SOP: ATAC-seq for cryopreserved nuclei preparations from fresh tissue
Date Modified: 02/11/2020
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The following protocol describes the isolation of nuclei from freshly harvested tissues, and subsequent preparation of ATAC-seq libraries for analysis of genome-wide open chromatin.

Chemicals Ordering Information

Item	Catalog Number	Manufacturer
Dimethyl Sulfoxide (DMSO Hybri-Max 5 x 10 mL)	D2650	Sigma-Aldrich
D-Sucrose	BP220-1	Fisher Scientific
$MgCl_2$ 1M (100 mL)	AM9530G	Ambion
Tris-HCl 1M pH 7.5 (1 L)	46-030-CM	Mediatech, Inc.
Complete EDTA Free Protease inhibitor Tablets, mini	04-693-132-001	Roche Applied Science
Phosphate buffered saline	10010023	ThermoFisher
Tris-HCl	10812846001	Sigma-Aldrich
NaCl	S9888	Sigma-Aldrich
IGEPAL CA-630	I8896	Sigma-Aldrich
DPBS, no calcium, no magnesium	14190250	ThermoFisher
Nextera DNA Sample Prep Kit	FC-121-1030	Illumina
SsoFast™ EvaGreen® Supermix	1725200	Bio-Rad
Qiagen MinElute PCR Purification Kit	28004	Qiagen
PCR Primers (custom sequences; see Table 1 below)		IDT
Milli-Q or Molecular Biology Grade Sterile Water		

Table 1. Oligo designs. A list of ATAC-seq oligos used for PCR. Primers were ordered from IDT. Stock solutions were prepared according to instructions from IDT (100 µM) and were stored at -20°C. Working solutions (25 µM) were also stored at -20°C.

Primer	Sequence (5' to 3')
1	AATGATACGGCGACCACCGAGATCTACACTCGTCGGCAGCGTCAGATGTG
2A	CAAGCAGAAGACGGCATACGAGATCTAGTACGGTCTCGTGGGCTCGGAGATGT
2B	CAAGCAGAAGACGGCATACGAGATTTCTGCCTGTCTCGTGGGCTCGGAGATGT
2C	CAAGCAGAAGACGGCATACGAGATGCTCAGGAGTCTCGTGGGCTCGGAGATGT
2D	CAAGCAGAAGACGGCATACGAGATAGGAGTCCGTCTCGTGGGCTCGGAGATGT
2E	CAAGCAGAAGACGGCATACGAGATCATGCCTAGTCTCGTGGGCTCGGAGATGT
2F	CAAGCAGAAGACGGCATACGAGATGTAGAGAGGTCTCGTGGGCTCGGAGATGT
2G	CAAGCAGAAGACGGCATACGAGATCCTCTCTGGTCTCGTGGGCTCGGAGATGT
2H	CAAGCAGAAGACGGCATACGAGATAGCGTAGCGTCTCGTGGGCTCGGAGATGT
2I	CAAGCAGAAGACGGCATACGAGATCAGCCTCGGTCTCGTGGGCTCGGAGATGT
2J	CAAGCAGAAGACGGCATACGAGATTGCCTCTTGTCTCGTGGGCTCGGAGATGT
2K	CAAGCAGAAGACGGCATACGAGATTCCTCTACGTCTCGTGGGCTCGGAGATGT
2L	CAAGCAGAAGACGGCATACGAGATATCACGACGTCTCGTGGGCTCGGAGATGT
2M	CAAGCAGAAGACGGCATACGAGATACAGTGGTGTCTCGTGGGCTCGGAGATGT
2N	CAAGCAGAAGACGGCATACGAGATCAGATCCAGTCTCGTGGGCTCGGAGATGT
2O	CAAGCAGAAGACGGCATACGAGATACAAACGGGTCTCGTGGGCTCGGAGATGT
2P	CAAGCAGAAGACGGCATACGAGATACCCAGCAGTCTCGTGGGCTCGGAGATGT
2Q	CAAGCAGAAGACGGCATACGAGATAACCCCTCGTCTCGTGGGCTCGGAGATGT
2R	CAAGCAGAAGACGGCATACGAGATCCCAACCTGTCTCGTGGGCTCGGAGATGT
2S	CAAGCAGAAGACGGCATACGAGATCACCACACGTCTCGTGGGCTCGGAGATGT
2T	CAAGCAGAAGACGGCATACGAGATGAAACCCAGTCTCGTGGGCTCGGAGATGT
2U	CAAGCAGAAGACGGCATACGAGATTGTGACCAGTCTCGTGGGCTCGGAGATGT
2V	CAAGCAGAAGACGGCATACGAGATAGGGTCAAGTCTCGTGGGCTCGGAGATGT
2W	CAAGCAGAAGACGGCATACGAGATAGGAGTGGGTCTCGTGGGCTCGGAGATGT
2X	CAAGCAGAAGACGGCATACGAGATTCGCCTTAGTCTCGTGGGCTCGGAGATGT

