Preparation and characterization of *Arabidopsis thaliana* nucleosome core particles containing the histone variant H2A.Z

A Thesis submitted to

Indian Institute of Science Education and Research, Pune in partial fulfilment of the requirements for the BS-MS Dual Degree Programme

by

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INDIAN INSTITUTE OF SCIENCE EDUCATION AND RESEARCH, PUNE

Certificate

This is to certify that this dissertation entitled "Preparation and characterization of *Arabidopsis thaliana* nucleosome core particles containing the histone variant H2A.Z" towards the partial fulfilment of the BS-MS dual degree programme at the Indian Institute of Science Education and Research, Pune represents study/work carried out by Afthab Saleem Poovalappil Arshad at the BRIC-Rajiv Gandhi Centre of Biotechnology (BRIC-RGCB), Thiruvananthapuram under the supervision of Dr. Dileep Vasudevan, Scientist F, BRIC-RGCB and the expertise of Dr. Gayathri Pananghat, Associate Professor, Department of Biology, Indian Institute of Science Education and Research, Pune, during the academic year 2024-2025.

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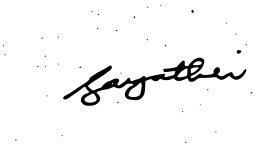

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This thesis is dedicated to my parents.

Declaration

I hereby declare that the matter embodied in the report entitled "**Preparation and characterization of *Arabidopsis thaliana* nucleosome core particles containing the histone variant H2A.Z** " are the results of the work carried out by me at the Structural Biology laboratory, BRIC-RGCB, Thiruvananthapuram under the supervision of Dr. Dileep Vasudevan, BRIC-RGCB and the same has not been submitted elsewhere for any other degree. Wherever others have contributed, every effort is made to indicate this clearly, with due reference to the literature and acknowledgement of collaborative research and discussions.

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List of Abbreviations

APS	Ammonium Persulphate
At	*Arabidopsis thaliana*
bp	base pairs
CIA	Chloroform-isoamyl alcohol
DMSO	Dimethyl Sulphoxide
DNA	Deoxyribonucleic acid
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic acid
HO	Histone Octamer
IPTG	Isopropyl 1-thio-β-D-galactopyranoside
kDa	kilo Daltons
L	Litres
M	Molar
NCP	Nucleosome Core Particle
PAGE	Polyacrylamide Gel Electrophoresis
PEG	Poly Ethylene Glycol
PMSF	Phenyl Methyl Sulphonyl Flouride
PTM	Post Translational Modification
SEC	Size Exclusion Chromatography
SDS	Sodium Dodecyl Sulphate
TAE	Tris base, Acetic acid and EDTA
TBE	Tris base, Boric acid and EDTA
TE	Trist EDTA
TEMED	Tetramethylethylenediamine

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Abstract

Histones perform a fundamental role in constituting the genetic structure of organisms. Histone variants replace some of these canonical histones, altering the nucleosome structure, leading to varying interactions within the constituent biomolecules and specialization of functions across species. The presence of histone variants and the characteristic changes to them compared to canonical histones have been implicated in structural and functional modifications and stability of DNA and genome as a whole, regulation of transcription in various cells, distribution of nucleosome arrangement along the genetic code, and DNA repair.

H2A.Z is a histone variant replacing H2A. Since its discovery decades ago, it has been discovered across a wide variety of living organisms, from simple protozoans and yeasts to highly advanced organisms like plants and even mammals like humans. This histone variant is the most evolutionarily conserved variant, showing a sequence similarity of ~90% across this aforementioned organism range. Despite having just close to 60% sequence similarity with its canonical counterpart, the occurrence of this histone variant being this widespread and conserved has great implications in functionality.

H2A.Z has been implicated in functions such as transcriptional regulation, chromosome regulation, and nucleosome stability. *Arabidopsis thaliana,* despite being a well-documented plant, studies on this variant from the plant are scarce. The focus of this thesis is, hence, on the preparation and characterization of *A. thaliana* nucleosome core particles containing H2A.Z. This work is expected to open up further research on understanding nuances due to H2A.Z incorporation in the structure and functional attributes of plant nucleosomes.

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I would like to extend my gratitude towards some of the exceptional faculty members I had the pleasure to interact with during my final months of stay at IISER Pune, who convinced me to continue with my thesis during a time of exceptional stress.

In particular, I would like to thank Dr. Gayathri Pananghat, whose guidance and faith in me was instrumental in regaining my passion for science when I was in dire need of it.

Concluding this, I would like to thank my parents, whose unconditional love, support and unwavering faith in me was a much needed pillar of stability during this period.

Contributions

Contributor name	Contributor role
Dileep Vasudevan	Conceptualization Ideas
Afthab Saleem Poovalappil Arshad, Sonali Ghosal	Methodology
Afthab Saleem Poovalappil Arshad, Sonali Ghosal	Software
Afthab Saleem Poovalappil Arshad, Sonali Ghosal	Validation
Afthab Saleem Poovalappil Arshad, Sonali Ghosal	Formal analysis
Afthab Saleem Poovalappil Arshad, Sonali Ghosal	Investigation
Dileep Vasudevan	Resources
Afthab Saleem Poovalappil Arshad, Sonali Ghosal	Data Curation
Afthab Saleem Poovalappil Arshad	Writing - original draft preparation
Sonali Ghosal, Dileep Vasudevan	Writing - review and editing
Afthab Saleem Poovalappil Arshad, Sonali Ghosal	Visualization
Sonali Ghosal, Dileep Vasudevan	Supervision
Dileep Vasudevan	Project administration
Dileep Vasudevan	Funding acquisition

This contributor syntax is based on the Journal of Cell Science CRediT Taxonomy[1].

[1] https://journals.biologists.com/jcs/pages/author-contributions

Introduction

○ Chromatin Organization

Genetic data is stored in the form of DNA in eukaryotic organisms in the cell nucleus, in various lengths ranging from hundreds of base pairs to billions. In eukaryotes, the genomic DNA is structured into a chromatin complex and situated within the nucleus, with a diameter of a few micrometers. (Kurumizaka *et al,* 2021). This structure is composed of repeating units of nucleosomes, which are complexes made by coiling of DNA around an octameric set of proteins called histones, and variations within these foundational blocks give rise to a myriad of biological phenomena.

○ Nucleosomes

Nucleosomes are the fundamental blocks of genetic material, ~10-12 nm in length, stabilized by electrostatic interactions and hydrogen bonding between its components. A stable nucleosome usually contains an octameric complex of the core histone proteins making up the nucleosome core. This histone octamer complex acts as a spool for DNA fragments about 145-147 base pairs in length to wrap around in left handed turns, and are themselves linked together with the linker histone H1 acting akin to a clip to prevent unspooling. (Luger *et al,* 1997). The nucleosome core mentioned above, along with the additional DNA required to connect the linker histone together makes up the chromatosome (McGinty and Tan, 2015). This structure usually has about 160-170 base pairs of DNA.The nucleosomes are linked to each other by DNA of differing length, which in a larger frame together form a “beads on a string” structure.

○ Histones

Histones are small, highly basic proteins which help in maintaining the structure and function of genetic material at the fundamental level. DNA wraps around two pairs of 4 core histones - H2A, H2B, H3 and H4, with a linker histone protein H1 /H5 holding

the coil together. The basic nature is owed to the heavy presence of Arginine and Lysine within these proteins.

The core histones are characterized by a “histone-fold”’ domain consisting of three α1-α3 helices, each alpha helix connected to the subsequent alpha helix by two L1-L2 loops respectively. binding of these L loops between core histones form the resulting H2A-H2B and H3-H4 dimers and these structural motifs are shaped like a “handshake” , due to overlapping crescent structures. (Ramaswamy & Ioshikhez, 2013).

The histone octamer ideally has a tetrameric set of H3 and H4 dimers, with the combined apparatus being flanked by two dimers of H2A and H2B (Kurumizaka *et al,* 2021). The initial step in the assembly of a stable nucleosome core particle involves the heterodimerization of H3 and H4, which then forms this tetramer by subsequent dimerization of these dimers. The histones H2A and H2B then dimerize themselves, and bind to both sides of the $(H3–H4)_2$ tetramer, thereby flanking them. (Ramaswamy & Ioshikhez, 2013).

Histones are also characterized by key structures called histone tails,mostly at the N terminal region, which extend from the core particle. Histone tails protrude out of the nucleosome core particle with each histone in the octamer set contributing their N terminal tail, and an additional couple of C terminal tails attributed to H2A (Bendandi *et al,* 2020), (McGinty & Tan, 2015).These structures bear responsibility for much of the post translational modifications present which in turn give rise to variant histones. It is to no surprise that histones, being such crucial building blocks of genetic code structure, seem to be strongly conserved across eukaryotes. Divergences that do occur whether in structural aspects of chromatin or cellular phenomena that can be attributed to histones are linked to these variants.

- **Variant Histones**

In addition to determination of the structural packaging of DNA within the nucleus, histones also play multiple roles in biological events, such as gene expression, DNA replication and DNA damage repair. The core histones can be extensively modified on their Histone tails and globular domains. These post translational modifications (PTMs) are various, leading to different consequences, such as acetylation or

methylation, changing binding sites for regulatory factors or neutralizing the charge of lysine residues.

Hence, histones have been historically divided into ‘canonical’ histones that are expressed only during the S-phase of the cell cycle and non allelic ‘variants’ that are inherently expressed during the cell cycle, among other distinguishing features. These aforementioned post translational modifications are responsible for the variable functions of histones and their modified variants across various biological aspects - structure and functions. These are what causes their unique chromatin neighbourhoods and often context variable functions like gene activations or silencing, in an enigmatically regulated manner. (Brewis & Wang, 2021).

Histone variants vary in their amino acid sequence from their canonical partners. Therefore, they can change the structural properties of the nucleosome, affecting their distribution on the chromatin and their interactions with different chromatin-associated proteins, thereby leading to various specialized functions. Consequently, they often have specialized functions in cell division, differentiation, DNA repair, transcription, and chromatin remodelling, while showing variable expressions with respect to uniformity across cells. (Talbert & Henikoff, 2021),(Martire & Banaszynski, 2020). A few of those variants are given below in **Table 1**.

Table 1: Histone variants and their functions.

Histone type	**Variant**	**Function**
H2A	H2A.X	DNA repair, transcriptional activation
	Macro H2A	Transcriptional silencing, involved in constitutive heterochromatin
	H2ABbd	Gene expression, Transcription activation
	H2A.Z	Highly conserved variant across eukaryotes with various functions.
H2B	H2BFWT	Testes-specific variant

	H2BV	Transcription activation
H3	CenH3	Kinetochore formation and chromosome segregation in centromeres
	H3.3	Transcriptional regulation

- **H2A.Z**

The histone variant H2A.Z is most widely conserved in evolution among all H2A variants showing an astounding sequence conservation of ~90% in between all these. H2A.Z shows just about ~60% sequence conservation with its canonical counterpart H2A, and how widespread this variant is within the living world. That implies crucial roles for the variant. H2A.Z variants have been discovered across the living world, from organisms like the protozoan parasite *Plasmodium falciparum*, *Tetrahymena thermophila*, the yeast *Saccharomyces cerevisiae*, and even in plants like *Arabidopsis thaliana* to *Homo sapiens*. A sequence comparison for H2A.Z and canonical H2A from *A. thaliana* has been provided in **Figure 1**. H2A.Z has been discovered to be involved in various biological events, some even functionally contradictory at a glance. some of the events this variant has found itself involved in would be gene activation and silencing, chromosome structuring and segregation, progression through the cell cycle etc (Zlatanova & Thakar, 2008),(Dhillon *et al,* 2006) and a few more varied, context-sensitive functions across species (Raisner & Madhani, 2006). Incorporation of H2A.Z has also been found to make the nucleosome more mobile, plastic and receptive to changes as a whole, with the energy barrier of DNA unwrapping showing considerable decrease. (Li *et al,* 2021).

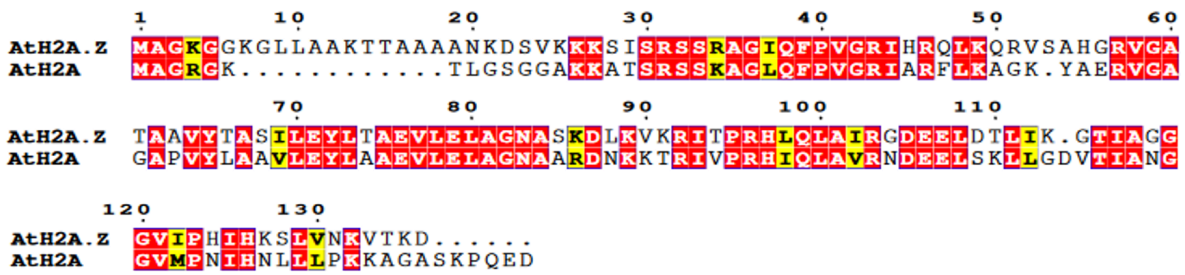


Figure 1: Sequence comparison of *At*H2A.Z with *At*H2A. The amino acid sequences of the histone variant *At*H2A.Z and the canonical histone *At*H2A aligned together and rendered using ESPript. The conserved residues are highlighted in red, while the semi-conserved residues are in yellow.

