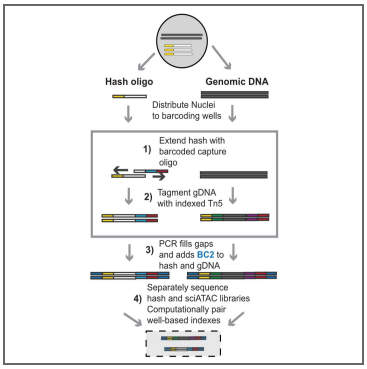

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Protocol status: Working

We use this protocol and it's working

SciPlex-ATAC (2-Level)

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ABSTRACT

Single-cell chromatin accessibility has emerged as a powerful means of understanding the epigenetic landscape of diverse tissues and cell types, but profiling cells from many independent specimens is challenging and costly. Here we describe a novel approach, sciPlex-ATAC-seq, which uses unmodified DNA oligos as sample-specific nuclear labels, enabling the concurrent profiling of chromatin accessibility within single nuclei from virtually unlimited specimens or experimental conditions. In this protocol, we describe the library preparation procedure for our 2-level version of sciPlexATAC-seq, starting from cell culture.

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PROTOCOL integer ID:
87966

Keywords: Chromatin, single cell genomics, gene regulation, ATAC, High throughput screens

Solutions to prepare in advance

1 **Fixation buffer**: (20mL)-Final concentrations
1.5% Formaldehyde
H20
1.25% dPBS

Nuclei buffer - (50mL)-Final concentrations
10mM TrisHCl
10mM NaCl
3mM MgCl2
Note: prepare an extra 5mL with 0.1% Tween20

CLB - (3mL)-Final concentrations
10mM TrisHCl
10mM NaCl
3mM MgCl2
0.1% NP40 --Add Fresh
0.1% Tween20 --Add Fresh

2x Tagment DNA buffer (TD) (2mL)-Final concentrations
20 mM Tris Ph 7.3,
10 mM MgCl2
20% DMF(added immediately before reaction)

4xCLB -- (PMID:25953818) (1mL)-Final concentrations
40mM TrisHCl
40mM NaCl
12mM MgCl2
0.4% NP40 --Add Fresh
0.4% Tween20 --Add Fresh

Stop Tag buffer (3mL)-Final concentrations
40mM EDTA
150uM spermidine

Tips

2

- Never exceed 600xG during spins (even for pulse spins).
- Never allow samples or reagents to sit at room temperature (always keep on ice, preferably within metal blocks).
- Samples should only exceed 4C during enzymatic steps/reactions.
- All mixing should be done with wide bore tips.
- When adding small volumes to nuclei (like primers or hashes) do not pipette up and down. Only release volumes.

Collect, hash, and fix nuclei

3 This protocol is written as for an experiment with cells grown in 96 well format.

- If using cells which grow in suspension, spin at 500xG for 5 minutes.
- Remove media by aspiration or by dumping into a waster container.
- Add 100uL of cold 1xPBS with a multichannel to every well to wash off residual media.
- Remove cold 1xPBS from wells via aspirating or dumping into a waste container.
- Add 50uL of Tryp-LE per well and place plate into a 37C incubator.
- Allow to incubate for 10-20 minutes based on the cell line.
- After cells are properly detached, add 150uL of cell culture medium to quench the reaction.
- Using a new set of tips for every well, transfer the 200uL volume cell suspension into a V-bottom 96 well plate. Make sure that the orientation is preserved between the 96 well culture plate and the 96 well V-bottom plate.
- Spin for 5 minutes at 300xG to pellet cells.
- Remove media by aspiration or by dumping into a waster container.
- Add 100uL of cold 1xPBS with a multichannel to each well to wash off residual media.
- Spin cells down again at 300xG for 5 minutes at 4C in the V-bottom plate to pellet cells.
- **NOTE**: Keep sample on ice and spin at 4C whenever possible from here on.
- Add 50uL of ice-cold CLB solution to each well of the 96 well V-bottom plate using a wide bore tip. Make sure to resuspend and mix using 3-5 strokes. Discard tips when finished.
- **Hashing**: To each well add 1 uL 10uM Hash transferred with preserved orientation from 96 well plate. The amount of hash added should be adjusted based on the number of cells/nuclei in each well. We typically target 0.5 pmol hash per 1000 cells. Adding too little hash can reduce the labeling efficiency.
- Hash oligos have the sequence: 5'-GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGXXXXXXXXXXBAAAAAAAAAAAAAAAAAAAAAAAAAAAAA-3', where 'X's represent the 10nt, well-specific hash ID.
- Stir with pipette tip to mix (do not pipette up and down).
- Incubate on ice for 5 minutes.
- Using a multichannel with wide-bore tips add 108uL of fixation buffer. Mix with 3 strokes to ensure complete permeation of the fixative.
- Incubate plate for 15 minutes on ice.
- Spin down cells for 5 minutes at 500xG at 4C.
- Pool all wells of fixed nuclei into 15ml conical.

