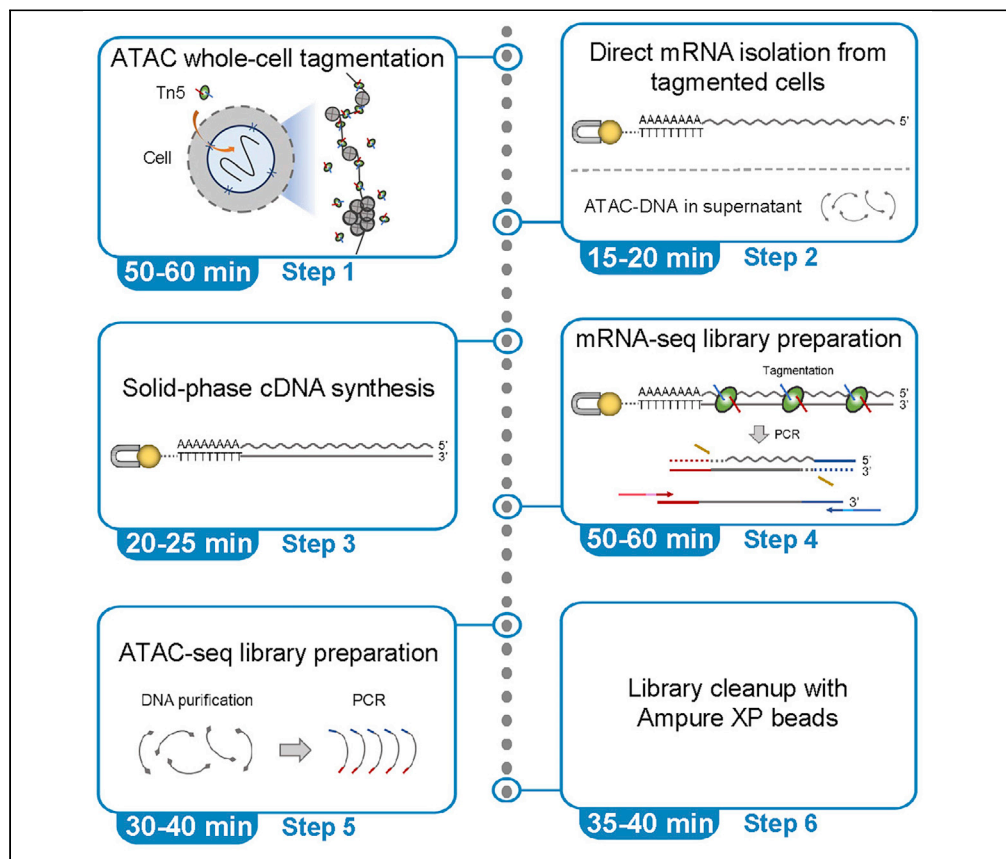


Protocol

Low-input ATAC&mRNA-seq protocol for simultaneous profiling of chromatin accessibility and gene expression



We present a simple, fast, and robust protocol (low-input ATAC&mRNA-seq) to simultaneously generate ATAC-seq and mRNA-seq libraries from the same cells in limited cell numbers by coupling a simplified ATAC procedure using whole cells with a novel mRNA-seq approach that features a seamless on-bead process including direct mRNA isolation from the cell lysate, solid-phase cDNA synthesis, and direct tagmentation of mRNA/cDNA hybrids for library preparation. It enables dual-omics profiling from limited material when joint epigenome and transcriptome analyses are needed.

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Highlights

A simple and fast dual-omics profiling protocol for low-input samples

Simultaneous measurement of open chromatin and gene expression from the same cells

Suitable for limited numbers of cells ranging from 5,000 to 20,000 cells

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Protocol

Low-input ATAC&mRNA-seq protocol for simultaneous profiling of chromatin accessibility and gene expression

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SUMMARY

We present a simple, fast, and robust protocol (low-input ATAC&mRNA-seq) to simultaneously generate ATAC-seq and mRNA-seq libraries from the same cells in limited cell numbers by coupling a simplified ATAC procedure using whole cells with a novel mRNA-seq approach that features a seamless on-bead process including direct mRNA isolation from the cell lysate, solid-phase cDNA synthesis, and direct tagmentation of mRNA/cDNA hybrids for library preparation. It enables dual-omics profiling from limited material when joint epigenome and transcriptome analyses are needed. For complete details on the use and execution of this protocol, please refer to Li et al. (2021).

BEFORE YOU BEGIN

The protocol below describes the specific steps for using 10,000 mouse embryonic stem cells (mESCs). However, we have also used this protocol for 5,000 and 20,000 mESCs with adjustment of reaction volumes in some of the steps.

Make sure the required materials and equipment are ready to use

1. If needed, order and prepare reagents and kits per manufacturer's instructions. The required reagents and kits are listed in [key resources table](#) below.
 - a. Confirm kit contents and make sure that you have the required components in sufficient amount.
 - b. Order and resuspend ATAC-seq PCR primers to 10 μ M working concentration.

Note: The sequences of universal i5 primer and index i7 primers were published previously ([Buenrostro et al., 2015](#)).

- c. Prepare 1% Digitonin – Digitonin (Promega cat# G9441) is supplied at 2% in DMSO. Dilute 1:1 with nuclease-free water to make a 1% (100 \times) stock solution. It can be kept at -20°C for up to 6 months. Avoid more than 5 freeze-thaw cycles.
2. Set up and save the thermocycler programs to be run in this protocol.

Note: See the detailed settings below in “[materials and equipment](#)” section.



- The day before the experiment, confirm that the Dynabeads® Oligo (dT)25 is in liquid suspension. If the Dynabeads® have dried in the vial, resuspend the beads in the buffer they are supplied in by placing the vial on a mixer overnight at 4°C.

Note: Store the vials of Dynabeads® Oligo (dT)25 upright to avoid drying of the beads.

- If multiple samples are processed at the same time, calculate in advance the total amounts of ATAC transposition mixture and reverse transcription reaction mix needed for the experiment.

Note: See instructions below in step 2 and step 23.

