# A Simple Method to Produce Sub-Nucleosome Complexes of High Purity *In Vitro*

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## Abstract

**With the identification of increasing number of chromatin modifiers, histone variants, histone post-translational modifications and their cross-talk, it is essential to validate these findings and interactions *in vitro* for which pure histone complexes are required. Although, the production of such complexes has been described earlier but still it remains a challenge for a non-specialist lab. Here we describe a protocol to quickly obtain large quantities of highly pure histones using bacterial expression system for GST pull-down and reconstitution experiments. In addition, we describe methods to quickly reconstitute and purify H2A/H2B dimers, H3/H4 tetramers and histone octamers for *in vitro* experiments. We demonstrate that these sub-complexes are properly folded and are hence, true representatives of the actual substrates *in vivo*. We also show that histones have a propensity to be non-specifically cleaved by proteases. Our results suggest that TEV protease is the most suitable protease while working with histones. The methodology described here should allow researchers to purify histone complexes in three days enabling functional and structural analyses of histone variants, mutants and post-translational modifications.**

## Keywords

## 1. Introduction

The eukaryotic genome is packaged inside the nucleus of only few microns in diameter [1] with the aid of highly

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basic proteins known as histones. The core histones comprise of H2A, H2B, H3 and H4. H1 is the linker histone. H2A and H2B form heterodimers and two copies each of H3 and H4 form a tetramer. Two H2A/H2B dimer and one H3/H4 tetramer form the octamer core around which ~147 bp of DNA is wrapped to give rise to nucleosome, which is the fundamental repeating unit of the chromatin [2]. Apart from the well-established fact now that the canonical histones undergo a variety of functionally important post translational modifications (PTM's), sequence divergent forms of the histones also exist which are known as histone variants [3]. In addition, novel histone modifiers and binding partners like histone chaperones are being identified with redundant or supposedly non-redundant functions.

With the identification of increasing number of chromatin modifiers, histone variants, histone post-translational modifications and their cross-talk, it is essential to validate these findings and interactions *in vitro* for which pure histone complexes are required. Large quantity of histones can be purified by isolation from cells [4], however, they are highly heterogeneous in terms of variants and PTM's [5] [6]. This does not allow the investigation of, for example, the possible contribution of a specific variant on structural organization of chromatin or the effect of a particular modification in recruiting chromatin modifiers or affecting nearby modifications. In such scenario use of recombinant histones becomes the method of choice to study the effect of histone variants or modified histones on the structure and stability of chromatin. Further, recombinant histones can be used to reconstitute nucleosome sub-complexes, H2A/H2B dimer, H3/H4 tetramers and H2A-H2B-H3-H4 octamers, which would fare as better representatives of *in vivo* substrates in *in vitro* assays.

Histones when expressed in bacteria form inclusion bodies and hence, their purification first requires solubilizing them with denaturing agents like urea or guanidinium chloride followed by ion exchange chromatography and gel filtration [7] [8]. Requirement of using multiple chromatography steps makes it technically and practically challenging for a non-specialist lab to employ these methods to purify recombinant histones. In addition, although, the production of nucleosome complexes with purified histones has been described earlier [9] it requires considerable expertise and resources to successfully execute the protocol. Further, a major challenge faced for the use of full length histone proteins for carrying out *in vitro* assays and reconstitution is their aggregation in commonly used buffers.

In the present study, we have evaluated various methods to purify histone proteins. We found that GST solubilizes histones enabling affinity purification and use in GST pull down assays. In addition, we came up with a simple 6xHis tag based purification system by modifying and combining existing protocols that enables quick reconstitution of H2A/H2B dimers, H3/H4 tetramers and histone octamers for *in vitro* experiments. Further, we biophysically characterize these complexes and demonstrate that these are properly folded.

## 2. Materials and Methods

### 2.1. Protein Expression and Fraction Preparation

Histone constructs in the desired vector were transformed in BL21 (DE3) pLysS. After overnight incubation, a single colony was inoculated from the plates of transformed BL21 (DE3) pLysS in 5 mL or 20 mL LB media and incubated at 37˚C until the OD600 reached between 0.3 and 0.6. Induction was carried out with 0.2 mM IPTG. The cultures were induced for 3 hrs at 37˚C or overnight at 18˚C. Post induction the bacteria were harvested and processed. The soluble and insoluble fractions of proteins were separated by re-suspending the cells in buffer containing 50 mM Tris-Cl pH 8.0, 0.5% Triton X-100 and 100 µg/ml lysozyme followed by three rounds of sonication, each for 30 seconds at 30% amplitude. The lysate was then centrifuged at 27000 g for 30 mins at 4˚C. The supernatant and pellet thus obtained contains the soluble proteins and the insoluble proteins respectively. The proteins were resolved by 18% SDS-PAGE followed by Coomassie staining (Brilliant Blue R250).

