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CHROMATIN MAPPING OF PLANKTONIC CRUSTACEAN DAPHNIA PULEX USING ATAC-SEQ

by

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Presented to the Faculty of the Honors College of

The University of Texas at Arlington in Partial Fulfillment

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for the Degree of

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April 18, 2022

ABSTRACT

CHROMATIN MAPPING OF PLANKTONIC

CRUSTACEAN DAPHNIA PULEX

USING ATAC-SEQ

Waris Muhammad Khuwaja, B.S. Biology

The University of Texas at Arlington, 2022

Faculty Mentor: Sen Xu

Assay for transposase accessible chromatin with high-throughput sequencing (ATAC-seq) is a method used to study the accessibility of chromatin in a eukaryotic DNA. In eukaryotes, the DNA is packaged into chromatin to fit in the nucleus. The accessibility of chromatin by the proteins is determined by its packing status. In this research, an adapted protocol of nucleic ATAC-seq was performed on *Daphnia pulex*, a planktonic crustacean, to optimize it and generate a chromatin map. ATAC-seq was performed on 150 and 500 *Daphnia pulex*, following three major steps: nucleic isolation and purification, transposition reaction, and polymerase chain reaction (PCR) library amplification. The TapeStation analysis results indicated that the optimum input for ATAC-seq of *Daphnia pulex* was 500 animals. The modification of the protocol will be useful for future investigation in not only the whole body but tissue-specific studies.

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CHAPTER 1

INTRODUCTION

1.1 *Daphnia pulex*

Daphnia pulex is a eukaryotic, planktonic crustacean that belongs to the genus *Daphnia*. *Daphnia* is a common water flea that gets its name from the jump-like movement that is caused due to the beat of the antennae that it uses for swimming (Stollewerk, 2010). During its normal lifespan, *Daphnia* reproduces by generating haploid eggs through asexual reproduction (parthenogenesis). Within three days, these eggs develop into a larva in the female brood chamber and are released into the water. It takes about four to six larval cycles before *Daphnia* develops into a sexually mature adult (Stollewerk, 2010). *Daphnia* can switch its mode of reproduction from parthenogenesis to sexual reproduction when it is in an extreme environment. Extreme environments that make *Daphnia* switch to sexual reproduction are overpopulation, shortened daylight, lack of nutrients, and low temperature. Under these extreme conditions, *Daphnia* produces male offspring (Zhang et al., 2016). Haploid resting eggs are then produced by the *Daphnia*. These haploid eggs are fertilized and after a long period of dormancy develop into a *Daphnia* (Stollewerk, 2010). Generating offspring sexually increases, the chances of survival due to increased genetic variability (Zhang et al., 2010). Cyclical parthenogenesis is common in *Daphnia*, but some lineages just reproduce asexually (obligate parthenogenesis). Based on season and external environment, *Daphnia* changes its mode of reproduction throughout the seasons. Due to its unique ability to perform cyclical parthenogenesis, *Daphnia* is often used as a model to

study the evolution (Stollewerk, 2010). Ability of *Daphnia pulex* to reproduce asexually makes performing ATAC-seq easier as all animals will have the same genetic material with no variation.

1.2 ATAC-seq

Assay for Transposase Accessible Chromatin with high-throughput sequencing (ATAC-seq) is a method to map the accessible chromatin genome-wide. This technique uses the Transposase enzyme (Tn5) that inserts sequencing adapters in the regions that are accessible in the chromatin (Buenrostro et al, 2015). In a eukaryotic cell, the long DNA is highly compressed to be stored in the small nucleus. To make the DNA fit into the nucleus, it is packaged into chromatin. The basic unit of the chromatin is a nucleosome. The nucleosome is a structure that consists of about 150 base-pair of double-stranded DNA wrapped around the histone protein cores. Even though the DNA is wrapped around the histones, some parts of the genomic DNA need to be accessible by the protein complexes for the DNA transcription, replication, and repair. To allow for gene expression, the status of chromatin needs to be dynamic or change statuses between packing and unpacking. DNA packing status is a status in which some regions are highly compressed so that they are stored in the small nucleus and a DNA unpacking status, in which nucleosomes are loosely packed to allow protein complexes to use the DNA templated for various processes. Chromatin regulating proteins or chromatin regulators remodel the histones to allow for accessibility of the chromatin by the protein complexes (Zhang et al, 2016). The purpose of ATAC-seq is to find what regions in the chromatin are in unpacking status, and therefore expressed, and what regions are in the packing status, and therefore not expressed.

