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ATAC-seq Assay with Low Mitochondrial DNA Contamination from Primary Human CD4+ T Lymphocytes

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Cover Letter

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Jaydev Upponi, Ph.D.
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September 18th, 2018

Dear Dr. Upponi,

Enclosed please find our manuscript entitled "An Improved ATAC-seq Protocol with Low Mitochondrial DNA Contamination to Map Genome-Wide Chromatin Architecture in Primary Human CD4+ T Lymphocytes", which we submit for publication to the Journal of Visualized Experiments. This submission follows our pre-submission correspondence. Below we provide a summary of our protocol, highlighting its novelty and implications.

ATAC-seq is a powerful tool in the field of epigenetics, a high throughput and relatively simple method to identify open and accessible chromatin. While ATAC-seq is widely used, current protocols are challenged by contaminating mitochondrial DNA reads that can comprise as much as 50% of sequencing reads. In our manuscript we present an improved ATAC-seq protocol that introduces a modified nuclei lysis buffer that is demonstrated to reduce mitochondrial DNA reads from an average of 50% to 3%. This allows for a large decrease in sequencing costs and higher quality data production from the ATAC-seq pipeline.

The main innovations of our work are:

The efficient isolation, freezing and activation of human primary CD4+ lymphocytes from patient whole blood, allowing for the collection of desired material at different times but simultaneous library construction and sequencing. This provides researchers with the flexibility to collect samples based on availability and research needs, while still avoiding batch bias in Tn5 transposase activity, library preparation, and sequencing.

An improved nuclei lysis buffer that minimizes contaminating mitochondrial DNA reads, allowing for a decrease in sequencing costs. A reduction from an average of 50% mitochondrial DNA reads to 3%, allowing for a 50% reduction in sequencing costs.

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Given the widespread use of ATAC-seq, our improved protocol will benefit a large range of research. This improved ATAC-seq protocol has been validated in various cell types, and has potential promises to improve single cell ATAC-seq library quality as well. The protocol will benefit from visual representation of CD4+ lymphocyte handling and nuclei lysis buffer preparation, making it of interest to the Journal of Visualized Experiments community. Thank you for your consideration.

Sincerely,

Christine S Cheng

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TITLE:
ATAC-seq Assay with Low Mitochondrial DNA Contamination from Primary Human CD4+ T Lymphocytes

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KEYWORDS:
Epigenomics, ATAC-seq, Tn5, CD4+ lymphocytes, nuclei, mitochondrial DNA contamination

SUMMARY:
Here, we present a protocol to perform an assay for transposase-accessible chromatin sequencing (ATAC-seq) on activated CD4+ human lymphocytes. The protocol has been modified to minimize contaminating mitochondrial DNA reads from 50% to 3% through the introduction of a new lysis buffer.

ABSTRACT:
ATAC-seq has become a widely used methodology in the study of epigenetics due to its rapid and simple approach to mapping genome-wide accessible chromatin. In this paper we present an improved ATAC-seq protocol that reduces contaminating mitochondrial DNA reads. While previous ATAC-seq protocols have struggled with an average of 50% contaminating mitochondrial DNA reads, the new lysis buffer introduced in this protocol reduces mitochondrial DNA contamination to an average of 3%. This improved ATAC-seq protocol allows for a near 50% reduction in the sequencing cost. We demonstrate how these high-quality ATAC-seq libraries can be prepared from activated CD4+ lymphocytes, providing step-by-step instructions from CD4+ lymphocyte isolation from whole blood through data analysis. This improved ATAC-seq protocol has been validated in a wide range of cell types and will be of immediate use to researchers studying chromatin accessibility.

INTRODUCTION:
The assay for transposase-accessible chromatin sequencing (ATAC-seq) has rapidly become the leading method for interrogating chromatin architecture. ATAC-seq can identify regions of

accessible chromatin through the process of tagmentation, the fragmenting and tagging of DNA by the same enzyme, to produce libraries with which sequencing can determine chromatin accessibility across an entire genome. This tagmentation process is mediated by the hyperactive Tn5 transposase, which only cuts open regions of chromatin due to nucleosomic steric hindrance. As it cuts, the Tn5 transposase also inserts sequencing adapters that allow for rapid library construction by PCR and next-generation sequencing of genome-wide accessible chromatin[1,2].

ATAC-seq has become the preferred method to determine regions of chromatin accessibility due to the relatively simple and fast protocol, quality and range of information that can be determined from its results, and small amount of starting material required. Compared to DNase-seq[3] (which also measures genome-wide chromatin accessibility), MNase-seq[4] (which determines nucleosome positions in open genome regions), and the formaldehyde-mediated FAIRE-seq[5], ATAC-seq is faster, cheaper, and more reproducible[1]. It is also more sensitive, working with starting material of as few as 500 nuclei, compared to the 50 million nuclei required for DNase-seq[3]. ATAC-seq also has the ability to provide more information about chromatin architecture than other methods, including regions of transcription factor binding, nucleosome positioning, and open chromatin regions[1]. Effective, single-cell ATAC-seq protocols have been validated, providing information on chromatin architecture at the single-cell level[6,7].

ATAC-seq has been used to characterize chromatin architecture across a wide spectrum of research and cells types, including plants[8], humans[9], and many other organisms. It has also been critical in identifying epigenetic regulation of disease states[7]. However, the most widely used ATAC-seq protocol includes the major drawback of contamination sequencing reads from mitochondrial DNA. In some data sets, this contamination level can be as high as 60% of sequencing results[1]. Here we present an improved ATAC-seq protocol that reduces the mitochondrial DNA contamination rate to just 3%, allowing reduction of around 50% in sequencing costs[10]. This is made possible by a streamlined process of CD4+ lymphocyte isolation and activation and an improved lysis buffer that is critical in minimizing mitochondrial DNA contamination.

This improved ATAC-seq protocol has been validated with a wide range of primary cells, including human primary peripheral blood mononuclear cells (PBMCs)[10], human primary monocytes, and mouse dendritic cells (unpublished). It has also been used successfully to interrogate melanoma cell lines in a clustered regularly interspaced short palindromic repeats (CRISPR) screen of non-coding elements[11]. Additionally, the data analysis package described in this protocol and provided on GitHub provides new and experienced researchers with tools to analyze ATAC-seq data. ATAC-seq is the most effective assay to map chromatin accessibility across an entire genome, and modifications to the existing protocol that are introduced here will allow researchers to produce high-quality data with low mitochondrial DNA contamination, reducing sequencing costs and improving ATAC-seq throughput.

PROTOCOL:

This improved protocol provides step-by-step instructions for performing ATAC-seq of CD4+ lymphocytes, from the starting material of whole blood through data analysis (**Figure 1**).

