ATAC-seq protocol- Atlantic salmon head kidney fresh cells

B Based on Buenrostro et al. (1) and Omni-ATAC Supplementary Protocol 1 (2) with following modifications:

1. **This protocol is based on starting with 75,000 viable cells, and 1.25ul of transposase enzyme.**
2. **Library clean-up using column purification will not remove primer-dimers. In addition, libraries often contain an excess of large fragments (> 1000 bp). 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**

Important: For the invitro ATAC-seq library preparation fresh Leukocytes were extracted using the following protocol
(https://data.faang.org/api/fire_api/samples/ABDN_SOP_Leukcoytes_ext_20210520.pdf) **and then proceeded with step 7 in this protocol.**

Product	Company	Reference	Comment
Common reagents :			
Tris-HCL 1M pH 7.4	Sigma-Aldrich	T2194-100ML	
NaCl	Sigma-Aldrich	S3014-500G	prepare 5M solution
PBS tablets	Sigma-Aldrich	P4417-50TAB	prepare 1 or 10X solution
Tween-20	Sigma-Aldrich	11332465001	
Trypan blue	BioRad	1450021	
Nuclease free water			
Specific reagents:			
MgCl2 1M	Sigma-Aldrich	M1028-100ML	
Digitonin	Promega	G9441	Dilute 1:1 with water to make a 1% (100x) stock solution
NP40	Sigma-Aldrich	11332473001	
cOmplete, Mini, EDTA-free Protease Inhibitor Cocktail	Sigma-Aldrich	4693159001	
100 µm Cell Strainer, Yellow (Sterile)	Starlab	CC8111-0102	
Illumina Tagment DNA Enzyme and Buffer	Illumina	20034197	
NEBNext® Ultra™ II Q5® Master Mix	NEB	M0544S	
IDT® for Illumina Nextera DNA Unique Dual Indexes	Illumina	20027213-20027216	
MinElute PCR purification kit	Qiagen	28004	
Agencourt Ampure XP beads	Beckman Coulter	A63880	

Bioanalyzer High Sensitivity kit and DNA Chips	**Agilent Technologies**	**5067-4626**	
Qubit High Sensitivity kit	**ThermoFisher**	**Q32854**	
Qubit assays tubes	**ThermoFisher**	**Q32856**	

Stock solutions, store at 4°C			
PBS (P4417-50TAB)			
Dissolve one tablet of PBS in 200 mL of Milli-Q water			
ATAC-RSB	**Final conc.**	**Vol for 50 mL**	
1 M Tris pH 7.4 (T2194-100ML)	10 mM	500 ul	
5 M NaCl (S3014-500G)	10 mM	100 ul	
1 M MgCl2 (M1028-100ML)	3 mM	150 ul	
dH2O		49.25 mL	
Detergents			
Digitonin (G9441) *is supplied at 2% in DMSO. Dilute 1:1 with water to make a 1% (100x) stock solution and aliquot in smaller volumes. Avoid more than 5 freeze thaw cycles. Can be kept at -20°C for up to 6 months.*			
Tween-20 (11332465001) *is supplied at 10%. Use at this concentration (100x stock). Store at 4°C*			
NP-40 (11332473001) *is supplied at 10%. Use at this concentration (100x stock). Store at 4°C*			
Same day buffer preparations		**ENTER NUMBER OF SAMPLES**	**3**
PBS + PIC	**Final conc.**	**Vol per sample (ul)**	**Vol for n samples (ul)**
PBS		5000	15000
Protease inhibitor cocktail tablet (1 tablet per 10 mL solution)	1	0.5	1.5
Total		5000	15000
ATAC-RSB-L	**Final conc.**	**Vol per sample (ul)**	**Vol for n samples (ul)**
ATAC-RSB		48.5	145.5
1% Digitonin	0.01%	0.5	1.5
10% Tween-20	0.10%	0.5	1.5
10% NP-40	0.10%	0.5	1.5

