# Strategies for the Purification of β-Clamp in Complex with the CTD of the DnaE of Pol III (α-Subunit)

**Atif A. Patoli** ( atifpatoli@gmail.com )
Institute of Microbiology, University of Sindh, Jamshoro https://orcid.org/0000-0002-3700-6751
**Bushra B. Patoli**
University of Sindh Faculty of Natural Sciences

**Research Article**

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# Abstract

Structural information on replisome components is required to understand highly co-ordinated events during DNA replication and repair in *Escherichia coli*. We aimed to purify complexes involving the replicative polymerase α subunit (DnaE) and β-clamp processivity factor (DnaN). DnaN and 255 amino acids long 6xhis tagged C-terminal domain of DnaE bearing consensus iCBM (DnaE905hM ) were over-expressed in *E. coli* B834 cells separately. Beta-clamp was obtained in soluble fraction while DnaE905hM appeared in insoluble fraction. The insoluble fraction containing over-expressed DnaEΔ905hM was solubilized. The denatured DnaE905hM was bound to affinity column followed by on-column re-folding and binding of the β-clamp. The purification of the complex was achieved on size exclusion column. The expected composition of complexes was a single β-clamp dimer (81.2 kDa) with one DnaE905hM (26 kDa) predicted to elute at ~180 ml and a complex of β-clamp dimer and two molecules of DnaE905M, predicted to elute at ~175 ml. A sharp peak was observed eluting at 173 ml, with more poorly defined, broader peaks eluting at 155, 205 and 220 ml. The analysis through SDS PAGE and Western blot technique using alkaline-phosphatase-conjugated Mouse Anti-Hexa-His antibodies and rabbit polyclonal anti β-clamp IgG antibody indicated most intense band for β-clamp in fraction 21 and DnaE905hM in fraction 22, presumably reflecting monomeric forms of each protein. The presence of fainter bands of both the β-clamp and DnaE905hM throughout fractions 15 to 19, seemed to cover a wide range of potential stoichiometric of complexes.


# Introduction

The maintenance of genomic stability and its accurate transmission to the next generation is essential to the proliferation of life. All life forms use specialized enzymes called DNA polymerases along with various other accessory proteins (collectively referred to as the replisome) for the replication of DNA (1) (2). These polymerases use circular sliding clamp proteins (processivity factors) to attain a tight topological grip on DNA. The bacterial processivity factor, the β-clamp, encircles DNA and increases the processivity of DNA polymerases by several orders of magnitude (1) (3). *E. coli* is currently known to possess five different DNA polymerases (4). Much attention is given to how cells control the actions of these DNA polymerases on β-clamp during the highly coordinated processivity and polymerase switches throughout replication and TLS respectively. Mutational, biochemical and structural information has revealed that β-clamp plays an integral role in the regulation of events during DNA replication and TLS (4) (5), however given the complex nature of these processes the roles of other components are also crucial. The structural information on the interacting regions for the various replisome subunits is key to understanding the highly co-ordinated processivity and polymerase switches during replication. In this respect, various co-crystal structures of β-clamp in complex with synthetic peptides from polymerases have been solved (6) (7). Although the peptide-clamp structures have contributed useful information on polymerase binding to β-clamp, these structures have not helped understand any secondary binding sites due to their very short length (8) (9). In E. coli the DNA Pol III is the major chromosomal replicative enzyme (10). The core polymerase is a 1:1:1 heterotrimer of α, ε and θ subunits (11). The α subunit

contains the DNA polymerase activity and is encoded by the dnaE gene (12). The DnaE protein is 1160 amino acids long with a molecular weight of 129.9 kDa (http://www.ecogene.org/geneInfo). The heterotrimer core polymerase is by itself slow, incorporating only 20ntd/s (11) and is weakly processive extending only 1–10 bases per binding event. For highly processive synthesis (~750 ntd/s), the core polymerase binds β-clamp, a ring shaped homodimer that encircles the duplex, whereupon it freely slides along it (11). Most of the proteins that bind to β-clamp have only one Clamp Binding Motif (CBM), however the replicative DNA polymerase III is known to possess two CBMs (13) (14). Using bioinformatics, yeast two-hybrid analysis and inhibition of protein–protein interaction by modified peptides, a consensus sequence (QL[SD]LF ) for the β- binding motif has also been proposed (15). Surface Plasmon Resonance has been used to show that replacing internal Clamp Binding Motif (i-CBM) in DnaE with the consensus sequence enhances its binding to β-clamp by 120-fold (13). An *in vivo* demonstration of the same is also shown by (16). Since the Pol III DnaE is known to have two β-clamp binding motifs within the CTD (13) (17) (14), a co-crystal of the DnaE with β-clamp, utilizing either or both of its β-clamp binding sites to bind β-clamp, is required to understand the structural basis of interaction between β-clamp and the replicative polymerase during replication. Attempts were therefore made to purify β-clamp in complex with C-terminal Domain (CTD) of DnaE for structural analysis. The current article entails the strategies to co-purify the β-clamp and DnaE-CTD complex and the formation of various stoichiometric complexes during purification.

