ATAC-seq protocol part 2: nuclear isolation, transposition, and library preparation

Authors:

Rose.Daniels@roslin.ed.ac.uk (Postdoc), queries regarding protocol

Diego.Perojil@ed.ac.uk (PhD researcher), queries regarding protocol

Daniel.Macqueen@roslin.ed.ac.uk (PI), all queries

This protocol describes a method to isolate nuclei using cryopreserved cell homogenates from salmonid embryos, and subsequent transposition and library preparation for ATAC-seq. This protocol can be performed in two or three days and is divided into “Day 1 – Tissue disruption, nuclei isolation, transposition and clean up”, “Day 2 – library amplification” and “Same day or next day – library clean-up and assessment”. Each section will have its own list of consumables.

This protocol is based on Buenrostro et al. (1) and Omni-ATAC Supplementary Protocol 1 (2), with the following modifications:

- This protocol is based on starting with flash frozed or cryopreserved cells.
- Library clean-up using column purification will not remove primer-dimers. In addition, libraries often contain an excess of large fragments (>1000bp). Library clean-up is therefore performed using double-sided bead purification. You can assess your amplified libraries prior to clean-up to determine if double-sided purification is needed.

1. Day 1 – Nuclei isolation, transposition and clean up.

Overview: The following steps describe how to isolate nuclei from cryopreserved salmonid embryos, as well as how to perform ATAC-seq nuclei transposition, and clean up of transposed DNA using columns prior to amplification.

Preparations:

- Pre-chill a fixed rotor centrifuge for 1.5 – 2ml tubes to 4°C
- Pre-book thermomixer and heat it to 37°C
- Prepare solutions and buffers (outlined below)

Reagents and consumables:

Make all reagents fresh on the day, except 2x ST which can be made the day before.

- 2x stock of salt-Tris solution (ST buffer) – make 10ml:
  - *146mM NaCl (Thermo Fisher Scientific, catalog no.AM9759)* ***(292ul)***
  - *10mM Tris-HCl pH 7.5 (Thermo Fisher Scientific, catalog no. 15567027)* ***(100ul)***
  - *1mM CaCl2 (VWR, E506-100ml)* ***(10ul)***
  - *21mM MgCl2 (Sigma-Aldrich, catalog no. M1028)* ***(210ul)***
  - *Nuclease-free wáter (VWR E476-500ml)* ***(9388ul)***
- 1x ST-buffer solution – make 5ml:
  - *Dilute 2x in nuclease-free water (1:1)*
- Working solution (TST) – make 10ml
  - *2x ST buffer (5ml)*
  - *1% Tween-20 (Sigma-aldrich, catalog no. P-7949)* ***(300ul)***
  - *2% BSA (New England Biolabs, catalog no. B9000S)* ***(50ul)***
  - *Nuclease free water* ***(4.65ml)***
  - *Proteinase Inhibitor Cocktail tablet (PIC)* ***(1 tablet)***
- PBS – make 10ml
  - PBS 10x (1ml)
  - Nuclease free water (9ml)
- PBS + PIC – make 10ml
  - PBS 10x (1ml)
  - Nuclease free water (9ml)
  - 1 PIC tablet

Make sure PIC tablets dissolve.

- *40um Falcon cell strainers*
- *Ice pellets*
- *Ice tray*
- *Dry ice*
- *Dry ice container (such as a polystyrene box)*
- *50ml Falcon tubes*

- *1.5ml Eppendorf tubes*
- *MinElute PCR purification kit*

1.1. Nuclei isolation workflow

1.1.1. Take the sample in dry ice to work space. When ready, place sample on ice, open cryotube and make up volume up to 1ml with ice cold PBS+PIC, pipette up and down until only a small clump of ice is left.
1.1.2. Centrifuge preparation at 4ºC for 5min at 500RCF.
1.1.3. Discard supernatant and resuspend pellet in 1ml cold PBS+PIC.
1.1.4. Centrifuge preparation at 4ºC for 5min at 500RCF.
1.1.5. Discard supernatant and resuspend pellet in 1ml cold TST solution.
1.1.6. Incubate on ice for 5min, gently pipetting up and down with a P1000 pipette set to 800ul. Avoid forming bubbles or froth.
1.1.7. Filter through a 40um filter into a 50ul Falcon tube.
1.1.8. Add an additional 1ml of TST buffer over filter.
1.1.9. Bring volume up to 5ml using 3ml of 1x ST buffer.
1.1.10. If liquid splashed the walls of the tube, give a quick (10s) spin at low speed in a swinging bucket centrifuge to collect all liquid at the bottom of the tube.

