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In vitro Characterization of Histone Chaperones Using Analytical, Pull-Down and Chaperoning Assays

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TITLE:

In vitro Characterization of Histone Chaperones Using Analytical, Pull-Down and Chaperoning Assays

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SUMMARY:

This protocol describes a battery of methods that includes analytical size-exclusion chromatography to study histone chaperone oligomerization and stability, pull-down assay to unravel histone chaperone-histone interactions, AUC to analyze the stoichiometry of the protein complexes, and histone chaperoning assay to functionally characterize a putative histone chaperone *in vitro*.

ABSTRACT:
Histone proteins associate with DNA to form the eukaryotic chromatin. The basic unit of chromatin is a nucleosome, made up of a histone octamer consisting of two copies of the core histones H2A, H2B, H3, and H4, wrapped around by the DNA. The octamer is composed of two copies of an H2A/H2B dimer and a single copy of an H3/H4 tetramer. The highly charged core histones are prone to non-specific interactions with several proteins in the cellular cytoplasm and the nucleus. Histone chaperones form a diverse class of proteins that shuttle histones from the cytoplasm into the nucleus and aid their deposition onto the DNA, thus assisting the nucleosome assembly process. Some histone chaperones are specific for either H2A/H2B or H3/H4, and some function as chaperones for both. This protocol describes how *in vitro* laboratory techniques such as pull-down assays, analytical size-exclusion chromatography, analytical ultra-centrifugation, and histone chaperoning assay could be used in tandem to confirm whether a given protein is functional as a histone chaperone.

INTRODUCTION:
Nucleosomes composed of DNA and histone proteins form the structural unit of chromatin and regulate several critical cellular events. Nucleosomes are dynamically repositioned and remodeled to make DNA accessible to various processes such as replication, transcription, and translation[1,2]. Histones that are highly basic either tend to interact with acidic proteins in the cellular milieu or undergo aggregation, thus leading to various cellular defects[3-5]. A group of dedicated proteins called histone chaperones aid the transport of histones from the cytoplasm to the nucleus and prevent aberrant histone-DNA aggregation events[6,7]. Fundamentally, most histone chaperones store and transfer histones onto DNA at physiological ionic strength, thereby aiding the formation of nucleosomes[8,9]. Some histone chaperones have a definite preference for the histone oligomers H2A/H2B or H3/H4[10].

Histone chaperones are characterized based on their ability to assemble nucleosomes dependent or independent of DNA synthesis[11]. For example, chromatin assembly factor-1 (CAF-1) is dependent while histone regulator A (HIRA) is independent of DNA synthesis[12,13]. Similarly, the nucleoplasmin family of histone chaperones is involved in sperm chromatin decondensation and nucleosome assembly[14]. The nucleosome assembly protein (NAP) family members facilitate the formation of nucleosome-like structures *in vitro* and are involved in the shuttling of histones between cytoplasm and nucleus[15]. Nucleoplasmins and NAP family proteins are both functional histone chaperones but do not share any structural features. Essentially, no single structural feature allows the classification of a protein as a histone chaperone[16]. The usage of functional and biophysical assays along with structural studies work best in characterizing histone chaperones.

This work describes biochemical and biophysical methods to characterize a protein as a histone chaperone that aids nucleosome assembly. First, analytical size-exclusion chromatography was carried out to analyze the oligomeric status and stability of histone chaperones. Next, a pull-down assay was performed to determine the driving forces and the competitive nature of histone chaperone-histone interactions. However, the hydrodynamic parameters of these interactions could not be accurately calculated using analytical size-exclusion chromatography because of the

protein's shape and its complexes that impact their migration through the column. Therefore, analytical ultracentrifugation was used, which provides in-solution macromolecular properties that include accurate molecular weight, the stoichiometry of interaction, and the shape of the biological molecules. Past studies have extensively used *in vitro* histone chaperoning assay to functionally characterize histone chaperones such as ySc S116[17], DmACF[18], ScRTT106p[19], HsNPM1[20]. Histone chaperoning assays were also used to functionally characterize the proteins as histone chaperones.

PROTOCOL:

1. Analytical size-exclusion chromatography to elucidate the oligomeric status and stability of histone chaperones

1.1. Analysis of the oligomeric status of histone chaperones

1.1.1. Equilibrate a 24 mL analytical size-exclusion chromatography (SEC) column with 1.2 column volume (CV), i.e., 28.8 mL of degassed SEC buffer [20 mM of Tris-HCl (pH 7.5), 300 of mM NaCl, and 1 mM of β-mercaptoethanol (β-ME)] at 4 °C (see **Table of Materials**).

NOTE: Column type, buffer composition, and buffer pH may be selected based on the protein of interest. The sample injection volume should not exceed 500 µL for a 24 mL column. Also, the column pressure needs to be maintained below 5 MPa.

1.1.2. From a higher concentration protein stock solution, prepare 500 µL of 0.5 mg/mL protein sample in degassed SEC buffer and inject it into the pre-equilibrated column using a 500 µL injection loop. Allow the chromatography run to proceed at an isocratic flow rate of 0.2-0.3 mL/min with the SEC buffer at 4 °C.

1.1.3. Monitor the elution profile of the protein by measuring absorbance at a wavelength of 280 nm. When dealing with proteins lacking aromatic residues, measure the absorbance at 214 nm.

1.1.4. Use the elution volume of the protein to calculate its approximate molecular weight in kDa using the standard calibration curve[21].

NOTE: Calibration curve is prepared by plotting the retention volume of known molecular weight proteins against the log of their respective molecular weights (log Mr), eluted using the same column.

1.2. Analysis of the thermal stability of the histone chaperones

1.2.1. Take 500 µL of 0.5 mg/mL of the protein sample prepared in degassed SEC buffer (same as used in 1.1.1) in individual microcentrifuge tubes and heat each tube to a particular

temperature ranging between 20 °C and 90 °C (20 °C, 40 °C, 60 °C, and 90 °C) for 10 min in a water bath.

1.2.2. Subsequently, centrifuge the heat-treated samples at 16,200 x *g* for 10 min at 4 °C, collect the supernatant with a micropipette, and inject each sample individually using a 500 µL injection loop into the analytical column, pre-equilibrated with the SEC buffer at 4 °C.

1.2.3. Allow the chromatography run to proceed at an isocratic flow rate of 0.2-0.3 mL/min with the SEC buffer at 4 °C.

1.2.4. Observe the position and height of the elution peaks and look for the appearance of additional peaks for the different samples.

1.3. Analysis of the chemical stability of the histone chaperones

1.3.1. To examine the salt stability of histone chaperones, incubate 500 µL of 0.5 mg/mL protein sample prepared in a Tris buffer [20 mM of Tris-HCl (pH 7.5), and 1 mM of β-ME] supplemented with increasing concentrations of NaCl (300 mM, 600 mM, 1 M, 1.5 M, and 2 M) in separate microcentrifuge tubes for 30 min at 4 °C. Centrifuge the samples at 16,200 x *g* for 10 min at 4 °C and retain the supernatant.

1.3.2. Next, load the protein samples in different NaCl concentrations individually, using a 500 µL injection loop into the analytical column pre-equilibrated with 1.2 CV (28.8 mL) of the respective buffer containing increasing NaCl concentrations at 4 °C.

