

COMMUNICATION

# A simplified method for reconstituting active *E. coli* DNA polymerase III

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## ABSTRACT

Genome duplication in *E. coli* is carried out by DNA polymerase III, an enzyme complex consisting of ten subunits. Investigations of the biochemical and structural properties of DNA polymerase III require the expression and purification of subunits including  $\alpha$ ,  $\epsilon$ ,  $\theta$ ,  $\gamma$ ,  $\delta'$ ,  $\delta$ , and  $\beta$  separately followed by *in vitro* reconstitution of the pol III core and clamp loader. Here we propose a new method for expressing and purifying DNA polymerase III components by utilizing a protein co-expression strategy. Our results show that the subunits of the pol III core and those of the clamp loader can be co-expressed and purified based on inherent interactions between the subunits. The resulting pol III core, clamp loader and sliding clamp can be reconstituted effectively to perform DNA polymerization. Our strategy considerably simplifies the expression and purification of DNA polymerase III and provides a feasible and convenient method for exploring other multi-subunit systems.

**KEYWORDS** *E. coli*, DNA polymerase III, coexpression, purification

## INTRODUCTION

Genetic information stored in genomic DNA must be duplicated from generation to generation and that is exactly where DNA replicase finds its place in the cell. Much of what is known about DNA replicase comes from investigation of replicases from prokaryotic and eukaryotic model organisms (Johnson and O'Donnell, 2005). Replicative DNA

polymerases of several organisms have been successfully reconstructed *in vitro* (Bruck and O'Donnell, 2000; Bruck et al., 2002; Bullard et al., 2002; Bruck et al., 2005).

In *E. coli*, replicative DNA polymerase III (referred to hereafter as DNA pol III) consists of three components, namely the pol III core, a sliding clamp and a clamp loader (Bruck and O'Donnell, 2000). The pol III core is made up of an  $\alpha$  subunit encoded by *dnaE*, an  $\epsilon$  subunit encoded by *dnaQ* and a  $\theta$  subunit encoded by *holE*. The  $\beta$  clamp encoded by *dnaN* is a ring-shaped dimer that encircles and slides along the DNA. The association of the pol III core with the  $\beta$  clamp makes it possible for pol III to replicate with speed and processivity. The clamp loader consists of five subunits *in vivo*, namely the  $\gamma$  (*dnaX*),  $\delta$  (*holA*),  $\delta'$  (*holB*),  $\chi$  (*holC*), and  $\psi$  (*holD*) subunits; however, *in vitro*, the  $\gamma$ ,  $\delta$  and  $\delta'$  subunits are sufficient for clamp loading (Naktinis et al., 1995; Onrust et al., 1995a, 1995b; Stukenberg and O'Donnell, 1995; Xiao et al., 1995).

Biochemical and structural studies on *E. coli* DNA pol III require expression and purification of all of the subunits, one at a time. The subunits obtained are then reconstituted *in vitro* to form the pol III core and the clamp loader. Here, we propose an alternative method for expressing and purifying pol III components based on the inherent interactions of their member subunits. A protein coexpression strategy (Tolia and Joshua-Tor, 2006) was applied in this study in an attempt to simplify the expression and purification process. Results showed that the purified pol III core, clamp loader and sliding clamp are jointly capable of performing DNA polymerization, showing that our method can successfully reconstitute *E. coli* DNA polymerase III. This method may be a useful tool for investigating DNA pol III in other organisms and for reconstituting other complex biochemical systems.