- On the day of the experiment, pre-chill microcentrifuge to 4°C and set thermomixer to 37°C.
- To prevent RNase contamination, follow best laboratory practices for handling RNA during reagents preparation and during the experiment; for example, wear disposable gloves and change them frequently, use sterile and RNase-free tubes and pipette tips with aerosol barriers, clean pipettors and the bench with RNaseZap® solution (Invitrogen cat# AM9780).

△ CRITICAL: Unless a safe stopping point is specified in the protocol, proceed immediately to the next step.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Chemicals, peptides, and recombinant proteins		
Digitonin	Promega	Cat# G9441
TWEEN® 20	Sigma-Aldrich	Cat# 11332465001
DPBS, no calcium, no magnesium	Gibco™	Cat# 14190250
SUPERase•In™ RNase Inhibitor (20 U/μL)	Invitrogen™	Cat# AM2696
Water, nuclease-free	Thermo Scientific™	Cat# R0581
Lithium chloride solution (8M)	Sigma-Aldrich	Cat# L7026-100ML
Ethylenediaminetetraacetic acid solution (0.5 M EDTA)	Sigma-Aldrich	Cat# 03690-100ML
Sodium Acetate Solution (3 M), pH 5.2	Thermo Scientific™	Cat# R1181
NEBNext® High-Fidelity 2x PCR Master Mix	NEB	Cat# M0541S
AMPure XP beads	Beckman Coulter	Cat# A63881
Ethyl alcohol, Pure	Sigma-Aldrich	Cat# E7023-500ML
Trypan Blue Solution, 0.4%	Thermo Fisher Scientific	Cat# 15250061
RNaseZap™ RNase Decontamination Solution	Invitrogen™	Cat# AM9780
Critical commercial assays		
Illumina Tagment DNA Enzyme and Buffer Large Kit	Illumina	Cat# 20034198
Dynabeads™ mRNA DIRECT™ Micro Purification Kit	Invitrogen™	Cat# 61021
SuperScript™ IV First-Strand Synthesis System	Invitrogen™	Cat# 18091050
Nextera XT DNA Library Preparation Kit	Illumina	Cat# FC-131-1024
Nextera XT Index Kit (24 indexes, 96 samples)	Illumina	Cat# FC-131-1001
MinElute PCR Purification Kit (250)	QIAGEN	Cat# 28006
Qubit™ dsDNA HS Assay Kit	Invitrogen™	Cat# Q32851
D1000 ScreenTape	Agilent	Cat# 5067-5582
D1000 Reagents	Agilent	Cat# 5067-5583
Deposited data		
Low-input ATAC&mRNA-seq data	This paper	GEO: GSE165478
Experimental models: cell lines		
E14 mouse embryonic stem cells	Kristian Helin Lab	RRID: CVCL_C320

(Continued on next page)

<i>Continued</i>		
REAGENT or RESOURCE	SOURCE	IDENTIFIER
<i>Oligonucleotides</i>		
Universal i5 primer: AATGATACGGCGACCACCG AGATCTACACTCGTCGGCAGCGTCAGATGTG	Buenrostro et al., 2013	Ad1_noMX
index i7 primer_Ad2.31: CAAGCAGAAGACGGCATA CAGATTAACCTTATGTCTCGTGGGCTCGGAGATGTG	Buenrostro et al. 2015	v2_Ad2.31_ATAAGTTA
index i7 primer_Ad2.32: CAAGCAGAAGACGGCATA CAGATCGAGTGATGTCTCGTGGGCTCGGAGATGTG	Buenrostro et al. 2015	v2_Ad2.32_ATCACTCG
index i7 primer_Ad2.34: CAAGCAGAAGACGGCATA CGAGATCTACCATTGTCTCGTGGGCTCGGAGATGTG	Buenrostro et al. 2015	v2_Ad2.34_AATGGTAG
index i7 primer_Ad2.35: CAAGCAGAAGACGGCATA CAGATACGTGCTCGTCTCGTGGGCTCGGAGATGTG	Buenrostro et al. 2015	v2_Ad2.35_GAGCACGT
index i7 primer_Ad2.36: CAAGCAGAAGACGGCATA CAGATTGACGAAAGTCTCGTGGGCTCGGAGATGTG	Buenrostro et al. 2015	v2_Ad2.36_TTTCGTCA
<i>Software and algorithms</i>		
Cutadapt v1.12	Martin, 2011	N/A
Bowtie2 v2.1.0	Langmead and Salzberg, 2012	N/A
samtools v1.3.1	Li et al., 2009	N/A
Picard tools v1.110	http://broadinstitute.github.io/picard	N/A
BEDtools v2.24.0	Quinlan and Hall, 2010	N/A
BedGraphToBigWig	http://hgdownload.soe.ucsc.edu/admin/exe/	N/A
STAR v2.5	Dobin et al., 2013	N/A
<i>Other</i>		
Refrigerated microcentrifuge	Eppendorf	Cat# 5425 R
MicroCL 21 Microcentrifuge	Thermo Fisher Scientific	Cat# 75002466
Ohaus™ Frontier™5306 Mini Centrifuge	Fisher Scientific	Cat# 02-112-305
DynaMag™-2 Magnet	Thermo Fisher Scientific	Cat# 12321D
Magnetic Separation Rack, 0.2 mL Tubes	EpiCypher	Cat# 10-0008
96-Well Aluminum Block For 0.2 mL Tubes	Universal Medical	Cat# 81001
Tube rotator	Labnet	Cat# H5500
ThermoMixer	Eppendorf	Cat# 5384000020
Qubit Fluorometer	Thermo Fisher Scientific	Cat# Q33216
2200 TapeStation System	Agilent	Cat# G2964AA
Applied Biosystems ProFlex PCR System	Thermo Fisher Scientific	Cat# 4484073
Countess™ II Automated Cell Counter	Thermo Fisher Scientific	Cat# AMQAX1000

MATERIALS AND EQUIPMENT

Alternatives: This protocol uses Qiagen MinElute PCR Purification Kit (cat# 28006) to purify ATAC-DNA. However, Zymo DNA Clean and Concentrator-5 Kit (cat# D4014) can be used instead.