- Pellet nuclei for 5 minutes at 500xG at 4C.
- Resuspend in 1mL of Nuclei Buffer with 0.1% Tween 20.
- Allow to rest on ice for 3 minutes.
- Spin down at 500xG for 5 minutes.
- Resuspend in 1mL of nuclei buffer.
- Spin down at 500xG for 5 minutes at 4C.
- Resuspend in 0.5 mL Nuclei Buffer for counting.
- take 5-10uL of resusupended nuclei, mix with equal volume of trypan blue, count cells and bring to final concentration of 2.5x10e6 cells/mL in Nuclei Buffer.

Hash Capture

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- Aliquot 2uL of diluted nuclei into each well (each well gets 5000 cells).
- Add 1uL of 25uM Nextera-OligoT Primers.
- Nextera-OligoT primers have the sequence: 5'-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGNNNNNNNNXXXXXXXXXXTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTVN-3'), 2here, 'X's represent a well specific barcode while 'N's reflect the unique molecular index (UMI).
- Incubate for 5 minutes at 55C.
- Incubate plate on ice for 5 minutes.
- Add 3 uL NEBNext High-Fidelity 2X PCR Master Mix (NEB).
- incubate plate for 10 minutes at 55C.
- Incubate plate on ice for 5 minutes. Current well volume is 6uL.

Indexted tagmentation

5

- prepare mixture 12uL 2xTD buffer per well (freshly added 20% DMF).
- Combine 12uL 2XTD with 6 uL of 4xCLB (per well).
- Add 18uL of mixture (TD+CLB) to each wells of plate and mix well (final well vol = 24 uL, with 1x CLB buffer).
- Add 1 uL indexed Tn5 to each well.

Note: Each well of the 96 well plate receives a unique combination of N5 and N7 indexed Tn5.

Tn5(N5_indices) are loaded with the one of eight oligos with the following sequences: 5'TCGTCGGCAGCGTCTCCACGCXXXXXXXXGCGATCGAGGACGGCAGATGTGTATAAGAGACAG-3', where 'X' indicates the index sequence.

Tn5(N7_indices) are loaded with the one of twelve oligos with the following sequences: 5'-GTCTCGTGGGCTCGGCTGTCCCTGTCCXXXXXXXXCACCGTCTCCGCCTCAGATGTGTATAAGAGACAG-3', where 'X' indicates the index sequence.

The complementary mosaic end (ME) sequence required for custom loading Tn5 is: 5'-/5Phos/CTGTCTCTTATACACATCT -3'

- Seal plate and incubate at 55C for 15 minutes.
- Upon completion place plate on ice (wait 1 minute to cool before adding stop solution).
- Add 25 uL of ice cold 40mM EDTA + 150uM spermidine (Stop Tag solution) to all wells (Do not pipette up and down).
- Pool all wells into FACS tube for sorting using wide bore tips.
- add DAPI to 3uM final (~5 min before sorting).

Fluorescence activated cell sorting (FACS) into PCR plate

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- Pre-load target plates (prepared 5 plates) with 12 uL reverse cross linking buffer (11 uL Elution buffer (qiagen), 0.5 uL 1%SDS, 0.5 uL 20mg/mL Proteinase K).
- Sort desired number of cells/well. Note: This will determine your expected doublet rate and overall recovered cells (e.g. sorting 20 cells per well gives an expected 9% doublet rate).
- Incubate all plates over night (13.5 hrs) at 65C to reverse crosslinks.
- At this point, plates can be stored at -20C until ready for PCR.

PCR amplification

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All wells will be subjected to a single reaction: It will capture both hash and ATAC libraries since all have both p5 and p7 nextera handles.

- Run the following PCR program (always use <25cycles):

72C for 5min
98C for 30sec
22 cycles of:
- 98C for 10sec
- 63C for 30sec
- 72C for 1min

72C for 5 min
hold at 4C

Note: if your libraries require more than 22 cycles, they will likely be of poor quality and contain many duplicate reads.

