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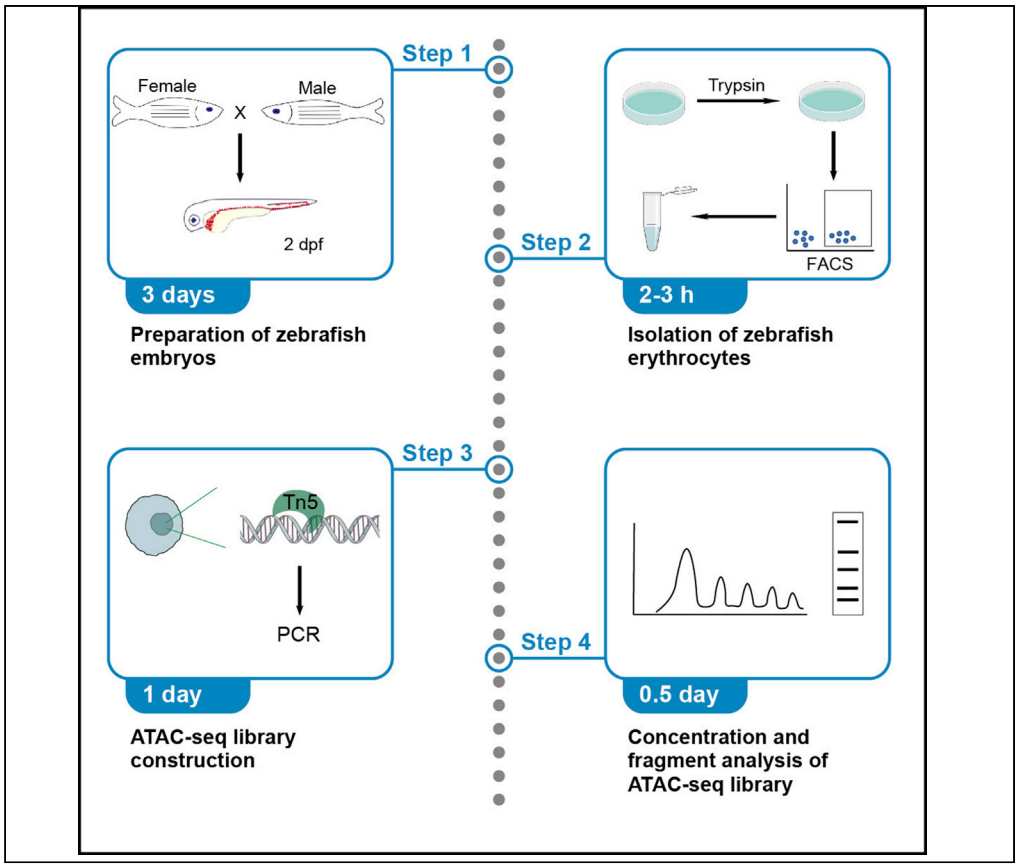
Protocol for isolation and ATAC-seq library construction of zebrafish red blood cells



Understanding chromatin dynamics in red blood cells (RBCs) is critical for exploring the differentiation process and homeostasis maintenance during erythropoiesis. Here, we describe a protocol for isolation of zebrafish erythrocytes labelled with *gata1*:dsRed by fluorescence-activated cell sorting. We detail steps for ATAC-seq library construction from the isolated RBCs and describe how to analyze the quality of the library. The library can then be used to assay genome-wide chromatin accessibility in these RBCs.

Publisher's note: Undertaking any experimental protocol requires adherence to local institutional guidelines for laboratory safety and ethics.

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Highlights

Isolation of zebrafish erythrocytes labelled by *gata1*:dsRed

ATAC-seq library construction of zebrafish erythrocytes

Analyze the quality of ATAC-seq library

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Protocol for isolation and ATAC-seq library construction of zebrafish red blood cells

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SUMMARY

Understanding chromatin dynamics in red blood cells (RBCs) is critical for exploring the differentiation process and homeostasis maintenance during erythropoiesis. Here, we describe a protocol for isolation of zebrafish erythrocytes labelled with *gata1*:dsRed by fluorescence-activated cell sorting. We detail steps for ATAC-seq library construction from the isolated RBCs and describe how to analyze the quality of the library. The library can then be used to assay genome-wide chromatin accessibility in these RBCs.

