ATAC-seq from freshly collected seabass embryos

Overview: This protocol describes a method used to prepare ATAC-seq libraries from seabass freshly collected embryos. *For embryo collection and dechorionation refer to the protocol https://data.faang.org/api/fire_api/samples/UNIPD_SOP_Embryos Dechorionation and Chorion Constituents Cleaning Dlab_20201012.pdf*

Day 1 – Embryo disruption, nuclei isolation, transposition and clean up

Embryo disruption and cell isolation

NB: In order to reflect seabass cell osmolarity, PBS 1X and RSB buffer were adjusted to 350 mOsm/kg by adding 2M NaCl.

1. Prepare a dounce tube with 700 ul of cold **RSB buffer (350 mOsm/kg)** on ice
2. Wash the collected embryos with 500 ul of cold **PBS 1X (350 mOsm/kg)** and **c**entrifuge at 500 g for 5 min at 8°C
3. Discard supernatant and resuspend embryos with 500 ul of cold **RSB buffer (350 mOsm/kg)**. Transfer the embryos to the dounce tube (total RSB volume 1200 ul)
4. To release cells, dounce with the loose (A) pestle until there is no resistance. Depending on stage, the number of pestle passes may vary and an additional filtration step through cell strainer might be needed, refer to Table 1.
5. Transfer the cell suspension in a 1,5 mL vial and pellet by centrifugation at 753g for 10 min at 8°C
6. Eliminate the supernatant and resuspend the pellet with 1mL of cold **PBS 1X (350 mOsm/kg)**
7. Centrifuge the suspension at 753g for 10 min at 8°C
8. Eliminate the supernatant and resuspend the pellet with 500ul of cold **PBS 1X (350 mOsm/kg**)
9. Count the cells with hemocytometer (5ul cell suspension + 5 ul PBS + 10 ul Trypan blue)

Stage	Dounce Pestle (A) N° passes	Cell strainer	DNAse treatment (min)
Late blastula	5	\	10
Mid gastrula	5	\	5
Early somitogenesis	8	\	5
Mid somitogenesis	14	\	5
Late somitogenesis	16	70 um	10
Post segmentation	18	70 um	10

Table 1. Stage-specific protocol conditions

DNAse treatment (using “RNase-Free DNase Set" from Qiagen)

10. Prepare a "**DNAse mix**" with 4 ul of resuspended DNAseI (10 kunits) and 5 ul of RDD buffer --> 9 ul/sample
11. Put a volume of cell suspension corresponding to 50.000 cells (from step 9) in a 1.5 ml vial
12. Add cold **PBS 1X (350 mOsm/kg)** to reach a total volume of 91 ul
13. Add 9 ul of "**DNAse mix**", mix gently and incubate at room temperature. Incubation time depends on developmental stage, refer to Table 1.
14. Add 900 ul of cold **PBS 1X (350 mOsm/kg)**
15. Pellet by centrifugation (610g, 5 min, 4°C)
16. Discard all supernatant, be careful to avoid cell loss

Nuclei isolation, transposase reaction and clean-up

17. Add 50 ul cold **ATAC-RSB-L** buffer and incubate on ice for 3 minutes
18. Wash out lysis with 1 mL cold **ATAC-RSB-W**, invert the tube at least 3 times to mix
19. Centrifuge nuclei at 800 g for 10 minutes at 4 ˚C
20. 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
21. Make the following transposase reaction mix:

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

22. Resuspend the pellet in 50 ul transposase reaction mix by pipetting up and down
23. Incubate the reaction at 37 ˚C for 30 minutes in a thermomixer with 1000 RPM mixing
24. Stop the reaction and purify the resulting DNA fragments with MinElute PCR purification kit
    24.1 Elute DNA in 23 ul EB buffer
25. SAFE STOP POINT - Store the eluted DNA at -20°C.

Day 2 – Library amplification

Initial PCR amplification

26. Save the following program (ATAC-PRE) on a thermal cycler with a heated lid:
    72°C for 5'
    98°C for 30''
    98°C for 10''
    63°C for 30'' } 5 cycles
    72°C for 1'
    4°C forever

27. Set up the following PCR reaction:
    27.1. Add 25ul of NEBNext® Ultra™ II Q5® Master Mix to each tube.
    27.2. Add 5 ul of Index from IDT® for Illumina Nextera DNA Unique Dual Indexes (UDI) Set
    27.3. Add 20 ul of transposed DNA (from step 24)

Reagent	**Vol per sample (ul)**
IDT® for Illumina Nextera DNA UDI	5
NEBNext Ultra II Q5 Master Mix	25
Transposed DNA	20
Total	50

28. Mix reagents, close tubes and centrifuge at 280 g for 1 min
29. Place the plate on the preprogrammed thermal cycler and run ATAC-PRE

qPCR assessment of final PCR cycles

30. Using 5 ul (10 %) of the Initially-amplified product, assemble 15 ul qPCR reactions in a qPCR plate to determine the appropriate number of additional cycles needed.
    30.1. Prepare a qPCR mix of nuclease free water, NEBNext® Ultra™ II Q5® Master Mix and 25X SYBRGreen for n samples +2 (n samples + 1 NTC + 1 additional).