# Materials And Methods

### Primers, Plasmids and Strains.

The primers used in this study were synthesized by eurofins MWG GmbH. The forward primer was designed to incorporate an N-terminal 6xHisTag into the DnaE construct. Table 1 **shows the details of the primers used in this study**. A pACYC184-11b (18) driven plasmid encoding full length β-clamp was donated by Dr. Karen A. Bunting (University of Nottingham UK). The plasmid was termed herein as pACYC11-*dnaN*. Plasmid pET11 (Novagen) was used for the expression of 6xHisTagged *dnaE905hM*. For cloning, DH5α chemically competent *E. coli* was used. For expression, chemically competent *E. coli B834* (DE3) cells containing an isopropyl β-D-1-thiogalactopyranoside (IPTG) inducible T7 RNA polymerase for expression of target genes cloned in pET vectors was used.

Table 1
Details of the primers used in this study. CATATG = *Nde*I, GGATCC = *Bam*HI

| Construct | *Primer* | Primer sequence |
|---|---|---|
| *dnaEΔ905H* | *E905h-f* | GGAATTCCATATGCATCATCATCATCATCATGCGTTAAAAGCGGCAG |
| 905–1160 (768bp) | *E905h-r* | CCCGGATCCTTATTAGTCAAACTCCAGTTCC |
| *dnaΔ905h-mut* | *BC-f* | GCGGAAGCTATCGGTCAGCTGGATCTGTTCGGCGTGCTCGCCGAAG |

**Amplification of** DnaE905.

Using primers E905f and E905r, (Table.1), and *E. coli* genomic DNA as template, the region of *dnaE* encoding C-terminal 255 residues was amplified with N-terminal 6x histidine. The amplified product was cloned into *pET-11* expression vectors using *Nde*I and *BamH*I restriction sites separately. The resulting plasmid was named pAP*dnaE905h.* DH5α cells were used for cloning and amplification of these plasmids. The presence of original wild type sequences of inserts in appropriate orientations was confirmed by DNA sequencing.

**Mutagenesis of** DnaE905h

Mutagenesis of the internal clamp binding motif (iCBM) in pAP*dnaE905h* was performed using a primer designed to incorporate the desired base changes (i-CBMCf, Table 1) and the Quickchange Multi-Site-Directed Mutagenesis Kit (Stratagene) as explained in (16). The resulting plasmid having desired mutations was termed as pAP-*dnaE905hM.*

# Protein Over-expression

**(a) Over-Expression of β-clamp.** Plasmid pACYC11-dnaN encoding full length β-clamp was transformed into *E. coli* B834 (DE3) expression strain [23]. Fresh transformants were grown in LB broth containing chloramphenicol (34 µg/ml) at 37°C. At OD600 = 0.6–0.8 the cells were induced with 0.1 mM IPTG, and were further incubated overnight at 25°C. The cells were then harvested by centrifugation, lysed by sonication in buffer A (50 mM HEPES pH 7.0, 200 mM NaCl, 20 mM imidazole). The soluble fraction was then obtained by centrifugation at 15,000 x g for 30 min at 4°C.

**(b) Over-Expression and Solubilization of DnaE905hM.** Plasmid pAP-*dnaE905hM* encoding C-terminal 255 residues of DnaE with N-terminal 6x histidine and bearing consensus iCBM was transformed into *E. coli* B834 (DE3) expression strain [23]. Fresh transformants were grown in LB broth containing ampicillin (100µg/ml) at 37°C. At OD600 = 0.6–0.8 the cells were induced with 0.1 mM IPTG, and were further incubated overnight at 25°C. The cells were then harvested by centrifugation, lysed by sonication in buffer A (50 mM HEPES pH 7.0, 200 mM NaCl, 20 mM imidazole). The in-soluble fraction was then obtained by centrifugation at 15,000 x g for 30 min at 4°C. The insoluble fraction containing over-expressed DnaE905h-mut was denatured in solublization buffer *(50 mM HEPES (pH 7.0), 1 M NaCl, 40 mM imidazole, 6Murea)*

# Affinity Column For On-column Re-folding And Complex Formation

A 5ml Nickel based HisTrap column (GE Healthcare) was used for Nickel-based affinity purification. The column was equilibrated with 10-column volume of Binding buffer *(50 mM HEPES (pH 7.0), 1 M NaCl, 40*

*mM imidazole, 6Murea)*. 10 ml of urea-denatured DnaE905hM was injected for binding, and the column was washed with 10-column volume of Re-folding buffer *(50 mM HEPES (pH 7.0), 1 M NaCl, 40 mM imidazole)* to achieve on-column refolding through a decreasing gradient of denaturant (programmed on AKTA Prime Plus system-GE Healthcare). 10ml of either clarified soluble fraction containing β-clamp was injected in the column manually at the rate of 0.5ml/min. The column was further washed in 10 column volume of Re-folding buffer and the complex was eluted in 5 column volume of elution buffer *(50 mM HEPES (pH 7.0), 1 M NaCl, 300 mM imidazole)* (programmed on AKTA Prime Plus system-GE Healthcare.

