SOP: ATAC-Seq Library Preparation and Size Selection

Description: This protocol describes ATAC-Seq library preparation from transposed DNA using PCR and subsequent size selection of the subnucleosomal fragment. The protocol is adapted from (Corces et al., 2017) including a size selection step as suggested by (Halstead et al., 2020). Nuclear DNA should have been prepared according to: https://data.faang.org/api/fire_api/samples/ROSLIN_SOP_ATAC_Seq_DNAIsolationandTagmentation_Frozen_Muscle_Tissue_20200720.pdf OR https://data.faang.org/api/fire_api/samples/ROSLIN_SOP_ATAC-Seq_DNAIsolationandTagmentation_Cryopreserved_Muscle_Nuclei_Preparations_20200720.pdf.

Materials List

Item	Catalog Number	Manufacturer
Qiagen MinElute PCR Purification Kit	28004	Qiagen
NEBNext Hi-Fi PCR mix	M0541s	New England Biolabs
SYBR Green Nucleic Acid Stain (10,000x concentrate)	S7563	Invitrogen
E-gel Size Select II 2% Agarose Gel	G661012	Thermo Fisher Scientific
E-Gel Sizing DNA Ladder and E-Gel Sample Loading Buffer	10488100 10482055	Thermo Fisher Scientific
D1000 High Sensitivity ScreenTape and reagents	5067-5584 5067-5585	Agilent
D5000 ScreenTape and reagents	5067-5588 5067-5589	Agilent

Equipment

Thermo Scientific E-Gel System	ThermoFisher Scientific
Agilent 2200 TapeStation System	Agilent
Roche Light Cycler qPCR machine	Roche 480

List of Primers for ATAC-Seq from (Corces et al., 2017). Ad2 primers contain the unique barcodes for each sample.

Ad1 noMX:	AATGATACGGCGACCACCGAGATCTACACTCGTCGGCAGCGTCAGATGTG
Ad2.1 TAAGGCGA	CAAGCAGAAGACGGCATACGAGATTCGCCTTAGTCTCGTGGGCTCGGAGATGT
Ad2.2 CGTACTAG	CAAGCAGAAGACGGCATACGAGATCTAGTACGGTCTCGTGGGCTCGGAGATGT
Ad2.3 AGGCAGAA	CAAGCAGAAGACGGCATACGAGATTTCTGCCTGTCTCGTGGGCTCGGAGATGT
Ad2.4 TCCTGAGC	CAAGCAGAAGACGGCATACGAGATGCTCAGGAGTCTCGTGGGCTCGGAGATGT
Ad2.5 GGACTCCT	CAAGCAGAAGACGGCATACGAGATAGGAGTCCGTCTCGTGGGCTCGGAGATGT
Ad2.6 TAGGCATG	CAAGCAGAAGACGGCATACGAGATCATGCCTAGTCTCGTGGGCTCGGAGATGT
Ad2.7 CTCTCTAC	CAAGCAGAAGACGGCATACGAGATGTAGAGAGGTCTCGTGGGCTCGGAGATGT
Ad2.8 CAGAGAGG	CAAGCAGAAGACGGCATACGAGATCCTCTCTGGTCTCGTGGGCTCGGAGATGT
Ad2.9 GCTACGCT	CAAGCAGAAGACGGCATACGAGATAGCGTAGCGTCTCGTGGGCTCGGAGATGT
Ad2.10 CGAGGCTG	CAAGCAGAAGACGGCATACGAGATCAGCCTCGGTCTCGTGGGCTCGGAGATGT
Ad2.11 AAGAGGCA	CAAGCAGAAGACGGCATACGAGATTGCCTCTTGTCTCGTGGGCTCGGAGATGT
Ad2.12 GTAGAGGA	CAAGCAGAAGACGGCATACGAGATTCCTCTACGTCTCGTGGGCTCGGAGATGT
Ad2.13 GTCGTGAT	CAAGCAGAAGACGGCATACGAGATATCACGACGTCTCGTGGGCTCGGAGATGT
Ad2.14 ACCACTGT	CAAGCAGAAGACGGCATACGAGATACAGTGGTGTCTCGTGGGCTCGGAGATGT
Ad2.15 TGGATCTG	CAAGCAGAAGACGGCATACGAGATCAGATCCAGTCTCGTGGGCTCGGAGATGT
Ad2.16 CCGTTTGT	CAAGCAGAAGACGGCATACGAGATACAAACGGGTCTCGTGGGCTCGGAGATGT
Ad2.17 TGCTGGGT	CAAGCAGAAGACGGCATACGAGATACCCAGCAGTCTCGTGGGCTCGGAGATGT
Ad2.18 GAGGGGTT	CAAGCAGAAGACGGCATACGAGATAACCCCTCGTCTCGTGGGCTCGGAGATGT
Ad2.19 AGGTTGGG	CAAGCAGAAGACGGCATACGAGATCCCAACCTGTCTCGTGGGCTCGGAGATGT
Ad2.20 GTGTGGTG	CAAGCAGAAGACGGCATACGAGATCACCACACGTCTCGTGGGCTCGGAGATGT
Ad2.21 TGGGTTTC	CAAGCAGAAGACGGCATACGAGATGAAACCCAGTCTCGTGGGCTCGGAGATGT
Ad2.22 TGGTCACA	CAAGCAGAAGACGGCATACGAGATTGTGACCAGTCTCGTGGGCTCGGAGATGT
Ad2.23 TTGACCCT	CAAGCAGAAGACGGCATACGAGATAGGGTCAAGTCTCGTGGGCTCGGAGATGT
Ad2.24 CCACTCCT	CAAGCAGAAGACGGCATACGAGATAGGAGTGGGTCTCGTGGGCTCGGAGATGT

