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ATAC-seq from nuclei from frozen tissue

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methods

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

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Abstract

Modified ATAC-seq method for frozen tissue, in this case brain tissue.

Guidelines

The Tn5 enzyme used in this experiment was from a locally produced batch from the Protein Science Facility at the Karolinska Institutet in Stockholm, which is first discussed in Picelli and others (2014). Thus, if the Tn5 is purchased through a company it may react differently.

Materials

- NEBNext High-Fidelity 2X PCR Master Mix - 250 rxns **New England Biolabs** **Catalog #**M0541L
- Digitonin, 40ul **Promega** **Catalog #**G9441
- Tn5 transposase with Nextera adapters loaded **Catalog #**UC-Macro-Tn5-Nextera adapter
- Zymo DNA Clean & Concentrator - 5 **Zymo Research** **Catalog #**D4014
- MACS SmartStrainers 30um **Miltenyi Biotec** **Catalog #**130-098-458
- Halt™ Protease Inhibitor Cocktail, EDTA-Free (100X) **Thermo Fisher** **Catalog #**78437
- SYBR™ Green I Nucleic Acid Gel Stain - 10,000X concentrate in DMSO **Thermo Fisher** **Catalog #**S7563
- Protease Inhibitor Tablets cOmplete Mini EDTA free **Roche** **Catalog #**11836170001

- KIMBLE 2mL Glass Dounce Tissue Grinder Set **Merck MilliporeSigma (Sigma-Aldrich)** **Catalog #**D8938
- 2 ml LoBind Tubes **Eppendorf** **Catalog #**0030108078
- 1.5 mL LoBind tubes **Eppendorf** **Catalog #**022431021
- Falcon® 100 mm TC-treated Cell Culture Dish **Corning** **Catalog #**353003

Equipment

DNA LoBind Tubes	NAME
Microcentrifuge tubes	TYPE
Eppendorf	BRAND
0030108051	SKU
https://online-shop.eppendorf.com/OC-en/Laboratory-Consumables-44512/Tubes-44515/DNA-LoBind-Tubes-PF-56252.html	LINK

- Qubit 2.0 Fluorometer
- Qubit® dsDNA HS Assay Kit **Thermo Fisher Scientific** **Catalog #**Q32854

Equipment

Countess 3 FL Automated Cell Counter	NAME
Automated Cell Counter	TYPE
Thermofisher scientific	BRAND
AMQAF2000	SKU
https://www.thermofisher.com/th/en/home/life-science/cell-analysis/cell-analysis-instruments/automated-cell-counters/models/countess-3-fl.html	LINK

Equipment

Thermomixer C	NAME
Eppendorf	BRAND
2231000667	SKU
https://www.pipette.com/2231000667-Promotion-Eppendorf-ThermoMixer-C-with-24×1-5-mL-SmartBlock-and-ThermoTop	LINK

Equipment

Dounce, 2ml	NAME
Homogenizer	TYPE
KIMBLE	BRAND
432-0250	SKU
https://se.vwr.com/store/product/561196/homogenisator-dounce-kimble	LINK
2 ml	SPECIFICATIONS

Equipment

Bio RS-24 Mini-rotator	NAME
mini-rotator	TYPE
BioSan	BRAND
RS-24	SKU
https://biosan.lv/products/-bio-rs-24-mini-rotator-for-test-tubes-with-timer/	LINK

Equipment

4200 TapeStation System	NAME
Electrophoresis tool for DNA and RNA sample quality control.	TYPE
TapeStation Instruments	BRAND
G2991AA	SKU
https://www.agilent.com/en/product/automated-electrophoresis/tapestation-systems/tapestation-instruments/4200-tapestation-system-228263	LINK

- 1 M Calcium Chloride (CaCl2) **Fisher Scientific Catalog #**BP510
- Mg(Ac)2
- Tris-HCl pH 7.5
- Sodium Chloride **Fisher Scientific Catalog #**S271
- Magnesium Chloride **Fisher Scientific Catalog #**AC223210010
- Molecular grade water nuclease-free
- NN-Dimethylformamide (DMF) solution **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**D4551
- Triton X-100 **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**T8787-50ML
- DTT **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**D0632
- Glycerol, 1000ml **Promega Catalog #**H5433
- EDTA (0.5 M), pH 8.0 **Life Technologies Catalog #**AM9260G
- 2-Mercaptoethanol **Merck MilliporeSigma (Sigma-Aldrich)**
- High Sensitivity D1000 Reagents **Agilent Technologies Catalog #**5067-5585
- High Sensitivity D1000 ScreenTape **Agilent Technologies Catalog #**5067-5584

	Amount	Reagents
	3 ml	1 M CaCl2
	0.6 ml	3 M Mg(Ac)2
	6 ml	1 M Tris pH 7.9

	89.2 ml	molecular grade water

6x Homogenization Buffer Stable Solution.