Alternatives: This protocol uses Agilent TapeStation to assess library size distribution. Alternatively, Agilent Bioanalyzer can be used.

Thermocycler program setup

- Save the following “RT” program on the thermal cycler:

Choose the preheat lid option and set to 100°C

Set the reaction volume to 20 µL

Incubation conditions

Steps	Temperature	Time	Cycles
Initial incubation	50°C	5 min	1
Reverse transcription	55°C	10 min	1
Hold	4°C	Forever	

- Save the following “TAG” program on the thermal cycler:

Choose the preheat lid option and set to 100°C

Set the reaction volume to 20 µL

Incubation conditions

Steps	Temperature	Time	Cycles
Tagmentation	55°C	5 min	1
Hold	10°C	Forever	

- Save the following “NXT PCR” program on the thermal cycler:

Choose the preheat lid option and set to 100°C

Set the reaction volume to 50 µL

PCR cycling conditions

Steps	Temperature	Time	Cycles
Gap filling	72°C	3 min	1
Initial denaturation	95°C	30 s	1
Denaturation	95°C	10 s	13–15 cycles
Annealing	55°C	30 s	
Extension	72°C	30 s	
Final extension	72°C	5 min	1
Hold	10°C	Forever	

- Save the following “ATAC PCR” program on the thermal cycler:

Choose the preheat lid option and set to 100°C

Set the reaction volume to 50 µL

PCR cycling conditions

Steps	Temperature	Time	Cycles
Gap filling	72°C	5 min	1
Initial denaturation	98°C	30 s	1
Denaturation	98°C	10 s	8–10 cycles
Annealing	63°C	30 s	
Extension	72°C	1 min	
Hold	4°C	Forever	

Recipes

- ATAC transposition mixture (10 μ L for 10,000 cells)

Reagent	Final concentration	Amount
DPBS	n/a	16.5 μ L
nuclease-free H ₂ O	n/a	2.5 μ L
TD Buffer	1 \times	25 μ L
10% Tween-20	1%	0.5 μ L
1% Digitonin	0.01%	0.5 μ L
TDE1	n/a	2.5 μ L
SUPERase ⁱⁿ ™ RNase Inhibitor (20 U/ μ L)	1 U/ μ L	2.5 μ L
Total	n/a	50 μL

Note: Prepare freshly at step 2 of the experiment. Store on ice before use.

- Reverse transcription reaction mix (20- μ L reaction)

Reagent	Final concentration	Amount
DEPC-treated water	n/a	12 μ L
5 \times SSIV Buffer	1 \times	4 μ L
10 mM dNTP mix (10 mM each)	0.5 mM each	1 μ L
100 mM DTT	5 mM	1 μ L
Ribonuclease Inhibitor (40 U/ μ L)	2 U/ μ L	1 μ L
SuperScript™ IV Reverse Transcriptase (200 U/ μ L)	10 U/ μ L	1 μ L
Total	n/a	20 μL

Note: Prepare freshly at step 23 of the experiment. Store on ice before use.

STEP-BY-STEP METHOD DETAILS

ATAC tagmentation using whole cells

⌚ Timing: 50–60 min

This step uses Illumina Tagment DNA Enzyme and Buffer Kit (Cat# 20034198) to fragment and tag open chromatin regions with adapter sequences.

Note: This step is based on Omni-ATAC protocol (Corces et al., 2017) with modifications.

⚠ **CRITICAL:** This step requires single cell suspension, as cell clumps can cause inaccurate cell counts and inefficient cell permeabilization.

⚠ **CRITICAL:** Cell viability needs to be above 90% and preferably around 95%.

⚠ **CRITICAL:** This step relies on a one-step membrane permeabilization and transposition. Different cell types may need different detergent or concentration for proper cell permeabilization. However, the combination of 0.01% digitonin and 0.1% Tween-20 is a good starting point.

1. Cell preparation
 - a. Wash the harvested mESCs once in cold DPBS, and then resuspend the cell pellet in cold DPBS.
 - b. Count the cells with a manual or automated cell counter. Trypan blue staining is recommended to assess cell viability.
 - c. Gently mix the cell suspension and pipette a volume corresponding to 1×10^4 cells into a new 1.5 mL Eppendorf tube.
2. Centrifuge the tube containing 1×10^4 cells at $500 \times g$ for 5 min at 4°C in a fixed angle centrifuge.

Note: While waiting for the centrifuge to stop, prepare ATAC transposition mixture as below and keep it on ice before use.

ATAC transposition mixture (10 μL for 10,000 cells)		
Reagent	Final concentration	Amount
DPBS	n/a	3.3 μL
nuclease-free H ₂ O	n/a	0.5 μL
TD Buffer	1 \times	5 μL
10% Tween-20	1%	0.1 μL
1% Digitonin	0.01%	0.1 μL
Tagment DNA Enzyme (TDE1)	n/a	0.5 μL
SUPERase [•] In™ RNase Inhibitor (20 U/ μL)	1 U/ μL	0.5 μL
Total	n/a	10 μL

Note: The volumes given here are for a single sample with 1×10^4 mESCs. For multiple samples, scale up and include 10% excess volume for pipetting losses.

△ CRITICAL: The ratio of cell number to TDE1 is crucial. 10 μL transposition mixture is used for 1×10^4 mESCs. Based on the actual cell number of the sample, adjust the volume of ATAC transposition mixture to use proportionally.

3. Remove all the supernatant carefully using two pipetting steps (aspirate down to 100 μL with a P1000 pipette then remove final 100 μL with a P200 pipette).
4. Resuspend the cell pellet in 10 μL ATAC transposition mixture by pipetting up and down 6 times.
5. Incubate the transposition reaction at 37°C for 30 min in an Eppendorf Thermomixer with shaking at 1000 RPM.

Note: While waiting for the incubation to complete, please prepare the following.