Total		50	150
ATAC-RSB-W	**Final conc.**	**Vol per sample (ul)**	**Vol for n samples (ul)**
ATAC-RSB		990	2970
10% Tween-20	0.10%	10	30
Total		1000	3000
Resuspension buffer (TET buffer, 16.5 ul per sample)	**Final conc.**	**Vol per sample (ul)**	**Vol for n samples (ul)**
Tris-HCL pH 8.0 (1 M)	10 mM	0.165	0.495
EDTA (0.5 M)	1 mM	0.033	0.099
Tween-20 (10 %)	0.05%	0.0825	0.2475
dH2O		16.2195	48.6585

Tissue disruption

1. Dissect out the entire head kidney (using scalpel, tweezers and spatula) and put into a 50 mL tube (tube 1) containing 20 mL extraction media (L15, 2% FBS and 0.02% EDTA, pH=8).
2. Take a 100 µm nylon mesh and fit it onto a new 50 mL tube
3. Using a sterile pipette or tweezers, transfer the head kidney from the tube 1 and place it on nylon mesh.
4. Working gently, squeeze the head kidney though the 100 µm nylon mesh using the rubber end of the plunger from a 2 mL sterile syringe. Continually apply **extraction media** from tube 3 to prevent cells from drying out and assist with their transit through the mesh. NOTE: Be very gentle, head kidney tissue will break up quite easily, but this may take 10 minutes.
5. Make the final volume of the disrupted cell suspension to 20 mL using additional **extraction media**
6. Count cells and allocate 75K cells in a 1.5ml loBind Eppendorf tube.

Nuclei isolation

7. Pellet cells (75k) by centrifugation (500g, 5 min, 4 ˚C)
8. Discard all supernatant using a p1000 followed by p100 pipette to avoid cell loss, be careful!
9. Wash- Resuspend with 1ml of PBS with PIC by pipetting gently. Centrifuge 5 mins, 500g, 4˚C. Remove the supernatant
10. Add 50 ul cold **ATAC-RSB-L** containing 0.1% Tween-20, 0.1% NP-40 and 0.01% Digitonin. Pipette up and down three times
11. Incubate on ice for 3 minutes
12. Wash out lysis with 1 mL cold **ATAC-RSB-W** with 0.1 % Tween-30 BUT NO NP-40 or Digitonin. Invert the tube 3 times to mix
13. Centrifuge nuclei for 10 minutes, 800 g, 4 ˚C
    13.1. Remember which direction you put the tube into the centrifuge to be able to predict where the pellet should lie
14. Aspirate supernatant carefully using a p1000 followed by p100 pipette. The nuclei pellet is usually very small and can only be observed as an opaque "shadow". Take care not to also aspirate the nuclei pellet!

Transposase reaction

Digestion

15. 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

16. Resuspend the pellet in 50 ul transposase reaction mix by pipetting up and down 6 times
17. Incubate the reaction at 37 ˚C for 30 minutes in a thermomixer with 1000 RPM mixing. (We used 200 RPM and it worked nicely)

Clean- up using MinElute PCR Purification kit from Qiagen

18. Centrifuge sample at 1000g briefly (2 minutes) to get rid of the cell debris, otherwise they cause blockage to spin column during DNA extraction.
19. Transfer supernatant into a new 1.5ml safe lock tube and proceed with the DNA purification with MinElute PCR purification kit (follow MinElute PCR purification kit protocol)
20. Elute DNA in 21 ul EB buffer
21. Eluted DNA can be stored at – 20 ˚C until ready to amplify

Library amplification

Determining the number of PCR cycles with qPCR

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 5 min extension at 72 ˚C to allow for extension of both ends of primers after transposition.

