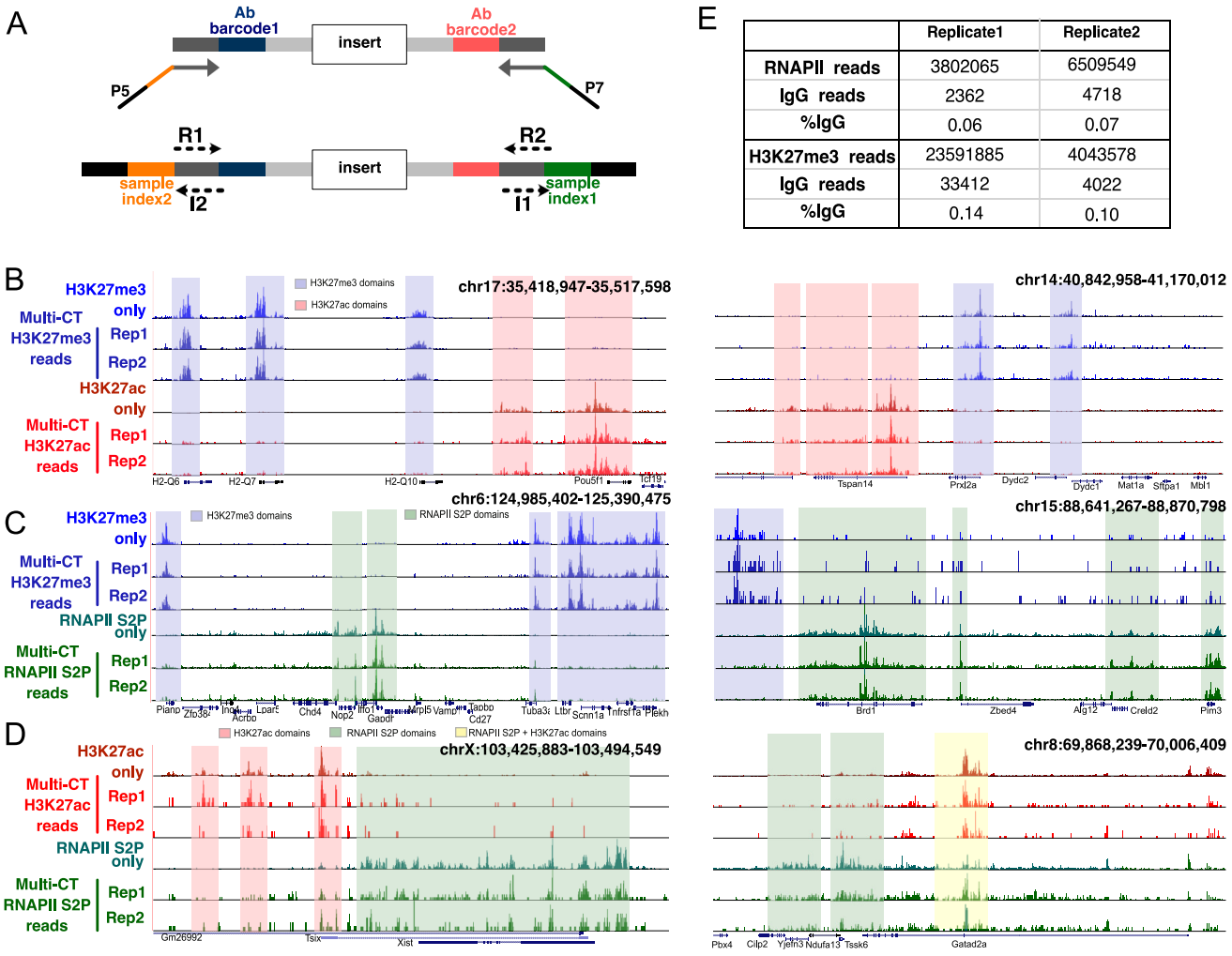

E

	Replicate1	Replicate2
RNAPII reads	3802065	6509549
IgG reads	2362	4718
%IgG	0.06	0.07
H3K27me3 reads	23591885	4043578
IgG reads	33412	4022
%IgG	0.14	0.10

Figure S1: Two Ab multi-CUT&Tag profiles, related to Figs. 1 and 2.