For complete details on the use and execution of this protocol, please refer to Ding et al. (2021).[1]

BEFORE YOU BEGIN

The protocol below describes the specific steps for the preparation of zebrafish single cell suspension for erythrocyte (i.e., RBC) isolation. The following ATAC-seq library construction is based on the Tn5 transposition of open chromatin regions.

Institutional permissions

Animal experiments and procedures were approved by the Ethical Review Committee in the Institute of Zoology, Chinese Academy of Sciences.

Preparation of reagents used in this experiment

Timing: 1–2 days

1. Prepare E3 embryonic medium: 5 mM NaCl, 0.17 mM KCl, 0.33 mM $CaCl_2$, 0.33 mM $MgSO_4$ in H_2O. The E3 medium can be kept at 25°C for 30 days.
2. Prepare digestion media: 0.5% trypsin in DPBS. Store at −20°C.
3. Prepare 100% Fetal Bovine Serum (FBS) and 1% FBS in DPBS. Store at −20°C.
4. Order TruePrep™ DNA Library Prep Kit V2 for Illumina (Vazyme, TD501), TruePrep™ DNA Index Kit V2 for Illumina (Vazyme, TD202) and VAHTS™ DNA Clean Beads (Vazyme, N411).
5. Prepare lysis buffer: 10 mM Tris-HCl (pH 7.4), 10 mM NaCl, 3 mM $MgCl_2$ and 0.15% NP-40. Store at −20°C.
6. Prepare chloroform/phenol/isoamyl-alcohol used for DNA extraction. The ratio of chloroform/phenol/isoamyl-alcohol is 25:24:1, the mixture was stored at 4°C.
7. Prepare TE (Tris-EDTA) buffer: 10 mM Tris-HCl (pH 8.0) and 1 mM EDTA (pH 8.0). Store at 4°C.

Preparation of zebrafish embryos

Timing: 3 days

8. Move one male and one female adult zebrafish with Tg (*gata1*:dsRed)[2] background in the mating cylinder at night.
9. In the next morning, after the zebrafish mating, collect the fertilized eggs into 90 mm culture dish and then raised in E3 embryonic medium in the incubator at 28.5°C until 2 days post fertilization (dpf).
10. Collect the Tg (*gata1*:dsRed) zebrafish embryos which have the specific fluorescence expression in the circulating RBCs under the fluorescence microscope. 75% of embryos were expected to be fluorescent.

Preparation for the cell sorting

Timing: 30 min

11. Warm the digestion medium at 28.5°C.
12. Prepare 1% FBS in DPBS and keep at 4°C.
13. Cool the centrifuge (eppendorf, 5424R) to 4°C.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Chemicals, peptides, and recombinant proteins		
Dulbecco's phosphate buffered saline (DPBS)	Gibco	14190144
Fetal bovine serum (FBS)	Gibco	10099141C
2.5% Trypsin	Gibco	15090046
NaCl	Macklin	S805275
KCl	Macklin	P816348
$CaCl_2$	Macklin	C805225
$MgSO_4$	Macklin	M813921
Chloroform	Guangzhou Chemical Reagent Factory	GD10
Phenol	Macklin	T917981
Isoamyl alcohol	Aladdin	M116196
Ethanol	Guangzhou Chemical Reagent Factory	HB15
Tris	Beyotime	ST761
EDTA	Aladdin	E116428
NaOAc	Amresco	0602
Glycogen	Thermo Fisher	R0551
TruePrep™ DNA Library Prep Kit V2 for Illumina	Vazyme	TD501
TruePrep™ DNA Index Kit V2 for Illumina	Vazyme	TD202
VAHTS™ DNA Clean Beads	Vazyme	N411
Carrier RNA	TransGen	GR101-06
Experimental models: Organisms/strains		
Zebrafish: Tg (*gata1*:dsRed)	Traver et al.[2]	N/A
Other		
1.5 mL microcentrifuge tube	Axygen	MCT-150-C
0.2 mL PCR tube	Axygen	PCR—02C
90 mm culture dish	Biosharp	BS-90-D
60 mm culture dish	Corning	430166
Cell strainers	BD Falcon	352235