Prepare EDTA-LiCl mixture by mixing 88 μL of 0.5 M EDTA with 275 μL of 8M LiCl. The mixture is stable at room temperature (21°C – 23°C) and can be reused in the future.

Prepare reagents for direct mRNA isolation from cells following step 7.

Thaw the components of SuperScript™ IV First-Strand Synthesis System that are frozen.

For each sample, prepare a mixture of 560 μL of Buffer PB (from Qiagen MinElute PCR Purification Kit) with 10 μL of 3 M sodium acetate, pH 5.2 in a labeled Eppendorf tube. Keep the mixture at room temperature (21°C – 23°C) before use in step 12.

6. Immediately upon completion of the incubation, quick spin the sample tube, then add 1 μL of EDTA-LiCl mixture (0.1 volume of the ATAC transposition mixture used in step 4) to stop the transposition reaction.

△ CRITICAL: Proceed immediately to step 8.

Direct mRNA isolation from the tagged cells

⌚ Timing: 15–20 min

This step uses Dynabeads™ mRNA DIRECT™ Micro Purification Kit (Cat# 61021) to isolate mRNA directly from the tagged cells.

Note: This step follows the manufacturer's manual with minor changes. https://assets.thermofisher.com/TFS-Assets/LSG/manuals/DynabeadmRNADIRECTMicro_UG_Rev004_20120514.pdf

7. Prepare reagents

- a. Prepare buffers from Dynabeads™ mRNA DIRECT™ Micro Purification Kit
 - i. Bring all buffers except the 10 mM Tris-HCl to room temperature before use. Store the 10 mM Tris-HCl on ice or at 2°C–8°C before use.
 - ii. Confirm that the Lysis/Binding Buffer has not precipitated. If any precipitation is observed, warm to room temperature and shake to dissolve.
- b. Wash Dynabeads® Oligo (dT)25 before use
 - i. Resuspend Dynabeads® Oligo (dT)25 by vortex for more than 30 s.

Note: Keep the Dynabeads® Oligo (dT)25 in liquid suspension during handling and storage to avoid reduced performance. If the Dynabeads® Oligo (dT)25 becomes dried out, resuspend the beads in the buffer they are supplied in by placing the vial on a roller or equivalent overnight at 2°C–8°C to restore their complete functionality.

- ii. Pipette 20 µL Dynabeads® Oligo (dT)25 into a 1.5 mL RNase-free Eppendorf tube.

Note: Use 20 µL Dynabeads® per sample with 1×10^4 cells. For multiple samples, include 5–10% excess volume for pipetting losses.

- iii. Add 1 mL of Lysis/Binding Buffer and mix.
- iv. Place the tube on a magnet for 1 min, then remove and discard the supernatant.
- v. Remove the tube from the magnet, then resuspend the beads in 20 µL of Lysis/Binding Buffer (the same volume as the Dynabeads® used in step ii).

8. Add 100 µL Lysis/Binding Buffer to the tagged cells from step 6. Perform a repeated passage of the solution through a pipette tip to obtain complete lysis.
9. Add 20 µL pre-washed Dynabeads® Oligo (dT)25 to the cell lysate and pipette up and down 2–3 times to mix.
10. Place the tube on a sample rotator at room temperature (21°C–23°C) for 5 min with continuous rotation to allow the mRNA to anneal to the Dynabeads®.

Note: While waiting, prepare reverse transcription reaction mix following step 23.

11. Quick spin the sample tube then place it on the magnet for 1 min.
12. Transfer the supernatant into the Eppendorf tube containing Buffer PB and sodium acetate mixture prepared earlier.

△ CRITICAL: Don't discard the supernatant, as it contains the tagged DNA for ATAC-seq library preparation. Store the mixture at room temperature before proceeding to step 42.

13. Remove the sample tube from the magnet and resuspend the Dynabeads®-mRNA complex in 100 µL Washing Buffer A by careful pipetting.
14. Place the sample tube on the magnet for 1 min, then remove and discard the supernatant.

15. Repeat steps 13 and 14 once.
16. Resuspend the Dynabeads®-mRNA complex in 100 µL Washing Buffer B.
17. Transfer the suspension into a RNase-free PCR tube.
18. Place the PCR tube on a magnet for 1 min, then discard the supernatant.
19. Resuspend the Dynabeads®-mRNA complex in 100 µL Washing Buffer B.
20. Place the PCR tube on the magnet for 1 min, then discard the supernatant.
21. Remove the tube from the magnet and resuspend the Dynabeads®-mRNA complex in 100 µL ice-cold 10 mM Tris-HCl.
22. Put the tube on ice and immediately proceed to step 24.

△ **CRITICAL:** Don't elute the isolated mRNA from the beads. The Dynabeads®-mRNA complex is used directly for reverse transcription.

Solid-phase cDNA synthesis

⌚ **Timing:** 20–25 min

This step uses SuperScript™ IV First-Strand Synthesis System (Cat# 18091050) for first-strand cDNA synthesis on beads with the bead-bound oligo (dT) as primer, resulting in mRNA/cDNA hybrids covalently linked to the Dynabeads.

Note: This step follows the manufacturer's manual with minor changes. https://assets.thermofisher.com/TFS-Assets/LSG/manuals/SSIV_First_Strand_Synthesis_System_UG.pdf

23. Prepare reverse transcription reaction mix as below and keep it on ice before use.

△ **CRITICAL:** Pre-warm the 5× SSIV Buffer to room temperature before use. Vortex and briefly centrifuge the buffer prior to preparing the reverse transcription reaction mix.

Reverse transcription reaction mix (20-µL reaction)		
Reagent	Final concentration	Amount
DEPC-treated water	n/a	12 µL
5× SSIV Buffer	1×	4 µL
10 mM dNTP mix (10 mM each)	0.5 mM each	1 µL
100 mM DTT	5 mM	1 µL
Ribonuclease Inhibitor (40 U/µL)	2 U/µL	1 µL
SuperScript™ IV Reverse Transcriptase (200 U/µL)	10 U/µL	1 µL
Total	n/a	20 µL

Note: The volumes given here are for a single 20-µL reverse transcription reaction. For multiple reactions, scale up and include 10% excess volume for pipetting losses.

