsing plasticware, which helps to reduce sample loss.

16.1 Take out pre-prepared annealed linker-barcodes 96 well plates go to step #3.1 , spin at 700 x g, Room temperature, 00:02:00 , thaw and bring to Room temperature 2m

16.2 Re-suspend ConA-bead bound nuclei with 2 mL **hybridisation mix** for 48 barcodes in each round

16.3 **Round 1** 1h 2m

- Add 40 µL of nuclei per well to **linker1-BC1** plate , 300 rpm, Room temperature, 00:30:00 , incubate on thermomixer (or 23 °C)
- Use multichannel pipettor to add 10 µL **block1**, pipetting up and down to mix, 300 rpm, Room temperature, 00:30:00 , incubate on thermomixer (or 23 °C)
- Centrifuge 300 x g, 00:02:00
- Use multichannel pipette (rinsed with 1% BSA in DPBS) to collect cells into reservoir (rinsed with 1% BSA in DPBS)

16.4 **Round 2** 1h 2m

- Add 55 µL of nuclei per well to **linker2-BC2** plate, 300 rpm, Room temperature, 00:30:00 , incubate on thermomixer
- Use multichannel pipette to add 10 µL **block2**, pipetting up and down to mix, 300 rpm, Room temperature, 00:30:00 , incubate on thermomixer (or 23 °C)
- Centrifuge 300 x g, 00:02:00
- Use multichannel pipette (rinsed tips with 1% BSA in DPBS) to collect nuclei into reservoir (also pre-rinsed with 1% BSA in DPBS)

16.5 **Round 3** 32m

- Add 70 µL of nuclei per well to **linker3-BC3** plate, 300 rpm, Room temperature, 00:30:00 , incubate on thermomixer (or 23 °C)
- Use multichannel pipette to add 10 µL **block3**, pipetting up and down to mix
- Centrifuge 300 x g, 00:02:00
- Use multichannel pipette (rinsed tips with 1% BSA in DPBS) to collect nuclei into reservoir (also pre-rinsed with 1% BSA in DPBS)

16.6 Transfer nuclei suspension to 5 mL tube, centrifuge 600 x g, 4°C, 00:03:00, remove and discard the supernatant 3m

16.7 Transfer the pellet into a new 1.5 mL protein lobind tube, wash twice with 0.5 mL **NIB** (no **RI**, **PIC**), invert to mix in between

16.8 **Ligation:** 30m

Re-suspend nuclei with 0.5 mL **Ligation buffer**,
300 rpm, 25°C, 00:30:00 , incubate on thermomixer

16.9 Briefly spin, and wash with 0.5 mL **NIB** (no **RI**, **PIC**),
re-suspend in 0.5 mL **NIB** (no **RI**, **PIC**),
filter through 30 µL strainer,
rinse strainer with 0.5 mL **NIB**

16.10 **(Optional)** Put strainer on top of a new tube, centrifuge 600 x g, 4°C, 00:03:00 3m

*

16.11 Collect all nuclei into one tube, manually count nuclei by labelling nuclei with trypan blue (Trypan Blue Solution, 0.4% **Thermo Fisher Catalog #**15250061)

16.12 Based on estimated collision rate, aliquot desired number of nuclei into each sub-library.

Reverse crosslinking and lysis 1h

17 Reversing the light fixation performed in go to step #8

Equipment:

- PCR machine

Preparing

17.1 **2x reverse cross-linking buffer**:

	A	B	C	D
	Reagents	Stocking	In buffer	/µL
	Tris pH 8.0	1M	100 mM	100
	NaCl	5M	100 mM	20
	SDS	10%	0.04%	4
	Nuclease-free water			876
			total	1000

- 1M Tris-HCl (pH 8.0) **Thermo Fisher Scientific Catalog #**15568025
- Sodium Chloride (5M) **Invitrogen - Thermo Fisher Catalog #**AM9760G
- SDS, 10% Solution, RNase-free **Thermo Fisher Catalog #**AM9822
- Nuclease-free water, not DEPC-treated **Life Technologies Catalog #**AM9932

17.2 **Reverse crosslinking and lysis**

17.3 Add **NIB** (go to step #1.8 Nuclei Isolation Buffer) to each sample to bring the volume to 20 µL in total

17.4 20 µL of **2× reverse cross-linking buffer**, 2 µL of [M] 20 mg/mL proteinase K (Proteinase K **Thermo Fisher Scientific Catalog #**EO0491 [M] 1 µg/µL in final), and 1.6 µL of **RI mix** (SUPERase RI:Protector 1:1) were mixed with each sample and incubated at 55 °C for 01:00:00 in PCR machine 1h

17.5 **OPTIONAL STOP POINT**
After adding lysis buffer, sub-libraries can be stored at -20 °C .