Materials List

Dounce 7mL Tissue Grinder with PYREX Pestles, Corning (VWR Cat# 22877-280)
GentleMACS dissociator from Mitenyi Biotec.
GentleMACS C-tubes (Mitenyi Biotec Cat# 130-093-237)
100 µm Steriflip 50mL Disposable Vacuum Filter System (Millipore Cat# SCNY00100)
Nalgene Cryo 1°C Freezing Container (Cat# 5100-0001)
Liquid Nitrogen Storage
Graduated pipets (5, 10, 25, 50 mL)
Micropipettes and tips (P2, P20, P200, P1000)
500 mL Corning 0.2 µM Filter System (Cat# 430758)
1 L Corning 0.2 µM Filter System (Cat# 430186)
0.2-ml PCR tubes
1.5-ml Eppendorf tubes
15 mL Corning Polypropylene Conical Centrifuge Tubes (Cat# 430766)
50 mL Corning Polypropylene Conical Centrifuge Tubes (Cat# 430828)
20 µM Steriflip 50mL Disposable Vacuum Filter System (Millipore Cat# SCNY00020)
CryoTube Vials, 1.8 mL (Nunc Cat# 368632)
Microscope (preferably with phase contrast)
Hemacytometer (Hausser Scientific Cat# 1492)
Refrigerated centrifuge (swing-out rotor) compatible with 15 mL Conical and 1.5-ml Eppendorf tubes
Thermal Mixer (ThermoFisher Cat# 13687711)
PCR thermal cycler
2100 Bioanalyzer Instrument from Agilent
Bioanalyzer High Sensitivity DNA Kit (Agilent Cat# 5067-4626)
PippinHT size-selection system from Sage Science
PippinHT 3% Agarose DNA size-selection cassette, 100-250bp (Sage Science Cat# HTG3010)

Stock Reagents

Unless otherwise noted, all buffers and stock solutions should be pre-chilled to 4°C (on ice) prior to use.

Sucrose buffer

Final concentration	*Stock concentration*	*Amount used from stock*
250mM D-Sucrose	0.5M D-Sucrose	250 mL
10mM Tris-HCl, pH 7.5	1M Tris-HCl, pH 7.5	5 mL
1mM $MgCl_2$	1M $MgCl_2$	0.5 mL

Molecular Biology Grade sterile H_2O to 500 mL
Filter sterilize with 500 mL 0.2 µM Filter System. Store at 4°C. Add Complete Protease Inhibitor Tablets (1 per 50mL solution) just prior to use.

ATAC-seq cell lysis buffer

Final concentration	*Stock concentration*	*Amount used from stock*
10mM Tris-HCl, pH 7.4	1M Tris-HCl, pH 7.4	50 µL
10mM NaCl	1M NaCl	50 µL
3mM $MgCl_2$	1M $MgCl_2$	15 µL
0.1% (v/v) IGEPAL CA-630		5 µL

Molecular Biology Grade sterile H_2O to 5 mL
Good for up to 1 week; recommend making fresh each time if possible.

