Fixed Cell ATAC-Seq Kit Manual

Catalog No. 53151

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Overview

The Assay for Transposase-Accessible Chromatin via Sequencing (ATAC-Seq) method was first introduced in 2013[1]. ATAC-Seq is a rapid assay of the epigenetic state by enabling identification of open chromatin regions. In the assay, intact nuclei are treated with a hyperactive Tn5 transposase mutant which is able to simultaneously tag the target DNA with sequencing adapters and fragment the DNA in a process termed "tagmentation"[2].

Because of the assay's speed, simplicity, sensitivity, and applicability to a wide range of sample types, ATAC-Seq has become a commonly used epigentic assay, and can serve as a gateway to further, more detailed, epigenetic analyses.

The Fixed Cell ATAC-Seq Kit provides the reagents necessary to produce 16 unique sequencing-ready Illumina®-compatible ATAC-Seq libraries from 50,000 - 100,000 formaldehyde-fixed cells per reaction.

Fixed Cell ATAC-Seq Advantages

- Assess the epigenetic profile of open chromatin regions in formaldehyde-fixed cell samples
- Yields next-gen sequencing-ready processed samples in 2 days
- Simple and rapid four-step protocol

product	format	catalog no.
Fixed Cell ATAC-Seq Kit	16 reactions	53151

Note: The Fixed Cell ATAC-seq Kit is for research use only. Not for use in diagnostic procedures.

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Kit Components and Storage

The kit contains sufficient reagents to produce 16 unique next-gen sequencing-ready ATAC-Seq libraries. The reagents in this kit have multiple storage temperatures. The Fixed Cell ATAC-Seq Kit is shipped at two temperatures, with one box on dry ice for components to be stored at -20°C, and a second box at room temperature for components to be stored at 4°C. Please store components according to the storage conditions below. All reagents are guaranteed stable for 6 months from date of receipt when stored properly.

Reagents	Quantity	Storage
Hypotonic Buffer	1.7 mL	RT
Hypotonic Quench	50 µL	4°C
Releasing Buffer	1.3 ml	RT
Proteinase K (10 µg/µl)	70 µl	-20°C
RNase A (10 µg/µl)	40 µl	-20°C
Protease Inhibitor Cocktail (PIC)	22 µL	-20°C
Assembled Transposomes	205 µl	-20°C
2X Tagmentation Buffer	425 µl	-20°C
10X PBS	500 µl	4°C
10% Tween 20	10 µl	RT
1.0% Digitonin	10 µl	-20°C
0.5 M EDTA	250 µL	RT
DNA Purification Columns SF	16 columns	RT
DNA Purification Binding Buffer	5.5 ml	RT
DNA Purification Wash Buffer	10 ml	RT
DNA Purification Elution Buffer	5 ml	RT
3 M Sodium Acetate	450 µl	RT
dNTP mix, 10 mM	40 µl	-20°C
5X Q5 Buffer	2 x 130 µl	-20°C
Q5 High-Fidelity DNA Polymerase (2U/µl)	10 µl	-20°C

Reagents	Quantity	Storage
i7 Indexed Primer 1	10 µl	-20°C
i7 Indexed Primer 2	10 µl	-20°C
i7 Indexed Primer 3	10 µl	-20°C
i7 Indexed Primer 4	10 µl	-20°C
i5 Indexed Primer 1	10 µl	-20°C
i5 Indexed Primer 2	10 µl	-20°C
i5 Indexed Primer 3	10 µl	-20°C
i5 Indexed Primer 4	10 µl	-20°C
SPRI Beads	1 ml	4°C

Additional Materials Required

- 100% Ethanol
- Deionized water
- Trypan Blue
- Pipettes and corresponding pipette tips
- Refrigerated Microcentrifuge
- Swing-bucket centrifuge
- Low-bind 1.5 ml microcentrifuge tubes
- 300 µl PCR tubes (PCR strip tubes are recommended)
- Bar magnet or magnetic plate
- 37°C incubator
- Microcentrifuge tube orbital rotator that can be place in 37°C incubator
- A dry block heater can be used instead of the 37°C incubator
- Thermal Cycler with heated lid
- Thermal Shaker or Thermomixer
- Ice and ice bucket

Note: We recommend fixing cells in fresh formaldeyde. Please see the Appendix at the end of this manual for details. Cells that were fixed in not freshly prepared formaldehyde work in the assay but yield less robust sequencing results.