I. PCR1

To set up a PCR reaction with a unique barcode for each sample, make master mix (MM) first and aliquot into tubes (one for each sample) then add one unique Ad2 primer (containing the barcode) per tube of sample.

Components	**Volume**	**4.5xMM1**	**Tube**	**Primers**
Transposed DNA	10ul		1	eg. Ad 2.4
Nuc free water	10ul	45	2	Ad 2.5
Ad1 primer 25um	2.5ul	11.25	3	Ad 2.8
Ad2 primer 25um	2.5ul		4	Ad 2.11
2xNEB Next Hi-Fi PCR mix	25ul	112.5		
Total volume		37.5ul/sample		
37.5ul MM1 + 2.5ul Ad2 + 10ul transposed DNA = 50ul total volume				

Run the following PCR1 program conditions:

ATACPCR

1. 72°C – 5min
2. 98°C – 30sec
3. 98°C – 10sec
4. 63°C – 30sec
5. 72°C – 1min

Go to step 3 4 additional times

6. 4°C for ever

II. Perform qPCR to determine the number of additional PCR amplification cycles are required for each sample.

Take a 5ul aliquot of the PCR product generated in above (I) and place the remainder in the fridge (or if storing for longer than a few hours place in the -20°C freezer**)**. First make master mix 2 (MM2) and aliquot into a 96 well qPCR plate before adding the template.

Use white Lightcycler plates and seals #04729692001.
*dilute SYBR stock 1:100 before use.

Components	Volume	6 x MM2 ul
PCR product	5ul	
Nuc free water	3.66ul	21.96
Ad1 primer 25um (10uM stock)	0.625	3.75
Ad2 primer 25um (10uM stock)	0.625	
2xNEB Next Hi-Fi PCR mix	5ul	30
*SyberGreen 100x (10.000x in stock)	0.09ul	0.54
Total	15ul/sample	
9.375ul MM2 + 0.625ul Ad2 + 5ul PCR product		

Spin down the plate at 3000g for 2 minutes.
Using a Roche LightCycler 480.
Switch on the machine and let it calibrate. Press the arrow button to eject the plate and press it again to put it back in.
Click on LightCycler Software and select 'new experiment from template' and select the ATAC-Q1 protocol.
Check reaction volume is set to 15ul and option for SYBR I/HRM dye.
Click the tick to run and save the experiment in ATAC-Seq folder.

The qPCR conditions are as follows:

1. 98°C – 30sec – 1cycle
2. 98°C – 10sec
3. 63°C – 30sec
4. 72°C – 1min

From point 2 to 4 – 20 cycles.

Once the run is complete:

The number of cycles N corresponds to 1/3 max fluorescence intensity – Greenleaf protocol.

Set the baseline then look at where the curve plateaus for each sample and that gives you the value for 100% fluorescence.

e.g. If curve plateaus at 52 then divide by 3 = 17.3 then look for 17.3 on the y-axis and read down on the x-axis to determine the number of cycles (e.g. 7 cycles more).