Prior to nuclei isolation:

1. Add protease inhibitor tablets to sucrose Buffer (1 tablet per 50 mL solution) and solubilize. Keep on ice.
2. Pre-label and open all sterile filters and GentleMACS C-tubes.
3. Work quickly using reagents maintained at appropriate temperatures.

Cryopreservation of nuclear prep samples using gentleMACS dissociator

1. Collect ~3mm square piece or roughly 1 gram of fresh tissue
   Note: Less material could be acceptable for ATAC-seq, which should work with a minimum of about 500 cells.
2. Transfer minced tissue into a gentleMACS C-tube with 10 ml of ice-cold sucrose buffer.
3. Mince tissue with scissors in in the gentleMACS C-tube. Pieces should be cut up to about 1/10 of the size of the square.
4. Homogenize tissue using gentleMACS Dissociator Program "E.01c Tube" *twice*.
5. Filter homogenate using 100 µm Steriflip Vacuum Filter System.
   Note: Pour slowly and check for large pieces as they can clog the filter. Gently scraping the filter surface can help dislodge clogs.
6. Bring volume to 9.9 ml with Sucrose buffer.
7. Add 1.1 mL DMSO to samples (10% final concentration), pipetting several times to adequately mix.
8. Aliquot into 5 cryotube vials, with approximately 1.5 mL in each vial.
9. Freeze at -80°C overnight in Nalgene Cryo 1°C Freezing Container, then move to -80°C freezer or -135°C liquid nitrogen for long-term storage.

Cryopreservation of nuclear prep samples using Dounce homogenizer

1. Ideally, keep equal weight between tissues (~3mm square tissue piece or roughly 1 gram)
2. Mince tissue with razor blade or scissors in polystyrene weighing dish.
3. Homogenize tissue with 3 mL Sucrose Buffer per gram tissue in Dounce tissue grinder.
4. Dounce approximately 5 times, slowly and smoothly, with loose (A) pestle.
5. Filter homogenate using 100 µm Steriflip Vacuum Filter system.
6. Bring up to 2.7 mL with Sucrose Buffer.
7. Add 0.3 mL DMSO to samples (10% final concentration), pipetting several times to adequately mix.
8. Aliquot into cryotube vials, freeze at -80°C overnight in Nalgene Cryo 1°C Freezing Container, then move to -80°C freezer or -135°C liquid nitrogen for long-term storage.

Prior to ATAC-seq:

1. Chill centrifuge *with swing-out rotor* to 4°C.
2. Prepare and chill ATAC-seq cell lysis buffer to 4°C. Ideally, cell lysis buffer should be prepared fresh.
3. Thaw cryopreserved nuclei preparations on ice.

ATAC-seq library generation from cryopreserved nuclei preparations

1. After thawing on ice, transfer nuclei preparations to 15 mL conical centrifuge tubes and centrifuge for 5 min, 500 RCF, 4°C.
2. Aspirate supernatant, gently resuspend pellet in 1 mL ice-cold DPBS, and centrifuge for 5 min, 500 RCF, 4°C.
3. Aspirate supernatant, gently resuspend pellet in 1 mL ice-cold ATAC-seq cell lysis buffer, and centrifuge for 10 min, 500 RCF, 4°C.
4. Aspirate supernatant and gently resuspend pellet in 1 mL cold DPBS.
   Note: Depending on the abundance of cells in the preparation, resuspending in smaller or greater volumes of DPBS may be necessary to accurately count nuclei.
5. Determine nuclei concentration using a hemocytometer.
   Note: For some samples, cells will be more commonly observed than nuclei. This may indicate incomplete cell lysis, which can be checked by staining with propidium iodide and Hoechst's.
6. Aliquot between 50,000 and 500,000 cells to 1.5 mL Eppendorf tubes and centrifuge for 5 min, 500 RCF, 4°C.
7. Carefully aspirate supernatant and gently resuspend pellet in ice-cold 50 µL transposition mix (25 µL TD buffer from Nextera DNA Sample Prep Kit, 2.5 µL TDE1 enzyme from Nextera kit, 22.5 µL ddH_2O).
8. Incubate nuclear pellet with transposition mix for 60 min, 37°C, 300 rpm.
9. Purify transposed DNA with MinElute PCR purification kit, and elute DNA in 10 µL Buffer EB.