1.3.3. Allow the chromatography run to proceed at an isocratic flow rate of 0.2-0.3 mL/min with 1 CV (24 mL) of the respective buffer at 4 °C.

1.3.4. Observe the position and height of elution peaks and look for the appearance of additional peaks for the different samples.

1.3.5. Similarly, for urea stability analysis, incubate 500 µL of 0.5 mg/mL protein sample prepared in a Tris buffer supplemented with increasing urea concentrations (1 M, 2 M, 3 M, 4 M, and 5 M) in separate microcentrifuge tubes for 16 h at room temperature. Centrifuge the samples at 16,200 x *g* for 10 min at room temperature and retain the supernatant.

1.3.6. Next, load the urea-treated protein samples individually using a 500 µL injection loop into the analytical column pre-equilibrated with 1.2 CV (28.8 mL) of the corresponding buffer containing different urea concentrations at room temperature.

1.3.7. Allow the chromatography run to proceed at an isocratic flow rate of 0.2-0.3 mL/min with 1 CV (24 mL) of the respective buffer at room temperature.

CAUTION: Do not perform the experiments with buffer containing urea at a lower temperature as urea tends to crystallize and damage the column.

1.3.8. Observe the position and height of the elution peaks and look for the appearance of additional peaks for the different samples.

2. Salt gradient-based pull-down assays to understand the type of interactions contributing to the complex formation between histone oligomers and a histone chaperone

2.1. For each reaction of pull-down assay, pipette 40 µL of Ni-NTA resin into a spin column and wash with sterile double-distilled water. Subsequently, equilibrate the resin with 100 CV (4 mL) of equilibration buffer [20 mM of Tris-HCl (pH 7.5), 300 mM of NaCl, 10 mM of imidazole, 10 µg/mL of BSA, and 1 mM of β-ME] (see **Table of Materials**).

NOTE: Pull-down can also be performed in a 1.5 mL microcentrifuge tube.

2.2. Prepare the sample by mixing 5 µM of the His-tagged histone chaperone with either 20 µM of histone H2A/H2B dimer or H3/H4 tetramer in the equilibration buffer. Incubate the sample on ice for 1 h.

NOTE: H2A/H2B dimer and H3/H4 tetramer are prepared from recombinant human histones[21], and the integrity of the oligomers is confirmed based on the estimated molecular masses by analytical ultracentrifugation (AUC). The same histone oligomers have been used for all experiments mentioned below.

2.3. Load the samples into separate pre-equilibrated spin columns with Ni-NTA resin from step 2.1, each labeled for a particular salt concentration, and keep the columns for 30 min at 4 °C. Centrifuge the columns at 1000 x *g* for 1 min.

2.4. Next, wash the columns with 100 CV (4 mL) of wash buffer [20 mM of Tris-HCl (pH 7.5), 50 mM of imidazole, 0.2% of Tween-20, and 1 mM of β-ME] containing different salt concentrations (i.e., 300 mM, 400 mM, 500 mM, 600 mM, 800 mM, and 1 M NaCl). Wash each column with a buffer having a particular salt concentration.

2.5. After the salt washing step, elute the protein from the different columns using 100 µL of elution buffer [20 mM of Tris-HCl (pH 7.5), 300 mM of NaCl, 300 mM of imidazole, and 1 mM of β-ME].

2.6. Subsequently, subject the eluted samples to 18% SDS-PAGE[22] and visualize the gel after staining with Coomassie Brilliant Blue R250 (see **Table of Materials**). Alternatively, you may directly load the resin onto the SDS-PAGE gel instead of eluting the bound protein from the Ni-NTA resin.

NOTE: Equilibration, wash, and elution buffer compositions and pH may be modified depending upon the protein of interest.

3. Competitive pull-down assay to identify the preference of a histone chaperone for H2A/H2B or H3/H4

3.1. Prepare spin column as described in step 2.1

3.2. Incubate 5 µM of the histone chaperone with 20 µM of H2A/H2B dimer in 300 µL of equilibration buffer (prepared in step 2.1) for 30 min on ice.

NOTE: The ratio of histone oligomer to histone chaperone in the reaction can be chosen based on known binding stoichiometry data; use five-fold excess histone if no information is available.

3.3. Centrifuge the histone chaperone-H2A/H2B complex at 16,200 x *g* for 5 min at 4 °C to remove any residue. Next, load the sample on the spin column pre-equilibrated with equilibration buffer (prepared in step 2.1) and incubate for 30 min at 4 °C.

3.4. Wash the column with 100 CV (4 mL) of wash buffer [20 mM of Tris-HCl (pH 7.5), 300 mM of NaCl, 50 mM of imidazole, 0.2% of Tween-20, and 1 mM of β-ME] to remove excess H2A/H2B dimer. Next, mix the histone chaperone-H2A/H2B complex with 20-60 µM of H3/H4 tetramer and incubate for 30 min on ice.

3.5. Rewash the column with 100 CV (4 mL) of wash buffer (prepared in step 3.4) to remove any unbound H3/H4 tetramer and elute the sample using elution buffer (prepared in step 2.5). Subject the eluted samples to 18% SDS-PAGE and visualize after staining with Coomassie Brilliant Blue R250.

NOTE: The assay could be reversed wherein, first, H3/H4 tetramer can be mixed with the chaperone, the complex allowed to bind to Ni-NTA beads, and the complex then be incubated with varying concentrations of H2A/H2B dimer.

4. Analytical ultracentrifugation - sedimentation velocity (AUC-SV) experiments to analyze the binding stoichiometry between histone chaperones and histones

4.1. Sample preparation for AUC

4.1.1. Dialyze the reconstituted histone H2A/H2B dimer, H3/H4 tetramer, and the histone chaperone separately through a 7 kDa cut-off dialysis tubing[23], against a dialysis buffer [20 mM of Tris (pH 7.5), 300 mM of NaCl, and 1 mM of β-ME] (see **Table of Materials**). To minimize background error due to buffer mismatch, perform dialysis extensively against the dialysis buffer, preferably three times over a period of 24 h.

NOTE: The initial OD_{280} of the protein samples should have a two to a three-fold higher value to achieve a final OD_{280} of 0.3-0.5. This is essentially done to nullify the effects of dilution.

4.1.2. Purify H2A/H2B dimer, H3/H4 tetramer, and the histone chaperone individually with the dialysis buffer, using analytical size-exclusion chromatography (as mentioned in step 1). Save the buffer from the run to prepare further dilutions later on and to use as a reference in the AUC cell.

4.2. Sample loading for AUC

4.2.1. Mix the purified proteins in a final volume of 450 µL using dialysis buffer from step 4.1.1 to reach an OD_{280} of 0.3-0.6. Mix the histone chaperone with H2A/H2B dimer or H3/H4 tetramer for complex formation in separate reaction tubes. Incubate the protein mixtures for 2-3 h.

NOTE: Alternatively, sedimentation data can be acquired with an interference optical scanning system in the analytical ultracentrifuge. Separately, for mixing purified proteins, fix the histone chaperone concentration and incubate it with increasing concentrations of the histone oligomers to obtain the exact stoichiometry.