24. Immediately before adding the reverse transcription reaction mix, place the tube containing the Dynabeads®-mRNA complex on the magnet for 1 min, then discard the supernatant.
25. Resuspend the Dynabeads®-mRNA complex in 20 µL reverse transcription reaction mix.
26. Incubate the reaction in a thermocycler with the "RT" program, 50°C for 5 min followed by 55°C for 10 min.

Note: When using a thermostable reverse transcriptase and the bead-bound oligo (dT) as primer for first-strand cDNA synthesis, an initial incubation at 50°C for 5 minutes is necessary before proceeding at the recommended reaction temperature.

△ **CRITICAL:** Proceed immediately once the incubation completes.

Note: While waiting, carry out the following.

Thaw Amplicon Tagment Mix (ATM), Tagment DNA Buffer (TD), Nextera PCR Master Mix (NPM), and index adapters on ice. These reagents are from Illumina Nextera XT DNA Library Preparation Kit and Nextera XT Index Kit.

Purify ATAC-DNA from step 12 following instructions in step 42.

27. Quick spin and place the sample tube on the magnet for 30 s, then discard the supernatant.
28. Remove the tube from the magnet and resuspend the beads in 100 μL of ice-cold 10 mM Tris-HCl.
29. Place the tube on the magnet for 1 min, then discard the supernatant.
30. Repeat steps 28 and 29 once.
31. Remove the tube from the magnet and put it on a cold metal block.
32. Add 5 μL ice-cold 10 mM Tris-HCl directly on the beads, and immediately proceed to step 33.

mRNA-seq library preparation

⌚ **Timing:** 50–60 min

This step uses Illumina Nextera XT DNA Library Preparation Kit (Cat# FC-131-1024) to directly tagment mRNA/cDNA hybrids on beads and then amplify the tagmented cDNA with PCR using index 1 (i7) adapter and index 2 (i5) adapter in Nextera XT Index Kit (Cat# FC-131-1001).

Note: This step follows the manufacturer's manual with minor changes. https://support.illumina.com/content/dam/illumina-support/documents/documentation/chemistry_documentation/samplepreps_nextera/nextera-xt/nextera-xt-library-prep-reference-guide-15031942-05.pdf

33. Add 10 μL Tagment DNA Buffer (TD) and 5 μL Amplicon Tagment Mix (ATM) directly on the beads.

△ **CRITICAL:** Tagmentation is sensitive to the input amount. The volumes given here have been validated for mRNA/cDNA hybrids generated from $1\text{--}2 \times 10^4$ mESCs.

34. Resuspend the beads by pipetting up and down 10 times to mix.
35. Place the sample tube in the preprogrammed thermocycler and run the "TAG" program (55°C for 5 min, then hold at 10°C).

△ **CRITICAL:** When the program reaches 10°C, immediately proceed to step 36 to inactivate the transposome.

36. Quick spin the sample tube, then add 5 μL Neutralize Tagment Buffer (NT) and mix by pipetting up and down 10 times.

Note: Check NT for precipitates. If present, vortex until all particulates are resuspended.

37. Incubate the sample tube at room temperature (21°C–23°C) for 5 min.
38. Add 5 μL i7 adapter, 5 μL i5 adapter, 15 μL Nextera PCR Master Mix (NPM) to the sample, then mix by pipetting up and down 10 times.

Note: Open only one index adapter tube at a time to prevent misplacing caps. If using Index Adapter Plate, add 10 μ L pre-paired i7 and i5 index adapters. If multiple samples are processed at the same time, please follow Illumina Index Adapters Pooling Guide to select index adapters for each sample.

39. Place the sample tube in the preprogrammed thermocycler and run the “NXT PCR” program.

PCR cycling conditions

Steps	Temperature	Time	Cycles
Gap filling	72°C	3 min	1
Initial denaturation	95°C	30 s	1
Denaturation	95°C	10 s	13–15 cycles
Annealing	55°C	30 s	
Extension	72°C	30 s	
Final extension	72°C	5 min	1
Hold	10°C	Forever	

Note: The PCR cycles needed is dependent on the input amount.

Note: While waiting for the PCR program to complete, set up ATAC PCR reaction following step 43.

40. Once “NXT PCR” program is complete, quick spin the sample tube then place it on the magnet for 1 min.

41. Transfer the supernatant containing the amplified mRNA-seq library into a new PCR tube.

⏸ **Pause point:** If you are stopping, store the supernatant at 2°C–8°C for up to 2 days or –20°C for longer time.

ATAC-seq library preparation

⌚ **Timing:** 30–40 min

This step uses Qiagen MinElute PCR Purification Kit (Cat# 28006) to purify ATAC-DNA from step 12, and then amplifies the purified DNA with a limited-cycle PCR using NEBNext® High-Fidelity 2x PCR Master Mix (Cat# M0541S) and 10 μ M ATAC-seq PCR primers (Buenrostro et al., 2015).

Note: ATAC-DNA purification (step 42) follows the manufacturer’s manual. <https://www.qiagen.com/us/resources/download.aspx?id=521feb84-9cfa-45ea-9a8f-54c2999aa9a9&lang=en>

Note: PCR amplification of the purified ATAC-DNA (Step 43) follows Omni-ATAC protocol (Corces et al., 2017) with slight modifications.

42. Purify ATAC-DNA from step 12 using Qiagen MinElute PCR Purification Kit.

- a. Mix and apply the sample to the MinElute column.
- b. Centrifuge for 1 min at 17,900 \times g at room temperature in a microcentrifuge.
- c. Discard flow-through and place the MinElute column back into the same tube.
- d. Add 750 μ L Buffer PE to the MinElute column and centrifuge at 17,900 \times g for 1 min.

Note: Add ethanol (96%–100%) to Buffer PE before use (see bottle label for volume).

- e. Discard flow-through and place the MinElute column back in the same tube. Centrifuge the column for an additional 1 min at maximum speed.

△ **CRITICAL:** Residual ethanol from Buffer PE will not be completely removed unless the flowthrough is discarded before this additional centrifugation.

