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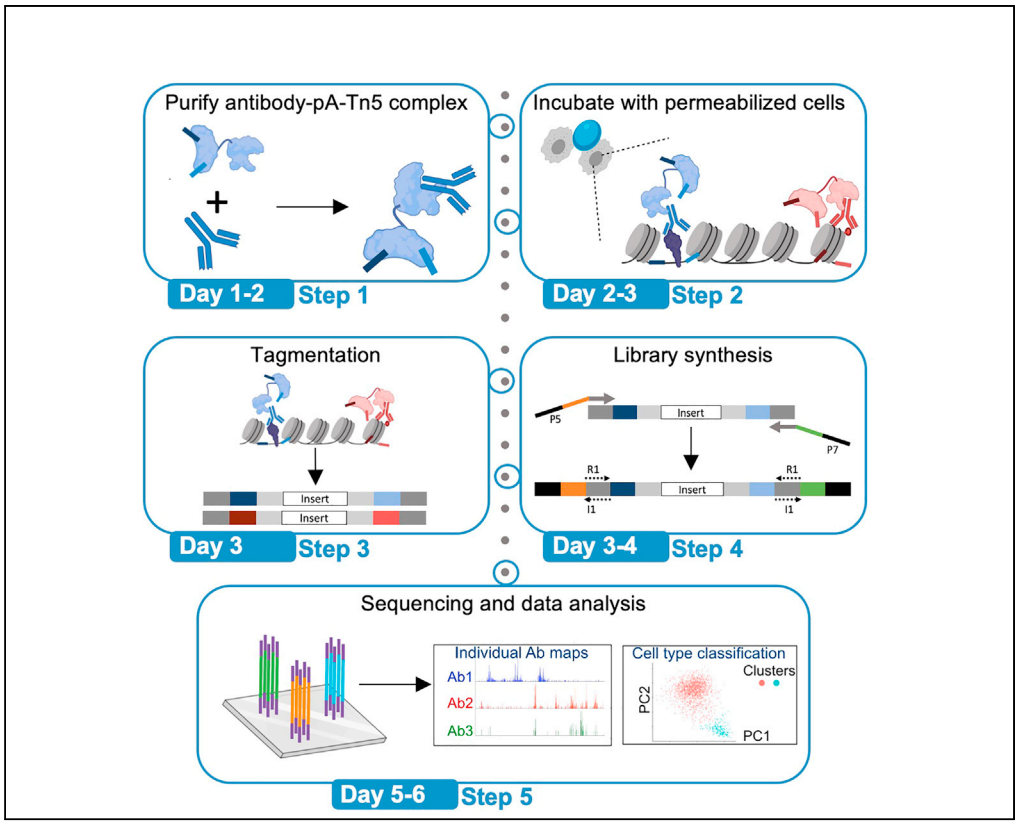
Multi-CUT&Tag to simultaneously profile multiple chromatin factors



Genome-wide chromatin mapping approaches typically focus on one protein at a time. We recently developed multi-CUT&Tag, which enables simultaneous mapping of multiple chromatin proteins in the same single cells or pools of cells. Using barcoded adapters loaded onto antibody-protein A-Tn5 transposase complexes, multi-CUT&Tag marks the locations of each chromatin protein and directly detects colocalization of different proteins in the same cell(s). Although slightly more laborious than CUT&Tag, multi-CUT&Tag provides a powerful option for generating multi-factor maps for epigenomic profiling.

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Highlights

Multi-CUT&Tag can map the locations of different chromatin proteins in the same cells

Multi-CUT&Tag directly detects co-localization of different epitopes at the same loci

Multi-CUT&Tag is easily adapted for single-cell chromatin mapping

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Multi-CUT&Tag to simultaneously profile multiple chromatin factors

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SUMMARY

Genome-wide chromatin mapping approaches typically focus on one protein at a time. We recently developed multi-CUT&Tag, which enables simultaneous mapping of multiple chromatin proteins in the same single cells or pools of cells. Using barcoded adapters loaded onto antibody-protein A-Tn5 transposase complexes, multi-CUT&Tag marks the locations of each chromatin protein and directly detects colocalization of different proteins in the same cell(s). Although slightly more laborious than CUT&Tag, multi-CUT&Tag provides a powerful option for generating multi-factor maps for epigenomic profiling.

For complete details on the use and execution of this protocol, please refer to Gopalan et al. (2021).

BEFORE YOU BEGIN

Multi CUT&Tag is designed for mapping multiple epitopes in the same cells using high-quality antibodies raised against each. We have tested antibodies recognizing H3K27me3, H3K27ac, and RNA Polymerase II phosphorylated on serine 2 in various combinations. We have performed multi-CUT&Tag in mouse embryonic stem cells and trophoblast stem cells (as described in the protocols below), but this method is expected to work in a wide range of mammalian cells, as previously observed for CUT&Tag (Kaya-Okur et al., 2019; Bartosovic et al., 2021; Wu et al., 2021). Before starting a bulk or single cell multi-CUT&Tag experiment:

1. Protein A-Tn5 transposase fusion protein (pA-Tn5) containing a six-histidine tag must be expressed and purified.
2. Make 5% (w/v) Digitonin solution in ultrapure water and store aliquots at –20°C.
3. Load pA-Tn5 with barcoded adapters as detailed below.
4. Generate antibody-pA-Tn5 conjugates by incubation of adapter-loaded pA-Tn5 with antibodies of interest, and removal of unbound antibodies and adapters, as described below.

Expression and purification of pA-Tn5 enzyme

Timing: 4 days

pA-Tn5 containing an N-terminal six-histidine tag is expressed and purified in *E. coli* for use in multi-CUT&Tag.

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5. Transform pET28-pA-Tn5 into BL21 (DE3) pLysS *E. coli* according to the manufacturer's recommendations (https://www.promega.com/-/media/files/resources/protcards/e-coli-competent-cells-quick-protocol.pdf?rev=81255b2b910545eb8c4c176dc722b7cf&sc_lang=en), selecting on LB agar plates supplemented with 25 mg/mL Chloramphenicol and 50 mg/mL Kanamycin. Use fresh transformants for expression and purification.
6. Inoculate a 5 mL starter culture of pET28-pA-Tn5 transformed BL21 (DE3) pLysS in LB supplemented with 25 mg/mL Chloramphenicol and 50 mg/mL Kanamycin. Grow at 37°C for ~12 h.
7. After overnight growth, dilute the starter culture into 1 L of 2× YT media and grow at 37°C for 2–3 h until the OD_{600} is approximately 0.6. Let the culture cool on ice and then add IPTG to a final concentration of 0.25 mM. Incubate culture for ~12 h at 17.5°C in a shaker.
8. Split culture into four 250 mL parts, centrifuge at 5,000 *g* for 10 min at 4°C, remove supernatant and resuspend each pellet in ~4 volumes (approximately 30 mL) of phosphate buffered saline (PBS). Transfer resuspended cells into 50 mL Oak Ridge centrifuge tubes and centrifuge at 5,000 *g* for 10 min in a Beckman Avanti J-25 centrifuge (or equivalent) at 4°C. Remove supernatant and flash-freeze pellets in liquid nitrogen.
9. Thaw and resuspend each pellet in 14 mL of HGX buffer supplemented with 1× Halt protease inhibitors. Sonicate samples 5–10 times in a Branson Sonifier for 10 s each at a 20% output setting, with 1 min on ice between each sonication cycle.

   Note: If solution is viscous, up to 14 mL additional HGX buffer can be added per pellet, followed by 2–3 additional sonication cycles.