A, Library construction and sequencing strategies for multi-CUT&Tag, depicting locations of Ab barcodes, sample indices, and sequencing primers for reads 1 and 2 (R1, R2) and index 1 and 2 (I1, I2) sequencing reads are shown. **B-D,** Genomic landscape showing single and double Ab multi-CUT&Tag profiles of H3K27me3+H3K27ac (B), H3K27me3+RNAPII S2P (C), and H3K27ac+RNAPII S2P (D). The shaded boxes represent domains enriched for the indicated epitopes. **E,** Table showing total aligned reads in double Ab multi-CUT&Tag profiles of RNAPII S2P+IgG and H3K27me3+IgG.

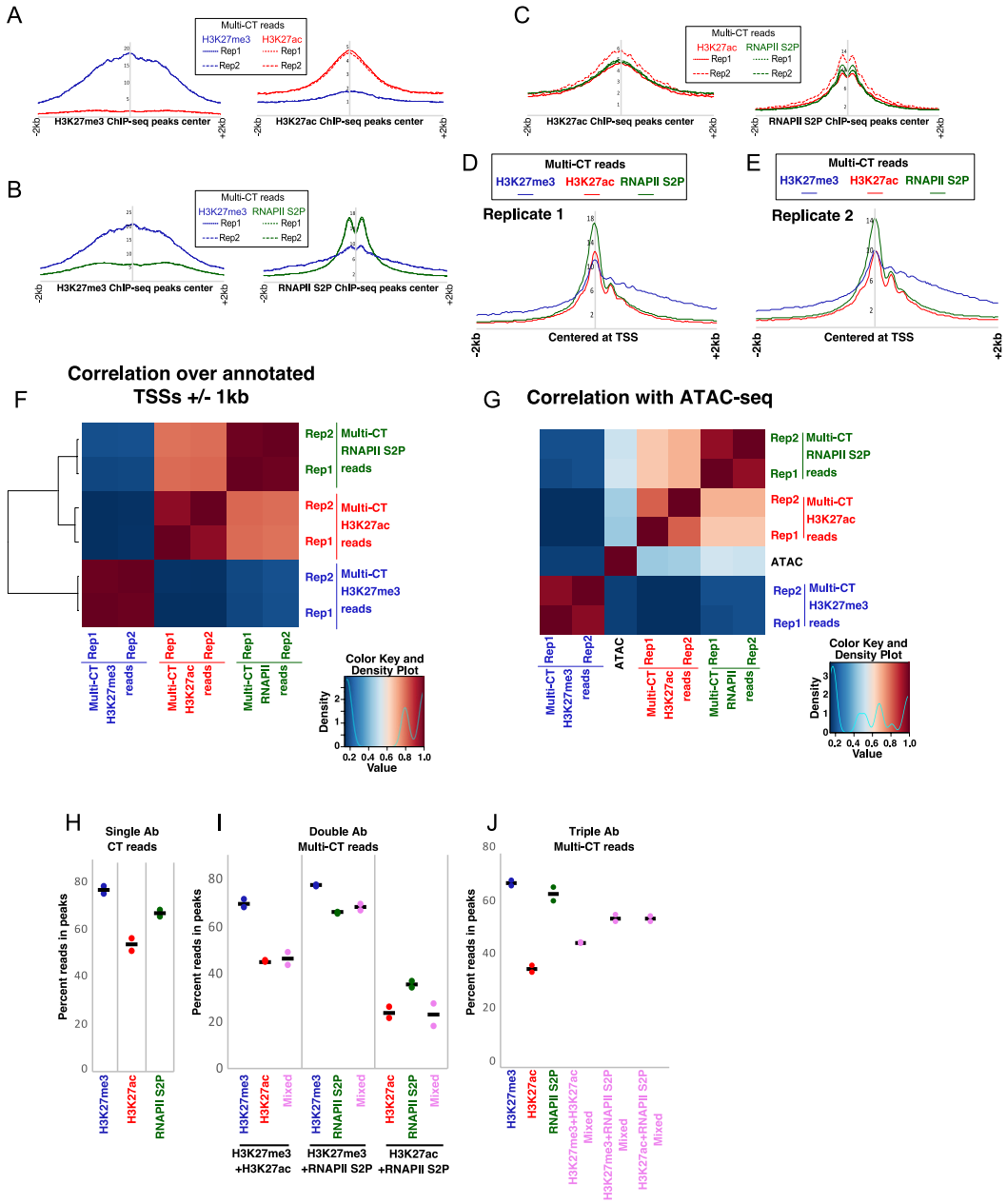

Figure S2: Specificity of multi-CUT&Tag maps, related to Figs. 1 and 2.