This is a non-critical step but avoiding it might lead to a slight loss of nuclei.

1.1.11. Transfer sample to a 15ml Falcon tube.
1.1.12. Centrifuge at 500RCF for 5min at 4ºC in a swinging bucket centrifuge.
1.1.13. Discard supernatant and resuspend in 1x ST buffer. Resuspension volume is dependant on size of pellet, usually in the range of 100-200ul.
1.1.14. Count nuclei using trypan blue and a haemocytometer.
1.1.15. Put forward 50.000 to 75.000 nuclei for the next step and make up the solution to 1ml with 1x ST buffer.
1.1.16. Centrifuge nuclei aliquot at 800g, for 10 minutes at 4ºC.

1.2. Transposase reaction and clean-up

1.2.1. Make the following transposase reaction mix:

Reagent	Volume per sample (ul)
2x Tagment DNA (TD) buffer	25
Transposase	1.25
PBS	16.5
Digitonin (1%)	0.5
Tween-20 (10%)	0.5
Nuclease free H_2O	6.25
Total	50

1.2.2. Discard supernatant and resuspend the pellet in 50ul of transposase reaction mix by pipetting up and down 6 times.
1.2.3. Incubate the reaction at 37ºC for 30 minutes in a thermomixer at 900RPM.
1.2.4. Stop the reaction and purify the resulting DNA fragments with MinElute PCR purification kit.
1.2.5. Elute DNA in 21ul EB (Present in MinElute PCR purification kit)
1.2.6. Eluted DNA can be stored at -20ºC until ready to amplify.

2. Day 2 – Library amplification

Overview: This section describes the process used to generate amplified libraries starting from eluted transposed DNA from salmonid embryos. To reduce GC and size bias in PCR, the appropriate number of PCR cycles is determined using qPCR to see when to stop the amplification prior to saturation. However, before we can do that we will do a pre-amplification step with 5min extension at 72°C to allow for extension at both ends of primers after transposition.

Reagents:

- 96 well PCR plate or PCR tube strip, depending on number of samples.
- PCR cap strips.
- qPCR plate.
- qPCR compatible cap strips.
- IDT® for Illumina® Nextera™ DNA UD Indexes.
- NEBNext® Ultra™ II Q5® Master Mix.
- 10.000x SYBR™Green.
- Nuclease free water.

Preparations:

- Make sure PCR and qPCR equipment will be available when needed.
- Thaw transposed DNA, indexes, Master Mix, and SYBR™Green.
- Invert the tubes to mix and spin down.

2.1. Save the following program (ATAC-PRE) on a thermal cycler with a heated lid:

Temperature (°C)	**Time**
Initial 5 min extension	
72	5min
98	30sec
Repeat 4 times (5 cycles total)	
98	10sec
63	30sec
72	1min
4	Hold forever

2.2. Set up the following PCR reaction:

Reagent	Volume per sample (ul)
IDT® for Illumina® Nextera™ DNA UD Indexes.	5
NEBNext® Ultra™ II Q5® Master Mix	25
Sample (Transposed DNA)	20
Total	50

2.3. Mix reagents, seal plate and centrifuge at 280RCF, 20ºC, 1min
2.4. Place the plate on the pre-programmed thermal cycler and run ATAC-PRE
2.5. Using 5ul (10%) of the pre-amplified mixture, run the following qPCR reaction to determine the number of additional cycles needed:

This plate can be prepared at the same time as the PCR-reaction in step 1

Dilute 10.000x SYBR™Green to 25x in NF-water (1:399)

Reagent	Volume per sample (ul)
Nuclease free water	3.76
NEBNext® Ultra™ II Q5® Master Mix	5
SYBR™Green 25x	0.24
IDT® for Illumina® Nextera™ DNA UD Indexes. (use the same ones as for original PCR reaction)	1
Pre-amplified sample	5
Total	15

2.6. When ATAC-PRE has finished, remove plate/tubes from the thermal cycler and add 5ul of each pre-amplified library to the qPCR reaction prepared in step 2.5.
2.7. Mix reagents, seal plate and centrifuge at 280RCF, 20ºC, 1min.
2.8. Cycle in a qPCR thermal cycler as follows:

Temperature (˚C)	**Time**
98	30 sec
Repeat 19 times (20 cycles in total)	
98	10 sec
63	30 sec
72	1 min
4	Hold forever

2.9. Manually assess the amplification profiles and determine the required number of additional cycles to amplify. The number of cycles should be equal to ¼ of max fluorescence (Fig. 1). This is to ensure that the amplification is stopped prior to saturation to avoid PCR bias.

