Method Article

Optimization of the Omni-ATAC protocol to chromatin accessibility profiling in snap-frozen rat adipose and muscle tissues

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ABSTRACT

ATAC-seq is a fast and sensitive method for the epigenomic profiling of open chromatin and for mapping of transcription factor binding sites [1]. Despite the development of the Omni-ATAC protocol for the profiling of chromatin accessibility in frozen tissues [2], studies in adipose tissue have been restricted due to technical challenges including the high lipid content of adipocytes and reproducibility issues between replicates. Here, we provide a modified Omni-ATAC protocol that achieves high data reproducibility in various tissue types from rat, including adipose and muscle tissues [3].

• This protocol describes a methodology that enables chromatin accessibility profiling from snap-frozen rat adipose and muscle tissues.
• The technique comprises an optimized bead-based tissue homogenization process that substitutes to Dounce homogenization, reduces variability in the experimental procedure, and is adaptable to various tissue types.
• In comparison with the Omni-ATAC protocol, the method described here results in improved ATAC-seq data quality that complies with ENCODE quality standards.

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**SPECIFICATIONS TABLE**

| Subject Area | Biochemistry, Genetics and Molecular Biology |
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| More specific subject area | Assay for Transposase-Accessible Chromatin using sequencing for assaying chromatin accessibility genome-wide |
| Method name | Ruptor-ATAC |
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| Resource availability | N/A |

*Method details

**Method Introduction**

The Assay for Transposase-Accessible Chromatin using sequencing (ATAC-seq), is a low-input, relatively fast and easy-to-perform method, which consequently has been widely used for mapping chromatin accessibility genome-wide and for investigating TF binding [1, 4]. It uses a genetically engineered hyperactive Tn5 transposase that simultaneously cleaves DNA in open chromatin regions and ligates adapters for high-throughput sequencing at these regions [5, 6]. While the Omni-ATAC protocol was developed to profile chromatin accessibility in frozen tissues [2], it has shown a few limitations. Notably, ATAC-seq analyses in adipose tissue or in tissue-derived adipocytes have been scarce, most likely due to the technical challenge posed by the high lipid content of adipocytes [7–11]. Moreover, low reproducibility between replicates has been observed, with variability in Irreproducible Discovery Rate (IDR) peaks and in transcription start site (TSS) enrichment scores across tissues [9]. In the Omni-ATAC protocol, tissues are homogenized using a Dounce homogenizer, involving a defined number of strokes with the pestles (e.g. 10 with the loose pestle, followed by 20 with the tight pestle), which may differ between tissue types (e.g. soft vs. hard tissues) [12]. Subsequently, the number of isolated nuclei can vary between tissue types, adding some sample-to-sample variability. To optimize the ATAC-seq method to frozen adipose tissue and gain high data reproducibility in various tissues, we modified the Omni-ATAC protocol to include an effective bead-based tissue homogenization process that is applicable to adipose tissue and skeletal muscle. Fig. 1 illustrates the differences between the old and the new protocol. The resulting Ruptor-ATAC protocol is suitable for high throughput studies, complies with the ENCODE quality standards for ATAC-seq data (https://www.encodeproject.org/atac-seq/#standards; [13]), and demonstrates improved ATAC-seq data quality based on metrics such as Usable Reads, IDR peaks, FRiP and TSS enrichment scores.

**Ruptor-ATAC protocol**

**Note:** All steps should be performed on ice or at 4°C. Pre-chill a centrifuge to 4°C. Frozen tissue fragments from rat (∼10 mg for muscle and ∼30 mg for adipose tissue) are used in this protocol.

**Required reagents and equipment:**

- 10-ml Micronics V-bottom screw cap tubes (Micronic, USA, Cat.# MP52318)
- Sucrose (Sigma-Aldrich, St. Louis, MO, USA)
Fig. 1. Comparison of the improved ATAC-seq method and the conventional Omni-ATAC method. Table of defined steps in ATAC-seq protocol using the conventional (Omni-ATAC) method versus our optimized protocol (Ruptor-ATAC).