A-C, Average enrichment of reads from double Ab multi-CUT&Tag for H3K27me3+H3K27ac (A), H3K27me3+RNAPII S2P (B), and H3K27ac+RNAPII S2P (C) over published ChIP-seq peaks (as in Figure 1) corresponding to each epitope. **D-E,** Average enrichment of reads for each Ab from triple Ab multi-CUT&Tag for replicate 1 (D) and replicate 2 (E) over transcription start sites (TSS). **F,** Correlation matrix of triple Ab multi-CUT&Tag maps. Pearson correlations were calculated using the normalized read counts around annotated TSSs (+/- 1kb). **G,** Correlation matrix of ATAC-seq and triple Ab multi-CUT&Tag maps. Pearson correlations were calculated using the normalized read counts around all ATAC and triple Ab multi-CUT&Tag peaks (+/- 250bp). **H-J,** Percent reads for H3K27me3, H3K27ac and RNAPII S2P within peaks from single (G), double (H), and triple (I) Ab multi-CUT&Tag. Dots indicate individual replicates; lines indicate the means.

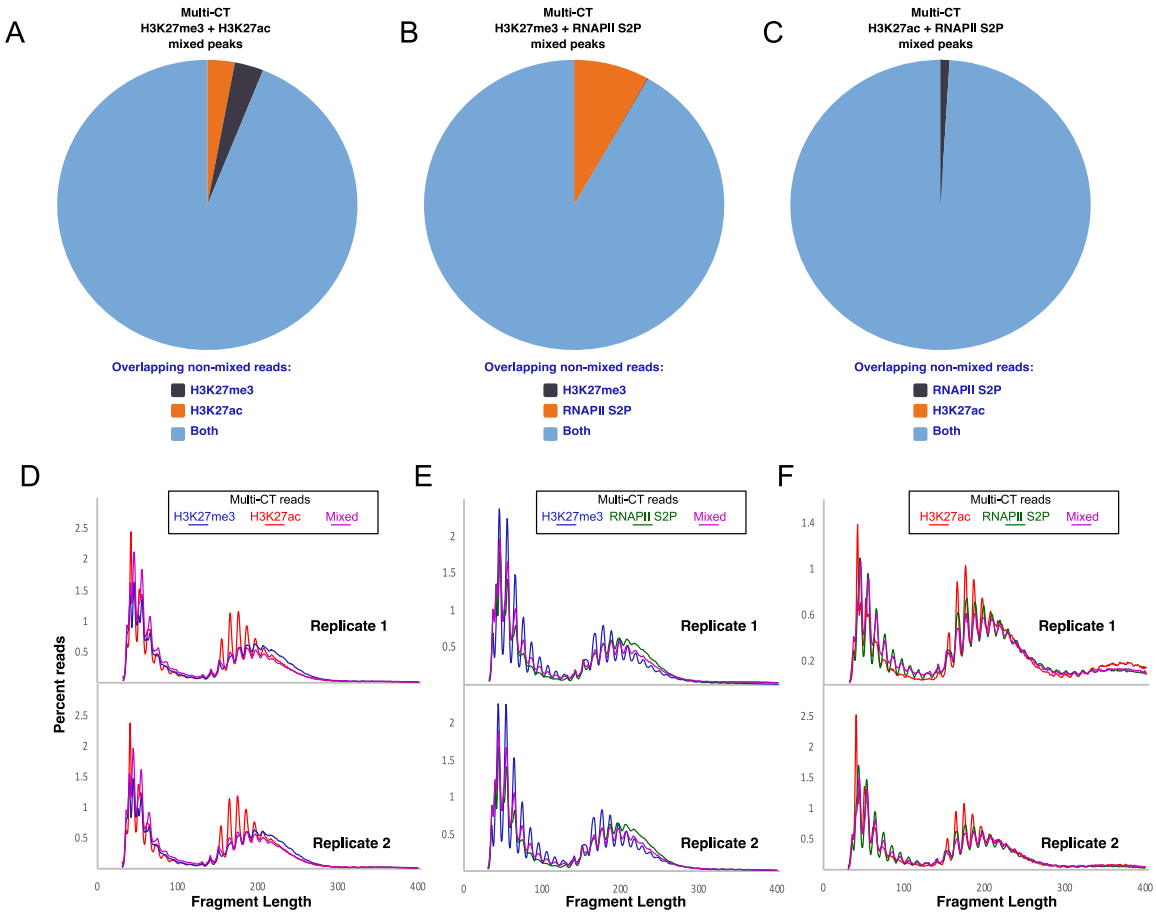

Figure S3: Size distribution of reads from multi-CUT&Tag, related to Fig. 3.