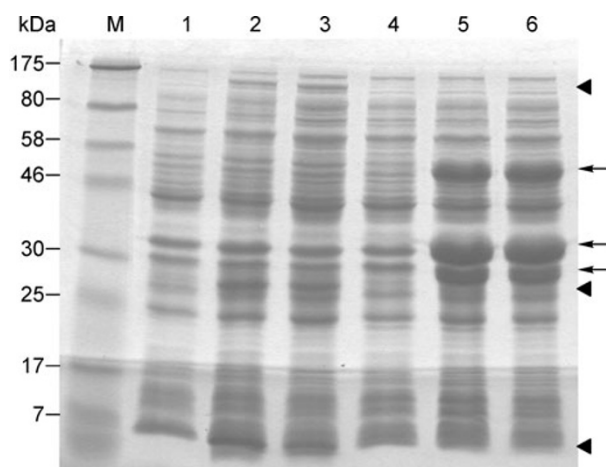
## RESULTS

## Construction of pol III core and clamp loader expression systems

We utilized Duet vectors (Merck) to coexpress the pol III core and clamp loader in this study. Both the pol III core and clamp loader consist of three genes; thus two coexpression vectors, pACYCDuet-1 and pETDuet-1, were used to construct plasmids for protein expression of each complex. To construct pol III core expression vectors, *dnaE* and *dnaQ* were cloned into the MCS-1 of pACYCDuet-1 and pETDuet-1, respectively. Appropriate selection of restriction sites ensured that HisTags were preserved for use in Ni-NTA affinity chromatography. *holE* was cloned into the MCS-2 of pETDuet-1, and lacked a HisTag or STag. As a result, purification of the  $\theta$  subunit (*holE*) depends on its binding to the  $\alpha$  (*dnaE*) or  $\epsilon$  (*dnaQ*) subunit. Likewise, *dnaX* and *holA* were cloned into the MCS-1 of pACYCDuet-1 and pETDuet-1, respectively. *holB* was cloned into the MCS-2 of pETDuet-1, and lacked a HisTag or STag. Similarly, purification of the  $\delta'$  subunit relies on its association with the  $\gamma$  or  $\delta$  subunit.

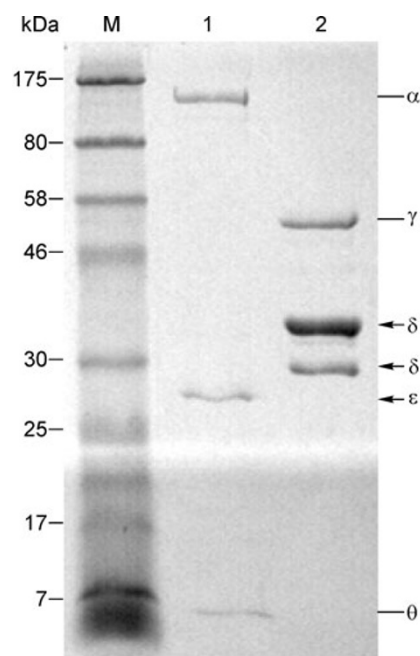
## Expression and purification of the pol III core and clamp loader

A small-scale expression test showed that the subunits of the pol III core or the clamp loader can be coexpressed in BL21 (DE3) in a soluble form (Fig. 1), and that the apparent



**Figure 1. Bacterial expression of *E. coli* DNA pol III core (lanes 1–3) and clamp loader (lanes 4–6).** In both groups of lanes, the first is the whole cell lysate of the uninduced sample (lanes 1 and 4), the second is the whole cell lysate of the IPTG-induced sample (lanes 2 and 5), and the third is the soluble fraction of the induced sample (lanes 3 and 6). The triangles on the right indicate the positions of the  $\alpha$ ,  $\epsilon$  and  $\theta$  sub-units, and the arrows show the positions of the  $\gamma$ ,  $\delta$  and  $\delta'$  subunits. The gel consisted of three layers of different gel concentrations (5%, 12%, and 15%) from top to bottom.

molecular weight of each subunit was consistent with its corresponding theoretical molecular weight. If these soluble subunits could associate *in vivo* in a manner strong enough to withstand Ni-NTA purification, then the expression, purification and reconstitution of the pol III core and clamp loader would be greatly simplified. To test this, we performed Ni-NTA purification of the pol III core followed by the clamp loader. Results showed that the pol III core and clamp loader are both capable of being co-purified (Fig. 2), suggesting that interactions among the  $\alpha$ ,  $\epsilon$  and  $\theta$  subunits and interactions among the  $\gamma$ ,  $\delta$  and  $\delta'$  subunits are strong.



**Figure 2. SDS-PAGE of the purified *E. coli* DNA pol III core and clamp loader.** Lane M: protein marker; lane 1: pol III core; lane 2: clamp loader. The gel consisted of three layers of different gel concentrations (5%, 12%, and 15%) from top to bottom.