- Tween-20, supplied at 10% (Sigma-Aldrich)
- 1M Tris- HCl pH 7.8 buffer (Thermo Fisher Scientific, Waltham, MA)
- 1M Tris-HCl pH 7.4 buffer (Thermo Fisher Scientific)
- 500 mM EDTA (Thermo Fisher Scientific)
- 1M CaCl2 (Thermo Fisher Scientific)
- 1M Mg(Ac)2 (Thermo Fisher Scientific)
- 1M MgCl2 (Thermo Fisher Scientific)
- 5M NaCl (Thermo Fisher Scientific)
- 14.3M β-mercaptoethanol (Sigma-Aldrich)
- Sterile nuclease-free, ultrapure distilled water (H2O; Thermo Fisher Scientific)
- cOmplete™ Protease Inhibitor Cocktail (mini-tablets; Thermo Fisher Scientific, Cat.# A32955)
- 2.8-mm ceramic beads (Omni International, Kennesaw, GA, USA, Cat.# SKU 19-646)
- Bead Ruptor Elite bead mill homogenizer with Omni BR Cryo cooling unit (Omni International, Cat.# SKU 19-042E)
- Liquid nitrogen
- Mini 20-μm pluriStrainer 100/PK (pluriSelect, Leipzig, Germany, Cat.# NC1423042)
- Countess II Automated Cell Counter or Cellometer K2 Image cytometer (Nexcelom, Lawrence, MA)
- Propidium Iodide (PI) dye (Sigma-Aldrich)
- Magnetic Rack (Thermo Fisher Scientific)
- Fixed Angle Centrifuge (Eppendorf 5418R)
- Eppendorf 5418R centrifuge
- 2X TD buffer (Illumina, Inc, San Diego, CA, Cat.# 20034198)
- Tn5 transposase (Illumina, Inc, San Diego, Cat.# 20034198)
- Thermomixer (USA Scientific, Ocala, FL, USA)
- DNA Clean and Concentrator kit - (Zymo Research, Irvine, CA, Cat.# 34014)
- Ethanol
- 0.2-ml PCR tubes
- NEBNext High Fidelity 2x PCR Master mix (New England Biolabs)
• Ad1_noMX PCR Primers (Eurofins Scientific)
• Ad2. x Indexing Primers (Eurofins Scientific)
• KAPA High-Fidelity 2X PCR Master Mix (Sigma-Aldrich)
• PCR thermal cycler
• Agencourt AMPure XP beads (Beckman Coulter, Indianapolis, IN, USA)
• Agilent 2100 Bioanalyzer High-Sensitivity DNA kit (Agilent Technologies, Santa Clara, CA, USA)
• Qubit dsDNA High Sensitivity Assay kit (Thermo Fisher Scientific)
• Novaseq 6000 (Illumina)

Step 1: Tissue homogenization

**Note:** Omni BR Cryo cooling unit is kept at 10°C ahead of time and throughout the tissue homogenization process using liquid nitrogen.

**Stock solution and buffer preparation**

Prepare 4x Homogenization Stock Buffer in advance and store at 4°C. Sterile filtration is recommended.

4x Homogenization Stock Buffer

| Reagent            | Final Concentration | Mass/Volume for 100 ml |
|--------------------|---------------------|------------------------|
| Sucrose            | 1.280 M             | 43.8 g                 |
| 1M Tris-HCl pH 7.8 | 40 mM               | 4 ml                   |
| 500 mM EDTA        | 400 mM              | 80 ml                  |
| 1M CaCl2           | 20 mM               | 2 ml                   |
| 1M Mg(Ac)₂        | 12 mM               | 1.2 ml                 |
| 14.3M β-mercaptoethanol | 668 μM        | 4.67 μl                |
| H₂O                | NA                  | Bring up to 100 ml     |

**Same day buffer preparation**

Prepare 1x Homogenization Buffer on the day of processing. Store at 4°C.

1x Homogenization Buffer

| Reagent                    | Final Concentration | Volume for 10 ml |
|---------------------------|---------------------|------------------|
| 4x Homogenization Stock Buffer | 1x                  | 2.5 ml           |
| Protease Inhibitor Cocktail | NA                  | 1 mini-tablet    |
| H₂O                        | NA                  | Bring up to 10 ml |

1. Transfer frozen tissue sample to a 1.1-ml micronic tube and add in 600 μl of cold 1x Homogenization Buffer. Resuspend by pipetting up and down.
2. Add three, 2.8-mm ceramic beads to the micronic tube.
3. Vortex micronic tube thrice for 5-10 seconds and keep it on ice.
4. Homogenize tissue sample in a Bead Ruptor Elite bead mill homogenizer with Omni BR Cryo cooling unit using 2 cycles of the following settings: speed: 1.0, time: 20 sec and speed: 2.1, time: 20 sec, dwell time: 20 sec at both speeds.