A-C, Pie chart showing homogenous reads in mixed peaks from double Ab multi-CUT&Tag for H3K27me3+H3K27ac (A), H3K27me3+RNAPII S2P (B), and H3K27ac+RNAPII S2P (C). **D-F,** Analysis of size distribution of homogeneous and mixed reads for both replicates from double Ab multi-CUT&Tag for H3K27me3+H3K27ac (D), H3K27me3+RNAPII S2P (E), and H3K27ac+RNAPII S2P (F).

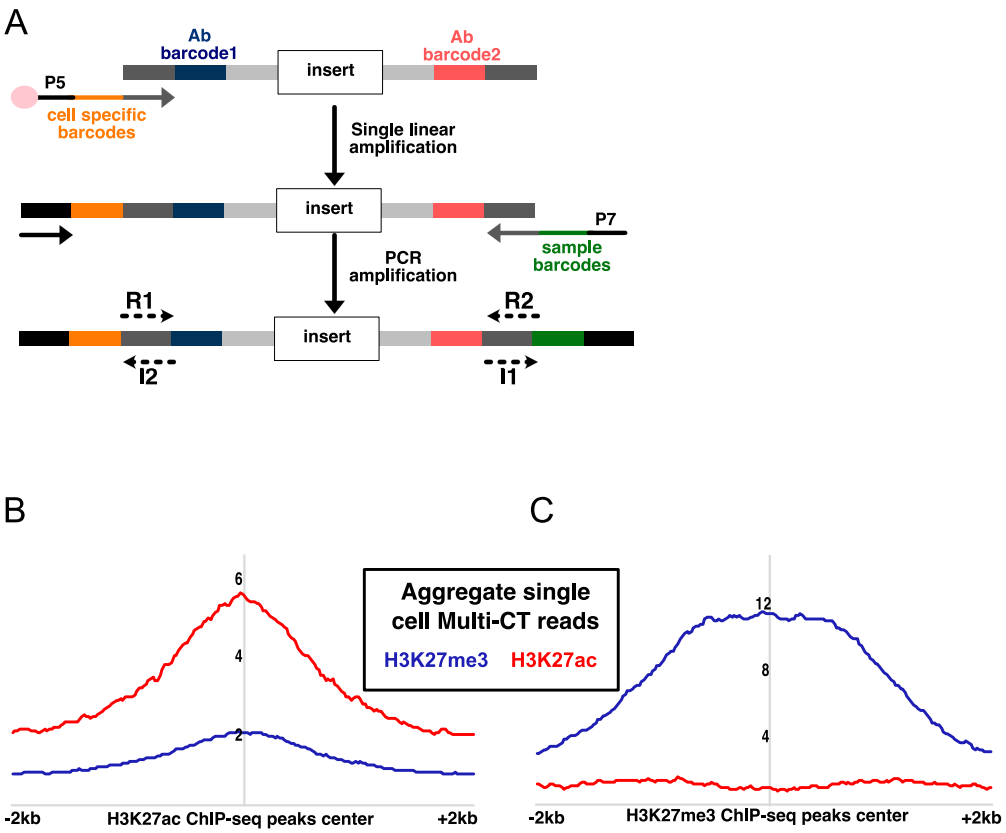

Figure S4: Workflow and specificity of scMulti-CUT&Tag, related to Fig. 4.

A, Library construction strategy for scMulti-CUT&Tag. The locations of cell specific barcodes (provided by 10X Genomics gel beads), Ab barcodes, sample barcodes, and sequencing primers for reads 1 and 2 (R1, R2) and index 1 and 2 (I1, I2) sequencing reads are shown. **B-C,** Average enrichment of reads from aggregate of scMulti-CUT&Tag over binding sites of H3K27me3 (B) and H3K27ac (C).

