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Single cell CUT&Tag-pro

Bingjie Zhang[1]

[1]New York Genome Center

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Bingjie Zhang

scCUT&Tag-pro is a multimodal assay for profiling histone modification coupled with the abundance of surface proteins in single cells. It was developed based on CUT&Tag (Kaya-Okur et al., 2019) and scASAP-seq (Eleni Mimitou et al., 2021). Our approach is compatible with the widely used 10x Genomics Chromium system, and complements recently introduced technologies for simultaneous CUT&Tag and transcriptomic profiling that leverage custom combinatorial indexing workflows.

Preprint: https://www.biorxiv.org/content/10.1101/2021.09.13.460120v1.abstract

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Bingjie Zhang 2021. Single cell CUT&Tag-pro. **protocols.io** https://dx.doi.org/10.17504/protocols.io.b2b7qarn

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Buffer preparation

1 **Staining buffer**:
2% BSA and 0.01% Tween in PBS

Isotonic permeabilization buffer (2 ml)
40 µl 1M Tris-HCl pH 7.4 (20 mM)

60 µl 5M NaCl (150 mM)
6 µl 1M MgCl2 (3 mM)
20 µl 10% NP-40 (0.1%)
20 µl 10% Tween-2 (0.1%)
40 ul 50x Proteinase inhibitor (Roche Complete Protease Inhibitor EDTA-Free tablet)
1800 µl dH2O

Wash buffer (50 ml)
1 ml 1 M HEPES pH 7.5 (20 mM)
1.5 ml 5 M NaCl (150 mM)
12.5 µL 2 M spermidine (0.5 mM)
Bring the final volume to 50 ml with dH2O, and add 1 Roche Complete Protease Inhibitor EDTA-Free tablet.

Antibody buffer (2ml):
40 µl 1 M HEPES pH 7.5 (20 mM)
60 µl 5 M NaCl (150 mM)
0.5 µL 2 M spermidine (0.5 mM)
8 µL 0.5 M EDTA (2 mM)
200 µl 10% BSA (final 1.0%)
1691.5 µl dH2O

High salt buffer (50 ml)
1 ml 1 M HEPES pH 7.5 (20 mM)
3 ml 5 M NaCl (300 mM)
12.5 µL 2 M spermidine (0.5 mM)
Bring the final volume to 50 ml with dH2O, and add 1 Roche Complete Protease Inhibitor EDTA-Free tablet.

Tagmentation buffer
Add 10 µl 1M MgCl2 to 1 mL high salt buffer.

Human TruStain FcX™ (Fc Receptor Blocking Solution): Biolegend (422301)

Fab Fragment Goat Anti-Mouse IgG: Jackson ImmunoResearch (115-007-003)

Tested antibodies:
H3K4me1(1:100, Abcam, ab8895)
H3K4me2 (1:100, Abcam, ab32356)
H3K4me3 (1:100, Abcam, ab213224)
H3K27ac (1:100, Abcam, ab177178)
H3K27me3 (1:100, Cell Signaling Technology, 9733)
H3K9me3 (1:100, Abcam, ab8898)
Phospho-Rpb1 CTD (Ser2/Ser5) (1:50, Cell Signaling, 13546)
Secondary antibody: guinea pig anti-rabbit (1:100, Novus Biologicals, NBP1-72763).

Cell surface protein antibody panel:

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TotalSeq-A conjugated antibodies and panels were obtained from BioLegend (399907)

- For specific cell type identification, a set of highly optimized antibody panel subsets is listed in our previous paper (Hao et al. 2021, Supplemental Table 2)

pAG-Tn5: EpiCypher (15-1017)

Bridge oligo A:
TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGNNNNNNNNNVTTTTTTTTTTTTTTTTTTTT
TTTTTTTTTT/3InvdT/

P5 primer:
AATGATACGGCGACCACCGAGATCTACAC

Example of an RPIx primer (TruSeq Small RNA handle, for ADT)
CAAGCAGAAGACGGCATACGAGAT**GTATCGCG**GTGACTGGAGTTCCTTGGCACCCGAGAATT
CCA

Example of an D7xx (TruSeq DNA handle, for HTO):
CAAGCAGAAGACGGCATACGAGAT**TCAAGATC**GTGACTGGAGTTCAGACGTGTGC

Guidelines

2 Centrifuge with a swinging bucket rotor is highly recommended for all centrifugation steps.

Surface Protein Antibody staining

3 Thaw the cryopreserved PBMCs into DMEM with 10% FBS.

Spin down at 4°C for 5 min at 400 g.

Remove the supernatant and wash cells with 1 ml PBS with 2% BSA, spin at 4˚C for 5 minutes at 400g.

Remove the supernatant and Resuspend the cells with 1 ml staining buffer.

Count cells and check the cell viability.

4 For each histone mark, 1~2 million cells are recommended for one experiment.

Centrifuge cells for 5 min at 400 g.

Resuspend in 200 µl staining buffer.

Add 10 µl Human TruStain FcX™ for blocking.