10. Spin lysate at 16,000 *g* for 15 min at 4°C in a Beckman Avanti J-25 centrifuge (or equivalent). Transfer supernatant to new 50 mL Oak Ridge centrifuge tubes and repeat centrifugation. Transfer supernatant to 50 mL conical centrifuge tubes and place on ice. Freeze 50 μL supernatant in a microcentrifuge tube for SDS-PAGE to test expression and purification.
11. Wash 2 mL (column bed volume) TALON beads in HGX by centrifuging at 500 *g* for 5 min at 4°C, remove the supernatant, resuspend in 10 bed volumes HGX buffer, centrifuge as before, and remove supernatant. Resuspend beads and add to pooled bacterial lysate (approximately 50 mL). Incubate for 90 min with mixing on a nutating mixer at 4°C.
12. Add beads to a suitable gravity flow column and allow flow through to drain. Freeze 50 μL of this "flow through" in a microcentrifuge tube for SDS-PAGE to test expression and purification.
13. Wash beads with 20 column volumes (40 mL) of HGX buffer with protease inhibitors and allow the buffer to flow through the column.
14. Perform five elutions with 2 mL of HGX supplemented with protease inhibitors and 250 mM imidazole. Combine eluates.
15. Dialyze for ~12 h at 4°C in 1 L Dialysis buffer. Recover dialyzed sample, which has reduced in volume to approximately 1.5–2.0 mL. Measure concentration using Bio-Rad Protein Assay reagent, according to the manufacturer's recommendations. Store at −20°C temporarily while assessing purity by SDS-PAGE and activity (as described in Picelli et al. (2014)). Subsequently, aliquot protein, flash freeze in liquid nitrogen, and store long-term at −80°C for at least 1 year.
16. If purified pA-Tn5 lacks sufficient purity (> ~70% of protein as assessed by SDS-PAGE and Coomassie staining) additional purification steps are performed by ion exchange chromatography. Dilute protein four-fold into $HN_{50}TE$ buffer. Freeze 20 μL in a microcentrifuge tube for SDS-PAGE to test purification.
17. Prewash 1 mL (column bed volume) Q Sepharose Fast Flow beads and, separately, 1 mL (column bed volume) SP Sepharose Fast Flow beads in $HN_{50}TE$ buffer. Bind pA-Tn5 to Q Sepharose Fast Flow in batch for 30 min on a nutating mixer at 4°C.
18. Add beads to a suitable gravity flow column and allow flow through to drain, saving the *unbound* fraction. Freeze 20 μL in a microcentrifuge tube for SDS-PAGE to test purification.
19. Next, bind to SP-Sepharose Fast Flow in batch for 30 min on a nutating mixer at 4°C. Add beads to a suitable gravity flow column and allow flow through to drain.

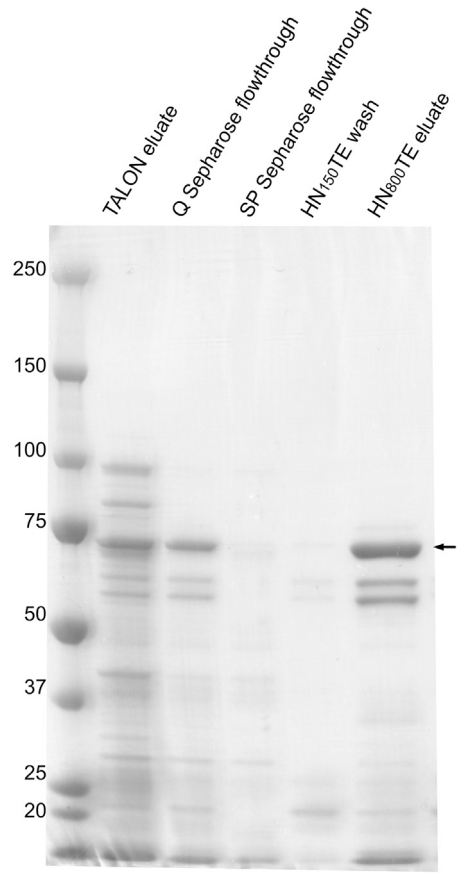

Figure 1. Coomassie-stained SDS-PAGE gel of pA-Tn5 at various purification steps

Indicated samples were run on a 4%–12% gradient SDS-PAGE gel and stained as described in the text. The full-length 73 kDa pA-Tn5 band is indicated with an arrowhead. Note: a few species of lower molecular weight than pA-Tn5, possibly representing prematurely truncated pA-Tn5 products, are often observed after all purification steps.

20. Wash with approximately 20 bed volumes (20 mL) of $HN_{50}TE$ and 10 bed volumes of $HN_{150}TE$ buffer. Freeze 20 µL of the first $HN_{150}TE$ wash in a microcentrifuge tube for SDS-PAGE to test purification.
21. Elute pA-Tn5 twice with three bed volumes each (6 mL total) of $HN_{800}TE$ buffer. Combine eluates and freeze 20 µL in a microcentrifuge tube for SDS-PAGE to test purification. Dialyze combined eluate for ~12 h at 4°C in 1 L Dialysis Buffer. Recover dialyzed pA-Tn5 (approximately 1.5–2.0 mL) and measure concentration using Bio-Rad Protein Assay reagent, according to the manufacturer's recommendations. The concentration of our final enzyme preparation was 1.55 µg/µL (21.1 µM). Quick freeze aliquots in liquid nitrogen and store long-term at −80°C, or short term at −20°C.
22. Perform SDS-PAGE on samples indicated above to assess purity. Coomassie stain gel (as in Figure 1) with SimplyBlue SafeStain, per the manufacturer's instructions.

Loading pA-Tn5 with barcoded adapters

Timing: 2 h

Load a batch of purified pA-Tn5 enzyme with adapters containing different barcodes for each antibody to be used. Adapter-loaded pA-Tn5 can be stored at −20°C for up to 6 months.

23. Anneal the adapter oligos:
    a. Assemble PCR tube 'A':

Component	Stock	Volume
P5_i5_1_Universal_Connector_A	100 μM	9 μL
Tn5MErev	100 μM	9 μL
Annealing buffer	10×	2 μL

    b. Assemble tube 'B'

Component	Stock	Volume
P7_i7_1_Universal_Connector_B	100 μM	9 μL
Tn5MErev	100 μM	9 μL
Annealing buffer	10×	2 μL

    c. Anneal the adapters

Steps	Temperature	Time
Initial	95°C	1 min
Ramp temperature down	25°C	at 0.1 °C/s
Hold	25°C	Forever

    d. Mix tubes A and B to generate a 45 μM mixture of annealed Tn5 adapters.

24. Heat ~100 μL of 100% glycerol to 90°C in thermocycler or heat block. Pipet 35 μL heated 100% glycerol to new tube and cool on ice.
25. Add 35 μL of 45 μM annealed Tn5 adapters to cooled 100% glycerol and flick extensively to mix.
26. Add 25 μL of 21.1 μM (monomer concentration) N-terminal 6-histidine (6-His) tagged pA-Tn5. Mix gently by flicking.

Note: The 21.1 μM concentration corresponds to our batch of purified pA-Tn5, but other concentrations will likely work so long as the total amount of pA-Tn5 enzyme is similar.

27. Incubate the mixture at RT for 60 min with occasional gentle flicking and then store the loaded pA-Tn5 enzyme at −20°C for up to 6 months.

Testing transposition activity

Timing: 4 h

This assay (adapted from Picelli et al. (2014)) tests the transposition activity of the oligo-loaded pA-Tn5 enzyme.