### 2.2. GST Affinity Purification

The fraction consisting of the soluble proteins was incubated with the equilibrated Glutathione conjugated beads for 2 hours at 4˚C. After incubation, the beads were washed with buffer containing 20 mM Tris-Cl pH 8.0, 200 mM NaCl, 1 mM EDTA pH 8.0, 0.5% Nonidet P-40, 2 µg/ml aprotinin, 1 µg/ml leupeptin, 0.7 µg/ml pepstatin and 25 µg/ml PMSF. The protein was eluted with the elution buffer containing 50 mM Tris-Cl pH 8.0 and 10 mM reduced glutathione.

## 2.3. Ni-NTA Affinity Purification

The insoluble pellets were dissolved in 50 mM Tris–HCl buffer (pH 8.0) containing 500 mM NaCl, 5% glycerol, and varying concentration of urea (see figure for more details), and were centrifuged at 27,000 g for 20 min at 4°C. The insoluble pellets were discarded. The supernatants containing the 6xHis-tagged histones were mixed with 50% slurry of nickel–nitrilotriacetic acid (Ni–NTA) agarose beads (Qiagen), and the samples were rotated at 4°C. After a 60 min rotation, the beads were washed with 50 mM Tris–HCl buffer (pH 8.0) containing 500 mM NaCl, 5% glycerol, 6 M urea, and 10 mM imidazole. The Ni–NTA agarose beads bound to the 6xHis-tagged proteins were washed further with the same buffer. The 6xHis-tagged histones were eluted by a linear gradient of imidazole from 5 to 300 mM. The eluted fractions were analysed by loading on 18% SDS-PAGE.

## 2.4. Reconstitution of H2A/H2B Dimer and H3/H4 Tetramer

To reconstitute the H2A/H2B dimer, the His6-tagged histones H2A and H2B or H3 and H4 in 10 mM HCl were mixed in a 1:1 stoichiometry. Then, the mixture was rapidly diluted three folds by adding 50 mM Tris–HCl buffer (pH 8.0) containing 10 mM DTT and 2 mM EDTA with continuous stirring. The diluted mixture is then dialyzed against 1 liter of 50 mM Tris–HCl buffer (pH 8.0) containing 10 mM DTT, 2 mM EDTA and 2 M NaCl for 1 h at room temperature, and the dialysis was continued overnight at 4°C. After an overnight dialysis, the impurities and excess histones were precipitated. The dialysate was then concentrated and loaded onto equilibrated gel filtration column and the peak fraction was collected. The salt concentration can be reduced by stepwise dialysis.

## 2.5. Reconstitution of Histone Octamer

The reconstituted H2A/H2B dimer and H3/H4 tetramer were mixed in 1:1 stoichiometry and dialysed overnight against 2 liter of 50 mM Tris–HCl buffer (pH 8.0) containing 10 mM DTT, 2 mM EDTA and 2 M NaCl at 4°C. The dialysate was then loaded onto equilibrated gel filtration column and the peak fraction was collected.

## 2.6. Mass Spectrometric Analysis

Protein spots of interest were manually excised and cut into fine pieces. These were destained, chemically reduced, alkylated and subjected to in gel digestion using 20 ng/μl Trypsin (Sigma) at 37°C for 16 - 18 hrs. Peptides thus obtained were extracted from gel pieces, vacuum dried and reconstituted in 10 μl of 0.1% TFA. Samples were then mixed with matrix solution (α-Cyano-4-hydroxycinnamic acid), dried and mass spectra were acquired on a Bruker Daltonics Ultraflex II in reflector positive ion mode. Database searching for protein masses was carried out using Mascot search engine (version 2.2.03) by comparing peptide masses with those in NCBInr protein database.

# 3. Results

## 3.1. Purification of Recombinant Histones Using Affinity Purification

Histones are highly insoluble when expressed in bacteria. One way of circumventing this issue is by co-expressing histones, for example H2A and H2B together in tandem, in bacteria which at times makes them soluble [10]. However, this method does not ensure that all histones expressed will be soluble [10]. As an alternative approach, we tried to use the commonly used GST tag as it often solubilizes protein and also enables use of affinity purification. Affinity purification may yield ~90% pure proteins after only one round of purification and is technically less challenging than ion-exchange chromatography.