While some of the functions attributed to H2A.Z seem contradictory at first glance, the explanation of this histone variant showing these context-dependent roles being attributed to varying PTMs makes the reasoning more palatable - PTMs such as acetylation and SUMOylation makes the variant more dynamic leading to the attributes of DNA repair and transcriptional activation, whereas attributes such as gene silencing can be owed to methylation and ubiquitination making the variant more stable (Peterson & Laniel, 2004),(Giaimo *et al*, 2019),(Sura *et al,* 2017),(March-Díaz, R., and Reyes, J.C , 2009),(Tsai *et al,* 2016),(Xiao *et al*, 2020)(Liu *et al*, 2023) (Zhou *et al,* 2009).

In fact, H2A.Z shows slight differences over various species and multiple isoforms even among the same species accounting for these changes, such as *Trypanosoma* H2A.Z having a distinct N terminal extension compared to other species, (Lowell *et al,* 2005) or *A. thaliana* showing 4 distinct Z variants (Deal *et al,* 2007). A list of H2A.Z isoforms has been appended below (**Table 2**). A comparison of the 4 H2A.Z variants from *A. thaliana* has been provided in **Figure 2**.

Table 2: Histone H2A.Z variant isoforms and their functions

Species	**H2A.Z variant(s)**	**Function(s)**
Saccharomyces cerevisiae	Htz1p	Transcription activation, repression of euchromatic genes, DNA repair (Menenghini *et a*l, 2007)
Tetrahymena thermophila	Hv1	Essential gene, correlated with transcriptional competence. (Liu *et al* ,1996), (Stargell et al, 1995)
Arabidopsis thaliana	HTA -4, -8, -9 -11	necessary for high-level expression for Flowering Locus C, represses gene expression by modulating promoter nucleosome structure

Homo Sapiens	H2A.Z, H2A.Z/F	Enriched in promoters of transcription start sites, gene expression, gene silencing
Mus musculus	H2Afz, H2Afv	Essential for early development, centromere structure

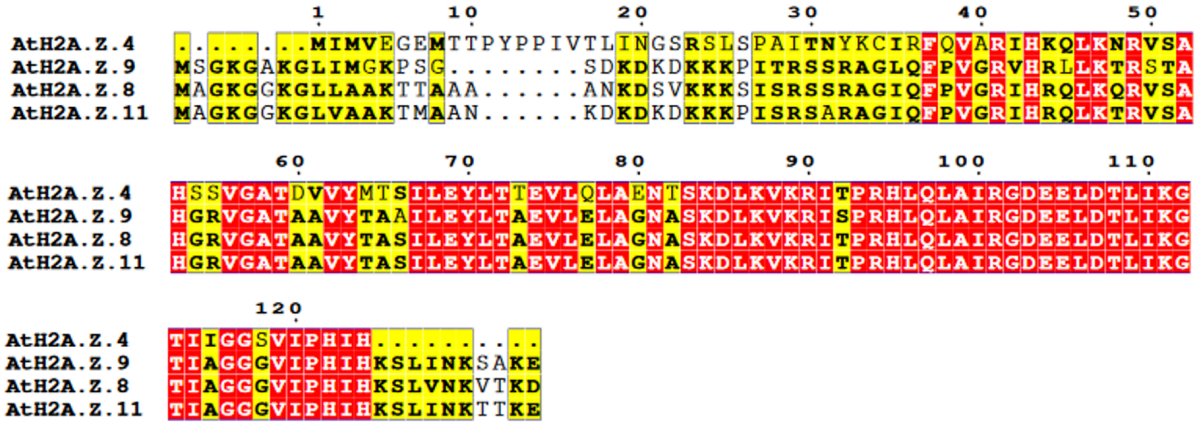


Figure 2: Sequence comparison of *At*H2A.Z isoforms. The amino acid sequences of the different isoforms of the histone variant H2A.Z found in *A. thaliana* aligned together and rendered using ESPript. The conserved residues are highlighted in red, while the semi-conserved residues are shown in yellow.

The *A. thaliana* H2A.Z variants seem more closely related to yeast rather than other plant H2As. Functionally, in this plant in particular, H2A.Z has been discovered to be required for high-level expression of the Flowering Locus C gene (Zlatanova & Thakar, 2008), and has a high affiliation with the functioning of Chromatin remodeling complexes like SWR1 and its subcomplexes. (Aslam *et al*, 2019) ,(Wu *et al,* 2005).

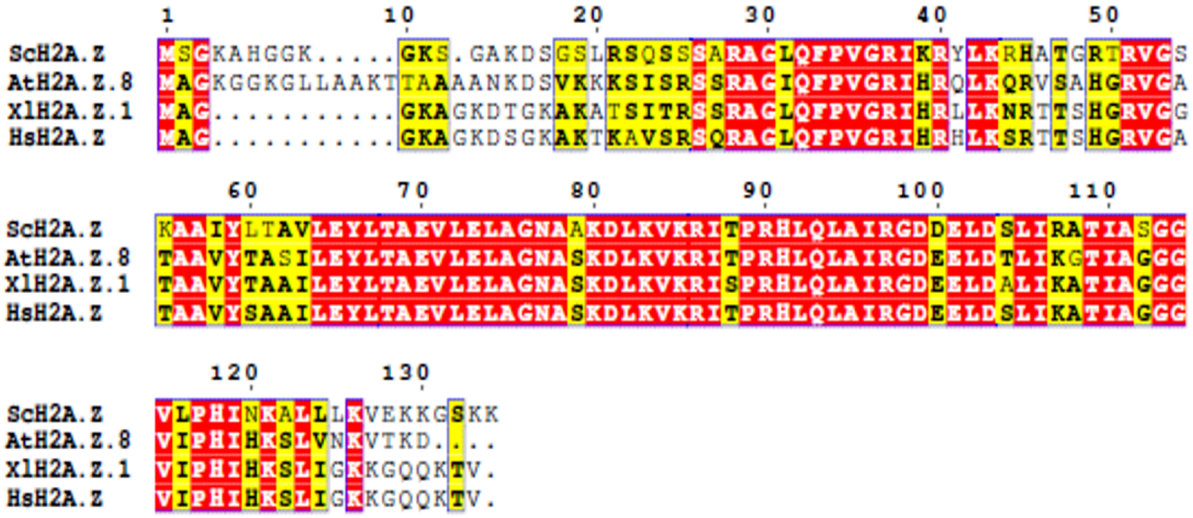


Figure 3: Sequence comparison of H2A.Z variants from different organisms. The amino acid sequences of H2A.Z from *Saccharomyces cerevisiae, Arabidopsis thaliana, Xenopus laevis and Homo sapiens* aligned together and rendered using ESPript. The conserved residues are highlighted in red, while the semi-conserved residues are shown in yellow.

The available structures of NCP from other organisms having H2A.Z show an overall similar structure to the canonical H2A version, albeit showing subtle differences in the structure, the predominant one being in the L1 loop region. Moreover, H2A.Z nucleosomes have shown lower thermal stability and get more readily lost from DNA during salt washes, compared to canonical H2A.

- ### Nucleosome Core Particle Reconstitution

Scaled-up amounts of recombinant histones and histone variants can be produced using a recombinant expression setup in bacterial competent cells by transforming corresponding vector constructs of histones of interest. These recombinant histones can be extracted from the competent cells as inclusion bodies. The inclusion bodies can be repeatedly washed in various buffers in the presence of detergents to extract a crude form of these histones. These can be further purified by size-exclusion chromatography in the presence of a denaturing agent such as urea, dialyzed repeatedly against water and then lyophilized to get purified histones for long-term storage (Luger et al., 1999).

A consequential combination of these lyophilized histone sets in equimolar ratios after solubilizing them could be done to refold these histones into an octamer. Optimal refolding of the octamer can be done by dialyzing against a refolding buffer with the high salt (2 M NaCl) in multiple cycles. This octamer complex can be further purified using a size-exclusion chromatography step (Luger et al., 1999; Dyer et al., 2003).

The length and sequence of DNA fragments used for assembly are crucial to obtain a stable and crystallizable NCP. One of the best-known DNA fragments optimal for this purpose is the “Widom 601” DNA in a 145 bp form discovered from a set of synthetic sequences by Widom and Lowary, known for its exceptional binding with histone octamer complexes (Lowary & Widom, 1998; Vasudevan et al., 2010).

In order to get sufficient amounts of DNA to prepare NCP, the competent cells like DH5α or HB101 transformed with a pUC57 vector, containing repeats of the 145 bp Widom 601 DNA fragments with EcoRV restriction sites between the stretches could be efficiently used (Vasudevan et al., 2010). The plasmid can be isolated by alkaline lysis method and subsequent extraction from the aqueous phase via phenol and Chloroform-isoamyl alcohol. The 145 bp fragment can be obtained via restriction digestion and PEG fractionation to remove the vector backbone, and further purification could be carried out by anion exchange chromatography (Dyer et al., 2003).

Mixing the histone octamer content and purified DNA fragments in equimolar stoichiometry in a high salt buffer and serially dialyzing against lower salt concentrations up to a no-salt buffer causes the DNA fragments to wrap around the octamer set, thereby reconstituting the NCP (Dyer et al., 2003; Vasudevan et al., 2010).

Rationale of the Project

The substitution of histone variants on the positions of canonical histones has been discovered to cause a variety of changes from the norm, including but not limited to the creation of specialized nucleosomes with differing structure, stability, function and position or distribution along the DNA. Preparation and characterization of NCPs containing these individual histone variants would assist in understanding the roles these proteins play in cellular machinery and events in the organism.

H2A.Z, despite showing ~60% sequence similarity with its canonical counterpart, shows a very high degree of sequence similarity between organisms - ~90% of the sequence has been discovered to be conserved along the evolutionary tree, from simple protists to high-functioning mammals. That, and the abundance of this variant in different forms in the living world across countless species, implies crucial roles beyond what has already been discovered so far.

Arabidopsis thaliana is a greatly documented model organism, and surprisingly, the 4 types of H2A.Z variants in this plant show a closer relation to yeast and other metazoan organisms than to other plants. So far, beyond the normal functions of H2A.Z, this variant has shown itself to be essential for flowering due to its involvement with Flowering Locus C. (Deal *et al, 2007*) Also, while H2A.Z has been discovered for decades and studies conducted across various organisms, *A. thaliana* seems rather understudied. Preparation and characterization of nucleosomes containing *At*H2A.Z could lead us a step closer to understanding any further enigmatic functions of this variant hidden within this model plant from a structure-perspective.

Objectives of the Project

The immediate objectives of the project are outlined as follows:

1. Expression of *A. thaliana* core histones H2B, H3, H4 and the non-allelic variant H2A.Z in a bacterial expression system
2. Purification of these histones by size-exclusion chromatography and lyophilization for long-term storage
3. Refolding of these histones to form a histone octamer containing variant H2A.Z
4. Preparation and purification of 145 bp “Widom 601” DNA
5. Reconstitution of NCP containing H2A.Z
6. *At*H2A.Z NCP quality analysis for further structural and functional studies

As part of this MS thesis project, recombinant expression of *A. thaliana* core histones H2B, H3 and H4 and the variant histone H2A.Z was done in a bacterial expression system and purified, which consequently underwent folding into a histone octamer and was then used to reconstitute an NCP containing H2A.Z with the help of Widom 601 DNA fragments which were isolated concurrently.

Materials

○ Molecular Biology Reagents:

Agarose, Acrylamide, Bis-Acrylamide, Amberlite resin, Ammonium persulphate (APS), Beta-mercaptoethanol (BME), Calcium chloride, Chloroform-isoamyl alcohol (CIA), Ethidium bromide (EtBr), Dithiothreitol (DTT), Ethylenediaminetetraacetic acid (EDTA), Glacial acetic acid, Glycerol, Guanidium hydrochloride, Imidazole, Isopropanol, Isopropyl β-D-1-thiogalactopyranoside (IPTG), Methanol, Phenol, Phenylmethylsulfonyl fluoride (PMSF), Polyethylene glycol 6000 (PEG 6000), Potassium acetate, Potassium chloride, Sodium acetate, Sodium chloride, Sodium hydroxide, Sodium dodecyl sulfate (SDS), Sucrose, Tris base, Triton X-100, and Urea (procured from Merck/Sigma-Aldrich), Protein marker ladder (procured from Bio-Rad).