28. Assemble the transposition reaction:

Component	Stock	Volume
Tn5 transposition buffer	5×	4 μL
Linearized plasmid DNA (any linearized plasmid DNA that is > 10 kb)	50 ng/μL	1 μL
Barcoded pA-Tn5 (from above)	n/a	1 μL
Water	n/a	14 μL

29. Incubate the reaction for 2 h at 37°C.
30. Add 0.3 μL proteinase K to stop the reactions and incubate for 10 min at 55°C.
31. Analyze the samples by agarose gel electrophoresis. Typically, all of the high molecular weight DNA is converted to fragments of average size 400–500 bp.

Coupling antibody of interest with loaded pA-Tn5

Timing: 9–17 h

Couple each antibody to be used to a differently barcoded pA-Tn5 enzyme.

32. In a 1.5 mL microcentrifuge tube, mix ~20 μL of loaded pA-Tn5 with ~30 μg of the antibody of interest.
33. Incubate for 4 h (or up to 12 h) for binding at 4°C in a nutating mixer.
34. Aliquot 80 μL of Dynabeads His-Tag Isolation and Pulldown into a 1.5 mL microcentrifuge tube, place on a magnetic rack for 2 min to pull the beads to the side of the tube and wash the beads with PBS.
35. Resuspend the washed beads in 20 μL PBS and add to the tube containing pA-Tn5 and antibody.
36. Incubate for 2–3 h at 4°C in a nutating mixer for capture of the His-tagged pA-Tn5-antibody complexes (along with some free pA-Tn5 that will not affect downstream steps).
37. Wash the beads in 200 μL of PBS. Repeat the wash step twice (totaling three washes).
38. To elute the antibody-pA-Tn5 complex, add 100 μL of 300 mM Imidazole in PBS. Incubate at 4°C for 60 min, in a nutating mixer. Place the tube in a magnetic rack for 2 min to pull beads to the side. Carefully pipette the eluate to a new microcentrifuge tube.
39. Perform two rounds of buffer exchange into PBS using Amicon Ultra- 0.5 mL Centrifugal filters following the manufacturer's protocol (https://www.emdmillipore.com/US/en/product/Amicon-Ultra-0.5-Centrifugal-Filter-Unit,MM_NF-UFC501096#anchor_UG). Elute the protein complex in 40 μL PBS.
40. Add 40 μL of 100% glycerol and gently mix to attain a final concentration of 50% glycerol.

Note: The final volume of the purified antibody-pA-Tn5 complex is 80 μL and has ~20 μL of loaded pA-Tn5 from step 27. We use ~1.5 μL of the pA-Tn5 enzyme per 100,000 cells in each CUT&Tag reaction and hence, we use 6 μL of the purified antibody-pA-Tn5 complex per reaction.

41. The purified antibody-pA-Tn5 complexes can be stored at −20°C for up to 3 months.

⚠ CRITICAL: At this step differentially barcoded purified antibody-pA-Tn5 complex can be used to perform Multi-CUT&Tag in bulk populations or in single cells, according to the appropriate protocol.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
H3K27me3 Monoclonal Antibody (G.299.10)	Invitrogen	Cat#MA5-11198
Anti-Histone H3 (acetyl K27) antibody	Abcam	Cat#ab4729
Phospho-Rpb1 CTD (Ser2) (E1Z3G) Rabbit mAb	Cell Signaling Technology	Cat#13499
Rabbit IgG	Abcam	Cat#Ab37415
Bacterial and virus strains		
BL21 DE3 pLysS bacteria	Novagen	Cat#69451

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Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Chemicals, peptides, and recombinant proteins		
Histidine tagged pA-Tn5	In this study	N/A (produced in Fazzio lab)
LB agar, powder	Thermo Fisher Scientific	Cat#22700025
Chloramphenicol	Millipore Sigma	Cat#R4408
Kanamycin	Millipore Sigma	Cat#K1377
IPTG	Thermo Fisher Scientific	Cat#15529019
Phosphate-Buffered Saline, 1× without calcium and magnesium	Corning	Cat#21-040-CV
Halt™ Protease Inhibitor Cocktail, EDTA-Free (100×)	Thermo Fisher Scientific	Cat#PI78439
Imidazole	Millipore Sigma	Cat#56750
TALON® Metal Affinity Resin	Takara Bio	Cat#635502
Q Sepharose® Fast Flow	Millipore Sigma	Cat#GE17-0510-01
SP Sepharose Fast Flow	GE Healthcare	Cat#17072901
Dithiothreitol (DTT)	Thermo Fisher Scientific	Cat#R0861
Glycerol	RPI Corp	Cat#G22020-4.0
Tryptone	Gibco	Cat#211701
Yeast Extract	Gibco	Cat#212750
Sodium chloride (NaCl)	Millipore Sigma	Cat#S9888
HEPES	Millipore Sigma	Cat#H3375
Triton™ X-100	Millipore Sigma	Cat#11332481001
Ethylenediaminetetraacetic acid tetrasodium salt dihydrate (EDTA)	Millipore Sigma	Cat#E6511
Potassium chloride (KCl)	Millipore Sigma	Cat#P9541
Quick Start™ Bradford 1× Dye Reagent	Bio-Rad Laboratories	Cat#5000205
SimplyBlue SafeStain	Thermo Fisher Scientific	Cat#LC6060
Dimethylformamide (DMF)	Cell Signaling Technology	Cat#12767S
Manganese(II) chloride solution (MnCl2)	Millipore Sigma	Cat#M1787
Dynabeads His-Tag Isolation and Pulldown	Invitrogen	Cat#10103D
Concanavalin A coated magnetic beads	Polysciences	Cat#21-1401
Calcium chloride (CaCl2)	Millipore Sigma	Cat#C4901
Spermidine trihydrochloride	Millipore Sigma	Cat#85580
Magnesium chloride (MgCl2)	Millipore Sigma	Cat#M8266
Digitonin	Millipore Sigma	Cat#D141
Proteinase K Solution	Bioline	Cat#BIO-37084
Phenol:Chloroform + Tris Buffer	Thermo Fisher Scientific	Cat#17908
Chloroform	Thermo Fisher Scientific	Cat#C298-500
Ethanol, 100%	Thermo Fisher Scientific	Cat#22-032-601
Glycogen from *Mytilus edulis*	Millipore Sigma	Cat#G1767
PhiX Control v3	Illumina	Cat#FC-110-3001
Critical commercial assays		
Amicon Ultra-0.5 Centrifugal Filter Unit	Millipore Sigma	Cat#UFC501096
NEBNext® High-Fidelity 2× PCR Master Mix	New England BioLabs	Cat#M0541
AMPure XP beads	Beckman Coulter	Cat#A63881
Qubit dsDNA HS Assay Kit	Invitrogen	Cat#Q32854
KAPA Library Quantification Kits	Roche	Cat#07960140001
NextSeq 500/550 High Output Kit v2.5 (150 Cycles)	Illumina	Cat#20024906
Sample Index Sets for Single Cell ATAC	10× genomics	Cat#PN-1000212
Chromium Next GEM Single Cell ATAC Reagent Kits v1.1	10× genomics	Cat#CG000209
Deposited data		
Raw and Analyzed data	GEO	GSE171554
Code for sequencing data analysis	GitHub	https://github.com/snehagopalan710/Bulk-Multi-CUT-Tag/tree/main

(*Continued on next page*)

