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Progenitor Cell Isolation From Mouse Epididymal Adipose Tissue and Sequencing Library Construction

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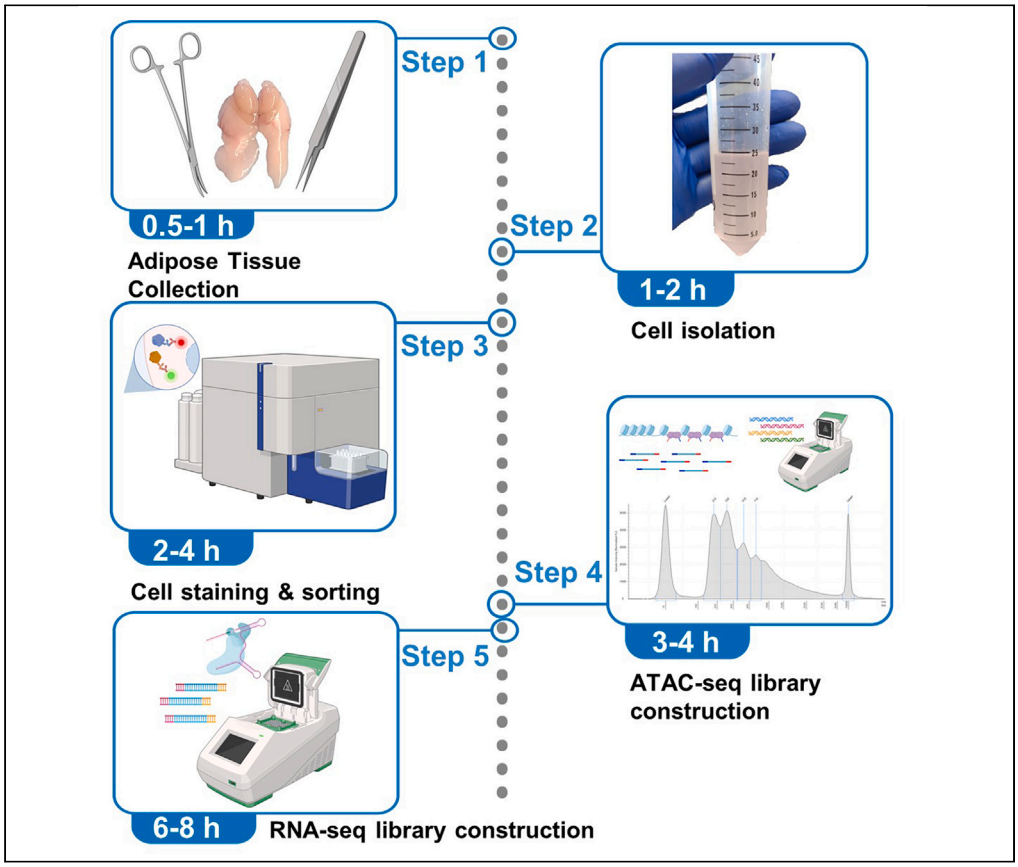

Here, we present a protocol to isolate progenitor cells from mouse epididymal visceral adipose tissue and construct bulk RNA and assay for transposase-accessible chromatin with sequencing (ATAC-seq) libraries. We describe steps for adipose tissue collection, cell isolation, and cell staining and sorting. We then detail procedures for both ATAC-seq and RNA sequencing library construction. This protocol can also be applied to other tissues and cell types directly or with minor modifications.

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 progenitors and other cells from mouse eVAT

Low-input techniques for bulk RNA-seq and ATAC-seq library construction

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Progenitor cell isolation from mouse epididymal adipose tissue and sequencing library construction

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SUMMARY

Here, we present a protocol to isolate progenitor cells from mouse epididymal visceral adipose tissue and construct bulk RNA and assay for transposase-accessible chromatin with sequencing (ATAC-seq) libraries. We describe steps for adipose tissue collection, cell isolation, and cell staining and sorting. We then detail procedures for both ATAC-seq and RNA sequencing library construction. This protocol can also be applied to other tissues and cell types directly or with minor modifications.

For complete details on the use and execution of this protocol, please refer to Liu et al. (2023).[1]

BEFORE YOU BEGIN

This protocol provides an easy and highly repeatable method for isolating progenitor cells from mouse eVAT and multiomic (RNA-seq and ATAC-seq) library construction methods using the isolated cells. Compared with other published protocols, this protocol requires a shorter enzymatic digestion time, resulting in less stress on the target cell populations. The optimized low-input library construction methods enable multiomic studies with limited cell numbers. Furthermore, with the detailed steps and the troubleshooting section, we are confident that this protocol can be easily followed.

