Experimental Protocol - ATAC-Seq of frozen pig muscle tissue samples

Summary

This protocol describes generation of ATAC-Seq libraries from frozen pig muscle tissue at the Roslin Institute. This experimental protocol was developed to investigate gene expression and regulation in developing muscle from large white x landrace piglets of varying sizes. A parallel RNA-Seq dataset was also generated for this study. The experiment was split into four steps described below.

STEP 1

Collection of frozen tissue samples for ATAC-Seq and RNA-Seq from large animals

(NB: Work quickly using reagents maintained at appropriate temperatures.)

1 Purpose / Introduction

To collect tissues for ATAC-Seq and RNA-Seq from large animals during post mortem for subsequent extraction of RNA and isolation of nuclei.

2 Equipment/Reagents/Materials

15ml Falcon tubes containing 5ml Sucrose Buffer with protease inhibitor tablet.

Cryotubes for snap freezing tissues.

Liquid Nitrogen dewar filled with liquid nitrogen.

No.11 and No. 22 Swan Morten Scalpels

Sterile Plastic Forceps

Sterile Scissors

Dry Ice

Wet Ice

Ziplock bags

Petri Dishes

3 Principle

Animals are euthanized according to University of Edinburgh protocol and a post mortem performed. Tissues are dissected out and cut into small pieces (~0.5cm diameter/200mg). For preservation of RNA, and for downstream isolation of nuclei from frozen tissue, the pieces of tissue are snap frozen separately in liquid nitrogen in 3-5 aliquots. A larger number of tissue pieces can be stored in one tube to use as a 'back-up' if the individual aliquots are insufficient, and/or for DNA isolation.

4 Procedure

4.1 At the farm all surfaces should be cleaned with disinfectant then RNaseZap and all participants should wear gloves and ensure they regularly change these gloves throughout the harvest.

4.2 Tissues should arrive from the post mortem team on a plastic petri dish. The tissue should be cleaned of any excess blood or gut contents (for GI tract tissues only) by dipping in sterile PBS using plastic forceps. A No. 22 Swan Morton disposable scalpel should then be used to chop the tissue into small pieces that are <0.5cm diameter in any direction. To secure the tissue for chopping either a No. 11 Swan Mortem Scalpel or sterile plastic forceps can be used. A set of new scalpels should be used for each sample.

4.3 Tissue pieces should be spread evenly and singly across cryovials for immediate preservation in liquid nitrogen. One aliquot can contain multiple pieces of tissue to be used as a 'back-up' should the single aliquots be insufficient. For transfer back to the lab decant the cryovials into dry ice and then then move to a -80°C freezer.

STEP 2

SOP: ATAC-Seq Isolation of Nuclear DNA from frozen tissue and tagmentation

Materials List

Item	Catalog Number	Manufacturer

Eppendorf Protein LoBind tubes 2.0ml PCR clean	0030108132	Eppendorf UK
Nextera DNA Sample Prep Kit	FC-121-1030	Illumina
Qiagen MinElute PCR Purification Kit	28004	Qiagen
TWEEN(R) 20,non-ionic, aqueous solution, 10% (w/v)	11332465001	Sigma Aldrich
$MgCl_2$ (1M)	AM9530G	Life Technologies
UltraPure 1M Tris-HCl Buffer, pH 7.5	15567027	Life Technologies
Trizma® hydrochloride solution, pH 7.8, 1M	T2569-100ML	Sigma Aldrich
EASYstrainer™ Cell sieve for 50ml tubes, 70µm mesh, blue, case of 50	542070	Greiner Bio-One Inc.
D-Sucrose 99.9% DNase-, RNase- and Protease-Free	10638403	Fisher Scientific UK Ltd
EDTA Stock Solution (500 mM, pH 8.0)	600215-100ml-CAY	Cambridge Bioscience
Calcium chloride, $CaCl_2$	15445389	Fisher Scientific UK Ltd
OptiPrep Density Gradient Medium	D1556-250ML	Scientific Laboratory Supplies Ltd.
Magnesium acetate solution, BioUltra, for molecular biology, approx. 1 M in H2O	63052-100ML	Sigma Aldrich