4.2.2. Assemble the cell with a double sector centerpiece and quartz windows for the AUC-SV experiment using an absorbance detector of the analytical ultracentrifuge as described previously in detail[24].

4.2.3. Fill 400 µL of the sample solution and 420 µL of dialysis buffer into the two sectors (sample and reference sectors, respectively) of the cell.

NOTE: A larger volume of buffer is used in the reference sector to keep the reference meniscus above the meniscus of the sample. However, while using an optical interference system, fill the two sectors with equal volume.

4.2.4. Weigh and accurately balance the cells and load them into a four-place titanium rotor (see **Table of Materials**). Align the cells using the marks provided at the bottom of the cells and the rotor. Load the rotor in the centrifuge, close the lid and allow to develop a vacuum until the pressure drops to less than 15 microns of Hg. The rotor temperature stabilizes to 20 °C (usually takes 2-2.5 h).

NOTE: AUC operating parameters include experimental temperature, rotor speed, the interval between scans, and the number of scans to be collected. In the case of SV experiments, the scan interval is given according to the protein's molecular mass; smaller proteins require larger time intervals between the scans. The rotor speed is also set according to the protein's expected molecular mass, and the experiment is conducted at 20 °C. The absorbance data is monitored at 280 nm.

4.2.5. To obtain the exact stoichiometry, keep the histone chaperone concentration constant and incubate with increasing concentrations of histone oligomers to attain saturation.

4.3. AUC data analysis

4.3.1. Perform the data analysis as previously described[25]. Briefly, calculate the density and viscosity for the buffer components using the program SEDNTERP[26] (see **Table of Materials**). Similarly, calculate the partial specific volume of the protein based on its amino acid composition, also using SEDNTERP.

4.3.2. Load the data from the AUC machine into the program SEDFIT[27] and define the meniscus (red line), the cell bottom (blue line), and data analysis boundaries (green lines). Choose continuous C(s) distribution as a model.

4.3.3. Next, set resolution maximum up to 100; set sedimentation coefficient (s), s min: 0 and s max: 10-15; set frictional ratio to 1.2 initially and opt to float to derive the ratio from the data; set confidence level (F-ratio; which determines the magnitude of regularization) to 0.68 for 1 sigma regularization; set partial specific volume, buffer density and buffer viscosity values as obtained from SEDNTERP.

4.3.4. Press **RUN** to allow the software to solve the Lamm equation[27]. Adjust the parameters if there is a significant data mismatch. After adjusting the parameters, press **FIT** to refine all parameters. Assess the quality of fit based on the root-mean-square deviation (RMSD) value, which should be less than 0.01 signal units.

4.3.5. Estimate the molecular masses of the peaks by choosing the option: show peak "Mw in c(s)" in the display function of the main toolbar, which will provide information about the 's' of the molecule/complex.

5. Plasmid supercoiling assay to confirm histone chaperoning function

5.1. Nucleosome assembly reaction

5.1.1. Mix 2 µM of H3/H4 tetramer and 4 µM of H2A/H2B dimer with increasing concentrations of the histone chaperone (1-6 µM) in an assembly buffer [20 mM of Tris HCl (pH 7.5), 1 mM of DTT, 1 mM of $MgCl_2$, 0.1 mg/mL of BSA, and 100 mM of NaCl] to a final volume of 50 µL. Incubate the mixture at 4 °C for 30 min.

5.1.2. Simultaneously, in a separate reaction, pretreat 500 ng of the negatively supercoiled pUC19 plasmid with 1 µg of topoisomerase I enzyme (see **Table of Materials**) in the assembly buffer in a final volume of 50 µL and incubate at 30 °C for 30 min.

NOTE: Topoisomerase I relaxes the supercoiled double-stranded plasmid DNA by generating a single-stranded nick. A topoisomerase I enzyme of eukaryotic origin, such as the commercially available wheat germ topoisomerase I or recombinantly expressed *Drosophila melanogaster* topoisomerase I, could be used.

5.1.3. Next, combine the H3/H4 tetramer, H2A/H2B dimer, histone chaperone mixture (from step 5.1), the relaxed plasmid DNA reaction mixture (from step 5.2), and incubate further at 30 °C for 90 min.

NOTE: Set up two control reactions for the assay; one having the histone chaperone and the relaxed plasmid DNA (but not the histones) and the other having the histone oligomers and the relaxed plasmid DNA (but not the histone chaperone).

5.1.4. Stop the assembly reaction by adding 100 µL of 2x stop buffer (40 mM of EDTA, 2% of SDS, and 0.4 mg/mL of proteinase K) and incubate at 37 °C for 30 min.

NOTE: Stop buffer deproteinizes the plasmid DNA by denaturation and proteolysis of bound histones.

5.2. Phenol-chloroform extraction and ethanol precipitation

5.2.1. Add an equal volume of Tris-saturated phenol in the tube containing the reaction mixture from step 5.1.4 and mix well, followed by centrifugation at 16,200 x *g* for 10 min at room temperature.

5.2.2. Gently collect the upper aqueous phase having the plasmid DNA with a micropipette and mix with an equal volume of chloroform. Vortex the mixture and centrifuge at 16,200 x *g* for 10 min at room temperature.

NOTE: Isoamyl alcohol could be included at this step to avoid a fuzzy interface between the aqueous and organic phases.

5.2.3. Next, collect the upper aqueous phase, add 1/10th volume of 3 M sodium acetate (pH 5.5) and 2.5 volumes of ice-cold ethanol (see **Table of Materials**). Mix the solution well by inverting the tube 3-4 times and keep the mixture in a -20 °C freezer for 30 min for complete precipitation of the plasmid DNA.

5.2.4. Centrifuge the sample from step 5.2.3 at 16200 x *g* for 10 min and gently discard the supernatant. Keep the tubes open at room temperature until even trace amounts of ethanol evaporate, leaving the precipitated plasmid DNA in the tube.

5.3. Perform the agarose gel electrophoresis to observe the plasmid supercoiling effect.

5.3.1. Dissolve the precipitated plasmid DNA from step 5.2.4 in sterile double-distilled water.

5.3.2. Resolve the samples on a 1% agarose gel in 1x Tris-acetate-EDTA (TAE) buffer (40 mM of Tris, 20 mM of acetic acid, and 1 mM of EDTA) (see **Table of Materials**).

5.3.3. Stain the gel with 0.2–0.5 µg/mL concentration of ethidium bromide and observe under UV to visualize the DNA bands on the gel.

REPRESENTATIVE RESULTS:

The recombinant N-terminal nucleoplasmin domain of the protein FKBP53 from *Arabidopsis thaliana* was subjected to analytical SEC. The elution peak volume was plotted against the standard curve to identify its oligomeric state. The analytical SEC results revealed that the domain exists as a pentamer in solution, with an approximate molecular mass of 65 kDa (**Figure 1A,B**). Further, the nucleoplasmin domain was analyzed for thermal and chemical stability in conjunction with analytical SEC. The nucleoplasmin sample subjected to heat-treatment up to 90 °C displayed no apparent shift in the elution volume and the peak height compared to the samples maintained at 20 °C, suggesting that the domain is highly thermostable (**Figure 1C**). Likewise, the nucleoplasmin domain displayed salt stability up to 2 M of NaCl (**Figure 1D**) and urea stability up to 4 M (**Figure 1E**). However, the nucleoplasmin pentamer started dissociating in higher urea concentrations.

