Supplementary Protocol #1 – Processing of frozen tissue fragments for ATAC-seq

Before you start the protocol:

1) All steps should be performed on ice or at 4°C. Pre-chill a swinging bucket centrifuge and a fixed angle centrifuge to 4°C.
2) Pre-chill all Dounces and pestles to 4°C in a fridge.
3) Pre-chill all tubes. For each sample you are processing, you will need:
    a. One 2 ml round-bottom LoBind tube for gradient separation
    b. One 1.5 ml LoBind tube for RNA homogenate
    c. One 2 ml Nunc Cryotube for extra nuclei
    d. One 50 ml conical for filtration step (often optional)
4) 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.
    a. Remember that the catalog number provided for iodixanol from Sigma comes as a 60% solution (not 100%).
5) Fill up a 2 L beaker with 500 ml sterile water to soak the used Dounces and pestles.

Isolation of Nuclei via Dounce Homogenization and Density Gradient Centrifugation:

1) Remove samples from liquid nitrogen storage and keep on dry ice until use.
2) Place 20 mg frozen tissue into a pre-chilled 2 ml Dounce containing 1 ml cold 1x HB and let thaw for 5 minutes.
    a. For >30 mg tissue, use 2 ml 1x HB. For 10-20 mg tissue, use 1 ml 1x HB. For 50 um tissue sections, use 0.5 ml 1x HB.
3) If you would like to collect RNA from the same sample, add 10 ul RiboLock per ml of 1x HB and mix well.
4) Dounce with "A" loose pestle until resistance goes away (~10 strokes).
5) Place "A" pestle into beaker with sterile water to soak for cleaning later.
    a. Optional – If residual un-homogenized tissue makes it difficult to Dounce, filter homogenate through a pre-chilled 50 ml conical using a 70 um bucket-style cell strainer filter prior to using tight pestle "B".
6) Dounce with "B" tight pestle for 20 strokes.
7) Place "B" pestle into beaker with sterile water to soak for cleaning later.
8) Filter during transfer using a 70 um Flowmi strainer and transfer homogenate to a pre-chilled 2 ml LoBind tube.
9) Place Dounce into beaker with sterile water to soak for cleaning later.
10) Pellet nuclei by spinning 5 min at 4°C at 350 RCF in a fixed angle centrifuge.
11) Remove all but 50 ul of supernatant (containing cytoplasmic RNAs) and transfer to a pre-chilled 1.5 ml LoBind tube. If the pellet is not clearly visible, you can leave more supernatant in the tube, up to 400 ul and add less of the 1x HB buffer in the next step.
12) Gently resuspend nuclei in a total volume of 400 ul 1x HB. If you only left 50 ul in the tube in the previous step, this means you should add 350 ul 1x HB. Make sure nuclei are fully resuspended without clumps.
13) Add 1 volume (400 ul) of 50% Iodixanol Solution and mix well by pipetting

14) Slowly layer 600 ul of 30% Iodixanol solution under the 25% mixture. To avoid mixing of layers, wipe the side of the pipette tip with a Kimwipe to remove excess Iodixanol solution from the external surfaces of the pipette tip.
15) Layer 600 ul of 40% Iodixanol solution under the 30% mixture. To avoid mixing of layers, wipe the side of the pipette tip with a Kimwipe to remove excess Iodixanol solution from the external surfaces of the pipette tip.
    a. During this step, you will need to gradually draw your pipette tip up to avoid overflowing the tube. However, the tip of your pipette must stay below the 30%-40% interface at all times.
16) In a pre-chilled swinging bucket centrifuge, spin for 20 min at 4°C at 3,000 RCF with the brake off. Handle tubes gently so as to not disturb the gradient.
    a. Iodixanol is meant to be used at higher speeds (10,000 RCF) but high-speed swinging bucket centrifuges are not always readily available so we perform this step at 3,000 g and have not had any issues.
17) Using a vacuum, aspirate the top layers down to within 200-300 ul of the nuclei band at the 30%-40% interface. Be careful not to get too close as you will disrupt the nuclei band.
18) Using a 200 ul volume, collect the nuclei band and transfer to a fresh tube. Do not aspirate more than 200 ul at this step as this can cause you to take too much of the 40% layer which sometimes contains debris.
19) Dilute nuclei by adding some volume of ATAC-RSB-Tween Buffer. Mix gently by pipetting. The precise volume of ATAC-RSB-Tween to add will depend on how many nuclei you have. If you don't dilute enough, it will be hard to get an accurate count. If you dilute too much, it will be similarly hard to get an accurate count. You should minimally add 200 ul of ATAC-RSB-Tween buffer to dilute the iodixanol as high concentrations of iodixanol can be too viscous for hemocytometers.