1. Isolation of CD4+ T cells from whole blood

NOTE: The starting material for this protocol is 15 mL of fresh whole blood collected using standard procedures, allowing the source of the starting material to be selected based on research requirements. Scale the protocol as needed. Pre-warm phosphate buffered saline (PBS) + 2% fetal calf serum (FCS) to room temperature (RT) and adjust the centrifuge to RT before starting the CD4+ T cell enrichment procedure.

1.1 Add 750 µL of human CD4+ T cell enrichment cocktail to 15 mL of whole blood in a 50 mL conical tube and mix gently by inversion. Incubate at RT for 20 min. When incubation is complete, add 15 mL of PBS + 2% FCS to the tube and mix gently by inversion.

1.2 Prepare a fresh 50 mL conical tube with 15 mL of density medium. Carefully layer the diluted blood sample onto the top of the density medium, being sure not to disrupt the density medium/blood interface that forms. Centrifuge for 20 min at 1200 x *g* and RT with acceleration set to 1 and the descending brake OFF.

NOTE: It is critical to set the acceleration to 1 and descending brake OFF on the centrifuge to avoid disruption of the cell layer in the density medium.

1.3 Collect the enriched CD4+ T cells from the density medium/plasma interface using a narrow stem transfer pipette. Transfer the collected cells to a fresh 50 mL conical tube. Centrifuge the collected CD4+ T cells for 8 min at 423 x *g* and RT.

NOTE: Make sure to return the centrifuge acceleration to 9 and descending brake to ON for step 1.3 and all of the following steps.

1.4 Discard the supernatant and wash the cell pellet twice with 50 mL of PBS + 2% FCS, centrifuging for 8 min at 423 x *g* and RT. Discard the final supernatant wash and suspend the washed cell pellet in 2 mL of PBS + 2% FCS.

1.5 Count cells using a hemocytometer. To continue with ATAC-seq, proceed to step 2.3. To freeze cells for later processing, proceed to step 1.6.

1.6 Add 1 mL of fresh freezing medium (90% FCS + 10% dimethyl sulfoxide) per 1 million cells. Aliquot 1 mL of cells in freezing medium in cryogenic safe tubes. Place tubes at -80 °C overnight in a slow-freezing container. The next day, transfer tubes to liquid nitrogen for long-term storage.

NOTE: 1 million CD4+ T cells will be isolated per 2 mL of original volume of whole blood. Freeze 500,000 cells in 1 mL aliquots of freezing medium. Make fresh freezing medium at every use.

2. Activate and purify CD4+ T cells

NOTE: This rapid protocol for activating and purifying CD4+ T cells only requires 48 h and results in 95% viable, activated CD4+ T cells. Cool the centrifuge to 4 °C before beginning the protocol.

2.1 Thaw 1 vial (500,000) of CD4+ T cells in a 37 °C water bath until the ice has just melted. Gently transfer cells to a 15 mL conical tube containing 9 mL of pre-warmed Roswell Park Memorial Institute-1640 (RPMI-1640) media supplemented with 10% FCS, hereafter referred to as complete RPMI.

NOTE: Do not let cells thaw completely to avoid exposure to DMSO.

2.2 Centrifuge for 6 min at 1500 x *g* and 4 °C. Discard the supernatant and gently suspend the pellet in 2 mL of complete RPMI. Repeat the spin down, discard the supernatant, and gently suspend cells in 0.5 mL of complete RPMI.

2.3. Count the cells using a hemocytometer. Adjust the density of the cell suspension with complete RPMI to plate 50,000 cells in 200 µL per well of a 96-well round bottom plate.

NOTE: From one frozen tube of 500K CD4+ T cells, plate 10 wells of 50,000 CD4+ T cells in 200 µL per well.

2.4 Prepare magnetic beads conjugated with human T-activator anti-CD3 and anti-CD28 antibodies.

2.4.1. Aliquot 12.5 µL of human T-activator CD3/CD28 beads per ATAC-seq sample to a 1.5 mL tube. Wash the beads with 1 mL of 1x PBS and place the tube on a magnet for 1 min.

2.4.2. Carefully remove and discard the clear supernatant, remove the tube from magnet, and suspend the beads in 13 µL complete RPMI per ATAC-seq sample.

NOTE: Calculate the amount of beads necessary based on the number of ATAC-seq samples being processed. This protocol requires 12.5 µL of CD3/CD28 beads per 500,000 cells, which constitutes one ATAC-seq sample.

2.5 Activate the CD4+ T cells. Collect 500,000 cells in 2.1 mL of complete RPMI medium in a 15 mL conical tube and add 12.5 µL of pre-washed human T-activator beads. Mix gently by inverting the tube.

2.6 Gently transfer the cells with beads to a sterile reservoir and plate 200 µL of cells with beads per well of round bottom 96-well plate using a multi-channel pipette. Incubate for 48 h in a 37 °C, 5% CO_2 incubator.

2.7 Post incubation, centrifuge the 96-well plate for 8 min at 423 x *g* and RT. Remove 100 µL of medium per well from the 96-well plate, collecting it to a conical tube before discarding to ensure no bead loss. Resuspend the cell pellet in the remaining 100 µL and collect all in a 1.5 mL tube.

NOTE: 10 wells of activated T cells will be collected in a 1 mL volume.

2.8 Perform CD4+ isolation. Add 50 µL of pre-washed CD4 conjugated beads to 500,000 cells in 1 mL of complete RPMI. Combine beads and cells by pipette. Incubate for 20 min at 4 °C in the fridge. Agitate beads and cell mixture every 6 min.

NOTE: This protocol is to be used for one sample of 500,000 cells. Adjust as necessary for two or more samples of 500,000.

2.9 When the incubation is complete, place the tube on the magnet for 2 min. Remove and discard the supernatant when clear. Wash the bead-bound cells by removing the tube from the magnet, suspending the bead-bound cells in 1 mL of PBS + 2% FCS, replacing the tube on the magnet for 1 min, and discarding the clear supernatant. Repeat this process for a total of 3 washes.

NOTE: Be careful not to discard any beads during washes.

2.10 After the final wash, place the tube on the magnet for 1 min. Remove and discard the supernatant and place the pellet on ice. Proceed to section 3 (ATAC-seq).

3. ATAC-seq

NOTE: In this step, nuclei are isolated from activated CD4+ T cells for ATAC-seq. The lysis buffer used in this protocol has been improved to be gentler on the nuclei, resulting in more efficient digestion and higher quality results. All centrifugation steps in section 3 are performed with a fixed-angle centrifuge maintained at 4 °C. Cool the centrifuge before beginning protocol.

3.1 Perform nuclei isolation. Resuspend beads and cells with cold lysis buffer (10 mM Tris-HCl, pH 7.4, 10 mM NaCl, 3 mM MgCl2, 0.03% polysorbate 20). Centrifuge immediately at 500 x *g* for 10 min at 4 °C. Remove and discard the supernatant.