A pull-down assay was performed to determine the type of interactions contributing to the complex formation between the histone chaperone (nucleoplasmin domain of AtFKBP53) and the histone oligomers H2A/H2B dimer and H3/H4 tetramer using a gradient salt wash. The interaction of the nucleoplasmin domain with H2A/H2B dimer was stable up to a salt concentration of 0.4 M NaCl (**Figure 2A**). In comparison, the association with H3/H4 was reasonably stable up to 0.7 M NaCl (**Figure 2B**). The ability of the chaperone-histone complexes to withstand high salt concentration suggests the role of hydrophobic interactions in stabilizing the complexes. The chaperone complex with H3/H4 being stable even in high salt concentrations offers a predominant role of hydrophobic interactions in the complex formation. The lower stability of the H2A/H2B-chaperone complex in high salt concentrations reveals a significant role for electrostatic interactions in the complex formation. In another experiment, the pull-down assay was used to examine whether the chaperone prefers either H2A/H2B dimer or H3/H4 tetramer. The results revealed that the chaperone binds to H2A/H2B dimer and H3/H4 tetramer simultaneously and irrespective of the order in which they are added to the chaperone (**Figure 2C,D)**. This indicated that the chaperone possesses separate sites for its interaction with the histone oligomers.

AUC-SV experiments (**Figure 3**) were performed to study the stoichiometry of interaction between histone oligomers and chaperones. AUC-SV data analysis provided a sedimentation coefficient (s) value of 5.4 S for the AtFKBP53 nucleoplasmin domain in complex with H2A/H2B that corresponded to a molecular mass of 104 kDa. The complex of the nucleoplasmin domain with H3/H4 gave a sedimentation coefficient value of 7.35 S, corresponding to 129 kDa. The estimated molecular mass of the complexes reveals that the pentameric nucleoplasmin forms complex with both H2A/H2B dimer and H3/H4 tetramer in a 1:1 stoichiometry.

It is essential to show that the protein can deposit histone oligomers onto DNA to confirm that it is a histone chaperone. Towards this end, a plasmid supercoiling assay was adopted (**Figure 4**). The relaxed circular plasmid was incubated with the histone oligomers H2A/H2B and H3/H4 with

the recombinant plant histone chaperones of the NAP family - AtNRP1 and AtNRP2[28]. The presence of the chaperone increased the amount of supercoiled plasmid, suggesting it could deposit histones onto the DNA to form nucleosomes, causing DNA supercoiling.

FIGURE LEGENDS:

Figure 1: Oligomeric state and stability of the nucleoplasmin domain of AtFBP53. (**A**) Analytical size-exclusion chromatography profile of the AtFKBP53 nucleoplasmin domain. (**B**) Calibration curve obtained using globular proteins of known molecular mass. The blue dots represent the molecular mass of the known proteins, whereas the red dot represents the AtFKBP53 nucleoplasmin domain. (440 kDa - ferritin, 158 kDa-aldolase, 75 kDa-con albumin, 44 kDa-ovalbumin, 6.5 kDa-aprotinin). (**C**) Analytical size-exclusion chromatogram of 500 µL of 0.5 mg/mL AtFKBP53 nucleoplasmin domain subjected to heat treatment at different temperatures: 20 °C (green), 40 °C (orange), 60 °C (black), 90 °C (light blue). (**D**) Analytical size-exclusion chromatogram of 500 µL of 0.5 mg/mL AtFKBP53 nucleoplasmin domain in buffers containing different NaCl concentrations: 0.3 M (purple), 0.6 M (red), 1.0 M (light blue), 1.5 M (green), 2.0 M (black). (**E**) Analytical size-exclusion chromatogram of the AtFKBP53 nucleoplasmin domain in buffers with different urea concentrations: 0 M (light blue), 1.0 M (pink), 2.0 M (black), 3.0 M (dark blue), 4.0 M (green), 5.0 M (brown). The nucleoplasmin pentamer shows high stability to thermal and chemical stress conditions. The figure is adapted from Reference[21].

Figure 2: Pull-down assays for the interaction of the nucleoplasmin domain of AtFKBP53 with histone oligomers. 18% SDS-PAGE images of the elution fractions from the assays are presented here. Pull-down assay for **(A)** 20 µM H2A/H2B dimer and **(B)** 20 µM H3/H4 tetramer with 5 µM AtFKBP53 nucleoplasmin domain in increasing concentrations of NaCl in the range of 0.3 M, 0.5 M, 0.6 M, 0.7 M, 0.8 M, 0.9 M, and 1.0 M. 5 µM AtFKBP53 FKBD was used as a negative control here. For the competitive binding experiment, **(C)** a mixture of 5 µM AtFKBP53 nucleoplasmin domain and 20 µM H3/H4 tetramer incubated with a range of 20-60 µM H2A/H2B dimer and **(D)** a mixture of 5 µM AtFKBP53 nucleoplasmin domain and 20 µM H2A/H2B dimer incubated with a range of 20-60 µM H3/H4 tetramer has been used. The label AtFKBP53 corresponds to the nucleoplasmin domain of AtFKBP53. Elution fractions show simultaneous binding of both the histone oligomers to the nucleoplasmin. The figure is adapted from Reference[21].

Figure 3: Analytical ultracentrifugation - sedimentation velocity (AUC-SV) experiment of histone oligomers, the nucleoplasmin domain of AtFKBP53, and their complexes. The AUC distance distribution *vs*. sedimentation coefficient (S) plot. The obtained sedimentation coefficient (s) values and molecular masses are also provided. The label AtFKBP53 corresponds to the nucleoplasmin domain of AtFKBP53. The estimated molecular masses reveal a 1:1 stoichiometry for the AtFKBP53 nucleoplasmin domain with the histone oligomers H2A/H2B dimer and H3/H4 tetramer. 450 µL of all the protein samples having an OD_{280} of 0.3-0.5 were used for the AUC-SV experiments. The figure is adapted from Reference[21].

Figure 4: Plasmid supercoiling assay. Plasmid supercoiling assay for the histone chaperones AtNRP1 and AtNRP2. 500 ng of pUC19 plasmid DNA was pretreated with 1 µg of Topoisomerase

I for the experiment. 4 µM AtNRP1, 4 µM AtNRP2, and a mixture of 4 µM H2A/H2B dimer and 2 µM H3/H4 tetramer were as control that shows no supercoiling activity when incubated with the pretreated pUC19 DNA. The lanes with a mixture of 4 µM H2A/H2B of dimer and 2 µM H3/H4 of tetramer and 4 µM each of AtNRP1 and AtNRP2 show the formation of supercoiled DNA upon incubation with the pretreated pUC19 DNA.

DISCUSSION:

This work demonstrates and validates a comprehensive set of methods for the biochemical and biophysical characterization of a putative histone chaperone. Herein, recombinantly expressed and purified NAP family proteins, AtNRP1 and AtNRP2, and the N-terminal nucleoplasmin domain of AtFKBP53 were used to demonstrate the methods. The same set of experiments could very well be used to delineate the functional attributes of previously uncharacterized histone chaperones from any organism.