# Size Exclusion Chromatography

A 26/60 Superdex 200 preparative column (GE Healthcare) was equilibrated in size exclusion buffer (HEPES 50 mM NaCl 250 mM ) and run according to Method programmed on AKTA Prime Plus system-GE Healthcare.

**SDS-PAGE and Western Blot analysis.** 10% poly-acrylamide gels (Severn Biotech) were set in 1.0 mm cassettes (Invitrogen) 1 X SDS running buffer was used. 15 µl of each protein sample was mixed with 5 µl of gel loading dye boiled and run. The program was set at 125 Volts for 1 hour 20 min. For western blotting. *Xcell II Blot* Module (Invitrogen) was used for western blotting the. The protein bands were transferred to nitrocellulose membrane in 1 X transfer buffer at 25 Volts for 1 hour 30 min. 10 ml of 5% milk protein in phosphate buffer saline (PBS)-Tween 20 were used as blocking buffer for 15 min. For 6xHisTagged *DnaE905hM* the blot was incubated with 1/1000 diluted alkaline-phosphatase- conjugated Mouse Anti-Hexa-His antibodies (Sigma) for 1 hour. The blot was washed with 10-15ml of PBS-Tween 20, three times for 5 min each time, and once with PBS. The blot was then developed using BCIP/NBT substrate (Sigma). For β-clamp the blot was incubated with 1.5 µl of rabbit polyclonal anti β-clamp IgG antibody (Primary antibody – donated by Dr. Jody Winter) for 1 hour. The blot was washed in a similar way as stated above. 1.5 µl of donkey anti-rabbit IgG alkaline phosphatase-conjugated antibodies (Secondary antibody) were then added. Once again the washing was achieved in a similar way. The blot was then developed using BCIP/NBT substrate (Sigma)

# Results

**Over expression of proteins.**

Initial attempts were made to over-express and purify DnaE905 singly, to combine with purified β-clamp for complex formation. For this the B834 (DE3) expression strains were transformed with pAP*dnaEΔ905h*. Freshly transformed cells were grown in 50ml LB broth containing appropriate antibiotics to OD600 ~ 0.6 at 37ºC and induced with 0.1mM IPTG at 25ºC for 4 hours. After induction a slight decrease in the cell growth rate was observed, as can be expected due to the burden on the cell of over-expression of most proteins. However, given the fact that DnaE905h contains two β-clamp binding motifs for β-clamp binding, this observation also raises the possibility of interference of the replication process due to sequestering of native β-clamp by over-expressed DnaE905h. The cells were lysed by

sonication in buffer containing 50 mM HEPES and 200 mM NaCl and the soluble fraction was obtained. The pre- and post-induction, soluble and insoluble samples were analyzed by SDS PAGE (Fig. 1). The DnaE905h was found in the insoluble fractions, presumably either due to rapid accumulation of over-expressed protein or its intrinsic nature, having been previously characterized as being unstructured by (5). Various induction and temperature conditions were therefore trialed but none of these yielded a soluble form of DnaE905h. The dnaE905h was therefore co-expressed with dnaN using co-expression strategy explained in (16) (19). As expected both proteins were seen in soluble fraction (Fig. 1). Maximum soluble expression was seen either after 4hrs or overnight induction with 0.1mM IPTG at $25^{O}C$, suggesting that the co-expressed β-clamp in the cells enhances the solubility of DnaE-CTD, presumably as a consequence of protein-protein interaction.

# Purification Of Dnae905h In Complex With β-clamp

Purification of DnaE905h in complex with β-clamp was initially attempted at small-scale using Talon affinity resin using a batch method. The flow through, washes and elution were analyzed by SDS-PAGE. However a desired co-elution of DnaE905h in complex with β-clamp was not observed. The DnaE905h were mostly retained on the resin and the presence of high amounts of β-clamp in the washes suggested that the interaction between DnaE-CTD with β-clamp was not sufficiently stable for co-elution to occur (Fig. 1).

# Purification Of Dnae905hm In Complex With β-clamp

To overcome this obstacle, the internal β-binding sequence of DnaE905h was replaced with its consensus sequence (QL[S/D]LF), which has previously been shown to enhance the interaction (13) (19). Mutant version termed dnaEΔ905hM was constructed via site-directed mutagenesis as explained in (16) (19). A DnaE905hM/β2-clamp complex was obtained following Talon purification (Fig. 1).The batch affinity purification was scaled up and the resulting complex was applied to a size exclusion column (*Superdex 26/60*). The traces obtained showed a sharp void peak which was analyzed by SDS-PAGE and western blot using anti6xHis and anti-β-clamp specific antibodies. The results demonstrated the presence of both DnaE905hM and β-clamp in the void volume as opposed to the expected position of a complex at ~ 180 ml predicted to contain one intact β-clamp (~ 81.2 kDa) and one DnaEΔ905hM (~ 26 kDa). (Fig. 2)

