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.
Protocol
Multi-CUT&Tag to simultaneously profile multiple chromatin factors

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https://doi.org/10.1016/j.xpro.2021.101100

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.
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](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<sub>600</sub> 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 dialed 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<sub>50</sub>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<sub>50</sub>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.
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   |
   | `TnSMRev`                  | 100 µM | 9 µL   |
   | Annealing buffer           | 10X    | 2 µL   |

   b. Assemble tube ‘B’

   | Component                  | Stock  | Volume |
   |----------------------------|--------|--------|
   | `P7_i7_1_Universal_Connector_B` | 100 µM | 9 µL   |
   | `TnSMRev`                  | 100 µM | 9 µL   |
   | Annealing buffer           | 10X    | 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                | 5X    | 4 µL   |
   | Linearized plasmid DNA (any linearized plasmid DNA that is > 10 kb) | 50 ng/µL | 1 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#MAS-11198 |
| Anti-Histone H3 (acetyl K27) antibody | Abcam | Cat#ab4729 |
| Phospho-Rpb1 CTD (Ser2) (E123G) Rabbit mAb | Cell Signaling Technology | Cat#13499 |
| Rabbit IgG          | Abcam  | Cat#Ab37415 |
| Bacterial and virus strains | Novagen | Cat#69451 |

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| 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, 1x without calcium and magnesium | Corning | Cat#21-040-CV |
| Halt Protease Inhibitor Cocktail, EDTA-Free (100x) | 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 1x Dye Reagent | Bio-Rad Laboratories | Cat#5000205 |
| SimplyBlue SafeStain | Thermo Fisher Scientific | Cat#LC6060 |
| Dimethylformamide (DMF) | Cell Signaling Technology | Cat#127675 |
| Manganese(II) chloride solution (MnCl2) | Millipore Sigma | Cat#M1787 |
| Dynabeads His-Tag Isolation and Pulldown | Invitrogen | Cat#10103D |
| ConcanaVan A coated magnetic beads | Polysciences | Cat#21-1401 |
| Calcium chloride (CaCl2) | Millipore Sigma | Cat#C4901 |
| Spermidine trihydrochloride | Millipore Sigma | Cat#S85580 |
| 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#C25-02.601 |
| 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 2x 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 | 10x genomics | Cat#PN-1000212 |
| Chromium Next GEM Single Cell ATAC Reagent Kits v1.1 | 10x 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 |

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## MATERIALS AND EQUIPMENT

### 2 × YT media

| Reagent          | Amount  |
|------------------|---------|
| Tryptone         | 16 g    |
| Yeast Extract    | 10 g    |
| NaCl             | 5 g     |
| ddH₂O            | Adjust to 1 L |
| **Total**        | 1 L     |

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

### 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-TnS | In this study | Table 1 |
|-------------------------------------|---------------|---------|
| Custom sequencing primers           | In this study | Table 2 |

### Software and algorithms

| 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 | 10x genomics | https://support.10xgenomics.com/single-cell-atac/software/release-notes/1-1 |
| Cutadapt, version 1.9    | 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://bioinfo.pasteur.fr/packages/packages/macs1.4.2 |
| Seurat, version 3.1.4     | Stuart et al. (2019) | https://github.com/satijalab/seurat/ |

### Other

| 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#G33238 |
| NextSeq 550 System                            | Illumina       | Cat#SY-415-1002 |
| Chromium Controller                           | 10x Genomics   | https://www.10xgenomics.com/instruments/chromium-controller |

### 2 × YT media

| Reagent          | Amount  |
|------------------|---------|
| Tryptone         | 16 g    |
| Yeast Extract    | 10 g    |
| NaCl             | 5 g     |
| ddH₂O            | 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     |
| ddH₂O                 | 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 (before use)   | 2 mL     |
| ddH₂O                 | n/a                 | 418 mL   |
| **Total**             | **n/a**             | **1,000 mL**|

Store at 4°C (without DTT) for up to 6 months

### HN50TE 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    |
| ddH₂O                 | n/a                 | 957 mL   |
| **Total**             | **n/a**             | **1,000 mL**|