○ Antibiotics:

- Chloramphenicol (25 mg/ml in ethanol and filter-sterilized)
- Ampicillin (100 mg/ml in MQ water and filter-sterilized)

○ Culture Media:

- **Luria-Bertani Medium: 1 Liter**

Ingredient	**Amount**
Bacto Tryptone	10 g
Yeast Extract	10 g
Sodium chloride	5 g
MilliQ water	Up to 1 L

The above ingredients were mixed into MilliQ water, made up to 1 L and autoclaved for use.

- **Luria-Bertani Agar: 1 Liter**

Ingredient	**Amount**
Bacto Tryptone	10 g
Yeast Extract	10 g
Sodium chloride	5 g
Agar	15 g
MilliQ water	Up to 1 L

The above ingredients were mixed into MilliQ water, made up to 1 L and autoclaved. The warm unsolidified agar medium was poured into sterile petri plates after mixing with the required antibiotics.

- **2XYT medium: 1 Liter**

Ingredient	**Amount**
Bacto Tryptone	16 g
Yeast Extract	10 g
Sodium chloride	5 g
MilliQ water	Up to 1 L

The above ingredients were mixed into MilliQ water, made up to 1 L and autoclaved for use.

- **Terrific Broth**

Ingredient	**Amount**
Bacto Tryptone	10 g
Yeast Extract	10 g
Sodium chloride	5 g
MilliQ water	Up to 1 L

After the ingredients were mixed and the volume made up to 1 Litre, the broth base was autoclaved, and the following sterile solutions were added per 1 litre of the broth base in a laminar air-flow hood.

- Pre-autoclaved NaH_2PO_4 (1 M): 17 mL
- Pre-autoclaved Na_2HPO_4 (1 M): 72 mL
- Pre-autoclaved Glycerol (50%): 8 mL
- Ampicillin (100 mg/ml): 1 mL

- **Stock Solutions:**

The following stock chemicals were prepared as per standard protocols.

- 0.5 M EDTA (pH 8.0)
- 1 M Tris-HCl (pH 6.8, 7.5, 8.0)
- 1.5 M Tris-HCl (pH 6.8, 8.8)
- 3 M Sodium acetate (pH 5.2)
- 5 M Sodium chloride
- 1 M DTT
- 40% Acrylamide
- 2% Bis-acrylamide
- 10% APS
- 10% SDS
- 4M Potassium chloride
- 40% PEG 6000

- 25% Sucrose

1. **4x SDS Sample Loading Dye Buffer**

Stock Solution	**Final concentration**	**Amount**
1M Tris-HCL (pH 6.8)	250 mM	20 ml
Glycerol	40%	16 ml
SDS	8%	3.2 g
Bromophenol Blue	0.4%	0.16 g
β-mercaptoethanol (14.2 M)	850 mM	2.378 ml
Milli-Q water		made up to 40 ml

2. **10x Tris-Glycine-SDS Buffer**

Stock Solution	**Amount**
Tris Base	30 g
Glycine	146 g
SDS	10 g
Milli-Q Water	made up to 1 L

3. **6x DNA Loading Buffer**

Stock Solution	**Final Volume**	**Amount**
1 M Tris-HCl (pH 7.5)	10 mM	200 µL
Glycerol	40%	16 mL
SDS	8%	3.2 g
Bromophenol Blue	0.4%	0.16 g

β-mercaptoethanol (14.2 M)	850 mM	2.378 mL
Milli-Q water		Made up to 40 ml

4. **50X Tris-EDTA Buffer**

Stock Solution	**Amount**
Tris Base	242 g
Glacial acetic acid	57 g
Sodium EDTA	37.2 g
Milli-Q water	made up to 1 L

5. **5X Tris-Borate Buffer**

Stock Solution	**Amount**
Tris Base	54 g
Boric Acid	27.5 g
0.5 M EDTA (pH 8.0)	20.7 mL
Milli-Q water	made up to 1 L

6. **Coomassie Protein Gel Staining Solution**

Stock Solution	**Amount**
Coomassie Brilliant Blue R 250	0.75 g
Methanol	225 mL
Acetic Acid	75 mL
Milli-Q water	made up to 500 mL

The solution was filtered through Whatman No.1 filter paper.

7. **Protein Gel Destaining Solution**

Stock Solution	**Amount**
Methanol	10 0mL
Acetic Acid	100 mL
Milli-Q water	made up to 1 L

8. **DNA Native-PAGE Staining Solution:**

Stock Solution	**Amount**
0.25X TBE	20 mL
SYBR DNA staining solution	2 µL

- **Gels:**

1. **SDS PAGE Gel Composition:**
   a. **18% Resolving Gel**

Stock Solution	**Volume**
1.5M Tris HCl (pH 8.8)	2250 µL
Acrylamide (40%)	4050 µL
Bis-Acrylamide (2%)	1350 µL
Milli-Q Water	1161 µL
SDS (10%)	90 µL
APS (10%)	9 µL
TEMED	9 µL
Final Volume	**9 mL**

b. 5% Stacking Gel

Stock Solution	**Volume**
1.5M Tris HCl (pH 6.8)	500 µL
Acrylamide (40%)	250 µL
Bis-Acrylamide (2%)	250 µL
Milli-Q Water	958 µL
SDS (10%)	20 µL
APS (10%)	20 µL
TEMED	2 µL
Final Volume	**2 mL**

- **BIO-RAD** Precision Plus Protein Standards were used as protein markers.

2. Native PAGE

a. 6% Native PAGE Gel

Stock Solution	**Volume**
5X TBE	1000 µL
Acrylamide (40%)	1500 µL
Bis-Acrylamide (2%)	750 µL
Milli-Q Water	6640 µL
APS (10%)	100 µL
TEMED	10 µL
Final Volume	**10 mL**

- ***E. coli* strains:**
  - BL21 (DE3) pLys S cells
  - LEMO21(DE3) cells
  - Rosetta Cells

- **Buffers Used:**

- **Inclusion Body Wash Buffer**

Stock Solution	Final concentration	Amount
1 M Tris-HCl (pH 7.5)	50 mM	50 mL
5 M NaCl	100 µM	20 µL
β-mercaptoethanol (14.26 M)	1 µM	70 µL
Milli-Q Water		made up to 1 L

- β-mercaptoethanol was added just before use
- Split this stock into two separate parts, with and without Triton X-100. And added 5 mL of Triton X-100 into one half.

- **SAUBE (Sod chloride, acetate, urea, BME & EDTA) - 1000 Buffer**

Stock Solution	Final Concentration	Amount
9 M Urea (de-ionized)	7 M	0.78 L
3 M Sodium Acetate (pH 5.2)	20 mM	6.65 mL
β-mercaptoethanol (14.26 M)	5 mM	0.35 mL
0.5 M EDTA	1 mM	2 mL
Milli-Q water		made up to 1 L

- Filtered and degassed, made with de-ionized urea stock
- β-mercaptoethanol was added freshly just before use

- **Crude Histone Unfolding Buffer**

Stock Solution	Final Concentration	Amount
Guanidinium HCl	7 M	133.742 g
3 M Sodium Acetate (pH 5.2)	20 mM	1.33 mL

1 M DTT	10 mM	2 mL
Milli-Q Water		made up to 200 mL

- DTT was added freshly just before use

- **Histone Octamer Unfolding Buffer**

Stock Solution	**Final Concentration**	**Amount**
Guanidinium HCl	7 M	133.742 g
1 M Tris-HCl (pH 7.5)	10 mM	2 mL
0.5 mM EDTA	1 mM	2 mL
Milli-Q Water		made up to 200 mL

- **Histone Octamer Refolding Buffer**

Stock Solution	**Final Concentration**	**Amount**
1 M Tris HCl (pH = 7.5)	10 mM	10 mL
NaCl	2 M	116.89 mL
β-mercaptoethanol (14.26 M)	10 mM	700 µL
Milli-Q		made up to 1 L

- β-mercaptoethanol was added freshly just before use.

- **Alkaline Lysis Solution I**

Stock Solution	**Final Concentration**	**Amount**
1 M Glucose	50 mM	50 mL
0.5 M EDTA	10 mM	10 mL
1 M Tris HCl (pH 8.0)	25 mM	25 mL
Milli-Q Water		made up to 1 L

- This solution was filtered using a 0.22-micrometer filter.

- **Alkaline Lysis Solution II**

Stock Solution	Final Concentration	Amount
5M NaOH	0.2 M	80 mL
10% SDS	1%	200 ml
Milli-Q Water		Made upto 2L

- This solution was filtered using a 0.22-micrometer filter.
- Fresh 10% SDS was made by dissolving 20 gm in 160 ml of water, heating it at 65 ^{o}C for 1 minute and the final volume made up to 200 ml.

- **Alkaline Lysis Solution III**

Stock Solution	Final Concentration	Amount
K-Acetate	4 M	785.2 g
Glacial Acetic Acid	2 M	228.8 mL
Milli-Q Water		made up to 2L

- This solution was autoclaved and stored at 4^{o}C

Equipment - Hardware and Software

- Biolynx Biosafety cabinet
- Thermo petriplate incubator
- New Brunswick Innova 44R shaker incubator
- Eppendorf Basic Biospectrometer
- Thermo Lynx 4000 centrifuge
- Sonics Vibracell probe-type sonicator
- Bio-Rad GelDoc system
- Millipore filtration unit
- Thermo NanoDrop One C spectrophotometer
- Bio-Rad NGC FPLC apparatus
- Cytiva AKTA Pure 25 M FPLC apparatus

Methodology

Histone Expression and Purification

○ Transformation of Histone Plasmid Constructs into Competent Cells

The transformation protocol for the expression constructs of *At*H2B, *At*H3, *At*H4 and the histone variant *At*H2A.Z in pET22b(+) vector were done as follows:

- *E. coli* BL21(DE3) pLys S competent cells were taken out from the -80 ºC freezer and kept to thaw on ice.
- Using a chilled pipette tip, mix and transfer 50 µL of competent cells to chilled tubes (50 µL for transformation and 50 µL for control).
- Under the hood, 10 ng of each plasmid were added to separate tubes with 50 µL competent cells. The contents of the tubes were gently mixed and then kept back on ice for 30 minutes.
- The cells were then given a heat shock at 42 ºC for 45 seconds. Immediately after, the cells were snap-chilled on ice for 2 minutes.
- Under the hood, 500 µL of LB broth medium was added. The cells were then incubated at 37 ºC for 1 hour in a shaking incubator.
- The cells were pelleted down at 6000 rpm for 2 mins. 300 µL of the supernatant was discarded, and the cells were resuspended in the remaining supernatant.
- The cells were then plated onto LB agar plates with 100 µg/mL ampicillin and incubated at 37 ºC overnight. Individual discrete colonies were obtained on the plates on the following day.

Note: The H2A.Z used in this study in AtH2A.Z.8

○ Histone Expression

- 2 discrete colonies were taken from the agar plates and inoculated into 5 mL of 2XYT in 15 mL falcon tubes, along with equivalent microlitres of antibiotic stocks - Ampicillin (and Chloramphenicol)

- This primary culture was incubated overnight at 37 ºC at 180 rpm in a shaker incubator.
- the next day, take 15 ml 2XYT and 15 µL of antibiotic in an Erlenmeyer flask. Take out 200 mL from this as a blank solution in a cuvette, and add 150 µL of primary culture into the flask for secondary culture.
- Shake the tubes at 37 ºC till the $O.D_{600}$ reaches 0.4-0.6 range.
- 1 mL of culture was taken out as uninduced control. Then, the cultures were induced with 0.5 mM IPTG and grown for 4 hours at 37 ºC at 200 rpm in a shaker incubator. After induction, 1 mL of the culture was taken to confirm histone expression using SDS-PAGE.
- All the cultures were centrifuged at 6000 rpm for 8 mins at 4 ºC. The supernatant was discarded, and the cell pellets were stored at -20 °C for further use.
- Wherever histone expression in BL21(DE3) was found to be suboptimal, alternate competent cells such as LEMO21(DE3) and Rosetta were used with the same expression protocol.
- Upon confirmation of test expression, large-scale expression was performed from the primary culture by repeating the above steps, where instead of a test culture, primary culture was instead taken to 4 Litres for large-scale expression. The cultures were induced with IPTG and, upon $O.D_{600}$ reaching ~0.6, were centrifuged and pelleted down with the same aforementioned parameters into storage at -20 ºC for further usage.

○ SDS-PAGE Analysis for confirmation of Histone Expression

- The 1 mL uninduced control and 1 mL IPTG-induced samples were pelleted at 6000 rpm for 2 minutes.
- The supernatant was discarded, and the pellets were resuspended in 30 µL of lysis buffer and mixed with 10 µL of 4X protein loading dye.
- The samples were then loaded onto an 18% SDS-PAGE gel and run at 120 V for 1 hour.