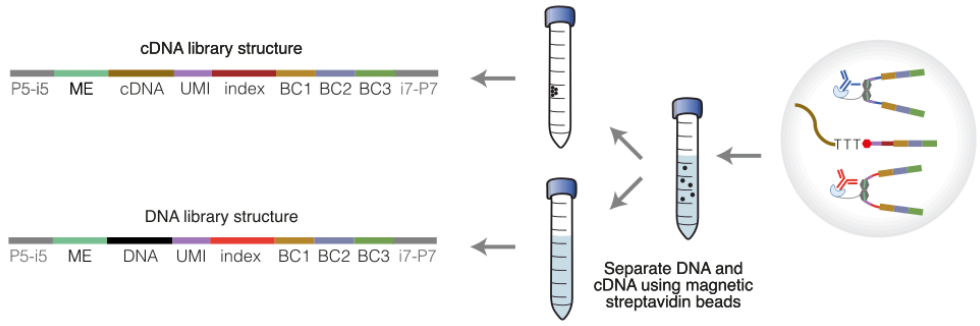
17.6 Add 2.5 µL of [M] 100 millimolar (mM) PMSF **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**P7626 to the reverse crosslinked sample to inactivate proteinase K and incubate at Room temperature for 00:10:00 . 10m

17.7 Attach to magnet and transfer supernatant into a new tube to remove ConA beads.

Myone C1 Streptavidin beads to separate tagged cDNA and gDNA

1h

18



Equipment:

- end-to-end-rotator

Wash

Dynabeads MyOne Streptavidin C1 **Invitrogen - Thermo Fisher Catalog #**65001 (10 µL for each sample, total 10 x N* µL) with 800 µL **1x B&W-T buffer** three times, and re-suspend in 45 µL **2x Binding & Washing buffer** + 1 µL SUPERaseIN RNase Inhibitor **Thermo Fisher Scientific Catalog #**AM2696 .

*where N is the number of samples

19 Add 46 µL re-suspended beads into each lysed sample (45.6 µL), mixed and rotated on an end-to-end rotator at

1h

10 rpm, Room temperature, 01:00:00 , end-to-end rotator

20 Put tubes on a magnetic stand to separate supernatant (transposed DNA fragments) and beads (cDNA).

RNA library preparation

3h 12m

21 **Equipment:**

- Qubit fluorometer

- PCR machine (thermocycler)
- end-to-end-rotator

Primers

	A	B
	Name	Sequence
	TSO	AAGCAGTGGTATCAACGCAGAGTGAATrGrG+G
	PA_F	CAGACGTGTGCTCTTCCGATCT
	PA_R	AAGCAGTGGTATCAACGCAGAGT
	P7_#1	CAAGCAGAAGACGGCATACGAGATGATCTGGTGACTGGAGTTCAG ACGTGTGCTCTTCCGATCT
	P7_#2	CAAGCAGAAGACGGCATACGAGATTCAAGTGTGACTGGAGTTCAG ACGTGTGCTCTTCCGATCT
	P7_#3	CAAGCAGAAGACGGCATACGAGATCTGATCGTGACTGGAGTTCAG ACGTGTGCTCTTCCGATCT
	P7_#4	CAAGCAGAAGACGGCATACGAGATAAGCTAGTGACTGGAGTTCAG ACGTGTGCTCTTCCGATCT
	P7_#5	CAAGCAGAAGACGGCATACGAGATGTAGCCGTGACTGGAGTTCAG ACGTGTGCTCTTCCGATCT
	P7_#6	CAAGCAGAAGACGGCATACGAGATTACAAGGTGACTGGAGTTCAG ACGTGTGCTCTTCCGATCT
	P7_#7	CAAGCAGAAGACGGCATACGAGATTTGACTGTGACTGGAGTTCAG ACGTGTGCTCTTCCGATCT
	P7_#8	CAAGCAGAAGACGGCATACGAGATGGAACTGTGACTGGAGTTCAG ACGTGTGCTCTTCCGATCT
	P5_#1	AATGATACGGCGACCACCGAGATCTACACTAGATCGCTCGTCGGC AGCGTCAGATGTGTAT
	P5_#2	AATGATACGGCGACCACCGAGATCTACACCTCTCTATTCGTCGGC AGCGTCAGATGTGTAT
	P5_#3	AATGATACGGCGACCACCGAGATCTACACTATCCTCTTCGTCGGC AGCGTCAGATGTGTAT
	P5_#4	AATGATACGGCGACCACCGAGATCTACACAGAGTAGATCGTCGGC AGCGTCAGATGTGTAT
	P5_#5	AATGATACGGCGACCACCGAGATCTACACGTAAGGAGTCGTCGGC AGCGTCAGATGTGTAT