Before you start, make sure all buffers are prepared freshly and kept on ice. The scissors and forceps used during eVAT collecting and processing need to be sterilized. The shaking incubator and the centrifuge can be turned on to reach the desired temperatures to reduce the overall processing time and handling stress.

This protocol has been verified on the *Tcf21* lineage progenitors in mouse eVAT. Minor modifications may be required if other cell types are being isolated, such as macrophages or endothelial cells.[2–4]

Institutional permissions

All animal experimentation in this protocol was approved by the Louisiana State University's Institutional Animal Care and Use Committee.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
BV421 rat anti-mouse Icam1 antibody (1:200 dilution)	BioLegend	Cat# 116141, RRID: AB_2876428
PE/Cy7 rat anti-mouse CD45 antibody (1:200 dilution)	BioLegend	Cat# 103114, RRID: AB_312979
APC rat anti-mouse Dpp4 antibody (1:200 dilution)	BioLegend	Cat# 137807, RRID: AB_10663403
Chemicals, peptides, and recombinant proteins		
Collagenase D	Roche	Cat# 11088882001
Dispase II	Roche	Cat# 04942078001
DMEM, high glucose	Corning	Cat# 10-013-CV
Bovine growth serum	VWR	Cat# 76324-910
HBSS	VWR	Cat# 02-0121-0500
HEPES	VWR	Cat# J848-100ML
BSA	VWR	Cat# 97061-420
TRIzol	Invitrogen	Cat# 15596018
SDS	Invitrogen	Cat# AM9822
Sodium chloride solution	Sigma	Cat# 59222C
Nonidet P-40	VWR	Cat# M158-100ML
Tris-HCl (pH = 7.4)	Sigma	Cat# T2194-100ML
Digitonin	Sigma	Cat# 300410
Calcium chloride solution	Sigma	Cat# 21115-100ML
Tween 20	Promega	Cat# H5152
Tris-HCl (pH = 8.0)	VWR	Cat# 97062
TE buffer	Sigma	Cat# 93283-100ML
Magnesium chloride	Sigma	Cat# M1028
Water	Ricca	Cat# R9145000-1G
DMF	Sigma	Cat# D4551
Red blood cell lysis buffer	Sigma	Cat# R7757
Ethanol	Koptec	Cat# V1001
PBS	Fisher	Cat# BP399-20
Critical commercial assays		
MinElute Reaction Cleanup Kit	QIAGEN	Cat# 28206
Phusion high-fidelity PCR master mix with HF buffer	NEB	Cat# M0531L
Illumina Tagment DNA Enzyme and Buffer Large Kit	Illumina	Cat# 20034198
miRNeasy Micro Kit	QIAGEN	Cat# 217084
Single Cell/Low-Input RNA Library Prep Kit for Illumina	NEB	Cat# E6420S
Qubit dsDNA HS and BR Assay Kits	Invitrogen	Cat# Q32854
Experimental models: Organisms/strains[a]		
Mouse: *Tcf21*^{MCM}; B6.129-*Tcf21*$^{tm3.1(cre/Esr1*)Eno}$	Acharya et al.[5]	N/A
Mouse: *R26*^{tdTomato}; B6.Cg-*Gt(ROSA)26Sor*$^{tm14(CAG-tdTomato)Hze}$/J	Jackson Laboratory	Cat# 007914
Mouse: *R26*^{eGFP}; FVB.Cg-*Gt(ROSA)26Sor*$^{tm1(CAG-lacZ,-EGFP)Glh}$/J	Yamamoto et al.[6]	N/A
Other		
Magnetic stand	Invitrogen	Cat# 12321D
Petri dish	VWR	Cat# 25384-164
1.5 mL centrifuge tube	VWR	Cat# 76332-068
2 mL centrifuge tube	VWR	Cat# 76332-058
50 mL centrifuge tube	VWR	Cat# 75845-202
5 mL polypropylene round-bottom tube	Falcon	Cat# 352063
14 mL polypropylene round-bottom tube	Falcon	Cat# 352059
Parafilm	Bemis	Cat# PM-992
10 μL low-retention filtered pipet tips	VWR	Cat# 76322-132

(*Continued on next page*)