Buffer recipes;

	A	B
	Amount	Reagents
	2.27084 ml	6x Homogenization Buffer stable
	3.78 ml	100mM PMSF
	0.28 ul	14.3 M B-mercaptoethanol
	1/2 tablet	Protease inhibitor (cOmplete Mini)

6x Homogenization Buffer Unstable Solution.

	Amount	Reagents
	500ul	1M Tris-Hcl ph 7.5
	100ul	5M NaCl
	150ul	1M MgCl2
	49.25ml	H2O

1x Homogenization Buffer Unstable Solution

	Amount	Reagents
	500ul	1M Tris-Hcl ph 7.5
	100ul	5M NaCl
	150ul	1M MgCl2
	49.25ml	H2O

ATAC-RSB

Amount	Reagents
8 ml	ATAC-RSB
8 ul	10% Tween

ATAC-RSB + 10% Tween

2X TD buffer:

Amount	Reagents
2 ml	1 M Tris-Hcl pH 7.5
1 ml	1 M MgCl2
20 ml	100% Dimethyl Formamide

2X TD buffer

(before the addition of dimethyl formamide, adjust the pH to 7.6 with 100% acetic acid)

DF buffer

Amount	Reagents
100 mM	HEPES (pH 7.2)
200 mM	NaCl
0.2 mM	EDTA
2 mM	DTT
2.00%	Triton X-100

	20.00%	Glycerol

2X DF buffer

	Primer	Sequence	Amount
	Tn5-A primer	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG	16 ul
	Tn5-rev primer	CTGTCTCTTATACACATCT	16 ul

Tube A

	Primer	Sequence	Amount
	Tn5-B primer	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG	16 ul
	Tn5-rev primer	CTGTCTCTTATACACATCT	16 ul

Tube B

Protocol materials

- Magnesium Chloride **Fisher Scientific** **Catalog #**AC223210010
- NN-Dimethylformamide (DMF) solution **Merck MilliporeSigma (Sigma-Aldrich)** **Catalog #**D4551
- Qubit 2.0 Fluorometer
- Tn5 transposase with Nextera adapters loaded **Catalog #**UC-Macro-Tn5-Nextera adapter
- Halt™ Protease Inhibitor Cocktail, EDTA-Free (100X) **Thermo Fisher** **Catalog #**78437
- Digitonin, 40ul **Promega** **Catalog #**G9441
- 1 M Calcium Chloride (CaCl2) **Fisher Scientific** **Catalog #**BP510
- Molecular grade water nuclease-free
- DTT **Merck MilliporeSigma (Sigma-Aldrich)** **Catalog #**D0632
- EDTA (0.5 M), pH 8.0 **Life Technologies** **Catalog #**AM9260G
- NEBNext High-Fidelity 2X PCR Master Mix - 250 rxns **New England Biolabs** **Catalog #**M0541L
- SYBR™ Green I Nucleic Acid Gel Stain - 10,000X concentrate in DMSO **Thermo Fisher** **Catalog #**S7563
- 2 ml LoBind Tubes **Eppendorf** **Catalog #**0030108078
- Falcon® 100 mm TC-treated Cell Culture Dish **Corning** **Catalog #**353003
- Zymo DNA Clean & Concentrator - 5 **Zymo Research** **Catalog #**D4014
- Protease Inhibitor Tablets cOmplete Mini EDTA free **Roche** **Catalog #**11836170001
- 1.5 mL LoBind tubes **Eppendorf** **Catalog #**022431021
- Sodium Chloride **Fisher Scientific** **Catalog #**S271
- High Sensitivity D1000 ScreenTape **Agilent Technologies** **Catalog #**5067-5584
- Tris-HCl pH 7.5
- Triton X-100 **Merck MilliporeSigma (Sigma-Aldrich)** **Catalog #**T8787-50ML
- 2-Mercaptoethanol **Merck MilliporeSigma (Sigma-Aldrich)**
- Qubit® dsDNA HS Assay Kit **Thermo Fisher Scientific** **Catalog #**Q32854
- Glycerol, 1000ml **Promega** **Catalog #**H5433
- High Sensitivity D1000 Reagents **Agilent Technologies** **Catalog #**5067-5585
- MACS SmartStrainers 30um **Miltenyi Biotec** **Catalog #**130-098-458
- KIMBLE 2mL Glass Dounce Tissue Grinder Set **Merck MilliporeSigma (Sigma-Aldrich)** **Catalog #**D8938
- Mg(Ac)2
- 50 ml Falcon tube

cOmplete™, Mini, EDTA-free (Protease Inhibitor) **Roche Catalog** ##11836170001)

Safety warnings

Regular lab safety rules apply. Ensure the use of googles during the usage of dry ice.