Step 2: Homogenate filtration and nuclei counting

1. Following homogenization, filter tissue homogenate through a mini 20-μm pluriStrainer. Wait for 20-30 sec.
2. Count nuclei in the filtrate using an Automated Cell Counter such as Countess II or Cellometer K2 Image cytometer according to the manufacturer’s instructions.
3. Aliquot nuclei for ATAC reaction.

**Note:** If the tissue homogenate does not pass through the strainer, vortex it quickly to let it pass. Adipose tissue homogenates do not pass easily through the strainer even after spinning. For such homogenates, use another strainer to filter.
Step 3: ATAC reaction

Same day buffer preparation

ATAC-seq Resuspension Buffer (ATAC-RSB; as described in [2] 28846090) containing 0.1% Tween-20

| Reagent          | Final Concentration | Volume for 50 ml |
|------------------|---------------------|------------------|
| 1M Tris-HCl pH 7.4 | 10 mM               | 500 μl           |
| 5M NaCl          | 10 mM               | 100 μl           |
| 1M MgCl₂         | 3 mM                | 150 μl           |
| 10% Tween-20     | 0.1%                | 500 μl           |
| H₂O              | NA                  | 49.75 ml         |

Transposition reaction mixture

| Reagent                  | Fold Dilution (x) | Volume for 50 μl |
|--------------------------|-------------------|------------------|
| 2x TD buffer             | 2x                | 25 μl            |
| PBS                      | 3x                | 16.7 μl          |
| 1% digitonin             | 100x              | 0.5 μl           |
| 10% Tween-20             | 100x              | 0.5 μl           |
| Tn5 transposase          | 33.33x            | 1.5 μl           |
| H₂O                      | NA                | 5.8 μl           |

1. Transfer approximately 75,000 nuclei into a fresh 2.0 ml Eppendorf tube and dilute them in 1 ml of cold ATAC-RSB containing 0.1% Tween-20. Mix by inverting tube 10 times.
2. Centrifuge nuclei for 10 min at 3,000 rpm at 4°C in an Eppendorf 5418R centrifuge.
3. Carefully aspirate supernatant (aspirate down to 100 μl with a p1000 pipette and remove final 100 μl with a p200 pipette).
4. Add 50 μl transposition reaction mixture to the nuclear pellet and resuspend by pipetting up and down 6 times.
5. Incubate transposition reaction at 37°C for 30 min using a thermomixer with 1,000 rpm mixing.
6. Immediately transfer to ice after incubation.
7. Purify transposed DNA using a DNA clean and concentrator kit as follows:
   7.1. In a 1.5-ml Eppendorf tube, add 5 volumes (250 μl) of DNA binding buffer to one volume of DNA sample (50 μl). Mix briefly by vortexing.
   7.2. Transfer mixture to Zymo-Spin Column in a collection tube. Centrifuge for 1 min at 8,000 rpm. Discard the flow-through.
   7.3. Add 300 μl of DNA wash buffer to the column. Centrifuge 1 min. Discard the flow-through. Repeat this step and centrifuge for 1 min at 8,000 rpm.
   7.4. Add 20 μl/22 μl of Elution Buffer or Ultrapure Distilled Water directly to the column matrix and incubate at room temperature for 1 min.
   7.5. Transfer the column to a 1.5-ml Eppendorf tube and spin for 1 min to elute the purified, transposed DNA.
8. Store at −20°C if necessary.

Step 4: ATAC-seq library preparation

1. PCR amplify purified, transposed DNA fragments by combining the following in a 0.2-ml PCR tube:
   20 μl transposed DNA
   1.0 μl nuclease-free H₂O
   2.0 μl Ad1_noMX primer (25 μM)
   2.0 μl Ad2. *Indexing Primer (25 μM)
   25 μl KAPA High-Fidelity 2X PCR Master Mix
Fig. 2. Quality control of ATAC-seq libraries. Fragment sizes for two representative ATAC-seq libraries, determined by Bioanalyzer. A size distribution plot and a gel electrophoresis-like image are presented for an adipose sample (A) and a muscle sample (B). Two molecular weight markers are used: a 35-bp and a 10,000-bp marker, which appear in green and in purple, respectively, in the gel-like image. Adapted from [3].