	A	B
	P5_#6	AATGATACGGCGACCACCGAGATCTACACACTGCATATCGTCGGC AGCGTCAGATGTGTAT
	P5_#7	AATGATACGGCGACCACCGAGATCTACACAAGGAGTATCGTCGGC AGCGTCAGATGTGTAT
	P5_#8	AATGATACGGCGACCACCGAGATCTACACCTAAGCCTTCGTCGGC AGCGTCAGATGTGTAT

Preparing

21.1 **1x B&W-T-RI** buffer:

- 1 mL **B&W-T** buffer (go to step #1.10 B&W-T buffer)
- 5 µL SUPERaseIN RNase Inhibitor **Thermo Fisher Scientific Catalog #**AM2696 .

(300 µL per sub-library)

21.2 **STE-RI** buffer:

- 1 mL **STE** buffer (go to step #1.9 STE buffer)
- 5 µL SUPERaseIN RNase Inhibitor **Thermo Fisher Scientific Catalog #**AM2696 .

(100 µL per sub-library)

21.3 **Template switch mix**:

	A	B	C	D
	Reagent	Stocking	Working 1x	1x /µL
	RT buffer	5x	1x	10
	Ficoll PM-400	20 %	4 %	10
	PEG 6000	50 %	15 %	15
	TSO primer	100 µM	2.5 µM	1.25
	dNTPs	25 mM/each	1 mM	2
	RNAse Protector	40 U/µL	0.5 U/µL	0.625
	Superase RI	20 U/µL	0.25 U/µL	0.625

	A	B	C	D
	Maxima H Minus RT	200 U/µL	10 U/µl	2.5
	Nuclease-free water			8
			total	50

- RT buffer (Maxima H minus RT)
- Ficoll PM-400 **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**F5415-50ML
- PEG 6000 (Poly(ethylene glycol)) **Bio Basic Inc. Catalog #**PB0432.SIZE.500g
- *TSO* (Template Switching Optimised) primer
- dNTP Set 100 mM Solutions **Thermo Fisher Scientific Catalog #**R0182
- Protector RNase Inhibitor **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**3335402001
- SUPERaseIN RNase Inhibitor **Thermo Fisher Scientific Catalog #**AM2696
- Maxima H Minus Reverse Transcriptase **Thermo Fisher Scientific Catalog #**#EP0741

This is an optimised Moloney murine leukemia virus Reverse Transcriptase (M-MuLV RT) which has terminal transferase activity. This means it can perform non-template addition of nucleotides to 3'-ends with a preference for dCTP. The resulting poly C overhang allows the TSO primer, which ends in 3 rGs, to anneal. This RT is also capable of template switching, meaning that it can read through the gap in the backbone between the end of the RNA template and the annealed TSO and continue elongating with the TSO as the template.