Reagent	**Vol per sample (ul)**
Nuclease free water	3.76
NEBNext Ultra II Q5 Master Mix	5
25X SYBRGreen (diluted*)	0.4
Total	9

* SYBR green is provided at 10,000 X. Make 1ul aliquots and freeze, then on the day of use add 399ul H2O and mix well.

    30.2 Distribute 9 ul of qPCR mix in each well.
    30.3 Add 1 ul of Nextera UDI index to each sample. IMPORTANT, this must be the same as was used for Initial PCR reaction.
    30.4 Add 5 ul of Initially amplified DNA (from step 29) or 5 ul of water (for NTC)

31. Seal the plate and centrifuge at 280g for 1 min
32. Run the following program on a Real-time thermal cycler:

98°C for 30''
98°C for 10''
63°C for 30'' } 20 cycles
72°C for 1'
30°C – Stop

33. Determine the required number of additional cycles to amplify. The number of cycles should equal ¼ of max fluorescence (Figure 2). This is to ensure that the amplification is stopped prior to saturation to avoid PCR bias.

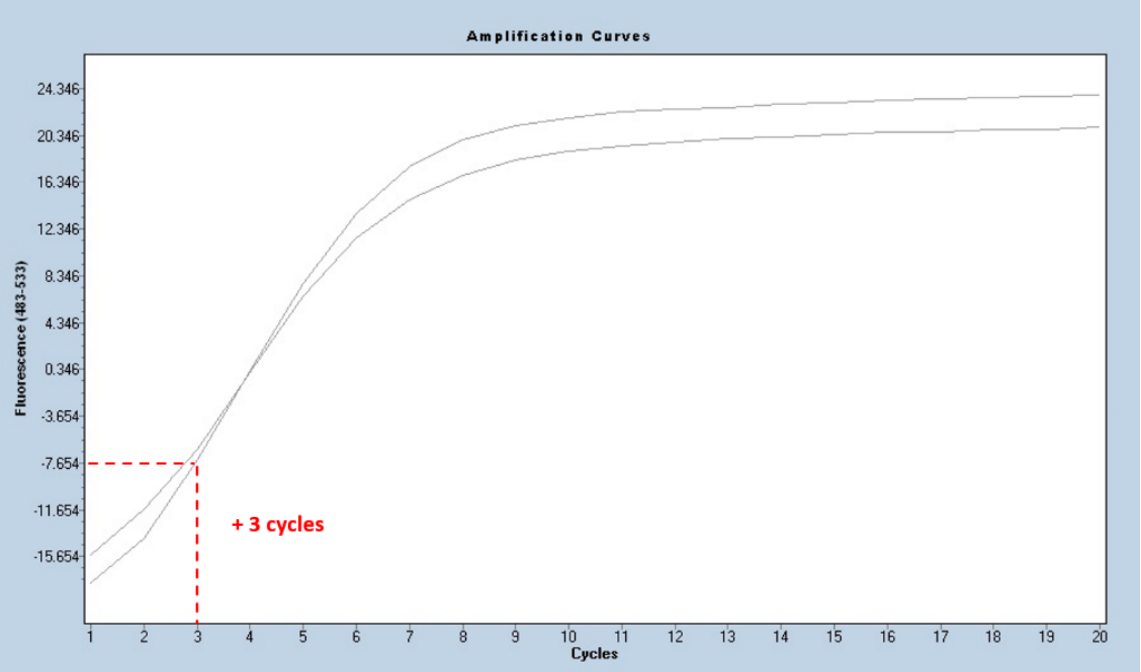

Figure 2. qPCR amplification plot showing ¼ of max fluorescence = 3 PCR cycles

34. Save the following program (ATAC-FINAL) on a thermal cycler with a heated lid

98°C for 30''
98°C for 10''
63°C for 30'' } n° cycles determined in step 33
72°C for 1'
4°C forever

35. Short-spin the PCR tubes containing your Initially-amplified product (45ul).
36. Place the plate on the thermal cycler and run ATAC-FINAL.