Continued		
REAGENT or RESOURCE	SOURCE	IDENTIFIER
Experimental models: cell lines		
E14 mouse embryonic stem cell line	Panning Lab, UCSF	RRID: CVCL_C320
5-4 mouse trophoblast stem cell line	Kalantry Lab, University of Michigan	N/A
Oligonucleotides		
Barcoded oligo for loading to pA-Tn5	In this study	Table 1
Custom sequencing primers	In this study	Table 2
Software and algorithms		
novoBarcode	Novocraft	http://www.novocraft.com/documentation/novobarcode/
Bowtie 2, version 2.4.1	Johns Hopkins University	http://bowtie-bio.sourceforge.net/bowtie2/index.shtml
Samtools, version 1.5	SAMtools	http://samtools.sourceforge.net/
Picard	Broad Institute	http://broadinstitute.github.io/picard/
HOMER software suite, v4.11	UCSD	http://homer.ucsd.edu/homer/
cellranger-atac, version 1.1.0	10× genomics	https://support.10xgenomics.com/single-cell-atac/software/release-notes/1-1
Cutadapt, version1.9	Martin (2011)	https://cutadapt.readthedocs.io/en/v1.9/guide.html
Bedtools, version 2.28.0	Quinlan and Hall (2010)	https://bedtools.readthedocs.io/en/latest/content/installation.html
Macs2, version 1.4.2	Zhang et al. (2008)	https://bioweb.pasteur.fr/packages/pack@macs@1.4.2
Seurat, version 3.1.4	Stuart et al. (2019)	https://github.com/satijalab/seurat/
Other		
1.5 mL Microfuge Tubes	USA scientific	Cat#1615-5500
PCR Tubes 0.5mL	Axygen	Cat#14-222-292
Magnetic Bead Separator	Invitrogen	Cat#12321D
Phase Lock Gel™	VWR	Cat#10847
Thermomixer	Eppendorf	Cat#2231000574
Thermocycler	Eppendorf	Cat#6311000010
Fragment Analyzer System	Agilent	n/a
Refrigerated centrifuge	Eppendorf	Cat#5404000537
Vortex mixer	Fisher Scientific	Cat#02215414
Tube Rotator & Rotisserie	VWR	Cat#10136-084
Nutating Mixer	Fisher Scientific	Cat#260100F
Cell counter	Bio-Rad TC20	Cat#1450102
Qubit Fluorometer	Thermo Fisher Scientific	Cat#Q33238
NextSeq 550 System	Illumina	Cat#SY-415-1002
Chromium Controller	10× Genomics	https://www.10xgenomics.com/instruments/chromium-controller

MATERIALS AND EQUIPMENT

2× YT media	
Reagent	Amount
Tryptone	16 g
Yeast Extract	10 g
NaCl	5 g
ddH_2O	Adjust to 1 L
Total	**1 L**

Autoclave at 121°C for 15 min. Store at 4°C for up to 6 months

HGX buffer		
Reagent	Final concentration	Amount
HEPES pH 7.2 (1 M)	20 mM	2 mL
NaCl (5 M)	800 mM	16 mL
Glycerol (50%)	10%	20 mL
Triton X 100 (10%)	0.2%	2 mL
ddH_2O	n/a	60 mL
Total	**n/a**	**100 mL**
Store at 4°C for up to 6 months		

Dialysis buffer		
Reagent	Final concentration	Amount
HEPES pH 7.2 (1 M)	20 mM	20 mL
NaCl (5 M)	200 mM	40 mL
EDTA (0.5 M)	0.2 mM	0.4 mL
Glycerol (100%)	50%	500 mL
Triton X 100 (10%)	0.2%	20 mL
DTT (1 M)	2 mM	2 mL (before use)
ddH_2O	n/a	418 mL
Total	**n/a**	**1,000 mL**
Store at 4°C (without DTT) for up to 6 months		

$HN_{50}TE$ buffer		
Reagent	Final concentration	Amount
HEPES pH 7.2 (1 M)	20 mM	20 mL
NaCl (5 M)	10 mM	2 mL
EDTA (0.5 M)	0.5 mM	1 mL
Triton X 100 (10%)	0.2%	20 mL
ddH_2O	n/a	957 mL
Total	**n/a**	**1,000 mL**
Store at 4°C for up to 6 months		

$HN_{150}TE$ buffer		
Reagent	Final concentration	Amount
HEPES pH 7.2 (1 M)	20 mM	20 mL
NaCl (5 M)	150 mM	30 mL
EDTA (0.5 M)	0.5 mM	1 mL
Triton X 100 (10%)	0.2%	20 mL
ddH_2O	n/a	929 mL
Total	**n/a**	**1,000 mL**
Store at 4°C for up to 6 months		

$HN_{800}TE$ buffer		
Reagent	Final concentration	Amount
HEPES pH 7.2 (1 M)	20 mM	2 mL
NaCl (5 M)	800 mM	16 mL
EDTA (0.5 M)	0.5 mM	0.1 mL
Triton X 100 (10%)	0.2%	2 mL
ddH_2O	n/a	79.9 mL
Total	**n/a**	**100 mL**
Store at 4°C for up to 6 months		

10× Annealing buffer		
Reagent	Final concentration	Amount
HEPES pH 7.2 (1 M)	100 mM	0.2 mL
NaCl (5 M)	500 mM	0.2 mL
EDTA (0.5 M)	10 mM	0.04 mL
ddH_2O	n/a	1.56 mL
Total	**n/a**	**2 mL**
Store at 4°C for up to 1 year		

5× Tn5 Transposition buffer		
Reagent	Final concentration	Amount
Tris pH 8.2 (1 M)	50 mM	0.5 mL
$MgCl_2$ (1 M)	25 mM	0.25 mL
DMF (100%)	50%	5 mL
ddH_2O	n/a	4.25mL
Total	**n/a**	**10 mL**
Store at 4°C for up to 1 year		

Binding buffer		
Reagent	Final concentration	Amount
HEPES pH 7.5 (1 M)	20 mM	2 mL
KCl (1 M)	10 mM	1 mL
CaCl2 (1 M)	1 mM	0.1 mL
MnCl2 (1 M)	1 mM	0.1 mL
ddH_2O	n/a	96.8 mL
Total	**n/a**	**100 mL**
Store at 4°C for up to 6 months		

Wash buffer		
Reagent	Final concentration	Amount
HEPES pH 7.5 (1 M)	20 mM	2 mL
NaCl (5 M)	150 mM	3 mL
Spermidine (1 M)	0.5 mM	0.025 mL
Protease Inhibitor cocktail	1×	Before use
ddH_2O	n/a	94.9 mL
Total	**n/a**	**100 mL**
Store at 4°C (without Protease Inhibitor) for up to 6 months		

Dig-wash buffer		
Reagent	Final concentration	Amount
Wash Buffer	See above	40 mL
Digitonin (5%)	0.05%	0.4 mL
Total	**n/a**	**40.4 mL**
make fresh		

Dig-med buffer		
Reagent	Final concentration	Amount
HEPES pH 7.5 (1 M)	20 mM	2 mL
NaCl (5 M)	300 mM	6 mL
Spermidine (1 M)	0.5 mM	0.025 mL

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Continued		
Reagent	Final concentration	Amount
Digitonin (5%)	0.01%	0.2 mL (Before use)
Protease Inhibitor cocktail	1×	Before use
ddH_2O	n/a	94.7 mL
Total	**n/a**	**100 mL**
Store at 4°C (without digitonin and Protease Inhibitor) for up to 6 months		

⚠ CRITICAL: Digitonin is toxic and causes Acute oral toxicity. Please use appropriate Personal Protective Equipment like masks, eyeshields and gloves while handling.

STEP-BY-STEP METHOD DETAILS

Bulk multi-CUT&Tag

Preparation of concanavalin A beads

Timing: 15 min

In this step, lectin coated magnetic beads are activated. Beads can be prepared 15 min before harvesting the cells.

1. Resuspend Concanavalin A coated magnetic beads and pipet 10 μL of beads into a 1.5 mL microcentrifuge tube per 100,000 cells to be profiled in all samples. Add 850 μL cold binding buffer.
2. Place the microcentrifuge tube containing concanavalin A beads on a magnetic stand for at least 2 min, until the solution is clear.

Note: For this and all bead separation steps, let the solution clear completely to avoid loss of beads.

