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Protocol status: In development
 We are still developing and optimizing this protocol

Created: Jun 11, 2019

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

MATERIALS

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

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Epigenomics, Single Cell,
Biotechnology

Tween-20: working stock is 10% (100X). Aliquots are stored at 4C.

IGEPAL-630: Prepare 10% (v/v) stock made with diH2O, store at Room Temperature (RT).

DAPI: Resuspend to 5 mg/mL in diH2O. 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

BEFORE START INSTRUCTIONS

Uniquely indexed transposomes (8 uM) should be prepared and loaded prior to sci-ATACseq protocol start. Refer to "sci Transposase Loading" protocol.

Sci- barcoded PCR primers should be prepared prior to sci-ATACseq protocol start. Refer to "sci Barcoded PCR Primer Preparation" 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 MgCl ₂	150 uL of 1M MgCl ₂
0.1 % Igepal	500 uL of 10% Igepal
0.1 % Tween	500 uL of 10% Tween
ddH ₂ O	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

2

Note

Note

Isolation of nuclei is dependent on the sample being used. Tissue should follow a dounce homogenization protocol, while liquid cell cultures 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

3

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

Note

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. $C_1V_1 = C_2V_2$

b. $(30\text{uL Total Volume per well})(70\text{uM Final Pitstop Conc.}) = (10\text{uL 2X TD Buffer})(X \text{ Pitstop2 Concentration})$

♣ $X = 210\text{uM}$

a. Determining volume of Pitstop2 to add to 2X TD Buffer mastermix:

♣ $C_1V_1 = C_2V_2$

♣ $(210\text{uM working concentration})(100\text{uL TD mastermix}) = (3000\text{uM Pitstop2 stock})(X \text{ Pitstop2 volume})$

i. $X = 7\text{uL Pitstop2 stock for each } 93\text{uL 2X TD buffer}$

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

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.