○ Preparation of Inclusion Bodies

- The frozen cells were thawed on ice for 15 min in falcon tubes.
- Inclusion body wash buffer without Triton-X was added to resuspend the pellets by vortexing.
- The cell suspension was then sonicated for 10 mins (3 secs on, 5 secs off cycle, 40% amplitude) until the solution became viscous.
- The sample was centrifuged at 10,500 rpm for 40 mins at 4 ^{o}C in 50 mL PPCO Oakridge tubes. The obtained pellet was then resuspended in a 15 mL inclusion body wash buffer containing 0.1% Triton X-100, sonicated for 10 mins, and centrifuged with the same parameters.
- The obtained pellet was again washed with 15 mL inclusion body wash buffer without Triton X-100
- The above steps with the inclusion body wash buffer were repeated, with the last wash cycle being without Triton X-100, and the final pellet of inclusion bodies was stored at -20 ^{o}C.

○ Purification of Histones by Size-Exclusion Chromatography

- A Cytiva HiLoad 26/600 Superdex 200 prep grade column (with a bed volume of 320 mL) was connected to a Bio-Rad NGC FPLC machine, and the column was washed with MilliQ water, followed by equilibration with 400 mL of SAUBE-1000 buffer at a flow rate of 1 mL/min.
- The stored inclusion body pellet was homogenized in 1 mL of DMSO by vigorous vortexing for 30 mins.
- Following that, 14 mL of crude histone unfolding buffer was added to the suspension, and it was vortexed vigorously for 1 hour to homogenize the pellet as extensively as possible.
- The solubilized histone mix was then triple-centrifuged at 10,500 rpm for 40 mins at 20 ^{o}C to remove particulate matter.
- The soluble supernatant was then injected into the 5 mL sample loop of the Bio-Rad NGC FPLC system and then eluted with 320 mL of SAUBE-1000 at a flow rate of 1 mL/min.
- The resultant fractions were then collected in fraction tubes (2.5 mL/fraction), and the resultant chromatogram was analyzed for protein peaks.

- The peak fractions were then run on an 18% SDS-PAGE gel to examine the quality of the fractions, and the fractions containing high concentrations of pure histones were pooled together.
- The pooled fractions were dialyzed against 5 L of Milli-Q water containing 5 mM beta-mercaptoethanol using dialysis tubing of 7 kDa cut-off.
- The dialysis was done at 4°C, with two water changes (with 5 mM beta-mercaptoethanol) after 4 hours each. The final dialysis step was allowed to proceed overnight.
- The dialyzed fractions were centrifuged at 10,500 rpm for 40 mins at 4°C to remove precipitated matter.
- The concentration of the samples was determined using OD_{276} reading using a NanoDrop Spectrophotometer.
- The supernatant was then aliquoted and flash-frozen using liquid nitrogen and then lyophilized and stored in a -80 °C freezer.

Refolding of Histone Octamer

- The lyophilized aliquots of each histone were dissolved in 1 mL of Histone Octamer Unfolding Buffer. The unfolding was allowed for ~30 minutes in the presence of DTT, with occasional mixing using a pipette, avoiding any strong disruption. OD276 was measured for each histone to measure concentration at the end of this period, as is given in **Table 3** below.

Table 3: Concentration of histones obtained after lyophilization

Histone Identity	Extinction Coefficient (M^{-1} cm^{-1})	Mass (kDa)	Concentration (mg/mL)	Molar Concentration (µM)
AtH2A.Z	2980	14.36	5.36	373
AtH2B	4470	16.40	29.65	1808
AtH3	2980	15.27	5.62	368
AtH4	5960	11.41	5.08	446

- 250 µM of each of the dissolved histones *At*H2A.Z, *At*H2B, *At*H3 and *At*H4 were added to a 35 ml tube. The volume was brought up to 17 mL by adding HO unfolding buffer, with the solution being gently mixed using a pipette.
- The solution was transferred to a dialysis tubing with a 7 kDa cut-off, and the dialysis bag was submerged in 800 mL of HO refolding buffer. The dialysis was performed at room temperature, with buffer changes after 4 and 5 hours, respectively, with the final dialysis cycle proceeding overnight.
- The dialyzed sample was centrifuged at 10,500 RPM for 30 min at 20 ºC to remove precipitates. The supernatant was transferred to a 30 kDa cut-off Amicon centrifugal concentrator and concentrated into a 5 mL sample.
- 1 mL each of the samples was aliquoted to 1.5 mL tubes and centrifuged at 13,000 RPM for 15 min at 20 ºC to remove precipitates. The supernatant was taken to a fresh tube.

○ Purification of Octamer by Size Exclusion Chromatography

- A HiLoad 16/600 Superdex 200 prep grade column was connected to the BioRad NGC FPLC apparatus, and the column was washed with autoclaved Milli Q water and then equilibrated with 150 mL HO refolding buffer at a flow rate of 0.5 mL/min.
- 5mL of the octamer sample was injected into the 5 mL sample loop of the apparatus and eluted out through the refolding buffer in the column at a flow rate of 0.5 mL/min. The eluted fractions corresponding to the protein peaks in the chromatogram were collected for an 18% SDS PAGE analysis.
- The pure fractions were pooled together, transferred to a 30 kDa cut-off Amicon centrifugal concentrator and concentrated into a 335 µL sample with a concentration of 4.54 mg/ml.
- The 335 µL was centrifuged at 12,000 RPM for 10 mins at 4 ºC to remove precipitates and were then stored in an equivalent amount of glycerol with a final concentration of 2.27 mg/ml at -20 ºC.

○ Preparation of 145 bp Widom 601 DNA

Transformation of pUC57 Widom '601' 145 bp DNA plasmid into *E. coli* DH5α competent cells

- *E. coli* DH5α competent cells were taken out of the -80 ºC freezer and kept to thaw on ice. 4 ng of pUC57 Widom '601' 145 bp DNA plasmid was added to the tube containing 50 µL of competent cells.
- The construct had eight repeats of 145 bp '601' DNA with internal EcoRV sites in a pUC57 vector. As expanded upon previously, the transformation protocol was followed, and the cells were then plated onto LB ampicillin (100 µg/mL) plates and incubated at 37 ºC overnight. Individual discrete colonies were obtained on the plates on the following day.

Plasmid DNA amplification

- Four discrete colonies from the transformed plate were inoculated into 15 mL of 2x YT media with ampicillin (100 µg/mL) and incubated at 37 ºC, 200 rpm for 4 hours.
- 5 mL of this primary culture was inoculated into 20 mL of TB media containing ampicillin ((100 µg/mL) and incubated at 37 ºC, 200 rpm until the OD600 reached 0.8.
- 10 mL of this culture was added to 1 L of TB media containing ampicillin (100 µg/mL) and incubated at 37 ºC, 150 rpm for 18 hours. The culture was then centrifuged at 7000 rpm for 6 mins at 20 ºC to obtain the cell pellet.

Plasmid isolation

- 5 mL Alkaline Lysis solution I was added to each tube containing the cell pellet and the cell pellet was resuspended in the solution by vortexing to obtain a homogenous solution.
- The solution was transferred to 500 mL PPCO centrifuge bottles, and Alkaline Lysis solution I was added to increase the volume to 50 mL. 100 mL of Alkaline Lysis solution II was added, and the solution was shaken vigorously for 2 mins and then incubated on ice for 20 mins with intermittent shaking every 5 mins. Then, 175 mL of Alkaline Lysis solution

III was added to the solution, and the bottles were gently mixed by invert-mixing for 2 mins.

- The bottles were then incubated on ice for 20 mins, with intermittent mixing every 5 mins. The solution was centrifuged at 10,000 g for 20 mins at 4 °C. The obtained supernatant was then filtered thrice through multiple layers of sterile cotton gauze into fresh 500 mL PPCO centrifuge bottles (6 cotton gauzes were stacked together and folded to get a total of 12 layers).
- To the filtered supernatant, 0.52 times the volume of isopropanol was added and mixed, and the solution was incubated for 30 mins. The solution was then aliquoted into 50 mL PPCO Oakridge tubes and centrifuged at 15,000 g for 30 mins at 20 °C. The supernatant was discarded, and the pellets were air-dried.
- The pellets were resuspended in 1 mL of TE (10/50) buffer and transferred to a single 50 mL PPCO tube, and TE (10/50) buffer was added to bring the final volume to 20 mL.
- To remove RNA contamination, 125 µL of RNase A (10 mg/mL) was added to the tube and incubated overnight at 37 °C, 160 rpm in a shaker incubator.
- The following day, the tube was centrifuged at 10,000 g at 20 °C for 30 mins, and the supernatant was transferred to a 50 mL Teflon tube. To the 20 mL DNA suspension, 10 mL of phenol was added, mixed by vortexing for 1 min, and then centrifuged at 15,000 g for 30 mins at 20 °C for phase separation.
- The aqueous phase thus obtained was transferred to a new Teflon tube, and the phenol extraction was repeated twice until the interface was clear. The aqueous phase was transferred to a new Teflon tube, and 10 mL of CIA (Chloroform – Isoamyl alcohol, 24:1) was added to remove the residual phenol. The tube was then centrifuged at 12,000 g for 10 mins at 20 °C, and the aqueous phase was then transferred into sterile 50 mL PPCO tubes.
- To the tube, 0.16 volume of 5 M NaCl (0.5 M NaCl final concentration) and 0.4 volume of 40% PEG 6000 (10% final concentration) were added, and the tube contents were mixed at 37 °C for 5 mins in a shaker incubator,

followed by 30 mins incubation on ice. The tube was centrifuged at 15,000 g at 4 °C for 30 mins.

- The supernatant was discarded, and the inside of the tube was wiped with sterile Kimwipes to remove PEG traces.
- The pellet in the tube was dissolved in 15 mL of TE (10, 0.1) buffer and incubated in a shaker incubator at 27 °C for 1 hour for complete dissolution.
- The DNA suspension from the tube was then transferred to a 50 mL Teflon tube, and 10 mL of CIA was added to remove residual PEG. The tube was then centrifuged at 12,000 g for 10 min at 20 °C.
- The aqueous layer was transferred to a fresh Teflon tube and repeated the CIA step. The DNA in the aqueous layer thus obtained was precipitated using 0.1 volume of 3 M Sodium Acetate (pH 5.2) and 2.5 volume of ice-cold absolute alcohol and incubated on ice for 30 mins. The tube was centrifuged at 15,000 g for 20 mins at 4 °C. The supernatant was discarded, and the pellet was air-dried overnight.
- The pellet was resuspended in 500 µL TE (10, 0.1) buffer, and the plasmid DNA concentration and purity were measured using a NanoDrop Spectrophotometer.

EcoRV digestion of plasmid DNA

- A reaction mix was set up in a 50 mL centrifugation tube containing 300 Units of EcoRV enzyme per 1 mg of plasmid DNA. The reaction mixture was incubated overnight at 37°C in a shaker, as is shown below in **Table 4**.

Table 4: Reaction mix used for EcoRV Digestion

Component	Stock Conc.	Working conc.	Volume
Plasmid DNA	6500 ng/ µl	2mg / mL	308 µL
EcoRV enzyme	15 units /µL	300 units	40 µL
10X H buffer	10X	1X	100 µL
NF Water	-	-	552 µL

- The total composition of the reaction mix added up to 1000 µL.

- A 6% Native PAGE assay was performed to confirm the completion of the digestion process.

PEG Fractionation of vector and 145 bp cut-out

- 0.374 volume of 40% PEG 6000 and 0.196 volume of 4 M NaCl were added to the plasmid digestion mix to give a final concentration of 9.5 % PEG and 0.5 M NaCl and mixed well. The tube was incubated on ice for 1 hour. The tube was then centrifuged at 16,000 g for 30 minutes, and the aqueous phase was transferred to a new 50 mL PPCO Oakridge tube.
- To the aqueous phase, 1/10th volume of 4 M NaCl and 2.5 times the volume of 100% cold ethanol were added and incubated on ice for 30 min. The tube was centrifuged at 15,000 g for 10 mins at 4 ºC. The supernatant was decanted carefully without disturbing the pellet, and the tube was carefully wiped using sterile Kimwipes.
- The pellet was then resuspended in 100 µL of TE (10, 0.1) buffer, and the DNA concentration and purity were measured using a NanoDrop Spectrophotometer. A 6% Native PAGE assay was performed to verify the absence of vector backbone

Reconstitution of the NCP

- For NCP reconstitution, histone octamer and DNA must be combined in equimolar stoichiometry. However, determining the accurate concentration determination of histone octamer is usually difficult.
- Therefore, multiple ratios of DNA:HO were screened for NCP reconstitution using the obtained concentration values of octamer and DNA. Thereby, the volume of DNA was maintained constant, while the volume of histone octamer was varied. The reconstitution was carried out in 200 µL dialysis buttons.
- NCP reconstitution was done using Dialysis buttons in a high salt to low salt gradient. Histone octamer and DNA were dissolved in 2 M KCl salt concentrations in 50 µL dialysis buttons to prevent aggregation and repeatedly dialyzed against dialysis buffers with gradually decreasing salt concentrations. The slow removal facilitated proper octamer-DNA interactions

where Histone interaction with K^+ and Cl^- ions were gradually replaced with interactions with the 145 bp DNA fragments.