Only do an extra 5-7 cycles, if any more are needed it's better to discard the library and repeat it.

III. PCR2 Once the number of additional cycles required is established place the remaining 45ul of sample back in the PCR machine.

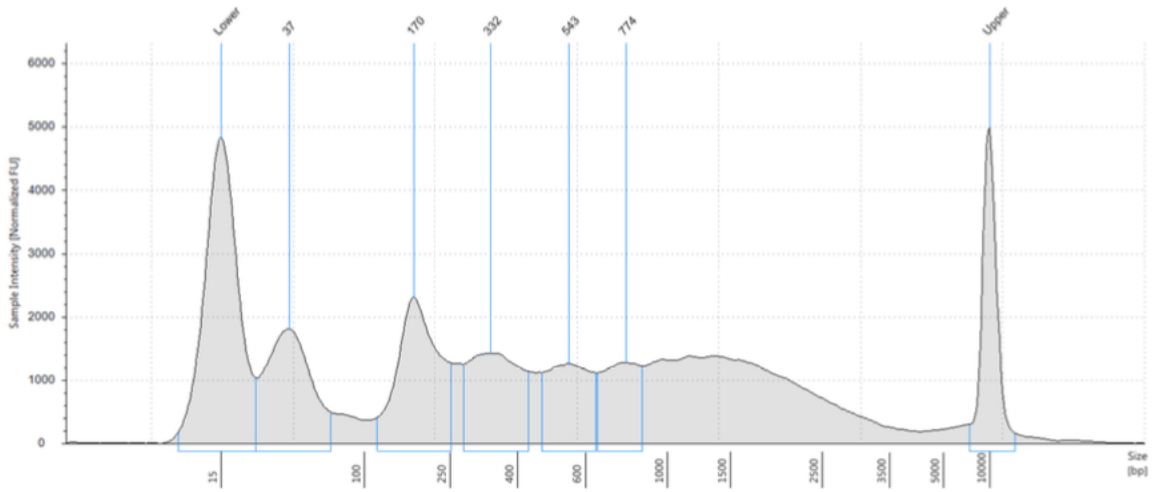
Program conditions: PCR **ATACPCR2** for each of remaining 45ul of sample from PCR1.

Samples
1. 98°C – 30sec 2. 98°C – 10sec 3. 63°C – 30sec 4. 72°C – 1min Go to 2 for an additional 5-7 cycles

Purify libraries using MinElute PCR purification kit (elute with 15 µL Buffer EB)

(Use a different Min Elute kit for Pre- and Post- Amplification)

Run an aliquot of each sample on the **D5000 screentape** and check that the library looks like this:



Sample Table

Well	Conc. [ng/ul]	Sample Description	Alert	Observations
D1	18.7			

Peak Table

There should be a peak at 150-250bp (the second peak from the lower marker above) which is the nucleosomal fragment.

IV. Size Selection

Size-select libraries for the subnucleosomal fragment (150-250 bp) on the Thermo Scientific E-Gel System.

Use a E-gel Size Select II 2% Agarose Gel with the E-Gel Sizing DNA Ladder and E-Gel Sample Loading Buffer.

Add sufficient E-Gel Sample Loading Buffer to each sample to make 25ul total volume and mix. Also mix the ladder by pipetting.

Take off the camera lid and switch on the machine at the back. Press the white button to open the lid.

Take well separators off the gel and click the gel into place with the top comb at the top of the machine.

Load 25ul of the E-Gel Sizing DNA ladder to the first, middle and last well.