There have been many methods such as MNase-seq, ChIP-seq, and DNase-seq that are used to find the accessible regions in the chromatin, but these techniques require an input of tens to hundreds of millions of cells. Due to this large input requirement, some rare and important cell subtypes cannot be acquired in a sufficient amount to conduct the chromatin analyses. ATAC-seq on the other hand uses the Tn5 transposase enzyme to cut and ligate adapters for high-throughput sequencing in the regions that are increasingly accessible in chromatin. ATAC-seq follows a simple protocol that can be conducted in hours and requires a standard size of 50,000 cells. ATAC-seq is also compatible with various methods for cell separation and isolation. ATAC-sew works for various species and several types of cells. Three steps that ATAC-seq follows are cell lysis, transposition, and amplification (Buenrostro et al, 2015).

1.3 Transposase Tn5 Enzyme

As mentioned above, Tn5 is used in ATAC-seq to access the accessible chromatin. Tn5 transposase is a bacterial enzyme that is used to integrate a DNA fragment into a genomic DNA, and it is used in eukaryotes to detect the nucleosome-free regions in the genomic DNA. Bacterial Tn5 promotes the transposition of Tn5 transposon (Sato et al, 2019). A transposon consists of two inverted IS50 sequences, IS50L and IS50R, that encode a Tn5 transposase and help in transferring strands in the DNA via a nucleophilic attack on target DNA with a 3' OH group of the Tn5 transposon. Tn5 works using a "cut and paste" mechanism that is widely used in genomic research. Tn5 can insert adaptors or barcodes into DNA so that the DNA can be used for PCR amplification and sequencing (Li et al, 2020).

1.4 Purpose of the Research

ATAC-seq has been used in many model organisms but still, this technique is not fully optimized in non-model organisms such as *Daphnia*. The purpose of this research was to perform an adapted protocol of nuclei ATAC-seq on *Daphnia pulex* to optimize it and generate a chromatin map.

CHAPTER 2

METHODOLOGY

2.1 Cell Extraction

For cell extraction, 500 and 150 instar *Daphnia pulex* from the clone PA32 were collected in two separate Eppendorf tube and flash-frozen in liquid nitrogen for 5 minutes. After 5 minutes, the frozen tissue sample was thawed on ice. Once thawed, 300 µL of MasterPure tissue and cell lysis solution, from Lucigen MasterPure Complete DNA and RNA Purification Kit, with 2.8µL of proteinase K (50 U/mL) was added to the tubes. The samples were then sheared using a pellet pestle motor. Once sheared, the samples were incubated at 65°C for 15 minutes with constant slow shaking. Next, the samples were cooled at 37°C for 5 minutes. Once the samples were cooled, 4µL of 1 mg/mL RNase A was added to the tubes. After adding RNase, A, the tubes were incubated at 37°C for 30 minutes. After incubation, the samples were placed on ice for 5 minutes (Kissane et al, 2021).

2.2 Nuclei Purification

For nuclei purification, 175µL of MPC protein precipitation reagent from Lucigen MasterPure Kit was added to each sample and mixed well with gentle pipetting. Next, the samples were centrifuged at 10,000 x g for 10 minutes in a centrifuge at 4°C. After centrifuging the samples, the supernatant from each tube was transferred to clean tubes. 1/3 of the transferred supernatant was then transferred to a new tube, each containing 1 mL

of resuspension buffer (RSB) from the MasterPure Kit and 0.1% Tween 20. Remaining 2/3 of the supernatant was stored at -20°C. 1/3 of supernatant that was treated with RSB, and Tween 20 was centrifuged at 500 x g for 10 minutes at a fixed-angle pre-chilled 4°C centrifuge to pallet the nuclei. Once the pallet was obtained, the supernatant was discarded. The pelleted nuclei were stored at -20°C until further processing (Kissane et al, 2021).