Store at 4°C for up to 6 months

### HN150TE 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    |
| ddH₂O                 | n/a                 | 929 mL   |
| **Total**             | **n/a**             | **1,000 mL**|

Store at 4°C for up to 6 months

### HN800TE 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     |
| ddH₂O                 | 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|
| ddH₂O                 | n/a                 | 1.56 mL|
| **Total**             | **n/a**             | **2 mL**|

Store at 4°C for up to 1 year

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**5× Tn5 Transposition buffer**

| Reagent               | Final concentration | Amount |
|-----------------------|---------------------|--------|
| Tris pH 8.2 (1 M)     | 50 mM               | 0.5 mL |
| MgCl₂ (1 M)           | 25 mM               | 0.25 mL|
| DMF (100%)            | 50%                 | 5 mL   |
| ddH₂O                 | n/a                 | 4.25 mL|
| **Total**             | **n/a**             | **10 mL**|

Store at 4°C for up to 1 year

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**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 |
| ddH₂O                 | n/a                 | 96.8 mL|
| **Total**             | **n/a**             | **100 mL**|

Store at 4°C for up to 6 months

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**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 |
| ddH₂O                 | n/a                 | 94.9 mL|
| **Total**             | **n/a**             | **100 mL**|

Store at 4°C (without Protease Inhibitor) for up to 6 months

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**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

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**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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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.

### Reagents

| Reagent                          | Final concentration | Amount       |
|----------------------------------|---------------------|--------------|
| Digitonin (5%)                   | 0.01%               | 0.2 mL (Before use) |
| Protease Inhibitor cocktail      | 1 x                 | Before use   |
| ddH₂O                            | n/a                 | 94.7 mL      |
| Total                            | n/a                 | 100 mL       |

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

| Primer                          | Sequence                                                                 |
|---------------------------------|--------------------------------------------------------------------------|
| P5_i5_1 Universal_Connector_A  | TCGTCGGCAGCGTCTCCACGCTAGCCTCGGATCGGAGCAG                                  |
| P5_i5_2 Universal_Connector_A  | TCGTCGGCAGCGTCTCCACGCTAGCCTCGGATCGGAGCAG                                  |
| P5_i5_3 Universal_Connector_A  | TCGTCGGCAGCGTCTCCACGCTAGCCTCGGATCGGAGCAG                                  |
| P5_i5_4 Universal_Connector_A  | TCGTCGGCAGCGTCTCCACGCTAGCCTCGGATCGGAGCAG                                  |
| P7_i7_1 Universal_Connector_B  | GTCTCAGTGCGCGCTCGGTCTCCCGGATCGGAGCAG                                    |
| P7_i7_2 Universal_Connector_B  | GTCTCAGTGCGCGCTCGGTCTCCCGGATCGGAGCAG                                    |
| P7_i7_3 Universal_Connector_B  | GTCTCAGTGCGCGCTCGGTCTCCCGGATCGGAGCAG                                    |
| P7_i7_4 Universal_Connector_B  | GTCTCAGTGCGCGCTCGGTCTCCCGGATCGGAGCAG                                    |
| TriSME Reverse                  | [phos]CTGTCCTTTATACACATCT                                                |

STORE at 4°C (without digitonin and Protease Inhibitor) for up to 6 months.
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 μL, 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 12 h 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:**

| 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   | 2.5 μL  |
| ATAC Reagent Kit, 10x Genomics                |         |
| ATAC buffer (Chromium Single Cell              | 7 μL    |
| ATAC Reagent Kit, 10x Genomics                 |         |
| 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 (10x Genomics) https://assets.ctfassets.net/an68im79xiti/6Be7TWXktEluNiaWgA0dj/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 10x 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 10x 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)

① 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 10x 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 10x Genomics’ ATAC seq protocol.

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

① 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, 10x 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.
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 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.
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 multi-CUT&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])(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/](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/5a7899179afccb65d3fe330debdfc3903a1199d/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 10x 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.

ACKNOWLEDGMENTS
We thank Yuqing Wang and Manuel Garber for performing single-cell multi-CUT&Tag analyses in the original publication (Gopalan et al., 2021). This work was supported by NIH grants R01HD072122 and R01HD093783 to T.G.F.

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.

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