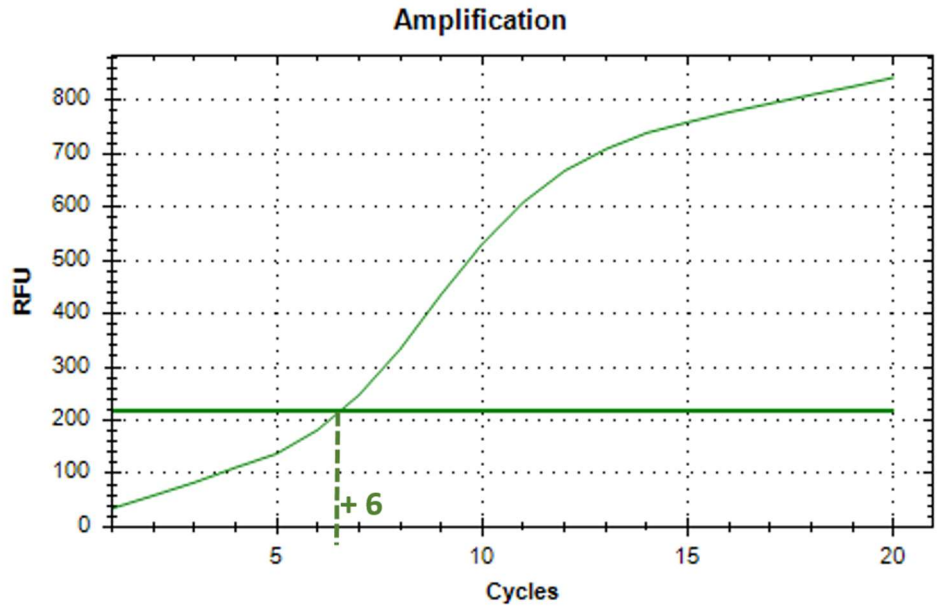

Figure 1. qPCR amplification plot showing 1/4 of max fluorescence = 6 additional PCR cycles

2.10. Save the following program (ATAC-AMP) on a thermal cycler with a heated lid:

Temperature (˚C)	Time
98	30 sec
# cycles determined by qPCR	
98	10 sec
63	30 sec
72	1 min
4	Hold forever

Choose the preheat lid option and set it to 100°C.

2.11. Seal plate and centrifuge at 280RCF, 20°C, 1min.

2.12. Place the plate on the pre-programmed thermal cycler and run ATAC-AMP.

2.13. Optional stop point – Continue directly to library clean-up or store the amplified libraries at 4°C for up to two days.

3. Same day or next day – Library clean-up and assessment

Overview: This section describes the methodology followed to perform double-sided bead purification on the previously amplified samples, in order to get rid of big (>670bp) and small fragments (<100bp). This is followed by assessment of libraries using a HS D5000 screentape.

Reagents and consumables:

- Magnet (for Eppendorf tubes or 96 well plate, depending on number of samples)
- Freshly prepared 80% ethanol.
- Room temperature Ampure XP beads.
- LoBind Eppendorf tubes or Sarstedt Microtest 96 well plate, conical bottom.
- Room temperature resuspension buffer (TET buffer):
  - Tris-HCl pH 8.0 (1M): 10mM final concentration. **16.5ul** for 100 samples.
  - EDTA (0.5M): 1mM final concentration. **3.3ul** for 100 samples.
  - Tween-20 (10%): 0.05% final concentration. **8.25ul** for 100 samples.
  - Nuclease free water: **1621.95ul** for 100 samples.

3.1. Thoroughly resuspend Ampure XP beads by vortexing for at least 1min.
3.2. Add 0.53x volume (23.9ul if you have 45ul) of beads to sample.

This will preferentially bind to longer DNA fragments).