We found the histones expressed robustly with the GST tag (Figure 1(a)), however, the expressed proteins were largely insoluble when growth temperature post-induction of recombinant protein expression was maintained at 37°C. The data of only H2A is shown but similar results were obtained for all the core histones (Figure 1(b)(i)). On attempting expression at 18°C, approximately 10% of the protein, as judged by densitometric analysis, was recovered in the soluble fraction (Figure 1(b)(ii)). Proteins were purified by affinity purification. The GST-Histone fusion proteins can be used for carrying out GST-pull down assays for investigating direct physical interactions of putative binding partners of histones.
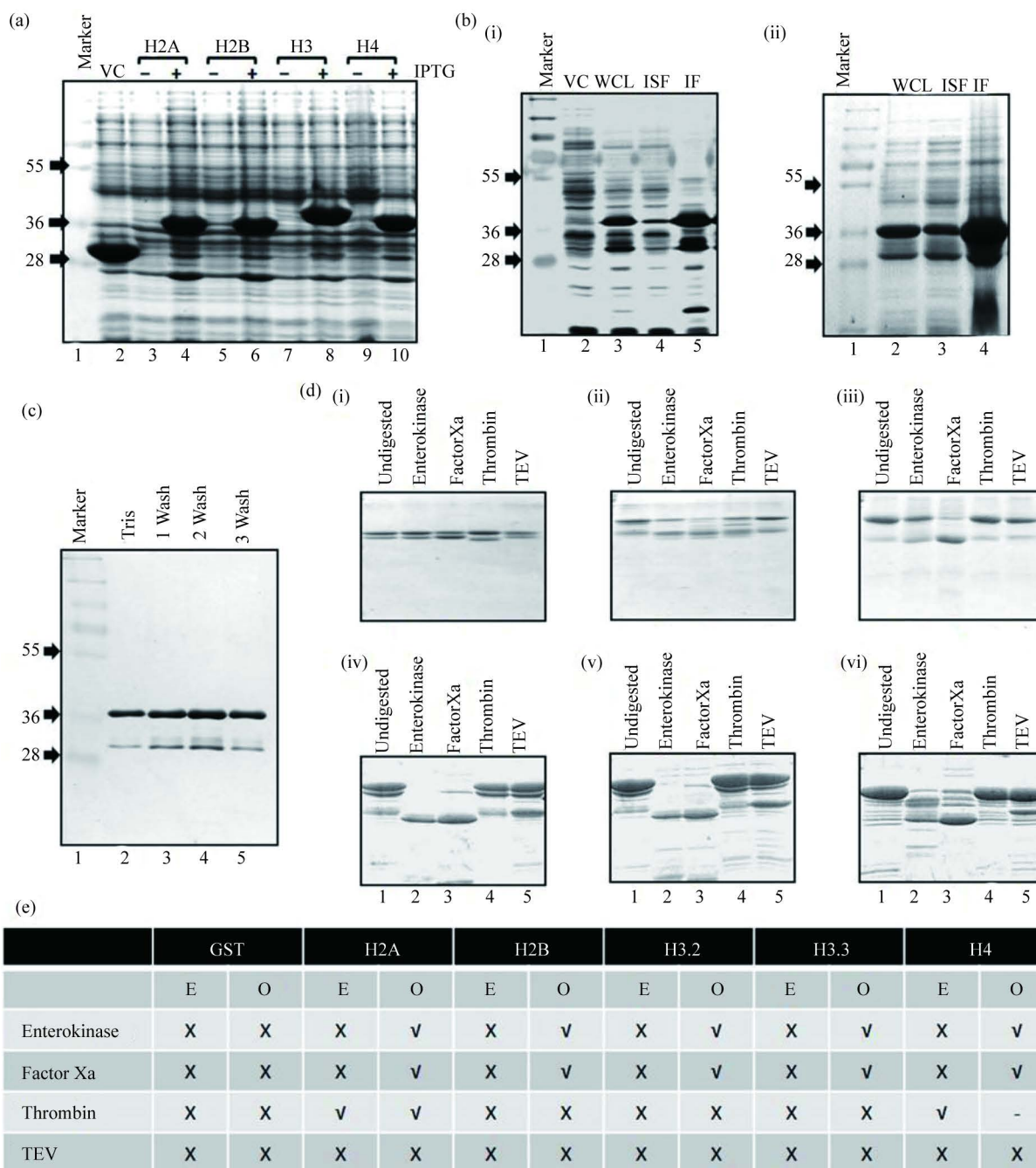
**Figure 1.** Expression and purification of GST tagged Histones; (a) Coomassie stained SDS gel loaded with whole cell lysates of BL21 (DE3) pLysS expressing GST Histones. (b)(i) Solubility analysis of GST-Histones expressed at 37°C. WCL-Whole cell lysate, ISF-Induced soluble fraction, IF-Insoluble fraction (ii) Solubility analysis of GST-Histones expressed at 18°C overnight. (c) SDS PAGE showing purified GST Histones. (d)(i) Proteolysis of GST tag with all the used enzymes. (ii)-(vi) Enzymatic action of proteases on GST tagged H2A, H2B, H3 and H4 histones. (e) Table summarised to show the protease activity of all the enzymes used on all the purified GST-Histones.

