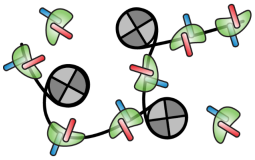
Jun 05, 2024 Version 1

Simplified ATAC-seq protocol on frozen brains of fruit flies V.1



Forked from Isolation of nuclei from frozen tissue for ATAC-seq and other epigenomic assays

This protocol is a draft, published without a DOI.

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Human Cell Atlas Method …

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External link: **https://www.ncbi.nlm.nih.gov/pubmed/28846090**

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

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Abstract

This protocol enables the isolation of nuclei from frozen tissues. These nuclei are suitable for use in ATAC-seq, single-cell ATAC-seq, ChIP-seq, HiC/3C, and many other assays.

Modified for a small amount of fruit fly brains from the omni-ATACseq protocol from Ryan Cortes

Guidelines

The quality of the tissue at the time of freezing is a major factor in the quality of data downstream. If freezing tissue for use later, you may want to consider cryopreserving 50 mg tissue chunks in BAM Banker cryopreservative. If using previously frozen tissue, the ischemic time or post mortem interval should be kept as short as possible.

Nuclei can be cryopreserved in BAM Banker. In our experience, freeze-thaw cycles do not adversely affect data quality as long as thawing is carried out on ice and freezing is performed using a slow-freeze freezing container. We have had success isolating nuclei from every tissue we have tried including difficult to work with tissues such as heart.

Materials

STOCK BUFFER INGREDIENTS

	A	B	C	D	E	F	G	H	I	J
	Stock (planned Conc.)	Name	chemical info	Formula mass (g/mol)	Volume (ml) of solution (planned) 2024.03.20	Mass (g) of solube (planned) 2024.03.20	Volume (ml) of solution (actual) 2024.03.20	Mass (g) of solube (actual) 2024.03.20	Actual Conc. (M) 2024.03.20	autoclave bottle before usage
	1	M Sucrose		342.30	100	34.23	100	34.34	1.00321355536079 46	1
	2	M KCl	BDH, potassium chloride, purity 99 - 100.5%, lot No. 118446, opened 2024.03.20	74.47	100	14.894	100	15.37	2.06391835638512 16	1
	1	M MgCl2 (hexahydrate)	dot scientific inc., magnesium Chloride hexahydrate, purity unknown, lot No. 61024-91729, opened 2019.06.18	203.3	100	20.33	100	20.44	1.00541072306935 56	1
	0.75	M Tricine-KOH pH 7.8		179.22	200	26.883	150	13.38	0.49771230889409 67	1
	2.5	M NaCl	Fisher Scientific, Sodium Chloride, putiry 99.3%, log No. 145475, already opened 2024.03.20	58.44	200	29.22	250	29.22	2	1
	60	% Iodixanol								
	0.5	M Spermidin		254.63	2	0.25463	2	0.257	0.50465381141263	

	A	B	C	D	E	F	G	H	I	J
		e							8	
	0.15	M Spermine		202.34	8	0.242808	8	0.2411	0.14894484530987448	
	1	M DTT		154.25	3	0.46275	3	0.468	1.01134521880064 82	
	10	% wt./vol. Tween-20	opened 2024.04.02	density: 1.095 g/mL at 25 °C	50					
	10	% wt./vol. IGEPAL® CA-630	opened 2024.04.04	density: 1.06 g/mL at 25 C	50					

STOCK BUFFER INGREDIENTS (dilute by density)

	A	B	C	D	E	F	G	H	I	J	K	L	M
	Stock (planned Conc.)	Name	chemical info	Density (g/mL at 25 C)	Volume (ml) of solution (planned) 2024.04.04	Mass (g) of solube (planned) 2024.04.04	Volume (mL) of solube (planned) 2024.04.04	Volume (mL) of water (planned) 2024.04.04	Mass (g) of solube (actual) 2024.04.04	Volume (mL) of solube (actual) 2024.04.04	Volume (mL) of water (actual) 2024.04.04	Sterile filter?	
	10	% wt./vol. Tween-20	opened 2024.04.02	1.095	50	5	4.566210045662101	45.4337899543379	5.005	4.570776255707763	45.42922374429224	yes	
	10	% wt./vol. IGEPAL® CA-630	opened 2024.04.04	1.06	50	5	4.716981132075471	45.283018867924525	5.005	4.721698113207546	45.278301886792455	yes	

STOCK BUFFERS

All stock solutions should be filtered using a 0.22 um PVDF filter system. All solutions except for the 50% Iodixanol solution are stable at 4°C for at least 6 months.