STEP-BY-STEP METHOD DETAILS

Isolation of zebrafish erythrocytes

Timing: 2–3 h

This step describes the procedures used for zebrafish erythrocytes isolation by FACS (Figure 1). The circulating erythrocytes which are labelled by *gata1*:dsRed can be sorted based on the fluorescent protein expression.[1]

1. Collect the Tg (*gata1*:dsRed) zebrafish embryos into the 60 mm culture dish.
   a. Add 1 mL DPBS into dish using a pipette.
   b. Shake the embryos gently and remove the DPBS using a pipette.
   c. Wash the embryos with DPBS twice.
2. Add the 1 mL digestion medium into the 60 mm culture dish and pipette up and down 10–20 times till the dissociated solution began turbidity.
3. Incubate the samples at 28.5°C for 10 min and pipette up and down several times.

   Note: The digestion process can be examined under a microscope until the formation of single cells.

4. Add the FBS into the 60 mm culture dish to a final concentration of 10% for 30 s to stop trypsin dissociation.
5. Transfer the sample into a 1.5 mL microcentrifuge tube and centrifuge (eppendorf, 5424R) at 300 × *g* for 5 min at 4°C.
6. Discard the supernatant and resuspend the cell pellet in DPBS containing 1% FBS.

   Note: Gentle with the pipetting during resuspension to keep the cells in good shape.

7. Centrifuge at 300 × *g* for 5 min at 4°C.
8. Repeat the steps 6 and 7.
9. Discard the supernatant, resuspend the cell pellet using DPBS containing 1% FBS and filter the cells in a new 1.5 mL microcentrifuge tube using cell strainers (BD Falcon, 352235).
10. Put the cell suspensions on ice.
11. The erythrocytes (*gata1*:dsRed^{+}) were sorted using MoFlo XDP (Beckman Coulter) and collected into 1% FBS in DPBS in a new 200 μL microcentrifuge tube.

    Note: The number of cells should be around 10,000–50,000 per tube.

12. Put the tube on ice and prepare to perform the lysis step.

Transposition reaction

Timing: 1–1.5 h

This step describes the procedures used for transposition of open chromatin regions. The amount of transposition enzyme and time for transposition reaction need to be optimized between different cell types.[3,4]

13. Centrifuge the tube containing the RBCs at 300 × *g* for 5 min at 4°C.

    Note: The cells should be harvested freshly and make sure these cells are placed on ice.

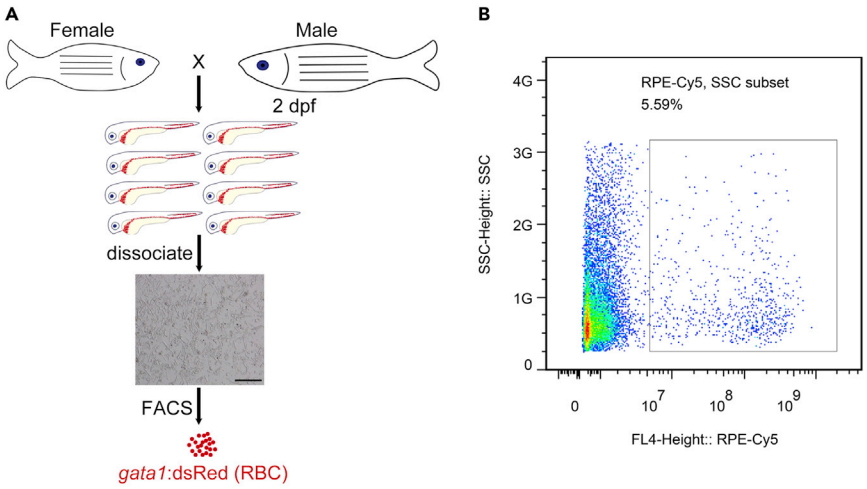

Figure1. Isolation of zebrafish erythrocytes

(A) The adult zebrafishes with Tg (*gata1*:dsRed) background were incrossed to acquire the embryos. The fertilized eggs were collected and then raised in E3 embryonic medium in the incubator at 28.5°C until 2 dpf. The circulating erythrocytes which are labelled by *gata1*:dsRed can be sorted based on the fluorescent protein expression. Scale bar, 50 μm.