2.3 Assembling Tn5 Transposome

To assemble Tn5 transposome, 9.4µL of 1x TE buffer and 0.6µL of 50µM duplex A stock were combined to dilute the duplex A to 3µM. 9.4µL of 1x TE buffer and 0.6µL of 50µM duplex B stock were combined to dilute the duplex B to 3µM. Refer to Table 2.3 for sequences of Duplex A and Duplex B. Once the duplexes were diluted, 2.4µL of Tn5, 0.6µL of 3µM duplex A, and 0.6µL of 3µM duplex B were combined in a tube to give 3.6µL of 200nM Transposome. The resulting transposome was further diluted to 100nM by combining 3.6µL of 200nM transposome and 3.6µL of the 1x TE buffer. Resulting solution of this dilution was 7.2µL of 100nM transposome.

Table 2.1: Sequence of Duplex A and Duplex B

Duplex Type	Sequence
Duplex A	5'-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG-3'
Duplex B	5'-GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG-3'

2.4 Transposition Reaction and Purification

The palleted nuclei was resuspended in the transposase reaction mix consisting of 5µL of 10x TD buffer, 2.5µL of 100nM transposome, and 42.5µL of ultrapure water. The Transposition reaction was incubated at 37°C for 30 minutes in a thermomixer using 1,000 rpm mixing speed. After incubation, the sample was purified using 1:1 volume Magbio

HighPrep PCR beads. To perform the purification, first, the beads were placed at 18°C-20°C for 30 minutes. Next, nuclease-free water was added to the ligation reaction to bring volume to 100 µL. Then, 100 µL of Magbio HighPrep PCR beads were added and mixed using a pipette. The reaction was incubated for 5 minutes at 18°C-20°C. After incubation, the tube was spin on a microcentrifuge and placed on a magnetic rack to separate beads and supernatant. After 5 minutes, when the solution was clear, the supernatant was discarded. After removing the supernatant, the fragments were washed by adding 200 µL of freshly prepared 80% ethanol solution to the tube while it was still on the magnetic rack and incubating it for 30 seconds. After 30 seconds, the supernatant was removed and discarded without disturbing the beads. This purification step with ethanol was repeated one more time. Once two washes with ethanol were performed, the beads were air dried for 5 minutes. After five minutes, the tube was removed from the magnetic rack and 23 µL of EB buffer was added to the tube and mixed by pipetting. The tube was incubated for 2 minutes at 18°C- 20°C. After incubation, the tube was placed back on the magnetic rack until the solution appeared clear. 20 µL of supernatant was transferred to a clean tube and beads were discarded. The purified sample was stored at -20°C until further processing (Kissane et al, 2021).

2.5 Library Amplification

Following the transposition reaction, library amplification was performed using NEB Q5 High-Fidelity DNA Polymerase. Library preparation was done by mixing 10 µL of 5x Q5 reaction buffer, 1 µL of 10 mM dTNPs, 2.5 µL of 25 µM ATAC-4 forward primer, 2.5 µL of 25 µM ATAC-4 reverse primer, 10.0 µL of transposed DNA, 0.5 µL of Q5 High-Fidelity DNA polymerase, and 23.5 µL of ultrapure water. Following PCR cycle was run:

a. 1 cycle

i. 5 minutes, 72°C

ii. 30 seconds, 98°C

b. 12 cycles

i. 10 second, 98°C

ii. 30 seconds, 63°C

iii. 60 seconds, 72°C

After the PCR cycle was complete, the sample was purified using 1:1 volume Magbio HighPrep PCR beads. To perform the purification, first, the beads were placed at 18°C-20°C for 30 minutes. Next, nuclease-free water was added to the ligation reaction to bring volume to 100 µL. Then, 100 µL of Magbio HighPrep PCR beads were added and mixed using a pipette. The reaction was incubated for 5 minutes at 18°C-20°C. After incubation, the tube was spin on a microcentrifuge and placed on a magnetic rack to separate beads and supernatant. After 5 minutes, when the solution was clear, the supernatant was discarded. After removing the supernatant, the fragments were washed by adding 200 µL of freshly prepared 80% ethanol solution to the tube while it was still on the magnetic rack and incubating it for 30 seconds. After 30 seconds, the supernatant was removed and discarded without disturbing the beads. This purification step with ethanol was repeated one more time. Once two washes with ethanol were performed, the beads were air dried for 5 minutes. After five minutes, the tube was removed from the magnetic rack and 23 µL of EB buffer was added to the tube and mixed by pipetting. The tube was incubated for 2 minutes at 18°C-20°C. After incubation, the tube was placed back on the magnetic rack until the solution appeared clear. 20 µL of supernatant was transferred to a

clean tube and beads were discarded. The purified library was stored at -20°C until further processing (Kissane et al, 2021).

Table 2.2: Sequence of ATAC-4 Forward and Reverse Primer

ATAC-4 Forward Primer Sequence	ATAC-4 Reverse Primer Sequence
5'-CAAGCAGAAGACGGCATACG AGATCCACTAAGGTCTCGTGGG CTCGGAGATGT-3'	5'-AATGATACGGCGACCACCG AGATCTACACCCACTAAGTCG TCGGCAGCGTCAGATGTG-3'

2.6 Next Generation Sequencing

Quality and size of ATAC library was evaluated using a High Sensitivity D1000 Screentape and High Sensitivity D1000 Reagents on a TapeStation. The library was sent for the Next Generation sequencing (Kissane et al, 2021).