Continued		
REAGENT or RESOURCE	SOURCE	IDENTIFIER
20 μL low-retention filtered pipet tips	VWR	Cat# 76322-134
200 μL low-retention filtered pipet tips	VWR	Cat# 76322-150
1250 μL low-retention filtered pipet tips	VWR	Cat# 76322-156
1250 μL wide orifice pipet tips	VWR	Cat# 89079-468
100 μm cell strainer	VWR	Cat# 76327-102
40 μm cell strainer	VWR	Cat# 76327-098
AMPure XP bead-based reagent	Beckman Coulter	Cat# A63881
D5000 ScreenTape	Agilent	Cat# 5067-5588

[a]In our study,[1] we used male mice at 2 weeks, 3 weeks, 7 weeks, and 24 weeks of age.

MATERIALS AND EQUIPMENT

Digestion buffer

Reagent	Stock concentration	Final concentration	Stock reagents needed to make 20 mL buffer
Collagenase D	0.25 U/mg	0.75 mU/μL	60 mg
Dispase II	1.0 U/mg	1 mU/μL	20 mg
$CaCl_2$	1 M	2 mM	40 μL
DMEM	-	-	19.96 mL
Total	-	-	**20 mL**

Note: Store at 4°C for up to 4 weeks

⚠ CRITICAL: The enzyme unit of Collagenase D and Dispase II may vary, please adjust usage accordingly.

Cell sorting buffer

Reagent	Stock concentration	Final concentration	Stock reagents needed to make 20 mL buffer
HEPES	1 M	25 mM	500 μL
BGS	-	3%	600 μL
BSA	10%	1%	2 mL
HBSS	-	-	16.9 mL
Total			**20 mL**

Note: Store at 4°C for up to 4 weeks

1× Lysis buffer

Reagent	Stock concentration	Final concentration	Stock reagents needed to make 1 mL buffer
Tris-HCl (pH = 7.4)	1 M	10 mM	10 μL
$MgCl_2$	1 M	3 mM	3 μL
NaCl	5 M	10 mM	2 μL
Nonidet P-40	10%	0.5%	50 μL
Tween-20	10%	0.1%	10 μL
Digitonin	5%	0.01%	2 μL
H_2O	-	-	923 μL
Total	-	-	**1 mL**

Note: Store at 4°C for up to 4 weeks

2× Tris-DMF tagmentation buffer			
Reagent	Stock concentration	Final concentration	Stock reagents needed to make 1 mL buffer
Tris-HCl (pH = 8.0)	1 M	20 mM	20 μL
$MgCl_2$	1 M	10 mM	10 μL
DMF	-	20%	200 μL
H_2O	-	-	770 μL
Total	-	-	1 mL

Note: Store at 4°C for up to 4 weeks

STEP-BY-STEP METHOD DETAILS

Adipose tissue collecting

Timing: 0.5–1 h

The following steps provide the details for collecting fresh mouse eVAT for adipose tissue progenitor cell isolation.

1. Sacrifice the mouse using a protocol approved by the performing institution, and spray 70% ethanol over the mouse for sterilization and to minimize contamination.
2. Dissect the skin and make an incision across the abdominal muscle with forceps and scissors.
3. Use forceps to hold the eVAT, slightly pull it up, and adjust the position to expose the entire eVAT pad, testis, and ductus deferens.
4. Dissect the eVAT together with the testis and epididymis while avoiding the ductus deferens (Figure 1).

Note: eVAT size varies at different ages and strains. For C57BL/6 mouse, we recommend collecting both sides of eVAT if the mouse is younger than 4 weeks and collecting one side if the mouse is over 8 weeks of age or under obese status to ensure getting enough cells for RNA-seq and ATAC-seq library construction. If a larger number of cells is needed for additional experiments, eVAT from littermates can be combined to increase the cell yield.

Pause point: At this point, the isolated eVAT can be placed in ice-cold PBS and kept on ice for at least 1 h if multiple samples need to be processed for one experiment. However, it is not recommended to keep the eVAT on ice for more than 6 h to ensure the cell yield and quality.

Adipose tissue processing and digesting

Timing: 30 min to 1.5 h

In this part, we describe the detailed procedures to isolate progenitor cells from the stromal vascular fraction of eVAT by enzymatic digestion. These steps provide an efficient and easy way to obtain large amounts of healthy progenitor cells for further applications.

5. Carefully rinse the eVAT in PBS to remove as much blood as possible.

Note: If viewed, remove any remaining undesired tissue, such as the epididymis or hairs.