3.2 Perform the transposase reaction. Suspend the isolated nuclei pellet with 50 µL of Tn5 transposase mix (**Table 1**). Incubate in a thermocycler for 30 min at 37 °C with a 40 °C lid, holding at 4 °C when complete.

3.3 After thermocycling, immediately perform a quick benchtop centrifuge spin down and place the tube on the magnet for 1 min to remove the beads from the product.

3.4 Transfer the clear supernatant from the tube on the magnet to a DNA purification column. Wash the column once with 250 µL of Buffer PB and twice with 750 µL of Buffer PE. Elute the sample in 10 µL of elution buffer. Proceed directly to step 3.5.

NOTE: This step amplifies the DNA fragments with the ligated adaptors, here referred to as the ATAC-seq library. In order to multiplex several ATAC-seq libraries to be run on one NGS lane, use unindexed Primer 1 for all samples and a different indexed Primer 2 for individual samples (**Table 2**). Primers are used at working concentrations of 1.25 µM.

3.5. Set up the initial PCR amplification reaction in a nuclease-free PCR tube, combining the components in the order and volume specified in **Table 3.** Place the PCR tube into the thermocycler and run the PCR amplification program with cycling conditions as specified in **Table 4**.

3.6. Monitor the reaction by qPCR. Set up the qPCR reaction in a nuclease-free PCR tube, combining the components in the order and amount specified in **Table 5.** Place in qPCR machine and cycle as specified in **Table 6**.

3.6.1 To determine the optimal number of additional amplification cycles for the remaining 45 µL reaction, create a plot with cycle number on the x-axis and relative fluorescence (RFU) on the y-axis. The optimal number of additional amplification cycles is one-third the number of cycles it takes for the qPCR reaction to reach plateau.

NOTE: Monitoring the reaction by qPCR allows for determination of the optimal number of PCR cycles desired to obtain optimal library fragment amplification while minimizing GC content and size bias.

3.7. Complete the final PCR amplification of the remaining 45 µL of PCR reaction. Place the PCR tube with the amplification reaction from step 3.5 back in the thermocycler and run the program as described in **Table 7**.

NOTE: For samples processed as described in this protocol, the optimal total number of PCR amplification cycles was determined to be 12.

3.8. After the amplification is complete, purify the libraries using a PCR cleanup kit following the manufacturer's protocol, eluting in 25 µL of the elution buffer.

NOTE: Amplified libraries can be stored at 4 °C for up to 48 h or frozen at -20 °C for long-term storage.

4. ATAC-seq library quality analysis

NOTE: It is important to validate the quality and quantity of ATAC-seq libraries before next-generation sequencing. The quality and quantity of the libraries should be assessed using commercially available kits (see **Table of Materials**).

4.1. Assess the quality and quantity of the ATAC-seq libraries using a microfluidics-based platform for sizing, quantification and quality control of DNA. See **Figure 2** for representative quality assessment results.

NOTE: The concentration of the ATAC-seq libraries must be >1 ng/µL to obtain quality sequencing results. On average, 30 nM in 25 µL was obtained.

5. Sequencing and data analysis

NOTE: This analysis pipeline allows users to control the quality of reads mapping procedure, adjust coordinates for experimental design, and call peaks for downstream analysis. The following are the commands lines and explanation of execution. The data analysis package is available at (https://github.com/s18692001/bulk_ATAC_seq).

5.1. Sequence the prepared libraries on a next generation sequencer to an average read depth of 42 million reads per sample.

5.2. Estimate the quality of sequencing reads by checking files generated by FastQC[12] software package using the command:
fastqc -o <output_directory> <fastq_file>

5.3. Trim the bases of reads by Trimmomatic[13] software, if needed, as determined by the FastQC quality check, using the command (for pair-ended):
java -jar <path to trimmomatic jar file> PE <input1> <input2> <paired output1> <unpaired output1> <paired output2> <unpaired output2> HEADCROP:<crop bases>

5.4. Align reads to human reference genome (hg38) by Bowtie2[14] software:
bowtie2 -x <reference genome> -1 <input pair 1> <input pair 2> -S <output SAM file>

NOTE: The argument -x is for the basename of the index for the reference genome, and -S is for the SAM format output.

5.5. Check thequality of mapped reads using Samtools[15] flagstat package:
samtools flagstat <BAM file>

5.6. Sort the mapped reads file and remove duplicates using Picard[16] tool:
java -jar picard.jar SortSam INPUT=<input SAM file name> OUTPUT=<output sorted BAM file name> SORT_ORDER=coordinate" and "java -jar picard.jar MarkDuplicates INPUT=<sorted BAM file> OUTPUT=<output BAM file without duplicates> REMOVE_DUPLICATES=TRUE

5.7. Index BAM file, trim the unused chromosome reads and shift the coordinates for the experimental reason of ATAC-seq[17]:

```
java -jar picard.jar BuildBamIndex INPUT=<BAM file>
samtools idxstats <Input BAM file> | cut -f 1 | grep -v chrY | grep -v chrM | grep -v chrUn |
xargs samtools view -b <Input BAM file> > <Output trimmed BAM file>
bedtools bamtobed -i <Input trimmed BAM file>  >  <Output trimmed BED file>
awk 'BEGIN {OFS = "\t"} ; {if ($6 == "+") print $1, $2 + 4, $3 + 4, $4, $5, $6; else print $1, $2 - 5,
$3 - 5, $4, $5, $6}' <Input trimmed BED file>  <Trimmed shifted BED file>
```

5.8. Delete the reads that are shifted to negative coordination:

```
awk '{if ($2 > 0) print $1 "\t" $2 "\t" $3 "\t" $4 "\t" $5 "\t" $6 }' <Input BED file>  <Output non-
negative coordination BED file>.
```

5.9. Convert the BED file to BAM file for following DiffBind[18] analysis:

```
bedtools bedtobam -i <Input BED file> -g <reference genome>  <Output BAM file>
```

5.10. Perform peak calling by DiffBind[18] software package:

```
macs2 callpeak -t <Input BAM file> -f BAM -g hs -nomodel --nolambda --keep-dup all --call-
summits --outdir <Output directory path> -n <Output name> -B -q 0.01 --bdg --shift -100 --
extsize 200
```

NOTE: After filtering, expect a median of 37 million reads per sample. Mitochondrial DNA contamination will range from 0.30%–5.39% (1.96% on average). There will be a low rate of multiply mapped reads (6.7%–56%, 19% on average) and a relatively high percentage of usable nuclear reads (60%–92%, 79% on average).