Realizing the importance of the OB-fold of DnaE905hM in stabilizing its interaction with β-clamp (20), SsDNA or primed DNA was assumed to play role in this enhanced interaction. The void peak was therefore analyzed for the presence of DNA using Agarose gel electrophoresis as explained in (16), as expected this confirmed the presence of DNA in the fractions corresponding to void peak (Fig. 3). In order to restrict the involvement of DNA in the aggregate formation the DNA removal was attempted through enzymatic digestion of contaminating DNA in the eluent after affinity chromatography. After treating the eluent from affinity chromatography with a commercial nuclease, Benzonase (Novagen) a dramatic decrease in the absorbance of the peak at 280 nm was seen (Fig. 2), suggesting a reduction in

contaminating DNA which absorbs strongly at this wavelength. This is consistent with the observed increase in absorption in fractions corresponding to lower molecular weight species. Despite various optimization attempts no defined peak corresponding to the DnaE905hM/β-clamp complex was observed.

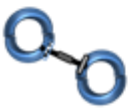
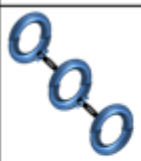
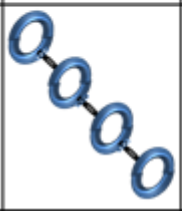
### On column refolding of DnaE905hM and binding of the β- clamp.

To overcome the problem of aggregate formation due to DNA binding, a refolding strategy for DnaE905hM was adopted, followed by on-column binding of the β- clamp. The insoluble fraction containing over-expressed DnaEΔ905hM was solubilized in buffer containing 6 M urea and bound to a nickel affinity column under denaturing conditions which was postulated to result in removal of contaminating DNA. Refolding was achieved on the column by gradually decreasing the urea concentration. Clarified lysate from cells over-expressing un-tagged β-clamp was then injected on to the column. The complex was eluted in buffer containing 300mM imidazole 50mM HEPES and 200mM NaCl. The analysis through SDS PAGE suggested the presence of both DnaE905hM and β- clamp (Fig. 4).

The fractions 10–16 were pooled and applied to a 10/300 Superdex 200 column. The size exclusion trace following refolding demonstrated increase in the UV absorption over a wide range of elution volumes (150–220 ml) with a defined peak at 173 ml. A relative reduction in the absorption of the void peak was also demonstrated, suggesting a reduction in the aggregate formation (Fig. 5). Elution volumes for individual components and expected complexes are calculated in Table 2. The most likely composition of complexes would be a single β-clamp dimer (~ 81.2 kDa) with one DnaE905hM (~ 26 kDa) predicted to elute at ~ 180 ml (equating to fraction 19) and a complex of β-clamp dimer and two molecules of DnaE905hM, predicted to elute at ~ 175 ml. A sharp peak is observed eluting at 173 ml, with more poorly defined, broader peaks eluting at 155, 205 and 220 ml. Analysis is complicated by a number of contaminating proteins presumably introduced with the lysate containing over-expressed clamp. The SDS-PAGE analysis shows the most intense band for β-clamp in fraction 21 and DnaE905hM in fraction 22, presumably reflecting monomeric forms of each protein (Fig. 5). Western blot analysis indicated the presence of fainter bands of both the β-clamp and DnaE905hM throughout fractions 15 to 19, covering a wide range of potential stoichiometries of complex (Fig. 5).

### Table 2

Elution volumes and corresponding fractions are calculated for individual components and expected complexes on a Superdex 26/60 column calibrated with known molecular weight standards.

| Expected complexes and individual components | Description | MW (kDa) | Superdex column (26/60) | | Expected complexes and individual components | Description | MW (kDa) | Superdex column (26/60) | |
|---|---|---|---|---|---|---|---|---|---|
| | | | A | B | | | | A | B |
|  | β-clamp monomer | 40.6 | ~205 | 21 |  | β-clamp dimer and DnaE905hM | 107.2 | ~180 | 19 |
|  | β-clamp dimer | 81.2 | ~190 | 20 |  | β-clamp dimer and 2 x DnaE905hM | 133.2 | ~175 | 18 |
|  | DnaE905hM | 26 | ~217 | 22 |  | 2 x β-clamp dimers and DnaE905hM | 188.4 | ~170 | 18 |
|  | β-clamp monomer and DnaE905hM | 66.6 | ~200 | 21 |  | 3 x β-clamp dimer and 2 x DnaE905hM | 295.6 | ~160 | 17 |
|  | β-clamp dimer and DnaE905hM | 107.2 | ~180 | 19 |  | 4 x β-clamp dimer and 3 x DnaE905hM | 402.8 | ~150 | 16 |

