Single cell CUT and Tag on 10x genomics platform

Marek Bartosovic[1], Goncalo Castelo-Branco[1]

[1]Karolinska Institute Stockholm

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10

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Marek Bartosovic

scCU&Tag on 10x platform uses scCUT&Tag protocol from Steven Henikoff's lab (Kaya-Okur et al., 2019), and scATAC-seq chromium platform (10x Genomics) to perform single-cell barcoding. The method can be used to obtain high quality data for tens of thousands of single cells with high specificity.

See also accompanying BiorXiv preprint https://www.biorxiv.org/content/10.1101/2020.09.02.279703v1 for more details.

scCUT&Tag can be performed on cell lines or freshly isolated cells from primary tissue.

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protocol

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CUT&Tag, single-cell CUT&Tag, scCUT&Tag, scATAC

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45134

Ultra pure DNAse/RNAse free water (ThermoFisher, 10977015)
1M Hepes (Alfa Aesar, J60712)
5M NaCL (Invitrogen, AM9759)
Spermidine (Sigma, S2626-1G)
Complete EDTA-free protease inhibitors (Sigma, 11873580001)
BSA powder (Sigma, A9418-50G)
0.5M EDTA (Invitrogen, AM9260G)
Digitonin powder (Merck, CAS 11024-24-1)
NP-40 (ThermoFisher, 85124)
1M MgCl2 (Invitrongen, AM9530G)
10% SDS (ThermoFisher, 15553027)
Proteinase K (Invitrogen, AM2546)
2x NEBNext High-Fidelity PCR master mix (NEB, M0541S)
SYBR green (dilute to 10x) (ThermoFisher, S7563)
Secondary antibody guinea pig anti-rabbit (Novus Biologicals, NBP1-72763)
pA-Tn5 pre-loaded with standard Tn5 adapter sequences (as in Kaya-Okur et al., 2020)

Primary antibody of choice

H3K4me3 (Diagenode, C15410030)
H3K27ac (Abcam, Ab177178)
H3K27me3 (Cell Signalling, 9733T)
H3K36me3 (Abcam, Ab9050)
Rad21 (GeneTex, GTX106012)
Olig2 (Novus Biologicals, NBP1-28667)

Oligonucleotide sequences:
Mosaic end-adapter A (Tn5ME-A) TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG
Mosaic end-adapter B (Tn5ME-B) GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG
Mosaic-end reverse oligonucleotides (Tn5MErev) 5'-[phos]CTGTCTCTTATACACATCT-3'

PCR primers (as in Buenrostro et al.) **adapter sequences.xlsx**

pA-Tn5 loading protocol **In house Tn5 assembly updated version.docx**

Buffers prep protocol **buffers_v4.xlsx**

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Tn5 loading 2h

1 Dilute Tn5ME-A, Tn5ME-B and Tn5ME-rev oligos to 100uM

Mix in two separate PCR tubes
1. 10ul Tn5ME-A + 10ul Tn5ME-rev
2. 10ul Tn5ME-B + 10ul Tn5ME-rev

Denature on a thermocycler for 5 min at 95°C, and cool down slowly on the thermocycler by ramping down by 0.1C/s

2 Prepare 2x dialysis buffer:

100mM	HEPES-KOH pH7.2
200 mM	NaCl
0.2 mM	EDTA
2mM	DTT*
0.2%	Triton-X
20%	Glycerol

* DTT is toxic and should only be added to the stock solution immediately prior to use (e.g. 45 ul buffer + 5 ul DTT)
Keep buffer at 4 °C

3 Mix Tn5 with the annealed oligonucleotides:

Tn5ME-A/Tn5ME-rev	2 ul
Tn5ME-B/Tn5ME-rev	2 ul
Glycerol	21.56 ul
2x Dialysis buffer	21.3 ul
Tn5 (3.5 mg/ml)	3.14 ul
total	50 ul

Mix gently with pipette and incubate for 1h at room temperature.
Store enzyme at -80C long term. Keep working stock at -20C.

Test small batch of Tn5 and if the loading is successful, scale up the loading reaction.