- Nuclease-free water, not DEPC-treated **Life Technologies Catalog #**AM9932

22 RNA library preparation

22.1 Wash beads three times using 100 µL **1x B&W-T buffer** with

SUPERaseIN RNase Inhibitor **Thermo Fisher Scientific Catalog #**AM2696

0.1 µL mix by inverting between washes

22.2 Wash beads once using 100 µL **STE** buffer with

SUPERaseIN RNase Inhibitor **Thermo Fisher Scientific Catalog #**AM2696

0.1 µL while tube on magnet, without re-suspending beads

22.3 Re-suspend beads with 50 µL **Template switch mix** by pipetting

22.4 Rotate beads 10 rpm, Room temperature, 00:30:00 , end-to-end-rotator 2h
Re-suspend by pipetting
Incubate for 01:30:00 at 42 °C in PCR machine

23 Pre-Amplification

23.1 After template switching, add 100 µL of **STE** to each tube to dilute the sample.
Remove the supernatant by placing the sample on a magnetic stand.
Wash beads with 200 µL of **STE** without disturbing the bead pellet

23.2 Beads were then re-suspended in 55 µL of **PCR mix**:

	A	B	C	D
	Reagent	Stocking	Working 1x	1x /µL
	Kapa HiFi PCR mix	2x	1x	27.5
	PA_F	10 µM	400 nM	2.2
	PA_R	10 µM	400 nM	2.2
	Nuclease-free water			23.1
			total	55

- HotStart ReadyMix (KAPA HiFi PCR kit) **Kapa Biosystems Catalog #**KK2601
- *PA_F* & *PA_R* primers
- Nuclease-free water, not DEPC-treated **Life Technologies Catalog #**AM9932

23.3 Run following programme to pre-amplify cDNA: 12m 15s
- 95 °C 00:03:00
- 12 cycles of:
  - 98 °C 00:00:30
  - 65 °C 00:00:45
  - 72 °C 00:03:00
- 72 °C 00:05:00

- 4 °C Hold

23.4 Purify by 0.8x Ampure XP beads **Beckman Coulter Catalog #**A63881 and elute in 32 µL 0.1x EB (Elution Buffer)

Measure concentration with Qubit

Note

Libraries made from ~1000-5000 cells and amplified for 12-15 cycles typically yield around 500 ng after purification

24 Tagging MEA and PCR amplification

24.1 Setup following reaction; incubate in PCR machine at 37 °C for 00:30:00 30m

	A	B
	1:20 pA-Tn5-MeA-MeR	2 µl
	4x Tag Buffer	5 µl
	Sample	Volume to give 50ng
	Nuclease-free water	To 20 µl

24.2 Add 2 µL 0.2% SDS mix and incubate at Room temperature for 00:10:00 10m to release tagged fragments; neutralise SDS with 1 µL 4% Triton X100

- SDS, 10% Solution, RNase-free **Thermo Fisher Catalog #**AM9822
- 10% Triton X-100 **Merck MilliporeSigma (Sigma-Aldrich)**

24.3 Amplify libraries with **NEBnext mix** as following 19m 45s

	A	B
	Sample	23 µl
	2x NEBnext mix	25 µl
	P5_#N 25µM	1 µl
	P7_#N 25uM	1 µl

Run the following programme to amplify libraries:
- 72 °C 00:10:00
- 98 °C 00:03:00
- 11 cycles of:
  - 98 °C 00:00:10
  - 65 °C 00:00:30
  - 72 °C 00:01:00
- 72 °C 00:05:00
- 4 °C hold

24.4 Purify the amplified library using 0.7x Ampure XP beads **Beckman Coulter Catalog #**A63881 three times and elute with 32 µL of EB.