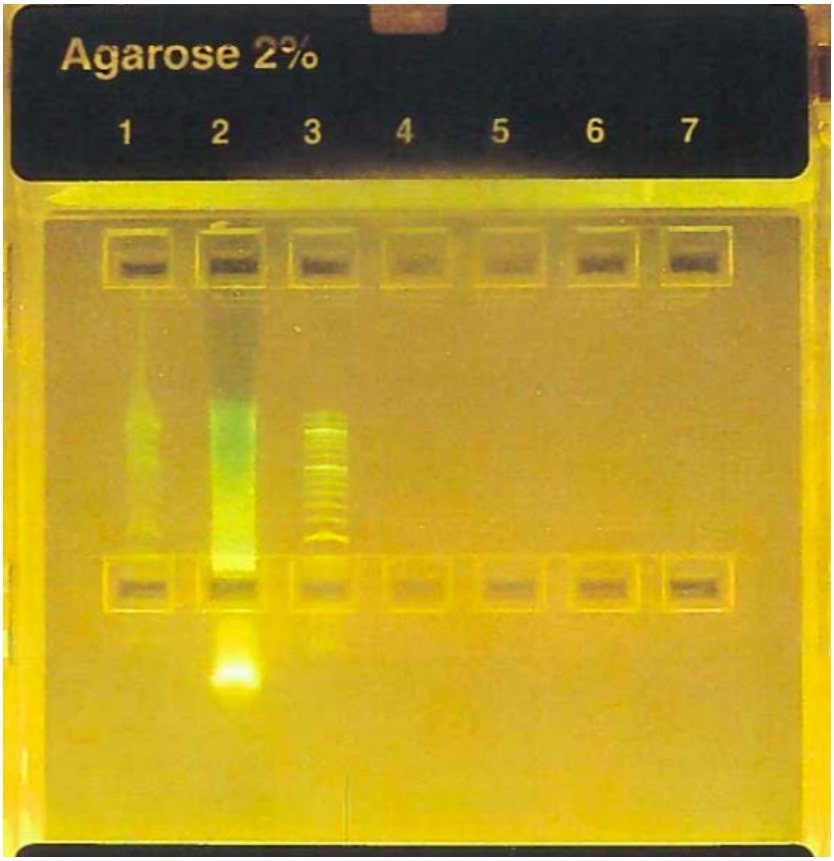
Add the samples spacing them evenly.

To each well that doesn't include sample or ladder and the second row add 40ul of water.

Select E-gel Size Select II 2% Gel from the list of options.

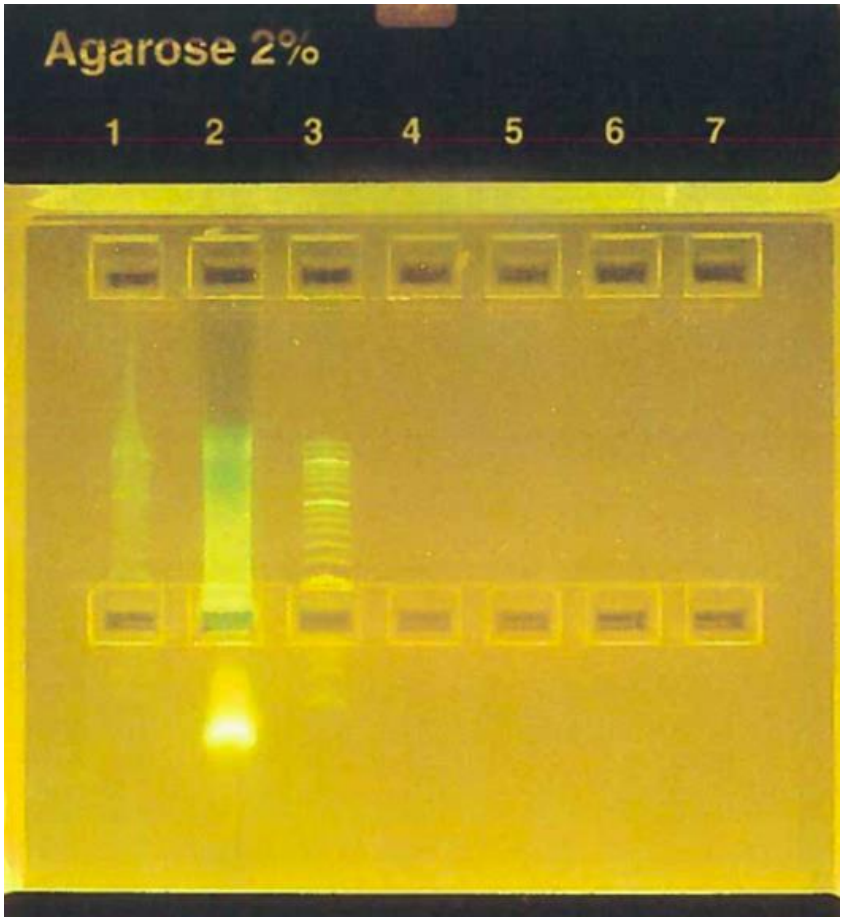
Set to run for 15 minutes and watch the time.

Press ‘pause’ before the size of band you want (150-250bp) reaches the wells in the second comb (reference line), take out the water (keep it) and replace with 20ul more water.