Transposition of Nuclei:

1) Count nuclei using Trypan blue staining (1:1 ratio of Trypan to sample) and a manual hemocytometer. We recommend using disposable hemocytometers for consistency but do not recommend automated cell counters.
2) We normally perform two technical replicates per sample. Each technical replicate should ideally have 50,000 nuclei, requiring 100,000 nuclei total. If you don't have at least 100,000 nuclei, follow this convention:
    a. More than 50,000 nuclei, still do 2 technical replicates using half of the volume for each replicate but reduce the volume of Tn5 transposase proportionately to the number of nuclei. Maintain all other transposition reaction volumes. For example, for 25,000 cells, use 1.25 ul of Tn5 transposase in a 50 ul total reaction volume. Replace omitted Tn5 volume with water.
    b. Less than 50,000 nuclei, only do 1 technical replicate and reduce the volume of Tn5 transposase proportionately to the number of nuclei. Maintain all other transposition reaction volumes. For example, for 25,000 cells, use 1.25 ul of Tn5 transposase in a 50 ul total reaction volume. Replace omitted Tn5 volume with water.
3) Label and chill 1.5 ml LoBind tubes according to how many tubes will be needed for transpositions.

4) Transfer 50,000 nuclei into a 1.5 ml LoBind tube containing 1000 ul of ATAC-RSB-Tween Buffer. If the total volume won't fit in a 1.5 ml tube, just reduce the amount of ATAC-RSB-Tween that you add to the tube to start.
5) Centrifuge nuclei for 10 minutes 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.
6) 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.
7) Add 50 ul ATAC-seq Reaction Mix to each tube and pipette up and down 6 times to resuspend nuclei pellet.
    a. 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.
8) Incubate reactions at 37°C for 30 min in a thermoshaker with 1000 RPM constant shaking.
9) After incubation, add 250 ul (5 volumes) of Binding Buffer from the Zymo DNA Clean and Concentrator 5 kit. Mix well by vortexing and inverting to collect any condensate from the lid.
10) Pulse centrifuge to collect volume in the bottom of the tube.
11) Either finish the cleanup protocol using the Zymo DNA Clean and Concentrator 5 kit or transfer the binding buffer transposition mix to -20°C for short term storage for up to 1 week.
    a. If you store the binding buffer transposition mix at -20°C, allow it to equilibrate to room temperature and mix well before proceeding with the Zymo DNA Clean and Concentrator clean up protocol.

Cleanup and Freezing Down Tubes:

1) If you would like to save extra nuclei for other assays or to potentially use in additional ATAC-seq experiments downstream:
    a. Pellet remaining nuclei by centrifugation for 10 min at 500 RCF at 4°C
    b. Carefully aspirate supernatant using two pipetting steps (p1000 then p200) as above.
    c. Gently resuspend nuclei pellet in 100 ul of cold BAM Banker media and transfer to a pre-chilled 2 ml Nunc cryovial.
    d. Slow-freeze nuclei in a freezing container and move to -80°C or liquid nitrogen storage the next day.
2) If you would like to keep homogenate for making RNA (or potentially protein) downstream:
    a. Store homogenate at -80°C.
3) Cleaning Dounces and pestles:
    a. Rinse all Dounces and pestles thoroughly with sterile water (2x) followed by 70% ethanol (2x).
    b. Let Dounces and pestles dry on a kimwipe or paper towel for a few hours to overnight.

Processing Homogenate to Make RNA (optional):

1) Pre-chill a fixed-angle centrifuge to 4°C.
2) Thaw homogenate on ice.
3) Transfer 150 ul to a 2 ml LoBind tube containing 1500 ul Trizol and mix.
4) Add 400 ul chloroform to the Trizol and mix by vortexing for 15 seconds.
5) Immediately spin in a pre-chilled fixed-angle centrifuge at 21,000 RCF for 15 minutes at 4°C.
6) Pipette clear aqueous layer (~650 ul) into a clean 1.5 ml LoBind tube.
7) Add an equal volume (650 ul) of 100% ethanol and mix well.
8) Pass all volume through a QIAgen RNeasy column using two centrifugation steps.
9) Follow the QIAgen RNeasy protocol and elute in 27 ul of elution buffer
10) Add 3 ul of 10x Turbo DNase Buffer and 1 ul of Turbo DNase enzyme.
11) Incubate at 37°C for 30 minutes.
12) Add 70 ul of RNase free water and 350 ul of QIAgen RLT Buffer and mix well.
13) Add 250 ul 100% ethanol and mix well.
14) Apply to column. Wash 2x with RPE. Elute in 20 ul RNase-free water.