- Pool all wells from PCR plate into 50 mL falcon tube.
- Purify pooled library using Zymogen clean and concentrate kit. Note: mix pool with 5 volumes of DNA binding buffer before passing through columns. Divide purification across 4 columns (to decrease the time this step takes).
- Elute products from each of the 4 columns in 25uL elution buffer (or water) and combine the elutions (100uL final).
- Measured DNA concentration with qubit dsDNA BR kit.

Gel extraction of final hash and ATAC libraries

8 Gel Extraction: Because the hash and ATAC sequencing libraries require different sequencing chemistry, they must be separately purified and sequenced. To segregate the two sequencing libraries, we run the combined library on a 1% gel, cut out the respective sizes and gel extract.

- Run half of eluted library material on 1% gel (want to load >1000ng total DNA).
- For the ATAC library, extract gel region ranging in size from 300-1200 (bp).
- For the Hash library, extract gel region corresponding to band at 200 bp.
- Perform gel extraction for both libraries following procedure for Zymoclean Gel DNA Recovery Kit (Zymo).
- Elute in 50 uL EB and measured DNA concentration with qubit dsDNA BR kit.

Sequencing scATAC library

9 NextSeq500 Sequencing (300 cycle high output):

- Dilute 2N NaOH to 0.1 NaOH (5 uL 2N to 95 uL nuclease free water)
- In a new 1.5 ml Low-bind tube, transfer 10 uL 0.1 NaOH and add 10 uL 2 nM pooled libraries.
- Incubate at room temperature for 5 minutes
- Add 980 uL HTI to dilute denatured libraries to 20 pM
- Dilute denatured libraries further to 1.5 pM loading concentration (112.5 uL 20 pM + 1387.5 uL HTI).
- Dilute custom primers to 0.6 uM

Custom Sequencing Primers

	A	B
	Read 1 Sequencing Primer (Extended Mosaic End A)	GCGATCGAGGACGGCAGATGTGTATAAGAGACAG
	Read 2 Sequencing Primer (Extended Mosaic End B)	CACCGTCTCCGCCTCAGATGTGTATAAGAGACAG
	Index 1 Sequencing Primer (Reverse Complement of Extended Mosaic End B)	CTGTCTCTTATACACATCTGAGGCGGAGACGGTG
	Index 2 Sequencing Primer	CTGTCTCTTATACACATCTGCCGTCCTCGATCGC

- Dilute primers in HT1 and load into sequencing kit according to the following table.

Reagent Loading positions:

	Kit Position	Primer	Vol. 0.6uM primer	Vol. HT1 (ul)	Load Vol. (ml)
	7	Read 1	9	1491	1.5
	8	Read 2	9	1491	1.5
	9	Index 1 *	9	1491	1.5
	9	Index 2 *	9	1491	1.5

- Load 1.5 mL of 1.5 pM sample library into position 10.

Sequencing Recipe: [Custom] NextSeq CPT 96complex 10barcode High

NOTE: This sequencing recipe includes the following customizations for indexed reads:

index 1: 8 cycles, 27 dark cycles, 10 cycles

Index 2: 10 cycles, 21 dark cycles, 8 cycles
The dark cycles are required to read through constant sequences separating the two indeces on the P5 and P7 ends of each molecule.

Sequencing run setup:

- Read Type: Paired end
- Read Length:
- Read 1: 75 (Note 75 maximizes use of reagents)
- Read 2: 75 (Note 75 maximizes use of reagents)
- Index 1: 18
- index 2: 18
- Custom Primers: (select all)

Sequencing hash library

10 Hash libraries can be sequenced on the NextSeq 500 platform (Illumina) using a 75 cycle kit.

- Dilute hash library to 2 nM based on qubit measurement.
- Dilute 2N NaOH to 0.1 NaOH (5 uL 2N to 95 uL nuclease free water)
- In a new 1.5 ml Low-bind tube, transfer 10 uL 0.1 NaOH and add 10 uL 2 nM hash library.
- Incubate at room temperature for 5 minutes
- Add 980 uL HTI to dilute denatured libraries to 20 pM
- Dilute denatured libraries further to 1.5 pM loading concentration (112.5 uL 20 pM + 1387.5 uL HTI).

Sequencing run setup:
NOTE: Sequencing of hash molecules does not require custom primers, nor a custom recipe
Read 1: 18 cycles,
Read 2: 52 cycles,
Index 1: 10 cycles,
Index 2: 10 cycles