   Optional stopping point: Store transposed DNA at -20°C.

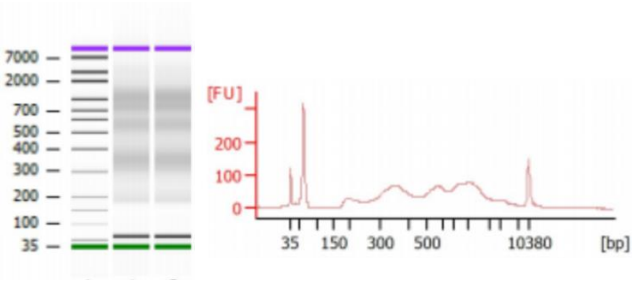
10. Add 40 µL PCR master mix (25.4 µL SsoFast™ EvaGreen® Supermix, 13 µL ddH_2O, 0.8 µL 25 µM Primer 1, 0.8 µL 25 µM Primer 2) to 10 µL eluted DNA and cycle as follows:

1x	5 min	72°C
	30 sec	98 °C
10 - 13x	10 sec	98 °C
	30 sec	63 °C
	1 min	72 °C

    Note: Fewer PCR cycles is desirable to minimize PCR duplication. Furthermore, if sufficient DNA concentration is obtained from fewer PCR cycles, it is a good indicator of library complexity. However, more cycles may be necessary to obtain sufficient DNA concentration in some samples.

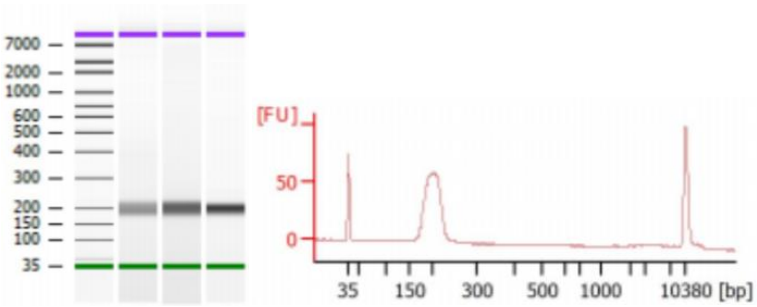
11. Purify libraries using MinElute PCR purification kit, and elute DNA in 10 µL Buffer EB.
12. Quantify and characterize libraries using Agilent Bioanalyzer High Sensitivity DNA chip.

<u>Note</u>: Libraries with concentrations less than 6 nM (150-250 bp range) should be considered for additional cycles of PCR. Keep in mind, this will increase PCR duplication rate after sequencing. If possible, repeating library construction may be preferable.



Example Bioanalyzer trace prior to size selection.

13. Size-select libraries for subnucleosomal fragments (150-250 bp) on the PippinHT system using a 3% Agarose DNA size-selection cassette.
14. Quantify library concentration and verify size-selection on Agilent Bioanalyzer High Sensitivity DNA chip.



Example Bioanalyzer traces after size selection.

15. Pool libraries with compatible primer indexes and submit for sequencing using a platform such as NextSeq 500 to generate paired-end reads. Ideally, 40 million reads should be obtained per each library in the pool (not including low-quality, mitochondrial, or duplicate alignments).