REPRESENTATIVE RESULTS:

From 15 mL of fresh whole blood, this protocol generates an average of 1 million CD4+ T cells. These can be frozen for later processing or used immediately. Viability of the CD4+ T cells, fresh or thawed, was consistently >95%. This method of CD4+ T cell isolation allows for flexibility in source material and collection time. This improved ATAC-seq protocol produces a final library of greater than 1 ng/µL for sequencing. Quality control performed using commercially available systems should demonstrate DNA fragments between 200 and 1000 bp (**Figure 2**). Sequencing should only be performed with high quality libraries.

All libraries were sequenced to an average depth of greater than 40 million read per sample. While previous ATAC-seq protocols have been challenged by contaminating sequencing reads from mitochondrial DNA that can range from 50%–60% of the total sequencing reads[1] this improved protocol eliminates the issue. Libraries prepared following this protocol contain on average only 3% mitochondrial reads (**Figure 3A**). The high percentage of usable reads is sufficiently constant across biological replicates (**Figure 3B**). The protocol was able to provide highly reproducible results across technical replicates (**Figure 4A, B**) as well as biological replicates (**Figure 4C, D**). Additionally, the protocol for CD4+ T cell activation presented takes 48 h rather than one week or more and results in consistent and efficient activation, as demonstrated by reproducible sequencing results (**Figure 4A, B**). Predicted ATAC-seq peaks are accurately called by the analysis pipeline (**Figure 4E**). Analysis of sequencing results identified clear changes in chromatin state during human T cell activation. Differentially accessible regions of open chromatin were identified between six samples before and after 48 h of activation (**Figure 5**).

FIGURE AND TABLE LEGENDS:

Figure 1: Experimental overview of the modified ATAC-seq protocol. (A) Sample acquisition and processing, from 15 mL of patient whole blood through CD4+ T cell isolation, plating and activation of the T cells, and nuclei isolation with the improved lysis buffer. (B) The transposase reaction and PCR amplification of the sequencing library. (C) Quality analysis, sequencing, and data analysis.

Figure 2: Representative high quality ATAC-seq libraries from a microfluidics-based platform for sizing, quantification and quality control of DNA. (**A**) Electronic gel image of samples B1 and D1 with banding between 200 and 1,000 base pairs. (**B and C**) Electropherogram trace result of samples B1 (B) and D1 (C), with peaks between 200 and 1,000 base pairs.

Figure 3: Decreased mitochondrial DNA contamination with the improved ATAC-seq protocol results in an increase in usable DNA sequencing reads. (A) Comparison of usable reads (purple), duplicate reads (green), and mitochondrial reads (red) from CD4+ T cell ATAC-seq profiling in the literature. (**B**) Comparison of usable reads (purple), duplicate reads (green), mitochondrial reads (red), and unmapped reads (blue) of CD4+ T cell ATAC-seq profiling from multiple healthy individuals (n = 22). This figure has been modified from Cheng et al.[10].

Figure 4: Improved ATAC-seq protocol reproducibility and accuracy. Scatter plots of chromatin accessibility (ATAC-seq signal, x and y-axes) for two replicate experiments of unstimulated (**A**; 36,486 Th peaks) or activated (**B**; 52,154 Thstim peaks) T cells demonstrates technical reproducibility. Chromatin accessibility for activated T cells from individuals IGTB1191 (y-axis) and IGTB1190 (x-axis) (**C**) and histogram of correlations between every pairs of individuals for the 52,154 Thstim peaks (**D**) demonstrates reproducibility between individuals. (**E**) ATAC-seq peaks called with our improved ATAC-seq protocol at chromosome 19 Q13.12. This figure has been modified from Cheng et al.[10].

Figure 5: Representative ATAC-seq results of changes in chromatin state after CD4+ T-cell activation. (A) Experimental overview (left) and nomenclature (right). (**B**) Differentially accessible regions of open chromatin (columns) in six samples (rows) before (top, Th) and after (bottom, Th_{stim}) 48 h activation of primary CD4+ T cells. This figure has been modified from Cheng et al.[10].

Table 1: Step 3.2 transposase reaction components.

Table 2: ATAC-seq oligos designs used for PCR.

Table 3: Step 3.5 initial PCR reaction mix.

Table 4: Step 3.5 initial PCR amplification cycling program.

Table 5: Step 3.6 qPCR reaction mix.

Table 6: Step 3.6 qPCR cycling program.

Table 7: Step 3.7 PCR cycling program for final PCR amplification.

DISCUSSION:

The modified ATAC-seq protocol presented in this article provides reproducible results with minimal mitochondrial DNA contamination. The protocol has been used to successfully characterize chromatin architecture of human primary PBMCs[10], human monocytes, mouse dendritic cells (unpublished), and cultured melanoma cell lines[11]. We anticipate this improved lysis condition has the potential to work for other cell types as well. It is also anticipated that this nuclei isolation protocol will be compatible with single nuclei ATAC-seq protocols, minimizing mitochondrial DNA contamination to improve sequencing results.

An additional benefit of this modified protocol is the ability to freeze batches of isolated CD4+ T cells from PBMCs at different times depending on the availability of patient samples. As ATAC-seq can then be performed concurrently on all samples, potential batch effect bias in the transposase reaction and sequencing is minimized[10]. It is critical that freezing medium be made fresh with each use, in order to maintain the high viability that is achieved with this freeze-thaw protocol. Viability of isolated CD4+ T cells should remain above 90% in order to avoid non-specific digestion in the transposase reaction[1].

Please note that further optimization may be required in order to use this protocol with variable cell types and quantities. Since the Tn5 transposase-to-nuclei ratio has been optimized for 500,000 nuclei in this protocol, if performing ATAC-seq with a different number of nuclei, the amount of Tn5 transposase should be adjusted accordingly. Over-lysis of nuclei due to an excess of Tn5 may lead to high background from closed chromatin and low complexity of sequencing libraries, while under-lysis may not provide a complete PCR amplified library[1]. In order to avoid these complications, it is advised to perform careful nuclei counting and optimize the Tn5 ratio as necessary. To further improve data quality, it is advised to optimize PCR amplification cycles by qPCR monitoring. If the final library undergoes too many amplification cycles, there may be bias introduced in the sequencing data[2]. It is recommended to perform proper quality control of the ATAC-seq libraries prior to next-generation sequencing in order to save time and money.

As we have demonstrated, a new lysis buffer is key to the reduction of mitochondrial DNA contamination and is effective on a wide range of cell types. While other protocols have addressed the issue of mitochondrial contamination through the addition of extra washing steps[19] or the intensive process of a CRISPR-mediated mitochondrial DNA depletion[20], this ATAC-seq protocol does not add reagents or experimental steps, which makes it a more accessible technique. Together with RNA-seq and single-cell sequencing, ATAC-seq is a powerful tool for exploring epigenetic regulation. This improved ATAC-seq protocol and data analysis package will help decrease sequencing costs and produce higher quality results.

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DISCLOSURES:
The authors have nothing to disclose.