DNA library amplification

15m 40s

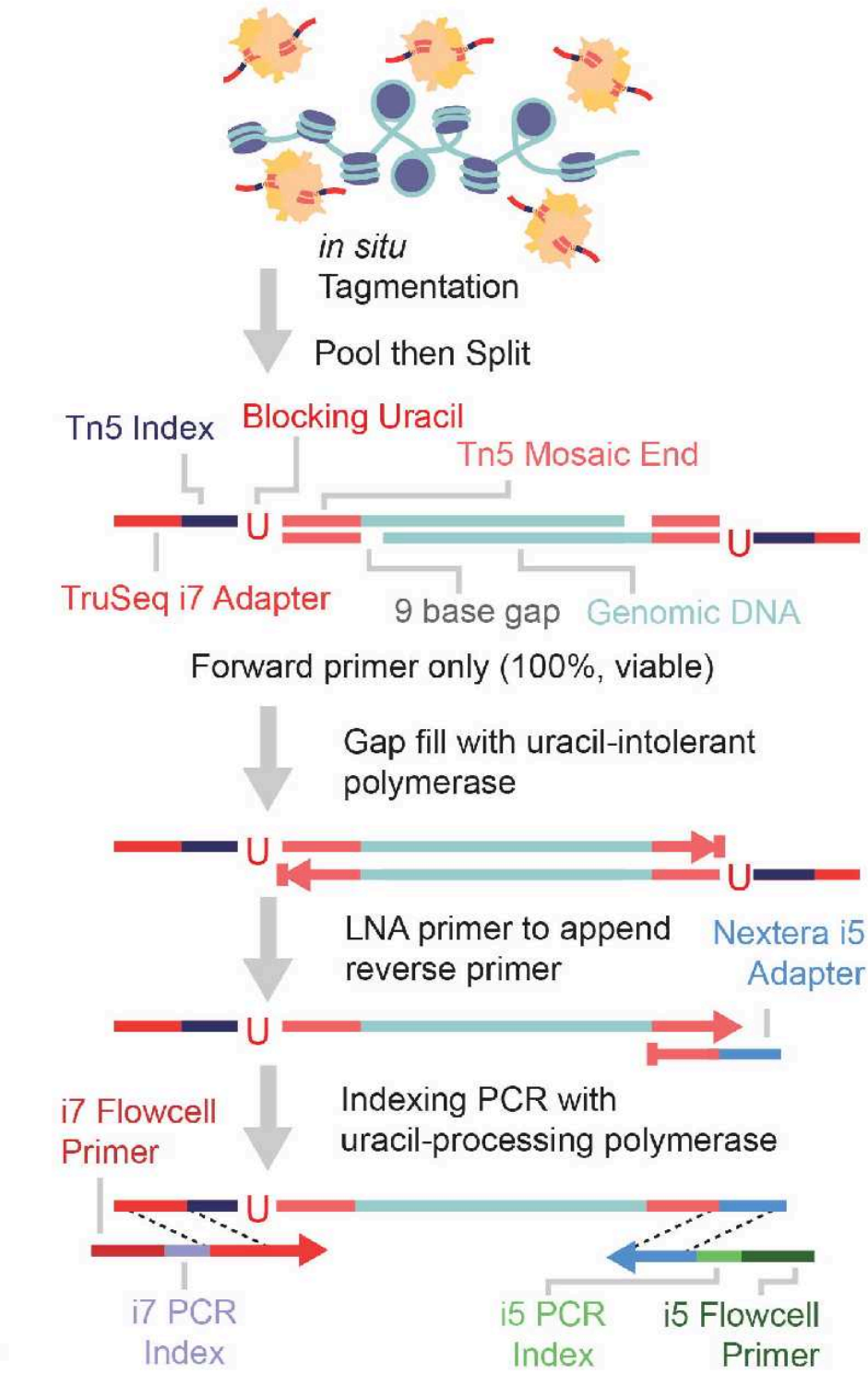
25 **Equipment:**
- PCR machine (thermocycler)
- Qubit fluorometer
- bioanalyzer

Primers
In addition to the *P7_#N* and P5_*#N* primers listed above in the RNA library prep section you will need:

	A	B
	Name	Sequence
	MEA_LNA	TCGTCGGCAGCGTC AGATGTGTA+TA+AG+AG+AC+AG/3InvdT/

Purify the DNA component using 1.2x Ampure XP beads **Beckman Coulter Catalog #**A63881, elute with 21 µL 0.1x EB buffer

26 **Add MEA by adapter switching**



Modified from Mulqueen et al. 2021 "[symmetrical strand single-cell combinatorial indexing (s3)]
uses single-adapter transposition to incorporate the forward primer sequence, the Tn5 mosaic end sequence and a reaction-specific DNA barcode. As with standard tagmentation workflows, extension through the bottom strand is then performed to provide adaptor sequences on both ends of each molecule; however, the s3 transposome complexes contain a uracil base immediately following the mosaic end sequence. Use of

complexes contain a uracil base immediately following the mosaic end sequence. Use of a uracil-intolerant polymerase therefore prevents extension beyond the mosaic end into the DNA barcode and forward adaptor sequence. A second template oligo is then introduced that contains a 3'-blocked locked nucleic acid (LNA) mosaic end reverse complement sequence with a reverse adaptor sequence 5' overhang. This oligo favorably anneals to the copied mosaic end sequence, due to the higher melting temperature of LNA, and acts as a template for the library molecule to extend through and copy the reverse adaptor. This results in all library fragments having both a forward and reverse adaptor sequence. The LNA-templated extension is carried out over multiple rounds of thermocycling to ensure maximum efficiency of reverse adaptor incorporation."