Thermal cycle as follows:

1 cycle: 72°C for 5 min; 98°C for 30 sec
10–11 cycles: 98°C for 10 sec, 63°C for 30 sec, 72°C for 1 min
2. Following amplification, transfer PCR tubes on ice or at 4°C to stop the reaction.
3. Purify PCR amplified library using Agencourt AMPure XP beads: perform a double-sided bead purification (to remove primer dimers and large >1,000 bp fragments) by following the steps described on the Kaestner Lab website under Resources Protocols (https://www.med.upenn.edu/kaestnerlab/protocols.html, **UPDATED** ATAC-seq Protocol - January 28, 2019)
4. Elute the purified library in 20 μl elution nuclease-free water buffer. Ensure that the column is dry prior to adding elution buffer to avoid ethanol contamination in the final library.
5. Store the purified libraries at −20°C if necessary.

Step 5: Library pooling and sequencing

Note: Prior to pooling, libraries are quantified and their quality is evaluated (see Method validation).

1. Pool all the purified libraries.
2. Sequence the pooled libraries using 100-bp paired-end reads on the Illumina Novaseq 6000.

Method validation

ATAC-seq library quality control

1. Determine the quantity of purified libraries using the Qubit dsDNA high sensitivity assay kit, which provides the DNA concentration measured on the fluorometer.
2. Assess the quality of purified libraries using an Agilent Bioanalyzer High-Sensitivity DNA kit, which gives information about the size distribution.

The nucleosome is comprised of a histone octamer that is complexed with approximately 147 bp of DNA [14]. Adapters are an additional 142 bp. A typical fragment size distribution plot shows an enrichment in nucleosome-free fragments (∼200 bp) and mono-nucleosome-bound fragments (∼300 bp), followed by di-nucleosome-associated regions (∼500 bp). Both adipose and muscle ATAC-seq libraries show the expected fragment size and nucleosome phasing (Fig. 2).

ATAC-seq data analysis and quality control

Required software:

- ENCODE ATAC-seq pipeline v1.7.0 (https://www.encodeproject.org/atac-seq/)
- FastQC [15]
Table 1
Comparison of the ATAC-seq data quality metrics obtained using Ruptor-ATAC (see in [3]) vs. those previously collected in mouse adipose and skeletal muscle tissue using Omni-ATAC [9]. Mapped reads, total number of reads minus number of unaligned reads. chrM reads, mitochondrial chromosome reads. Usable reads, number of mapped reads minus number of low mapping quality, duplicate, and mitochondrial reads. % Usable reads, Usable reads to Total reads ratio. TSS enrichment, TSS enrichment score. IDR Peaks, Irreproducible Discovery Rate on more than two replicates. FRiP, Fraction of reads in peaks. N.a., not available.