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Figure 1

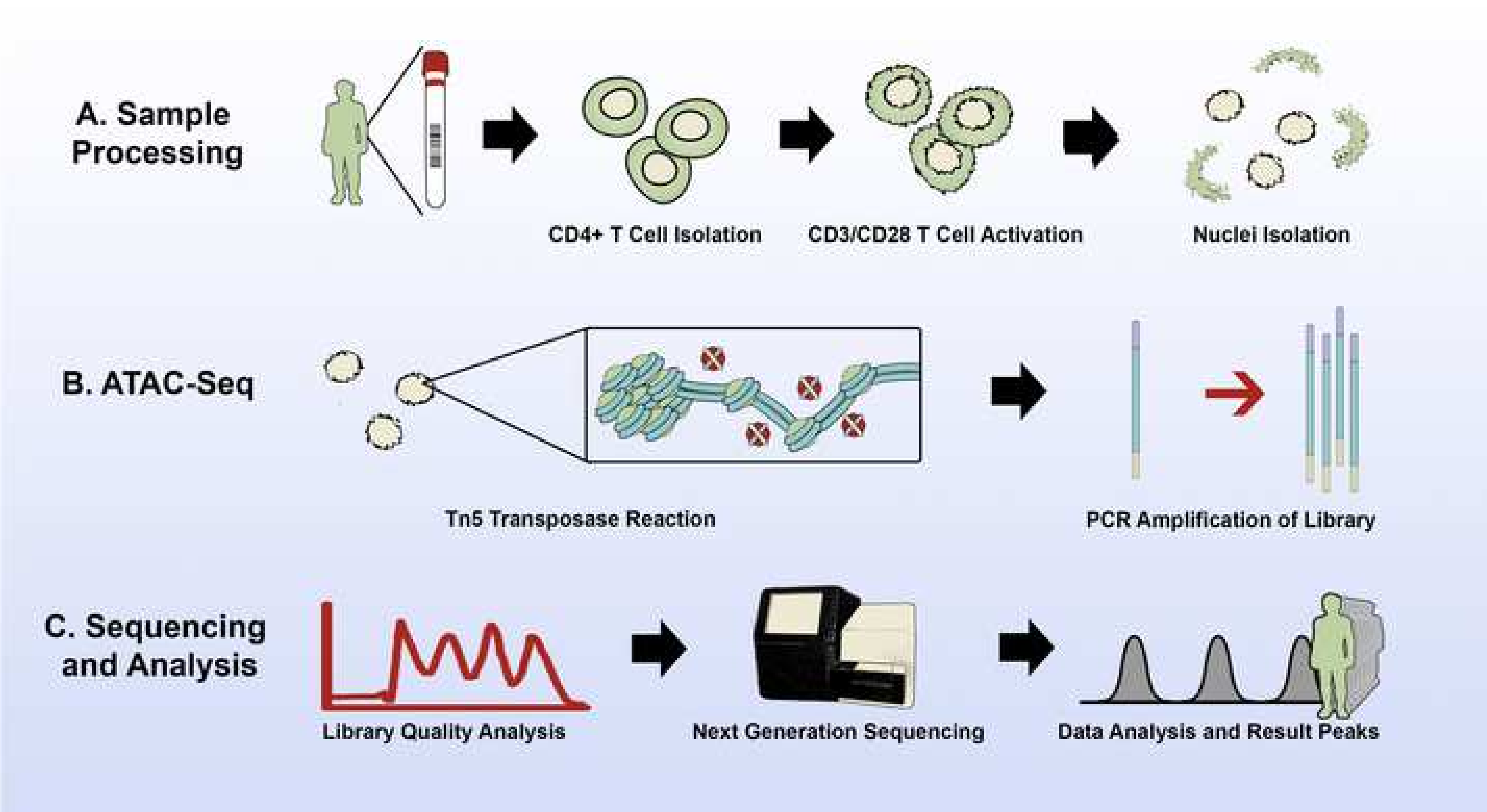
A. Sample Processing
CD4+ T Cell Isolation
CD3/CD28 T Cell Activation
Nuclei Isolation
B. ATAC-Seq
Tn5 Transposase Reaction
PCR Amplification of Library
C. Sequencing and Analysis
Library Quality Analysis
Next Generation Sequencing
Data Analysis and Result Peaks

Figure 2

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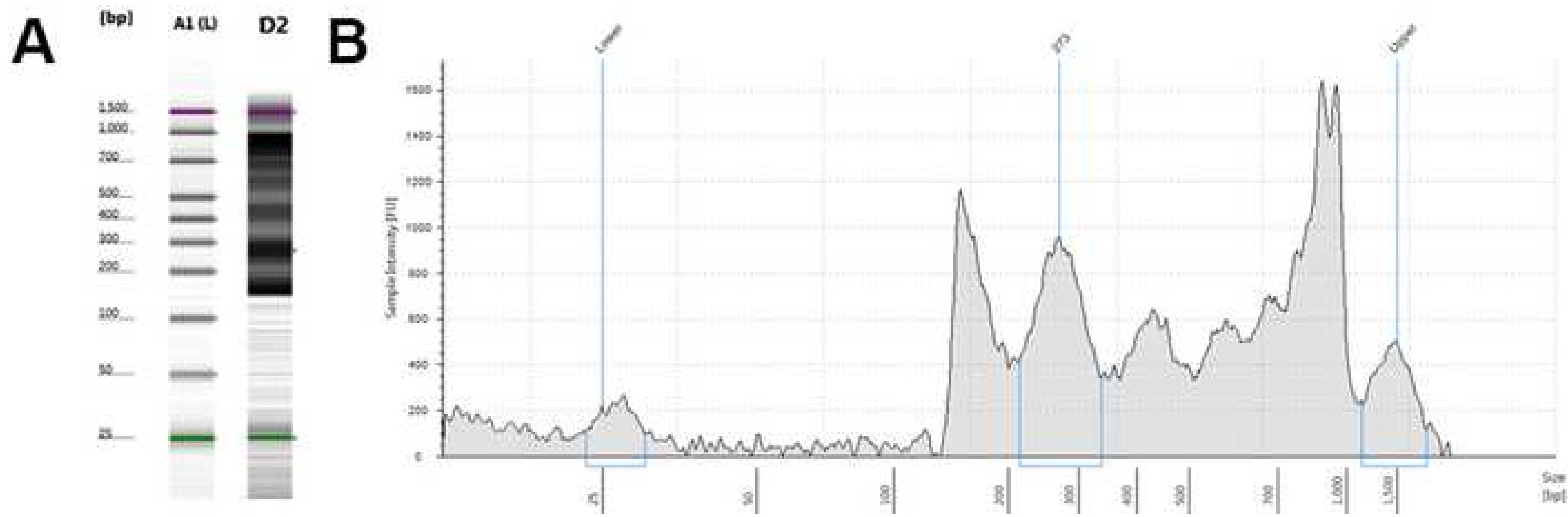