- The dialysis buttons were submerged in 200 mL of TCS-0.85. The dialysis was performed at room temperature with continuous stirring, with three buffer changes after 3 hours each to reduce the salt concentration gradually.
- The buffer changes proceeded stepwise, from TCS-0.65 to TCS-0.45, then to TCS-0.25. The fourth dialysis step in TCS-0.25 was allowed to proceed overnight. The buffer was changed to TCS-0, and dialysis was performed for another 3 hours. The dialyzed NCP solutions were then transferred to 1.5 mL tubes and centrifuged at 13,000 rpm for 10 mins at 4 ^oC to remove any precipitates. The supernatant was transferred to fresh 1.5 mL tubes. The concentration of the nucleosomes was measured using OD_{260} readings using a NanoDrop Spectrophotometer, as given in **Table 5** below.

Table 5: NCP reconstitution mixture composition

Component	**1 : 1.6 ratio**	**1 : 2 ratio**	**1 : 2.2 ratio**	**Final Conc.**
NF water	2.89 µL	23.88 µL	29.51 µL	-
4M KCl	31.20 µL	30.84 µL	24.55 µL	2 M KCl
1M DTT	0.65 µL	0.65 µL	0.65 µL	10 mM DTT
~19.76 µM HO	5.2 µL	6.57 µL	7.23 µL	
42.36 µM DNA	3.06 µL	3.06 µL	3.06 µL	2 µM DNA
Total Volume	65 µL	65 µL	65 µL	

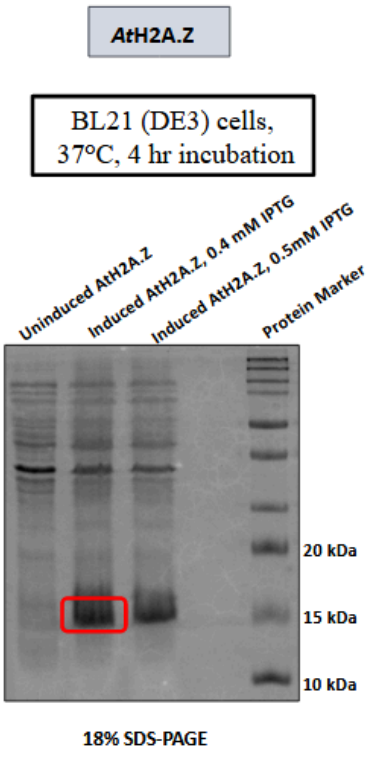
Results and Discussion

Results

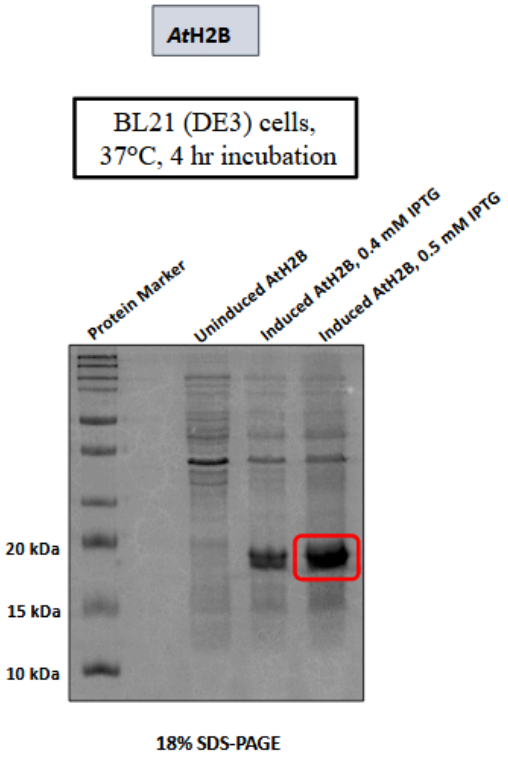
Expression and Purification of *Arabidopsis thaliana* Histones

- **Histone Expression**

E coli. BL21 (DE3) competent cells were initially transformed with pET22b(+) plasmid vector constructs of *Arabidopsis thaliana* histones H2B, H3, H4 and histone variant H2A.Z. The primary cultures were induced by IPTG when $O.D_{600}$ reached 0.4-0.6 and then incubated in a shaker at 37 °C for 4 hours, upon which they were pelleted down. Induced and uninduced samples were run through an 18% SDS PAGE analysis, with varying expression levels. When the expression levels seemed suboptimal, alternate competent cell BL21(LEMO) was taken, with the same steps repeated as above. After confirming sufficient expression levels, mass culture was done for the recombinant histones, in 4 L cultures from the primary media.



a)



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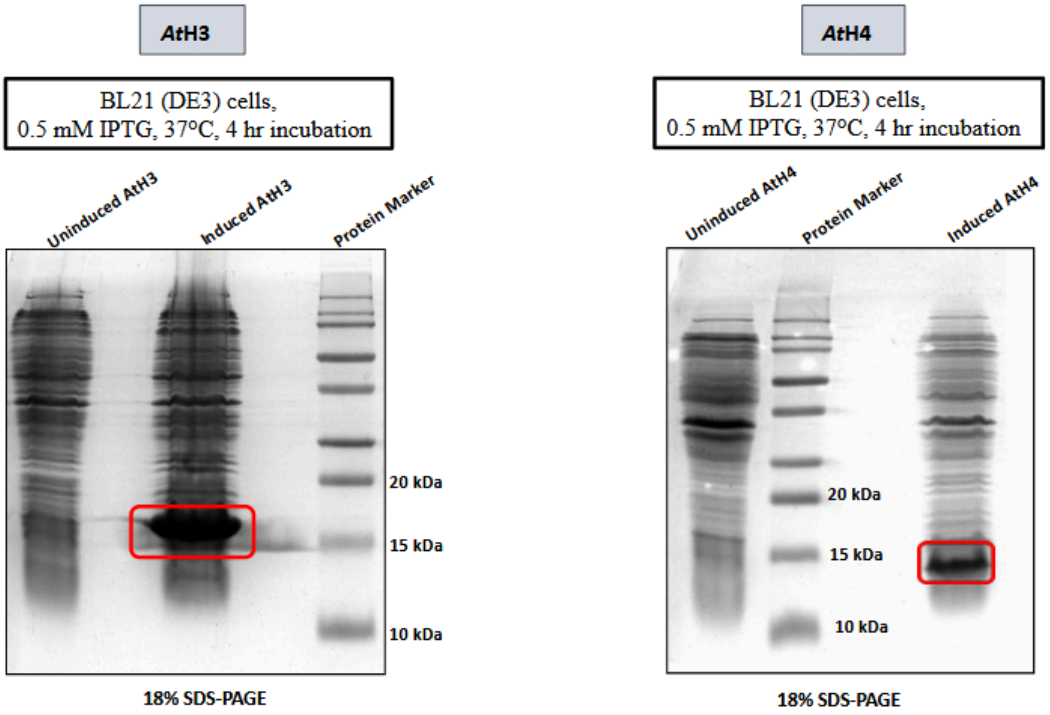
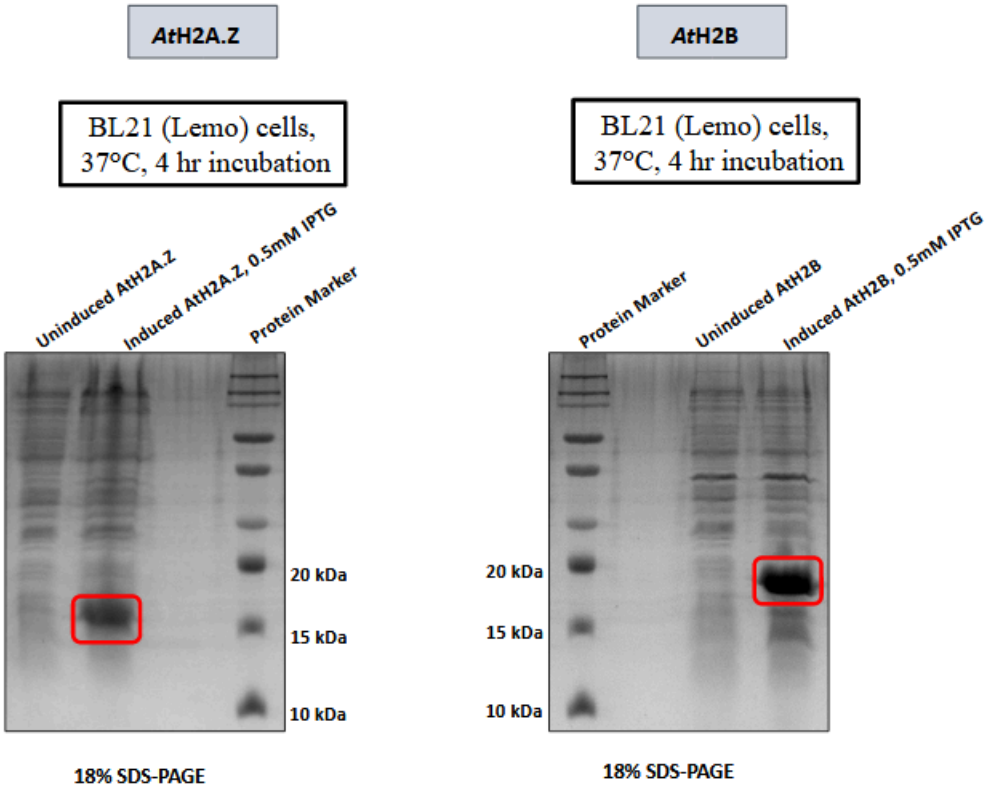


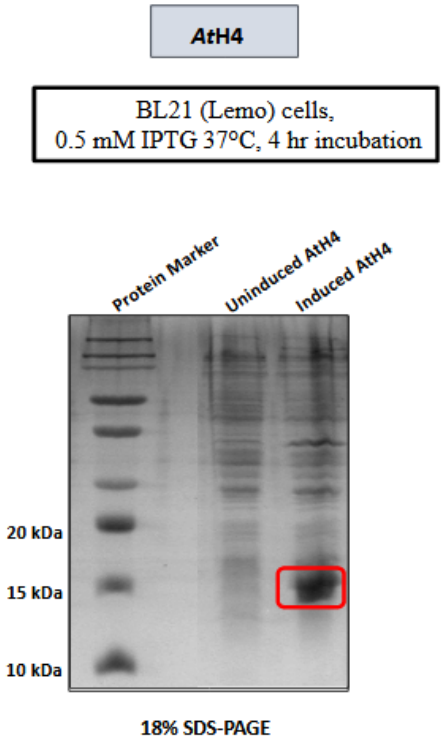
c) d)

Figure 4: SDS PAGE images of test expression in BL21(DE3) for a) *At*H2A.Z; b) *At*H2B; c) *At*H3; d) *At*H4.

The test expressions were repeated in Lemo cells except for *At*H3.



a) b)



c)

Figure 5: SDS PAGE image analysis of construct test expressions in Lemo21(DE3) competent cells for a) *At*H2A.Z; b) *At*H2b; c) *At*H4.

The mass cultures were pelleted down, and the sediment was stored at -20°C for further use.

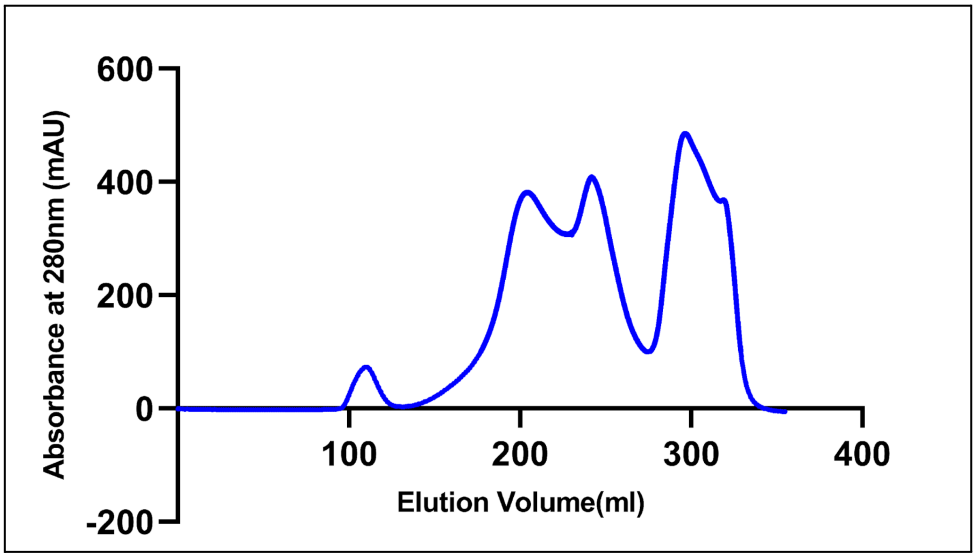
- **Preparation of Inclusion Bodies**

The proteins were in the form of insoluble inclusion bodies after the mass culture and were stored at -20 °C prior to further purification. The inclusion bodies were purified by successive steps of lysis of the cell pellets in wash buffer, sonication and centrifugation in repeating, alternating cycles with and without Triton X-100, with the first and last cycles lacking this non-ionic detergent. The final purification cycle and pellets mostly contained inclusion bodies of our histones of interest.