CHAPTER 3

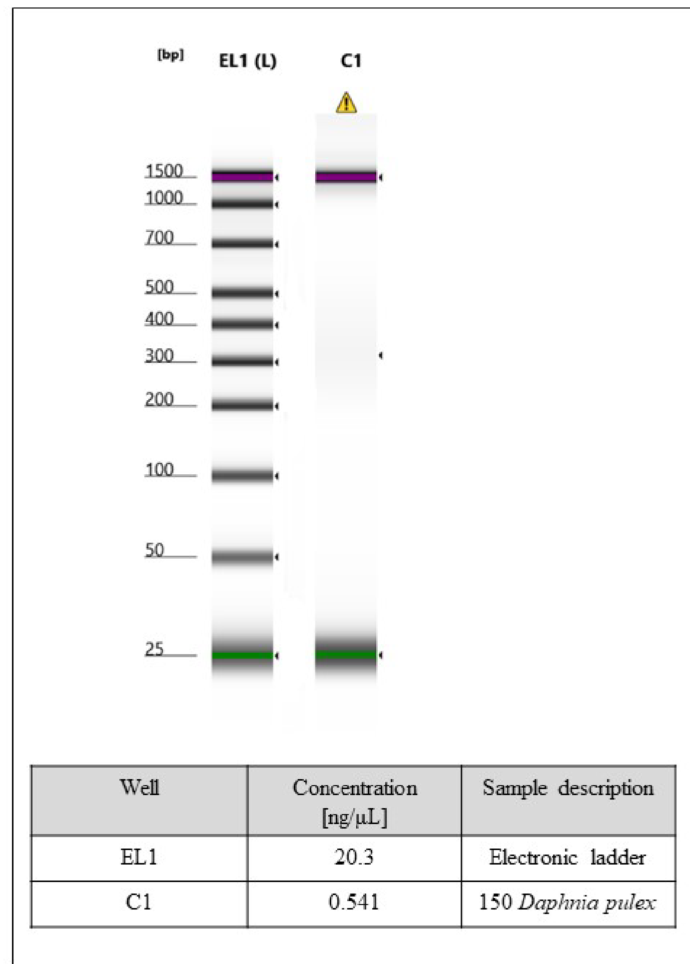
RESULTS

3.1 TapeStation Quality and Size Check

Quality and size results were obtained from the TapeStation. For the sample of 150 *Daphnia* pulex, a peak of concentration 0.541 ng/µL and molarity 2.62 nmol/L was observed at 318 base-pairs size regions, integrating 100% of the area. For the sample of 500 *Daphnia pulex*, two peaks were observed. First peak of concentration 4.75 ng/µL and molarity 24.7 nmol/L was observed at 296 base-pairs size regions, integrating 86.28% of the area. Second peak of concentration 0.755 ng/µL and molarity 0.437 nmol/L was observed at 2654 base-pairs size region, integrating 13.72% of the area.

3.2 TapeStation Electrophoresis for the Sample of 150 *Daphnia pulex*

The TapeStation performed the electrophoresis on the amplified library. Two wells that were loaded to TapeStation contained electronic ladder (EL1) and amplified ATAC library of 150 *Daphnia pulex* (C1). A very light mark was observed around the 300-base pair region on the gel.

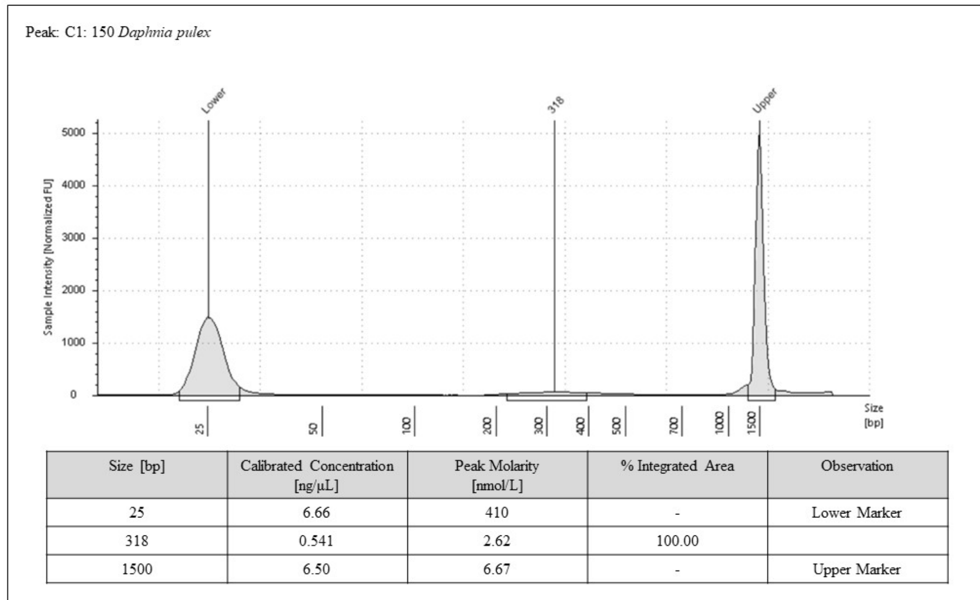


Well	Concentration [ng/µL]	Sample description
EL1	20.3	Electronic ladder
C1	0.541	150 *Daphnia pulex*

Figure 3.1: Electrophoresis for the Sample of 150 *Daphnia pulex*

3.3 TapeStation Peak Analysis for the Sample of 150 *Daphnia pulex*

The TapeStation analysis of the amplified library of 150 *Daphnia pulex* showed a weak peak at 318-base pair. This peak had the concentration of 0.541 ng/µL and molarity of 2.62 nmol/L. The peak integrated 100.00% of the area.