Figure 4

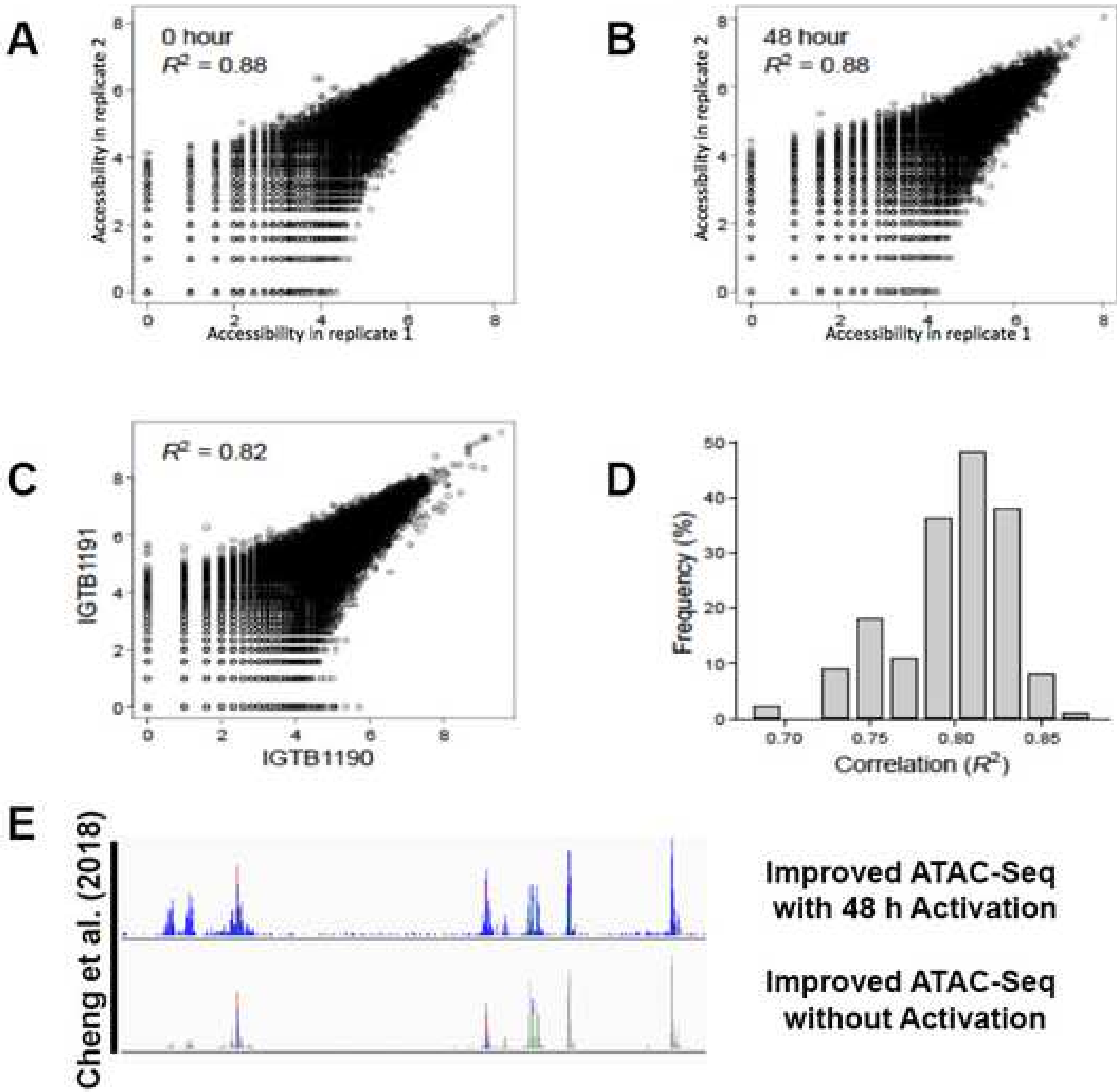
A
0 hour
R^2 = 0.88
Accessibility in replicate 2
Accessibility in replicate 1
B
48 hour
R^2 = 0.88
Accessibility in replicate 2
Accessibility in replicate 1
C
R^2 = 0.82
IGTB1191
IGTB1190
D
Frequency (%)
Correlation (R^2)
E
Cheng et al. (2018)
Improved ATAC-Seq with 48 h Activation
Improved ATAC-Seq without Activation

Figure 5

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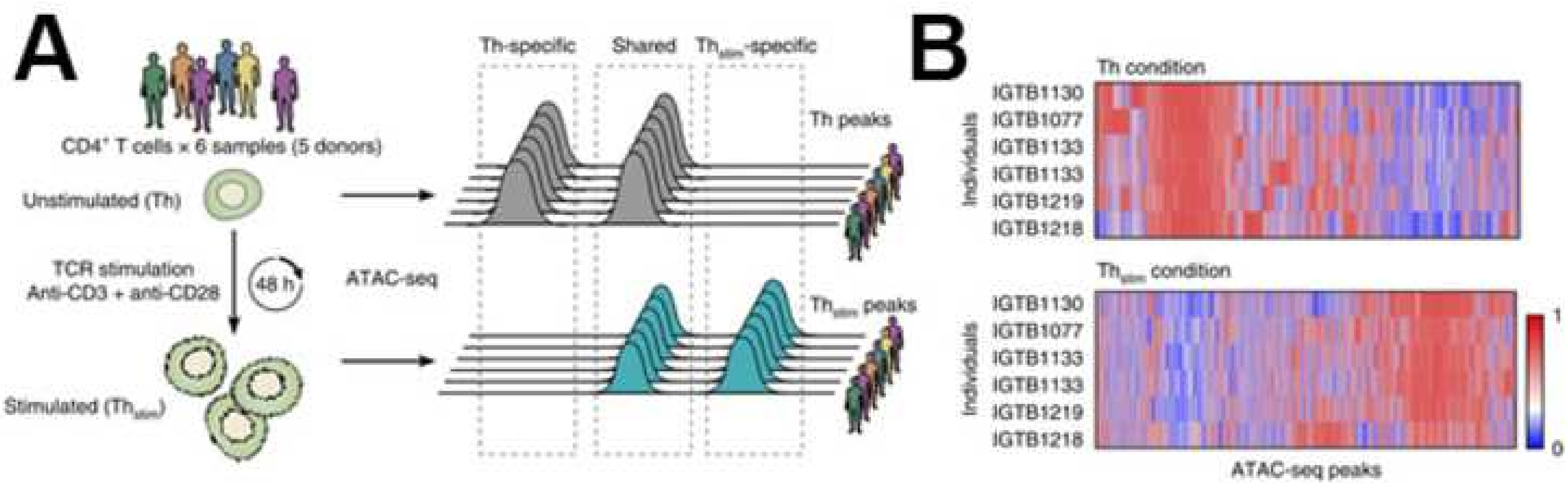