Incubate for 15 min on ice.

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For experiments involving cell hashing, go to step 5. Otherwise, go to step 6.

5 This website could be used to plan experiments: https://satijalab.org/costpercell/. It can help determine the number of hashes, the number of cells to load and check the expected doublet rates. (In the following protocol, five hashes are used and aim to have 15,000 cells in one 10x lane)

Distribute the cells evenly into 5 tubes.

Add 2 µl hashing antibody and mix with gentle pipetting.

Incubate for 20 min on ice.

Wash cells three times with 400 µl staining buffer, spin at 4˚C for 5 minutes at 400g.

After washing, resuspend all the cells in the 5 tubes with 200 µl staining buffer.

For the conjugation of cell hashing antibody to oligonucleotides, please see the detailed protocol here: https://citeseq.files.wordpress.com/2019/03/cite-seq_hyper_conjugation_190321.pdf

Hashtag barcoding antibody-oligos
HTO1:
/5AmMC12/GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTAGGACCATCCAABAAAAA
AAAAAAAAAAAAAAAAAAAAAA*A*A
HTO2:
/5AmMC12/GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTACATGTTACCGTBAAAAA
AAAAAAAAAAAAAAAAAAAAAA*A*A
HTO3:
/5AmMC12/GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTAGCTTACTATCCBAAAAA
AAAAAAAAAAAAAAAAAAAAAA*A*A
HTO4:
/5AmMC12/GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTTCGATAATGCGABAAAAA
AAAAAAAAAAAAAAAAAAAAAA*A*A
HTO5:
/5AmMC12/GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTGAGGCTGAGCTABAAAAA
AAAAAAAAAAAAAAAAAAAAAA*A*A

6 Prepare antibody pool (panel or titrated amounts) according to the manufacture.

Add the antibody pool and incubate for 30 min on ice.

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Wash cells three times with 1 ml staining buffer, spin down at 4˚C for 5 minutes at 400g.

Resuspend the cells in 100 µL staining buffer

7 Add 4 µl Fab Fragment Goat Anti-Mouse IgG and mix with gentle pipetting.

Incubate on ice for 15 min.

Wash cells three times with 1 ml staining buffer, spin down at 4˚C for 5 minutes at 400g.

After the final wash, cells are resuspended in 200 µl PBS and ready for fixation.

Fixation and permeabilization

8 Add 1.25 µl 16% formaldehyde (0.1% final concentration) and incubate 5min at room temperature. Invert occasionally.

Quench by adding 12 µl 1.25M glycine.

Centrifuge cells for 5 min at 400 g.

Discard the supernatant and repeat wash twice with 1 ml ice-cold PBS.

9 Resuspend the cell pellet with 200 µl isotonic permeabilization buffer and mix by pipetting.

Incubate on ice for 7 min.

Add 1 ml wash buffer and centrifuge cells for 5 min at 800 g.

Resuspend the cells with 150 µl antibody buffer.

Antibody incubation

10 Add 1.5 µg primary antibody to each tube and mix well.

Place the samples on a rotator and incubate **Overnight** at 4°C.

11 The next day spin down for 5mins at 800 g and remove the supernatant.

Wash cells one time with 150 µl washing buffer, spin down for 5 min at 800g.

Discard the supernatant and resuspend the cells with 150 µl wash buffer.

12 Add 1.5 µg secondary antibody to each tube and mix well. 1h

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Incubate for 01:00:00 at room temperature on a rotator.

Spin down for 5 min at 800 g and remove the supernatant.

Wash cells two times with 150 µl washing buffer, spin down for 5 min at 800g.

pAG-Tn5 incubation

13 For each sample, prepare 150 µl diluted pAG-Tn5 by mixing 7.5 µl pAG-Tn5 with 142.5 µl high salt buffer. 1h

Resuspend the cells with 150 µl diluted pAG-Tn5.

Incubate for 01:00:00 at room temperature on a rotator.

Spin down for 5 min at 800 g and remove the supernatant.

Wash cells two times with 150 µl high salt buffer, spin down for 5 min at 800g.

Discard the supernatant and resuspend the cells with 100 µl Tagmentation buffer.

Tagmentation

14 Place the samples on a PCR machine with 50°C heated cap to incubate for 01:00:00 at 37 °C 1h

Prepare 1× Diluted Nuclei Buffer (10x Genomics scATAC kit).

Add 4 µl of 0.5 M EDTA to stop the tagmentation.

Spin down for 5 min at 1000 g and remove the supernatant.

Loading to 10x

15 Resuspend the cells with 30 µl 1× Diluted Nuclei Buffer

Count cells and adjust the cell concentration with 1× Diluted Nuclei Buffer according to the 10x table.

> We normally have 100K ~ 200K cells at this stage. I prefer to adjust the cell concentration to ~2500 cells/µl.