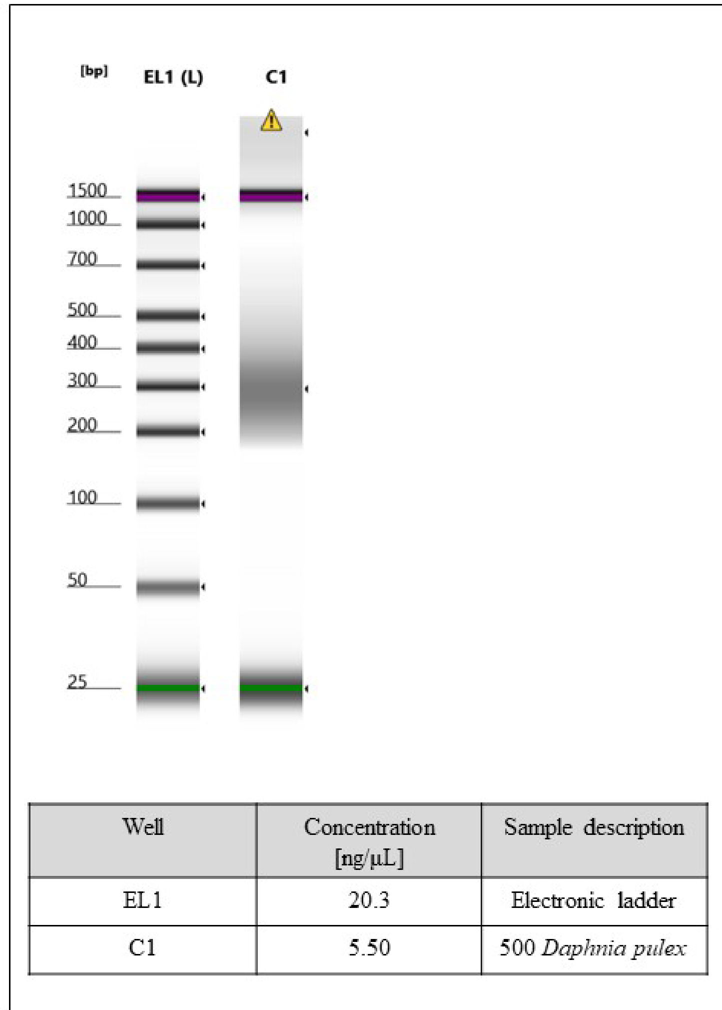


Size [bp]	Calibrated Concentration [ng/µL]	Peak Molarity [nmol/L]	% Integrated Area	Observation
25	6.66	410	-	Lower Marker
318	0.541	2.62	100.00	
1500	6.50	6.67	-	Upper Marker

Figure 3.2: Peak Analysis for the Sample of 150 *Daphnia pulex*

3.4 TapeStation Electrophoresis for the Sample of 500 *Daphnia pulex*

The TapeStation performed the electrophoresis on the amplified library. Two wells that were loaded to TapeStation contained electronic ladder (EL1) and amplified ATAC library of 500 *Daphnia pulex* (C1). A dark mark was observed around the 300-base pair region on the gel. Another very light mark was observed above the 1500 bp region.