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

Temperature (˚C)	Time
72	5 min
98	30 sec

Repeat 4 times (5 cycles in total)	
98	10 sec
63	30 sec
72	1 min
4	Hold forever

Set up the following PCR reaction:

Reagent	Volume per sample (ul)	For n samples
Nextera dual indexes	5	
NEBNext® Ultra™ II Q5® Master Mix	25	
Sample (Transposed DNA)	20	
Total	50	

22. Mix reagents, seal plate and centrifuge at 280 g, 20 ˚C (room temperature), 1 min
23. Place the plate on the preprogrammed thermal cycler and run ATAC-PRE
24. Using 5 ul (10 %) of the pre-amplified mixture, run qPCR to determine the number of additional cycles needed. This plate can be prepared at the same time as the PCR-reaction
25. Make the following qPCR reaction mix

Reagent	Volume per sample (ul)
Nuclease free H2O	3.76
Nextera dual indexes	1
NEBNext® Ultra™ II Q5® Master Mix	5
25x SYBRGreen	0.24
Pre-amplified sample (add in step 5)	5
Total	15

26. When ATAC-PRE has finished, remove plate/tubes from the thermal cycler and add 5 ul of each pre-amplified library to the qPCR reaction prepared in step 25.
27. Mix reagents, seal plate and centrifuge at 280 g, 20 ˚C (room temperature), 1 min
28. Cycle 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

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

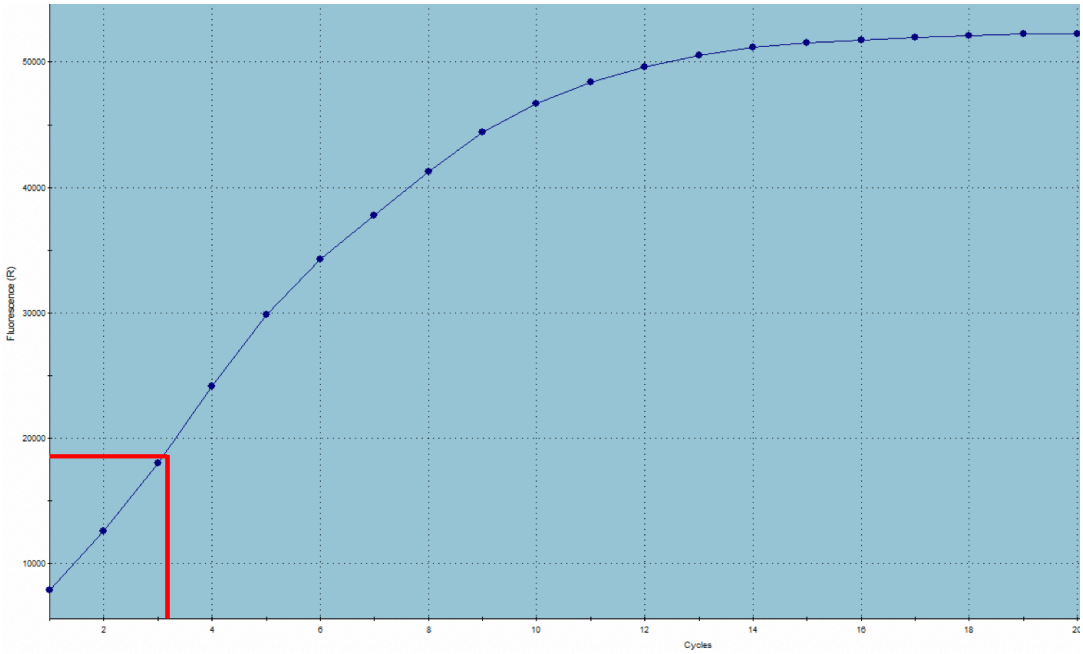

Figure 1: qPCR amplification plot showing ¼ of max fluorescence = around 4 additional PCR cycles

30. 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 to 100 ˚C

31. Run the remaining pre-amplified transposed DNA with required number of additional cycles on PCR:
    a. Seal plate and centrifuge at 280 g, 20 ˚C (room temperature), 1 min
    b. Place the plate on the **preprogrammed** thermal cycler and run ATAC-AMP
    c. You can continue directly to library clean-up or store the amplified libraries at 4 ˚C for up to two days