2x TD Buffer	25 µL
TN5 Enzyme	5 µL
Nuclease Free Water	20 µL
Total Volume	50 µL

Ad1_noMX: AATGATACGGCGACCACCGAGATCTACACTCGTCGGCAGCGTCAGATGTG
Ad2.1_TAAGGCGA CAAGCAGAAGACGGCATACGAGATTCGCCTTAGTCTCGTGGGCTCGGAGATGT
Ad2.2_CGTACTAG CAAGCAGAAGACGGCATACGAGATCTAGTACGGTCTCGTGGGCTCGGAGATGT
Ad2.3_AGGCAGAA CAAGCAGAAGACGGCATACGAGATTTCTGCCTGTCTCGTGGGCTCGGAGATGT
Ad2.4_TCCTGAGC CAAGCAGAAGACGGCATACGAGATGCTCAGGAGTCTCGTGGGCTCGGAGATGT
Ad2.5_GGACTCCT CAAGCAGAAGACGGCATACGAGATAGGAGTCCGTCTCGTGGGCTCGGAGATGT
Ad2.6_TAGGCATG CAAGCAGAAGACGGCATACGAGATCATGCCTAGTCTCGTGGGCTCGGAGATGT
Ad2.7_CTCTCTAC CAAGCAGAAGACGGCATACGAGATGTAGAGAGGTCTCGTGGGCTCGGAGATGT
Ad2.8_CAGAGAGG CAAGCAGAAGACGGCATACGAGATCCTCTCTGGTCTCGTGGGCTCGGAGATGT
Ad2.9_GCTACGCT CAAGCAGAAGACGGCATACGAGATAGCGTAGCGTCTCGTGGGCTCGGAGATGT
Ad2.10_CGAGGCTG CAAGCAGAAGACGGCATACGAGATCAGCCTCGGTCTCGTGGGCTCGGAGATGT
Ad2.11_AAGAGGCA CAAGCAGAAGACGGCATACGAGATTGCCTCTTGTCTCGTGGGCTCGGAGATGT
Ad2.12_GTAGAGGA CAAGCAGAAGACGGCATACGAGATTCCTCTACGTCTCGTGGGCTCGGAGATGT
Ad2.13_GTCGTGAT CAAGCAGAAGACGGCATACGAGATATCACGACGTCTCGTGGGCTCGGAGATGT
Ad2.14_ACCACTGT CAAGCAGAAGACGGCATACGAGATACAGTGGTGTCTCGTGGGCTCGGAGATGT
Ad2.15_TGGATCTG CAAGCAGAAGACGGCATACGAGATCAGATCCAGTCTCGTGGGCTCGGAGATGT
Ad2.16_CCGTTTGT CAAGCAGAAGACGGCATACGAGATACAAACGGGTCTCGTGGGCTCGGAGATGT
Ad2.17_TGCTGGGT CAAGCAGAAGACGGCATACGAGATACCCAGCAGTCTCGTGGGCTCGGAGATGT
Ad2.18_GAGGGGTT CAAGCAGAAGACGGCATACGAGATAACCCCTCGTCTCGTGGGCTCGGAGATGT
Ad2.19_AGGTTGGG CAAGCAGAAGACGGCATACGAGATCCCAACCTGTCTCGTGGGCTCGGAGATGT
Ad2.20_GTGTGGTG CAAGCAGAAGACGGCATACGAGATCACCACACGTCTCGTGGGCTCGGAGATGT
Ad2.21_TGGGTTTC CAAGCAGAAGACGGCATACGAGATGAAACCCAGTCTCGTGGGCTCGGAGATGT
Ad2.22_TGGTCACA CAAGCAGAAGACGGCATACGAGATTGTGACCAGTCTCGTGGGCTCGGAGATGT
Ad2.23_TTGACCCT CAAGCAGAAGACGGCATACGAGATAGGGTCAAGTCTCGTGGGCTCGGAGATGT
Ad2.24_CCACTCCT CAAGCAGAAGACGGCATACGAGATAGGAGTGGGTCTCGTGGGCTCGGAGATGT

Nuclease Free Water	11.9 µL
100 µM Custom Nextera Primer 1 (Table 2)	0.6 µL
NEBNext High-Fidelity 2x PCR Master Mix	25 µL
ATAC-Seq Library	10 µL
25 µM Custom Nextera Primer 2 (Table 2)	2.5 µL
Total Volume	50 µL

CYCLE STEP	TEMPERATURE	TIME
Extension	72 °C	5 min
Initial Denaturation	98 °C	30 s
Denaturation	98 °C	10 s
Annealing	63 °C	30 s
Extension	72 °C	1 min
Hold	4 °C	Infinity

CYCLES

1

1

10

1

PCR Reaction Aliquot	5 µL
PCR Cocktail from Table 3 with 0.6x Syber Green	10 µL

CYCLE STEP	TEMPERATURE	TIME	CYCLES
Initial Denaturation	98 °C	30 s	1
Denaturation	98 °C	10 s	
Annealing	63 °C	30 s	20
Extension	72 °C	1 min	
Hold	4 °C	Infinity	1

CYCLE STEP	TEMPERATURE	TIME
Initial Denaturation	98 °C	30 s
Denaturation	98 °C	10 s
Annealing	63 °C	30 s
Extension	72 °C	1 min
Hold	4 °C	Infinity

CYCLES

1

As Determined

1

Name
1X PBS, Sterile
5810/5810 R Swing Bucket Refrigerated Centrifuge with 50 mL, 15 mL, and 1.5 mL Tube Buckets
96 Well Round Bottom Plate
Agilent 4200 Tape Station System
Cryotubes
DMSO
Dynabeads Human T-Activator CD3/CD28
Dynabeads Untouched Human CD4 T Cells Kit
Dynamagnet
FCS
High Sensitivity DNA Kit
Magnesium Chloride, Hexahydrate, Molecular Biology Grade
MinElute PCR Purification Kit (Buffer PB, Buffer PE, Elution Buffer)
Mr. Frosty
NaCl, Molecular Biology Grade
NEBNext High Fidelity 2X PCR Master Mix
Nextera DNA Library Preparation Kit (2X TD Buffer, Tn5 Enzyme)
Nuclease Free Sterile dH20
Polysorbate 20 (Tween20)
PowerUp SYBR Green Master Mix
Precision Water Bath
QIAquick PCR Purification Kit
Qubit dsDNA HS Assay Kit
Qubit FlouroMeter
Rosette Sep Human CD4+ Density Medium
Rosette Sep Human CD4+ Enrichment Cocktail
RPMI-1640
Sterile Resevoir
T100 Thermocycler with Heated Lid
Tris-HCL