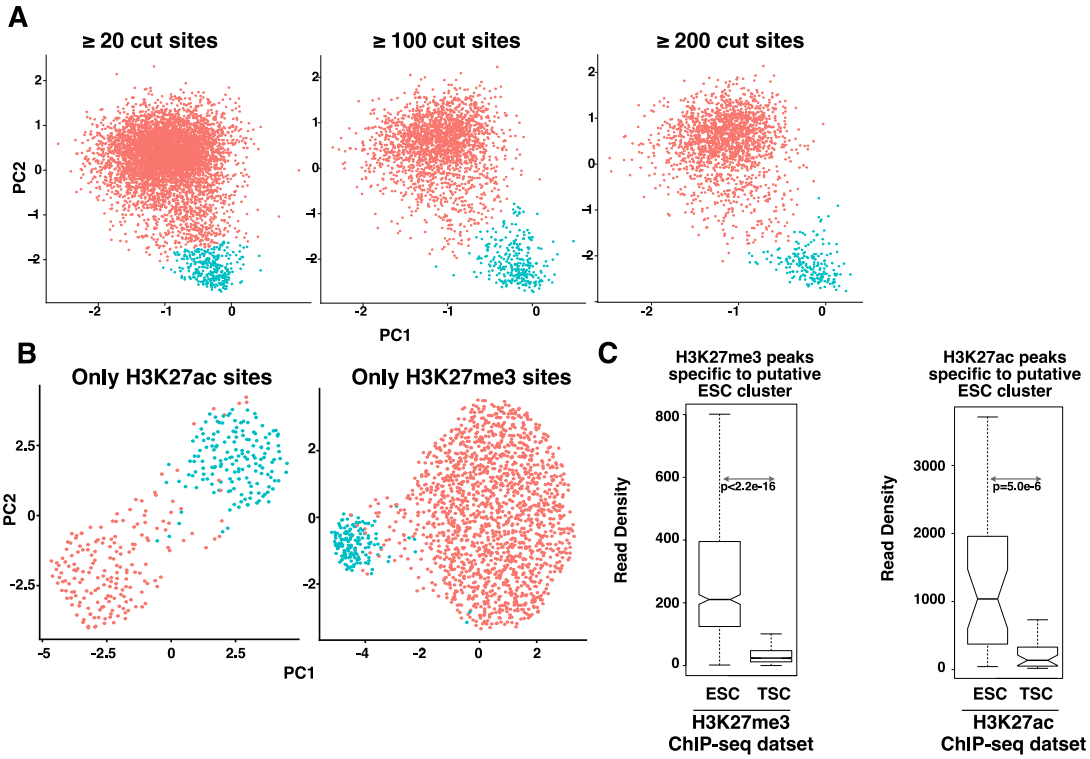

Figure S5: Unbiased clustering of scMulti-CUT&Tag, related to Fig. 5.

A, LSI plots showing cell type-specific clusters from cells with ≥20, ≥100, and ≥200 unique reads. Clusters from higher thresholds were generally similar overall to clusters using lower thresholds, but higher thresholds generally increased separation of clusters at the expense of including fewer cells. **B,** LSI plots showing cell type-specific clusters from H3K27ac and H3K27me3 specific reads. **C,** Read density from independent ESC or TSC ChIP-seq datasets within ESC-specific peaks specific to each antibody from single-cell multi-CUT&Tag. Two-sided Wilcoxan Rank-Sum Tests were used to test for significance.

Table S1. Oligonucleotides used in study, related to Fig. 1.

Name	Function	Sequence
P5_i5_1_Universal_Connector_A	Barcoded oligo for loading to pA-Tn5 (used for H3K27ac)	TCGTCGGCAGCGTCTCCACGCTATAGCCTGCGATCGAGGACGGCAGATGTGTATAAGAGACAG
P5_i5_2_Universal_Connector_A	Barcoded oligo for loading to pA-Tn5 (used for RNAP II)	TCGTCGGCAGCGTCTCCACGCATAGAGGCGCGATCGAGGACGGCAGATGTGTATAAGAGACAG
P5_i5_3_Universal_Connector_A	Barcoded oligo for loading to pA-Tn5 (used for H3K27me3)	TCGTCGGCAGCGTCTCCACGCCCTATCCTGCGATCGAGGACGGCAGATGTGTATAAGAGACAG
P5_i5_4_Universal_Connector_A	Barcoded oligo for loading to pA-Tn5 (used for IgG)	TCGTCGGCAGCGTCTCCACGCGGCTCTGAGCGATCGAGGACGGCAGATGTGTATAAGAGACAG
P7_i7_1_Universal_Connector_B	Barcoded oligo for loading to pA-Tn5 (used for H3K27ac)	GTCTCGTGGGCTCGGCTGTCCCTGTCCCGAGTAATCACCGTCTCCGCCTCAGATGTGTATAAGAGACAG
P7_i7_2_Universal_Connector_B	Barcoded oligo for loading to pA-Tn5 (used for RNAP II)	GTCTCGTGGGCTCGGCTGTCCCTGTCCTCTCCGGACACCGTCTCCGCCTCAGATGTGTATAAGAGACAG
P7_i7_3_Universal_Connector_B	Barcoded oligo for loading to pA-Tn5 (used for H3K27me3)	GTCTCGTGGGCTCGGCTGTCCCTGTCCAATGAGCGCACCGTCTCCGCCTCAGATGTGTATAAGAGACAG
P7_i7_4_Universal_Connector_B	Barcoded oligo for loading to pA-Tn5 (used for IgG)	GTCTCGTGGGCTCGGCTGTCCCTGTCCGGAATCTCCACCGTCTCCGCCTCAGATGTGTATAAGAGACAG
Tn5MErev	Reverse Primer for loading to pA-Tn5	[phos]CTGTCTCTTATACACATCT
Read1	Custom read1 primer for multi-CUT&Tag	TCGTCGGCAGCGTCTCCACGC
Read2	Custom read2 primer for multi-CUT&Tag	GTCTCGTGGGCTCGGCTGTCCCTGTCC
Index1	Custom Index1 primer for multi-CUT&Tag	GGACAGGGACAGCCGAGCCCACGAGAC
Index2	Custom Index2 primer for multi-CUT&Tag	GCGTGGAGACGCTGCCGACGA
PE read1	Read1 for sequencing phiX	ACACTCTTTCCCTACACGACGCTCTTCCGATCT
PE read2	Read2 for sequencing phiX	CGGTCTCGGCATTCCTGCTGAACCGCTCTTCCGATCT