Chromatin Opening and Cell Permeabilization - Day 1

NOTE: This protocol is written for one single reaction of 50,000 cells per reaction. We recommend 50,000 to 100,000 cells per reaction.

You will need to add 1 µL Proteinase Inhibitor Cocktail to 99 µL Hypotonic Buffer per reaction, prepared fresh.

Place Hypotonic Quench on ice for step 7. You will need 2 µL per sample.

1. Pre-heat thermal shaker with microcentrifuge tube adapter to 62°C.
2. Prior to retrieving cells, add 1 µL of Proteinase Inhibitor Cocktail to 99 µL room temperature Hypotonic Buffer (per sample).
3. Put your fixed cell samples on ice and do not thaw. We recommend 50,000 cells per sample.
4. Resuspend each cell pellet in 100 µL room temperature Hypotonic Buffer by gently pipetting in a 1.5 mL microcentrifuge tube.
5. Place the microcentrifuge tube containing the cells into the preheated thermal shaker, set the RPM to 600, press start, and incubate for 10 minutes.
6. During the 10 minute incubation, label 1 PCR tube per sample, and keep on ice.
7. After 10 minutes, remove the microcentrifuge tube containing the cells from the thermal shaker and add 2 µL of cold Hypotonic Quench directly to the tube. At this stage you now have processed lysed cells.
8. Place each microcentrifuge tube containing the cells in the orbital rotator and place inside-inside the 37°C incubator. Set the orbital rotator to 10 RPM and incubate for 15 minutes.

   NOTE: As an alternative, the microcentrifuge tubes can be placed on a dry heat block set to 37°C, while gently flicking the tubes every 10 minutes.

9. During the 15 minute incubation, make the Tagmentation Reaction Buffer and keep on ice.

   NOTE: The Assembled Transposomes will be added to the Tagmentation Reaction Buffer at a later step. Keep the Assembled Transposomes at -20°C until it is required. In step 12 below you will add 12 µL of Assembled Transposomes to this Tagmentation Reaction Buffer.

Tagmentation Reaction Buffer	**Per Sample**
Reagent	**Volume**
2X Tagmentation Buffer	25 µL
10X PBS	2 µL
1% Digitonin	0.5 µL
10% Tween 20	0.5 µL
Total Volume	28 µL

10. Retrieve the sample tubes from the incubator and centrifuge at 1000 x g for 5 minutes at 4°C.
11. Carefully remove supernatant, leaving behind 15 µL, ensuring the cell pellet is not disturbed. Discard supernatant. Leave some supernatant, approximately 15 µL in the tube containing the cell pellet.
12. Add 12 µL of Assembled Transposomes to the ice-cold Tagmentation Reaction Buffer from step 9. Mix by pipetting up and down.

Tagmentation Reaction - Day 1

13. Add 40 µL of Tagmentation Reaction Buffer containing the Assembled Transposomes to each sample. Gently pipette to resuspend cells in the Tagmentation Reaction Buffer. Keep on ice.
14. Transfer 50 µL of the resuspended pellet in the Tagmentation Reaction Buffer to the corresponding pre-labelled and pre-chilled PCR tube from step 6. Quickly pulse spin the PCR tubes in mini-centrifuge to collect all liquid together in the bottom of the tubes.
15. Incubate the PCR tubes at 37°C with no shaking for 60 minutes.
16. After the incubation, quickly spin the PCR tubes with a single pulse on a mini-centrifuge to collect all liquid together in the bottom of the tubes and add 4.2 µL of 0.5 M EDTA per sample to the PCR tubes. Gently vortex the PCR tubes for 1 second at low speed and set the tubes at room temperature for 15 minutes.
17. During the incubation, label a low-bind microcentrifuge tube, corresponding to the PCR tubes.
18. After the incubation, transfer all of the tagmented DNA to the corresponding microcentrifuge tube labeled in the previous step.