# Discussion

### Complexes of variable stoichiometry are formed between DnaE905hM and β-clamp in the absence of DNA.

Given the dynamic action and complexity of the replication process, structural information on the interacting regions for the various replisome subunits is key to understanding the highly co-ordinated processivity and polymerase switches during replication. Since β-clamp has previously been purified (9) therefore, initial attempts were made to purify the DnaE905 of pol III. To facilitate purification and detection the 6xHis-Tag was fused to N-terminus of DnaE905. Despite various purification attempts the 6xHis-Tagged-DnaE905 was found in the insoluble fraction. The co-expression of DnaE905h with β-clamp was therefore attempted and shown to solubilize the DnaE905h, presumably due to protein-protein interaction providing a stabilizing effect on this largely disordered domain. Subsequent purification of the complex proved problematic due to complex instability during the purification process. Complex stability was achieved by mutating iCBM of DnaE905h (DnaE905hM), however the purification was hindered due to contaminating DNA resulting in the formation of larger aggregates. A refolding strategy for DnaE905hM, followed by on-column binding of the β-clamp permitted the removal of DNA, however increase in the absorption over a wide range of fractions, and analysis of those fractions suggested the formation of complexes of variable stoichiometry between DnaE905hM and β-clamp in the absence of DNA. Although in the absence of DNA the over all affinity of OB-mutant and WT polymerase for β-clamp

is shown to remain same (20), however data presented by (13) suggest that affinity of eCBM to β-clamp is lesser than iCBM in the absence of DNA. The purification of complex in the absence of DNA may well result in the inability of the eCBM to interact with β-clamp. This could result in a complex of DnaE905hM with β-clamp (MW. 107.2 kDa) utilizing only one of its β-clamp binding sites, described in table 2. Due to their same molecular weight as for the one utilizing both β-clamp binding sites, both types of complexes were predicted to elute in the same fraction i.e. fraction 19 (Table 2). On the other hand the vacant protein-binding site on that β-clamp perhaps might recruit another DnaE905hM as described in table 2. This was predicted to elute in the fraction (Fraction 18 on 26/60 Superdex 200). SDS PAGE and western blot analysis demonstrated the presence of both DnaE905hM and β-clamp in fraction 18, however SDS PAGE showed the presence of various contaminating protein in fraction 18

# Declarations


### Funding :

The project was funded by HEC Pakistan and University of Sindh, Jamshoro, and executed in the School of Biology, Queens Medical Centre, University of Nottingham, Nottingham, NG7 2UH, UK.


### Conflicts of interest/Competing interests :

The authors of the manuscript declare no conflict of interest.

### Availability of data and material :

The project was executed in the School of Biology, Queens Medical Centre, University of Nottingham, Nottingham, NG7 2UH, UK.

### Code availability :

Not Applicable

### Authors' contributions :

A.A. Patoli designed the project. Cloning of various constructs i.e. experimental work was done by A.A. Patoli and B. Patoli. Both the authors contributed in writing the manuscript.

### Ethics approval :

Authors declare that the current research work does not involve any human participant or animal model.