3. After the solution has completely cleared (about 2 min), remove and discard the supernatant by pipetting without disturbing the beads.
4. Remove microcentrifuge tube from magnetic stand and resuspend the concanavalin A beads in 1 mL binding buffer by gently pipetting the solution. Place it on magnetic stand for 2 min.
5. After the solution has completely cleared, remove and discard supernatant without disturbing beads.

Table 1. Barcoded oligos for loading to pA-Tn5 (antibody-specific barcodes in bold)

Primer	Sequence
P5_i5_1_Universal_Connector_A	TCGTCGGCAGCGTCTCCACGC**TATAGCCT**GCGATC GAGGACGGCAGATGTGTATAAGAGACAG
P5_i5_2_Universal_Connector_A	TCGTCGGCAGCGTCTCCACGC**ATAGAGGC**GCGAT CGAGGACGGCAGATGTGTATAAGAGACAG
P5_i5_3_Universal_Connector_A	TCGTCGGCAGCGTCTCCACGC**CCTATCCT**GCGA TCGAGGACGGCAGATGTGTATAAGAGACAG
P5_i5_4_Universal_Connector_A	TCGTCGGCAGCGTCTCCACGC**GGCTCTGA**GCG ATCGAGGACGGCAGATGTGTATAAGAGACAG
P7_i7_1_Universal_Connector_B	GTCTCGTGGGCTCGGCTGTCCCTGTCC**CGAGTA AT**CACCGTCTCCGCCTCAGATGTGTATAAGAGACAG
P7_i7_2_Universal_Connector_B	GTCTCGTGGGCTCGGCTGTCCCTGTCC**TCTCCGGA**C ACCGTCTCCGCCTCAGATGTGTATAAGAGACAG
P7_i7_3_Universal_Connector_B	GTCTCGTGGGCTCGGCTGTCCCTGTCC**AATGAG CG**CACCGTCTCCGCCTCAGATGTGTATAAGAGACAG
P7_i7_4_Universal_Connector_B	GTCTCGTGGGCTCGGCTGTCCCTGTCC**GGAATC TC**CACCGTCTCCGCCTCAGATGTGTATAAGAGACAG
Tn5ME Reverse	[phos]CTGTCTCTTATACACATCT

Table 2. Custom sequencing primers

Primer	Used for	Sequence
Read 1	Custom read 1 primer for multi-CUT&Tag	TCGTCGGCAGCGTCTCCACGC
Read 2	Custom read 2 primer for multi-CUT&Tag	GTCTCGTGGGCTCGGCTGTCCCTGTCC
Index 1	Custom index 1 primer for multi-CUT&Tag	GGACAGGGACAGCCGAGCCCACGAGAC
Index 2	Custom index 2 primer for multi-CUT&Tag	GCGTGGAGACGCTGCCGACGA
PE Read 1	Read1 for sequencing PhiX	ACACTCTTTCCCTACACGACGCTCTTCCGATCT
PE read 2	Read 2 for sequencing PhiX	CGGTCTCGGCATTCCTGCTGAACCGCTCTTCCGATCT

6. Repeat steps 4 and 5.
7. Remove microcentrifuge tube from magnetic stand and resuspend beads in 10 µL binding buffer.

Binding of cells to magnetic beads

Timing: 15 min

Cells are harvested and bound to the prepared concanavalin A-coated beads.

8. If required, trypsinize and resuspend cells to achieve a single cell suspension. Count and add appropriate number of cells to a 1.5 mL microcentrifuge tube in PBS. We typically use 100,000 cells for each multi-CUT&Tag bulk experiment.
9. Centrifuge the cells for 5 min at 600 *g*, 4°C. Discard supernatant.
10. Add 1 mL Wash buffer and resuspend the cell pellet by gentle pipetting.
11. Centrifuge the cells for 5 min at 600 *g*, 4°C. Discard the supernatant.
12. Resuspend the cell pellet in 0.5 mL Wash buffer and add the activated Concanavalin A coated beads (10 uL, from the previous step).
13. Incubate cells/beads for 15 min at 25°C in an end-over-end rotator.

Incubating permeabilized cells with purified antibody-pA-Tn5 complex(es)

Timing: 4–12 h

In this step, differentially barcoded purified antibody-pA-Tn5 complex is added to permeabilized cells and allowed to bind to the chromatin proteins to be mapped.

14. Place the microcentrifuge tube containing Concanavalin A bead-bound cells on a magnetic stand for 2 min. Then carefully remove all liquid by pipetting.
15. Resuspend cells in 100 µL cold Dig-wash Buffer containing 2 mM EDTA.

Note: Gentle pipetting is preferable to vortexing at this step. Take care to prevent the introduction of bubbles during resuspension.

16. Add 6 µL of each of the differentially barcoded purified antibody-pA-Tn5 complex to the cells.
17. Incubate on nutating mixer at 4°C for 4 h (or up to 12 h) incubation.

Tagmentation

Timing: 1.5 h

In this step, antibody-tethered pA-Tn5 will insert unique barcodes into the DNA around the site of pA-Tn5 recruitment.

18. Place the microcentrifuge tube on a magnetic stand for at least 2 min, until the solution is clear.

19. After the solution has completely cleared, remove and discard the supernatant without disturbing the beads.
20. Remove microcentrifuge tube from magnetic stand and resuspend the beads in 1 mL Dig-med Buffer by gently pipetting. Incubate on nutating mixer for 5 min and then place it on magnetic stand for 2 min.
21. Discard supernatant without disturbing beads. and repeat step 20.
22. After discarding the supernatant, resuspend the bead-bound cells in 300 μL Dig-med Buffer containing 10 mM MgCl2 while gently vortexing.
23. Incubate bead-bound cells at 37°C for 60 min to allow transposition of adapters into DNA.

Purification of DNA

Timing: 2 h or 14 h

In this step, the transposed DNA fragments are purified prior to PCR amplification.

24. To stop tagmentation and solubilize DNA fragments, add 10 μL 0.5 M EDTA, 3 μL 10% SDS and 1 μL of 20 mg/mL Proteinase K to each sample.
25. Vortex on full speed and incubate for 60 min at 50°C or 12h at 37°C.
26. Add 300 μL of Phenol-Chloroform-Isoamyl alcohol (listed as Phenol:Chloroform +tris buffer in Key Resources) to the tube and mix by full-speed vortexing. Transfer to a phase-lock tube, and centrifuge for 3 min at 16,000 *g* at 25°C.

Note: Phenol and Chloroform are chemical hazards and should be handled with caution in a chemical hood.

27. Add 300 μL chloroform to the same phase lock tube (to the aqueous phase), mix by inversion and centrifuge for 5 min at 16,000 *g* at 25°C.
28. Remove the aqueous phase to a new tube, add 0.5 μg/μL glycogen, and mix by vortexing well.
29. Add 750 μL of 100% ethanol and mix by vortexing. Incubate at −20°C for 15 min.

Pause point: Sample can also be stored in −20°C for at least several days.

30. Centrifuge for 15 min at 4°C at 16,000 *g*.
31. Carefully remove and discard the supernatant without disturbing the pellet.

⚠ CRITICAL: The pellet is hardly visible, so be careful when removing the supernatant not to disturb the pellet.

32. Wash the pellet in 1 mL 100% ethanol and carefully remove and discard the supernatant.
33. Centrifuge for 1 min at 16,000 × *g*, 4°C, to spin down any residual ethanol.
34. Discard any residual supernatant by pipet.
35. Air dry ~5 min at 25°C.

⚠ CRITICAL: Do not overdry samples.