6. Transfer the eVAT into a pre-chilled 2 mL centrifuge tube and add 500 μL digestion buffer.

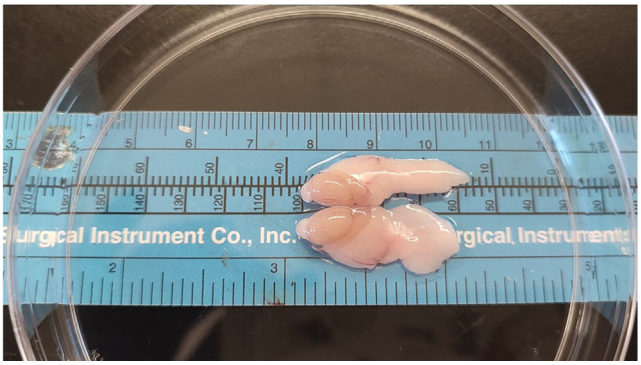

Figure 1. Example of collected eVAT with the associated testis and epididymis from an 8-week-old mouse

Note: The digestion buffer added in this step facilitates mincing and keeps the eVAT from drying. The amount of digestion buffer added is adjustable based on the size of the eVAT. For smaller eVAT (<1 g), 500 μL digestion buffer is enough. For eVAT larger than 1 g, especially eVAT from obese mice, use a 5 mL centrifuge tube and add 1 mL digestion buffer or split the eVAT into several 2 mL tubes, then add 500 μL digestion buffer into each tube.

7. Mince the eVAT with scissors into ~1 mm^3 pieces and keep the tube on ice until all samples are processed.
8. Add more digestion buffer. For larger eVAT, transfer the suspension to a 5 mL or 14 mL Falcon tube.

Note: For eVAT <0.5 g, use 1 mL of digestion buffer in total. For eVAT >0.5 g, add 1 mL digestion buffer per 0.5 g of tissue.

⚠ CRITICAL: Make sure the volume of the suspension is between 40% and 60% of the tube capacity (Figure 2) to ensure sufficient agitation of the minced eVAT for efficient digestion.

9. Place the centrifuge tube horizontally in a pre-heated shaking incubator at 37°C, 150 rpm for 10–15 min.

Note: Use parafilm to seal the cap to prevent potential leakage. Mount the tube firmly in the shaking incubator with tape.

10. Remove the tube from the incubator. Shake the tube briefly or use a wide-bore pipette tip to pipet the suspension 10 times to facilitate tissue dissociation.

Note: If adipocytes are desired, allow the cell suspension to stand on the benchtop for 1 min. The upper adipocyte layer can be transferred to a 1.5 mL tube with a wide-bore pipette tip for further processing.

11. Transfer the suspension to a pre-chilled 50 mL centrifuge tube and add 10–20 mL ice-cold PBS to stop the digestion (Figure 3A).
12. Filter the cell suspension with a 100 μm cell strainer then a 40 μm cell strainer.

Note: More PBS can be used to wash the cell strainer to increase the cell yield.

13. Centrifuge the filtered cell suspension at 600 × g for 5 min at 4°C.
14. Carefully remove the upper lipid layer and the supernatant without disturbing the cell pellet mainly containing the stromal vascular cells (SVCs) and immune cells.