The first section of the methods part involves investigating the oligomeric state and stability of a histone chaperone. Several reports indicate that histone chaperones exhibit considerable diversity in their oligomeric state. For example, human CAF-1 exists as a monomer[29]. NAP family members exist as dimer or tetramer[29-31]. Nucleoplasmins reveal pentameric and often decameric oligomeric states[32,33]. An analytical SEC experiment can determine the oligomeric state of a histone chaperone, and AUC-SV experiments can confirm the same. Several of the histone chaperones are known to be highly stable under various thermal and chemical stress conditions[33,34]. The thermal and chemical stability features of histone chaperones could also be explored in conjunction with analytical SEC. Further, circular dichroism spectroscopy could be effectively used for in-depth analysis of the changes in the secondary structure of the chaperone when subjected to increasing temperatures or higher concentrations of a chemical agent.

The second section of methods covers pull-down assays that could examine the fundamental interactions that aid the association of histone oligomers with the chaperone by using a salt-gradient approach and a competitive pull-down assay to identify the histone oligomer preference of a chaperone. If the complex falls apart with a slight increase in salt concentration, that would suggest a major contribution of electrostatic interactions in stabilizing the complex. An intact complex in high salt would suggest a significant role for hydrophobicity in stabilizing the complex[35]. The competitive pull-down assay could be easily employed to determine the specificity or preference of a histone chaperone to a specific histone oligomer class. Based on their preference towards histone oligomers, histone chaperones can be classified into three categories such as H2A/H2B chaperones, H3/H4 chaperones, and H2A/H2B-H3/H4 chaperones[10,36]. In addition, if necessary, isothermal titration calorimetry (ITC) could be used to understand the histone oligomer specificity of a given chaperone and understand the thermodynamic characteristics of their interactions.

The third part of the methods section covers the investigation of the interaction stoichiometry between a histone chaperone and the histone oligomers. In general, the different families of histone chaperones differ considerably for the stoichiometry of their association with H2A/H2B or/and H3/H4[21,28,37-38]. AUC-SV experiment aids in obtaining sedimentation coefficient (s) and

molecular mass of a protein or its complex, which becomes very useful in accurately estimating the stoichiometry in the complex formation. Alternatively, ITC can also be used to examine stoichiometry.

The fourth segment of the methods section focuses on investigating the nucleosome assembly function of histone chaperones. Histone chaperones play a crucial role in nucleosome assembly, which regulates vital cellular processes such as replication, transcription, and DNA repair[39]. Plasmid supercoiling assay that is typically employed for the *in vitro* assessment of histone chaperoning activity of histone chaperones are elaborated in this section.

It may be noted that not all histone chaperones are thoroughly structured. Few are known to have intrinsically disordered regions[40,41]. Therefore, thermal and chemical stability assays may not be suitable for such proteins. Further, histone chaperones from different organisms have different oligomeric states and differential abilities to bind to histones. Therefore, this protocol may be a good starting point but would entail modifications as necessary.

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We acknowledge the help of Ms. Sudeshna Sen and Ms. Annapurna Sahoo for their help with histone preparation. The discussions with our colleagues Dr. Chinmayee Mohapatra, Mr. Manas Kumar Jagdev, and Dr. Shaikh Nausad Hossain are also acknowledged.

DISCLOSURES:
No conflict of interest was declared.