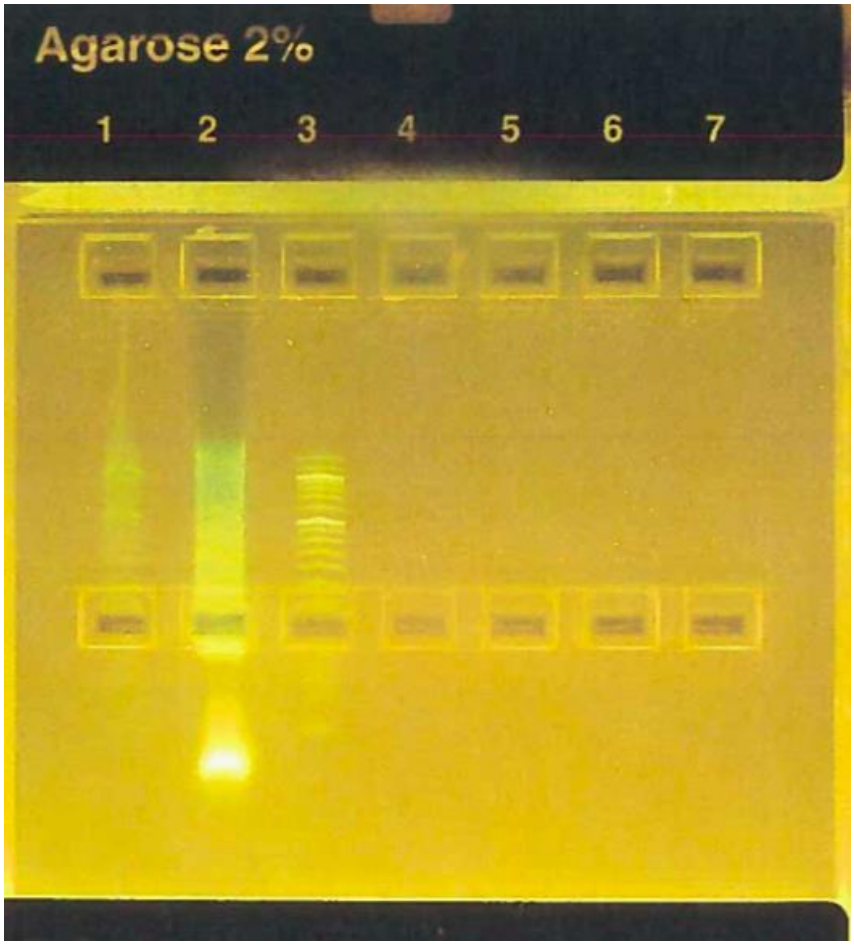


Resume run then stop run as soon as the band you want enters the well (this will only be 30 secs or so)

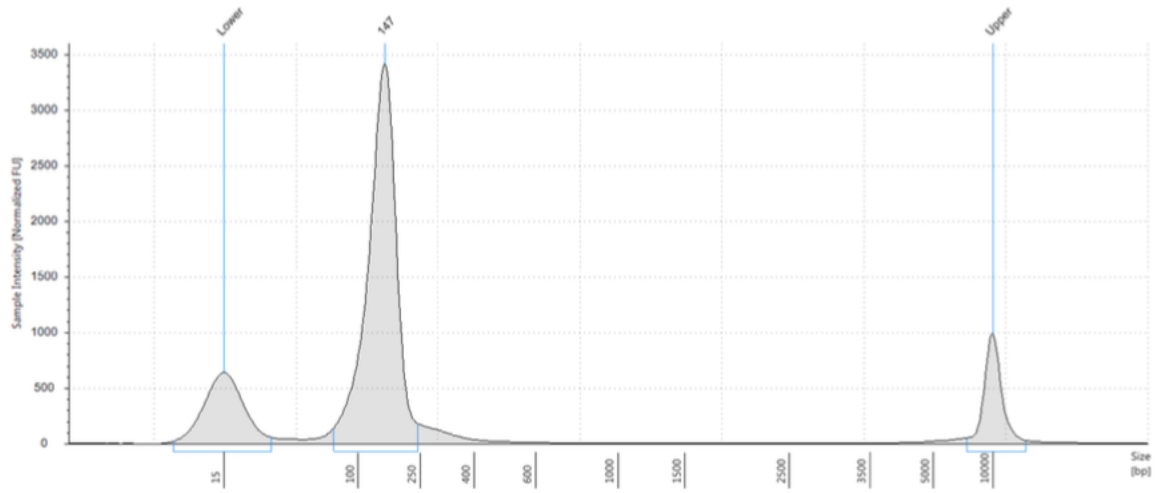
Pause when the size you want (150-200bp) hits the well in the second comb remove the sample and transfer to a new tube then add 20ul of new water and resume run.