Library clean-up

Remove small fragments and fragments above 670 bp

32. Resuspend Ampure XP beads by vortexing well (> 1 min)
33. Add 0.55x volume (22.5 ul) of beads to sample

34. Mix well by pipetting. Be gentle to avoid bubbles
35. Incubate at room temperature for 5 min
36. Place on magnetic rack and let it stand for 5 min (until the supernatant is clear of beads)
37. Transfer the supernatant to a new tube/plate
38. Add another 1.3x original volume (58.5 ul) Ampure beads to the supernatant
39. Mix well by pipetting. Be gentle to avoid bubbles
40. Incubate at room temperature for 15 min
41. Place on magnetic rack and let it stand for 5 min (until the supernatant is clear of beads)
42. Remove and discard supernatant
43. Wash the beads 2x with freshly made 80 % ethanol while plate/tube is still on the magnetic rack:
    a. Add 100 ul 80 % ethanol over beads
    b. Wait 1 minute
    c. Remove ethanol
    d. Repeat 1x
44. Pulse spin the samples and remove the last bit of ethanol while plate/tube is still on the magnetic rack
45. Air dry beads (until you see small cracks in the beads, do not wait longer)
46. Remove samples from the magnet
47. Add 16.5 room temperature Resuspension Buffer. Resuspend beads by pipetting
48. Rehydrate at room temperature for a minimum of 2 minutes
49. Place on magnetic rack and let it stand for 5 min (until the supernatant is clear of beads)
50. Transfer the supernatant (eluted DNA) to a LoBind Eppendorf tube or 96 well plate

Library assessment

51. Add 1 ul of library to 3 ul nuclease-free H_2O (1:4 dilution)
52. Use 1 ul of diluted library to measure DNA library concentration with Qubit High Sensitivity Kit
53. Use 1 ul of diluted library to validate DNA fragment size distribution with Tapestation or Bioanalyzer High Sensitivity DNA kit
    a. The DNA fragment size distribution should to some extent follow a nucleosome pattern (Fig. 2) with the most prominent peak being at about 200 bp.
    b. If you still have an excess of primer-dimers in your samples, try an addition clean-up with MinElute PCR purification column and elute with 10 ul
    c. If you still have an excess of long fragments in your samples (>1000 bp), do an additional round of bead purification

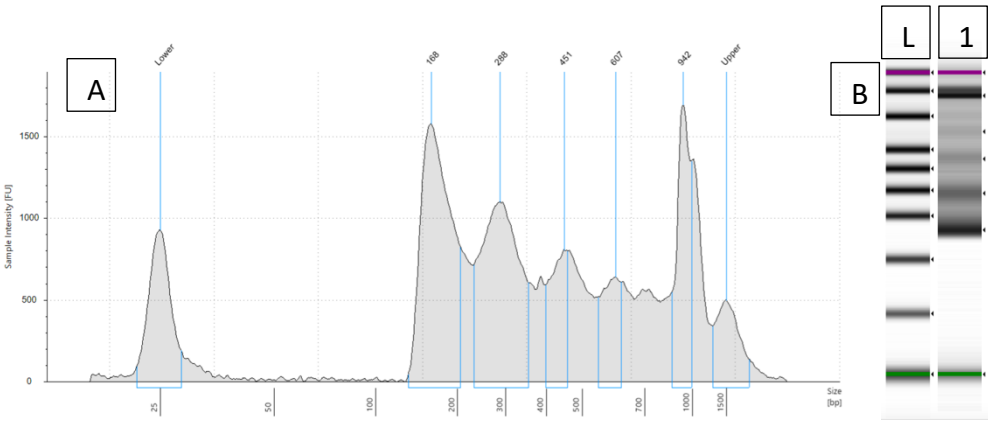

Figure 2: TapeStation validation of cleaned ATAC library ready for sequencing. A.The peaks after 168bp are indicative of the periodicity of the chromatin structure and show nucleosome-free, mononucleosome, dinucleosome and multinucleated fragments. Frequency of peaks occurring at 150-180bp, concordant with the length of DNA wrapped around each nucleosome. B. Nucleosome banding also seen from gel image.

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.