## 3.2. Removal of GST Tag from Recombinant Proteins

GST being of 26 kDa size and a homodimer itself is expected to interfere with the hetero-dimerisation of histones. This makes cleavage of the GST tag essential for carrying out H2A/H2B dimerisation or H3/H4 tetramerisation. We tested the efficacy of some commonly used proteases for histone purification. Incubation of only

GST protein did not lead to any cleaved products (**Figure 1(d)(i)**) as expected. However, to our surprise, we found proteases like FactorXa and Enterokinase, non-specifically cleave the histones as can be seen with the disappearance of the band corresponding to GST fusion and appearance of band corresponding to the GST protein (**Figures 1(d)(ii)-(vi)**). By mass spectrometry we confirmed that the protein we purified were indeed GST-Histone fusion. On screening for different proteases, TEV protease was found not to cleave histones non-specifically. The expected and observed pattern of protease activity is tabulated in **Figure 1(e)**. Although, thrombin is expected to cleave H4 protein but the cleavage site is just after four amino acids. This makes the band shift difficult to be perceived on SDS-PAGE (**Figure 1(d)(vi)**). Post GST removal the monomeric histones were found to precipitate that is commonly observed with full length histones. This limits their use in downstream reconstitution experiments. However, for purification of smaller peptides of histones, which might be soluble, our results suggest that TEV protease should be used.

## 3.3. 6xHis Tag Is Better Suited for Affinity Purification of Histones

We opted to use N-terminal 6xHis tag for histone purification keeping in consideration that a small tag like 6xHis is not expected to interfere in the oligomerization of histones (N-terminal tails of histones protrude out of the nucleosomes). Unlike the case with GST tag, this would enable reconstitution without the need of removal of affinity tag.

We used pET28a vector to express histones in BL21 (DE3) pLysS. The recombinant protein expression was robust (**Figure 2(a)**). On analyzing different fractions, the histones were found to be completely part of insoluble fraction at both strategies were employed. Urea was the preferred denaturant to solubilize proteins from the insoluble fraction as GdmHCl doesn't allow solubilized 6xHis proteins to bind to Ni-NTA column. Upon further titration with different urea concentrations, 6 M urea was found as the optimal denaturant concentration to solubilize these proteins (**Figure 2(c)**). The solubilized proteins robustly bound to Ni-NTA beads (**Figure 2(d)**). Elution with buffers containing increasing concentration of imidazole suggested that histones could be very efficiently eluted with 150 mM imidazole concentration with almost no non-specific proteins (**Figure 2(e)**). Thus,
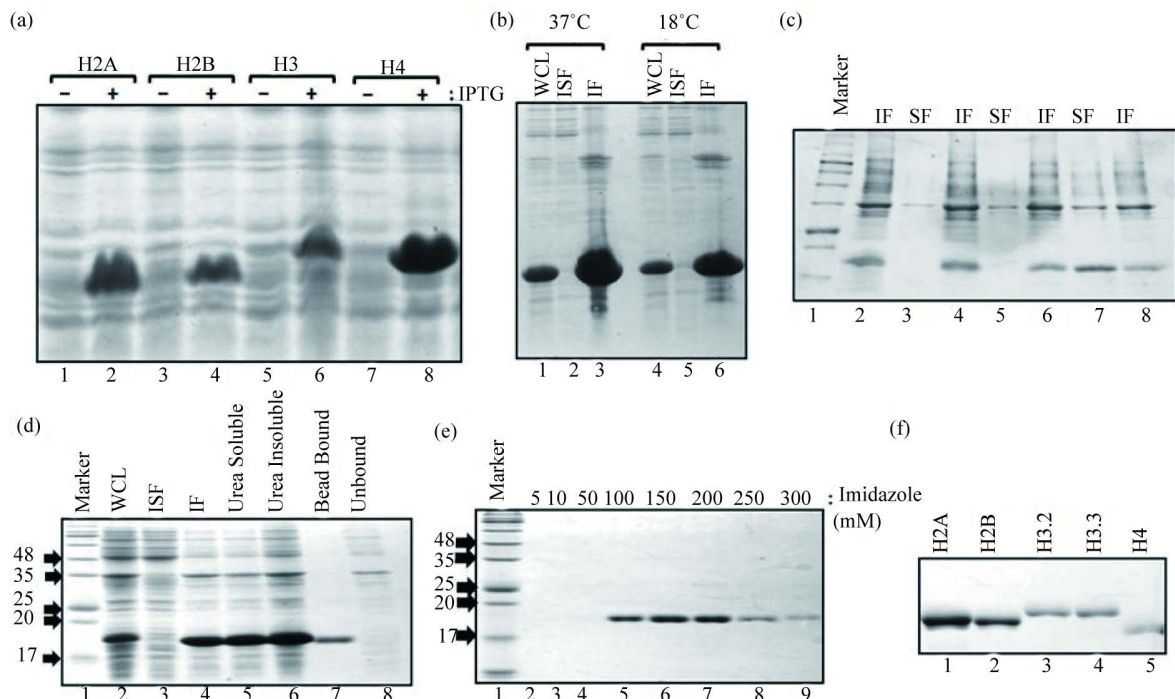