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Figure 1

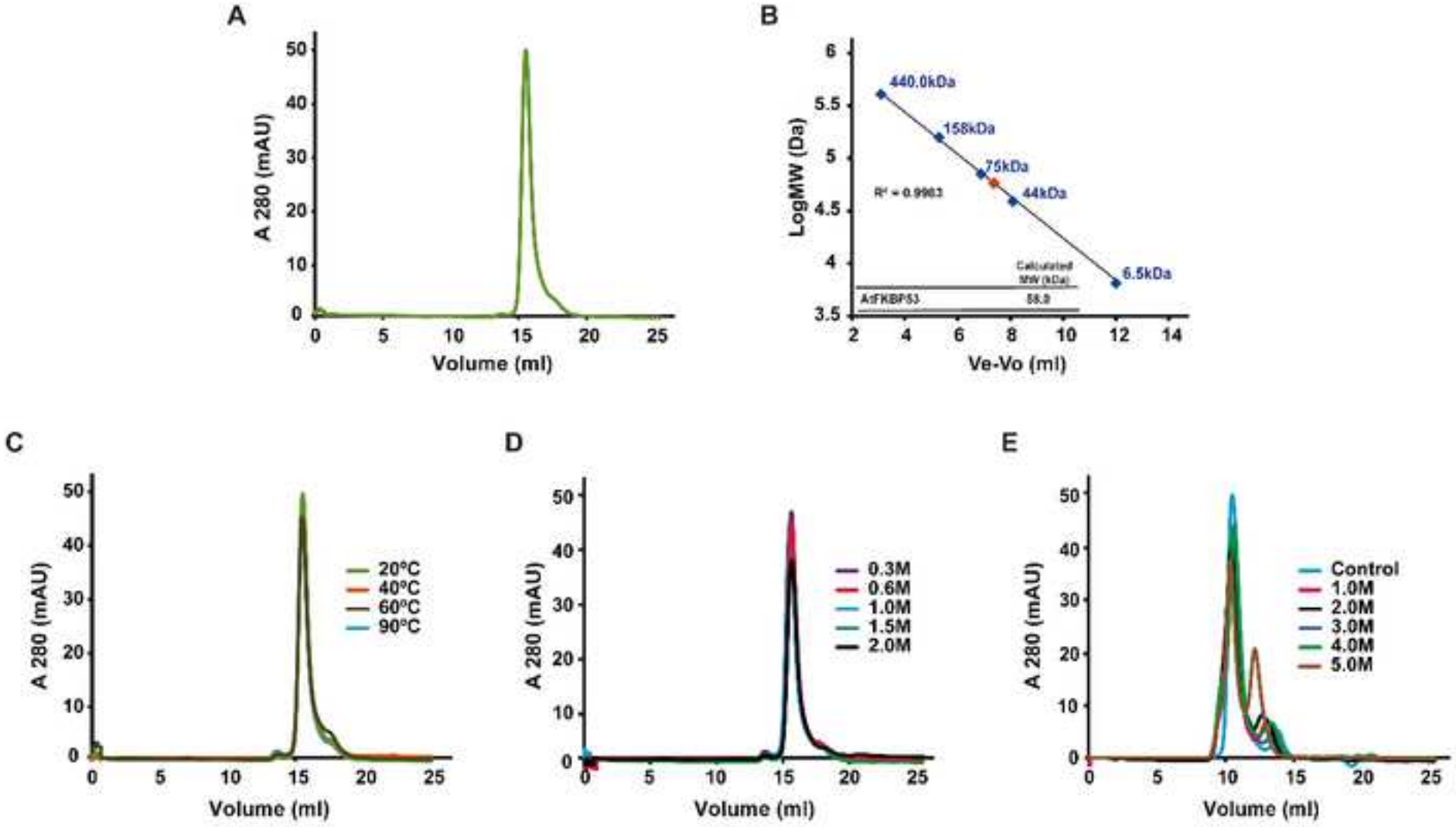


Figure 2

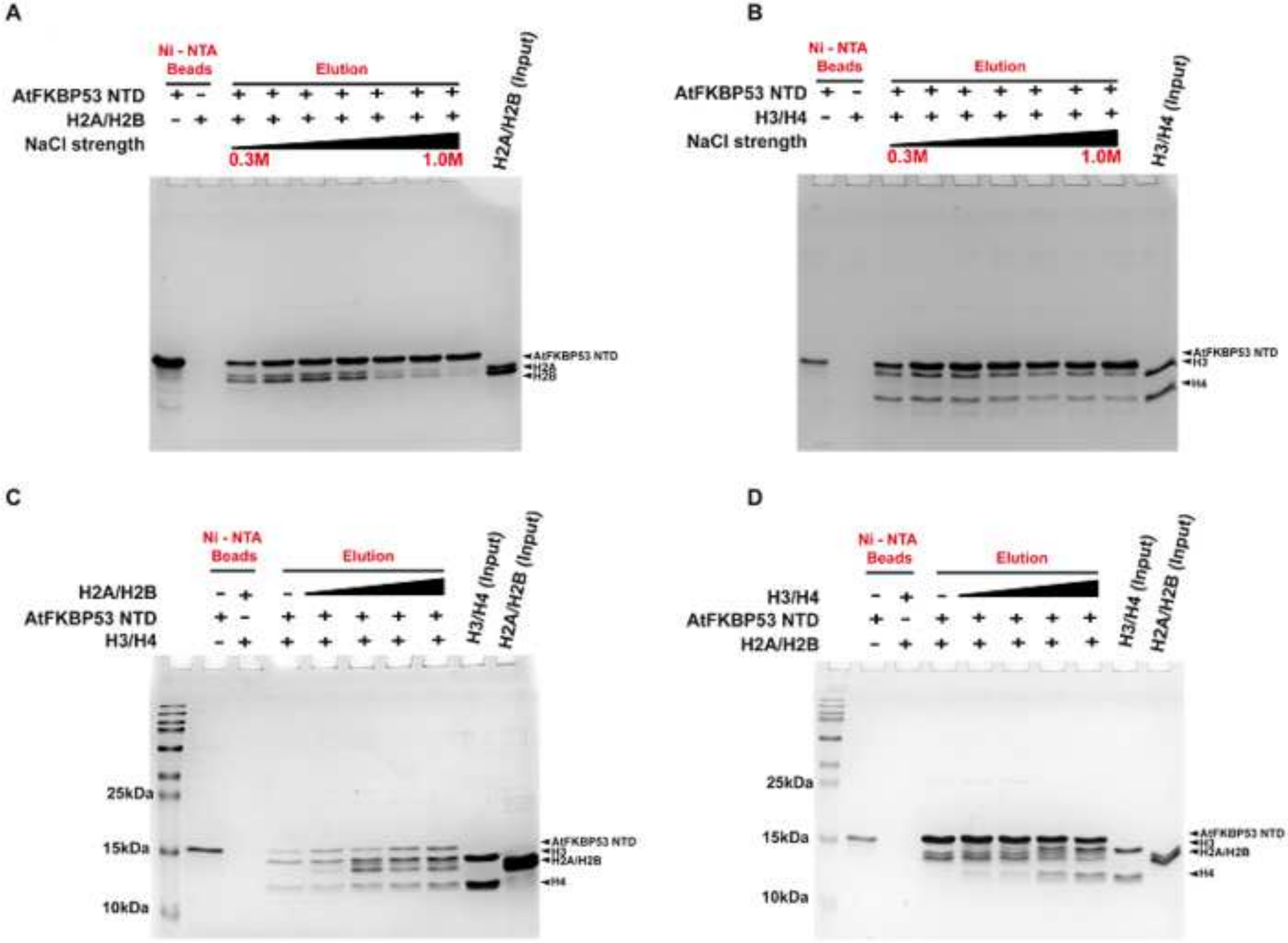


Figure 3

Click here to access/download;Figure;Figure 3.tif

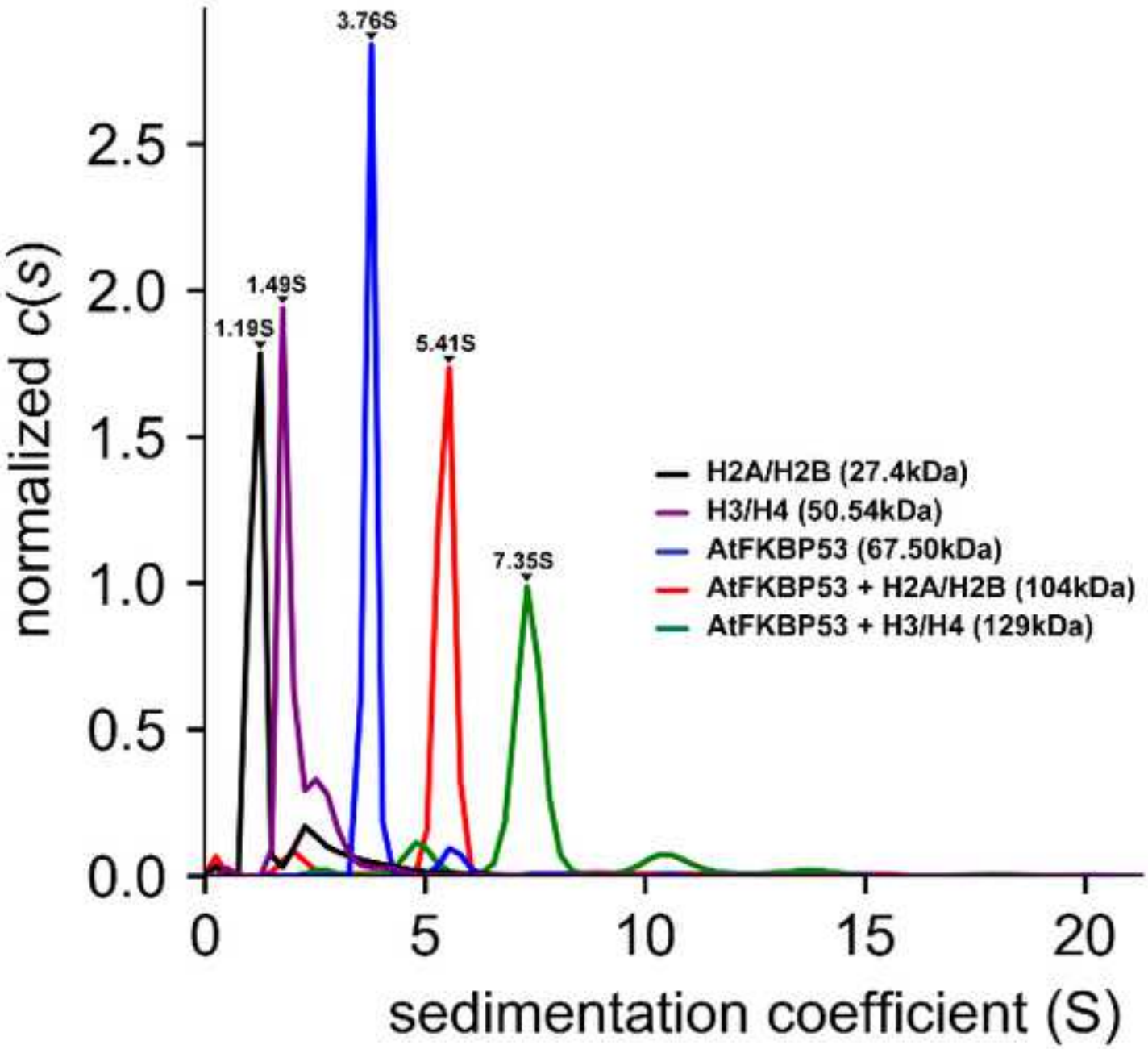
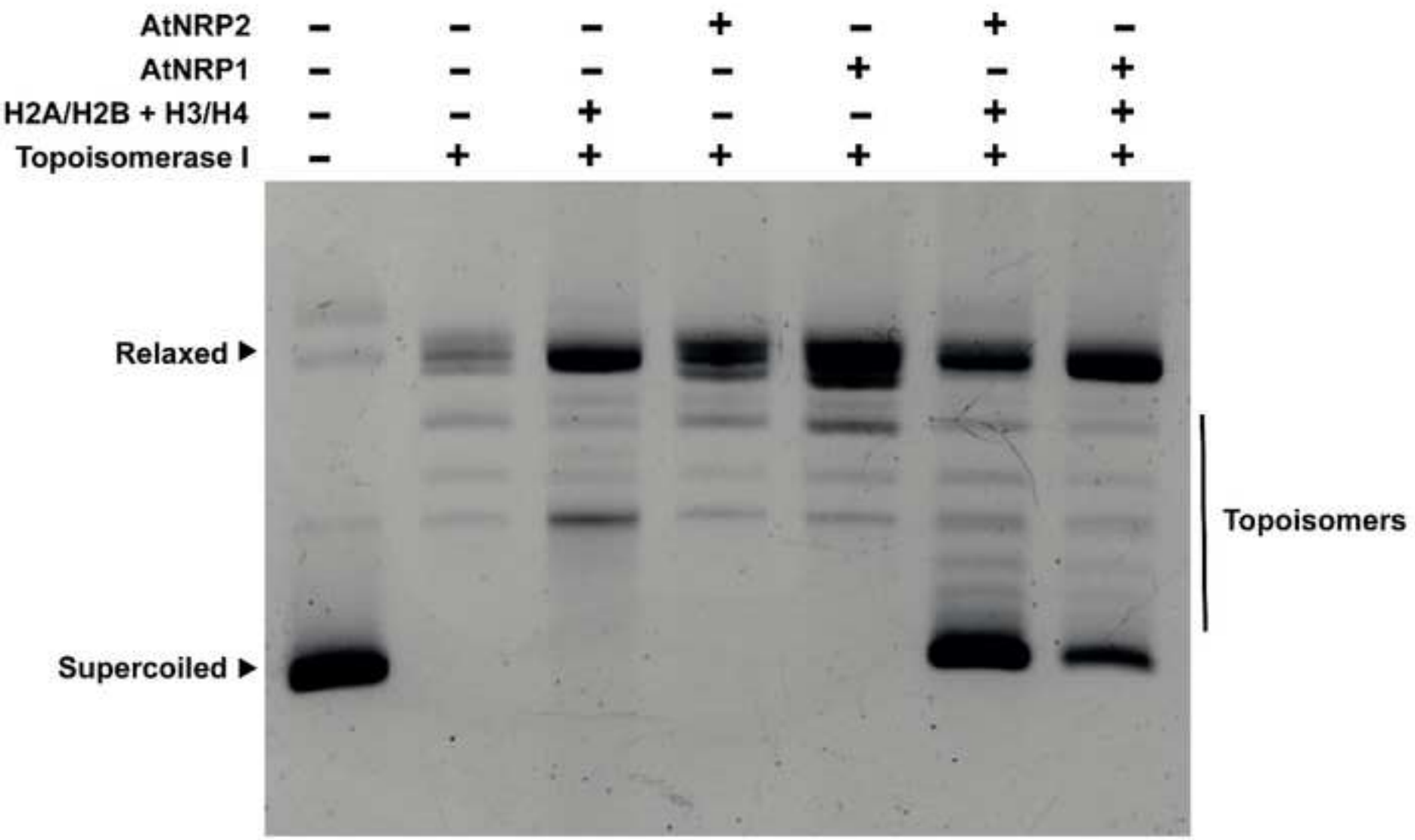


Figure 4

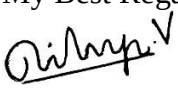


Bhubaneswar
15/November/2021

Dear Editor,

Thank you for the comments on our manuscript with the title "*In vitro* characterization of histone chaperones using analytical, pull-down and chaperoning assays". Herewith, we are submitting the second revised version of our manuscript, wherein, changes have been made as required and replies have been given to the comments that were provided in the manuscript word file. A revised version of the Table of Materials has also been uploaded. We hope you will find the changes made and the responses acceptable.

My Best Regards,



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02/November/2021

Dear Editor and Reviewers,

Many thanks for your feedback and comments on our manuscript with the title "*In vitro* characterization of histone chaperones using analytical, pull-down and chaperoning assays". Herewith, we have provided a point-wise response to all your queries. We hope you will find the responses and the revised manuscript acceptable.

My Best Regards,
Dileep

Comments from the Editor:

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.
Author response: We have thoroughly proofread the manuscript and made changes as necessary to rectify grammar and spelling issues.

2. Please provide a Summary to clearly describe the protocol and its applications in complete sentences between 10-50 words: "Here, we present a protocol to …"
Author response: As per your suggestion, we have added a summary section in the end of the article

3. Please also include the following in the Introduction along with citations:
a) The advantages over alternative techniques with applicable references to previous studies