(B) FACS results of *gata1*:dsRed$^+$ cells in Tg (*gata1*: dsRed) embryos at 2 dpf.

14. Discard the supernatant gently without disturbing the cell pellet.
15. Add 100 μL cold DPBS to the tube and resuspend the cell pellet on ice.
16. Centrifuge at 300 × *g* for 5 min at 4°C.
17. Discard the supernatant gently without disturbing the cell pellet.
18. Add 50 μL cold lysis buffer (10 mM Tris-HCl (pH 7.4), 10 mM NaCl, 3 mM $MgCl_2$ and 0.15% NP-40) into the cell pellet.
    a. Resuspend the pellet in the lysis buffer.
    b. Incubate for 5 min on ice.
19. Centrifuge and discard the supernatant to get the cell pellet (about 2 μL).
20. Add the transposition reaction system combining 5×TTBL, TTE Mix (components of TruePrep™ DNA Library Prep Kit V2 for Illumina, Vazyme, TD501) and H_2O immediately to the cell pellet and pipette up and down gently for several times.

Transposition reaction system

Reagent	Amount
Cell pellet	2 μL
5×TTBL	10 μL
TTE Mix	5 μL
H_2O	33 μL

21. Incubate the sample at 37°C for 30 min.

Note: The time of transposition reaction is critical for the successful transposition of open chromatin regions. DNA extraction need to be performed immediately after the incubation step to avoid the over digestion.

DNA extraction

Timing: 1 day

This step describes the procedures used for DNA extraction after Tn5 incubation.

22. Prepare the extraction mix in a 1.5 mL microcentrifuge tube.

Extraction mix	
Reagent	Amount
Carrier RNA (20 ng/μL)	4 μL
TE buffer	206 μL
chloroform/phenol/isoamyl-alcohol	260 μL

23. Transfer the DNA into the extraction mix. The total volume of the solution will be 520 μL (50 μL of DNA and 470 μL of extraction mix).
24. Vortex and incubate at 25°C for 3 min.
25. Centrifuge at 12,000 × *g* for 15 min at 4°C.
26. Transfer the supernatant into a new 1.5 mL microcentrifuge tube, add the ethanol (1,300 μL), NaOAc (3 M, 48 μL), and Glycogen (3 μL) into the tube.
27. Shake the tube with hand to mix the sample and put it at −80°C. The sample can be kept at −80°C for 1 day.
28. Centrifuge at 12,000 × *g* for 15 min at 4°C.
29. Discard the supernatant and wash pellets with 75% ethanol without disturbing the pellet.
30. Centrifuge at 12,000 × *g* for 5 min at 4°C.
31. Discard the supernatant, air dry for 5 min and add 24 μL H_2O to resolve DNA.

DNA library construction

Timing: 1.5 h

This step describes the procedures used for DNA library construction.

32. Prepare the PCR reaction master mix to amplify transposed DNA fragments. The DNA was amplified by PCR using TruePrepTM DNA Index Kit V2 for Illumina (Vazyme, TD202).

PCR reaction master mix	
Reagent	Amount
DNA product	24 μL
5×TAB	10 μL
N5XX	5 μL
N7XX	5 μL
PPM	5 μL
TAE	1 μL

PCR cycling conditions			
Steps	Temperature	Time	Cycles
Extension of both ends of the primer	72°C	3 min	1
Initial Denaturation	98°C	30 s	1
Denaturation	98°C	15 s	12–15 cycles
Annealing	60°C	30 s	
Extension	72°C	3 min	
Final extension	72°C	5 min	1
Hold	4°C	forever	

Note: After PCR reaction, the PCR mix can be stored at −20°C for 1 day or proceed with step 33.