Prepare the master mix (10x scATAC v1.1):
Cells in 1× Diluted Nuclei Buffer: 8 ul
ATAC buffer B: 7 ul

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Barcoding reagent B: 56.5 ul
Reducing agent B: 1.5 ul
Barcoding Enzyme: 2 ul
1 µM bridge oligo A: 0.5 µl

Please follow the 10x Chromium single-cell ATAC protocol (v1.1) to load the 10x Chromium chip.

For the GEM incubation, please use the following PCR program (step 2.5a):
40 °C for 5 min
72 °C for 5 min
98 °C for 30 s
12 cycles of (98 °C for 10 s, 59 °C for 30 s and 72 °C for 1 min)
ending with hold at 15 °C.

Post GEM Incubation Cleanup

16 Please follow the 10x Chromium single-cell ATAC protocol (v1.1) from step 3.1a to step 3.1n

During silane bead elution (Step 3.1o), if cell hashing is done, please elute beads in 46.5 µl of Elution Solution I. Otherwise elute in 43.5 µL of Elution Solution I. The extra volume is used for the cell hashing and surface protein tags library.

3.1p. Pipette mix without introducing bubbles.
3.1q. Incubate 1 min at room temperature.
3.1r. Centrifuge briefly. Place on the magnet•Low until the solution clears.
3.1s.Transfer 40 µl sample to a new tube strip for CUT&Tag library, transfer 3 µl sample to another tube strip for ADT library, if cell hashing is done, transfer the remaining 3 µl sample to a third tube strip for HTO library.

3.2a. Vortex the SPRIselect reagent until fully resuspended. Add 48 µl SPRIselect reagent to each sample. Pipette mix thoroughly.
3.2b. Incubate 5 min at room temperature.
3.2c. Centrifuge briefly. Place on the magnet•High until the solution clears.

For ADT and HTO: During SPRI cleanup (Step 3.2d), the supernatant is saved and the short DNA derived from antibody oligos is purified with 2.0× SPRI beads:

- Transfer the supernatant to a new tube and add 32 µl SPRIselect reagent to obtain a final SPRI volume of 2X SPRI.
- Incubate 10 minutes at room temperature.
- Place on the magnet•High until the solution clears.
- Carefully remove and discard the supernatant.
- Add 200 µl 80% ethanol to the pellet. Wait 30 sec.
- Remove the ethanol.
- Add 200 µl 80% ethanol to the pellet. Wait 30 sec.
- Remove the ethanol.
- Centrifuge tube briefly and return it to the magnet.

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- Remove and discard any remaining ethanol.
- If cell hashing is done, resuspend in beads in 85 µl water. Otherwise, resuspend in beads in 43 µl water.
- The eluted DNA is combined with the 3 µL left aside in step 3.1s to be used as input for protein tag amplification. (For both ADT and HTO, the final volume is 42 µl + 3 µl = 45 µl)

For CUT&Tag:
Follow the protocol from Step 3.2e onwards

Library Construction

17 **For CUT&Tag:**
Follow the protocol in step 4
The final PCR amplification is done with 14-20 PCR cycles depending on the antibody. Follow the standard protocol for PCR product purification.

Amplify ADT sequencing library:
Prepare 100ul PCR reaction with purified ADT:
- 45 µl purified ADT fraction
- 50 µl 2x KAPA Hifi PCR Master Mix.
- 2.5 µl TruSeq Small RNA RPIx primer (containing i7 index) 10 µM.
- 2.5 µl P5 oligo at 10 µM

PCR program:
95˚C 3 min
95˚C 20 sec |
60˚C 30 sec | ~ 12~16 cycles
72˚C 20 sec |
72˚C 5 min

Amplify Cell Hashing HTO sequencing library:
Prepare 100ul PCR reaction with purified HTO:
- 45 µl purified Hashtag fraction
- 50 µl 2x KAPA Hifi PCR Master Mix.
- 2.5 µl TruSeq DNA D7xx_s primer (containing i7 index) 10 µM.
- 2.5 µl P5 oligo at 10 µM

PCR program:
95˚C 3 min
95˚C 20 sec |
64˚C 30 sec | ~ 12~16 cycles
72˚C 20 sec |
72˚C 5 min

Purify ADT and HTO PCR products using 1.6X SPRI purification.

18 Run 1 µl sample on the Agilent Bioanalyzer High Sensitivity DNA chip to determine fragment

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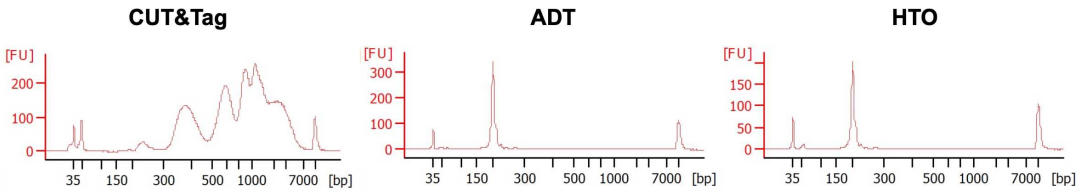
size.

Representative traces:



PBMC scCUT&Tag-pro of H3K27me3

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