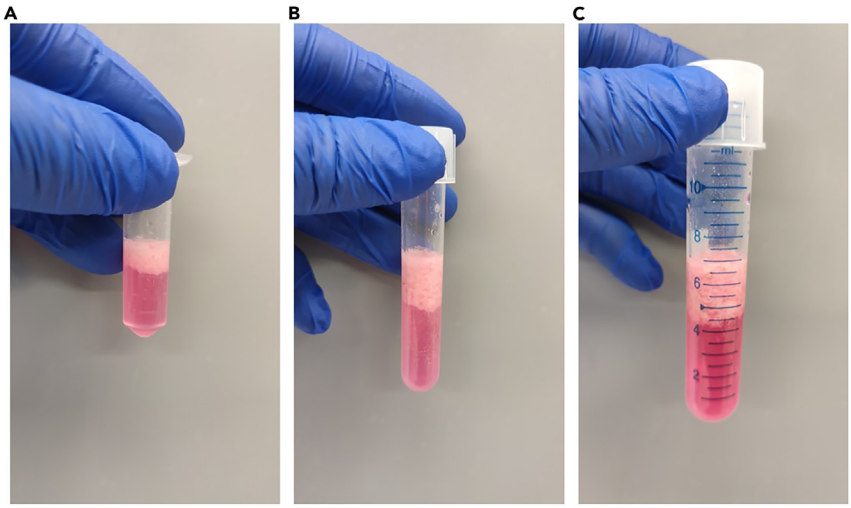

Figure 2. Examples of different amounts of eVAT, types of tubes, and digestion buffer volume
(A) A 2 mL tube with 1 mL digestion buffer for <0.5 g eVAT.
(B) A 5 mL Falcon tube with 2 mL digestion buffer for ~1.0 g eVAT.
(C) A 14 mL Falcon tube with 5 mL digestion buffer for ~2.5 g eVAT.

Note: After this step, if your target cell population has a fluorescent marker, like eGFP or tdTomato, please go to step 21 directly.

Optional: Resuspend the cell pellet in 1 mL red blood cell lysis buffer for 1 min on ice. Add 10–20 mL ice-cold PBS, then centrifuge at 600 × g for 5 min at 4°C.

Cell staining

Timing: 1–2 h

The following part is to label target progenitor cell populations before cell sorting. If the desired population expresses reporters like eGFP or tdTomato, this part can be skipped.

15. Resuspend the cell pellet in 100 μL cell ice-cold sorting buffer containing 0.5 μL (1:200) fluorophore-conjugated antibodies, such as BV421-anti-Icam1, APC-anti-Dpp4, and PE/Cy7-anti-CD45, to separate different subpopulations of the *Tcf21* lineage progenitors.

Note: For larger cell pellets, 200 μL sorting buffer or more can be used to resuspend.

16. Transfer the suspension to a 1.5 mL centrifuge tube and incubate in the dark for 20 min at 4°C.
17. Add 1 mL ice-cold PBS and centrifuge at 500 × g for 5 min at 4°C.
18. Discard the supernatant and resuspend the cell pellet in 1 mL ice-cold cell sorting buffer.
19. Centrifuge at 500 × g for 5 min at 4°C.
20. Discard the supernatant and resuspend the cell pellet in 300 μL ice-cold cell sorting buffer.

Note: Check the cell density under a microscope before proceeding to cell sorting. Dilute if the cell density is too high.

FACS

Timing: 1–2 h

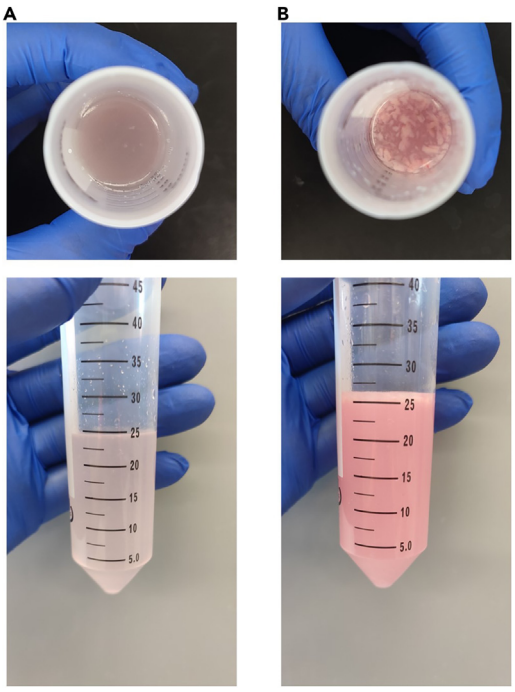

Figure 3. Example of digested eVAT after adding ice-cold PBS

(A) A well-digested sample.

(B) An incompletely digested sample. Undigested adipose tissue can be seen in the top floating layer.

This step is to sort out and collect the desired cell population for further study.

21. Perform FACS to sort out the desired cell population.

Note: For RNA-seq, collect 10,000 target cells into 700 μL TRIzol reagent directly. For ATAC-seq, collect 10,000 target cells into 1 mL cell sorting buffer. Library construction using fewer cells is possible, but the protocol may need further optimization.

ATAC-seq library construction

DNA tagmentation and purification

22. Centrifuge the sorted cells at 1,000 × g for 10 min at 4°C.
23. Carefully remove the supernatant without disturbing the cell pellet.

Note: The cell pellet might be hard to see. We recommend using a pipet to remove the last 100 μL supernatant. 1–2 μL residual is acceptable.