Releasing and Decrosslinking Tagmented DNA - Day 1

19. Centrifuge the microcentrifuge tubes at 12,000 x g for 3 minutes at 4°C.

    TIP: If the microcentrifuge tubes are all oriented in the centrifuge with their hinges facing outward, the pellet will be on the hinge side of the tube. At this step, the tagmented DNA is in the pellet, this is the ATAC-Seq reaction sample.

20. The protocol steps continue at room temperature after the centrifugation in Step 19. Carefully remove and discard 42 µL of the supernatant. The pellet will be hard to see. 10 µL should be left behind, be very careful not to disturb the pellet.

21. Add 75 µL of room temperature Releasing Buffer to the pellet, and resuspend by gently pipetting 20 to 30 times. At this point, you are releasing the tagmented DNA from the pellet.

22. Give the sample a quick vortex at low setting. Then, incubate the resuspended pellet at room temperature for 30 minutes. Every 10 minutes during this 30 minute incubation, gently vortex on low setting for 1 second. At this point there is no active protein, and the DNA is released from the pellet.

23. Centrifuge the tubes at 17,500 x g for 15 minutes at 4°C. This is removing the large molecular weight fragments.

    During this step, thaw the Proteinase K and RNaseA at room temperature. Once thawed, place on ice. Label PCR tubes for each sample for the next steps.

24. Retrieve sample tubes from the centrifuge and transfer 56 µL of the supernatant to the corresponding new PCR tube.

25. Add 2 µL Proteinase K and 2 µL RNase A directly to each sample.

26. Gently vortex the PCR tubes for 1 second at low speed and quickly spin the PCR tubes with a single pulse in a mini-centrifuge to collect all liquid at the bottom of the tubes.

27. Put PCR tubes into a thermal cycler and run the following overnight decrosslinking program with the volume set at 60 µL and the lid temperature set to 105°C:

    Decrosslinking Program
    37°C for 30 minutes
    55°C for 4 hours
    65°C for 12 hours
    65°C hold

    NOTE: The 65°C hold is so you can continue the assay the following day. This is the end of Day 1. The DNA Purification must be done on the following day, not more than 18 hours later.

DNA Purification - Day 2 (3 - 4 hours)

28. Label a low-bind 1.5 mL tube to correspond with the sample.
29. Add 300 µL of room temperature DNA Purification Binding Buffer to each labeled microcentrifuge tube.
30. Add 5 µL Sodium Acetate directly to each microcentrifuge tube containing the DNA Purification Binding Buffer.
31. Retrieve the PCR tubes containing the decrosslinked and tagmented DNA from the thermal cycler. Let the tubes sit at room temperature for 1 minute before proceeding to the next step.
32. Gently spin the tubes with a single pulse on a mini-centrifuge to collect all liquid at the bottom of the tubes.
33. Trasfer all 60 µL of the sample from the PCR tube to the corresponding 1.5 mL tube containing the DNA Purification Binding Buffer. The color should be yellow. If the color is anything other than yellow, add 3 M Sodium Acetate in 5 µL increments until the color is yellow. Pipette up and down to mix the sample.
    a. For each sample, place a labeled DNA Purification Column into a collection tube.
    b. Transfer each sample to its corresponding column, close the cap, and centrifuge at 17,000 x g for 1 minute.
    c. Discard the flow-through and return the collection tube to the column.