**Figure 2.** Expression and purification of 6X His tag histones; (a) 18% SDS PAGE showing the expression of 6xHis-Histones. (b) Solubility analysis of His tag Histones at 37°C and 18°C. (c) Solubilisation of histones with different urea concentrations. Lanes 2, 4, 6, 8 correspond to insoluble fractions and lanes 3, 5, 7 to fractions solubilized with 2 M, 4 M and 6 M urea. (d) Complete profile of purification strategy for 6x His tagged histones. (e) Elution profile of tagged histones at different concentrations of imidazole. (f) SDS PAGE showing all the purified histones.

all the core histones could be very efficiently and conveniently purified with this one step purification process.

## 3.4. Histone Monomers Are Not Properly Folded in Solution

To remove denaturant from the purified proteins, the eluted fraction was overnight dialyzed against 50 mM phosphate buffer. However, often it led to the precipitation of majority of histones in a concentration dependent manner. Histones being highly basic proteins dialyzing them against an acidic solution may enhance solubility. Hence, histones were dialyzed against 10 mM HCl to remove urea. By this method the precipitation could be circumvented irrespective of the concentration of histones. For carrying out subsequent analysis the concentrated histones could be diluted in phosphate or tris buffer without precipitation. Afterwards, we wanted to see whether the histone monomers attain their native confirmation on removal of the denaturant. Circular dichroism spectra showed a dip at around 200 nm for all the histone monomers suggesting that the monomers were not folded properly and were largely in random coil (**Figure 3(a)**). This might be attributed to the fact that the alpha 2 helix of histones is largely hydrophobic in nature.

## 3.5. Histone Oligomers Can Be Conveniently Reconstituted from Affinity Purified Histones

Next, we wanted to reconstitute H2A/H2B dimers and H3/H4 tetramers as 1) they would be better substrates for carrying out *in vitro* assays, 2) would be properly folded owing to the burial of hydrophobic residues and 3) would be easier to use for octamerisation (discussed later).

In this regard, we used the protocol of rapid dilution of histones for forming oligomers which was previously described [5]. We found that even H3/H4 tetramers could be *in vitro* reconstituted directly from 10 mM HCl (**Figure 3(c)**) without urea denaturation by this method which was not previously reported. Post rapid dilution the protocol involves binding of the mix to heparin column and subsequent elution [5]. We found dialyzing the rapid dilution mix against 2 M NaCl leads to precipitation of excess histone monomers leaving the properly folded dimers in soluble form thus eliminating the requirement of Heparin column. In ideal scenario, with usage of equimolar histones, there should be no or minimum precipitate post dialysis. The precipitate (if formed) could be removed by centrifuging the sample at 10,000 g for 20 mins. The dimerization could be confirmed by loading the soluble fraction onto a gel filtration column. The dimers thus obtained were purified by size-exclusion chromatography using HiLoad 16/60 Superdex-200 gel filtration column (GE). The dimer eluted at a volume of 82 - 84 mL (**Figure 3(b)(i)**) consistent with previous reports [2]. The molecular weight of H2A and H2B being very similar (H2A-14119.5Da, H2B-13906.1Da) (**Figure 3(b)(ii)**), the peak fractions from gel filtration were resolved onto AUT-PAGE, which separates proteins on the basis of hydrophobicity, to confirm the formation of heterodimers (**Figure 3(b)(iii)**).