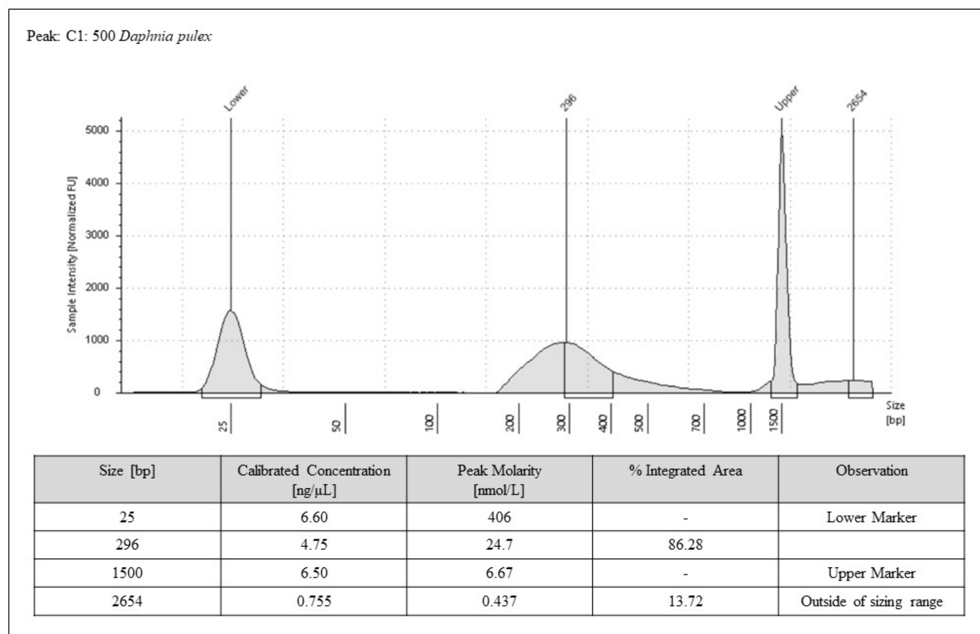


Well	Concentration [ng/µL]	Sample description
EL1	20.3	Electronic ladder
C1	5.50	500 *Daphnia pulex*

Figure 3.3: Electrophoresis for the Sample of 500 *Daphnia pulex*

3.5 TapeStation Peak Analysis for the Sample of 500 *Daphnia pulex*

The TapeStation analysis of the amplified library of 500 *Daphnia pulex* showed a strong peak at 296 bp. This strong peak had the concentration of 4.75 ng/µL and molarity of 24.7 nmol/L. The strong peak integrated 86.28% of the area. Another weak peak was observed at 2654 bp. This weak peak had the concentration of 0.755 ng/µL, molarity of 0.437 nmol/L, and integrated 13.72% of the area.



Size [bp]	Calibrated Concentration [ng/µL]	Peak Molarity [nmol/L]	% Integrated Area	Observation
25	6.60	406	-	Lower Marker
296	4.75	24.7	86.28	
1500	6.50	6.67	-	Upper Marker
2654	0.755	0.437	13.72	Outside of sizing range

Figure 3.4: Peak Analysis for the Sample of 500 *Daphnia pulex*

CHAPTER 4

DISCUSSION

4.1 Interpretation of Results

In a eukaryotic chromatin, the DNA is wrapped in a nucleosome. Each nucleosome contains approximately 150 bp of DNA is wrapped around the histone octamer with 20-90 bp DNA linker separating each nucleosome. During ATAC-seq, Tn5 transposase adds adapters randomly in the DNA. This can occur within the 20-95 bp region between the nucleosomes, creating short fragments of less than 90 bp and larger ones where a nucleosome is sandwiched between the cuts made by the Tn5 in DNA. In addition to this, during the library preparation, 135 bp adapter is added to each end. This creates the library fragments starting around 200 bp to 1,000 bp. (Carlson, 2021). A successful ATAC-seq that contains the necessary DNA fragments will show a peak around 200 bp when the TapeStation analysis is performed.

The results with 150 *Daphnia pulex* showed a peak at 318 bp with a concentration of 0.541ng/µL. This indicates that the library of the sample of 150 *Daphnia* had the required fragments, but the concentration was low. The reason for the low concentration could be the less starting material. Due to the low concentration, the sequencing could not be carried out for this sample of 150 *Daphnia*.

The results with 500 *Daphnia* pulex showed two peaks. The first peak was at 296 bp with concentration of 4.75 ng/µL. Second peak was located at 2654 bp with the concentration of 0.755 ng/µL. The first peak showed that the targeted fragments were present in the library of 500 *Daphnia*. The first peak also had a concentration enough for the sequencing and therefore the library was sent for next generation sequencing. The second peak could indicate the presence of DNA that was not fragmented. This could be due to inadequate Tn5 during the transposition reaction. Since the concentration of the second peak was low, it will have no effect on the sequencing.

4.2 Hyperactive Tn5 Transposase

Tn5 transposase plays a vital role in ATAC-seq as the whole process is dependent on the mechanism of how Tn5 works by cutting and adding adapters to the nucleosome-free regions. To improve the ATAC-seq technique and increase its efficiency, it is necessary to understand what is involved in the transposition reaction.