Buffers preparation 30m

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4

scCUT&Tag buffers preparation

5% Digitonin	Prepare aliquotes and store at -20
1g	Digitonin powder
20 ml	DMSO

20 % BSA	Filter through 0.45 um filter	always use fresh
2g	BSA	
10ml	water	

2x Wash buffer (25ml)		Store at 4C and use within one week	
Final		Stock	Amount
40 mM	Hepes pH 7.5	1 M	1 ml
300 mM	NaCl	5 M	1.5 ml
1 mM	Spermidine	2 M	12.5 ul
2x	Protease inh.	tablet	1 tablet
	water		22.5 ml

Antibody buffer (2ml/sample)				
2x		1 ml	4ml	Wash buffer
2 mM		8ul	32 ul	EDTA (500 mM)
0.05%		20ul	80 ul	Digitonin (5%)
0.01 %		2ul	8ul	NP-40 (10%)
1%		100ul	400ul	BSA (20%)
		870 ul	3480 ul	Water

Dig -Wash-BSA (2ml/sample)				
2x		1ml	4ml	Wash buffer
0.05%		20 ul	80 ul	5% Digitonin
0.01%		2 ul	8 ul	10% NP-40
1%		100 ul	400 ul	20% BSA
		878 ul	3512 ul	water

Dig300 -Wash-BSA (2ml/sample)				
1x		1ml	4ml	Wash buffer
0.05%		20 ul	80 ul	5% Digitonin
0.01%		2 ul	8 ul	10% NP-40
1%		100 ul	400 ul	20% BSA
300 mM		60 ul	240 ul	5 M NaCl
		818 ul	3272 ul	water

Tagmentation buffer (200 ul/sample)			
1x		750 ul	2x wash buffer
10 mM		15 ul	MgCl2 (1M)
0.05%		15 ul	Digitonin (5%)
0.01 %		1.5 ul	NP-40 (10%)
300 mM		45 ul	NaCl (5M)
		637.5 ul	water

Nuclei preparation and primary antibody incubation 30m

5 Dissociate your tissue/ cell line into a single-cell (single-nuclei) suspension and count your cells using manual counting chamber. We generally use between 150,000-250,000 cells as input from fresh samples and up to 500,000 nuclei extracted from frozen tissue.

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If FACS sorting from a dissociated tissue, sort directly into eppendorf tubes with 500 ul of the antibody buffer cooled to 4 degrees.

6 Centrifuge cells/nuclei for 5 minutes at 300x g. Resuspend in 200 ul of Antibody buffer and centrifuge again 3 minutes at 600x g.

Perform all incubations and centrifugations in 0.5ml standard eppendorf tubes.

Use swinging bucket rotor centrifuge with adapters for 0.5ml tubes. Centrifugation in fixed angle rotor centrifuge leads to higher nuclei loss during centrifugation.

7 Prepare 1:50 dilution of primary antibody in 200 ul of antibody buffer per sample.

Resuspend the pelleted nuclei in 100 ul of the antibody buffer

We have validated following antibodies work for scCUT&Tag:

H3K4me3 (Diagenode, C15410030)
H3K27ac (Abcam, Ab177178)
H3K27me3 (Cell Signalling, 9733T)
H3K36me3 (Abcam, Ab9050)
Rad21 (GeneTex, GTX106012)
Olig2 (Novus Biologicals, NBP1-28667)

8

Incubate the cells with primary antibody overnight at 4 degrees on rotating wheel or roller with slow rotation speed.

Overnight

Secondary antibody incubation 1h 30m

9 Centrifuge the nuclei, 3minutes at 600x g.

Remove supernatant. Wash once with 200ul of Dig-wash buffer.

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10 Centrifuge 3 minutes at 600x g

Remove the supernatant.

11 Prepare 200ul of 1:50 diluted secondary antibody per sample in Dig-Wash-BSA buffer.

Resuspend the nuclei in 200 ul of diluted secondary antibody.

> We use guinea pig anti rabbit secondary antibody for rabbit primary.
>
> Guinea pig anti-rabbit (Novus Biologicals, NBP1-72763)

12 Incubate 1 hour **01:00:00** rotating at room temperature. 1h

pA-Tn5 incubation 1h 30m

13 Centrifuge 3 minutes at 600x g

Remove the supernatant. Resuspend in 200 ul of Dig-300-BSA wash.

14 Repeat the wash-centrifugation 2 more times for total of 3 washes

15 Prepare 200 ul of 1:100 diluted pA-Tn5 in Dig-300-BSA buffer per sample.

16 Resuspend the pellet in 200 ul of diluted pA-Tn5

17 Incubate for 1 hour **01:00:00** rotating at room temperature 1h

Tagmentation 1h 30m

18 Centrifuge 3 minutes at 300x g

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Remove the supernatant. Resuspend in 200 ul of Dig-300-BSA wash.