| Ruptor-ATAC protocol | Sample ID | Gender | Total reads | Mapped reads | chrM Reads | Usable Reads | % of Usable Reads | AVERAGE % of Usable Reads | TSS Enrichment | AVERAGE TSS Enrichment | Number of Peaks | IDR Peaks | AVERAGE IDR Peaks | FRiP |
|----------------------|-----------|--------|-------------|--------------|------------|--------------|-------------------|------------------------|----------------|----------------------|----------------|----------|---------------------|------|
| Adipose-1            | Male      | 186920128 | 152454036   | 3325180      | 152247883  | 81.5         | 90.9              | 11.31                  | 11.18          | 131709               | 52307          | 48311    | 0.21                |
| Adipose-2            | Male      | 154260126 | 147601827   | 2934811      | 145736711  | 94.5         | 11.92             | 134503                 | 53745          | 166811              | 46164          | 0.22     |
| Adipose-3            | Male      | 171080622 | 160714303   | 2968216      | 158805941  | 92.8         | 11.12             | 161960                 | 54458          | 169572              | 34883          | 0.18     |
| Adipose-4            | Male      | 151594180 | 144181953   | 3878928      | 141673087  | 93.5         | 12.29             | 169572                 | 34883          | 169572              | 34883          | 0.18     |
| Adipose-5            | Male      | 143599246 | 134322230   | 2566141      | 132644041  | 92.3         | 9.25              | 169572                 | 34883          | 169572              | 34883          | 0.18     |
| Muscle-1             | Male      | 181961570 | 168227640   | 2542107      | 166550226  | 91.5         | 90.1              | 16.64                   | 20.75          | 207168              | 92617          | 98196    | 0.40                |
| Muscle-2             | Male      | 239434514 | 216391974   | 3753295      | 213924262  | 89.3         | 18.52             | 200610                 | 94235          | 207911              | 84195          | 0.41     |
| Muscle-3             | Male      | 196768302 | 176279821   | 3257512      | 174089894  | 88.5         | 17.63             | 207911                 | 84195          | 208890              | 107660         | 0.52     |
| Muscle-4             | Male      | 200874760 | 185106070   | 5941583      | 181097830  | 90.2         | 25.18             | 208680                 | 112272         | 208580              | 112272         | 0.52     |
| Muscle-5             | Male      | 249010484 | 229770406   | 5103892      | 226359211  | 90.9         | 25.78             | 208580                 | 112272         | 208580              | 112272         | 0.52     |
| Omni-ATAC protocol   |           |         |            |              |            |             |                   |                        |                |                     |                |          |                     |      |
| ATAC-7 (Adipose)     | Female    | 170643536 | 166598249   | 1204915      | 61090112   | 35.8         | 36.4              | 13.03                   | 12.83          | 28827               | 28827          | n.a.     |
| ATAC-8 (Adipose)     | Female    | 165896236 | 135431464   | 1175420      | 61493964   | 37.1         | 12.63             | n.a.                   | 28827          |                     |                |          |
| ATAC-59 (Muscle)     | Male      | 117557552 | 115960450   | 1920255      | 44835398   | 38.1         | 29.0              | 6.32                   | 7.92           | n.a.                | 14722          | n.a.    |
| ATAC-60 (Muscle)     | Male      | 214824060 | 211626739   | 2469962      | 42681444   | 19.9         | 9.52              | n.a.                   | 14722          |                     |                |          |
• Trimmomatic [16]
• Bowtie2 [17]
• Picard tools (https://broadinstitute.github.io/picard/)
• MACS2 [18]
• HOMER
• Integrated Genomic Viewer (IGV 2.3.61)
• DiffBind package (v1.2.4) [19]

1. Process the ATAC-seq data (trim, align, filter, and quality control the data) using the ENCODE ATAC-seq pipeline v1.7.0. Table 1 presents the ATAC-seq metadata and mapping statistics obtained using the Ruptor-ATAC protocol, along with the ATAC-seq metrics previously acquired by Liu et al. using the Omni-ATAC protocol [9]. Reproducible peaks range from 44,673 to 54,458 in adipose, and from 84,195 to 112,272 in muscle samples (IDR peaks). TSS enrichment values are generally well above the cutoff (>9; https://www.encodeproject.org/atac-seq/#standards), and the fraction of reads in called peak regions (FRIP) scores are mostly greater than 0.2, denoting the high quality of the ATAC-seq data.

2. For all samples, read quality is assessed using FastQC.
3. Trimmomatic is used to remove adapters and low-quality base pairs and reads are identified by FastQC.
4. Reads for each sample are aligned to the rat genome (rn6.0) using Bowtie2.
5. After alignment, reads mapping to the mitochondrial genome are removed.
6. As a general measure of sensitivity to Tn5 transposase fragmentation, ATAC-seq signal is defined as number of transposase cuts mapping to each bp (or a total count of cuts mapping to a selected genomic interval). The cuts are defined as 5’ ends of ATAC-seq reads, with additional shifting by +4 bp and -5 bp for reads mapping to the plus and minus strands, respectively [4] (Buenrostro 2013). Duplications are removed by Picard tools.
7. MACS2 is applied on each merged bam file to call peaks (with option –nomodel –extsize 200 –shift 100).
8. HOMER is used to annotate ATAC-seq peaks to the various genomic regions (promoter, 5' UTR, exon, intron, 3' UTR, downstream, and intergenic), and assign them to the nearest gene based on rat genome assembly rn6. Notably, promoters are defined as 3 kb upstream and downstream from the TSS. Downstream regions correspond to the 3 kb DNA region located downstream from the transcription termination site.
9. All sequencing tracks, bigWig files are viewed using the Integrated Genomic Viewer. Fold-change of ATAC-seq signal is calculated using DiffBind package in R. Interpretation is limited to peaks that exceed a L2FC of 1 (FDR < 0.01) in either direction.

Declaration of Competing Interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Supplementary material and/or Additional information

None

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