Author response: In this article, we have described a battery of well-established methods that can be used in tandem to characterize proteins belonging to the family of histone chaperones and not any particular technique that is superior over other established methods. Therefore, it has not been possible to collate advantages for all the methods described. Besides, a mention of the advantages for all of the methods discussed would make the article a lot lengthier. Therefore, we have mentioned the advantages of only those techniques which are superior to other available techniques.

b) A description of the context of the technique in the wider body of literature
Author response: Again, a description of the context of the all the techniques in the introduction section would make the article very lengthy. The methods that have been included are well established techniques that are used for a wide array of experiments to study proteins and protein complexes in general. However, the histone chaperoning assay is exclusive to histone chaperones and has been used extensively to study this class of proteins.

c) Information to help readers to determine whether the method is appropriate for their application
Author response: All of the methods described here, except histone chaperoning assay are well established techniques that are routinely used for protein-protein interaction studies. The methods that have been described form a battery of biophysical and biochemical techniques that can be used to characterize known histone chaperones or unknown proteins with putative histone chaperone functions. Only histone chaperoning assay is specific to the class of histone

chaperones. This has been clearly mentioned in the introduction and discussion section of the manuscript.

4. JoVE cannot publish manuscripts containing commercial language. Please remove all commercial language from your manuscript and use generic terms instead. Please include all the commercial terms in the Table of Materials. e.g., Superdex, Cytiva, Invitrogen, SigmaPrep, Sigma, An-60Ti, Beckman Coulter, etc.
Author response: We have removed all commercial terms. Not mentioning the terms Superdex and An-60Ti, will actually compromise the description of protocols. However, we are following your instruction.