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		For 200 ml stock solution		
Stock	*Name*	*Final Conc.*	*Fold Dilution (x)*	*Total Vol. (ul)*
1	M Sucrose	0.26	3.87	51706.50
2	M KCl	0.03	77.36	2585.33
1	M MgCl2	0.01	193.40	1034.13
0.75	M Tricine-KOH pH 7.8	0.02	36.26	5515.36
-	Water	-	-	139158.69
			Total Vol. (ul)	200000.00

Diluent Buffer		For 100 ml stock solution		
Stock	*Name*	*Final Conc.*	*Fold Dilution (x)*	*Total Vol. (ul)*
2	M KCl	0.15	13.33	7500.00
1	M MgCl2	0.03	33.33	3000.00
0.75	M Tricine-KOH, pH 7.8	0.12	6.25	16000.00
-	Water	-	-	73500.00
			Total Vol. (ul)	100000.00

50% Iodixanol Solution		For 50 ml stock solution		
Stock	*Name*	*Final Conc.*	*Fold Dilution (x)*	*Total Vol. (ul)*
-	Diluent Buffer	1	-	8333.33
60	% Iodixanol	50	1.20	41666.67
**Remake monthly for stability			Total Vol. (ul)	50000.00

ATAC-RSB Buffer		For 500 ml stock solution		
Stock	*Name*	*Final Conc.*	*Fold Dilution (x)*	*Total Vol. (ul)*
1	M Tris-HCl pH 7.5	0.01	100.00	5000.00
5	M NaCl	0.01	500.00	1000.00
1	M MgCl2	0.003	333.33	1500.00
-	Water	-	-	492500.00
			Total Vol. (ul)	500000.00

1M Sucrose		For 300 ml stock solution		
Stock	*Name*	*Final Conc.*	*Fold Dilution (x)*	*Total*
-	Sucrose (Powder)	1000	-	102.69 g
	H2O			235.5 ml
			Total Vol. (ul)	300000.00

Same Day Buffers – should be prepared fresh each day

**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.

1x Homogenization Buffer Unstable Solution				
Stock	*Name*	*Final Conc.*	*Fold Dilution (x)*	*Vol per 12 samp. (ul)*
1.0341	x HB Stable Solution	1	1.03	24175.00
1	M DTT	0.001	1000.00	25.00
500	mM Spermidine	0.5	1000.00	25.00
150	mM Spermine	0.15	1000.00	25.00
10	% NP40	0.3	33.33	750.00
-	cOmplete Protease Inhibitor	-	-	0.50 Tablets
			Total Volume (ul)	25000.00

30% Iodixanol Solution				
Stock	*Name*	*Final Conc.*	*Fold Dilution (x)*	*Vol per sample (ul)*
-	1x Homog. Buffer Unstable	-	-	240.00
50	% Iodixanol Solution	30	1.67	360.00
			Total Volume (ul)	600.00

40% Iodixanol Solution				
Stock	*Name*	*Final Conc.*	*Fold Dilution (x)*	*Vol per sample (ul)*
-	1x Homog. Buffer Unstable	-	-	120.00
50	% Iodixanol Solution	40	1.25	480.00
			Total Volume (ul)	600.00

ATAC-RSB-Tween Buffer				
Stock	*Name*	*Final Conc.*	*Fold Dilution (x)*	*Vol per sample (ul)*
-	ATAC-RSB	-	-	2970.00
10	% Tween-20	0.1	100.00	30.00
			Total Volume (ul)	3000.00

ATAC-seq Rxn Mix	
Reagent	*Vol per sample (ul)*
H2O	5
PBS	16.5
2x TD	25
1% Digitonin	0.5
10% Tween-20	0.5
Tn5	2.5

Order List

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
Sucrose	Sigma	S7903-250G
NP40	Roche (Sigma)	11332473001
Tricine	Sigma	T0377-25G
Potassium Hydroxide (KOH)	Sigma	P5958-250G
cOmplete Protease Inhibitors	Roche	11697498001
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	Roche (Sigma)	11332465001
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