- f. Place the MinElute column in a clean 1.5 mL microcentrifuge tube.
- g. Add 11 μ L Buffer EB (10 mM Tris·Cl, pH 8.5) to the center of the membrane to elute DNA. Let the column stand for 1 min, and then centrifuge for 1 min.

△ **CRITICAL:** Ensure that the elution buffer is dispensed directly onto the center of the membrane for complete elution of bound DNA.

- h. Repeat step 42 g once.

▮ **Pause point:** If you are stopping, store the purified ATAC-DNA at -20°C .

43. Amplify the purified ATAC-DNA with NEBNext® High-Fidelity 2 \times PCR Master Mix and ATAC-seq PCR primers (Buenrostro et al., 2015).
 - a. Add 20 μ L DNA, 2.5 μ L universal i5 primer (10 μ M), 2.5 μ L index i7 primer (10 μ M), and 25 μ L NEBNext HiFi 2 \times PCR Master Mix into a PCR tube.

Note: If multiple samples are processed at the same time, make sure that each sample uses a different index i7 primer (Buenrostro et al., 2015). If ATAC-seq and mRNA-seq libraries will be sequenced together, please follow Illumina Index Adapters Pooling Guide to select the indexes compatible with those used in step 38 for mRNA-seq libraries.

- b. Mix, quick spin, then place the PCR tube in the preprogrammed thermocycler and run the "ATAC PCR" program.

Note: The PCR cycles needed is dependent on the input amount. The optimal PCR cycles can be determined by qPCR amplification after initial 5 cycles according to Omni-ATAC protocol (Corces et al., 2017).

PCR cycling conditions

Steps	Temperature	Time	Cycles
Gap filling	72°C	5 min	1
Initial denaturation	98°C	30 s	1
Denaturation	98°C	10 s	8–10 cycles
Annealing	63°C	30 s	
Extension	72°C	1 min	
Hold	4°C	Forever	

▮ **Pause point:** If you are stopping, store the PCR product at 2°C – 8°C for up to 2 days or -20°C for longer time.

Clean up mRNA-seq and ATAC-seq libraries

⌚ **Timing:** 35–40 min

This step uses Agencourt AMPure XP beads (Cat# A63881) to clean up the amplified mRNA-seq library from step 41 and ATAC-seq library from step 43 with single-sided bead purification.

Note: Bring AMPure XP beads to room temperature for at least 30 minutes before use.

44. Vortex AMPure XP beads and add 1.8 \times beads to each sample (90 μ L beads for 50 μ L PCR).
45. Gently pipette the entire volume up and down 10 times to mix thoroughly.
46. Incubate at room temperature (21°C – 23°C) for 15 min.

Note: Thaw Illumina Resuspension Buffer (RSB) at room temperature.

Note: Prepare fresh 80% ethanol 400 μ L per sample.

47. Place the PCR tubes on the magnetic stand for 5 min or until the liquid is clear.
48. Remove and discard all supernatant without disturbing the beads.
49. With the PCR tubes on the magnetic stand, add 200 μ L freshly prepared 80% ethanol without disturbing the beads.
50. Incubate at room temperature for 30 s, then remove and discard all the supernatant.
51. Repeat steps 49 and 50 for a total of two ethanol washes.

Note: Use a P10 pipette to remove and discard residual ethanol after the second wash.

52. With the PCR tubes on the magnetic stand, air-dry the beads at room temperature (21°C–23°C) for 8 min.

Note: Do not overdry the beads, as it may result in lower DNA recovery. If the beads appear cracked, they have been overdried.

53. Add 32.5 μ L Resuspension Buffer (RSB) directly on the beads.
54. Remove the tubes from the magnetic stand, then resuspend the beads thoroughly by gently pipetting the entire volume up and down 10 times.
55. Incubate at room temperature (21°C–23°C) for 2 min.
56. Place the PCR tubes on the magnetic stand for 5 min or until the liquid is clear.
57. Transfer 30 μ L of clear supernatant into a new Eppendorf tube labeled with corresponding library name.

▣▣ Pause point: If you are stopping, store the purified libraries at –20°C.

Library quality control and sequencing

⌚ **Timing:** 20–30 min

This step checks library quality and quantity followed by Illumina sequencing.

58. Quantify the libraries using Qubit® Fluorometer with Qubit dsDNA HS Assay Kit (Cat# Q32851) according to manufacturer's User Guide (https://assets.thermofisher.com/TFS-Assets/LSG/manuals/Qubit_dsDNA_HS_Assay_UG.pdf).
59. Determine library size distribution using Agilent TapeStation with D1000 ScreenTape & Reagents following the manufacturer's User Manual (https://www.agilent.com/cs/library/usermanuals/public/ScreenTape_D1000_QG.pdf).

Note: Alternatively, Agilent Bioanalyzer can be used.

60. Sequence the libraries on Illumina sequencer following the manufacturer's instructions.

Note: Paired-end sequencing is recommended with sequencing depth following ENCODE guideline for bulk ATAC-seq and mRNA-seq.

EXPECTED OUTCOMES

Low-input ATAC&mRNA-seq protocol simultaneously generates ATAC-seq and mRNA-seq libraries from the same cells. The library yield largely correlates with the input cell number and PCR cycles

