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sci-ATAC-seq HTAN

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ABSTRACT

Single-cell combinatorial indexing ATAC-seq (Sci-ATAC-seq) workflow fro human cells/tissue.

This protocol is the up-to-date Adey Lab protocol that incorporates the use of Pitstop2

EXTERNAL LINK

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MATERIALS

NAME	CATALOG #	VENDOR
Magnesium Chloride	AC223210010	Fisher Scientific
IGEPAL-CA630	I3021 SIGMA-ALDRICH	Sigma Aldrich
Triton X-100	T8787-50ML	Sigma Aldrich
Tween-20	P-7949	Sigma-aldrich
Sodium Chloride	S271-3	Fisher Scientific
Agencourt Ampure XP	A63880	Beckman Coulter
4,6-Diamidino-2-Phenylindole, Dihydrochloride (DAPI)	D1306	Thermo Fisher Scientific
Pierce Preotease Inhibitor Tablets, EDTA-Free	A32955	Thermo Fisher Scientific
Tris-HCl	AM9855	Life Technologies
1X PBS, cell culture grade		Thermo Fisher Scientific
Potassium Chloride	P9541	Sigma Aldrich
EDTA	AM9261	Invitrogen - Thermo Fisher
Qiagen Protease	NC9221823	Fisher Scientific
Pitstop 2	SML1169-5MG	Sigma Aldrich
Nextera DNA Flex Library Prep	20018705	Illumina, Inc.
QIAquick PCR Purification Kit	28106	Qiagen
Uniquely Indexed Transposomes	View	
Sci- Barcoded PCR Primers	View	

MATERIALS TEXT

Tween-20: working stock is 10% (100X). Aliquots are stored at 4C.
IGEPAL-630: Prepare 10% (v/v) stock made with diH20, store at Room Temperature (RT).
DAPI: Resuspend to 5 mg/mL in diH20. Aliquot and store at -20C.
Pitstop2: Resuspend in 3mM in DMSO. Aliquot and store at -20C.

Supplies List:

- 96-well PCR plates (Eppendorf, 951020427)
- 35 um cell strainer (VWR, 21008-948)
- High Sensitivity DNA Chip (Agilent, 5067-4627)

Instrument List:

- Table top centrifuge cooled to 4C with rotors for spinning 1) 96-well plates, and 2) 15 mL falcon tubes at 600 rcf
- Fluorescence Activated Cell Sorter (FACS)
- Thermomixer (55C incubations at 300 rpm)
- Real-Time PCR instrument (Bio-Rad CFX Connect)
- DNA fluorometer or spectrophotometer (Qubit Fluorometer is used in this protocol)
- Agilent Bioanalyzer
- Sequencing: NextSeq 500 using custom chemistry protocol

Prepare Buffers and Pre-chill

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Final Concentration	For Construction
10 mM Tris, pH 7.5	500 uL of 1M Tris, pH7.5
10 mM NaCl	100 uL of 5M NaCl
3mM MgCl2	150 uL of 1M MgCl2
0.1 % Igepal	500 uL of 10% Igepal
0.1 % Tween	500 uL of 10% Tween
ddH20	to 50 mL
Protease Inhibitor	2 tablets

Table 1. Nuclear Isolation Buffer (NIB) formulation.

Note: NIB keeps for ~1 month at 4C. For most preparations, 15 mL is sufficient.

Nuclear Isolation

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Note
Isolation of nuclei is dependent on the sample being used. Tissue should follow a dounce homogenization protocol, while liquid cell cutures can be pelleted and resuspended directly in NIB.

Generalized nuclear isolation steps:

1. Pellet **10 ml** liquid cultured cells in **4 °C** centrifuge at 500 rcf for **00:10:00 min**
2. Aspirate pellet and resuspend in **2 ml NIB**
3. Incubate on ice for **00:10:00 min**
3. Pellet liquid cultured cells in **4 °C** centrifuge at 500 rcf for **00:05:00 min**
4. Aspirate pellet and resuspend **500 µl NIB**
5. Run total volume through 35 um filter
6. Add DAPI to final concentration of 15 ug/mL (ie **3 µl 5 mg/mL DAPI** for every 1 mL of sample).

Perform 1st sort

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1. Per well use **5 µl NIB** and **5 µl TD buffer (2X)** from Illumina
2. Sort 2,000-5,0000 nuclei into each well using fuoresence activated cell sorting (FACS)

Nuclei count may vary by prep, but ensure that numbers *within* a prep are constant, ie all wells receive the same number of cells. Keep in ice or in 96-well plate chiller the whole time.