Hyperactive Tn5 transposase is used in the ATAC-seq. The invention of hyperactive Tn5 transposase is a great advancement in the library preparation as it allows for fragmentation of double stranded DNA and ligates synthetic oligonucleotides at both ends. Wild type Tn5 transposon is a combination of two almost identical insertion sequence, IS50L and IS50R. Each of these IS50 sequences contain two inverted 19 bp end sequences, an open sequence, and an inside sequence. Because end sequences in wild type Tn5 transposon had low activity, they were replaced by a hyperactive mosaic end sequence (5'-[phos]CTGTCTCTTATACACATCT-3'). A complex of transposase with 19 bp mosaic end sequence is the only necessary component for the transposition to occur, given

that the sample DNA is long enough to bring together these two sequences to form an active Tn5 transposon homodimer (Picelli et al, 2014).

4.3 Advantages, Limitation, and Applications of ATAC-seq

Compared to other techniques used to study the chromatin, ATAC-seq has several advantages when it comes to time for the process, cell input, and accuracy. The use of transposase in ATAC-seq significantly reduces the time to 2-3 hours to get the DNA fragments, compared to DNase-seq and Mnase-seq that takes 2-3 days. Apart from the reduced time, the protocol for ATAC-seq is very easy to follow and gives a higher success rate with lower input. ATAC-seq reduces input size by 1,000 times as ATAC-seq requires as low as 500 cells compared to 50 million cells required for Dnase-seq. Another advantage of ATAC-seq is that it uses paired-end sequencing technology to map nucleosome positioning. Paired-end sequencing can sequence both ends of the DNA, hence making it more accurate. One limitation of ATAC-seq is that Tn5 simultaneously fragments and tags the DNA through "cut and paste" mechanism. There are random adaptor joints at each end of the fragmented DNA, and this leads to a 50% probability that there will be a same adapter at both ends of the same fragments. This means that half of the generated fragments cannot be used for library amplification and sequencing (Sun et al, 2019).

Since the discovery of ATAC-seq, it is widely used in research related to the field of genetics. ATAC-seq has been used in epigenetics to study cancer by finding the target genes. This technique is also used study chromatin landscape in various diseases such as type 2 diabetes, osteoarthritis, Alzheimer's disease, coronary artery disease, and Parkinson's disease. Apart from diseases, ATAC-seq is also used to compare different

species by evaluating chromatin accessibility. ATAC-seq can also be used in plants, fungi, and protozoa to study the environmental interactions (Luo et al, 2022).

4.4 Future Research

ATAC-seq will be further explored in the lab to create chromatin maps of *Daphnia pulex* that will be useful in many processes such as microinjection. The mechanism of Tn5 will be studied to enhance the efficiency and increase the probability of Tn5 attaching a different adapter to each end of the same fragment. If efficiency of Tn5 is increased, single-cell ATAC-seq will be carried out on Daphnian embryo to study how accessibility of the chromatin changes throughout the lifespan.

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BIOGRAPHICAL INFORMATION

Waris Muhammad Khuwaja will be graduating in Fall 2022 from the University of Texas at Arlington with an Honors Bachelor of Science in Biology. Waris was born in Pakistan and then moved to the United Arab Emirates before migrating to the United States in 2017. Waris has always found the field of biology very interesting, and he is fascinated by how simple structures can perform complex processes. This was also the reason why Waris joined Dr. Xu's lab at UTA to explore and contribute to the field of genetics.

Throughout his time at UTA, Waris has been involved in various extracurricular activities. Waris has served as an Undergraduate Teaching Assistant, Honors College Senator, Honors College Council's Vice-President, Honors College Council's President, Honors Advocate, and Student Government's Supreme Court's Associate justice at UTA. Waris strives to enhance his leadership experience further and get involved in his community.

After graduation, Waris will apply for medical school and hopes to be a cardiologist one day. Waris wants to enter the field of medicine to serve his community and help people in developing countries around the globe.