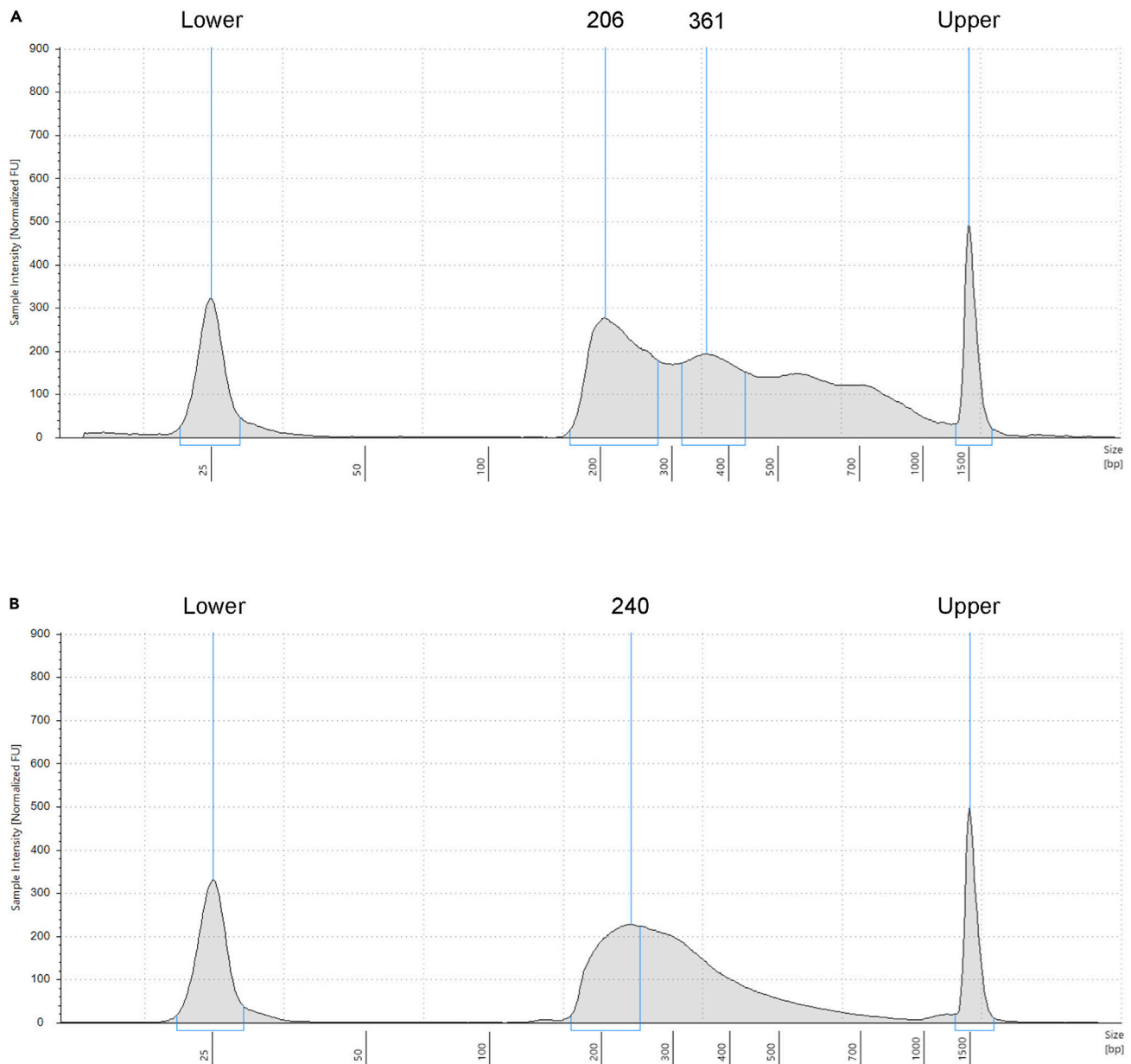


Figure 1. Fragment size distribution of the libraries

Representative TapeStation traces of ATAC-seq (A) and mRNA-seq (B) libraries.

used for library amplification. Using 1×10^4 mESCs, the ATAC-seq library yield was ~ 275 ng with 10 cycles of PCR, and the mRNA-seq library yield was ~ 125 ng with 15 cycles of PCR. Regarding library size distribution, the ATAC-seq library exhibited nucleosome laddering pattern (Figure 1A), and the mRNA-seq library showed a single peak around 250 bp (Figure 1B). After sequencing, the ATAC-seq data displayed good signal enrichment at open chromatin regions (e.g., transcription start sites), while the mRNA-seq data showed read coverage at expressed genes (Figure 2).

QUANTIFICATION AND STATISTICAL ANALYSIS

ATAC-seq fastq files were filtered to remove all entries with a mean base quality score below 20 for either read in the read pair. Adapters were removed via Cutadapt v1.12 (Martin, 2011) with parameters "-a CTGTCTCTTATA -A CTGTCTCTTATA -O 5 -q 0 -m 30". The remaining