Tagmentation following 1st Sort

4 Spin down 8-well strips in table top centrifuge immediately after sorting finishes.
Store covered and on ice until the full plate is complete.
Note: to cover strips as they finish sorting, I tend to tear up aluminum plate covers into single-strip width.
Addition of Pitstop2 in 2X TD Buffer
Make sure to test the volume in a well following sort. Sorting a high count of nuclei (2-5K/well increases to volume by 5-10uL, respectively). If unadjusted, this will lower your TD buffer concentration and decrease Tn5 activity.
In 2X TD Buffer, add Pitstop2 reagent before addition into wells.
Example: Pitstop2 addition for a 5K nuclei sort (adding 10uL to each well which contains 10uL [NIB+2X TD Buffer] and 10uL of sorted nuclei; 30uL Final Volume).
Determining concentration of Pitstop2 for TD buffer addition:
a. C1V1=C2V2
b. (30uL Total Volume per well)(70uM Final Pitstop Conc.)=(10uL 2X TD Buffer)(X Pitstop2 Concentration)
♣ X=210uM
a. Determining volume of Pitstop2 to add to 2X TD Buffer mastermix:
♣ C1V1=C2V2
♣ (210uM working concentration)(100uL TD mastermix) = (3000uM Pitstop2 stock)(X Pitstop2 volume)
i. X= 7uL Pitstop2 stock for each 93uL 2X TD buffer

• Add 1uL 8uM uniquely indexed transposome to each well
• Seal plate and incubate at 55C for 15 minutes with gentle shaking (on eppendorf thermomixer, ~300 rpm).
• Plate plate on ice immediately to stop reaction. Keep samples on ice to prevent over-transposistion and nuclei lysis.
• Pool all wells, while maintaining everything on ice.
• Add 2uL/per mL pooled sample of DAPI (5mg/mL) and bring to sorter for second sort.

2nd Sort Plate Setup: Preparing Xie Buffer

5 Preparing Second Plate Xie Buffer (8.5uL/well):
Volume
Reagent:
6 mL

0.05M Tris-HCl pH 7.8
40 uL
0.5 EDTA
200 uL
1M KCl
200 uL
10% Triton X-100
300 uL
Qiagen Protease
3.26 mL
H2O
10 mL
Total

• Add 2.5 uL of 10 uM i5 Indexed PCR Primer and 2.5 uL of 10 uM i7 Indexed PCR Primer prior to sort.

2nd Sort Protocol

6 • Thaw RT-PCR reagents on ice before second sort
• Sort X nuclei per well (X is dependent on number of wells tagmented in first sort, as a linear trend)
• 1 plate = 22 nuclei/well
• 1.5 plates = 33 nuclei/well
• 2 plates = 44 nuclei/well etc...
• Using same gates as first sort:
• Sort X nuclei per well with modified sort settings:
o "Single cell" rather than "Normal"
o This leads to a higher abort count (less efficient sorting) but is far more precise

• As each 8-well strip (containing the Xie buffer) is completed, replace with a new prepared strip.
• Spin down the completed 8-well stip in table-top centrifuge beside the machine.
• Cover the strips and store on ice until all sorting is completed.
• Note: to cover strips as they finish sorting, I tend to tear up aluminum plate covers into single-strip width.
• Keep sorted samples on ice to prevent transposases cross-reacting with other nuclei.

Transposase Denaturation and PCR setup

7 • Spin down 8-well strip tubes
• Cover plate and hold on ice until sorting is complete.
• Digest remaining Tn5 and then denature the Qiagen serine protease on Eppendorf Thermocyclers:
• **55 °C** **00:20:00**

70 °C **00:30:00**

7.1 Add 13.5 ul total of PCR Master Mix to each well:

5 µl 5X KAPA GC buffer

0.75 µl 10 mM dntps

0.5 µl KAPA non-hotstart HiFi

0.25 µl 100X SYBR Green I

8 Perform PCR on the Biorad CFX Connect:

72 °C **00:05:00**

98 °C **00:00:30**

63 °C 00:00:30

72 °C 00:01:00

Plate Read

72 °C 00:00:20

Pull once majority of wells begin to plateaus, for sciATAC libraries amplify between 14-19 cycles

00:00:00

Library Clean-up and Quantification

9 Pool 10uL for each well.
Ran full pool volume through Qiaquick PCR column following manufacturer's protocol.
Eluted in 32 uL 10mM Tris HCl pH 8
Quantified 2 uL in 2:200 dsDNA HS Qubit assay
• This is to ensure that the library amount will be visible on a gel.
Use Qubit reading to dilute to ~4ng/uL and run 1uL on HS Bioanalyzer chip
o Run 1uL of sample at 4ng/uL library dilution on Bioanalyzer High-Sensitivity DNA chip (following manufacturer's protocol)
o Quantify library from the range of 100-1000bp
o Dilute this down to 1nM concentration for sequencing.