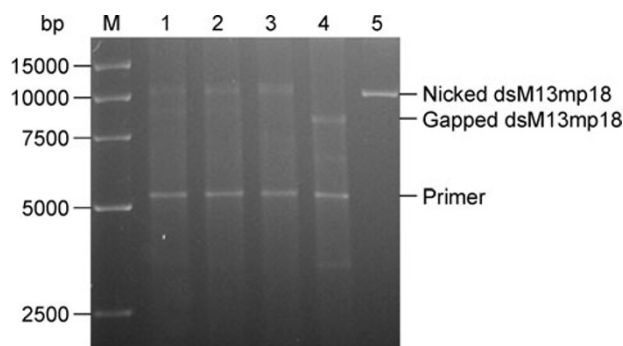
During the purification process, it is important to adjust the NaCl and imidazole concentrations to optimize removal of unwanted proteins. Some of these unwanted proteins bind nonspecifically to the Ni-NTA resin, while others associate with target proteins via protein-protein interactions. In our experiments non-targeted proteins were largely removed since interactions of target proteins with the resin and interactions among subunits were much stronger than non-specific interactions.

Stoichiometry of the subunits is another important factor, which cannot be controlled during the expression and purification process. We tried to further purify the complex using MonoQ (GE Healthcare), but unfortunately the subunits underwent degradation during purification. Considerable

effort is required to overcome or reduce degradation during subsequent purification processes.

### DNA polymerization activity assays for the purified pol III core and clamp loader

To test the DNA polymerization activity of the obtained pol III core and clamp loader, a gap filling experiment was conducted. The DNA substrate used was a double-stranded M13mp18 with a gap of about 2 kb. The  $\beta$  clamp, clamp loader and pol III core were added in turn to the reaction system to fill the gap in the substrate DNA. Differences in migration rates between gapped and nicked DNA during electrophoresis make it possible to monitor gap filling activity (Fig. 3). As the activity of the purified pol III core and clamp loader were quite strong, 5 nmol/L of pol III core and clamp loader was sufficient for the gap filling activity assay. Figure 3 shows that most of the gapped DNA was filled to the nicked form after 2 h of incubation at 37°C, confirming that the pol III core and clamp loader obtained in this study had DNA polymerization activity.



**Figure 3. Filling in of the gapped dsM13mp18 by *E. coli* DNA pol III components.** M: DL15000; lanes 1, 2 and 3: DNA substrate after filling with 5 nmol/L of the pol III core, 5 nmol/L of the clamp loader and 10 nmol/L of  $\beta$  for 30 min, 1 h and 2 h, respectively; lane 4: DNA substrate; lane 5: nicked dsM13mp18. Primer: the larger fragment of dsM13mp18.

## DISCUSSION

In this report, we have presented a coexpression and purification strategy for obtaining *E. coli* DNA pol III core and clamp loader and a gap filling experiment that verifies its catalytic activity. The simplicity and effectiveness of our strategy provides a useful tool for studying DNA pol III from other bacteria.

In each of the complexes of three subunits, one subunit ( $\theta$  in pol III core and  $\delta'$  in clamp loader) bears no affinity tag. The subunits associate strongly *in vivo* during expression to form a complex that is capable of withstanding the ensuing harsh purification process. The copy numbers of pACYCDuet-1 (10–12 per cell) and pETDuet-1 (about 40 per cell) are known to be different (Merck). In addition, the mRNA stability of each

gene and the liability of each subunit to degradation might also be different. Thus, it is quite unlikely that the actual production of each subunit will be the same. In our experiment, the HisTags of the two subunits are designed for Ni-NTA purification while the protein without a HisTag helps to balance stoichiometry during Ni-NTA purification.

The strategy is also flexible in that for coexpression and purification of either of the triple subunits, one subunit can be removed during plasmid construction without affecting purification as two of the three subunits have affinity tags. The subunit can then be replaced by its counterpart from other bacteria during the gap filling experiment to test the interchangeability of the subunits from two DNA pol III systems. This system provides an alternative strategy when *in vivo* complementation experiments for the DNA pol III subunit do not work (Wechsler and Gross, 1971; Horiuchi et al., 1978; Strauss et al., 2004).

