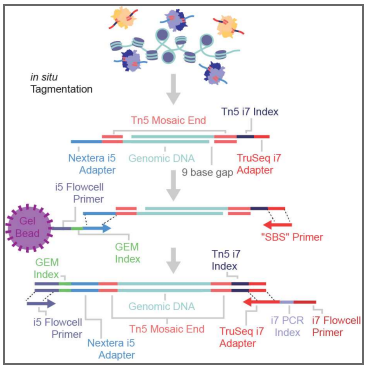

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Protocol status: Working
We use this protocol and it's working

Ten(10)X-compatible Combinatorial Indexing ATAC sequencing (txci-ATAC-seq)

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ABSTRACT

The txci-ATAC-seq method is a large-scale single-cell ATAC-seq technique that combines the Tn5-based pre-indexing with the 10X Chromium-based microfluidic barcoding. This molecular hashing strategy enables the profiling of up to 200,000 nuclei across multiple samples in a single emulsion reaction.

MATERIALS

Loading Tn5

- Annealing Buffer:

Reagent	Final Concentration	Per 10 ml
1M Tris-HCl, pH8.0	40 mM	400 µl
5M NaCl	50 mM	100 µl
H2O		9.5 ml

- Sequences of Tn5 linker oligos (The ‘N’ bases shown in the Tn5ME-B sequence represent the Tn5 barcodes):

Linker Oligo	Sequence 5’ -> 3’
Tn5ME-A	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG
Tn5ME-B	CGTGTGCTCTTCCGATCTNNNNNNNNAGATGTGTATAAGAGACAG
Tn5MErev	[phos]CTGTCTCTTATACACATCT

- Sequences of Tn5ME-B barcode:

Row	1	2	3	4	5	6	7	8	9	10	11	12
A	GAACCGCG	AGGTTATA	TCATCCTT	CTGCTTCC	GGTCACGA	AACTGTAG	GTGAATAT	ACAGGCGC	CATAGAGT	TGCGAGAC	GACGTCTT	AGTACTCC

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Keywords: Single-cell, ATAC-seq, Chromatin accessibility, Combinatorial indexing, Molecular hashing

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Row	1	2	3	4	5	6	7	8	9	10	11	12
B	TGG CCG GT	CAA TTA AC	ATA ATG TG	GCG GCA CA	CTA GCG CT	TCG ATA TC	CGT CTG CG	TAC TCA TA	ACG CAC CT	GTA TGT TC	CGC TAT GT	TAT CGC AC
C	TCT GTT GG	CTC ACC AA	TAT TAG CT	CGC CGA TC	TCT CTA CT	CTC TCG TC	CCA AGT CT	TTG GAC TC	GGC TTA AG	AAT CCG GA	TAA TAC AG	CGG CGT GA
D	ATG TAA GT	GCA CGG AC	GGT ACC TT	AAC GTT CC	GCA GAA TT	ATG AGG CC	ACT AAG AT	GTC GGA GC	CCG CGG TT	TTA TAA CC	GGA CTT GG	AAG TCC AA
E	ATC CAC TG	GCT TGT CA	CAA GCT AG	TGG ATC GA	AGT TCA GG	GAC CTG AA	TGA CGA AT	CAG TAG GC	AGC CTC AT	GAT TCT GC	TCG TAG TG	CTA CGA CA
F	TAA GTG GT	CGG ACA AC	ATA TGG AT	GCG CAA GC	AAG ATA CT	GGA GCG TC	ATG GCA TG	GCA ATG CA	GTT CCA AT	ACC TTG GC	CTT ATC GG	TCC GCT AA
G	GCT CAT TG	ATC TGC CA	CTT GGT AT	TCC AAC GC	CCG TGA AG	TTA CAG GA	GGC ATT CT	AAT GCC TC	TAC CGA GG	CGT TAG AA	CAC GAG CG	TGT AGA TA
H	GAT CTA TC	AGC TCG CT	CGG AAC TG	TAA GGT CA	TTG CCT AG	CCA TTC GA	ACA CTA AG	GTG TCG GA	TTC CTG TT	CCT TCA CC	GCC ACA GG	ATT GTG AA

Isolation of nuclei from cell lines

Buffers to make beforehand

- Omni Resuspension Buffer (RSB; filter and store at 4°C):

Reagent	Final Concentration	Per 50 ml
1M Tris-HCl, pH 7.5	10 mM	500 µl
5M NaCl	10 mM	100 µl
1M MgCl2	3 mM	150 µl
H2O		49.25 ml

- Omni TD Buffer (filter and store at -20°C):