        NOTE: 100% Ethanol must be added to the DNA Purification Wash Buffer before the first use, to a final concentration of 80% (40 mL of 100% Ethanol to a new bottle of DNA Purification Wash Buffer).

    d. Add 750 µL of DNA Purification Wash Buffer to the column and cap the column. Centrfuge at 17,000 x g for 1 minute.
    e. Discard the flow-through and the collection tube. Transfer the column to an empty microcentrifuge tube. Centrifuge the empty tube with column at 17,000 x g for 2 minutes to remove ay remaining DNA Purification Wash buffer. Discard the microcentrifuge tube after spinning.
    f. Transfer each column to a new microcentrifuge tube and add 35 µL DNA Purification Elution Buffer to the center of the column matrix. Cap the column and incubate at room temperature for 1 minute.
    g. Centrifuge at 17,000 x g for 1 minute. Discard the column. DNA purification is complete. This is now the purified tagmented DNA. The purified DNA can be stored at -20°C for up to 3 days or you may proceed directly to the PCR amplification.

PCR Amplification of Tagmented DNA

Note: If a kit such as the KAPA Real-Time Library Amplification Kit is used, an initial 72°C extension step is essential.

1. Set up the PCR reactions by adding the components in the order shown below. If libraries are to be multiplexed for sequencing on the same flow cell, ensure that a unique i5 and/or i7 index combination is used for each.

   Each sample will require a combination of one i7 Indexed Primer and one i5 Indexed Primer in the PCR amplification reaction. There are 4 x 4 = 16 unique combinations of i7/i5 primers for a total of 16 samples that can be multiplexed. These Indexed Primers are based on Illumina's Nextera adapters.

   Per reaction:

Use one i7 Indexed Primer	**And use one i5 Indexed Primer**
i7 Indexed Primer 1 = i7 N701	i5 Indexed Primer 1 = i5 N501
i7 Indexed Primer 2 = i7 N702	i5 Indexed Primer 2 = i5 N502
i7 Indexed Primer 3 = i7 N703	i5 Indexed Primer 3 = i5 N503
i7 Indexed Primer 4 = i7 N704	i5 Indexed Primer 4 = i5 N504

Reagent	Volume
Tagmented DNA	33.5 µl
i7 Indexed Primer (25 µM)	2.5 µl
i5 Indexed Primer (25 µM)	2.5 µl
dNTPs (10 mM)	1 µl
5X Q5 Reaction Buffer	10 µl
Q5 Polymerase (2 U/µl)	0.5 µl

2. Perform PCR using the following program on a thermal cycler (with a heated lid):

   72°C 5 minutes

   98°C for 30 seconds

   7 to 10 cycles of: 98°C for 10 seconds, 63°C for 30 seconds, 72°C for 1 minute

   Hold at 10°C.

   During the PCR cycles, take SPRI beads out of 4°C and warm to room temperature.

3. Once the PCR reaction is complete, fully resuspend the SPRI beads by gently vortexing on low setting for 1-2 seconds. Use the SPRI beads immediately after resuspension to avoid beads settling at the bottom of the tube.

   Perform double-sided SPRI clean-up with 25 µl SPRI bead solution for right side clean up and 25 µL for left side clean up as follows:

   a. Prepare 400 µL of 80% ethanol per sample from 100% ethanol and nuclease free water.

   b. Uncap the PCR tubes and add 25 µL of resuspended room temperature SPRI beads to each tube containing the PCR product.

   c. Vortex the tubes briefly to mix.

   d. Incubate the samples at room temperature for 2 minutes.

   e. Apply magnet to sample tubes and wait 5 minutes for beads pellets to form.

   f. Keepping the tubes on the magnet, carefully uncap the tubes and transfer the supernatant to new PCR tubes, taking care to avoid the bead pellets. The tubes with the beads may be discarded.

   g. Vortex the SPRI beads to resuspend. Add 25 µL of SPRI beads to each tube containing the supernatant.

   h. Vortex the tubes briefly to mix.

   i. Incubate the samples at room temperature for 5 minutes.

   j. Apply magnet to sample tubes and wait 5 minutes for bead pellets to form. Remove and discard supernatant.