## MATERIALS AND METHODS

### Construction of expression vectors

*dnaE* (GeneID:6061238) was amplified from *E. coli* K-12 with an upstream primer (5'-CGCGGATCCGATGTCTGAACCACGT-3' containing a *Bam*HI site) and a downstream primer (5'-CGAGCTCTAGTCAAACCTCCAGTT-3' containing a *Sac*I site). The PCR product was digested with *Bam*HI and *Sac*I and ligated to the MCS-1 of pACYCDuet-1 (Merck) to produce pACYCDuet- $\alpha$ . *dnaQ* (GeneID:6061081) was amplified from *E. coli* K-12 with an upstream primer (5'-CGGAATTCGATGAGCACTGCAATT-3' containing an *Eco*RI site) and a downstream primer (5'-GGGAAGCTTTATGCTCGCCAGA-3' containing a *Hind*III site). The PCR product was digested with *Eco*RI and *Hind*III and ligated to the MCS-1 of pETDuet-1 (Merck) to produce PETDuet- $\epsilon$ . *holE* (GeneID:6060582) was amplified from *E. coli* K-12 with an upstream primer (5'-GGAATTCATATGCTGAAGAATCTGG-3' containing an *Nde*I site) and a downstream primer (5'-CGGCTCGAGTTATTTAAGTTTGGGC-3' containing an *Xho*I site). The PCR product was digested with *Nde*I and *Xho*I, and ligated to the MCS-2 of PETDuet- $\epsilon$  to produce PETDuet- $\epsilon\theta$ . After the above three genes were confirmed by DNA sequencing, pACYCDuet- $\alpha$  and PETDuet- $\epsilon\theta$  were co-transformed into *E. coli* BL21(DE3) competent cells to produce BL21(DE3)/Duet  $\alpha\epsilon\theta$  for expressing the pol III core.

*dnaX* (GeneID:6059726) was amplified from pQE30-*dnaX* (constructed previously by Li et al. (2008)) with an upstream primer (5'-CGGAATTCGATGAGTTATCAGGT-3' containing an *Eco*RI site) and a downstream primer (5'-GGGAAGCTTTCATTCCTTTTTTG-3' containing a *Hind*III site). The PCR product was digested with *Eco*RI and *Hind*III, and then ligated to the MCS-1 of pACYCDuet-1 to produce pACYCDuet- $\gamma$ . The *holA* (GeneID:6061561) was amplified from pQE30-*holA* (constructed previously by Li et al. (2008)) with an upstream primer (5'-CGGAATTCGATGATTCGGTTGTA-3' containing an *Eco*RI site) and a downstream primer (5'-GGGAAGCTTTCACCGTCGATAA-3' containing a *Hind*III site). The PCR product was digested with *Eco*RI and *Hind*III, and ligated into pETDuet-1 to produce pETDuet- $\delta$ . *holB* (GeneID:6058796) was amplified from pQE30-*holB* (constructed previously by Li et al. (2008)) with an upstream primer (5'-GGAATTCATATGATGATGGTATCCAT-3' with

an *NdeI* site) and a downstream primer (5'-CGGCTCGAGTCAAA-GATGAGGAA-3' with an *XhoI* site). The PCR product was digested with *NdeI* and *XhoI*, and then ligated to the MCS-2 of pETDuet- $\delta$  to produce pETDuet- $\delta\delta'$ . After the above three genes were confirmed by DNA sequencing, pACYCDuet- $\gamma$  and pETDuet- $\delta\delta'$  were co-transformed into *E. coli* BL21(DE3) to produce BL21(DE3)/Duet $\gamma\delta\delta'$  for expressing the clamp loader.

M15/pQE30-*dnaN* (GeneID: 6058510, constructed previously (Li et al., 2008)) was used to express the  $\beta$  clamp.