36. Dissolve the pellet in 23 μL of 10 mM Tris-HCl pH8 containing 0.1 mg/mL RNase A.
37. Incubate for 10 min at 37°C.

PCR amplification of the multi-CUT&Tag library

Timing: 1 h

A low amount of DNA is recovered during the previous step, which needs to be amplified. Full P5/P7 adapter sequences for sequencing are also added during this PCR step.

Note: When amplifying multi-CUT&Tag DNA, the initial extension prior to melting is needed to fill in the gap left during the transposition and allow productive amplification cycles.

38. Prepare PCR mix as below, using universal or barcoded i5 and i7 primers (Buenrostro et al., 2013) and mix gently.

Component	Stock	Volume
Purified DNA (From step 37)	n/a	21 μL
i5 primer	10 μM	2 μL
i7 primer	10 μM	2 μL
NEBNext HiFi 2× PCR Master mix	2×	25 μL

39. PCR:

PCR cycling conditions			
Steps	Temperature	Time	Cycles
Initial extension	72°C	5 min	1
Initial Denaturation	98°C	30 s	1
Denaturation	98°C	10 s	17–20 cycles
Annealing/ Extension	63°C	10 s	
Final extension	72°C	1 min	1
Hold	8°C	Forever	

⚠ CRITICAL: Additional PCR cycles may be needed (dependent on the type of cell, number of cells used, antibody used, etc.), and can be determined by quantitative PCR using KAPA Library Quantification Kits (according to manufacturer's protocol). Approximately 5–10 ng of library is needed for an Illumina sequencing run.

⚠ CRITICAL: It is essential to keep the number of PCR cycles constant among samples that are to be compared

Post PCR cleanup

Timing: 1 h

These steps remove the excess primers and isolate the appropriate sizes of DNA fragments for sequencing.

40. To the same tube from the previous step, add 55 μL AMPure XP beads (1.1 volumes) and mix by pipetting. Incubate at 25°C for 10 min.
41. Place on magnet and carefully pipet off the liquid. Without disturbing the beads, wash the beads twice with 200 μL of 80% ethanol.
42. Perform a quick (1–2 s) spin in a mini centrifuge, replace tubes on the magnet, carefully remove all remaining liquid, and air dry the beads for 2–3 min.
43. Add 25 μL 10 mM Tris-HCl pH 8 to the beads and vortex on full. Incubate at 25°C for 5 min.
44. Place on magnet for 2 min, transfer the supernatent a fresh 1.5 mL microcentrifuge tube.

45. Determine the size distribution (by Fragment analyzer) and concentration (by Qubit or KAPA Library Quantification Kit) of the libraries before sequencing.

Single cell multi-CUT&Tag

Incubating permeabilized cells with purified antibody-pA-Tn5 complex(es)

Timing: 12 h

46. This step allows binding of differentially labeled antibody-pA-Tn5 complexes to the respective chromatin epitopes in the bulk population of cells, prior to single cell isolation and barcoding.
    a. Cells were trypsinized and washed in PBS. After cell counting, 200,000 cells were used as starting material.
    b. Centrifuge cells at 600 *g* for 5 min at 4°C.
    c. Remove the supernatant and resuspend the cells in 1 mL Wash buffer. Transfer the cells to 1.5 mL microcentrifuge tube.
    d. Centrifuge cells at 600 *g* for 5 min at 4°C to remove the supernatant. Resuspend cell pellet in 100 μL cold Dig-wash Buffer containing 2 mM EDTA.
    e. Add each of the differentially barcoded purified antibody-pA-Tn5 complexes (12 μL) to the cells.
    f. Place on nutating mixer at 4°C for ~12 h incubation to allow antibodies to bind to chromatin.

Tagmentation

Timing: 1.5 h

In this step, antibody-tethered pA-Tn5 will insert unique barcodes into the DNA around the site of pA-Tn5 recruitment.

    g. Centrifuge cells at 600 *g* for 5 min at 4°C.
    h. Remove the supernatant and resuspend the cells in 1 mL Dig-med Buffer by gently pipetting. Incubate on nutating mixer for 5 min.
    i. Repeat the wash steps 52 and 53 three times (for a total of four washes).
    j. Count the cells again during the last wash, as some cells can be lost during the wash steps.
    k. Resuspended cells in Dig-med Buffer at 5000 cells/μL and incubate on ice for 5 min.
    l. To start tagmentation, add an equal volume of Dig-med Buffer containing 20 mM MgCl2 and incubate samples at 37°C for 60 min.
    m. After 1 h, 2.5 μL of the tagmentation reaction (2500 cells/μL) is used for a targeted cell recovery of 4000 cells. It is mixed into a tube as below:

Component	Volume
Tagmentation reaction (From step 57)	2.5 μL
Diluted Nuclei Buffer (Chromium Single Cell ATAC Reagent Kit, 10× Genomics)	2.5 μL
ATAC buffer (Chromium Single Cell ATAC Reagent Kit, 10× Genomics)	7 μL
50% glycerol	3 μL
5 M NaCl	0.5 μL

GEM generation & barcoding

Timing: 1 h

This step allows single cell isolation by generation of Gel Beads-in-emulsion (GEMs), using a Chromium Next GEM Single Cell ATAC Library & Gel Bead Kit (10× Genomics) https://assets.ctfassets.net/an68im79xiti/6Be7TWXkltEluNIaWgA0dj/37c815979660f6a718328d525914a5d5/CG000209_Chromium_NextGEM_SingleCell_ATAC_ReagentKits_v1.1_UserGuide_RevF.pdf

n. Prepare the barcoding master mix, by mixing 61.5 μL of barcoding reagent, 1.5 μL of Reducing Agent B and 2 μL of barcoding enzyme.
o. Load the samples and Gel beads to Chromium Chip E following the 10× Genomics User protocol (step 2 of Chromium Next GEM Single Cell ATAC Reagent Kits v1.1 User Guide, linked above). After GEM generation in the 10× Genomics Chromium Controller, the GEMs are transferred to a PCR tube.
p. Incubate the GEMs in a thermocycler with following program:

PCR cycling conditions

Steps	Temperature	Time	Cycles
Initial	72°C	5 min	1
Initial Denaturation	98°C	30 s	1
Denaturation	98°C	10 s	1 cycle
Annealing/Extension	59°C	10 s	
Final extension	72°C	1 min	1
Hold	15°C	forever	

⚠ CRITICAL: The use of a single amplification cycle at this step differs from the manufacturer's recommendations for scATAC-seq (linked above), which recommends >10 cycles of linear amplification to introduce cell-specific barcodes. We found that elimination of all but one amplification cycle at this step was necessary to prevent incorporation of uninserted Tn5 adapters into libraries during PCR.

Post GEM incubation cleanup (according to manufacturer's protocol)

Timing: 2 h

Silane magnetic beads remove leftover biochemical reagents from the post GEM reaction mixture. AMPure beads eliminate unused barcodes from the sample.

q. Post GEM cleanup is performed first using Dynabeads MyOne SILANE beads according to the 10× Genomics' ATAC seq protocol (step 3 of Chromium Next GEM Single Cell ATAC Reagent Kits v1.1 User Guide).
r. An additional round of cleanup is then performed using AMPure XP beads according to 10× Genomics' ATAC seq protocol.

Single cell multi-CUT&Tag library construction (according to manufacturer's protocol)

Timing: 2 h

Single cell libraries with cell-specific and sample-specific barcodes are generated in this step.

s. Construct libraries using sample indexing primers (Single Index Kit, 10× Genomics), by performing 14 cycles of PCR according to manufacturer's protocol (step 4 of Chromium Next GEM Single Cell ATAC Reagent Kits v1.1 User Guide).
t. Perform a double-sided size selection post sample index PCR using AMPure XP beads according to manufacturer's protocol.