Before start

The pre-experimentation steps are important
Also it is important that all the primers have been ordered and reconstituted beforehand.

Pre-experimentation

1 All steps should be performed on ice or at 4 °C . 1m

2 Pre-chill all Dounces and pestles to 4 °C in a fridge or on ice 10m

3 Pre-chill all tubes. 10m

For each sample you are processing, you will need:
(i) One 2 mL 2 ml LoBind Tubes **Eppendorf Catalog #**0030108078 per sample
(ii) Three 1.5 mL LoBind tubes **Eppendorf Catalog #**022431021 per sample
(iii) one PCR tube per sample
(iv) One 50 ml Falcon tube for filtration step per sample

4 Prepare buffers. 1h 30m
i) 6x Homogenization Buffer Stable Solution.
ii) 6x Homogenization Buffer Unstable Solution.
iii) 1x Homogenization Buffer Unstable Solution.
iv) ATAC-RSB
v) ATAC-RSB + 10% Tween
vi) 2X TD buffer
vii) 2X DF buffer

4.1 i) 6x Homogenization Buffer stable Solution. 20m

	A	B
	Amount	Reagents
	3 ml	1 M CaCl2
	0.6 ml	3 M Mg(Ac)2
	6 ml	1 M Tris pH 7.9
	89.2 ml	molecular grade water

6x Homogenization Buffer Stable

4.2 ii) 6x Homogenization Buffer Unstable Solution. 20m

	Amount	Reagents
	2.27084 ml	6x Homogenization Buffer stable
	3.78 ml	100mM PMSF
	0.28 ul	14.3 M B-mercaptoethanol
	1/2 tablet	Protease inhibitor (cOmplete Mini)

6x Homogenization Buffer Unstable Solution

cOmplete™, Mini, EDTA-free (Protease Inhibitor) **Roche Catalog** ##11836170001)

4.3 ii) 1x Homogenization Buffer Unstable Solution. 20m

	Amount	Reagents
	2.166645 ml	6x Homogenization Buffer Unstable Solution
	4.16 ml	1 M sucrose
	2.6 ul	500 mM EDTA
	130 ul	10.00% NP10
	6.540755 ml	H2O

1x Homogenization Buffer Unstable Solution

4.4 iii) ATAC-RSB: 20m

	A	B
	Amount	Reagents
	500ul	1M Tris-Hcl ph 7.5
	100ul	5M NaCl
	150ul	1M MgCl2

	A	B
	49.25ml	H2O

ATAC-RSB

4.5 iv) ATAC-RSB + 10% Tween 20m

	Amount	Reagents
	8 ml	ATAC-RSB
	8 ul	10% Tween

ATAC-RSB + 10% Tween

4.6 v) 2X TD buffer 20m

	A	B
	Amount	Reagents
	2 ml	1 M Tris-Hcl pH 7.5
	1 ml	1 M MgCl2
	20 ml	100% Dimethyl Formamide

2X TD buffer

(before the addition of dimethyl formamide, adjust the pH to 7.6 with 100% acetic acid)

4.7 vi) 2X DF buffer 20m

	A	B
	Amount	Reagents
	100 mM	HEPES (pH 7.2)
	200 mM	NaCl

	A	B
	0.2 mM	EDTA
	2 mM	DTT
	2.00%	Triton X-100
	20.00%	Glycerol

2X DF buffer

5 Tn5 assembly reaction 2h

5.1 1h

	A	B	C
	Primer	Sequence	Amount
	Tn5-A primer	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG	16 ul
	Tn5-rev primer	CTGTCTCTTATACACATCT	16 ul

Tube A

	A	B	C
	Primer	Sequence	Amount
	Tn5-B primer	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG	16 ul
	Tn5-rev primer	CTGTCTCTTATACACATCT	16 ul