Reagent	Final Concentration	Per 50 ml
1M Tris-HCl, pH 7.5	20 mM	1 ml
1M MgCl2	10 mM	0.5 ml
Dimethyl Formamide	20%	10 ml
Sterile water		38.5 ml

- Freezing buffer stock solution (FB stock; filter and store at -20°C):

Reagent	Final Concentration	Per 50 ml

Reagent	Final Concentration	Per 50 ml
1M Tris-HCl, pH 8.0	50 mM	2.5 ml
1M Mg(OAc)2	5 mM	0.25 ml
50% Glycerol	25%	25 ml
0.5M EDTA	0.1 mM	0.01 ml
Sterile water		22.24 ml

Buffers to make on the day of the experiment

- RSB Lysis Buffer (200 µl per sample):

Reagent	Final Concentration	Per 200 µl
RSB	~1x	194 µl
10% Igepal-CA630	0.1%	2 µl
1% Digitonin	0.01%	2 µl
10% Tween-20	0.1%	2 µl

- RSB Washing Buffer (1 ml per sample):

Reagent	Final Concentration	Per 1 ml
RSB	~1x	990 µl
10% Tween-20	0.1%	10 µl

- Freezing buffer working solution (FBW; 1 ml for every 3 millions of nuclei):

Reagent	Final Concentration	Per 1 ml
FB stock	~1x	975 µl
1M DTT	5 mM	5 µl
Protease Inhibitors (Sigma P8340)	2% (v/v)	20 µl

txci-ATAC-seq protocol

Buffers to make beforehand

- TMG washing buffer (50 ml):

Reagent	Final Concentration	Per 50 ml
0.2M Tris-acetate pH 7.8	10 mM	2.5 ml
1M Magnesium acetate	5 mM	0.25 ml

Reagent	Final Concentration	Per 50 ml
50% Glycerol	10%	10 ml
Sterile water		37.25 ml

- Loading Buffer was made by mixing buffer 1 (5x) and buffer 2 below (5x):

1. Buffer1 (5x):

Reagent	Final Concentration	Per 1000 µl
0.2M Tris-acetate pH 7.6	50 mM	250 µl
1M Magnesium acetate	25 mM	25 µl
Dimethyl Formamide	50%	500 µl
H2O		225 µl

2. Buffer2 (5x):

Reagent	Final Concentration	Per 1000 µl
100% Glycerol	50%	500 µl
5M NaCl	100 mM	20 µl
1M Tris-HCl, pH 7.5	50 mM	50 µl
0.5M EDTA	0.1 mM	0.2 µl
1M DTT	1 mM	1 µl
H2O		428.8 µl

3. Loading Buffer:

Reagent	Final concentration	Per 250 µl
5x Buffer1	1x	50 µl
5x Buffer2	1x	50 µl
H2O		150 µl

- Omni Resuspension Buffer (RSB; filter and store at 4C):

Reagent	Final Concentration	Per 50 ml
1M Tris-HCl, pH 7.5	10 mM	500 µl
5M NaCl	10 mM	100 µl
1M MgCl2	3 mM	150 µl
H2O		49.25 ml

- 10% (100 mg/mL) BSA:

Dissolve 1 g powdered Fraction V or molecular biology grade BSA in 10 mL of

distilled H_2O. Then, store in aliquots at −20°C.

Buffers to make on the day of the experiment

- PBSB (containing 0.04% BSA) (2 ml):

Reagent	Final concentration	Per 2 ml
PBS	~1x	1960 µl
20 mg/ml BSA	0.4 mg/ml	40 µl

- RSB washing buffer (4 ml per sample):

Reagent	Final Concentration	Per 4 ml
RSB	~1x	3920 µl
10% Tween-20	0.1%	40 µl
10% BSA	0.1%	40 µl

- Loading buffer supplemented with SBS oligo (LBS):

Reagent	Final Concentration	Per 150 µl
Loading Buffer	~1x	140 µl
75 µM SBS Oligo	5 µM	10 µl

Note: SBS oligo sequence (5’-3’) is CGTGTGCTCTTCCGATCT

- 300 µM DAPI solution:

Reagent	Final Concentration	Per 200 µl
10.9 mM DAPI	300 µM	5.5 µl
H2O		194.5 µl

Note: Add 1 µl of 300 uM DAPI to each 100 µl nuclei to make a final concentration of 3 µM for staining.