SAFE STOP POINT - After Final PCR amplification, keep PCR product at 4˚C until you proceed with size selection.

Same day or another day – Library size selection

Library size selection

37. Resuspend Ampure XP beads by vortexing well (> 1 min)
38. Add 0.55x volume (24.75 ul) of beads to sample. Mix well by pipetting. Be gentle to avoid bubbles
39. Incubate at room temperature for 10 min
40. Place on magnetic rack and let it stand for 5 min (until the supernatant is clear of beads)
41. **Transfer the supernatant** to a new tube/plate and add another 1.3x original volume (58.5 ul) of Ampure beads to the supernatant
42. Mix well by pipetting. Be gentle to avoid bubbles
43. Incubate at room temperature for 10 min
44. Place on magnetic rack and let it stand for 5 min (until the supernatant is clear of beads)
45. Remove and **discard supernatant**
46. Wash the beads 2x with freshly made 80 % ethanol while plate/tube is still on the magnetic rack:
    - 46.1 Add 100 ul 80 % ethanol over beads
    - 46.2 Wait 1 minute
    - 46.3 Remove ethanol
    - 46.4 Repeat 1x
47. Remove samples from the magnet and allow the tubes to air dry (30sec-2min)
48. Add 16.5 room temperature TET Buffer. Resuspend beads by pipetting
49. Rehydrate at room temperature for a minimum of 2 minutes
50. Place on magnetic rack and let it stand for 5 min (until the supernatant is clear of beads)
51. Transfer the supernatant (eluted DNA) to a LoBind Eppendorf tube or 96 well plate

Library quality assessment

52. Measure DNA library concentration with Qubit High Sensitivity Kit
53. Use 1 ul of diluted library to validate DNA fragment size distribution with Bioanalyzer High Sensitivity DNA kit .

The DNA fragment size distribution should to some extent follow a nucleosome pattern (Figure 3) with the most prominent peak being at about 200 bp.

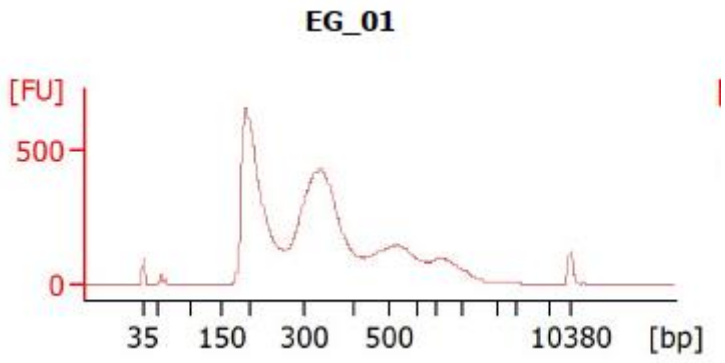

Figure 3. Example of Bioanalyzer trace after library preparation and size selection

BUFFERS

Buffers that can be prepared in advance (store at 4°C)

ATAC-RSB (store at 4°C)	**Final conc.**	**Vol for 50 mL**
1 M Tris pH 7.4	10 mM	500 ul
5 M NaCl	10 mM	100 ul
1 M MgCl2	3 mM	150 ul
dH2O		49.25 mL

Detergents		
Digitonin *is supplied at 2% in DMSO. Dilute 1:1 with water to make a 1% (100x) stock solution. Avoid more than 5 freeze thaw cycles. Can be kept at -20°C for up to 6 months.*		
Tween-20 *is supplied at 10%. Use at this concentration (100x stock). Store at 4°C*		
NP40 *is supplied at 10%. Use at this concentration (100x stock). Store at 4°C*		

TET buffer (16.5 ul per sample) (store at 4°C)	**Final conc.**	**Vol per sample (ul)**	**Vol for 100 samples**
Tris-HCL pH 8.0 (1 M)	10 mM	0.165	16.5
EDTA (0.5 M)	1 mM	0.033	3.3
Tween-20 (10 %)	0.05%	0.0825	8.25
dH2O		16.2195	1621.95

Buffers to be prepared the same day (keep on ice)

ATAC_RSB_L	**Final conc.**	**Vol per sample (ul)**
ATAC-RSB	1x	48.50
1% Digitonin	0.01%	0.50
10% Tween-20	0.10%	0.50
10% NP-40	0.10%	0.50
	TOTAL	50.00

ATAC-RSB-W	**Final conc.**	**Vol per sample (ul)**
ATAC-RSB	1x	990.00
10% Tween-20	0.10%	10.00
	TOTAL	1000.00