IGEPAL(R) CA-630, for molecular biology	I8896-50ML	Sigma Aldrich
Phenylmethanesulfonyl fluoride,>=98.5% (PMSF)	P7626-1G	Sigma Aldrich
2-Mercaptoethanol,>=99.0%	M6250-100ML	Sigma Aldrich
GentleMACS C-tubes	130-093-237	Mitenyi Biotec
Trypan Blue Solution 0.4%	15250061	Gibco

Equipment

GentleMACS Dissociator	Mitenyi Biotec
Bucket centrifuge with swing out rotor, chill setting and adaptor for 2ml eppendorf tubes.	Eppendorf
Thermomixer	Starlabs

I. Prior to ATAC-seq:

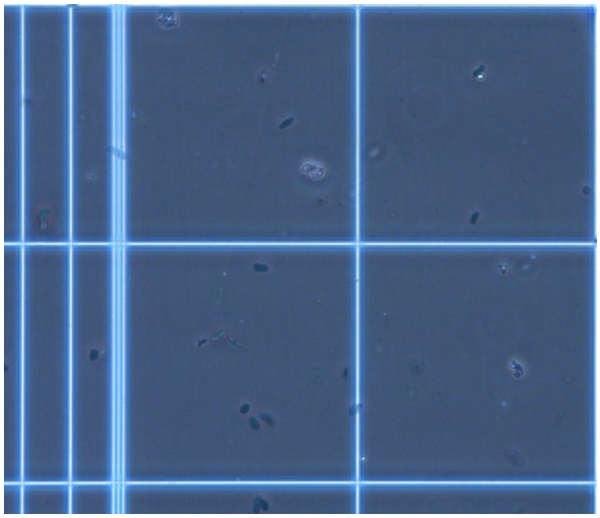
1. Make up the ATAC-Seq buffers the afternoon before and store in the fridge (see 'ATAC-Seq Buffers' table below).
2. Pre-chill centrifuge with the adaptors for 2ml tubes and swing-out rotor to 4°C.
3. Prepare and chill ATAC RSB buffer to 4°C (ideally, should be made fresh).
4. Label as many GentleMACS C-tube (Mitenyi Biotec Cat# 130-093-237) as required, one per sample.

II. Nuclei extraction from frozen muscle tissue:

NB: Throughout the protocol keep all buffers chilled and work quickly. Using centrifuge with swing-out rotor for all spins.

1. Take 200mg of frozen tissue.

2. Using a scalpel blade, chop the tissue into small pieces on a petri dish kept on dry-ice.
3. Place the tissue pieces in a GentleMACS C-tube and keep C-tube on dry ice until all the samples are prepared.
4. Place the C-tube on wet ice and add 1ml 1XHB buffer (+Protease Inhibitor Cocktail (PIC)) then allow to thaw for three minutes on ice.
5. Place the C-tubes on the GentleMACS dissociator two at a time and run the m_muscle_0.1_0.1 (equivalent to 'E0.1c Tube') programme twice.
6. Remove the tubes immediately and place back on wet ice.
7. Filter the sample through a 70µm corning cell strainer into a 50ml conical falcon tube (conical NOT flat-bottomed), first wetting the filter with 400ul of 1XHB buffer.
8. Rinse the C-tube with 1ml 1XHB buffer (+PIC) and put this through the filter.
9. Spin the cell solution down at 3000g for 5 minutes, remove the supernatant and resuspend the pellet in 400ul 1XHB buffer (+PIC) (the pellet may be invisible at this stage).
10. Transfer the sample to a 2ml "Protein Lo-Bind eppendorf tube".
11. Add 400µl of 50% Iodixanol solution to the 400µl of cell solution (final 25% Iodixanol)
12. Proceed with iodixanol gradient:
    - Slowly add 600µl of 29% Iodixanol solution **under** the previous mixture.
    - Slowly add 600µl of 35% Iodixanol solution **under** the 29% Iodixanol solution
13. In the swinging bucket centrifuge spin the 2ml tubes for 25 minutes at 3155g (max speed) at 4^{o}C with no break.
14. A thin "whitish" band should appear between layer 2 and 3 (**from the top**). Collect this band (200µl) and transfer it to a new collection tube.
15. Take an aliquot of 10µl for nuclei purity evaluation (microscope) and counting: +trypan blue -> microscope -> Purity and counting.
16. Check that the nuclei (they should stain blue with Trypan blue) look like this:

 x30 magnification

17. *Take 50.000 nuclei as determined by nuclei counting. For muscle tissue this usually means proceeding with all you have as there are less nuclei than for liver.*
18. (*Optional*) *Transfer the 50.000 nuclei to a new tube containing 1ml of ATAC-RSB + 0.1%Tween20 (and proceed to step 20).*
19. If using all nuclei prep (as expected for muscle) add 1ml of ATAC-RSB Buffer + 0.1%Tween20 to the whole sample.
20. Centrifuge the nuclei for 10 minutes at 500g at 4°c.
21. Aspirate supernatant and continue with tagmentation (the pellet may be invisible at this stage).

III. Tagmentation

1. Gently resuspend pellet in 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 Molecular Biology Grade Sterile H20
2. Incubate pellet with transposition mix for 60 min, 37°C, 300 rpm on thermomixer.
3. Purify transposed DNA with MinElute PCR purification kit (elute DNA with 15 µL Buffer EB)

    Optional stopping point: store transposed DNA at -20°C

ATAC-Seq buffers

6XHB Stable buffer		
Reagents	**Final conc**	**Volume for 100ml**
1M CaCl2	30 mM	3 ml
1M Mg(Ac)2	18 mM	1.8 ml
1M Tris-HCl pH7.8	60 mM	6 ml
Molecular Biology Grade Sterile H20	NA	Up to 100ml
6XHB UnStable buffer (kept in the fridge) : 650 µl/sample		
Reagents	**Final conc**	**Volume for 1sample (ul)**
6XHB Stable buffer	6X	650
PMSF 100mM (Protease Inhibitor)	0.1 mM	0.65
B-mercaptoethanol 14.3M	1 mM	0.045
1XHB UnStable buffer (kept in the fridge) : 2 ml/sample		
Reagents	**Final conc**	**Volume for 1sample (ul)**
6XHB Unstable buffer	1X	333.33
Sucrose 1M	18 mM	640
EDTA 500mM	60 mM	0.4
IgepalCA630 10%	0.1% or 0.4%	20
Molecular Biology Grade Sterile H20	NA	1006.27
50% Iodixinol Solution : 400 µl/sample		
Reagents	**Final conc**	**Volume for 1sample (ul)**
6XHB Unstable buffer	1X	66.67
Iodixanol stock solution (60%)	0.5	333.33
29% Iodixinol Solution : 600 µl/sample		
Reagents	Final conc	**Volume for 1sample (ul)**
6XHB Unstable buffer	1X	100
Sucrose 1M	160 mM	96
Iodixanol stock solution (60%)	29%	290
Molecular Biology Grade Sterile H20	NA	114
35% Iodixinol Solution : 600 µl/sample		
Reagents	**Final conc**	**Volume for 1sample (ul)**
6XHB Unstable buffer	1X	100
Sucrose 1M	160 mM	96
Iodixanol stock solution (60%)	35%	350
Molecular Biology Grade Sterile H20	NA	54
ATAC-RSB Buffer		
Final Concentration	**Stock Concentration**	**Amount Used from Stock (for 5ml)**
10 mM Tris-HCl, pH 7.4	1M Tris-HCl, pH 7.4	50ul
10 mM NaCl	5M NaCl	10ul
3mM MgCl2	1M MgCl2	15ul
Molecular Biology Grade Sterile H20 to 50ml		4.925ml
On morning before use to 5ml of RSB buffer add:		
0.1%(v/v) Tween 20	10% Tween 20	5ul

STEP 3

ATAC-Seq Library Preparation and Size Selection

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.

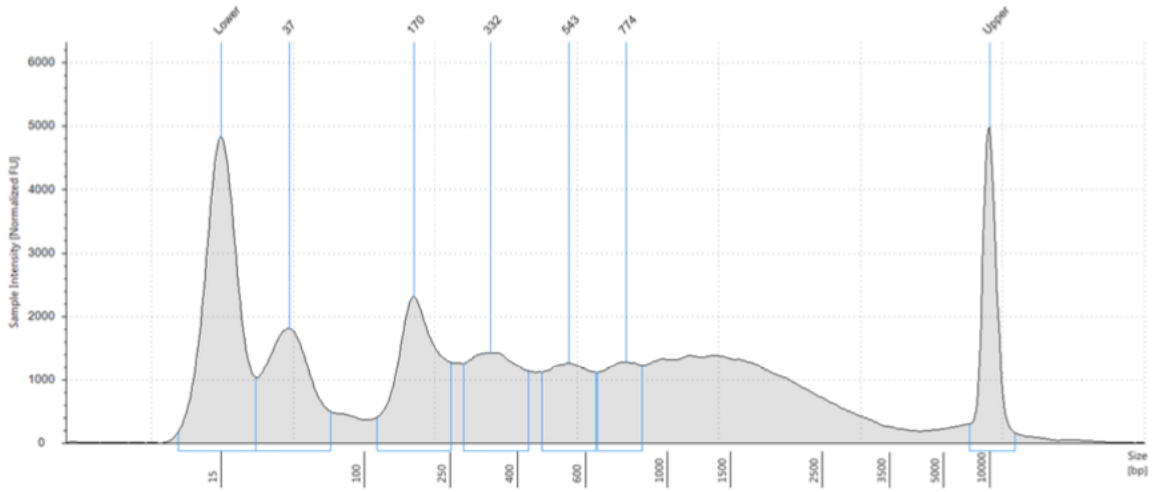
Samples
1. 98°C – 30sec 2. 98°C – 10sec

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/µl]	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.

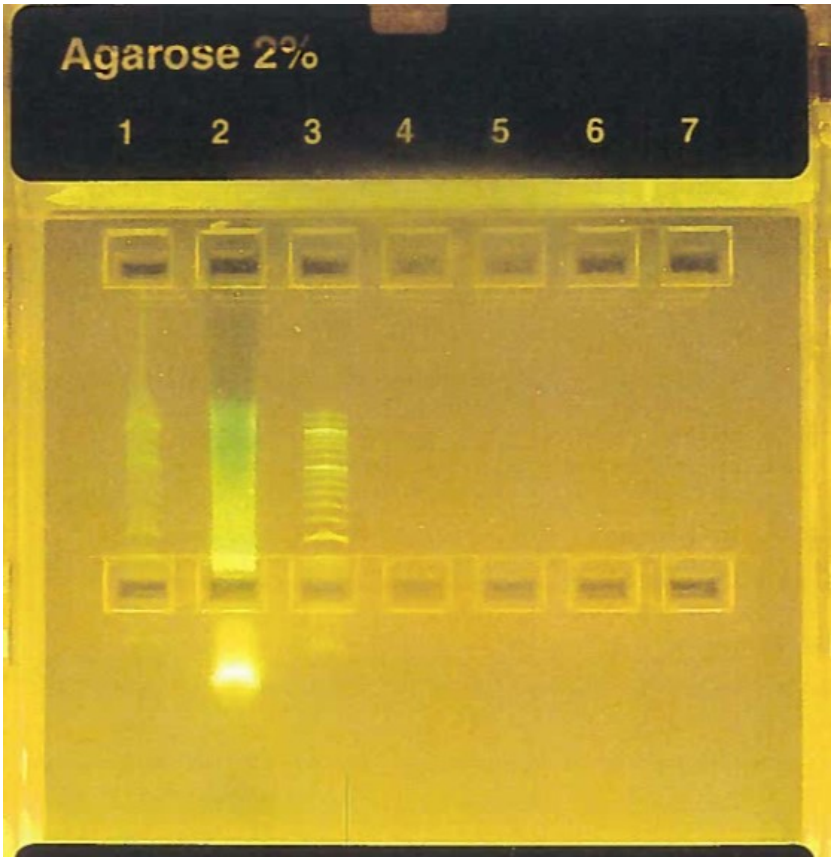
Add the samples spacing them evenly.

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.

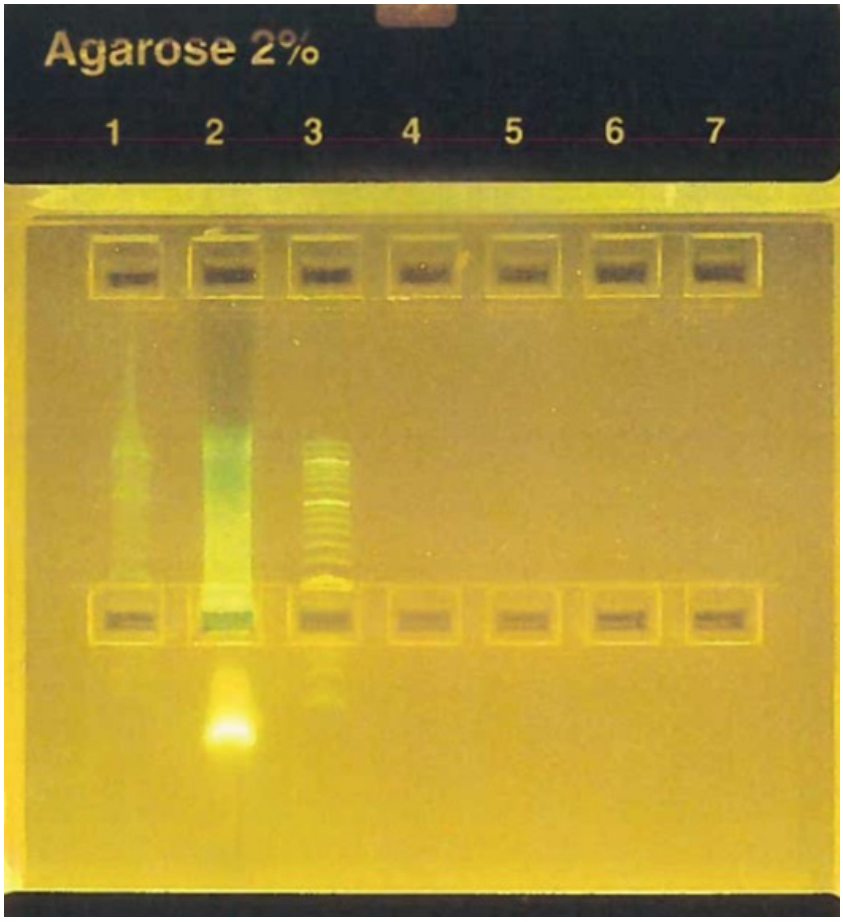
Set to run for 15 minutes and watch the time.

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.

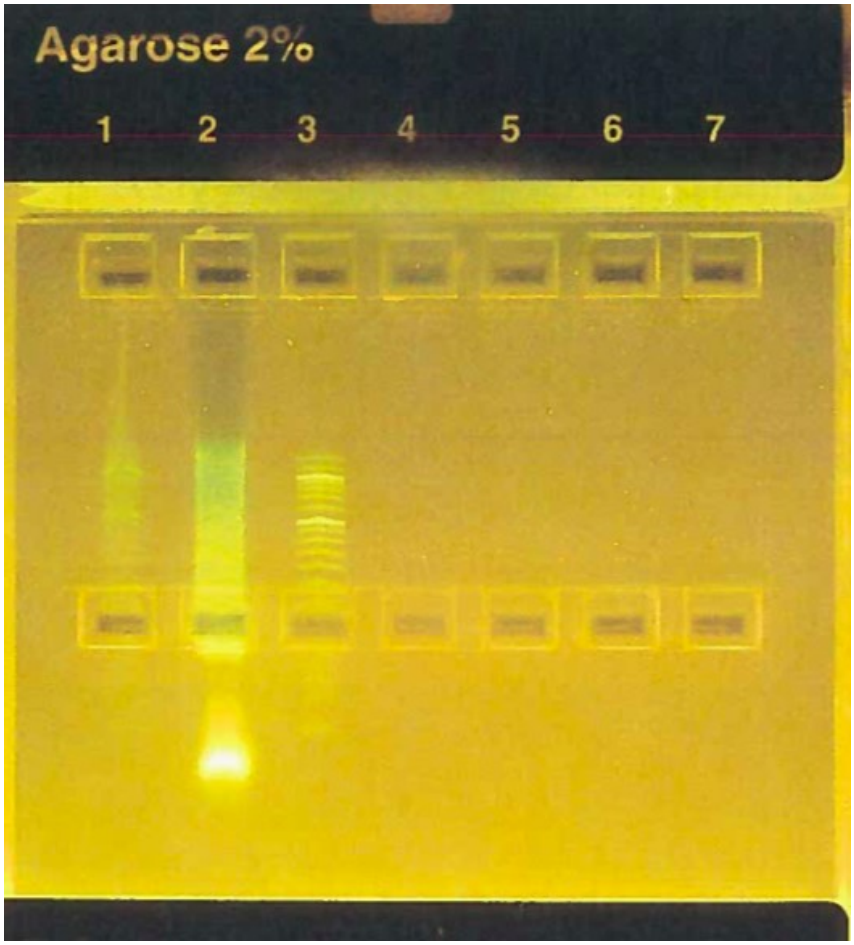


Resume run then stop run as soon as the band you want enters the well (this will only be 30 secs or so)

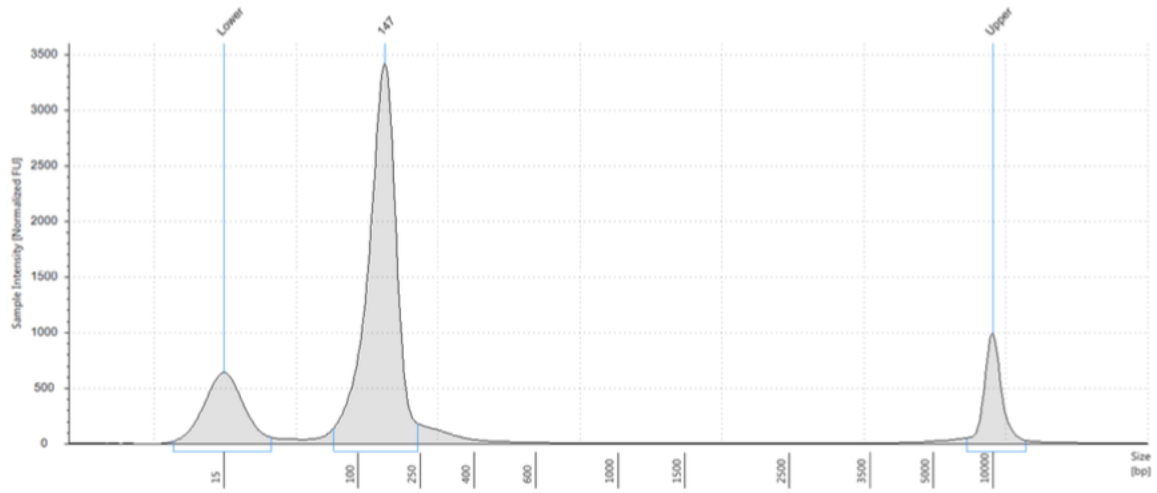
Pause when the size you want (150-200bp) hits the well in the second comb remove the sample and transfer to a new tube then add 20ul of new water and resume run.



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:

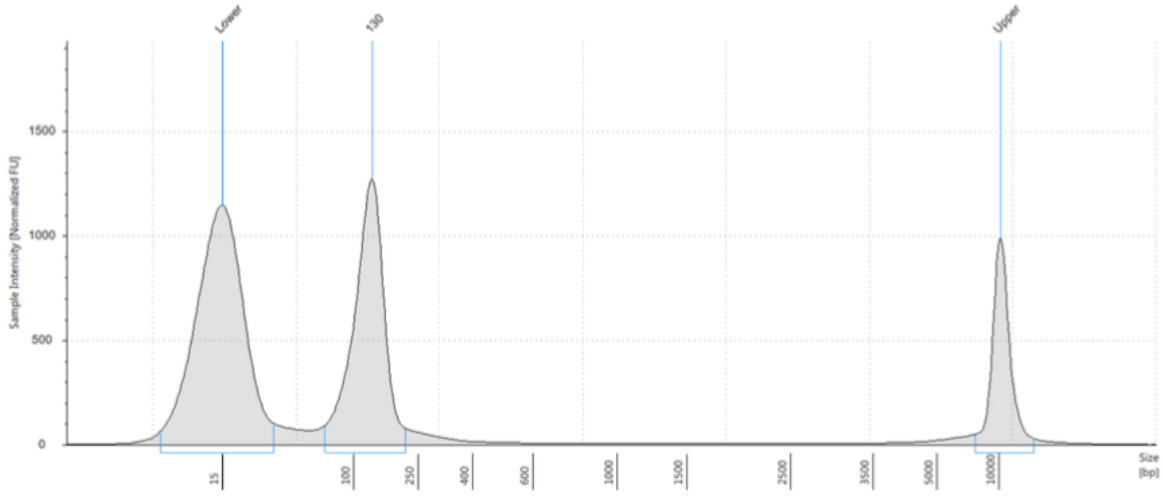


Sample Table

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.