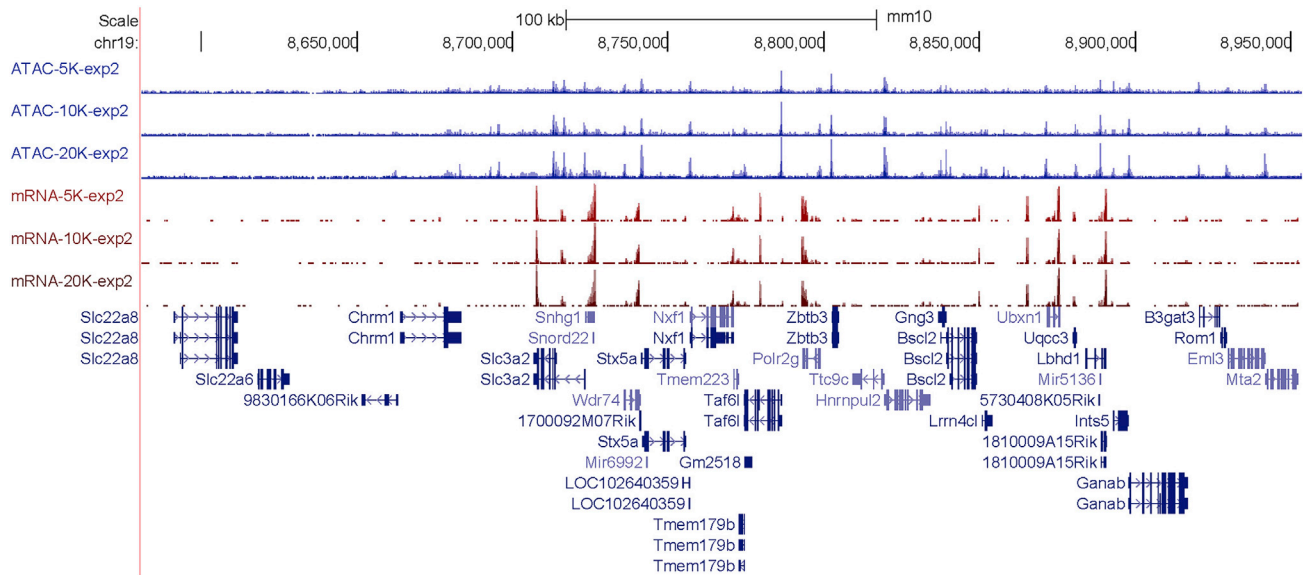


Figure 2. UCSC genome browser view of ATAC-seq and mRNA-seq coverage tracks

filtered and trimmed read pairs were mapped against the mm10 reference assembly via Bowtie2 v2.1.0 (Langmead and Salzberg, 2012) with parameters “-X 2000 -fr -end-to-end -very-sensitive”, followed by filtering with samtools v1.3.1 (Li et al., 2009) at MAPQ5. Reads mapped to chrM were ignored in all downstream analysis. Duplicate mapped read pairs were removed by Picard tools MarkDuplicates.jar (v1.110) (<http://broadinstitute.github.io/picard>) with parameter “REMOVE_DUPLICATES=TRUE”. Only the 9 bp at the 5' end of each read was retained for downstream analysis. Coverage tracks for genome browser views were generated using BEDtools v2.24.0 (Quinlan and Hall, 2010) genomeCoverageBed with depth normalized to 10 million read ends per sample and then converted to bigWig format with UCSC utility bedGraphToBigWig (<http://hgdownload.soe.ucsc.edu/admin/exe/>).

RNA-seq fastq files were filtered to remove all entries with a mean base quality score below 20 for either read in the read pair. Filtered read pairs were mapped against the mm10 reference assembly via STAR v2.5 (Dobin et al., 2013) with parameters “-outSAMattrIHstart 0 -outFilterType BySJout -alignSJoverhangMin 8 -limitBAMsortRAM 55000000000 -outSAMstrandField intronMotif -outFilterIntronMotifs RemoveNoncanonical”. Coverage tracks were generated with STAR v2.5 with parameters “-runMode inputAlignmentsFromBAM -outWigType bedGraph -outWigStrand Unstranded -outWigNorm RPM”, followed by conversion to bigWig format via UCSC utility bedGraphToBigWig.

Note: Use appropriate reference genome assembly according to the species of the samples.

Note: Alternatively, BWA-MEM can be used for alignment of ATAC-seq data.

LIMITATIONS

Low-input ATAC&mRNA-seq protocol requires fresh live cells and is thus not suitable for frozen samples. The mRNA-seq library generated with this protocol is not strand specific.

TROUBLESHOOTING

Problem 1

Low yield of ATAC-seq library (step 58)

Potential solution

Below are the possible causes and potential solutions.

Inefficient cell permeabilization.

- a. Make sure the cells are in single-cell suspension. Large cell clumps are difficult to permeabilize. (step 1)
- b. The detergent and its concentration may need optimization for the cell type of interest. To ensure success, please first determine the optimal condition for cell permeabilization by trypan blue staining. Briefly, treat the cells with varying detergent and concentration, then examine the percentage of blue cells in trypan blue staining to empirically determine the minimal concentration required for efficient permeabilization (>95% blue cells). (step 4)

Low transposition efficiency.

Tn5 transposase requires magnesium ions as cofactor in the transposition reaction. To get rid of chelating agent EDTA in the cell suspension, wash the cells in PBS buffer without EDTA before transposition reaction. (step 1)

Insufficient PCR cycles.

Increase the PCR cycles for ATAC-seq library amplification. (step 43b)

Low recovery during library cleanup.

Don't overdry the AMPure XP beads after the ethanol washes. (step 52)

Problem 2

Poor signal-to-noise ratio of ATAC-seq data

Potential solution

The possible causes and potential solutions are as below.

Low cell viability.

Make sure the cells are alive with more than 90% viability. (step 1)

Suboptimal amount of TDE1 used.

Optimize TDE1 amount to use for the cell type of interest at a given cell number. (step 4)

Problem 3

Low yield of mRNA-seq library (step 58)

Potential solution

The possible causes and solutions are as below.

RNA degradation.

- a. Follow best practices of RNA handling to avoid RNase contamination during the experiment.
- b. Consider adding more RNase inhibitor in ATAC transposition mixture and reverse transcription reaction mix. (step 4 and 23)

Overtagmentation or undertagmentation of mRNA/cDNA hybrids.

Overtagmentation leads to fragments too small to be recovered by AMPure beads purification, while undertagmentation results in fragments too large to be amplified efficiently by PCR. Successful tagmentation highly depends on the ratio of the sample input to the amount of Amplicon Tagment Mix (ATM) used. Try different amount of ATM for the cell type of interest at a given cell number. (step 33)

Insufficient PCR cycles.

Increase the PCR cycles for mRNA-seq library amplification. (step 39)

Low recovery during library cleanup.

Don't overdry the AMPure XP beads after the ethanol washes. (step 52)

Problem 4

The fragment size of mRNA-seq library is too small or too large (step 59)

Potential solution

Avoid overtagmentation or undertagmentation of mRNA/cDNA hybrids as mentioned above. (step 33)

Problem 5

Excessive large DNA fragments (> 1 Kb) in ATAC-seq library (step 59)

Potential solution

Conduct size selection with AMPure XP beads to remove large fragments. (step 44)

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Ruifang Li (lir1@mskcc.org).

Materials availability

This study did not generate new unique reagents.

Data and code availability

The datasets generated during this study are available at NCBI Gene Expression Omnibus (GEO) with accession number GSE165478.

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AUTHOR CONTRIBUTIONS

R.L. conceived the project and performed the experiments. R.L. and S.A.G. analyzed and interpreted the data. R.L. wrote the manuscript, and P.A.W. and S.A.G. reviewed and edited the manuscript.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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