Table S2. Read counts for multi-CUT&Tag libraries, related to STAR Methods.

Experiment	Raw reads	Epitopes
DoubleAb_AM_rep1	15670828	H3K27ac, H3K27me3
DoubleAb_AM_rep2	23210456	H3K27ac, H3K27me3
DoubleAb_AP_rep1	7890307	H3K27ac, RNAPII_Ser2P
DoubleAb_AP_rep2	3529709	H3K27ac, RNAPII_Ser2P
DoubleAb_PM_rep1	25779060	RNAPII_Ser2P, H3K27me3
DoubleAb_PM_rep2	33124783	RNAPII_Ser2P, H3K27me3
TripleAb_APM_rep1	32863407	H3K27ac, RNAPII_Ser2P, H3K27me3
TripleAb_APM_rep2	63496502	H3K27ac, RNAPII_Ser2P, H3K27me3
SingleAb_P_rep1	14977230	RNAPII_Ser2P
SingleAb_P_rep2	20828490	RNAPII_Ser2P
SingleAb_M_rep1	7135724	H3K27me3
SingleAb_M_rep2	10287107	H3K27me3
SingleAb_A_rep1	21970708	H3K27ac
SingleAb_A_rep2	19275066	H3K27ac
SingleCell_AM	288336450	H3K27ac, H3K27me3
DoubleAb_IM_rep1	30457152	IgG, H3K27me3
DoubleAb_IM_rep2	6077676	IgG, H3K27me3
DoubleAb_IP_rep1	10753883	IgG, RNAPII_Ser2P
DoubleAb_IP_rep2	11345061	IgG, RNAPII_Ser2P

Methods S1. Detailed Multi-CUT&Tag Protocol, related to STAR Methods.

Multi-CUT&Tag Protocol

Buffers to be made:

1) **10X Annealing buffer:** 100 mM HEPES pH 7.2, 500 mM NaCl, 10 mM EDTA.
2) **Binding buffer:** 20 mM HEPES pH 7.5, 10 mM KCl, 1 mM $CaCl_2$, 1 mM $MnCl_2$.
3) **Wash Buffer:** 20 mM HEPES pH 7.5, 150 mM NaCl, 0.5 mM Spermidine, 1× Protease inhibitor cocktail.
4) **Dig-wash Buffer:** 20 mM HEPES pH 7.5, 150 mM NaCl, 0.5 mM Spermidine, 0.05% Digitonin, 1× Protease inhibitor cocktail.
5) **Dig-med Buffer:** 20 mM HEPES pH 7.5, 300 mM NaCl, 0.5 mM Spermidine, 0.01% Digitonin, 1× Protease inhibitor cocktail.

Step 1: Loading pA-Tn5 with barcoded adapters.

1) In a PCR tube 'A', add 9 ul of 100 uM P5_i5_1_Universal_Connector_A, 9 ul of Tn5MErev and 2 ul of 10X annealing buffer.
2) In tube 'B', add 9 ul 100 uM P7_i7_1_Universal_Connector_B, 9 ul of 100 uM Tn5MErev, and 2 ul 10X annealing buffer.
3) To anneal the adapters, heat tubes to 95 °C for 1 minute and slowly ramp down to 25 °C. Hold at 25 °C.
4) Then mix tube A and B (45 uM Mixed Annealed Tn5 Adapters).
5) Heat ~100 ul of 100% glycerol to 90 °C in thermocycler or heat block. Pipet 35 ul heated 100% glycerol to new tube and cool on ice.
6) Add 35 ul of 45 uM Annealed Tn5 Adapters to cooled 100% glycerol and flick to mix.
7) Add 25 ul of 21.1 uM N-terminal 6-histidine (6-His) tagged pA-Tn5. Mix gently by flicking.
8) Incubate at RT for 60 minutes with occasional gentle flicking and then store the loaded pA-Tn5 enzyme at -20 °C.