Loading Tn5

1 Resuspend Tn5ME-A, Tn5ME-B, and Tn5MErev in the annealing buffer to a final concentration of 100 µM.

2 Prepare annealed linker A: Mix one volume of Tn5ME-A with one volume of Tn5MErev in a PCR tube.
e.g. 100 µl Tn5ME-A + 100 µl Tn5Merev.

3 Prepare annealed linker B: Mix one volume of each barcoded Tn5ME-B with one volume of Tn5MErev on a 96-well plate.
e.g. 10 µl Tn5ME-B (Index A1) + 10 µl Tn5MErev.
Table1_Barcoded_Tn5MEB.xlsx

4 Mix briefly by pipetting and run the following PCR program in a thermocycler for annealing oligos.

	Temperature	Time
	95 °C	5 min
	Slowly Cool down to 65 °C	-0.1 °C/sec
	65 °C	5 min
	Slowly Cool down to 4 °C	-0.1 °C/sec

The annealed oligos can be kept at -20°C for long-term storage.

5 Add 1 µl of each annealed linker (A and B) to 20 µl of the Tn5 stock (0.3 mg/ml) on a 96-well plate with a unique annealed linker B in each well.

6 Mix briefly by pipetting, and then incubate at 23°C for 30 minutes in a thermomixer at 350 rpm.

7 Store at -20°C.

Isolation of nuclei from cell lines

8 Remove approximately $10x10^6$ cells from culture.

Note

The nuclei isolation protocol was adapted from Corces MR, et al. 2017.

CITATION

Corces MR, Trevino AE, Hamilton EG, Greenside PG, Sinnott-Armstrong NA, Vesuna S, Satpathy AT, Rubin AJ, Montine KS, Wu B, Kathiria A, Cho SW, Mumbach MR, Carter AC, Kasowski M, Orloff LA, Risca VI, Kundaje A, Khavari PA, Montine TJ, Greenleaf WJ, Chang HY (2017). An improved ATAC-seq protocol reduces background and enables interrogation of frozen tissues..
LINK
https://doi.org/10.1038/nmeth.4396

9 Pellet the cells at 500 RCF at 4°C for 5 min in a swinging-bucket centrifuge.

10 Aspirate supernatant.

11 Resuspend pellet in 200 µl RSB Lysis Buffer.

12 Incubate on ice for 3 minutes.

13 Add 1 ml RSB Washing Buffer.

14 Take 10 µl nuclei and dilute it with 40 µl of Omni TD buffer, then count the nuclei on a hemocytometer by adding 50 µl Trypan blue solution to the diluted nuclei (Note: we found that adding the RSB-resuspended nuclei straight to Trypan blue solution will cause inflation of nuclei, and diluting nuclei in Omni TD buffer before exposure to Trypan blue improves the nuclei integrity).

15 Pellet the remaining nuclei in RSB Washing Buffer at 500 RCF for 10 min at 4°C in a fixed-angle centrifuge.

16 Resuspend nuclei pellet in FBW at ~3 million nuclei/ml.

17 Snap-freeze nuclei in liquid nitrogen, and then transfer the cryovials to a liquid nitrogen dewar (or -80°C) for long-term storage.

Isolation of nuclei from lung tissue

18 The following protocol can be used to isolate nuclei from lung tissues.

CITATION

Nikita Joshi, Alexander Misharin. Single-nucleus isolation from frozen human lung tissue for single-nucleus RNA-seq. protocols.io.
LINK
https://protocols.io/view/single-nucleus-isolation-from-frozen-human-lung-ti-zu8f6zw

txci-ATAC-seq: Preparing nuclei

19 Take out flash-frozen nuclei (~3 million in 1 ml) from liquid nitrogen for each sample and thaw in a water bath at 37°C for about 1 min.

20 Add 3 ml RSB washing buffer to an empty 15 ml tube for each sample.

21 Transfer 1 ml nuclei stored in the freezing buffer to the 15 ml tube containing 3 ml RSB washing buffer.

22 Pellet the nuclei at 500 RCF for 10 min at 4°C.

23 Resuspend nuclei with 1 ml RSB washing buffer and then transfer to a 1.5 ml LoBind tube through Flowmi (40 micron).

24 Pellet the nuclei at 500 RCF for 5 min at 4°C in a fixed-angle centrifuge.

25 Resuspend nuclei with 100 µl of 1X PBSB for each sample.

26 Count nuclei with DAPI:

26.1 Add 1 µl of 300 µM DAPI to 100 ul nuclei;

26.2 Incubate on ice for 5 mins;

26.3 Add 10 µl stained nuclei to the countess slide to count nuclei.

txci-ATAC-seq: 96 barcoded Tn5 transposition

27 Prepare TD mix:

	Reagent	Final Concentration	Per Rxn	X120 rxn (in a 2ml tube)
	2X Nextera TD Buffer*	1X	12.5 µl	1500
	1% Digitonin	0.01%	0.25 µl	30
	10% Tween-20	0.1%	0.25 µl	30

*Omni TD buffer can be used to replace the Illumina Nextera TD buffer.

