High-level Expression and Purification of DNA and DNase Free Taq DNA Polymerase

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Authors’ contributions

This work was carried out in collaboration between all authors. Author MNM designed the study, performed the statistical analysis, wrote the protocol and wrote the first draft of the manuscript. Authors JM and SDN managed the analyses of the study and performed the experiments. Authors MNM, SDN and SB managed the literature searches. All authors read and approved the final manuscript.

ABSTRACT

Aim: Taq DNA polymerase from Thermus aquaticus is a key enzyme in the field of molecular biology that has been mostly used in polymerase chain reaction (PCR). Our aim is to produce standard grade Taq DNA polymerase free from censorious impurities like DNase, DNA and other contaminating proteins.

Place and Duration of Study: The experiments were performed at the Molecular Diagnostic Division of Bhat Biotech India (P) Ltd., Bangalore from February 2017 to January 2018.

Methodology: The recombinant Taq DNA polymerase clone was confirmed by PCR and DNA sequencing followed by BLAST analysis. The recombinant protein was in soluble form and was expressed by E. coli DH5α strain. The enzyme was extracted using the boil-lysis method and followed by purification with ion exchange chromatography and silica column chromatography to remove the contaminating protein, DNase and DNA. The yield of the protein was also calculated.

Results: In our laboratory, high-quality Taq DNA polymerase was purified using ion exchange
1. INTRODUCTION

Polymerase Chain Reaction (PCR) and its expanding variants have revolutionised the field of molecular biology and biotechnology and are indispensable in the field of recombinant DNA technology, molecular diagnosis, genetic analysis, forensic studies, etc., This technique ubiquitously requires a thermostable enzyme, Taq DNA polymerase, which has a deoxyribonucleic acid polymerisation ability at high temperature thus, it has a huge market potential.

Taq DNA polymerase was primarily isolated from a thermophilic bacterium, *Thermus aquaticus*. In *T. aquaticus* the expression of native Taq DNA polymerase is quite low but the applications and demand are tremendous hence, later it was cloned into expression vectors which facilitate the production of a large amount of protein in bacterial expression system [1]. The Taq DNA polymerase gene has 2499 base pairs, the full-length protein has about 832 amino acids and its predicted molecular weight is nearly 94 kDa [2]. Several articles are available about the overexpression of Taq DNA polymerase in *E. coli* expression system. Many researchers have also worked on the purification system and tried to obtain pure enzyme, for that several methods has been followed by them to obtain high grade purified enzyme from the heat treated clarified lysate. Their methods included precipitation with polyethyleneimine followed by ion exchange chromatography [3]; precipitation with ammonium sulphate subsequently dialysed with storage buffer [4]; precipitation with ethanol and dissolved in the storage buffer [5], nucleic acid precipitation with polymin P, followed by phenyl sepharose and heparin-sepharose column chromatography [6]; freezing and high temperature thawing of the culture filtrate followed by dialysis against storage buffer [7].

The pitfalls in the Taq DNA polymerase purification system as reported by many researchers are the time consumption, low yield, host contaminating protein and DNA and sometimes nuclease activity. All these strategies must be considered for developing a novel purification protocol to get excellent quality of Taq DNA polymerase. An attempt has been made in our laboratory to purify high-quality Taq DNA polymerase using ion exchange chromatography columns, with a resulting yield of about 45-50 mg/L.

2. MATERIALS AND METHODS

2.1 Confirmation of Clone

The Taq DNA polymerase gene cloned in the pLoxGentrc vector was transformed by heat shock method into *E. coli* DH5α competent cells that were prepared by CaCl₂ method [8]. The transformed cells were plated on Luria Bertani (LB) agar plates containing 100 µg / mL ampicillin and incubated at 37°C overnight. The insert of a clone was confirmed by PCR with gene-specific primers followed by sequencing. The sequences of the forward and reverse gene-specific primers were 5' ATGAGGGGGATGCTGCCCCTCT 3' and 5' TCACCTCTTGCGGAGACCAG 3' respectively. The 2499 bp amplicon was visualised on a 1% agarose gel. The amplicon was gel eluted using Geneasy gel extraction kit (Bhat Biotech India Pvt. Ltd.) and sequenced. The sequence was further confirmed by NCBI BLAST analysis.

2.2Expression and Purification of Taq DNA Polymerase

The transformed colony was inoculated in 200 mL of LB broth supplemented with 100 µg / mL ampicillin, incubated overnight at 37°C in an incubator shaker and used as a primary culture. This culture was used as pre-inoculum to inoculate 2 litres of LB broth containing 100 µg / mL ampicillin in the ratio of 1:10 and incubated at 37°C until the broth reached an OD₆₀₀ of 0.6. The culture was induced with 1.0 mM Isopropyl β-D-1-thiogalactopyranoside (IPTG) and incubated for 16 h at 37°C. The culture was harvested by centrifugation at 8000 rpm for 10 min and washed with 200 mL of Taq buffer A (20 mM Tris, pH 7.9; 50 mM Glucose; 1 mM EDTA). The cells were resuspended in 40 mL of Taq buffer A supplemented with 4 mg / mL lysozyme.

Conclusion: The use of a silica column to remove the residual DNA is a remarkable step in obtaining an unequalled quality of Taq DNA polymerase.

Keywords: Taq DNA polymerase; Thermus aquaticus; DNase; PCR; BLAST; chromatography.

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chromatography columns and silica column, with a resulting yield of about 45-50 mg/L and the activity was found to be 1.5 U/µL.