   k. Keeping the tubes on the magnet and with the magnetic beads still pelleted, add 180 µL fresh 80% ethanol to each well, taking care not to disturb the pelleted beads. Incubate for 30 seconds, aspirate, and repeat with another 180 µL of 80% ethanol.

   l. Aspirate the supernatant completely and air dry the beads until the beads are no longer shiny (1 - 3 minutes).

   m. Remove tubes from the magnet and elute DNA by adding in 20 µL of DNA Purification Elution Buffer to each tube.

   n. Vortex tubes briefly to mix and incubate for 5 minutes for bead pellet to form.

   o. Place the PCR tubes on the magnet, carefully remove eluate and transfer to fresh, labelled PCR tubes.

4. At this stage, libraries are ready for quantification and sequencing. Use a library quantification kit for next-generation sequencing to quantify the library (e.g. Roche Kapa Biosystems, Catalog No. KR0405). PCR amplified libraries can also be analyzed to assess size distribution with a Bioanalyzer, TapeStation, or similar instrument to assess size distribution.

Index Primers and Sample Sheet Information

Index 1 (i7) Primers

CAAGCAGAAGACGGCATACGAGAT[i7]GTCTCGTGGGCTCGG

Index 2 (i5) Primers

AATGATACGGCGACCACCGAGATCTACAC[i5]TCGTCGGCAGCGTC

i7 Index	i7 Sequence	Sample Sheet
N701	TCGCCTTA	TAAGGCGA
N702	CTAGTACG	CGTACTAG
N703	TTCTGCCT	AGGCAGAA
N704	GCTCAGGA	TCCTGAGC

i5 Index	i5 Sequence	Sample Sheet (NovaSeq v1.0 Reagent Kits, MiSeq, HiSeq 2000/2500)
N501	TAGATCGC	TAGATCGC
N502	CTCTCTAT	CTCTCTAT
N503	TATCCTCT	TATCCTCT
N504	AGAGTAGA	AGAGTAGA

i5 Index	i5 Sequence	Sample Sheet (NovaSeq v1.5 Reagent Kits iSeq, MiniSeq, NextSeq, HiSeq 3000/4000)
N501	TAGATCGC	GCGATCTA
N502	CTCTCTAT	ATAGAGAG
N503	TATCCTCT	AGAGGATA
N504	AGAGTAGA	TCTACTCT

Sequence for Read 1 and Read 2 adapter trimming: CTGTCTCTTATACACATCT.

References

1. Buenrostro, J. D., *et al.* (2013) *Nat. Methods* 10: 1213-1218.
2. Adley, A., *et al.* (2010) *Genome Biol.* 11.
3. Corces, M. R. *et al.* (2017) *Nat. Methods* 14: 959-962.
4. Zhang, H., *et al.* (2022) *BMC Genomics* 23: 214.

Recommended Cell Fixation Protocol

Note: The protocol below is designed to produce aliquots of 50,000 formaldehyde-fixed cells per reaction.

Best results are obtained when fresh, methanol-free formaldehyde is used. We recommend 16% formaldehyde that is provided in an ampule such as Pierce™ 16% Formaldehyde (w/v), Methanol-free Catalog Number 28906 available from Thermo Fisher Scientific. If preparing your own Formalehyde Solution, the formaldehyde must be added fresh, just before using the solution.

PREPARE: **Cell Fixation Buffer**

Compound	Initial Concentration	Final Concentration
Formaldehyde Solution (formaldehyde must be added right before using if preparing your own)	16%	11%
NaCl	5 M	100 mM
EDTA, pH 8.0	250 mM	1 mM
HEPES, pH 7.5	1 M	50 mM
Water	Quantity Sufficient	Quantity Sufficient