DNA fragments length purification and sequencing

Timing: 1.5 h

This step describes the procedures used for DNA fragments length purification and sequencing.

33. Vortex the VAHTSTM DNA Clean Beads (Vazyme, N411).
    a. Add 60 μL (1.2×) DNA Clean Beads into the PCR mix.
    b. Pipette up and down to mix the beads.

    Note: The VAHTSTM DNA Clean Beads need to be taken from the fridge and let it reach the 25°C before use.

34. Incubate at 25°C for 5 min.
35. Put the reaction tube on a magnetic rack to separate the beads and liquid (about 5 min).
36. Gently discard the supernatant, do not disturb the beads.
37. Add 200 μL freshly prepared 80% ethanol into the tube, do not disturb the beads.
38. Incubate at 25°C for 30 s.
39. Gently discard the supernatant.
40. Repeat the steps 37–39.
41. Open the cover and air dry for 3–5 min.

    Note: There should be no ethanol left because it will hamper the downstream steps. The following steps are to purify the PCR product.

42. Remove the PCR tube from the magnetic rack.
    a. Add 52 μL H_2O to the PCR tube.
    b. Pipette up and down to mix the beads.
    c. Incubate at 25°C for 2 min.
43. Put the tube on a magnetic rack to separate the beads and liquid (about 5 min).
44. Transfer the 50 μL supernatant into a new PCR tube.

    Note: The following steps are to remove large fragment of DNA (more than 1,000 bp).

45. Add 25 μL (0.5×) DNA Clean Beads into the PCR tube and pipette up and down several times.
46. Incubate at 25°C for 5 min.
47. Put the tube on a magnetic rack to separate the beads and liquid (about 5 min).
48. Transfer the supernatant into a new PCR tube.
49. Add 50 μL (1×) DNA Clean Beads into the PCR tube and pipette up and down several times.
50. Repeat the steps 34–41.

    Note: If the magnetic beads are cracked after super dry, extend the following incubation time appropriately.

51. Remove the PCR tube from the magnetic rack.
    a. Add 22 μL H_2O to the PCR tube.
    b. Pipette up and down to mix the beads.
52. After incubation for 2 min, put the tube on a magnetic rack to separate the beads and liquid.
53. Transfer the supernatant into a new PCR tube, store at −20°C.