## 3.6. H2A/H2B Dimers and H3/H4 Tetramers Are Better Substrates for Octamer Reconstitution than Histone Monomers

Once we were able to obtain properly folded H2A/H2B dimers and H3/H4 tetramers, we next wanted to test whether these complexes can be used to reconstitute histone octamers. Equimolar ratio of H2A/H2B dimer and H3/H4 tetramer were mixed and were dialysed against 2 M NaCl. As the H2A/H2B and H3/H4 complexes are independently stable under high NaCl concentrations, no precipitation was observed. This is unlike the use of histone monomers where errors in quantification can lead to large amount of protein precipitation (discussed later). Post dialysis the histone octameric core particle was formed which could be confirmed and purified by loading onto size exclusion chromatography column with elution at an expected volume ~65 ml (**Figure 3(d)**). The excess H2A/H2B dimer eluted at 84 mL.

## 3.7. Histone Oligomers Are Properly Folded

Post reconstitution and purification, secondary structure validation of the H2A/H2B dimer and H3/H4 tetramer were carried out by far-UV CD. The spectra dip at 222 and 208 nm, characteristic of helices confirmed that the histone oligomers, which has a high proportion (40%) of helical structure (**Figure 4(a)**), is properly folded (**Figure 4(b)(i)**) for H2A/H2B dimer, (c)(i) for H3/H4 tetramer]. The greater dip at 208 nm compared to 222 nm highlights the greater proportion (~60%) of randomly coiled structure of the dimer and tetramer. The tertiary structure of the H2A/H2B dimer and H3/H4 tetramer was validated by near UV CD and fluorescence spectros-

copy. H2A has three tyrosine residues at position 39, 50 and 57 and H2B have five tyrosine residues at position 37, 40, 42, 83 and 121. Histones do not contain tryptophan, hence, the fluorescence of histone proteins is majorly attributed to the tyrosine. The bell shaped curve with peak near 278 nm in the near-UV CD spectra is in agreement with signature tyrosine peak between 275 and 282 nm (**Figure 4(b)(iii)**). The dimers exhibited a fluorescence emission maximum at 305 nm (**Figure 4(b)(ii)**) which is expected for tyrosine. Similar pattern was observed for the H3-H4 tetramers (**Figure 4(c)(ii)**).