1.034x Homogenization Buffer Stable Solution - 200 ml (2014 ul/sample)

	A	B	C	D	E	F
	Stock	*Name*	*Final Conc.*	Actual stock conc.(made on 2024.03.20)	actual *Fold Dilution (x)*	actual *Total Vol. (ml)*
	1	M Sucrose	0.26	1.00	3.846153846153846	52.000000000000001
	2	M KCl	0.03	2.06	68.66666666666667	2.912621359 2233006
	1	M MgCl2	0.01	1.01	101	1.980198019

	A	B	C	D	E	F
						8019802
	0.75	M Tricine-KOH pH 7.8	0.02	0.50	25	8
	-	Water	-	-	-	135.1071806209747

Diluent Buffer - 100 ml (16.4 ul/sample)

	A	B	C	D	E	F
	Stock	*Name*	*Final Conc.*	Actual stock conc.(made on 2024.03.20)	actual *Fold Dilution (x)*	actual *Total Vol. (ul)*
	2	M KCl	0.15	2.06	13.733333333333334	7.281553398058252
	1	M MgCl2	0.03	1.01	33.66666666666667	2.97029702970297
	0.75	M Tricine-KOH, pH 7.8	0.12	0.50	4.166666666666667	24
	-	Water	-	-	-	65.74814957223879

50% Iodixanol Solution - 50 ml (Remake monthly for stability) (840 ul/sample)

	A	B	C	D	E
	Stock	*Name*	*Final Conc.*	*Fold Dilution (x)*	*Total Vol. (ml)*
	-	Diluent Buffer	1	-	8.33333
	60	% Iodixanol	50	1.20	41.66667

ATAC-RSB Buffer - 500 ml (2970 ul/sample)

	A	B	C	D	E	F
	Stock	*Name*	*Final Conc.*	Actual stock conc.(made on 2024.03.20)	actual *Fold Dilution (x)*	actual *Total Vol. (ml)*
	1	M Tris-HCl pH 7.5	0.01	0.50	50	10
	5	M NaCl	0.01	2	200	2.5
	1	M MgCl2	0.003	1.01	336.6666666666667	1.4851485148514851
	-	Water	-	-	-	486.01485148514854

SAME DAY BUFFERS

1x Homogenization Buffer Unstable Solution

**Note – cOmplete Protease Inhibitors come as tablets. It is difficult to use less than 1/2 tablet so we prepare the 1x Homogenization Buffer Unstable Solution in batches of 12 as outlined below.

	A	B	C	D	E	F	G
	Stock	*Name*	*Final Conc.*	*Fold Dilution (x)*	*Vol per 12 samp. (ul)*	Vol (ul) made 2024.04.24 (14.4 sample)	Vol (ul) made 2024.05.01 (10 sample, assuming 200 ul per sample)
	1.0341	x HB Stable Solution	1	1.03	24175.00	29010	2417.5
	1	M DTT	0.001	1000.00	25.00	30	2.5
	500	mM Spermidine	0.5	1000.00	25.00	30	2.5
	150	mM Spermine	0.15	1000.00	25.00	30	2.5
	10	% NP40	0.3	33.33	750.00	900	75
	-	cOmplete Protease Inhibitor mini tablets	-	-	2.50 Tablets	3 tablets	0.25 tablet
		TOTAL VOLUME			25000	30000	2500

30% Iodixanol Solution

	A	B	C	D	E	F
	Stock	*Name*	*Final Conc.*	*Fold Dilution (x)*	*Vol per sample (ul)*	Vol (ul) made 2024.04.04 (12 samp.)
	-	1x Homog. Buffer Unstable	-	-	240.00	2880
	50	% Iodixanol Solution	30	1.67	360.00	4320

40% Iodixanol Solution

	A	B	C	D	E	F
	Stock	*Name*	*Final Conc.*	*Fold Dilution (x)*	*Vol per sample (ul)*	Vol (ul) made 2024.04.04 (12 samp.)
	-	1x Homog. Buffer Unstable	-	-	120.00	1440
	50	% Iodixanol Solution	40	1.25	480.00	5760