### Consent to participate :

Yes

### Consent for publication

Yes

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# Figures

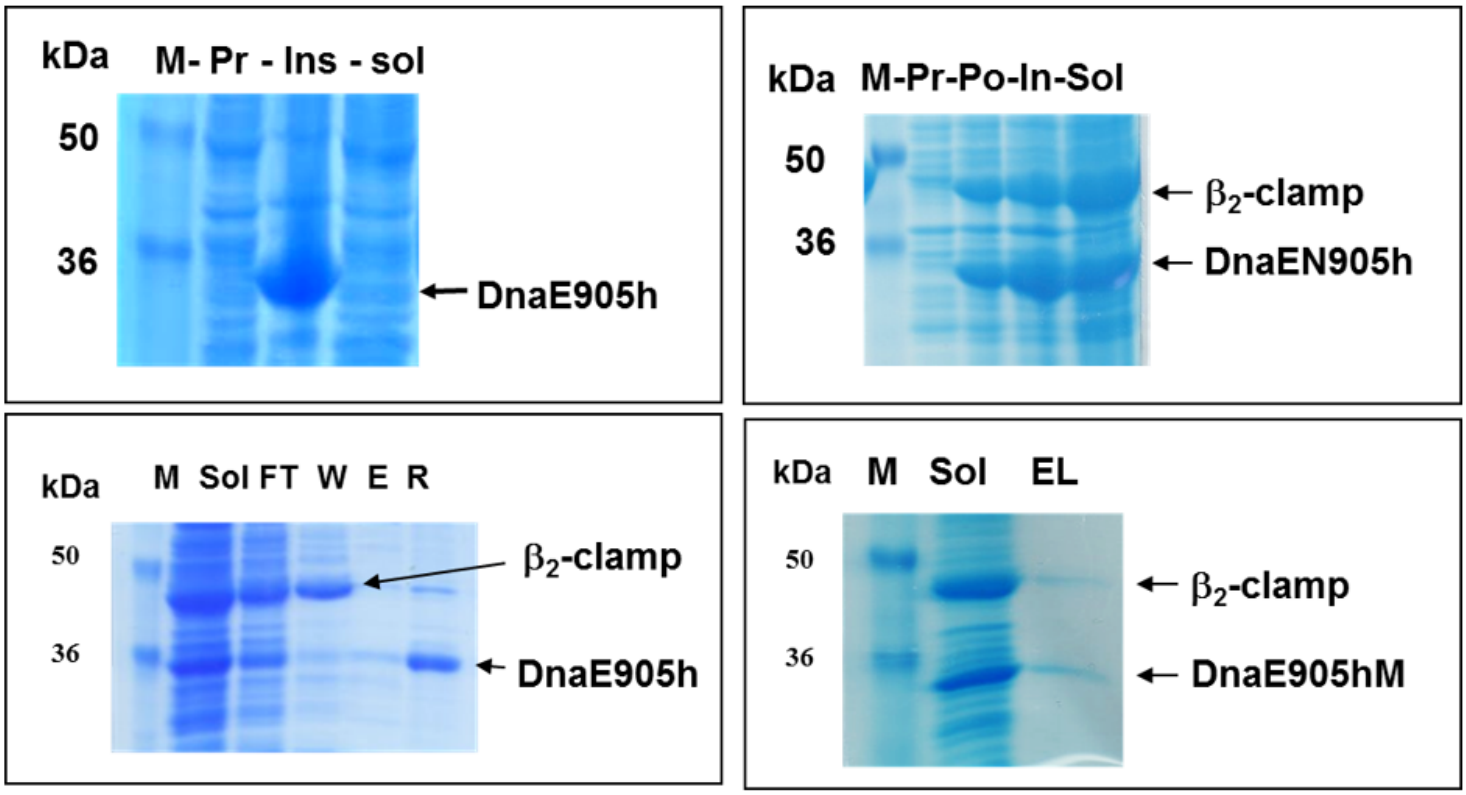



**Figure 1**

(Top Left) SDS-PAGE gel showing the soluble and insoluble fractions of DnaE905h. (Top Right) SDS-PAGE gels showing the co-expression of DnaE905h and β– clamp. (Bottom Left) SDS-PAGE gel showing the retention of DnaE905h on Talon resin and dissociation