19 Repeat the wash-centrifugation 2 more times for total of 3 washes

20 Resuspend the nuclei in 200ul of tagmentation buffer. Incubate 1hour **01:00:00** at 37 degres in water bath or thermomixer. 1h

Tap the tubes 3-4 times during the incubation to prevent nuclei sedimentation

The tagmentation buffer does not contain BSA, so excessive clumping of nuclei can occur, depending on the sample. If that is the case, 1% final BSA can be added to the tagmentation buffer.

21 Prepare 1x Diluted nuclei buffer (DNB) supplemented with 2%BSA

Prepare STOP buffer by mixing 200ul of Dig-300 buffer with 10 ul of 500mM EDTA per sample.

The 10x scATAC kit provides 20x diluted nuclei buffer (DNB). We routinely prepare 2x DNB by mixing 900ul of dH_2O + 100ul of 20xDNB and store at -20 degrees. Then on the day of experiment we mix 500ul of 2x DNB + 400ul of dH_2O + 100ul of 20%BSA to prepare 1xDBB+2%BSA.

We have successfully used 1xPBS + 1%BSA instead of 1x DNB+2%BSA. We did not observe any difference in nuclei clumping or data quality.

22 ⚠

Add 200ul of STOP buffer and mix well by pipetting up and down several times.

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Final 0.5 % final BSA, critical, otherwise the nuclei would clump during the centrifugation

23 Centrifuge for 3 minutes at 300x g.

Wash the nuclei with 200 ul of 1xDNB+BSA
Step 23 includes a Step case.

qPCR cycle check of bulk library

qPCR cycle check (optional) 2h

step case

qPCR cycle check of bulk library

At this stage success of tagmentation can be checked by generating bulk library from part of the sample and performing qPCR.

24 While nuclei are in 200ul of DNB, take 10% (20 ul) in a new tube, add 73 ul of water, 5 ul of 10%SDS and 2ul of proteinase K. Mix well by pipetting up and down 5x.

Keep the remaining nuclei on ice for about 2 hours during optional cycle check. This does not influence efficiency of scCUT&Tag.

25 Incubate for 30 minutes **00:30:00** at 50 degrees. 30m

26 Purify the DNA using ZYMO DNA Clean an Concentrator-5 kit. Use 1:5 ratio of binding buffer.

27 Elute the DNA in 25 ul of elution buffer.

28 Mix qPCR reaction:

Fw primer ATAC*	2ul
Rev primer ATAC*	2ul
water	8.5 ul

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10xSYBR	2.5 ul
2x NEBnext MM	25 ul
eluted DNA template	10ul
total	50 ul

*Indexed primers described by Buenrostro, J.D. et al. Single-cell chromatin accessibility reveals principles of regulatory variation. Nature 523:486 (2015).

29 Run qPCR program:

1. 72C 5min
2. 98C 1min
3. 98C 15s
4. 63C 10s (data collection)
5. GOTO 3 39x

30 Typical successful experiment has CT value in < 15 and can be easily distinguished from failed runs where CT > 20.

Counting the nuclei

31 Centrifuge the nuclei for 3 minutes at 300x g.

Resuspend the nuclei in 15-25ul of1xDNB+2%BSA

32 Count the nuclei using manual counting chamber.

Use 2ul of Nuclei suspension + 8ul of trypan blue in 2 replicates

33 Calculate nuclei concentration and refer to the 10x scATAC-seq manual regarding concentration and volume required for chromium chip loading for desired yield.

scCUT&Tag is compatible with

Chromium Single Cell ATAC Reagent Kits (v1)
or
Chromium Next GEM Single Cell ATAC Reagent Kits (v1.1)

10x scATAC-seq protocol 1h 30m

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34 Skip Step 1 in the scATAC-seq manual, start at Step 2 GEM Generation and barcoding

If using scATAC-seq kit **v1.1**, thaw **ATAC buffer B**

35 ⚠

Prepare master mix:

scATAC v1:

Nuclei suspension	15 ul
Barcoding reagent	61.5 ul
Reducing agent B	1.5 ul
Barcoding Enzyme	2 ul

scATAC v1.1:

Nuclei suspension	8 ul
ATAC buffer B	7 ul
Barcoding reagent B	56.5 ul
Reducing agent B	1.5 ul
Barcoding Enzyme	2 ul

scATAC v1.1 requires ATAC buffer B from Step 1 to be added to the master mix.

36 Load the 10x chromium chip according to manufacturers instructions in the 10x scATAC-seq kit in Step 2

37 Continue with the protocol exactly as in manufacturers instructions in the 10x scATAC-seq from Step 3 onwards

38 For final PCR amplification, use standard chromium scATAC-seq kit amplification protocol, with 16-20 PCR cycles depending on the antibody.

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