## 4. Discussion

We have described a very convenient and robust method to express, purify and reconstitute sub-nucleosomal complexes of histones for carrying out *in vitro* studies. The proteins were purified by one step affinity chromatography. GST tag could be used to solubilise and purify histones, however, due to the precipitation of monomeric histones post GST tag removal the strategy was not useful especially considering that only 10% of the
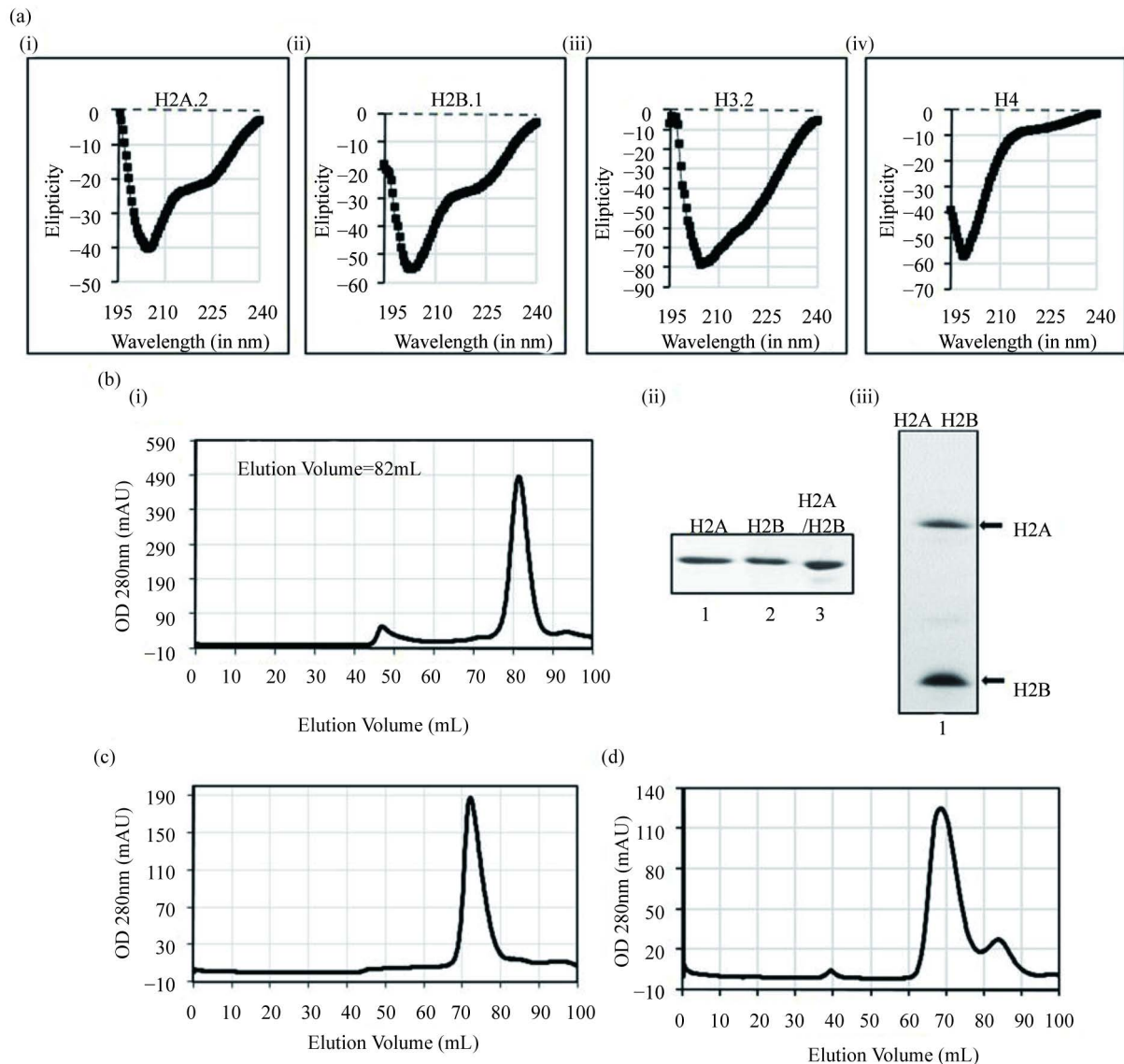


**Figure 3.** Reconstitution of histone dimer, tetramer and octamer: (a)(i)-(iv) Far-UV CD spectra of all the purified histone monomers showing their unfolded state. (b)(i) Graph corresponding to elution profile of H2A/H2B dimer, eluting at 82 ml. (ii) SDS PAGE of the above collected dimer. (iii) AUT PAGE of H2A/H2B dimer, showing distinctly resolved H2A and H2B. (c) Elution profile of reconstituted H3/H4 tetramer eluting at 72 ml. (d) Histone octamer elution peak at 65 ml.
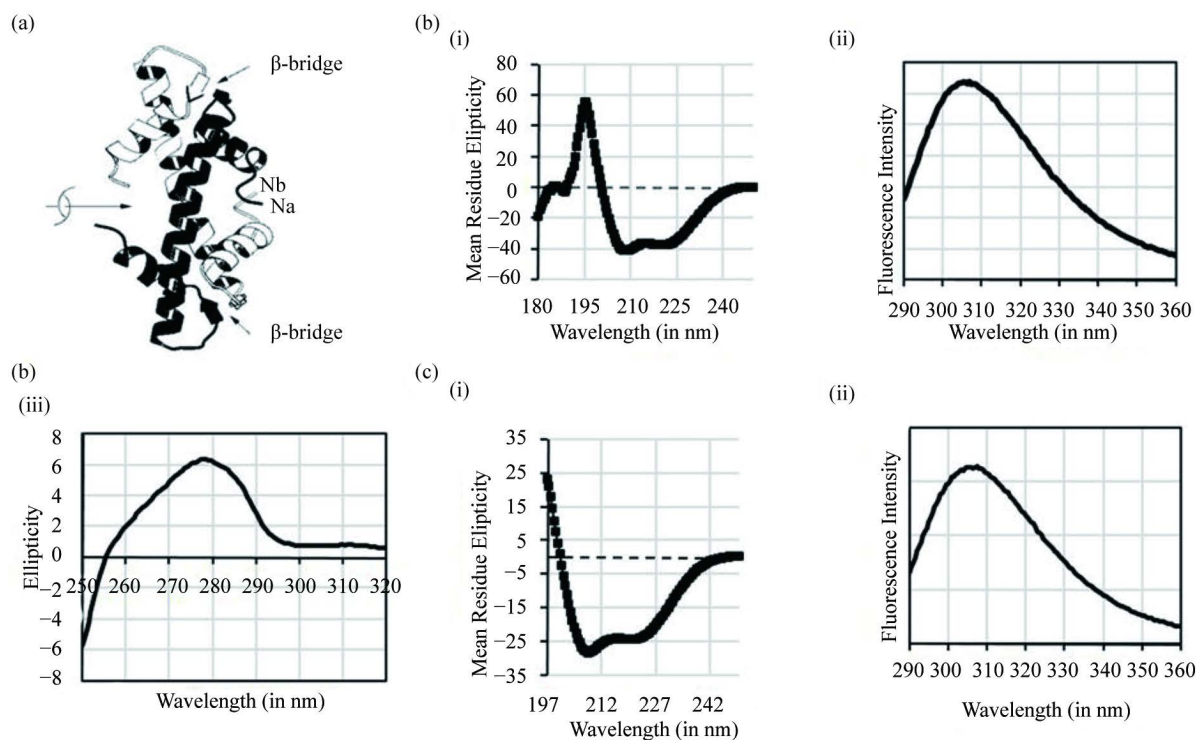
**Figure 4.** Secondary structure analysis of histone oligomers: a) Pictorial representation of structure of a histone H2A/H2B dimer. (b)(i) Far UV CD spectra of reconstituted dimer. (ii) Fluorescence spectral scan of reconstituted dimer. (iii) Near UV CD spectra for tertiary structure determination of dimers. (c)(i) Far UV CD spectra of reconstituted H3/H4 tetramer. (ii) Fluorescence spectral scan of tetramer.