Step 2: Coupling antibody of interest with loaded pA-Tn5.

1) Mix ~20 μL of loaded pA-Tn5 with ~40 μL of antibody of interest, aiming for approximately 2:1 pA-Tn5:Ab molar ratio.
2) Incubate overnight for binding at 4 °C in a nutating mixer.
3) Take 80 μL of Dynabeads His-Tag Isolation and Pulldown (Invitrogen, 10103D) and wash the beads with phosphate buffered saline (PBS).
4) Add the washed beads in 20 μL PBS to the tube containing pA-Tn5 and antibody.
5) Keep for 2-3 hours for binding at 4 °C in a nutating mixer.
6) Wash the beads in 200 μL of PBS. Repeat the wash step twice.
7) To elute the antibody-pA-Tn5 complex, add 100 μL of 300mM Imidazole in PBS. Incubate at 4 °C for 1 hour, in a nutating mixer.
8) Perform buffer exchange using Amicon Ultra- 0.5mL Centrifugal filters (Millipore, UFC501096) following the manufacturer's protocol.
9) The purified antibody-pA-Tn5 complexes can be stored at -20 °C in PBS + 50% glycerol.

Bulk multi-CUT&Tag

Step 3: Binding the cells to Concanavalin A-coated beads.

1) Resuspend Concanavalin A coated magnetic beads (Polysciences) and use ~10 μL of beads for 100,000 cells.
2) Wash the Concanavalin A coated beads twice in Binding buffer to activate the beads.
3) Trypsinize cells to single cell suspension and wash in PBS. We used ~100,000 cells for each multi-CUT&Tag bulk experiment.
4) Wash the cells in Wash buffer. Resuspend in 0.5 mL wash buffer and add the activated Concanavalin A coated beads.
5) Incubate cells/beads for 15 minutes at room temperature in an end-over-end rotator.

Step 4: Incubating permeabilized cells with purified antibody-pA-Tn5 complex(es).

1) Place the tube containing Concanavalin A coated beads bound cells on a magnetic stand until the solution clears and carefully remove liquid by pipetting.
2) Resuspend cells in 100 μL cold Dig-wash Buffer containing 2 mM EDTA.
3) Add 2 μg of each differentially barcoded purified antibody-pA-Tn5 complex to the cells.
4) Place on nutator at 4 °C for overnight incubation.

Step 5: Tagmentation

1) Wash the cells with 1 mL of Dig-med Buffer. Repeat the wash step two additional times.
2) Add 300 μL Dig-med Buffer containing 10 mM $MgCl_2$ while gently vortexing.
3) Incubate at 37 ºC for 1 hr.

Step 6: Purification of DNA.

1) To stop tagmentation and solubilize DNA fragments, add 10 μL 0.5M EDTA, 3 μL 10% SDS and 1 μL of 20 mg/mL Proteinase K to each sample.
2) Vortex on full speed and incubate for 1 hr at 50 ºC.
3) Add 300 μL of Phenol-Chloroform-Isoamyl alcohol to the tube and mix by full-speed vortexing. Transfer to a phase-lock tube, and centrifuge for 3 min room temperature at 16000 g.
4) Add 300 μL chloroform to the aqueous phase and centrifuge for 5 min at 16000 g.
5) Remove aqueous phase to a new tube and add 750 μL of 100% ethanol and 0.5 μg/μL glycogen to it. Incubate at -20 ºC for 15 mins and centrifuge for 15 min at 4 °C at 16000 g.
6) Carefully remove the liquid without disturbing the pellet. Wash the pellet in 1 mL 100% ethanol.
7) After air drying the pellet, dissolve it in 23μL of 10 mM Tris-HCl pH8 containing 1/100 RNAse A. Incubate for 10 min at 37 °C.

Step 7: PCR amplification of multi-CUT&Tag library.

1) To 21 μL of purified DNA, add 2 μL each of the barcoded i5 primer (10 μM) and i7 primers, using a different combination for each sample. Add 25 μL of NEBNext HiFi 2x PCR Master mix and mix gently.
2) Place in a thermocycler with the following program.
   Step 1: 72 °C for 5 min
   Step 2: 98 °C for 30 sec
   Step 3: 98 °C for 10 sec
   Step 4: 63 °C for 10 sec
   Repeat Steps 3-4: 17 times
   Step 5: 72°C for 1 min
   Step 6: Hold at 8 °C

Step 8: Post PCR cleanup.