24. Add 8.5 μL ice-cold 1× lysis buffer and pipette 10–20 times to fully resuspend the cell pellet.
25. Incubate the lysate on ice for 20 min to disrupt the cell membrane.

Note: 20 min is for SVCs. Optimization of the lysis time needs to be done for different cell types.

26. Add 12.5 μL 2× Tris-DMF tagmentation buffer and 2 μL Nextera Tn5 transposase (TDE1). Pipette to fully mix.

Note: DO NOT vortex to mix the lysate. Adjust the enzyme usage if you are using more cells.

27. Incubate in a pre-heated ThermoMixer C at 700 rpm and 37°C for 30 min.

⚠ CRITICAL: Optimize the tagmentation time for your cell type to ensure the best tagmentation outcome.

28. Add 0.5 μL 10% SDS, pipette to mix well, and incubate on ice for 5 min to stop the tagmentation reaction.
29. Use the QIAGEN MinElute Reaction Cleanup Kit to purify the tagmented DNA fragments following the manufacturer's instruction (Protocol can be found HERE).

Pause point: The purified DNA fragments can be stored at 4°C for up to 1 week or at −20°C for 4 weeks before PCR amplification.

DNA fragments amplification

Timing: 1.5–2 h

The following steps in this part provide the step-by-step details to finish the ATAC-seq library construction.

30. Prepare the PCR reaction to amplify the DNA fragments as follows.

PCR reaction master mix	
Reagent	Volume/ μL
Tagmented DNA	20
2× Phusion PCR master mix	25
100 mM F primer (Ad1)*	0.625
100 mM R primer (Ad2.1-2.24)*	0.625
H_2O	3.75
Total	**50**

Note: *Primer sets were adopted from Ming et al. (2021).[2]

31. Run PCR with the following setting in a thermocycler.

PCR cycling conditions			
Steps	Temperature/ °C	Time	Cycles
Extension of both ends of the primer	72	3 min	1
Initial Denaturation	98	30 s	1
Denaturation	98	15 s	9–12
Annealing	60	30 s	
Extension	72	3 min	
Final extension	72	5 min	1
Hold	4	∞	-

Note: Annealing cycle number needs optimization for your input cell type and numbers.

Pause point: At this point, the amplified DNA fragments can be stored at 4°C for 24 h or at −20°C for 1 week.

32. After PCR amplification, add 55 μL (1.1×) AMPure XP beads, pipette to mix well, and transfer to a 1.5 mL centrifuge tube.

Note: The AMPure XP beads are stored at 4°C and must be placed at 25°C for 30 min before use.

33. Incubate on bench top for 5 min.
34. Place the tube on a magnetic stand for 5 min or until the liquid phase becomes clear.
35. While the tube remains on the magnetic stand, carefully remove the supernatant by pipetting without disturbing the bead pellet.
36. Gently add 200 μL freshly made 80% ethanol without disturbing the bead pellet and incubate for 30 s.
37. Remove ethanol and repeat steps 36–37 to wash the bead pellet twice in total.
38. Remove residual ethanol with a pipette and air dry the bead pellet with the cap open for up to 5 min while the tube is on the magnetic stand.

Note: DO NOT over-dry (the beads start to crack) the beads. Elute the DNA when the beads are still dark brown without visible liquid. Otherwise, the DNA yield will be lower.

39. Remove the tube from the magnetic stand, add 25 μL TE buffer or nuclease-free H2O, and mix well by gentle pipetting.
40. Incubate on bench top for 3 min.
41. Place the tube back on the magnetic stand for 5 min or until the liquid phase becomes clear.
42. Carefully transfer 23 μL supernatant to a new 1.5 mL tube.
43. Analyze the DNA concentration and the size distribution with an Agilent TapeStation or a Bioanalyzer.

Note: The DNA library can be further sequenced or stored at −80°C for up to 12 months.

RNA-seq library construction

Timing: 6–8 h

This part is for extracting the total RNA from the sorted cells and constructing the RNA-seq library to be sequenced.

The RNA-seq libraries are constructed with a commercial RNA extraction kit (QIAGEN 217084, protocol can be found HERE) and an RNA Library Prep Kit (NEB E6420S, protocol can be found HERE) following the manufacturer's instructions.

EXPECTED OUTCOMES

With this protocol, cells from eVAT can be isolated. With FACS, different cell types (e.g., pre-adipocytes, endothelial cells, fibroblasts, mesenchymal progenitor/stem cells) can be separated and collected.