5. Please use SI units as much as possible and abbreviate all units: L, mL, µL, cm, kg, etc. Use h, min, s, for hour, minute, second. Please use centrifugal force denoted as x g for all centrifugation steps.
Author response: We have made changes wherever necessary. Relative centrifugal force (rcf) has been denoted as g, throughout the manuscript.

6. Please add more details to your protocol steps. Please ensure you answer the "how" question, i.e., how is the step performed? Readers of all levels of experience and expertise should be able to follow your protocol.
Author response: We have taken all efforts to provide as much details as possible.

Please provide the volumes and composition of equilibration, wash, elution buffer, etc.
Authors response: Suitable changes have been made in the revised version.

Step 1.1.2, 1.2.3: What was the column pressure?
Author response: This has been added to the revised version.

Step 1.1.4: Which protein was used as the standard for preparing the standard curve?
Author response: We have made additions in the figure legend to specify the proteins that make up the standard curve.

Step 2.6, 3.5: Please provide more details as to how the SDS-PAGE was run and how Coomassie blue staining was done. Alternatively, provide references to previously published protocols describing how this can be done.
Author response: As it is too much to elaborate how a basic experiment like SDS-PAGE and gel staining was done, we have provided suitable reference.

Step 3.4: How was the protein-protein titration done? What was the endpoint measurement for completion of titration?
Author response: This is not a typical titration experiment and does not have a specified endpoint. For protein titration experiments, typically a five to ten-fold excess of the binding partner is added to the protein under study to understand the protein-protein interaction. To avoid confusion, we have removed the word "titration".

Step 4.1.2: How was protein dialysis done? Please describe all the associated steps. Alternatively, provide references to previously published protocols describing how this can be done.
Author response: Again, it is too much to elaborate how a basic experiment like dialysis was done. We have added suitable reference.

Step 4.2.1: What is the initial concentration of protein used to prepare the 450 µL volume solution.
Author response: The initial concentrations of the protein samples need not be specified. Essentially, one can start with a protein concentration having two to three-fold higher OD280 to achieve a final OD280 of 0.3-0.5. This is essentially done to nullify the effects of dilution. This has been added to the revised version.

Step 4.2.2: If this step needs to be filmed, please provide all details as to how the cell was assembled.
Author response: We will film it in short. However, we have not provided a detailed protocol for cell assembly because it is already available in JoVE. Therefore, we have added a reference for the same.

Step 4.3.1: Software steps must be more explicitly explained ('click', 'select', etc.). Please add more specific details (e.g., button clicks for software actions, numerical values for settings, etc.). If using long scripts and long command, please include as supplementary file. We need actions to show how the software is used.
Author response: We have made some changes and have added a reference article describing the software steps in detail.

7. Please include a single line space between each step, sub-step, and note in the protocol section. Please highlight up to 3 pages of the Protocol (including headings and spacing) that identifies the essential steps of the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol. Remember that non-highlighted Protocol steps will remain in the manuscript, and therefore will still be available to the reader.
Author response: Instructions have been followed. And now we have highlighted sections in the manuscript that would be covered in the video.

8. Please do not number the results. Only the protocol steps should be numbered.
Author response: We have removed the numbers from the results section.

9. Figures: Please use SI abbreviations for all units e.g., mL, °C, etc. Please maintain a single space between the numeral and (abbreviated) unit. For example: 5 mL, 100 °C, 5 M, 6 h, etc.
Author response: We have made the necessary changes.

10. Please discuss all the figures in the representative results section.
Author response: All figures have been discussed in the results section. Separately, figure legends have been modified for ease of understanding, in line with the reviewer's suggestion.

11. Please include the following in the Discussion section in detail with citations:
a) Critical steps within the protocol
b) Any modifications and troubleshooting of the technique
c) Any limitations of the technique
d) The significance with respect to existing methods
e) Any future applications of the technique
Author response: Critical steps within the protocol have been covered in the methods section itself. Mentioning them again in the discussion section would lead to redundancy in text making the article bulky. All of these techniques are well established techniques. We have not reported any new technique in this article. We have focussed on using these techniques to biophysically

and biochemically define histone chaperones. Therefore, significance of the techniques with respect to existing methods, modifications of the techniques as well as future applications of the techniques become irrelevant. Limitations of the techniques, if any, have been covered in the discussion section. Troubleshooting of all the techniques would be too broad to discuss and are beyond the scope of this article.

12. Please add all items (plastic and glassware, solvents, equipment, software, etc.) in the Table of Materials so that it serves as a handy reference for users to get everything ready for the protocol.
Author response: A revised table of materials has been provided.

Comments from the Reviewers:

Reviewer #1:
Manuscript Summary:
The manuscript entitled "In vitro characterization of histone chaperones using analytical, pull-down and chaperoning assays" by Bobde et al. is good work. The authors have well presented their work, and it will be useful for a researcher working in the chromatin field, specifically on histone chaperones. The most commonly used histone chaperoning assay techniques like analytical size-exclusion chromatography, pull-down assay, Analytical ultracentrifugation, and plasmid supercoiling assay are presented nicely with suitable experimental examples with proper explanation.

Minor Concerns:
The histone chaperones as diverse structural folds. Some histone chaperones are folded into the globular structure, and many are known to exist as intrinsically disordered proteins (IDP). So it is not good to generalize the thermal stability and Urea stability for all the histone chaperones. The author can modify their statement accordingly.
Author response: We thank the reviewer for his valuable comments. Indeed, urea and thermal stability assays cannot be performed to characterize histone chaperones that are intrinsically disordered. We have added a note on this in the revised version of the manuscript in the last paragraph of the Discussion section and have added suitable references.

Reviewer #2:
Manuscript Summary:
The MS entitled "In vitro characterization of histone chaperones using analytical, pull-down and chaperoning assays". The study describes the characterization of Histone chaperones through a number of in-vitro experiments pull-down assays, analytical size-exclusion chromatography, analytical ultra-centrifugation, and histone chaperoning assay. This study provides a detailed insight of the in vitro characterization of Histone chaperones. The work is significant and may be useful for the research community. The manuscript is well written and organized. However, I have minor concern with some of the points, which I feel needs to be addressed.

Minor Concerns:
1. The MS has some grammatical errors and some of the sentences are not clear, author is suggested to proof read the whole MS.

Author response: We have gone through the entire manuscript thoroughly and have made corrections wherever necessary.

2. The Figure legends are less explanatory. I suggest the authors to explain the figure parameters in detail in the figure legends.
Author response: Suitable changes have been made to better represent all the figures.

3. The source of histones dimer and tetramer and confirmation of its oligomeric state may be included in the MS.
Author response: This information has been added to the revised version as a Note to section 2.2.

4. The experiments are done on Arabidopsis chaperones. Is there any modification required when applying these protocols to the organism specific chaperones?
Author response: A note pertaining to this has been added in the first paragraph of the discussion section of the revised manuscript.

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Lead author	Ruchir C. Bobde
Title of targeted journal	JoVE
Publisher	MyJoVE Corporation
Expected publication date	Dec 2021
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