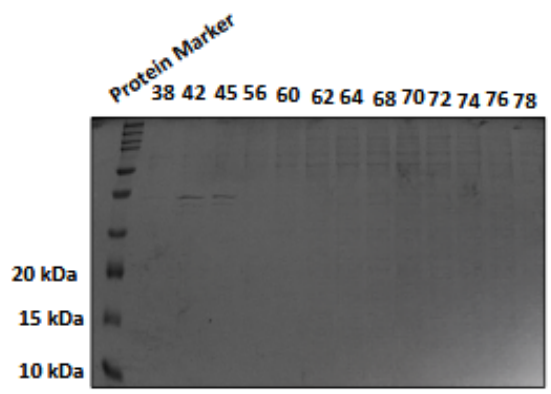
- **Size-Exclusion Chromatography for the Histones**

The inclusion bodies were homogenized and solubilized in a Crude Histone Unfolding Buffer by repeated mixing under denaturing conditions provided by DMSO. After triple centrifugation of the resultant suspension and removal of pellets, the dissolved histones supernatant mix were injected in the FPLC apparatus set up with a HiLoad 26/600 mm Superdex 200 prep grade column and was eluted out in SAUBE-1000 buffer. Excluding the first peak accounting for the contaminated regions, the fractions corresponding to the absorbance peaks were taken and analyzed with SDS PAGE.

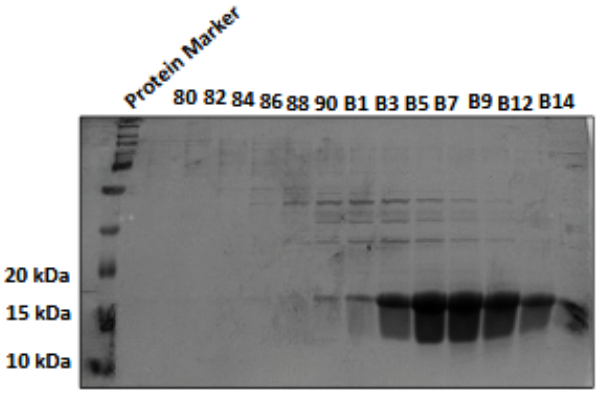
The stretches of fractions corresponding to the best expression were pooled together and dialyzed using a SnakeSkin membrane for three cycles against double distilled water with BME added, with 2 litres of water in each run. The three dialysis runs lasted for 4 hours, 4 hours and overnight, respectively. The $O.D_{276}$ was checked using a NanoDrop for each final histone, noted down and flash-frozen using liquid nitrogen. The flash-frozen samples were lyophilized and stored at -80^{o}C for further use.



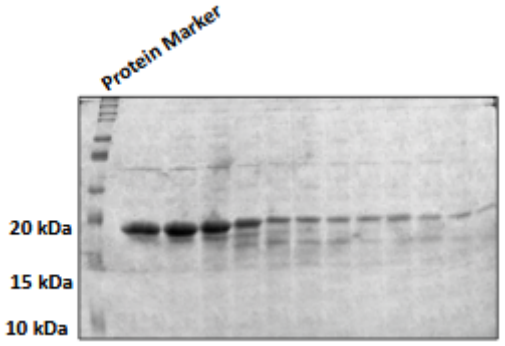
(a)



(b)

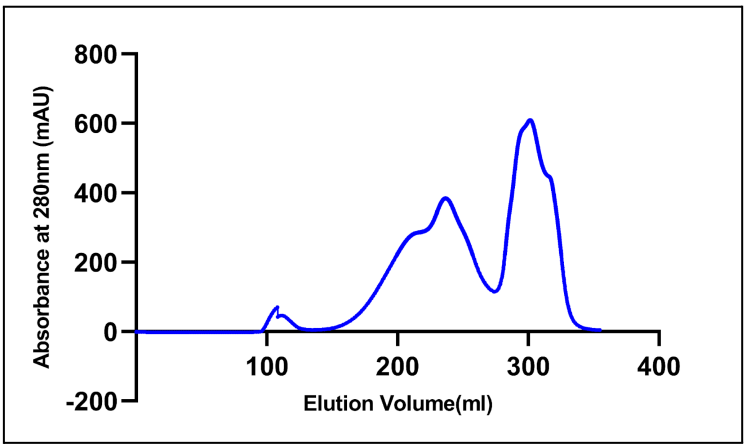


(c)

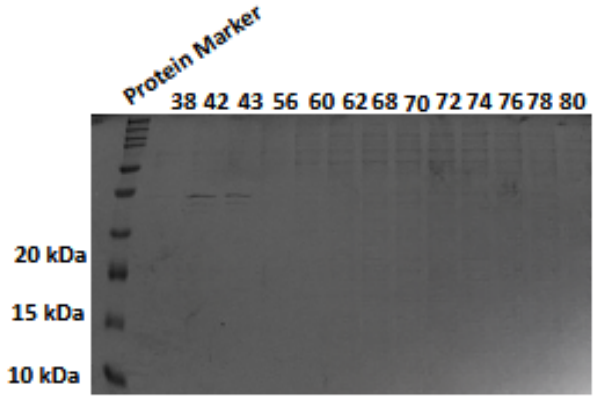


(d)

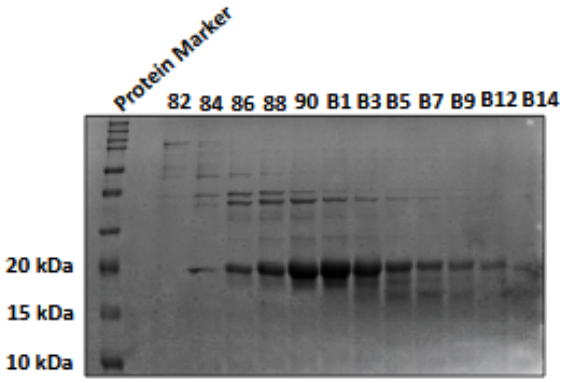
Figure 6: Size-exclusion chromatographic purification of *At*H2A.Z. (a) shows the purification profile for *At*H2A.Z by SEC using a Superdex 26//600 prep grade column. **(b), (c)** and **(d)** show images for the 18% SDS-PAGE analysis of protein fractions corresponding to the elution peaks. **(c)** and **(d)** shows the stretch of fractions containing the histone variant.



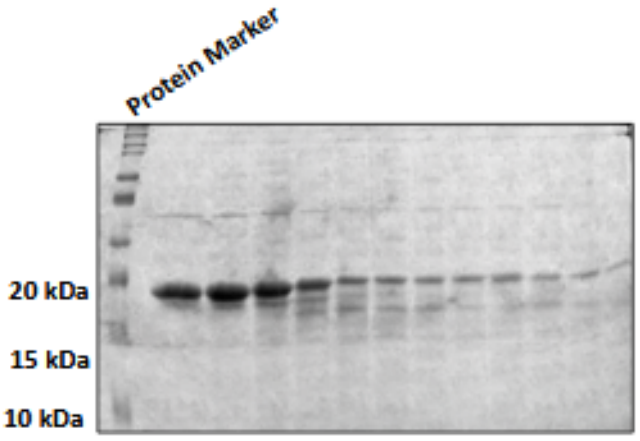
(a)



(b)

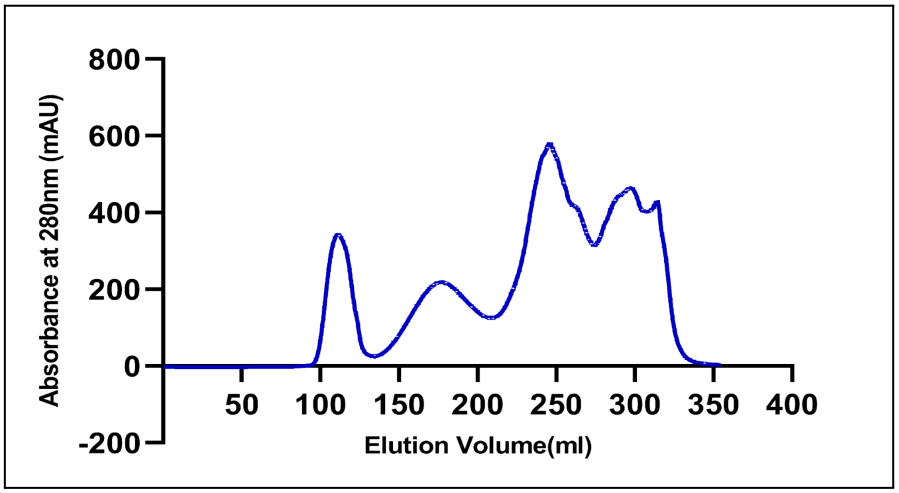


(c)

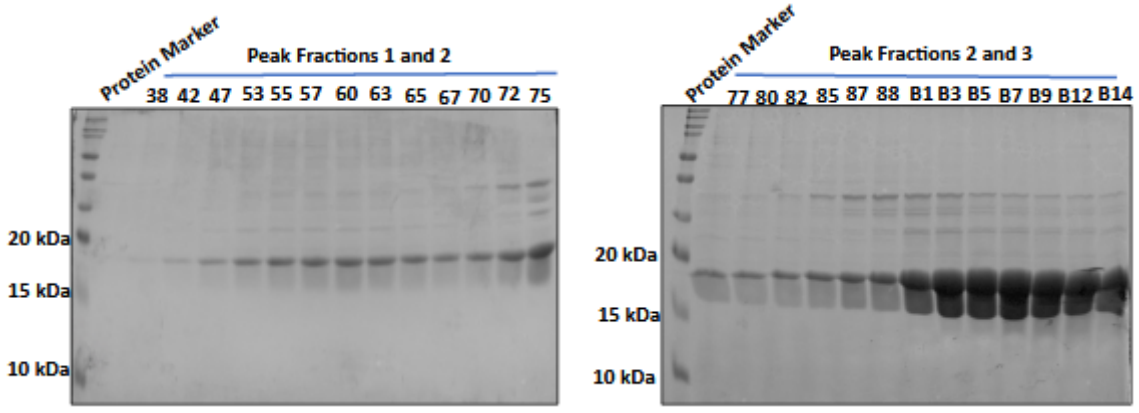


(d)

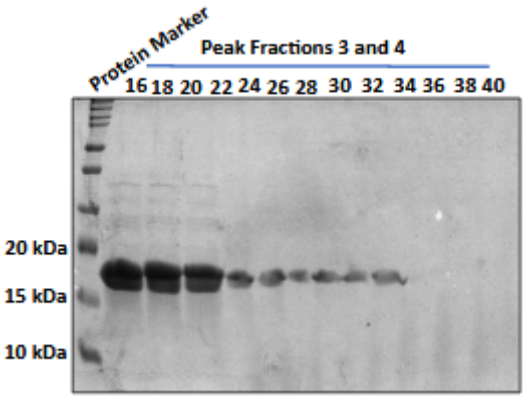
Figure 7: Size-exclusion chromatographic purification of *At*H2B. (a) shows the purification profile for *At*H2B by SEC using a Superdex 26//600 prep grade column. **(b), (c)** and **(d)** show images for the 18% SDS-PAGE analysis of protein fractions corresponding to the elution peaks. **(c)** and **(d)** shows the stretch of fractions containing H2B.