protein is solubilised on GST tag incorporation. Nonetheless, the strategy can be useful to carry out *in vitro* GST pull down assays. Another striking thing which emerged from our data is the degradation of histones by proteases FactorXa and Enterokinase, which have propensity to non-specifically cleave at basic residues. Hence, care is to be taken while choosing the protease to remove tags from fusion proteins consisting of histones. TEV protease was found to be the best suited for this purpose. For obtaining recombinant histones in bulk amount, Ni-NTA affinity purification under denaturing conditions was very convenient and robust technique. In addition, 6xHis tag being small and as N-terminus of histones are not involved in formation of the complexes, the His-tagged proteins could be directly used in reconstitution experiments without removal of tag. Further, we documented that the reconstituted complexes are properly folded which makes them useful for carrying out *in vitro* studies pertaining to histone variants/mutants and modifications. During the formation of octamer equimolar ratio of the core histones assemble. Accurate quantification of histone monomers is crucial to prevent precipitation of proteins and successful reconstitution. However, histones lack tryptophan and have very less tyrosine and phenylalanine. This makes their accurate quantification difficult using colorimetric approach. In this regard use of reconstituted H2A/H2B dimers and H3/H4 tetramers for histone octamer reconstitution is more convenient rather than adding all the four histone monomers stoichiometrically. In our experience using the reconstituted dimers and tetramers stoichiometrically for octamerisation gives more consistent results than using the individual monomers. The histone octamer elutes at 65 mL. The H3/H4 tetramer elutes at 72 mL, whereas the H2A/H2B dimer elutes at 84 mL. Hence, it is advised to use a little excess of H2A/H2B dimer during octamer reconstitution as they are easier to distinguish from the octamer peak during gel filtration as compared to H3/H4 tetramer peak.

## 5. Conclusion

We have described a protocol in which histones can be purified and its oligomeric structures reconstituted in very few steps in three days. Histones can be purified in bulk by affinity purification and then via use of rapid dilution method followed by dialysis, H2A/H2B dimers and H3/H4 tetramers can easily be reconstituted. For the

octamerisation, the reconstituted and purified dimers and tetramers are mixed proportionately followed by dialysis. These reconstituted and purified octamers which have their native structure are better substrates for use in *in vitro* assays. We also showed that adding GST tag partially solubilises the otherwise insoluble histones which can be used for *in vitro* assays. Further, histones have a propensity to be non-specifically cleaved by proteases and TEV protease should be opted for while working with histones.

## Acknowledgements

## Conflict of Interest

The authors hereby declare no conflicts of interest.

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