Tube B

95 °C to 25 °C
cooling -0.1°C/second

~1 hour on PCR machine

Olgio Annealing program

5.2 TN5 Assembly 1h

	A	B
	Amount	Reagent
	25 ul	Tn5
	15.5 ul	Tube A
	15.5 ul	Tube B
	33 ul	2X DF buffer

Tn5 Assembly Mix

Room temperature 01:00:00

Tn5 from the Protein Science Facility at the Karolinska Institutet in Stockholm (Picelli *et al.*,2014)
or

Tn5 transposase with Nextera adapters
loaded **Catalog #**UC-Macro-Tn5-Nextera adapter

Nuclei extraction and filtration

6 Materials for the cutting of tissue on dry ice. 1m

6.1 - Gloves
- White warm gloves
- Dry ice
- Blades and handle
- Forceps
- Ethanol spray
- Cell culture dish

1m

7 In the most sterile way possible, cut a small piece of tissue, half a pea size or so, and leave it in the petri dish with a marking on the lid, in the dry ice. Weigh it and cut again if needed. 20m

Make sure to use the ethanol to clean everything and be careful not to cut yourself.

8 Add 2 mL 1X HB buffer into the dounce, which is sitting in the ice. 2m

Add 0.2 µL 1 M DTT and and 1 µL 100X Halt protease inhibitor

9 Place 20 mg frozen tissue into a pre-chilled 2 ml Dounce containing 1 ml cold 1x HB and let thaw for 00:05:00 . 5m

10 Dounce with “A” loose pestle until resistance goes away (~10 strokes). 2m

Put the A pestle into the beaker of water

11 Dounce with “B” tight pestle for 20 strokes. 2m

Put the B pestle into the beaker of water

12 Pour everything from the dounce into a 30 um MACS smartstrainer which is sitting on top of a labelled 50 ml falcon tube sitting in ice. 2m

MACS SmartStrainers 30um **Miltenyi Biotec Catalog #**130-098-458

13 Let it drip through for 10-15 minutes. 15m

14 Transfer to a labelled 2 mL Lobind Eppendorf tube, already cold from sitting in ice. 5m

15 To pellet the nuclei, centrifuge 900 rpm, 4°C, 00:10:00 10m

15.1 Transfer the supernatant to a new tube without disturbing the pellet 10m

Repeat the centrifugation

16 Discard supernatant 5m

17 Resuspend the nuclei in 200 µL of ATAC-RSB + 10% Tween 5m

18 Count the amount of cells using the cell counter in the cell lab. 20m

18.1 Put 10 µL Trypton blue onto a piece of parafilm and mix with 10 µL of the sample

18.2 Take 10 µL of the mix and pipette into a cell counting cell

18.3 Measure on the cell counting machine, making sure to adjust for the smaller size of the nuclei.

The machine will think they are dead cells.

19 Calculate the amount of nuclei to use for the next step;
(500000 nuclei) 5m

19.1 ATAC-seq requires 50,000 cells for the experiment. So for example if the number of nuclei is;

4.76×10^7

then the calculation will look like this;

50000 / 47600 = 1.10

20 Turn on the thermomixer to 37 °C 1m

ATAC-seq 27m

21 Add the calculated amount of cells into a 2 mL lobind eppendorf tube with 1 ml ATAC-RSB + 10% Tween. 5m

So taking the example above one would take 1.10 ul of nuclei in the 200 µL ATAC-RSB + 10% Tween to the new tube.

22 Centrifuge nuclei for 00:10:00 900 rcf, 4°C, 00:10:00 10m

22.1 Here one can also save the nuclei for later. Simply spin the tube down with the new tube of diluted nuclei, discard the supernatant, add 300 µL of the and put in the -20° freezer. 10m

00:10:00 900 rcf, 4°C, 00:10:00

23 Discard most of the supernatant. Leave a bit in the bottom to ensure that there is enough for the reaction and that the pellet stays intact 2m

24 Add the reaction mix to the pellet and remaining water and resuspend the pellet in the mix 5m

	A	B
	Amount	Reagents
	25 ul	2X TD buffer
	16.5 ul	1X PBS (cold)
	0.5 ul	1% digitonin
	0.5 ul	10% Tween -20
	2.5 ul	TN5 assembly

Reaction Mix

25 Put the tube into the thermomixer at 1000 rpm, 37°C, 00:30:00 30m

26 Take the tubes out and immediately proceed to the column clean up 2m

27 Use the Zymo DNA clean & concentrator to clean the nuclei 30m

Zymo DNA Clean & Concentrator - 5 **Zymo Research Catalog #**D4014

27.1 Add 2-7 volumes of the DNA binding buffer to each volume of DNA sample

So in this case the volume is approximately 50 µL , so add 300 µL of DNA binding buffer

27.2 Transfer mixture to a provided Zymo-spin column in a collection tube

27.3 Centrifuge 10000 x g, 00:00:30
Discard supernatant

27.4 Add 200 µL DNA wash buffer to the column

Centrifuge 10000 x g, 00:00:30

27.5 repeat the wash step

27.6 Trasnfer the column to a 1.5 low-bind eppendorf tube.

Add 21 µL 2 DNA Elution buffer to the column.