(a)

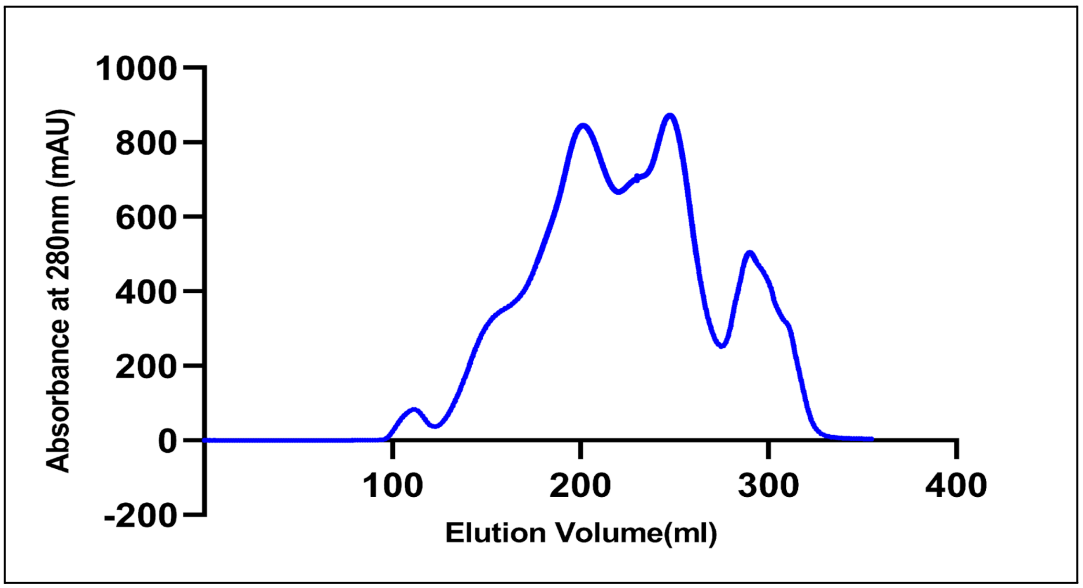


(b) **(c)**

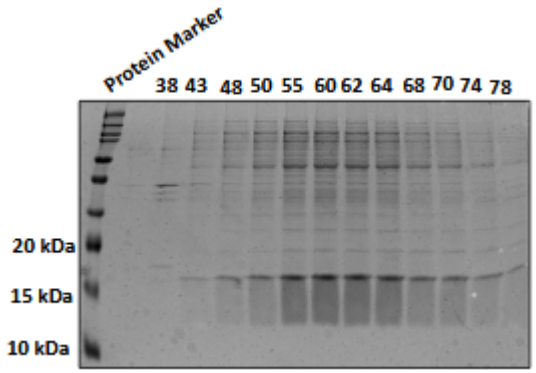


(d)

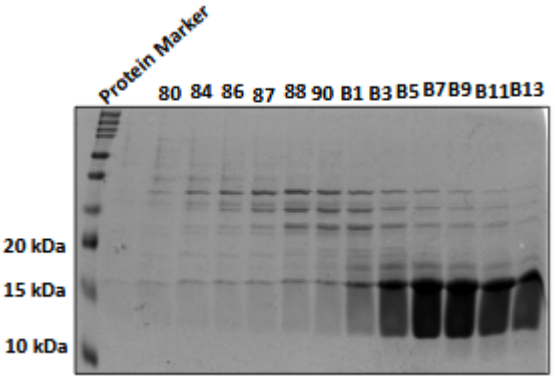
Figure 8: Size-exclusion chromatographic purification of *At*H3. (a) shows the purification profile for *At*H2B by SEC using a Superdex 26//600 prep grade column. **(b), (c)** and **(d)** show images for the 18% SDS-PAGE analysis of protein fractions corresponding to the elution peaks. **(c)** and **(d)** shows the stretch of fractions containing more H3.



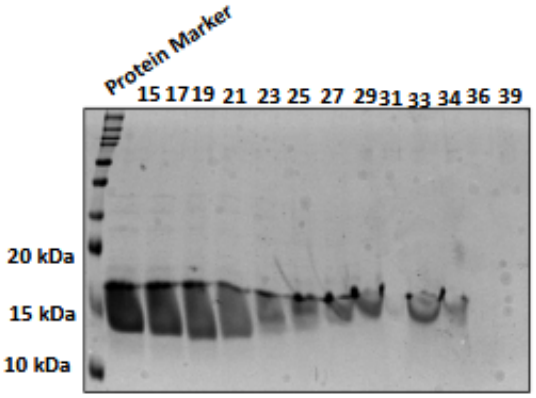
(a)



(b)

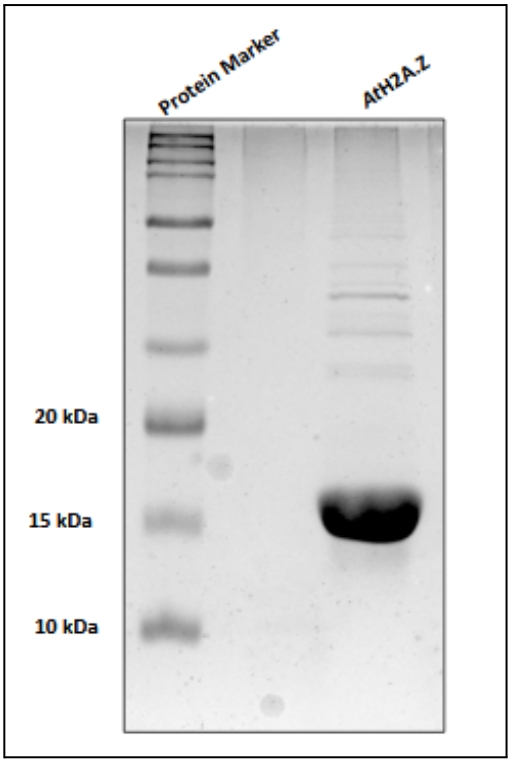


(c)

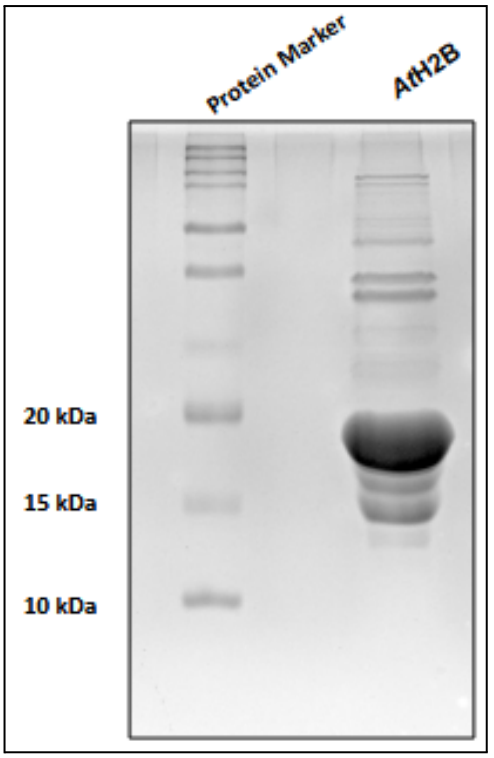


(d)

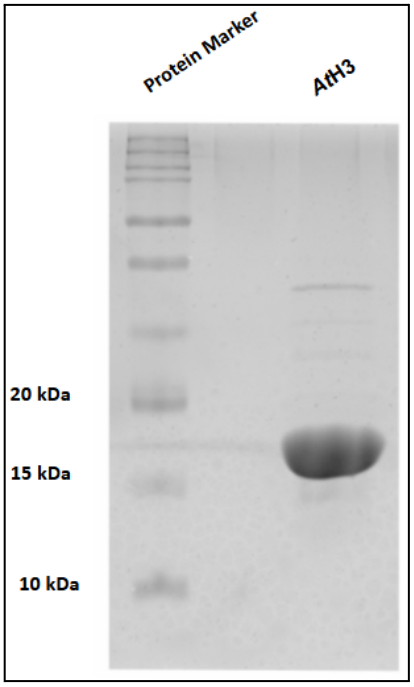
Figure 9: Size-exclusion chromatographic purification of *At*H4. (a) shows the purification profile for *At*H2B by SEC using a Superdex 26//600 prep grade column. **(b), (c)** and **(d)** show images for the 18% SDS-PAGE analysis of protein fractions corresponding to the elution peaks. **(c)** and **(d)** shows the stretch of fractions containing more H4.


Protein Marker
AtH2A.Z
20 kDa
15 kDa
10 kDa

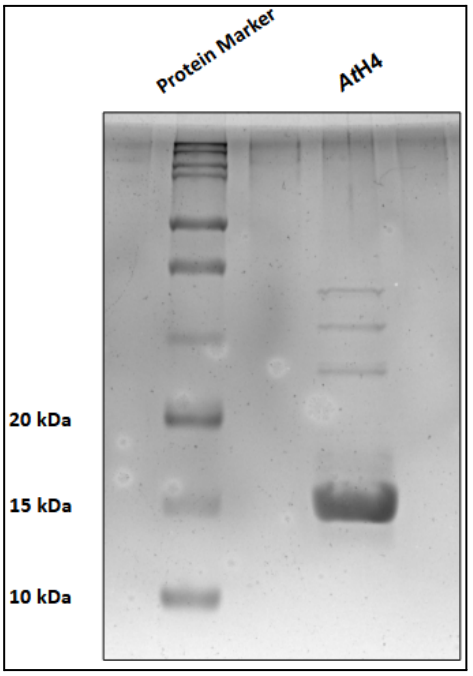
(a)


Protein Marker
AtH2B
20 kDa
15 kDa
10 kDa

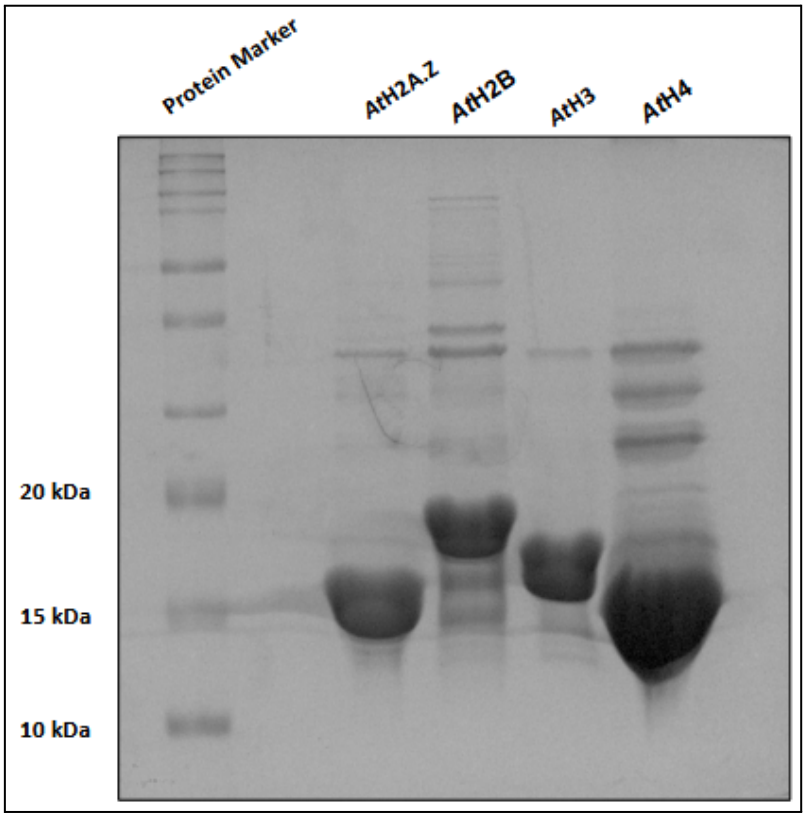
(b)


Protein Marker
AtH3
20 kDa
15 kDa
10 kDa

(c)


Protein Marker
AtH4
20 kDa
15 kDa
10 kDa

(d)



(e)

Figure 10: 18% SDS-PAGE analysis of lyophilized histones aliquoted from dialyzed samples. images (**a**), (**b**), (**c**) and (**d**) show the discrete samples of *At*H2A.Z, *At*H2B, *At*H3, *At*H4, respectively. (e) shows the comparative analysis of the purified histones together.

Refolding of Histone Octamer containing H2A.Z

The lyophilized histone aliquots were dissolved individually in Histone Octamer Unfolding buffer, in the presence of DTT. The histones were combined in near equimolar stoichiometry to the limiting histone concentration and then triple dialyzed against the refolding buffer with BME in a 4-hour, 5-hour and overnight cycle with each buffer change. The dialyzed samples were centrifuged to remove any precipitates and then concentrated by centrifugation in a 15 ml Amicon concentrator in refolding buffer. The OD_{276} reading for the histones was taken, and the concentration was estimated, before taking for purification by SEC.