ATAC-RSB-Tween Buffer

	A	B	C	D	E	F	G
	Stock	*Name*	*Final Conc.*	*Fold Dilution (x)*	*Vol per sample (ul)*	Vol (ul) made 2024.04.04 (12 samp.)	Vol (ul) made 2024.05.01 (11 samp., assuming

	A	B	C	D	E	F	G
							1000 ul per sample)
	-	ATAC-RSB	-	-	2970.00	35640	10890
	10	% Tween-20	0.1	100.00	30.00	360	110

ATAC-seq Reaction Mix

	A	B	C	D
	Reagent	*Vol per sample (ul)*	Vol (ul) made 2024.04.24 (11 samp.)	Vol (ul) made 2024.05.01 (11 samp.)
	H2O	5.25	57.75	57.75
	PBS	16.5	181.5	181.5
	2x TD Buffer	25	275	275
	2% Digitonin	0.25	2.75	2.75
	10% Tween-20	0.5	5.5	5.5
	Tn5 Transposase	2.5	27.5	27.5

PCR master mix (MM)

	A	B	C	D
	Reagent	*Vol per sample 1x50 ul run (ul)*	Vol (ul) made 2024.04.25 (10 + 1 samp.)	Vol (ul) made 2024.05.05 (10 + 1 + 1 samp.)
	H2O	25.5	280.5	306
	2 X KAPA high fidelity butter	10	110	120
	dNTPs	1.5	16.5	18
	Hifi enzyme	1	11	12
	TOTAL MM VOLUME	38	418	456

Reagents used in this protocol

	A	B	C
	Item	**Supplier**	**Cat Number**
	Eppendorf 2 ml Lo-Bind tubes	Sigma	Z666556-250EA
	Eppendorf 1.5 ml Lo-Bind tubes	Sigma	Z666548-250EA
	Nunc cryovials	Thermo	375418PK
	Iodixanol (comes at 60%)	Sigma	D1556-250ML
	Digitonin	Promega	G9441
	Sucrose	Sigma	S7903-250G
	IGEPAL® CA-630 (identical with NP40, which was discontinued)	Sigma	I8896-50ML
	Tricine	Sigma	T0377-25G
	Potassium Hydroxide (KOH)	Sigma	P5958-250G
	cOmplete Protease Inhibitors mini tablets (1/5 size of the full tablets cat. 11697498001)	Roche	11836170001
	MgCl2	Ambion (Thermo)	AM9530G
	KCl	Ambion (Thermo)	AM9640G
	DTT	Thermo	R0861
	Spermidine	Sigma	S2501
	Spermine	Sigma	S3256-1G
	70 um Flowmi cell strainers	Fisher	03-421-228
	70 um bucket-style cell strainers	BD Falcon	352350
	Tris-HCl pH 7.5	Invitrogen	15567-027
	NaCl	Ambion (Thermo)	AM9759
	Tween 20	Sigma	P2287-100ML
	H2O	Invitrogen	10977-015
	Dounce Tissue Grinder Set	Sigma	D8938-1SET
	INCYTO Disposable hemocytometers	Fisher	22-600-100
	BAM Banker	Wako Chemicals	302-14681
	RiboLock	Thermo	EO0384
	0.22 um PVDF Filter Units (500 ml)	Millipore	SCGVU05RE
	0.22 um PVDF Filter Units (50 ml)	Millipore	SE1M179M6

	A	B	C
	Tn5 Transposase (TDE1)	Illumina	15027865
	2x TD Tagment DNA Buffer	Illumina	15027866

Before you start the protocol:

1 All steps should be performed on ice or at 4 °C. Pre-chill a fixed angle centrifuge to 4°C.

2 Pre-chill all tubes. For each sample you are processing, you will need: (i) one 2 ml cryo tube with 10 1.6 mm beads for bead homogenization (ii) one 1.5 ml round-bottom LoBind tube for nuclei pelleting (iii) one 1.5 ml round-bottom LoBind tube for collecting purified DNA

3 Prepare all buffers. For faster dissolution, crush protease inhibitor tablets prior to addition to 1x Homogenization Buffer Unstable Solution. DTT, Spermidine, Spermine, and digitonin are stored at -20°C. All other detergents, ATAC-RSB, and other buffers are stored at 4 °C. Do not prepare transposition mix ahead of time.