Upon the completion of the library construction, the size distribution pattern of the ATAC-seq library should look like Figure 4.

LIMITATIONS

This protocol was optimized for mesenchymal progenitor cells from mouse white adipose tissue, especially eVAT. If used on other species, organs/tissues, or cell types, the tissue digestion time, the number of cells needed for ATAC-seq library construction and the cell lysis and tagmentation time may need to be further optimized.

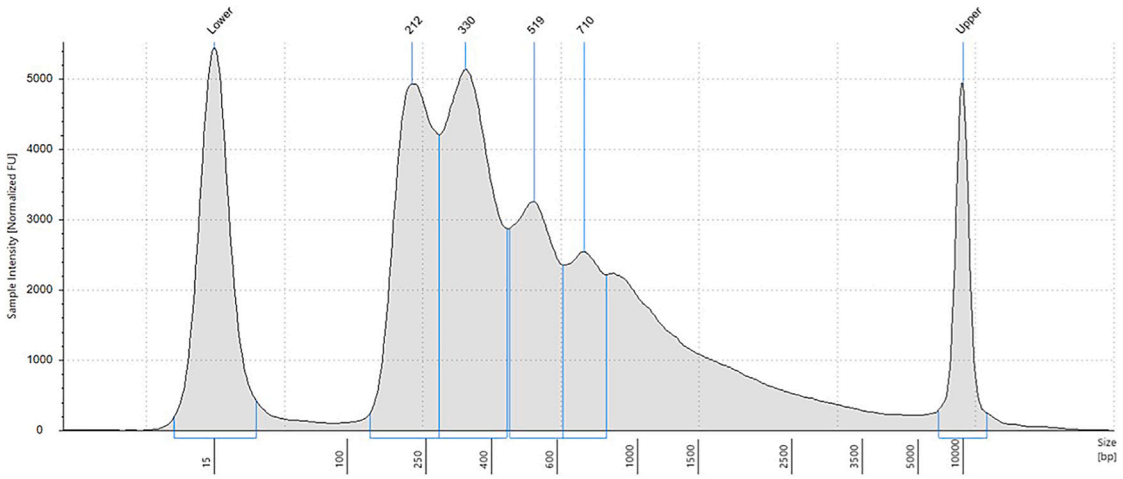

Figure 4. An example of a good ATAC-seq library pattern

TROUBLESHOOTING

Problem 1

Adipose tissue digestion is incomplete (related to step 7 and step 9 to step 11, Figure 3B).

Potential solution

- Make sure the adipose tissue is finely minced before proceeding to digestion.
- Increase the digestion time up to 20 min.
- Check if you are using the right size tube. Ideally, the suspension volume is less than 60% of the tube capacity for efficient digestion.
- Re-digest the undigested adipose tissue with additional digestion buffer while placing the well-digested tissue slurry on ice.

Problem 2

Cell pellets cannot be seen after centrifuge (related to step 22).

Potential solution

- Make sure the cell sorting buffer contains 1% BSA.
- Use Low-Bind centrifuge tubes.
- Process the adipose tissue immediately after collection without storing at 4°C for too long to reduce cell death.
- Reduce the sorting speed to improve the cell viability.

Problem 3

No peaks can be identified in ATAC-seq libraries (related to step 30 and step 39).

Potential solution

- Monitoring the beads while they are being air dried in step 38. Do not over-dry the beads.
- If the beads start to crack in step 38, incubate longer when eluting the DNA fragments from the beads in step 40.
- Make sure to mix the PCR reaction mix thoroughly in step 30.

Problem 4

A single peak smaller than 200 bp is seen in ATAC-seq libraries (related to step 43, Figure 5).