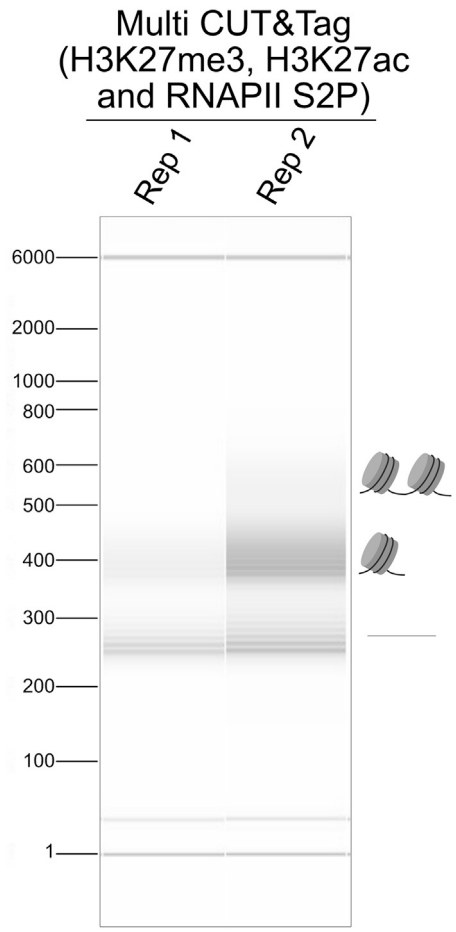

Figure 2. Library size distribution by fragment analyzer of triple antibody multi-CUT&Tag for H3K27me3, H3K27ac, and RNAPII S2P

Multi-CUT&Tag libraries tend to display peaks corresponding to subnucleosomal fragments plus a nucleosome ladder: mononucleosomes, dinucleosomes, etc. Fragment sizes corresponding to each class, plus adapters, are indicated.

u. Dilute 1 μL of the library to determine its concentration using KAPA Library Quantification Kit (According to manufacturer's protocol). The library size distribution can be determined by Fragment Analyzer (According to manufacturer's protocol) (Figure 2), prior to sequencing.

Note: ~5 ng of library is needed for Illumina sequencing run.

Sequencing of multi-CUT&Tag libraries

Timing: 24 h

Paired-end Illumina sequencing of multicell or single cell multi-CUT&Tag libraries is performed using custom sequencing primers.

47. Paired-end sequencing is performed on an Illumina NextSeq 500, using a 150 cycle sequencing kit. We used manual mode on the NextSeq 500 for sequencing.
48. Custom read primers for library and PhiX Control v3 were mixed together for the run (Table 2). Custom indexing primers were used for indexing run (Table 2).

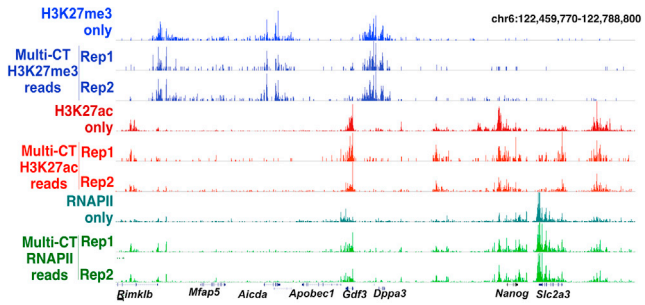

Figure 3. Genomic landscape comparing single antibody CUT&Tag and triple antibody multi-CUT&Tag for H3K27me3, H3K27ac and RNAPII S2P.

49. The following sequencing parameters were used for the run: read 1–72 cycles, read 2–72 cycles, index 1–8 cycles, index 2–8 cycles (for bulk multi-CUT&Tag) or 16 cycles (for single cell multi-CUT&Tag).

Note: PhiX Control v3 was added at 20%–30% per reaction, due to the sequence homogeneity of the initial sequencing cycles, which read through adapter sequences that are identical in all reads. Addition of a spike-in library like PhiX Control v3 is a quality control step that offers multiple benefits during cluster generation, sequencing, and read alignment. It is optional but highly recommended.

EXPECTED OUTCOMES

Figure 2 shows examples of typical multi-CUT&Tag library size profiles. For antibodies that recognize histone marks and RNA polymerase II, a clear nucleosomal pattern is expected, with peaks of fragment sizes corresponding to sub-nucleosome, mono-nucleosome, di-nucleosome, and so on. However, it is also possible to see a "flattened" profile, in which the nucleosomal peaks are less prominent in some cases.

The library yield largely correlates with the input cell number, antibodies used, number of epitopes present, and PCR cycles used for library amplification. Using 100,000 mouse embryonic stem cells, the bulk multi-CUT&Tag library yield was in the range of 50–250 ng with 17 cycles of PCR.

After sequencing, the multiCUT&Tag data should display high signal to noise and be enriched at expected regions (H3K27me3 at repressed promoters and H3K27ac and RNA Polymerase II at the active promoters/genes) (Figure 3). Where possible, comparison of multi-CUT&Tag maps with orthogonal maps of each epitope (e.g., ChIP-seq) is useful for assessment of library quality.

Data analysis

The analysis of the reads can vary based on the questions being asked. We provide these basic analyses just as a general set of recommendations. This does not represent a comprehensive list of analyses that one might do with multi-CUT&Tag data to address other questions.

Bulk multi-CUT&Tag (as described in https://github.com/snehagopalan710/Bulk-Multi-CUT-Tag/tree/main):

Paired-end reads from each sample are split based on their antibody specific barcodes on both ends of the fragment using novoBarcode (http://www.novocraft.com/documentation/novobarcode/demultiplexing-barcodedindexed-reads-with-novobarcode/).

The first 42 bases of the reads are trimmed to remove the antibody barcodes and the bases common to all Tn5 adaptor sequences.

The reads are then aligned to the mouse genome (mm10) using Bowtie2 with the parameters -N 1 and -X 2000.

Duplicates are removed using Picard (http://broadinstitute.github.io/picard/).

Reads with low quality scores (MAPQ < 10) are removed.

The remaining mapped reads can then be processed using the HOMER software suite (Heinz et al., 2010) or similar tools.

Single cell multi-CUT&Tag (as described in https://github.com/snehagopalan710/Bulk-Multi-CUT-Tag/blob/5a7899179fafcbb65d3fe330debdfc3903a1199d/scMulti-CUT&Tag%20analysis):

Use cellranger-atac/1.1.0 for BCL file conversion and demultiplexing.

The reads are considered valid only if both antibody barcodes are within the Tn5 barcode list and the cell barcode is in the whitelist provided by 10× genomics. Perform valid read extraction and barcode correction using a custom script included in the link above.

Remove potential read-though adaptors using cutadapt/1.9 (Martin, 2011).

Align reads to the mm10 genome with bowtie2/2.4.1. Remove low-quality reads using samtools (Li et al., 2009; Ai et al., 2019) with -q 30.

Read pairs are considered as duplicates of the same DNA fragment if they meet two criteria: their cell barcodes are identical, and they have identical start and end locations in the genome.

After deduplication using a custom script linked above, separate unique cut sites according to the antibody barcodes for independent downstream process.

Extend the cut sites by 100 bp on each end using bedtools/2.28.0 (Quinlan and Hall, 2010) and call peaks using the MACS2 (Zhang et al., 2008) 'call- peak' command in macs/1.4.2 with–nomodel–broad flags.

> ***Note:*** Newer versions of sequence analysis software, including samtools, MACS2, bowtie2 can also be used.

LIMITATIONS

Multi-CUT&Tag utilizes a custom sequencing strategy (as described), which prevents pooling multi-CUT&Tag libraries with libraries that require standard Illumina sequencing primers.