of β-clamp from the complex during the washing steps (Bottom Right) SDS PAGE gels showing the presence of of DnaE905hM and β-clamp in the eluent after affinity chromatography. M=Marker, Pr = Pre-induction, Po = Post Induction, Ins = Insoluble fraction, Sol=Soluble fraction, FT= Flow through, S=Soluble fraction, W=Wash, E=Eluent, R=Resins after elution.

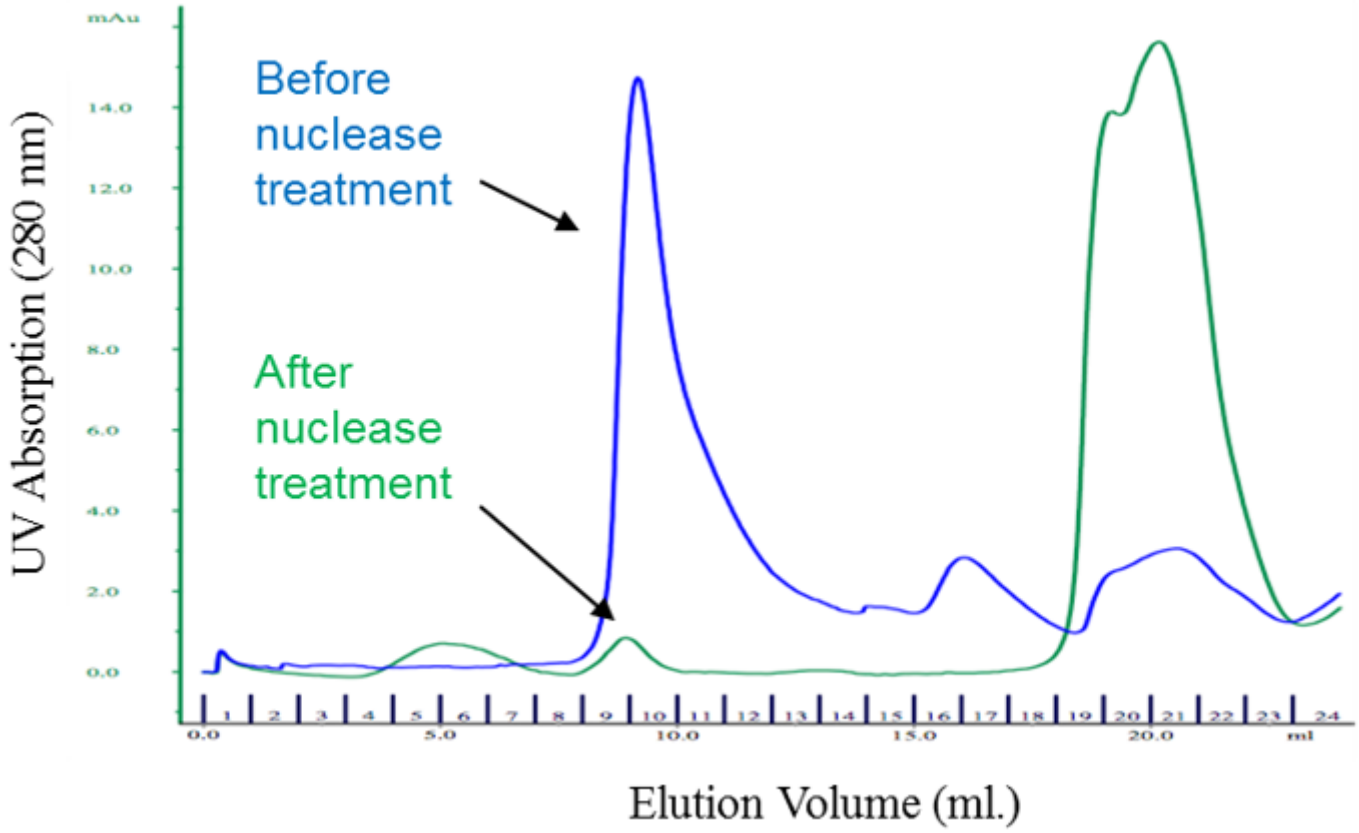
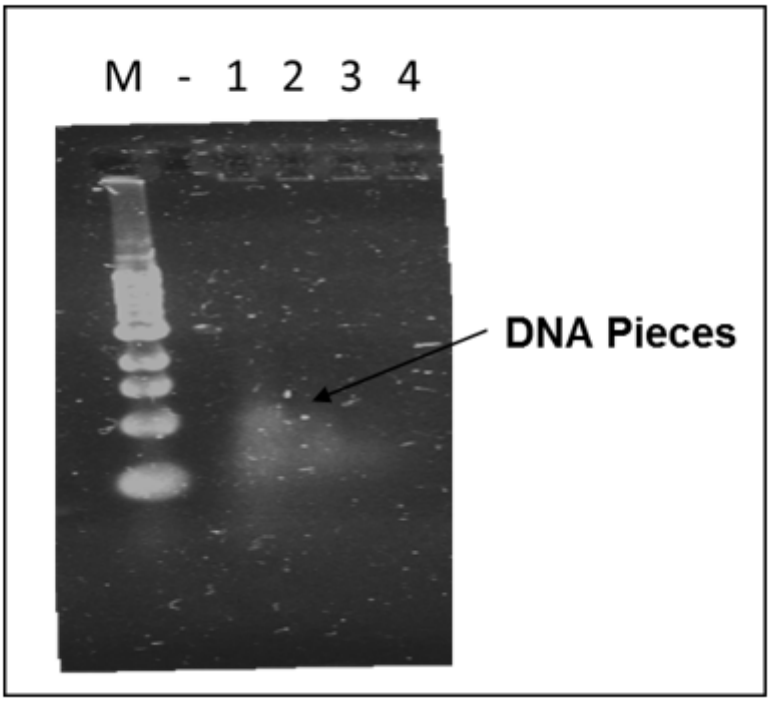