1. Bring the cell suspension concentration to 1 - 2 x 10^6 cells/mL with complete cell culture medium in a 15 mL tube (<10 mL of suspension) or 50 mL tube (10 - 30 mL of cells). If <1 x 10^6 cells, use 0.5 mL of the medium in a 1.5 mL tube.
2. Gently vortex the cell suspension, add 1:10 (vol:vol) 10X Cell Fixation Buffer drop by drop with a micropipettter and rotate at a low speed for 10 min. at room temperature.
3. Stop the reaction by gently vortexing, and add 2.5 M glycine in the ratio of 1:20 (vol:vol). Invert the tubes a few times and incubate on ice for 5 min.
4. *Perform the remaining steps at 4°C or on ice.* Spin the tubes at 800 x g for 5 min at 4°C and discard the supernatant.
5. Resuspend the pellet gently with 5 mL of ice-cold 1X PBS and incubate for 2 min. on ice.
6. Repeat steps 4 and 5 with a volume of 1 mL of ice-cold 1X PBS that will bring the cell density to 1 x 10^6 cells/mL. Aliquot 50,000 cells into precooled 1.5 mL tubes labeled for long-term storage.

   NOTE: We recommend aliquoting cells into 50,000 - 100,000 cell pellets. This eliminates the need to count and aliquot stored cells after they are thawed. Cell pellets are then snap frozen, and can be thawed directly in Hypotonic Buffer at 62°C for the assay.

7. Spin the tubes at 1,200 x g at 4°C and remove as much of the supernatant as possible without disturbing the cell pellet. Snap freeze the tubes in liquid nitrogen, and store at -80°C.

Troubleshooting Guide

Question	Answer
Why did my reaction fail?	Pipetting 10 - 30 times and vortexing every 10 minutes in the Tagmentated DNA Releasing step 22 is essential. The experiment will not work without this.
Why is there high background in the sequencing data?	Yes, high background is expected with fixed cells in ATAC-Seq. The background is always higher in fixed cells versus native cells. Please see reference 4, Zhang *et al* 2022.
Why was no library produced?	Sample loss is very likely. Be sure to leave behind 15 µL of sample at removal of Hypotonic Buffer stage (step 11) and after the high speed spin/buffer exchange following tagmentation into Releasing Buffer (step 19).
Can all the reagents and kit components, including master mixes and enzymes, thaw out and be prepared at room temperature?	Everything except the Assembled Transposomes and Q5 DNA polymerase can be thawed at room temperature. The Assembled Transposomes and Q5 DNA Polymerase are formulated with glycerol and will not need thawing, just put these on ice. The other components can be frozen again and thawed again for future reactions, so not all 16 reactions need to be done simultaneously.
Does sonicating fixed samples generate better results?	No. We did not observe improvement in data generated with the assay by sonicating fixed cell samples.
What is the expected final library size, and what should the library traces look like on a fragment analyzer?	Library fragments should range from around 250 bp to 1000 bp in length with an oscillation period ~150 bp. To see example traces, please visit the kit web page at activemotif.com/atac-seq-kit
How many sequencing reads are needed per sample?	Typically 30 million paired-end reads is sufficient, with 20 million being a minimum. The number of reads is dependent both on the sample genome and the end goal of the ATAC-Seq assay. If the sample genome is very large, or if more advanced analysis is required, more sequencing reads may be needed.
Can I multiplex more than 16 samples using the ATAC-Seq Kit?	The Fixed Cell ATAC-Seq Kit is supplied with 4x4 unique dual indexes for 16 unique samples. The indexed primers in the kit are identical to the Illumina Nextera primers corresponding to N701-N704 and N501-N504. If you would like to multiplex more than 16 samples our Nextera™-Compatible Multiplex Primers (96 plex) kit (Cat. No. 53155) enables multiplexing up to 96 reactions. These primers are provided at a concentration of 25 µM to be used directly in our Kits. You could also purchase and combine other Illumina Nextera primers at the same concentration (25 µM) as those in the kit.

Technical Services

If you need assistance at any time, please call or send an e-mail to Active Motif Technical Service at one of the locations listed below.

Active Motif North America

Toll free: 877.222.9543
Direct: 760.431.1263
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