Isolation of Nuclei via Bead Homogenization and Centrifuging

4 move samples from liquid nitrogen, or -80 °C freezer onto On ice (wet ice) wet ice 2 mins before homogenization. Add 200 µL HB to the tube wall, vortex and spin down to get the sample off the tube wall.

4.1 if samples were slow-frozen in Bambanker, put the sample on On ice (wet ice) until the Bambanker thaws, then spin down sample 00:05:00 at 4 °C at 750 RCF in a fixed angle centrifuge, and remove supernatant. Add 200 µL HB and 10 1.6 mm beads to the tube. 5m

4.2 if samples are fresh, put the sample on on On ice (wet ice) until homogenization (200 µL pre-chilled HB should have been added to the tube before dissection.

5 Homogenize in BeadRupter Elite bead mill homogenizer using 2 cycles of the following: 1m 20s

Speed 1.0 for 00:00:20

Dwell for 00:00:20

Speed 2.0 for 00:00:20

Dwell for 00:00:20

6 Pellet nuclei by spinning 00:05:00 at 4 °C at 350 RCF in a fixed angle centrifuge. 5m

7 Re-suspend the nuclei in 1000 µL of ATAC-RSB-Tween Buffer

8 Centrifuge nuclei for 00:10:00 at 500 RCF at 4 °C in a fixed angle centrifuge. At this point, the pellet should be clearly visible if 50,000 nuclei were used. Pellets of as few as 10,000 nuclei should be visible.

Transposition of Nuclei

9 Using a p1000 pipette, remove all but the last 100 ul of supernatant. Remove last 100 ul with p200 pipette set to 200 ul using a single fluid pipetting motion. Place the tip of your pipette on the opposite side of the tube to where the nuclei pellet is located during this final aspiration step.

10 Add 50 ul ATAC-seq Reaction Mix to each tube and pipette up and down 6 times to resuspend nuclei pellet.

10.1 Unlike the published ATAC-seq protocols, you do not need to do an individual lysis step in this protocol because the nuclei are exposed to NP40 throughout the Douncing portion of the protocol.

11 Incubate reactions at 37 °C for 00:30:00 in a heat block (ideally should use a thermoshaker with 1000 RPM constant shaking).

12 After incubation, follow instructions of Qiage Minelute DNA clean-up Kit to purify the DNA

13 Store purified DNA under -20 °C , if not used for PCR for a more than a week. Otherwise the purified DNA can be stored at 4 °C temporarily

DNA Library Amplification

14 Remove PCR reagents from freezer to thaw at Room temperature ; dNTPs, 2X KAPA High fidelity buffer (yellow lid), and primer plate. Once all traces of ice are gone, vortex and spin down. Turn on thermal cycler.

15 Remove Hifi enzyme from freezer and place on wet ice. Make PCR master mix (MM)

PCR master mix (MM)

	A	B	C	D
	1	*Reagent*	*Vol per sample 1x50 ul run (ul)*	Vol (ul) made 2024.04.25/04.29 (10 + 1 samp.)
	2	H2O	25.5	280.5
	3	2 X KAPA high fidelity butter	10	110
	4	dNTPs	1.5	16.5
	5	Hifi enzyme	1	11
	6	TOTAL MM VOLUME	38	418

16 Mix and spin down master mix, keep cold, and add 38 uL to each reaction tube.

17 Visually inspect primer plate to ensure evaporation has not occurred in the wells you intend to use. Spin down in plate spinner. Carefully open foil to needed wells.

17.1 primer plate in use:

	A	B	C	D	E
		2024.04.29			
	plate number	3 + 7	H2O	25.5	280.5
	well number	1-10	2 X KAPA high fidelity butter	10	110

18 Add 2 uL of primer mix to each reaction. Make sure to record which index primers are used!

19 Add 10 uL of cleaned tagmentation product. Mix and spin down. Make sure to close PCR tube lids tightly with a marker end.

20 Place tubes in thermal cycler, making sure lid is tightly against your tubes, and run program ML2.

(Optional) Cleanup and Freezing Down of Extra Nuclei

21 If you would like to save extra nuclei for other assays or to potentially use in additional ATAC-seq experiments downstream: