FR-AgEncode: a French pilot project to enrich the annotation of livestock genomes

ATAC-SEQ Protocol

INRA
Division of Animal genetics

This protocol for ATAC-SEQ preparation has been tested on liver, spleen and CD4+/CD8+ cells from pigs, goats, cattle and chickens.

Written by:
Adeline Goubil (adeline.goubil@jouy.inra.fr)

November 2015

I. Cell dissociation

Around 1 hour, at room temperature. If working from cells go directly to step II.

Required reagents
- PBS 1X for Cell Culture (Eurobio; Cat # CS1PBS01-01)
- RPMI 1640 (Eurobio; Cat # CM1RPM00-01)
- StemPro® Accutase® (Life technologies; Cat # A1110501)
- Trypan blue solution (Thermo fisher Scientific; Cat # T10282)

Required material
- Pipettes and tips
- Forceps Dumont
- Scalpel and scalpel blades
- Petri dishes (90mm)
- Falcon® tubes 50ml
- 10ml, 20 ml pipettes
- Pipet-aid
- Cell strainer 70µm (BD Biosciences Dutscher; Cat # 352350)
- 1.5 ml, 2ml Eppendorf tubes
- Centrifuge machine for 50ml Falcon® tubes at room temperature
- 37 °C oven or 37 °C water bath
- Microscope, Malassez cell and hand cells counter
-Timer
- Marker pen
- Tube holder
- Ice bucket

Prior to start
- Get ice
- Warm a 37°C water bath
- Warm the Stempro® Accutase®, the PBS and the RPMI 1640 at 37°C

1. In a petri dish, dilacerate the fresh tissue using scalpel blades as fine as you can dissect
2. In a 50 ml Falcon® tube wash the dilacerate tissue using 37°C PBS to remove the circulating blood as much as possible. For that, add PBS to the pieces, agitate well, decant and remove the supernatant using a 10 ml pipette, repeat until the supernatant is transparent. Remove the supernatant
3. Add 10 to 20ml of StemPro® Accutase® previously warmed at 37°C and incubate for 10 minutes with moderate agitation on a rotating wheel at 37°C or in a 37°C water bath
4. Using a 20ml pipette and a pipet-aid, pipet up and down to homogenize the suspension (if it is well done, you won't have problem to do it with a 10ml pipette)
5. Filter through a cell strainer of 70µm into a new 50ml Falcon® tube. Fill tube to 50 ml with 37°C RPMI 1640
6. Centrifuge at 250g for 5 minutes
7. Remove the supernatant
8. Make an additional PBS wash to make sure to remove the remaining Accutase®, centrifuge at 250g for 5 minutes and remove supernatant
9. Fill tubes with 50 ml 37 °C RPMI 1640
10. Evaluate cell quality and count the cells

1ml from step 9. is diluted 5 to 10 times, depending on the species and tissue, and then evaluated by microscope

11. Count and Organize 50 000 cells aliquots in new 1.5 ml Eppendorf tubes

50 000 cells are the amount of alive cells recommended to use by Buenrostro and Al. In our experiment with chickens spleen and liver we have tested both 50 000 and 150 000 cells aliquots.

Proceed immediately to step II.

II. Process to ATAC-Seq preparation
Around 1 hour, on Ice

Required reagents
- PBS 1X for Cell Culture (Eurobio; Cat # CS1PBS01-01)
- Lysis Buffer (store up to 1 week at 4°C):
  10 mM Tris.Cl, pH 7.4
  10 mM NaCl
  3 mM $MgCl_2$ (Sigma-Aldrich; Cat # 63069-100ML)
  0.1 % (v/v) Igepal ® CA-630 (Sigma-Aldrich; Cat # I8896-50ML)
  Filtered the solution using a sterile filtration unit 0.22 µm

Required material
- Pipettes and tips
- Centrifuge machine for 2 ml tubes 4°C
- Pens
- Tube holder
- Ice bucket

Prior to start
- Get ice
- Cold a PBS aliquot and the lysis buffer on ice

1. Centrifuge 50,000 cells 5 min at 500g, 4°C
2. Remove and discard supernatant. Wash cells once with 50 µl of cold PBS buffer. Centrifuge 5 min at 500g, 4°C
3. Remove and discard supernatant. Gently pipet up and down to resuspend the cell pellet in 50 µl of cold lysis buffer. Centrifuge immediately 10 min at 500g, 4°C
4. Discard the supernatant, and immediately continue to transposition reaction.

Be sure to keep the cells pellet set on ice

III. Transposition Reaction
Around 1 hour, on Ice

Required reagents
- TD Buffer from Nextera DNA Library Preparation kit (Illumina; Cat # FC-121-1030)
- Tn5 Transposas from Nextera DNA Library Preparation kit (Illumina; Cat # FC-121-1030)
- Nuclease free water (Sigma; Cat # 95284-100ML)

Required material
- Pipettes and tips
- 1.5 ml, 2ml Eppendorf tubes
- 37 °C water bath
- Timer
- Pens
- Tube holder
- Ice bucket
-Vortex

Prior to start
- Get ice
- Warm a 37°C water bath

1. Prepare a transposition reaction solution to be kept on ice as follows:

2x TD Buffer	25 µl	25 µl
Tn5 Transposas	2.5 µl	1 µl
Nuclease Free H2O	22.5 µl	24 µl
	50 µl Total	*50 µl Total*

2.5 µL Tn5 Transposas is the amount of enzyme recommended to use by Buenrostro and Al. In our experiment, with the different tissues and species we have tested, we have used both 2.5 and 1 µl Tn5 Transposas.

2. Distribute 50 µl of the transposition reaction solution previously made to each cells aliquot
3. Gently pipette to resuspend nuclei in the transposition reaction mix
4. Incubate the transposition reaction at 37°C for 30 min

Immediately purify using a Qiagen MinElute® PCR Purification Kit

IV. Qiagen MinElute® PCR Purification Kit

Around 30 min, at Room temperature

Required reagents
- Qiagen MinElute® PCR Purification Kit (Qiagen; Cat # 28004)
Prior to use add ethanol (96-100%) to buffer PE concentrate; add 1:250 volume pH indicator I to Buffer PB.
- 3 M sodium acetate, pH 5.0

Required material
- Pipettes and tips
- 1.5 ml Eppendorf tubes
- Centrifuge machine for 2 ml tubes 4°C
-Timer
- Pens
- Tube holder

Prior to start
- Get MinElute columns stored at 4°C

1. Add 5 volumes (250 µl) of Buffer PB to 1 volume (50 µl) of the transposition reaction and mix
Checks that the color of the solution is yellow otherwise add 10µl 3 M sodium acetate, pH 5.0 and mix.
2. Place a MinElute column in a provided 2 ml collection tube properly labeled
3. To bind DNA, apply the sample to the MinElute column and centrifuge for 1 min at 13000 rpm
4. Discard flow through and place the MinElute column back in the same tube
5. To wash, add 750 µl Buffer PE to the MinElute column and centrifuge for 1 min at 13000 rpm
6. Discard flow through and place the MinElute column back in the same tube. Centrifuge the column for an additional 1 min at max speed
7. Place the MinElute column in a clean labeled 1.5 ml Eppendorf centrifuge tube
8. To elute DNA, add 10 µl Buffer EB, let the column stand for 1 min and then centrifuge for 1 min at 13000 rpm

DNA is stored at -20°C or kept on Ice and processed to PCR amplification

V. PCR Amplification
Around 4 hour, on ice

Required reagents
- Nuclease free water (Sigma; Cat # 95284-100ML)
- 25 µM PCR Primer 1(custom-synthesized by Integrated DNA technologies (IDT); cf sequences **Annexe 1**)
- 25 µM PCR Barcoded Primer 2 (custom-synthesized by Integrated DNA technologies (IDT); cf **Annexe 1**)
- NEBNext High Fidelity 2 x PCR Master Mix (New England Biolabs (NEB); Cat # M0541S)
- 100× SYBR Green I

Required material
- Pipettes and tips
- 0.2 ml PCR tubes
- PCR thermal cycler
- QuantStudio 12K flex (Life Technologies)
- 1.5 ml Eppendorf tubes
- Centrifuge machine for 2 ml tubes 4°C
- Pens
-Tube holder
- Ice bucket
-Vortex

Prior to start
- Get ice

1. To amplify transposed DNA fragments, combine the following in a 0.2 ml PCR tube:

10 µl transposed DNA
10 µl nuclease-free H_2O
2.5 µl 25 µM PCR Primer 1 (=Ad1_noMX)
2.5 µl 25 µM Barcoded PCR Primer 2 (=Ad2.x)
25 µl NEBNext High Fidelity 2 x PCR Master Mix
50 µl Total

You might need to pay close attention when library are made to be multiplexed on a same line when sequenced. Indexes need to be chosen properly, they could be more or less compatible between each other.

2. Amplify as follows:

1 cycle :	5 min	72°C
	30 sec	98°C
5 cycles:	10 sec	98°C
	30 sec	63°C
	1 min	72°C

3. Perform qPCR

To reduce GC and size bias in PCR, the appropriate number of PCR cycles (*N*) is determined using qPCR, allowing us to stop amplification prior to saturation.
To calculate the additional number of cycles needed, plot linear Rn versus cycle and determine the cycle number that corresponds to one-third of the maximum fluorescent intensity.

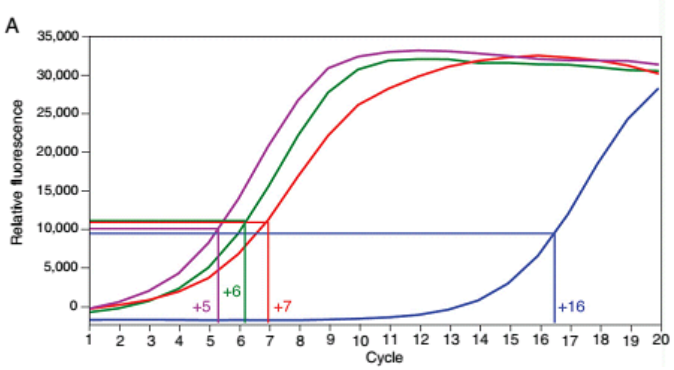

Figure 1: Representative amplification plot demonstrating the correct number of additional cycles to perform for four ATAC-seq libraries.

- Combine the following in a 0.2 ml PCR tube

5 µl	of previously PCR-amplified DNA
4.41µl	nuclease-free H2O
0.25 µl	25 µM PCR Primer 1(=Ad1_noMX)
0.25 µl	25 µM Barcoded PCR Primer 2(=Ad2.x)
0.09 µl	100× SYBR Green I
5 µl	NEBNext High-Fidelity 2× PCR Master
15 µl	*Total*

- Using the QuantStudio 12K flex instrument, cycle as follows:

1 cycle:	30 sec	98°C
20 cycles:	10 sec	98°C
	30 sec	63°C
	1 min	72°C

- Calculate the additional number of cycles *N* needed

4. Run the remaining 45 µl PCR reaction to the cycle number *N* determined by qPCR as follows:

1 cycle :	30 sec	98°C
N cycles:	10 sec	98°C
	30 sec	63°C
	1 min	72°C

DNA is stored at 4°C or immediately purify using a Qiagen MinElute® PCR Purification Kit

VI. Qiagen MinElute® PCR Purification Kit

Around 30 min, at room temperature

Required reagents
- Qiagen MinElute® PCR Purification Kit (Qiagen; Cat # 28004)
- 3 M sodium acetate, pH 5.0

Required material
- Pipettes and tips
- 1.5 ml Eppendorf tubes
- Centrifuge machine for 2 ml tubes 4°C
-Timer
- Pens
- Tube holder

Prior to start
- Get MinElute columns stored at 4°C

1. Add 5 volumes (250 µl) of Buffer PB to 1 volume (45 µl) of the PCR reaction and mix.
Checks that the color of the solution is yellow otherwise add 10µl 3 M sodium acetate, pH 5.0 and mix.
2. Place a MinElute column in a provided 2 ml collection tube properly labeled
3. To bind DNA, apply the sample to the MinElute column and centrifuge for 1 min at 13000 rpm
4. Discard flow through and place the MinElute column back in the same tube
5. To wash, add 750 µl Buffer PE to the MinElute column and centrifuge for 1 min at 13000 rpm
6. Discard flow through and place the MinElute column back in the same tube. Centrifuge the column for an additional 1 min at max speed
7. Place the MinElute column in a clean1.5 ml centrifuge tube
8. To elute DNA, add 20 µl Buffer EB, let the column stand for 1 min and then centrifuge for 1 min at 13000 rpm

DNA is stored at -20°C or immediately process to AMPure XP beads purification

VII. AMPure XP beads purification; 1.6 v.

Around 1 hour, at room temperature

Required reagents
- EB Buffer
- Agencourt AMPure XP - PCR Purification (Beckman Coulter; Cat # A63880)
- EtOH 96-100 %
- Nuclease free water (Sigma; Cat # 95284-100ML)
- RSB from Nextera DNA Library Preparation kit (Illumina; Cat # FC-121-1030)

Required material
- Pipettes and tips
- 1.5 ml Eppendorf tubes
- DynaMag™-2 Magnet (Thermo Fisher Scientific; Cat # 12321D)
- Centrifuge machine for 2 ml tubes 4°C
- Vortex
- Timer
- Pens
- Tube holder
- Vortex

Prior to start
- Get AMPure XP beads out of the fridge 30 min prior to start
- Make fresh EtOH 70 %

1. Add and mix 80 µl EB Buffer to the previous 20 µl sample and transfer the solution in a new Eppendorf tube filled with 160 µl AMPure XP beads. Pipet up and down 10 times. Vortex and quick spin if needed
2. Incubate at Room Temperature for 15 min
3. Apply the tube on the DynaMag™-2 Magnet rack and stand for 5 min until the solution is clear
4. Remove the supernatant. Be careful to not disturb the beads
5. Wash beads with cold 250µl EtOH 70 % freshly made
6. Incubate for 1 min and remove the EtOH
7. Let the beads dry for 10 to 15 min or until the beads look “cracked”
8. Resuspend beads in 40 µl RSB; Remove tube from the rack and pipet up and down 20 times. Vortex and centrifuge
9. Incubate 2 min at Room temperature
10. Apply the tube on the DynaMag™-2 Magnet rack and let stand for 5 min until the solution is clear
11. Get 38 µl of the solution and put it in a new labeled 1.5 ml Eppendorf tube. Do not disturb the beads

VIII. Quality Control

Around 2 hours, at room temperature

Required reagents
- Agilent High Sensitivity DNA Kit (Agilent Technologies; Cat # 5067-4626)
- Qubit® dsDNA HS Assay Kit (Thermo Fisher Scientific; Cat # Q32851)

Required material
- Qubit® Fluorometer (Thermo Fisher Scientific)
- Agilent 2100 Bioanalyzer
- Pipettes and tips
- Centrifuge machine for 2 ml tubes 4°C
- Vortex
- Tube holder
- Ice bucket

Prior to start
- Get ice
- Get the Agilent High Sensitivity DNA Kit out of the fridge 30 min prior to start

1. Monitor RNA yield using a Qubit® Fluorometer
2. Assess RNA integrity using an Agilent 2100 Bioanalyzer and High sensitivity DNA kit

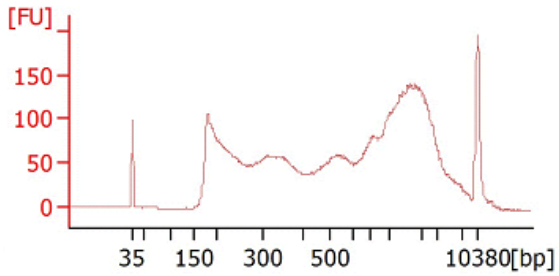

Figure 2: Example of an Agilent Bioanalyzer Electropherogram from GM 12878 lymphoblastoid cells after Atac-seq preparation treatment.

If a 50-60 bps peak still remained repeat steps **VII.** and **VIII.**

Annexe

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	AAGCAGAAGACGGCATACGAGATCAGCCTCGGTCTCGTGGGCTCGGAGATGT
Ad2.11_AAGAGGCA	AAGCAGAAGACGGCATACGAGATTGCCTCTTGTCTCGTGGGCTCGGAGATGT
Ad2.12_GTAGAGGA	AAGCAGAAGACGGCATACGAGATTCCTCTACGTCTCGTGGGCTCGGAGATGT
Ad2.13_GTCGTGAT	AAGCAGAAGACGGCATACGAGATATCACGACGTCTCGTGGGCTCGGAGATGT
Ad2.14_ACCACTGT	AAGCAGAAGACGGCATACGAGATACAGTGGTGTCTCGTGGGCTCGGAGATGT
Ad2.15_TGGATCTG	AAGCAGAAGACGGCATACGAGATCAGATCCAGTCTCGTGGGCTCGGAGATGT
Ad2.16_CCGTTTGT	AAGCAGAAGACGGCATACGAGATACAAACGGGTCTCGTGGGCTCGGAGATGT
Ad2.17_TGCTGGGT	AAGCAGAAGACGGCATACGAGATACCCAGCAGTCTCGTGGGCTCGGAGATGT
Ad2.18_GAGGGGTT	AAGCAGAAGACGGCATACGAGATAACCCCTCGTCTCGTGGGCTCGGAGATGT
Ad2.19_AGGTTGGG	AAGCAGAAGACGGCATACGAGATCCCAACCTGTCTCGTGGGCTCGGAGATGT
Ad2.20_GTGTGGTG	AAGCAGAAGACGGCATACGAGATCACCACACGTCTCGTGGGCTCGGAGATGT
Ad2.21_TGGGTTTC	AAGCAGAAGACGGCATACGAGATGAAACCCAGTCTCGTGGGCTCGGAGATGT
Ad2.22_TGGTCACA	AAGCAGAAGACGGCATACGAGATTGTGACCAGTCTCGTGGGCTCGGAGATGT
Ad2.23_TTGACCCT	AAGCAGAAGACGGCATACGAGATAGGGTCAAGTCTCGTGGGCTCGGAGATGT
Ad2.24_CCACTCCT	CAAGCAGAAGACGGCATACGAGATAGGAGTGGGTCTCGTGGGCTCGGAGATGT

Annexe 1: Oligo designs. A list of ATAC-seq oligos used for PCR.

References

Buenrostro JD, Giresi PG, Zaba LC, Chang HY and Greenleaf WJ, 2013. Transposition of native chromatin for multimodal regulatory analysis and personal epigenomics. *Nature methods*. 10(12):1213-1218. doi:10.1038/nmeth.2688.

Buenrostro JD, Wu B, Chang HY and Greenleaf WJ, 2015. ATAC-seq: A Method for Assaying Chromatin Accessibility Genome-Wide. *Curr. Protoc. Mol. Biol.* 109:21.29.1-21.29.9. doi: 10.1002/0471142727.mb2129s109