CITATION

Corces MR, Trevino AE, Hamilton EG, Greenside PG, Sinnott-Armstrong NA, Vesuna S, Satpathy AT, Rubin AJ, Montine KS, Wu B, Kathiria A, Cho SW, Mumbach MR, Carter AC, Kasowski M, Orloff LA, Risca VI, Kundaje A, Khavari PA, Montine TJ, Greenleaf WJ, Chang HY (2017). An improved ATAC-seq protocol reduces background and enables interrogation of frozen tissues..

LINK

https://doi.org/10.1038/nmeth.4396

28 Thaw a 96-well plate preloaded with 5 µl of barcoded Tn5 on ice. Mix by brief shaking at 1400 rpm for 30 seconds, spin for a minute at 2000 RCF at 4°C, and carefully unseal the aluminum foil seal.

29 Dilute nuclei to 2857 nuclei/µl in PBSB and then mix 7 µl diluted nuclei with 13 µl TD mix for each well.

30 Add 20 µl nuclei/TD mix mixture to each well of the 96-well plate containing 5 µl of barcoded Tn5 per well (total 25 µl).

31 Seal the plate using Bio-Rad Microseal B film.

32 Mix by shaking at 1000 rpm for one minute.

33 If liquid splashes to the seal, briefly spin at 500 RCF for 10 sec.

34 Incubate at 37°C for 60 min in a thermocycler block with a heated lid (47°C).

35 Thaw TMG washing buffer on ice.

36 Remove the plate from the thermocycler.

37 Briefly centrifuge at 500 RCF for 10 sec at 4°C.

38 Incubate the plate on ice for 5 min.

39 Pool nuclei in a LoBind 12-tube strip and then transfer them to a 15 ml conical tube preloaded with 400 ul of TMG.

40 Add 50 µl/well of TMG to the first row of the plate and pipette them throughout the whole plate to wash out the residual nuclei remaining in the plate.

41 After washing the last row of the plate, the TMG was transferred to the same conical tube that was used to collect the barcoded nuclei.

42 Centrifuge nuclei at 500 RCF for 10 min at 4°C.

43 Remove most of the supernatant.

44 Resuspend nuclei with 500 µl of TMG, then transfer to a 1.5 ml LoBind Tube through Flowmi.

45 Centrifuge at 500 RCF for 5 min at 4°C.

46 Remove most of the supernatant and resuspend the nuclear pellet with 30 µl of LBS.

47 Count nuclei with a hemocytometer.

48 Take the volume of solution containing the desired number of nuclei and dilute it with the LBS to make a total of 15 µl.

49 Use the 15 µl dilution as input into the 10X Chromium droplet generator – follow Step 2, page 24 of the Chromium Single Cell ATAC kit instructions (10x Document CG000209 Rev D) to complete the assay.

txci-ATAC-seq: Modification of 10X Chromium protocol

50 For Step 2.5. GEM Incubation:

50.1 a. Incubate in a thermal cycler with the following protocol (Lid temperature at 105°C).

	Temperature	Time
	72 °C	00:05:00
	98 °C	00:00:30
	98 °C	00:00:10
	59 °C	00:00:30
	72 °C	00:01:00; Go to step 3, repeat 7X (Total 8 cycles)
	15 °C	Hold

50.2 b. Store at 15°C for up to 18 h or at −20°C for up to 1 week, or proceed to the next step.

51 For Step 4.1 Sample Index PCR

51.1 c. Add 2.5 µl of customized i7 TruSeq primer (25 µM) containing an 8 bp custom barcode to each 10X library. Record assignment. Pipette mix and centrifuge briefly.

Table2_TrueSeq_i7_Primer.xlsx

51.2 d. Incubate in a thermal cycler with the following protocol (Lid temperature at 105°C).

	Temperature	Time
	98 °C	00:00:45
	98 °C	00:00:20
	67 °C	00:00:30
	72 °C	00:00:20; Go to step 2, repeat 4X (Total 5 cycles)
	72 °C	00:01:00
	4 °C	Hold

Sequencing

52

	Sequencing Read	Cycles
	Read 1N	51 bp
	i7 index (I1)	8 bp
	i5 index (I2)	16 bp
	Read 2N	78 bp