3.3. Mix well by pipetting but be gentle to avoid bubbles.
3.4. Incubate at room temperature for 10min, while mixing gently every 5min.
3.5. Touch spin to collect liquid, then place tube on magnetic rack and allow to stand for 5min (until supernatant is clear of beads)
3.6. Transfer the supernatant to a new tube and another 1.3x original volume (58.5 ul if you had 45ul) Ampure beads to the supernatant.
3.7. Mix well by pipetting but be gentle to avoid bubbles.
3.8. Incubate at room temperature for 15 min while mixing gently every 5 minutes.
3.9. Place on magnetic rack and let it stand for 5 min (until the supernatant is clear of beads).
3.10. Remove and discard supernatant.
3.11. While the tubes are still on the magnet, and without disturbing the pellet, wash beads with 100ul of freshly prepared 80% ethanol, remove ethanol and discard. Repeat once.
3.12. Remove the tubes from the magnet, touch spin the samples, place back on the magnet and carefully remove the last traces of ethanol.
3.13. Remove samples from the magnet and allow the tubes to air dry between 30 sec-2min. Do not wait until you see small cracks in the beads!.
3.14. Add 16.5ul TET Buffer. Resuspend beads by pipetting.
3.15. Rehydrate at room temperature for a minimum of 2 minutes.

3.16. Place on magnetic rack and let it stand for 5 min (until the supernatant is clear of beads).

3.17. Transfer the supernatant (eluted DNA) to a LoBind Eppendorf tube or 96 well plate.

3.18. Add 1ul of library to 3ul of nuclease-free water (1:4 dilution)

3.19. Use 1ul of diluted library to measure DNA library concentration with Qubit High Sensitivity Kit.

3.20. Use 1ul of diluted library to validate DNA fragment size distribution with Tapestation using a HS D5000 screen tape. The DNA fragment size distribution should to some extent follow a nucleosome pattern with the most prominent peak being at about 200bp. (Figure 2.)

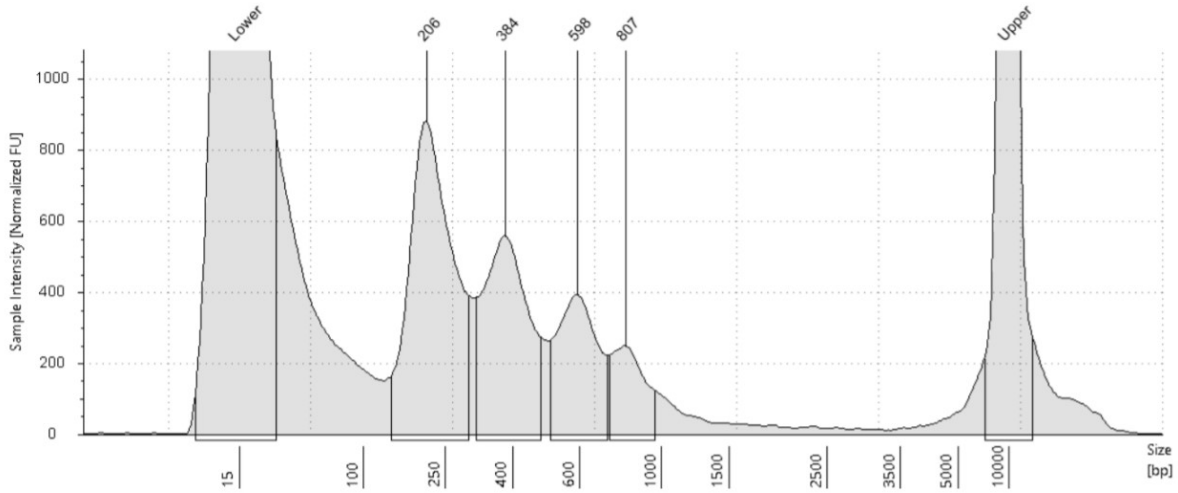

Figure 2. Example of a Tapestation profile after library preparation and clean up. This shows a very well represented nucleosome pattern with the most prominent peak at ~200bp.

Bibliography

1. Buenrostro JD, Wu B, Chang HY, Greenleaf WJ. ATAC-seq: A Method for Assaying Chromatin Accessibility Genome-Wide. Curr Protoc Mol Biol. 2015 Jan 5;109:21.29.1–9.

2. Corces MR, Trevino AE, Hamilton EG, Greenside PG, Sinnott-Armstrong NA, Vesuna S, et al. An improved ATAC-seq protocol reduces background and enables interrogation of frozen tissues. Nat Methods. 2017 Oct;14(10):959–962.