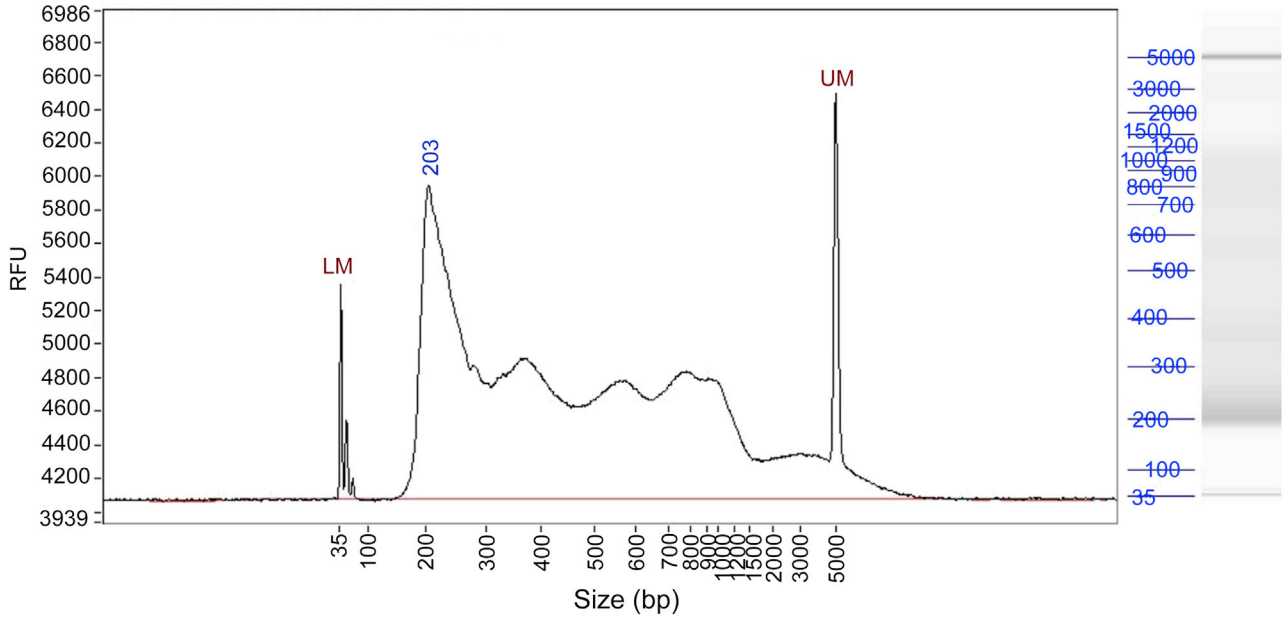

Figure2. Example result from fragment analysis of ATAC-seq library

DFN-915 analysis showing the nucleosomal "laddering" pattern of the ATAC-seq library. The expected size range is 200 bp–1,000 bp, and number of peaks is around 5. The LM (lower marker) indicates 35 bp and the UM (upper marker) indicates 5,000 bp. The ladders are quantified using Agilent 5400.

54. Analyze the DNA concentration using Real-time Quantitative PCR (QPCR) and quality of ATAC-seq library using DFN-915 analysis based on both the ladders in the agarose gel and the numbers of bands.
55. The DNA libraries can be sequenced using Illumina NovaSeq with pair end 150 bp (PE150).

EXPECTED OUTCOMES

For successful ATAC-seq library construction, the concentration of libraries is around 10–20 nmol/L. Figure 2 shows an example of size and laddering pattern of ATAC-seq library.

LIMITATIONS

This protocol was optimized for zebrafish erythrocytes. If used on other type of cells, the dissociation protocol, the time of cell lysis and enzyme digestion need to be optimized.

TROUBLESHOOTING

Problem 1

The viability of cell suspension is low (related to step 11).

Potential solution

- Reduce the time of trypsin digestion and pipette up and down gently.
- Make sure the 0.5% trypsin, 100% FBS and 1% FBS in DPBS were store at −20°C before use.

Problem 2

Inefficient elution from VAHTSTM DNA Clean Beads by measuring the DNA concentration (related to step 54).

Potential solution

- Make sure there is no remaining ethanol after air dry.
- If the magnetic beads are cracked after super dry, extend the following incubation time of beads with H_2O. On the other hand, reduce the time of air dry to avoid cracking of beads.

Problem 3

The main peak of DNA fragments is smaller than 200 bp (related to step 54).

Potential solution

- The expected size range of DNA fragments is 200 bp–1,000 bp. If the library size is smaller, reduce transposition reaction time.
- Increase the number of input cells to avoid the over digestion.

Problem 4

The main peak of DNA fragments is larger than 1,000 bp (related to step 54).

Potential solution

- Extend the transposition reaction time.
- Decrease the number of input cells to increase the digestion efficiency.

Problem 5

The shape of ATAC-seq peaks is suboptimal (related to step 54).

Potential solution

- Cells should be intact and freshly prepared to guarantee good quality of ATAC-seq library. Generally, the cell viability after FACS should be greater than 85%, which can be estimated using trypan blue staining.
- A dead cells stain (such as DAPI) can also be used to remove dead cells during FACS.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Feng Liu (liuf@ioz.ac.cn).

Materials availability

All the materials used in this protocol are commercially available.

Data and code availability

This study did not generate any unique data sets or code.

ACKNOWLEDGMENTS

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AUTHOR CONTRIBUTIONS

Y.D. and F.L. designed the study and wrote the manuscript. Y.D. performed most of the experiments.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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