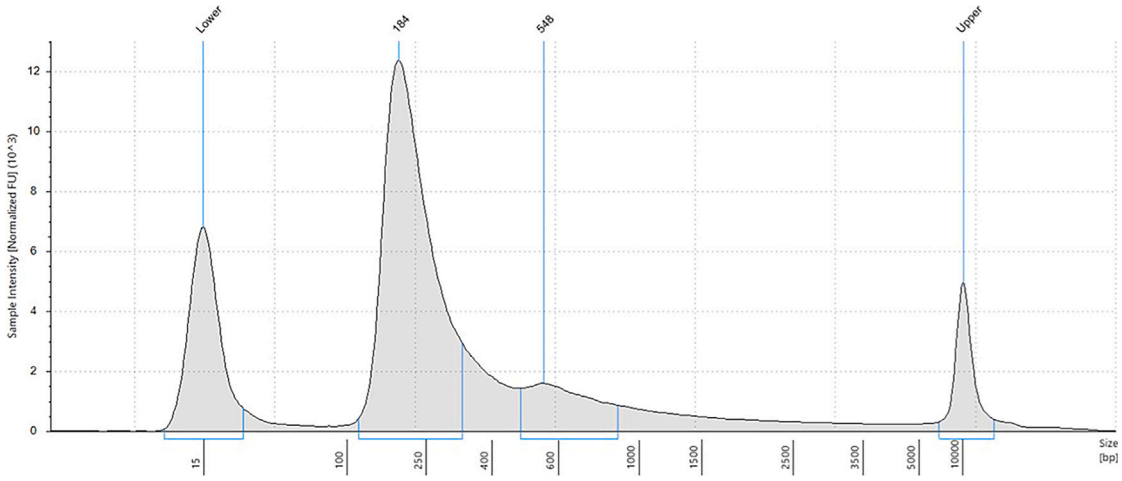

Figure 5. An example of a low-quality ATAC-seq library pattern

Potential solution

- Check the steps that may cause cell death. Improve cell viability.
- Reduce the tagmentation time or reduce the Tn5 transposase usage.

Problem 5

Dominant peaks larger than 1,000 bp are present in ATAC-seq libraries (related to step 43, Figure 6).

Potential solution

- Remove as much residual buffer as possible in step 23 to improve tagmentation efficiency.
- Increase the tagmentation time.
- Increase Tn5 transposase usage.
- Utilize the AMPure XP beads to perform the size selection to remove the DNA fragments larger than 1000 bp.

Note: All samples to be compared should be treated in the same way to ensure consistency.

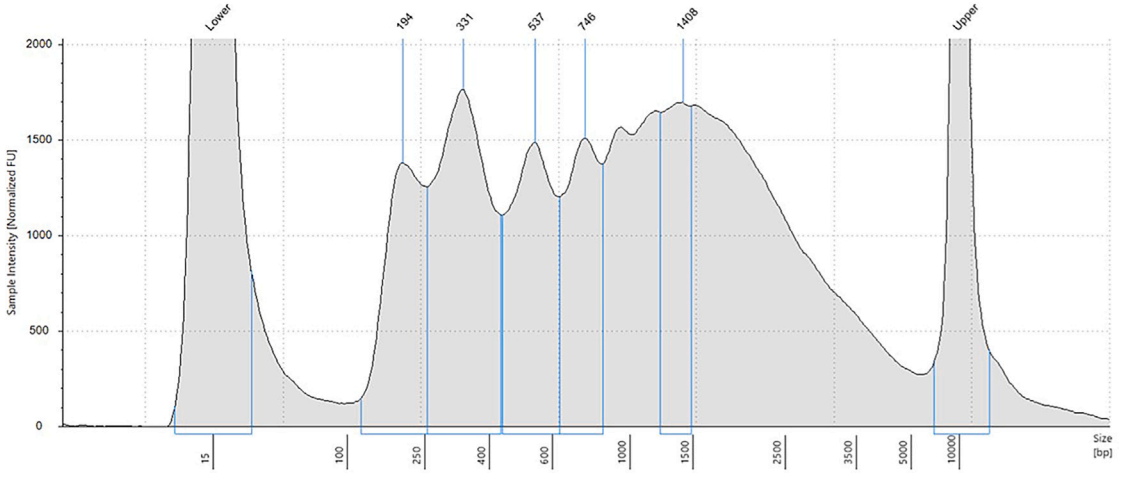

Figure 6. An example of incompletely tagmented ATAC-seq library pattern

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Dr. Xing Fu (xfu1@agcenter.lsu.edu).

Materials availability

This protocol did not generate new unique reagents. All the reagents and materials used in this protocol are commercially available.

Data and code availability

- This protocol did not generate new datasets, nor data analyzing codes.

ACKNOWLEDGMENTS

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AUTHOR CONTRIBUTIONS

Q.L., C.L., Y.L., L.W., X.Z., B.D., and J.Z. performed the experiments, wrote the protocol, and created the figures. Q.L., C.L., B.D., P.G., M.S., and F.A. analyzed the data. J.M.S., C.A.S., J.F., J.S., and X.F. reviewed, edited, and revised the protocol.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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