Centrifuge 10000 x g, 00:00:30

28 Can stop here and start the next day if necessary.
Leave samples in 4°

29 Set up PCR; 20m

	A	B
	Amount	Reagent
	2.5 ul	Primer AD1
	2.5 ul	Primer AD2.#
	25 ul	NEBNext Master Mix

	A	B
	20 ul	sample

PCR

NEBNext High-Fidelity 2X PCR Master Mix - 250 rxns **New England Biolabs Catalog #**M0541L

29.1 PCR program - ATAC-seq pre-amplification 40m

	A	B	C
	Tempuratur e	Time	Cycle
	72°	5 minutes	1
	98°	30 seconds	-
	98°	10 seconds	5
	63°	30 seconds	-
	72°	1 minute	-
	72	5 minutes	1
	4	infinate	-

ATAC-seq pre-amplification

30 Remove PCR tubes from the machine and put immediately onto ice. 1m

31 Proceed immediately to the qPCR amplification to determine additional cycles step 1m

32 qPCR amplification to determine additional cycles 1h 30m

prepare a mix or master mix

	A	B	C
	Reagent	1X	6.5X
	Molecular grade water	3.76 ul	24.5 ul
	Primer AD1	0.5 ul	6.5 ul
	Primer AD2.#	0.5 ul	0.5 ul *
	25x SYBR green (in DMSO)	0.24 ul	1.56 ul
	2x NEBNext Master Mix	5 ul	32.5 ul

qPCR mix

SYBR™ Green I Nucleic Acid Gel Stain - 10,000X concentrate in DMSO **Thermo Fisher Catalog #**S7563

NEBNext High-Fidelity 2X PCR Master Mix - 250 rxns **New England Biolabs Catalog #**M0541L

32.1 qPCR program

	A	B	C
	Tempuratur e	Time	Cycle
	98°	30 seconds	1
	98°	10 seconds	20
	63°	30 seconds	-
	72°	1 minute	-

qPCR program

32.2 qPCR extra setup options 1m

- 6 machine not 6

- standard
- SYBR green

- no melt curve

- turn off ROX

- Fill in sample list

33 Determine the required amount of cycles that each sample needs in addition 5m

33.1 Looking at the final amplification curve, determine the max fluorescence where the graph plateaus

33.2 Determine 1/3 of that number.

For example if the max is 1400000 then 1/3 would be 433333

33.3 Check the graph for how many cycles line up with this number from the curve.

In the case above it would be 6 because of where the line was

34 Continue the PCR with the additional amount of cycles skipping the beginning parts of the program

34.1 # of addtional cycles 40m

	A	B	C
	Tempuratur e	Time	Cycle
	98°	10 seconds	Based on calculation
	63°	30 seconds	-
	72°	1 minute	-
	4°	Infinate	1

Additional cycles

35 Take the tubes out and immediately proceed to the column clean up 1m

36 Use the Zymo DNA Clean & Concentrator to clean the nuclei 30m

Zymo DNA Clean & Concentrator - 5 **Zymo Research Catalog #**D4014

36.1 Add 2-7 volumes of the DNA binding buffer to each volume of DNA sample 1m

So in this case the volume is approximately 50 ul, so add 300 ul of DNA binding buffer

36.2 Transfer mixture to a provided Zymo-spin column in a collection tube 1m

36.3 Centrifuge 10000 x g, 00:00:30 1m
Discard supernatant

36.4 Add 200 µL DNA wash buffer to the column 2m

Centrifuge 10000 x g, 00:00:30

36.5 Repeat the wash step 2m

36.6 Transfer the column to a 1.5 low-bind Eppendorf tube. 1m

Add 21 ul **Sterile water** buffer to the column.

Centrifuge 10000 x g, 00:00:30

37 Quality control
TapeStation (DS1000) and Qubit quantification 1h