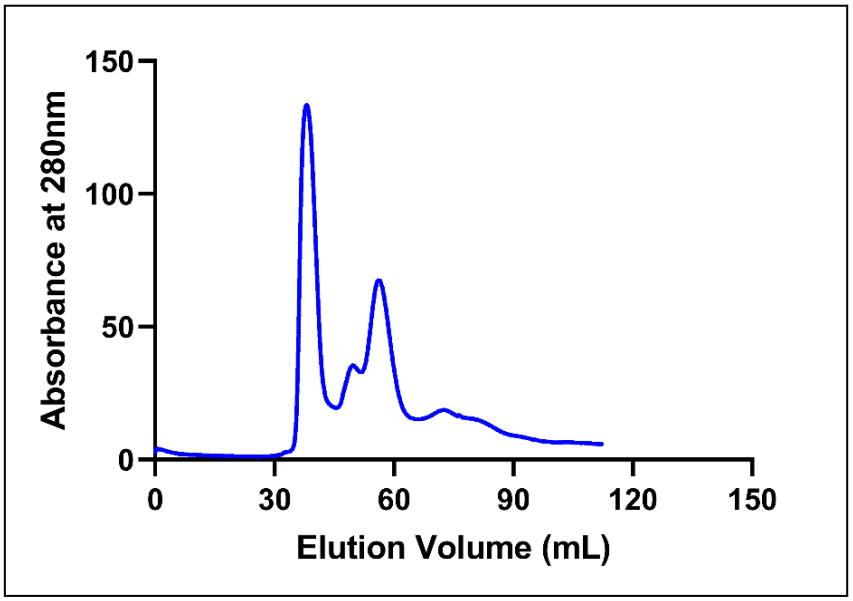


Figure 11: Size-exclusion chromatographic purification of Histone Octamer containing *At*H2A.Z. The chromatogram shows very steep peaks indicating presence of our proteins of interest.

The double-centrifuged, concentrated octamer sample was moved through a 16//600 Sephacryl column in an FPLC apparatus in the Refolding buffer. 18% SDS-PAGE analysis was performed on the fractions corresponding to the peaks to verify octamer formation, indicated by the presence of all 4 proteins H2A.Z, H2B, H3 and H4. The fractions, thereby having a high concentration of pure octamer, were collected for NCP reconstitution.

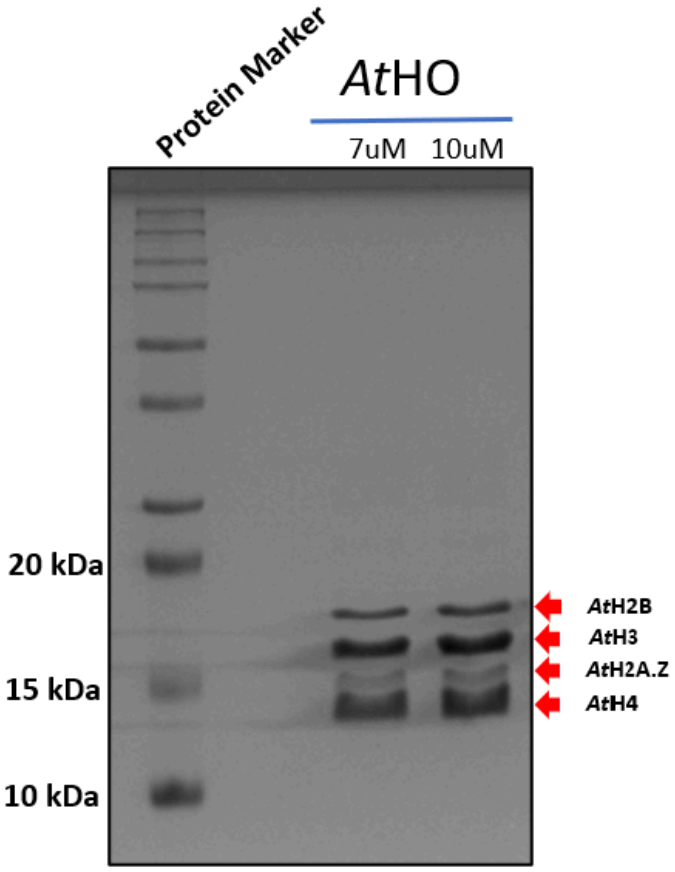


Figure 12 : 18% SDS PAGE analysis for purified histone octamer containing *At*H2A.Z. The sample for SDS-PAGE was from the pooled fraction from the above chromatogram in Figure 11.

Preparation and purification of 145bp Widom 601 DNA

E. coli DH5∝ cells were transformed with pUC57 145bp Widom 601 DNA sequence plasmid containing 8 repeats of the sequence with internal EcoRV sites. The colonies were grown in primary culture into 1 L of secondary culture for plasmid isolation.

The plasmids were isolated from the homogenized cultures via repeated alkaline lysis and high-speed centrifugation. EcoRV digestion was performed overnight to isolate the 145 bp fragments. This was followed by a 6% Native PAGE assay to verify the fragment's presence.

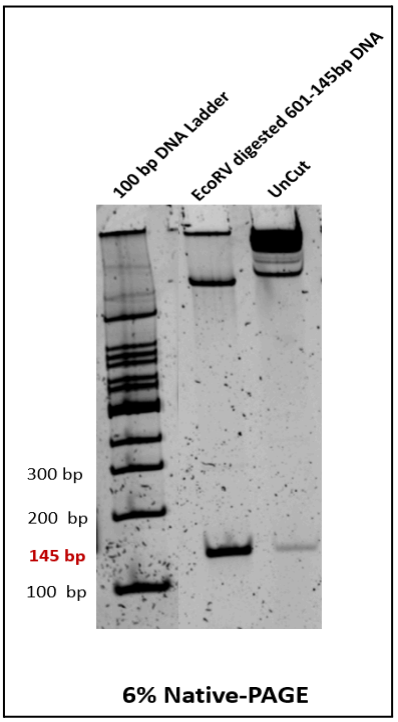


Figure 13: 6% Native PAGE analysis for the EcoRV digestion experiment. The image shows a lane with uncut plasmid and the other with EcoRV digestion showing the 145 bp DNA released from the vector backbone.

Removal of the vector backbone to extract the fragments was done by PEG fractionation. Then a 6% Native PAGE assay was performed to verify the segregation of fragments in the aqueous phase and vector backbone in the pellet after the high-speed centrifugation. This aqueous phase showed negligible vector backbone contamination.

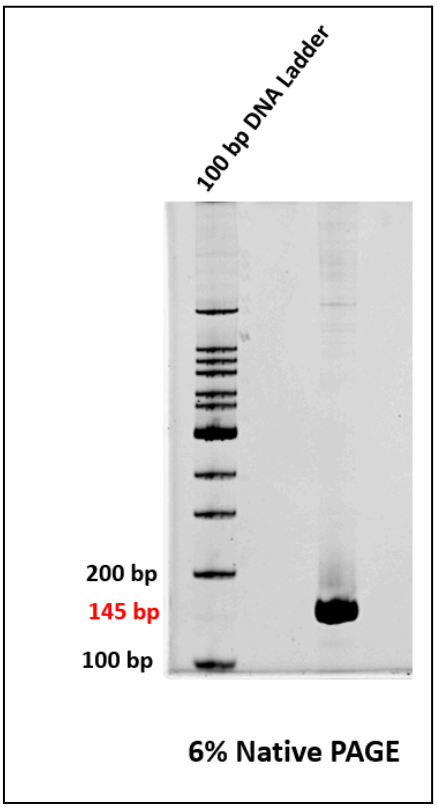


Figure 14: 6% Native PAGE analysis to check the purity of the 145 bp DNA after PEG fractionation. The 145 bp DNA preparation appeared pure.

Reconstitution and Analysis of *Arabidopsis thaliana* Nucleosome Core Particles containing H2A.Z

Ideally, the reconstitution of the NCP happens upon combining an equimolar stoichiometry of Histone Octamer to DNA, though this varied slightly in execution due to difficulty in getting the exact concentration of the octamer. Hence, varying amounts of histone octamer were added to DNA, being the limiting factor, with very slight variations in amounts for optimization of stoichiometry. The DNA was combined with the octamer in ratios of 1:1.6, 1:2 and 1: 2.2. The reconstitution was carried out as described earlier. The quality of the reconstituted NCP was verified using a 6% Native PAGE assay.

Along with the nucleosome, there was a significant formation of entities migrating slower, visible as bands in the Native PAGE analysis, indicating the requirement of further optimization for obtaining NCP without additional components.

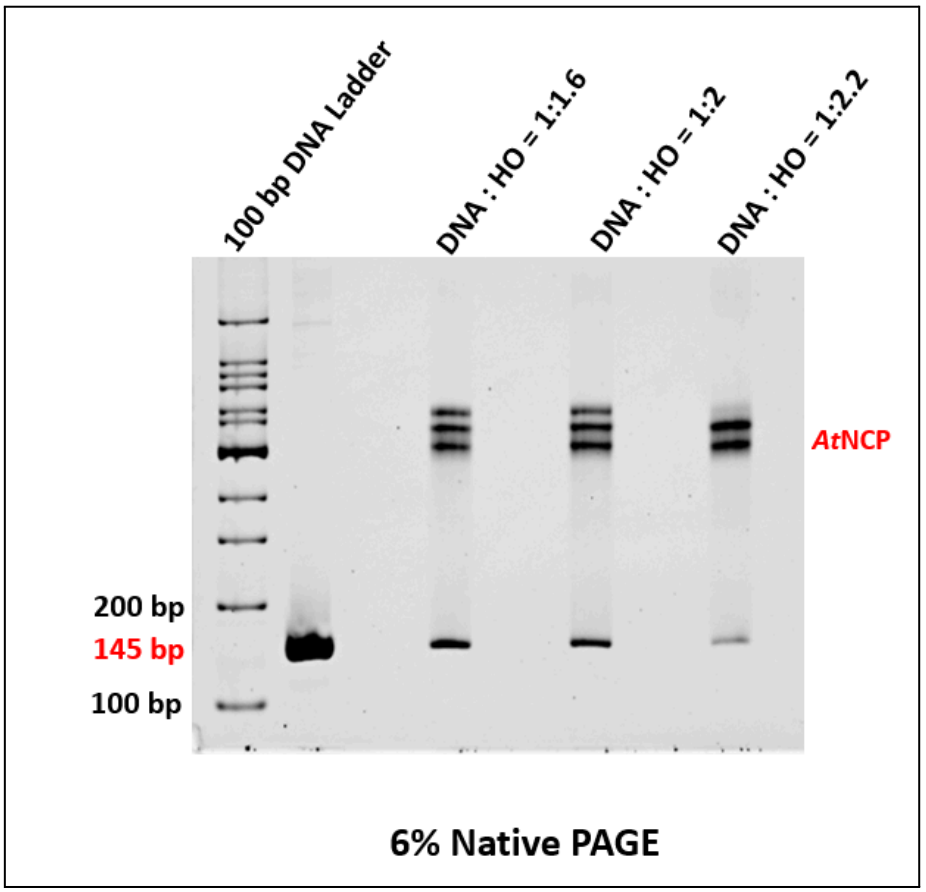


Figure 15: 6% Native PAGE analysis for the reconstituted NCP. The DNA to HO ratio used for the mixtures is mentioned above the wells.

Discussion

Nucleosome Core Particles are fundamental repeating structures making up the chromatin, thereby being an integral part of the genetic code. Histone variants replacing or working in conjunction with their canonical counterparts alter the histone octamer core, which fundamentally alters the structural integrity, accessibility, stability and functional features of the nucleosome core particle, thereby making it very dynamic and active for cellular events. Preparation and characterization of these NCPs by reconstituting them *in vitro* using recombinant techniques can assist in understanding the functional dynamics and specialized nuances borne out of histone variant incorporation.

While H2A.Z is a histone variant that has been known for decades and *Arabidopsis thaliana* is a very well-studied model plant, the studies intermingling both are scarcer

than optimal. Reconstitution of NCPs containing *At*H2AZ.8 was an attempt to mitigate this and had a few challenges.

Histones were expressed in small testing scales and, upon satisfactory expression, scaled up in multiple bacterial expression systems, depending on the need. The bulk of the project was spent on careful expression and purification of these raw materials, whereas the steps involving refolding of the histone octamer and reconstitution of NCP are less time-consuming, albeit needing much optimization to hit the right ratios to get homogeneous complexes.

Reconstitution of the NCP was performed at 145 bp DNA to histone octamer ratios of 1:1.6, 1:2 and 1:2.2. The ratios were calculated based on the estimated concentrations of DNA and histone octamer. These 'off' ratios had to be done because the histone octamer concentration estimations were never accurate. Plus, the 145 bp DNA had very minimal contamination with the vector backbone after PEG fractionation. Ideally, if the DNA and octamer concentrations were estimated correctly, a 1:1 ratio would work perfectly.

The wells from the native PAGE analysis after reconstitution showed additional bands migrating slower than the expected size on the reconstituted *At*NCP containing *At*H2A.Z.8 (~500 bp), indicating the presence of other possible entities where more than one octamer could be bound to the 145 DNA. This could happen if the histone octamer used were in excess. In such a case, optimizing the reconstitution ratio for DNA and histone octamer should give pure NCP.

Another possibility is that some percentage of the prepared histone octamer would have lost a histone dimer (during or prior to reconstitution), and the reconstituted material would not be a full NCP and might be hexasomes lacking one H2A.Z-H2B dimer and bound to 145 bp DNA in different conformations. If that is the case, the histone octamer will have to be prepared freshly and in pure form before proceeding with NCP reconstitution.

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