Citation

Mulqueen RM, Pokholok D, O'Connell BL, Thornton CA, Zhang F, O'Roak BJ, Link J, Yardımcı GG, Sears RC, Steemers FJ, Adey AC
(2021). High-content single-cell combinatorial indexing.

https://doi.org/10.1038/s41587-021-00962-z LINK

26.1 **Gap filling** reaction 10m

20 µL sample (use all of the sample) + 8 µL

NEBNext® High-Fidelity 2X PCR Master Mix **New England Biolabs Catalog #**M0541

run: 72 °C 00:10:00

26.2 **Adapter switching** reaction 1m 10s

Add 3 µL [M] 1 micromolar (µM) *MEA_LNA*

- run: 98 °C 00:00:30
- 10 cycles of :
  - 98 °C 00:00:10
  - 59 °C 00:00:20
  - 72 °C 00:00:10

26.3 **Amplification** reaction 4m 30s

Add:

- 50 µL Q5U 2x readymix DNA polymerase

- 15 µL H_2O
- 2 µL *P7_#N* ([M] 25 micromolar (µM))
- 2 µL P5_*#N* ([M] 25 micromolar (µM))

run: 98 °C 00:00:30

- 10 cycles of:
  - 98 °C 00:00:10
  - 55 °C 00:00:20
  - 72 °C 00:00:30
- 72 °C 00:03:00
- 4 °C hold

27 Take out 50 µL into a new tube and store at -20 °C as a backup, and add extra cycles (4-10 cycles, depending on the sample) to the remaining libraries to generate enough material for sequencing.

28 Purify libraries with 0.7x Ampure XP beads **Beckman Coulter Catalog #**A63881 twice, elute with 32 µL EB, check concentration with Qubit, and run samples on Bioanalyzer.

Sequencing

29 Use a 150 bp paired-end sequencing kit with the following customised parameters:

- Read 1 with **Nextera adapter** for 100 bp. (TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG)
- Read 2 with **Illumina Truseq adapter** for 200 bp. (GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT)
- Read 2 contains 128 bp for cell barcodes and UMI sequences, plus 72 bp for genomic sequence.
- i7 index is 6bp
- i5 index is 8bp

Normally, we add 10% PhiX spike-in for Illumina sequencing machines, and 2% PhiX spike-in for Element AVITI machine.

Data Processing & Analysis

30 Data processing can be performed with our analysis pipeline.
See the README file in the pipeline repository for additional details.

Software

nf_mtr_seq	NAME
https://github.com/Rugg-Gunn-Lab/nf_mtr_seq	REPOSITORY
https://doi.org/10.5281/zenodo.1504690	SOURCE LINK

Protocol references

3XFlag-pATn5: https://dx.doi.org/10.17504/protocols.io.8yrhxv6
CUT&Tag: https://doi.org/10.1038/s41467-019-09982-5
Split-seq: https://www.science.org/doi/10.1126/science.aam8999
Split-seq protocol: https://www.seeliglab.org/uploads/5/9/9/7/59974251/split-seq_protocol_v3.0
Share-seq: https://doi.org/10.1016/j.cell.2020.09.056
Paired-seq: https://doi.org/10.1038/s41594-019-0323-x
Paired-Tag: https://doi.org/10.1038/s41592-021-01060-3
Adapter switch S3: https://doi.org/10.1038/s41587-021-00962-z

Citations

Step 26

Mulqueen RM, Pokholok D, O'Connell BL, Thornton CA, Zhang F, O'Roak BJ, Link J, Yardımcı GG, Sears RC, Steemers FJ, Adey AC. High-content single-cell combinatorial indexing.
https://doi.org/10.1038/s41587-021-00962-z