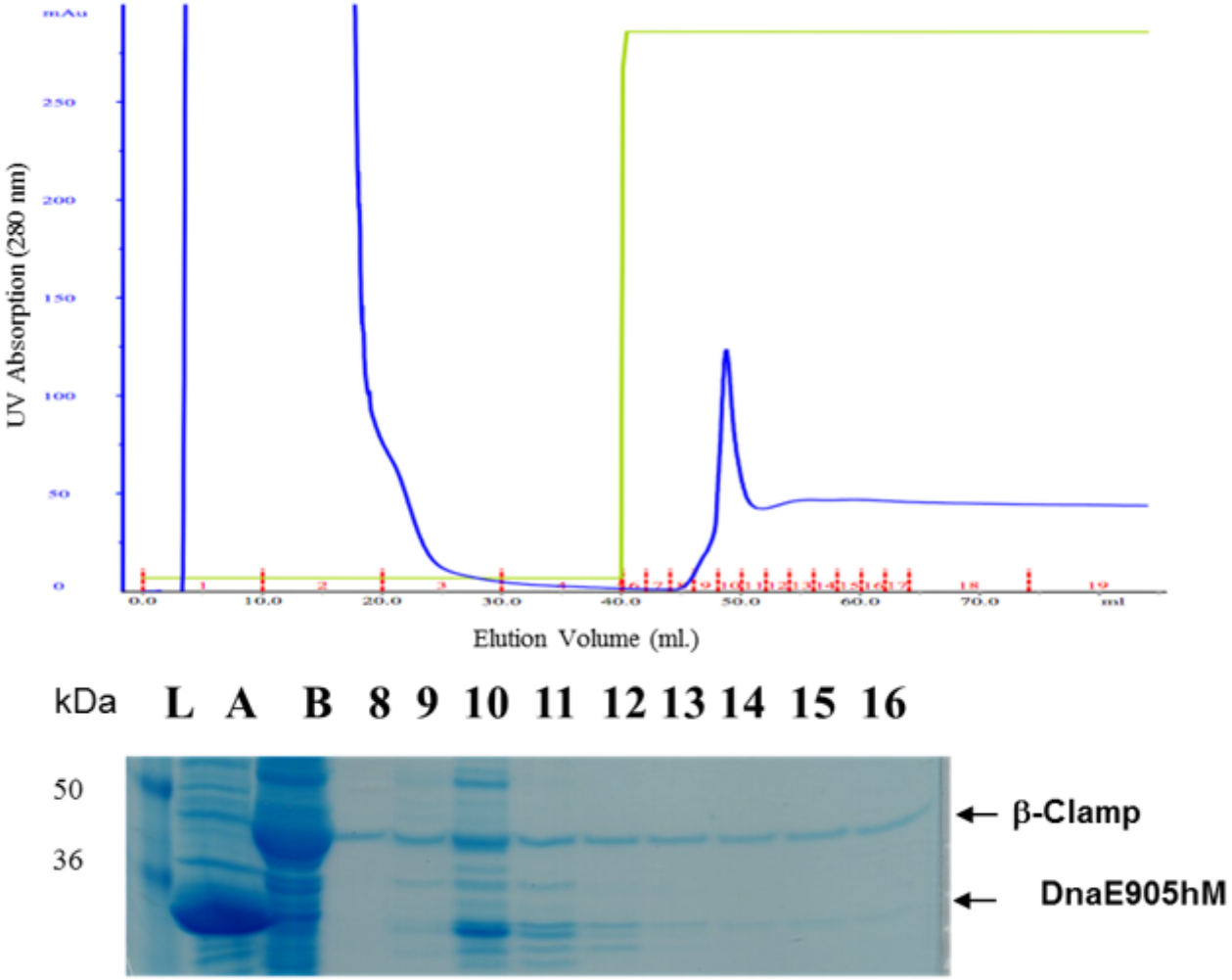

**Figure 2**

(Blue) The size exclusion chromatography traces of DnaE905hM in complex with β-clamp demonstrating the void peak. (Green) The size exclusion chromatography traces demonstrating the degradation of DNA by nuclease (Benzonase). The decrease in the absorbance of void peak suggests the nuclease degrades co-eluting DNA.



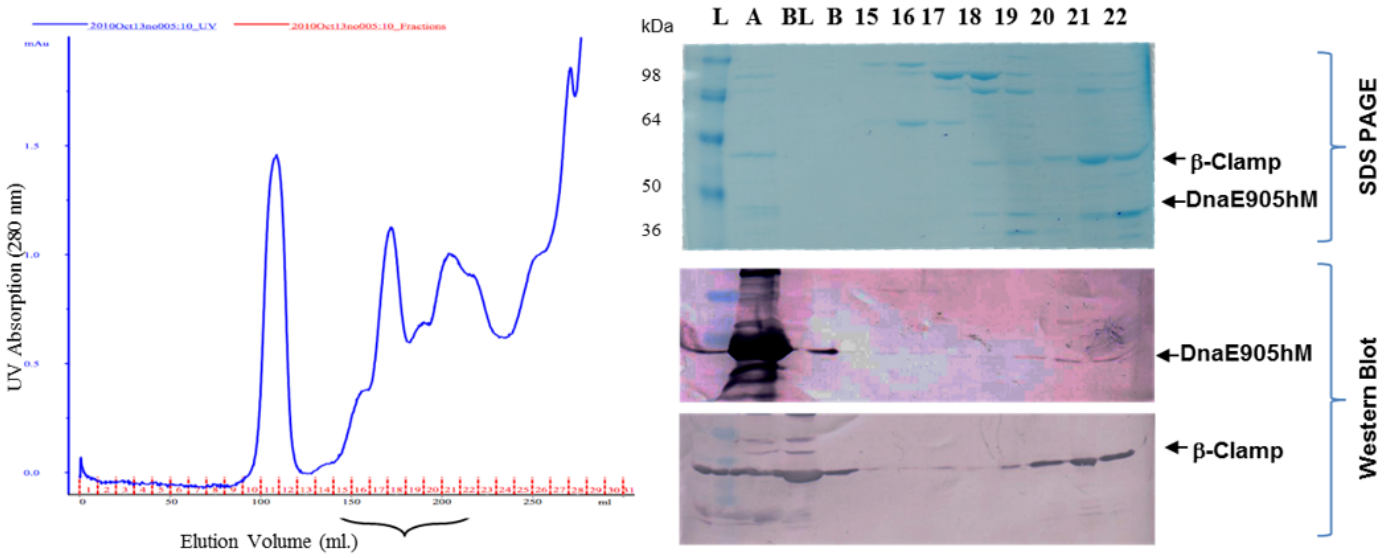

## Figure 3

1% agarose gel confirming the presence of DNA in the void volume. M=Marker, E=Eluent, Fractions 1-4.




## Figure 4

(Top) The chromatography traces from nickel affinity column showing absorption peak at fraction 10. (Bottom) SDS PAGE gels showing presence of DnaE905hM and β-clamp in the eluent after affinity chromatography.




## Figure 5

(Left) A trace from a size exclusion column for purification of DnaE905hM/ β-clamp complex demonstrating an increase in the UV absorption over a wide range of volume. (Right) SDS PAGE gel to show the presence of DnaE905h-mut with β-clamp over a range of fractions and Western blots to demonstrate the presence of DnaEΔ905hM and β-clamp using anti-His and anti-β antibodies respectively