### Expression and purification of *E. coli* DNA pol III components

For expression of the pol III core, BL21(DE3)/Duet  $\alpha\epsilon\theta$  was streaked onto a solid LB plate containing 100  $\mu\text{g}/\text{mL}$  ampicillin and 34  $\mu\text{g}/\text{mL}$  chloramphenicol and incubated overnight at 37°C. Cells from a single clone were then grown in LB in the presence of 100  $\mu\text{g}/\text{mL}$  ampicillin and 34  $\mu\text{g}/\text{mL}$  chloramphenicol at 37°C. As the OD<sub>600</sub> reached about 0.8, 2 mL of the bacterial culture was inoculated in 200 mL LB in the presence of 100  $\mu\text{g}/\text{mL}$  ampicillin and 34  $\mu\text{g}/\text{mL}$  chloramphenicol at 37°C and cultured until the OD<sub>600</sub> reached about 0.8. IPTG was added to a final concentration of 0.4  $\mu\text{mol}/\text{L}$  and induction continued for 3 h. Cells were collected by centrifuging at 4000 *g* for 10 min at 4°C, and were then re-suspended in binding buffer (20 mmol/L Tris-Cl, pH 7.9; 500 mmol/L NaCl; 5 mmol/L imidazole), lysed by ultrasonication and centrifuged with a SS-34 rotor at 16,000 rotation/min for 30 min at 4°C. The supernatant was loaded onto a Ni-NTA column (GE Healthcare) equilibrated with binding buffer. 3–5 volumes of binding buffer was applied to equilibrate the column and 3–5 volumes of wash buffer (20 mmol/L Tris-Cl, pH 7.9; 500 mmol/L NaCl; 90 mmol/L imidazole) was used to wash out unwanted proteins. Finally, the target protein was eluted with 3–5 volumes of elution buffer (20 mmol/L Tris-Cl, pH 7.9; 500 mmol/L NaCl; 250 mmol/L imidazole). The eluted proteins were concentrated via ultra-filtration and stored in storage buffer (50 mmol/L Tris-Cl, pH 7.5; 100 mmol/L NaCl; 5 mmol/L DTT; 0.025 mmol/L EDTA; 20% glycerol (*v/v*)) at -20°C. Protein concentration was determined using Thermo Scientific Pierce Coomassie Plus Assay Reagent (Thermo) according to the manufacturer's instructions.

Procedures for the expression and purification of the clamp loader were the same as those for the pol III core, while  $\beta$  clamp was expressed and purified as described by Li et al. (2008).

### Preparation of the gapped dsM13mp18 DNA substrate

The gapped dsM13mp18 DNA substrate was prepared according to the method of Kunkel (Kunkel, 1985) with a minor adjustment. The dsM13mp18 was digested with *EcoRI* and *BsrGI*, and the resultant larger fragment was recycled. Competent annealing was conducted with the larger fragment (in excess) and the ssM13mp18 in SSC buffer, denatured for 10 min at 94°C and slowly cooled to room temperature. The annealed mixture was then utilized as the substrate for DNA pol III polymerization reactions. Nicked dsM13mp18 was prepared as a marker by digesting dsM13mp18 with *Nb.BtsI* (NEB #R0707).

### DNA pol III gap filling experiment

The gap filling reaction was conducted according to the method of Bruck and O'Donnell (Bruck and O'Donnell, 2000) with some

adjustments. The volume of the reaction mixture was 20  $\mu\text{L}$  and contained 20 mmol/L Tris-Cl (pH 7.5), 0.1 mmol/L EDTA, pH 8.0, 5 mmol/L DTT, 40  $\mu\text{g}/\text{mL}$  BSA, 2 mmol/L ATP, 8 mmol/L  $\text{MgCl}_2$ , 4% glycerol (*v/v*) and 60  $\mu\text{mol}/\text{L}$  dNTP, 80 ng DNA substrate, 5 nmol/L pol III core (calculated at a stoichiometry of  $\alpha:\epsilon:\theta = 1:1:1$ ), 5 nmol/L clamp loader (calculated at a stoichiometry of  $\gamma:\delta:\delta' = 1:1:1$ ), and 10 nmol/L  $\beta$ . The mixture was preincubated for 5 min at room temperature and then incubated at 37°C for different lengths of time. The reaction was terminated with 1  $\mu\text{L}$  10% SDS (*w/v*), prior to the addition of 0.5  $\mu\text{L}$  20 mg/mL Proteinase K (NEB) to digest the proteins. The mixture was then subjected to electrophoresis in a 1% agarose gel (*w/v*) in TAE buffer at a constant 18 V for 20 h. The gel was soaked in 0.5  $\mu\text{g}/\text{mL}$  ethidium bromide (Promega) for 10 min and photographed with Alphamager 2200.

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