Some DNA binding proteins, such as transcription factors, perform less well in the multi-CUT&Tag procedure, similar to their poorer performance relative to histone modifications and RNAPII in standard CUT&Tag (Kaya-Okur et al., 2019).

Negative controls used in ChIP-seq studies, such as non-specific IgG, perform poorly in multi-CUT&Tag, preventing usage of such controls as background models for peak finding (Gopalan et al., 2021).

TROUBLESHOOTING

Problem 1

Low yield of purified pA-Tn5 enzyme

Potential solution

The bacterial pellet size can vary depending on how well chilled the bacteria was prior to IPTG addition, the precise incubation temperature, or other factors. If the cell lysate is viscous after sonication, it will negatively impact bead binding. Additional sonication steps or increasing the volume of the HGX buffer during sonication may improve performance and yield.

Problem 2

Low yield of multi-CUT&Tag library

Potential solution

Below are the possible causes and potential solutions.

Inefficient cell permeabilization.

Make sure the cells are in a single-cell suspension. Large cell clumps are difficult to permeabilize. The digitonin concentration is important and needs to be added fresh.

Low transposition efficiency.

pA-tn5 transposase requires magnesium ions for transposition. The washes after Tn5 binding are important to wash away unbound pA-Tn5 and to remove the chelating agent EDTA.

Insufficient PCR cycles.

PCR cycles can be increased for multi-CUT&Tag library amplification, although excessive amplification may lead to an increase in duplicated reads.

> *Note:* Spike-in DNA can also be used to determine the optimal number of PCR cycles needed to bring the library to an ideal molarity for sequencing (Li et al., 2021). A spike-in has the additional benefit of serving as an internal control for later normalization purposes.

Low recovery during library cleanup.

Do not over dry the AMPure XP beads after the ethanol washes, as this can inhibit elution.

Problem 3

The fragment size distribution of the multi-CUT&Tag library is too small or too large.

Potential solution

Perform size selection with AMPure XP beads to remove large fragments. Make sure the AMPure XP slurry is at 25°C before adding to the library.

Problem 4

Low signal to noise in resulting data.

Potential solution

The antibody used may not have been sufficiently specific. Different antibodies can be tested and more extensive or stringent washes before tagmentation may help.

Problem 5

High abundance of read duplicates, especially for single cell data.

Potential solution

The high duplication rate is expected in the single cell multi-CUT&Tag and may require higher sequencing depth. The duplicate reads are removed during the analyses. The remaining reads are typically highly specific for enriched regions.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Thomas G. Fazzio (Thomas.Fazzio@umassmed.edu).

Materials availability

This study did not generate new unique reagents.

Data and code availability

Data generated and analyzed during this study are available at Gene Expression Omnibus (GEO), with the accession GSE171554. Code for sequencing data analysis can be found at https://github.com/snehagopalan710/Bulk-Multi-CUT-Tag/tree/main.

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AUTHOR CONTRIBUTIONS

S.G. and T.G.F. designed the study. S.G. optimized the multi-CUT&Tag approach and performed all experiments. S.G. and T.G.F. wrote the manuscript.

DECLARATION OF INTERESTS

The authors declare no competing interests.

REFERENCES

Ai, S., Xiong, H., Li, C.C., Luo, Y., Shi, Q., Liu, Y., Yu, X., Li, C., and He, A. (2019). Profiling chromatin states using single-cell itChIP-seq. Nat. Cell Biol. *21*, 1164–1172. https://doi.org/10.1038/s41556-019-0383-5.

Bartosovic, M., Kabbe, M., and Castelo-Branco, G. (2021). Single-cell CUT&Tag profiles histone modifications and transcription factors in complex tissues. Nat. Biotechnol. *39*, 825–835. https://doi.org/10.1038/s41587-021-00869-9.

Buenrostro, J.D., Giresi, P.G., Zaba, L.C., Chang, H.Y., and Greenleaf, W.J. (2013). Transposition of native chromatin for fast and sensitive epigenomic profiling of open chromatin, DNA-binding proteins and nucleosome position. Nature Methods *10*, 1213–1218. https://doi.org/10.1038/nmeth.2688.

Gopalan, S., Wang, Y., Harper, N.W., Garber, M., and Fazzio, T.G. (2021). Simultaneous profiling of multiple chromatin proteins in the same cells. Mol. Cell *81*, 4736–4746.e5. https://doi.org/10.1016/j.molcel.2021.09.019.

Heinz, S., Benner, C., Spann, N., Bertolino, E., Lin, Y.C., Laslo, P., Cheng, J.X., Murre, C., Singh, H., and Glass, C.K. (2010). Simple combinations of lineage-determining transcription factors prime cis-regulatory elements required for macrophage and B cell identities. Mol. Cell *38*, 576–589. https://doi.org/10.1016/j.molcel.2010.05.004.

Kaya-Okur, H.S., Wu, S.J., Codomo, C.A., Pledger, E.S., Bryson, T.D., Henikoff, J.G., Ahmad, K., and Henikoff, S. (2019). CUT&Tag for efficient epigenomic profiling of small samples and single cells. Nat. Commun. *10*, 1930. https://doi.org/10.1038/s41467-019-09982-5.

Li, H., Handsaker, B., Wysoker, A., Fennell, T., Ruan, J., Homer, N., Marth, G., Abecasis, G., and Durbin, R. (2009). The sequence alignment/map format and SAMtools. Bioinformatics *25*, 2078–2079. https://doi.org/10.1093/bioinformatics/btp352.

Li, Y., Nakka, K., Olender, T., Gingras-Gelinas, P., Wong, M.M., Robinson, D.C.L., Bandukwala, H., Palii, C.G., Neyret, O., Brand, M., et al. (2021). Chromatin and transcription factor profiling in rare stem cell populations using CUT&Tag. STAR Protoc. *2*, 100751. https://doi.org/10.1016/j.xpro.2021.100751.

Martin, M. (2011). Cutadapt removes adapter sequences from high-throughput sequencing reads. EMBnet J. *17*, 10–12. https://doi.org/10.14806/ej.17.1.200.

Picelli, S., Björklund, A.K., Reinius, B., Sagasser, S., Winberg, G., and Sandberg, R. (2014). Tn5 transposase and tagmentation procedures for massively scaled sequencing projects. Genome Res. *24*, 2033–2040. https://doi.org/10.1101/gr.177881.114.

Quinlan, A.R., and Hall, I.M. (2010). BEDTools: a flexible suite of utilities for comparing genomic features. Bioinformatics *26*, 841–842. https://doi.org/10.1093/bioinformatics/btq033.

Stuart, T., Butler, A., Hoffman, P., Hafemeister, C., Papalexi, E., Mauck, W.M., Hao, Y., Stoeckius, M., Smibert, P., and Satija, R. (2019). Comprehensive integration of single-cell data. Cell *177*, 1888–1902.e21. https://doi.org/10.1016/j.cell.2019.05.031.

Wu, S.J., Furlan, S.N., Mihalas, A.B., Kaya-Okur, H.S., Feroze, A.H., Emerson, S.N., Zheng, Y., Carson, K., Cimino, P.J., Keene, C.D., et al. (2021). Single-cell CUT&Tag analysis of chromatin modifications in differentiation and tumor progression. Nat. Biotechnol. *39*, 819–824. https://doi.org/10.1038/s41587-021-00865-z.

Zhang, Y., Liu, T., Meyer, C.A., Eeckhoute, J., Johnson, D.S., Bernstein, B.E., Nusbaum, C., Myers, R.M., Brown, M., Li, W., and Liu, X.S. (2008). Model-based analysis of ChIP-seq (MACS). Genome Biol. *9*, R137. https://doi.org/10.1186/gb-2008-9-9-r137.