Company	Catalogue Number	Comments
Gibco	10010023	Can use other comparable products.
Eppendorf	22625501	Can use other comparable products.
Thermo Scientific	163320	Can use other comparable products.
Agilent	G2991AA	Suggested for quality assessment.
Thermo Scientific	374081	Can use other comparable products.
Sigma	D8418	Can use other comparable products.
Invitrogen Life Technologies	11131D	Critical Component
Invitrogen Life Technologies	11346D	Critical Component
Invitrogen Life Technologies	12321D	Critical Component
Gemini Bio Products	100-500	Can use other comparable products.
Agilent	50674626	Suggested for quality assessment.
Sigma	M2393	Can use other comparable products.
Qiagen	28004	Critical Component
Thermo Scientific	5100-0001	Can use other comparable products.
Sigma	S3014	Can use other comparable products.
New England BioLabs	M0541	Critical Component
Illumina	FC1211030	Critical Component
Gibco	10977015	Can use other comparable products.
Sigma	P9416	Can use other comparable products.
Applied Biosystems	A25780	Critical Component
Thermo Scientific	TSGP02	Can use other comparable products.
Qiagen	28104	Critical Component
Invitrogen Life Technologies	Q32851	Suggested for quality assessment.
Invitrogen Life Technologies	Q33226	Suggested for quality assessment.
Stem Cell Technologies	15705	Critical Component
Stem Cell Technologies	15022	Critical Component
Gibco	11875093	Can use other comparable products.
Thermo Scientific	8096-11	Can use other comparable products.
BioRad	1861096	Can use other comparable products.
Sigma	T5941	Can use other comparable products.

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Author(s): Hannah D Rickner, Simon Niu, Christine S Cheng

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Vineeta Bajaj, Ph.D.
Review Editor
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October 30th, 2018

Dr. Vineeta Bajaj,

We thank the editors and reviewers for their constructive comments on the manuscript and have made edits to address their points.

In particular, all commercial language has been removed from the manuscript and confined to the Table of Materials.

We believe the manuscript is now suitable for publication in JOVE.

Thank you.

Christine Cheng

Assistant Professor

Department of Biology

Boston University

Editorial Comments

UPDATED 11/5/18

1. The editor has formatted the manuscript to match the journal's style. Please retain the same.

We thank the editor for these corrections.

2. Please address all the specific comments marked in the manuscript.

These comments have been addressed in the manuscript or here in the rebuttal.

3. Please change the title to reflect the highlighted portion of the protocol.

The emphasis on low mitochondrial DNA contamination in the title is necessary. Modifications to the original ATAC-seq protocol that we demonstrate are all aimed towards this goal of reducing mitochondrial contamination, and will be the primary interest for readers. We have deleted a description of ATAC-seq from the title instead to make is shorter.

4. Notes cannot be filmed hence highlighting is removed. Highlights are adjusted to form a cohesive story. Please check.

The highlighted script forms a cohesive story.

5. Please refer to the figures in the order of their numbering. So figure 1 should be referred before 2 and so on. Please do the same with the tables as well.

Thank you for bringing this to our attention. The error has been fixed.

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A title has been clarified or each figure and table.

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.

The manuscript has been carefully reviewed.

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3. Figure 1: Please remove commercial language (Tween20).

Commercial language has been removed.

4. Figure 4: Please change the time unit "hr" to "h".

Time unit has been corrected.

5. Please upload each Table individually to your Editorial Manager account as an .xls or .xlsx file.

Apologies for the confusion on this point, tables have been uploaded as separate documents to the Editorial Manager.

6. Tables: Please use the micro symbol µ instead of u and use the temperature unit (°C) instead of "C".

Micro symbols and temperature units have been corrected.

7. Table of Equipment and Materials: Please sort the items in alphabetical order according to the Name of Material/ Equipment.

Table of Materials has been sorted alphabetically.

8. Please provide an email address for each author.

An email address has been provided for each author.

9. Keywords: Please remove commercial language (Tween20, Illumina). Please ensure that there are at least 6 keywords or phrases.

Commercial language has been removed, there are 6 keywords or phrases.

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The summary has been rewritten to more clearly describe the protocol.

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All commercial language has been removed from the manuscript and contained to the Table of Materials.

13. 3.1.3: What is used to wash and what volume? Please specify.

This step has been rewritten for clarity.

14. 4.2.3.1: What is washed?

This step has been rewritten for clarity.

15. Please combine some of the shorter Protocol steps so that individual steps contain 2-3 actions and maximum of 4 sentences per step.

The protocol steps have been reworked to combine shorter steps and provide more clarity.

16. There is a 2.75 page limit for filmable content. Please highlight 2.75 pages or less of the Protocol (including headings and spacing) that identifies the essential steps of the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol.

Less than 2.75 pages of protocol has been highlighted for filmable content.

17. Discussion: Please discuss any limitations of the technique.

The current limitations of the protocol are now more clearly highlighted as a discussion on the necessity of re-optimizing the protocol if used with other cell types or cell volumes, and in different contexts, such as single cell RNA-seq.

Reviewer #1 Comments:

In Figure 2, the large DNA fragment around 1000kb is not typical in ATAC-seq library and might be misleading. I suggest author to provide several more image to help readers have a better idea of how ATAC-seq library looks like.

We agree that the large DNA fragment around 1000 kb is not typical in an Agilent BioAnalyzer trace of ATAC-seq libraries. However, the traces presented in this paper are from the Agilent TapeStation, and often show such a band at 1000 kb. This is because the sizing range of the High Sensitivity D1000 ScreenTape we use from TapeStation only go up to 1,000 bp, any DNA fragments bigger than 1000bp is compressed into the dark band that's shown at 1,000 to 1,500bp (the upper marker). Thus this is due to the limitation of the TapeStation not the quality of our ATAC-seq libraries. All quality control of the samples presented in the paper was done with Agilent TapeStation. We also agree that more examples are warranted, and at the reviewer's suggestion have provided two samples' gel electropherograms and traces to give readers a better idea of what to expect from their ATAC-seq libraries.

In Figure3, only one result from improved protocol are shown. As author did a bunch of experiments, it is necessary to show the results from several replicates to demonstrate the reproducibility of method.

We have included a figure on biological replicates to demonstrate the reproducibility of the method.

As several previous publications addressed this question in other ways, it is important to discuss and compare them in the discussion part (Wu, J. et al. 2016, Nature; Corces, MR. et al.

2017, Nature Method).
We thank the reviewer for reminding us of these papers and the methods they employed to address the question of mitochondrial contamination, and we have included them in our discussion.

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Title: Genetic determinants of coaccessible chromatin regions in activated T cells across humans

Author: Rachel E. Gate et al

Publication: Nature Genetics

Publisher: Springer Nature

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