1) Add 55 μL Ampure XP beads (1.1 volumes) and mix by pipetting. Incubate at room temperature 10 min.
2) Place on magnet and withdraw the liquid. Without disturbing the beads, wash the beads twice with 200 μL of 80% ethanol.

3) Perform a quick spin, replace tubes on the magnet, remove all liquid, and air dry the beads for 2-3 min.
4) Add 25 µL 10 mM Tris-HCl pH 8 to the beads and vortex on full. Incubate at room temperature for 5 minutes.
5) Place on magnet and withdraw the liquid to a fresh tube.
6) Determine the size distribution (by Fragment analyzer) and concentration (by Qubit or KAPA Library Quantification Kit (Roche)) of the libraries before sequencing.

Single cell multi-CUT&Tag

Step 3: Incubating permeabilized cells with purified antibody-pA-Tn5 complex(es).

1) ESCs and TSCs were trypsinized and washed in PBS. ~200,000 cells were used as starting material.
2) Wash the cells in Wash buffer. Resuspend cells in 100 µL cold Dig-wash Buffer containing 2 mM EDTA.
3) Add 4 µg of each differentially barcoded purified antibody-pA-Tn5 complex to the cells.
4) Place on nutator at 4 °C for overnight incubation for antibodies to bind to chromatin.

Step 4: Tagmentation

1) Wash the cells with 1 mL of Dig-wash Buffer. Pellet the cells at 600 g for 3 minutes at 4°C and discard the supernatant. Repeat the wash step 3 times.
2) Count the cells again during the last wash, as some cells are lost during the wash steps.
3) Resuspended cells in Dig-med Buffer at 5000cells/µL and incubate on ice for 5 minutes.
4) To start tagmentation, add an equal volume of Dig-med Buffer containing 20 mM MgCl2 and incubate samples at 37 °C for 1 hour.
5) After one hour, 2.5 µL of the tagmentation reaction (2500 cells/µL) are used for a targeted cell recovery of 4000 cells and mixed with 2.5 µL of Diluted Nuclei Buffer, 7 µL of ATAC buffer (both from the Chromium Single Cell ATAC Reagent Kit, 10X Genomics), 3 µL of 50% glycerol and 0.5 µL of 5M NaCl.

Step 5: GEM Generation & Barcoding (according to manufacturer's protocol).

1) Barcoding master mix was prepared by mixing 61.5 µL of barcoding reagent, 1.5 µL of Reducing Agent B and 2 µL of barcoding enzyme.
2) Load the samples and Gel beads to Chromium Chip E following 10X Genomics' User protocol. GEMs were transferred to a PCR tube.
3) Incubate the GEMs in a thermocycler with following program:
   Step 1: 72 °C for 5 min
   Step 2: 98 °C for 30 sec
   Step 3: 98 °C for 10 sec
   Step 4: 59 °C for 10 sec
   Step 5: 72°C for 1 min
   Step 6: Hold at 15 °C
   Note: the use of a single amplification cycle at this step differs from the manufacturer's recommendations for scATAC-seq, 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.

Step 6: Post GEM Incubation Cleanup (according to manufacturer's protocol).

1) Post GEM cleanup was performed first using Dynabeads MyOne SILANE according to 10X Genomics' ATAC seq protocol.
2) Perform another round of cleanup using Ampure XP beads according to 10X Genomics' ATAC seq protocol.

Step 7: Single cell multi-CUT&Tag library construction (according to manufacturer's protocol).

1) Construct libraries using sample indexing primers (Single Index Kit, 10X Genomics), by performing 14 cycles of PCR according to manufacturer's protocol.
2) Perform a double-sided size selection post sample index PCR using Ampure XP beads according to manufacturer's protocol.
3) Determine the size distribution (by Fragment analyzer) and concentration (by Qubit or KAPA Library Quantification Kit (Roche)) of the libraries before sequencing.

Sequencing of multi-CUT&Tag libraries.

1) Paired-end sequencing was performed on an Illumina NextSeq 500
2) Custom read and index primers (Sup. Table 1) were used and the sequencing parameters were as follows: 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 sc multi-CUT&Tag).
3) PhiX DNA was added to 20-30%, due to the sequence homogeneity of the initial sequencing cycles, which read through regions of the adapter that are identical in all reads.