Collect the next fragment and repeat as above, replace the water and watch to see if the bright 150-200bp band runs past the well, if it does run it back into the well using ‘Reverse E-gel’ programme and collect.



Run an aliquot of the collected samples (separately first to check the size of each fragment then combine those that are the correct size, usually the first fragment and reverse E-Gel fragment) on the **D1000 HS screentape** and check that the combined library looks like this:

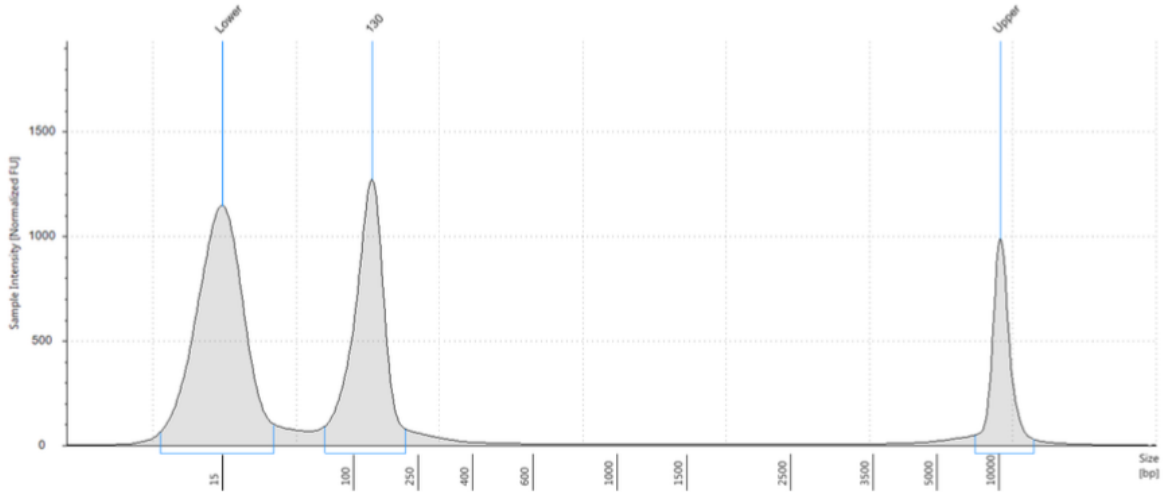


Sample Table

Well	Conc. [pg/µl]	Sample Description	Alert	Observations
C1	1140	Ad2.24 Fragment 1		

V. Pooling

Using the Illumina Pooling Calculator https://support.illumina.com/help/pooling-calculator/pooling-calculator.htm work out pooling volumes. Check the pool quantity and fragment size on the **D5000 HS screen tape.**



Sample Table

Well	Conc. [pg/µl]	Sample Description	Alert	Observations
B1	454	ATAC-Seq Library Pool	⚠	Caution! Expired ScreenTape device (used after two weeks of first use)

Submit the library pool for sequencing using a platform such as the Illumina NovaSeq to generate paired-end 50 bp reads. Ideally, >60 million reads should be obtained per each library in the pool (not including low-quality, mitochondrial, or duplicate reads). A low depth run on the NextSeq can be undertaken first to ascertain if library preparation has been successful, although this approach can underestimate the proportion of mitochondrial reads.

References

Corces, M. R., Trevino, A. E., Hamilton, E. G., Greenside, P. G., Sinnott-Armstrong, N. A., Vesuna, S., et al. (2017). An improved ATAC-seq protocol reduces background and enables interrogation of frozen tissues. *Nat. Methods* 14, 959–962. doi:10.1038/nmeth.4396.

Halstead, M. M., Kern, C., Saelao, P., Chanthavixay, G., Wang, Y., Delany, M. E., et al. (2020). Systematic alteration of ATAC-seq for profiling open chromatin in cryopreserved nuclei preparations from livestock tissues. *Sci. Rep.* 10, 5230. doi:10.1038/s41598-020-61678-9.