The use of a silica column to remove the residual DNA is a remarkable step in obtaining an unequalled quality of Taq DNA polymerase.
and incubated at 37°C for 15 min. After cell lysis, cell lysate was loaded onto 12% SDS-PAGE and observed for the presence of Taq DNA polymerase by Coomassie Brilliant Blue (CBB) staining [8]. The cell lysate was subjected to DNase treatment for 30 min at 37°C. Further, 40 mL of Taq buffer B (10 mM Tris, pH 7.9; 50 mM KCl; 1 mM EDTA; 0.5% Tween; 1 mM PMSF) was added and incubated for 1h at 80°C. After that cell lysate was centrifuged at 10,000 rpm for 20 min at 4°C to remove the cell debris. The supernatant was collected and passed through Q-sepharose column pre-equilibrated with Taq Q buffer (25 mM Tris, pH 7.9; 1mM EDTA; 0.5% Tween 20; 10% glycerol; 1 mM dithiothreitol (DTT); 1 mM phenylmethane sulfonyl fluoride (PMSF)) supplemented with 25 mM KCl. The Q-sepharose column was washed with Taq Q buffer containing 25 mM KCl. The protein was eluted with a stepwise gradient of KCl concentration from 25 - 500 mM in Taq Q buffer. The collected fractions were checked on 12% SDS-PAGE followed by silver staining [8] for the presence of Taq DNA polymerase. The fractions containing the protein were pooled and dialysed in Taq S buffer (20 mM (4-2-hydroxyethyl)-1-piperazineethanesulfonic acid) (HEPES), pH 6.9; 1 mM EDTA; 0.5% Tween 20; 10% glycerol; 1 mM DTT; 1 mM PMSF) containing 25 mM KCl. The dialysed sample was loaded onto S-sepharose column pre-equilibrated with Taq S buffer containing 25 mM KCl. The S-sepharose column was washed with 60 mL of Taq S buffer with 25 mM KCl. The protein was eluted with a stepwise gradient of KCl concentration varying from 25 - 500 mM in Taq S buffer. At each step, 2 mL fractions were collected and checked using 12% SDS-PAGE followed by silver staining [8] for the presence of Taq DNA polymerase.

2.3 Purification of Taq DNA Polymerase by Silica Column Chromatography

The fractions containing the protein were pooled and passed through the silica column. The flow-through was collected and analysed for the presence of Taq DNA polymerase using 12% SDS-PAGE. The flow-through was further dialysed against Taq storage buffer (50 mM Tris, pH 8.0; 100 mM NaCl; 0.1 mM EDTA; 1% Triton X 100; 5 mM DTT; 50 % glycerol). The in-house purified protein along with the commercial Taq DNA polymerase was loaded onto 12% SDS-PAGE and silver stained [8] to examine the purity of the purified protein as compared to the commercial one.

2.4 PCR Assay for Bacterial DNA Contamination

A PCR reaction was performed using universal primer for bacterial 16S rDNA and malB (maltose binding protein) primer to check for the E. coli genomic DNA contamination. The 16S rDNA bacterial primers generated a 1487 bp amplicon using forward primer 5’ AGAGTTTATCCTG GCTAG 3’ and reverse primer 5’GTTACCTTGATCGACTT 3’. The PCR was performed using following conditions; initial denaturation at 97°C for 3 min followed by 35 cycles of 94°C denaturation for 1 min, 55°C annealing for 1 min, 72°C extension for 90 sec followed by final extension at 72°C for 10 min. The forward 5’ GATGCGTGACCTGTTTTTA 3’ and reverse primer 5’ ACACCGAATTCCCCC 3’ amplified a target of 491 bp fragment of malB in E. coli genome. The PCR conditions for the amplification of malB included initial denaturation at 97°C for 3 min followed by 35 cycles of 94°C denaturation for 45 sec, 57°C annealing for 45 sec, 72°C extension for 45 sec followed by final extension at 72°C for 10 min.

2.5 DNase Activity Assay

DNase activity assay was carried out with purified Taq DNA polymerase to ensure that the purified protein was free from DNase enzyme. The plasmid was taken as the template to determine the DNase activity of the purified protein. The assay consisted of the following discrete reactions with appropriate controls as follows; (i) plasmid alone as control, (ii) plasmid incubated with DNase buffer as control, (iii) plasmid incubated with DNase enzyme and buffer, (iv) plasmid incubated with DNase buffer and purified in-house Taq DNA polymerase enzyme and (v) plasmid incubated with DNase buffer and commercial Taq DNA polymerase enzyme. All the reaction tubes were incubated overnight at 37°C.

2.6 Activity Assay and Unit Determination of Taq DNA Polymerase

This study was carried out using standard commercial Taq DNA polymerase with various concentrations ranging from 0.25 U/µL to 2 U/µL. Each 50 µL PCR reaction was performed using a cloned plasmid containing 164 bp of HCV genome as a template. The samples were loaded and run on a 1.5% of agarose gel and the band intensity of amplicon generated using
commercial Taq DNA polymerase was calculated using ImageJ software. A standard curve was prepared using peak area produced by the software for various concentrations of commercial enzyme. Using this standard curve, the units were extrapolated for in-house Taq DNA polymerase.

2.7 Activity Assay with Different Clinical Samples

The enzyme activity assay of the purified Taq DNA polymerase was performed using isolated DNA from different clinical samples. The Acinetobacter spp. was tested in the clinical sample using primers of rpoB gene of amplicon size 397 bp. The presence of uropathogenic E. coli (UPEC) was assessed in the urine sample by amplifying a 615 bp region of chuA gene which encodes the outer membrane of heme utilisation receptor protein in UPEC. The presence of Mycobacterium tuberculosis was examined in the sputum sample by amplifying a 236 bp fragment of early secretory antigen Esat6 gene.

3. RESULTS

The presence of the Taq DNA polymerase gene within the recombinant plasmid was confirmed by PCR with gene-specific primers and 2499 bp amplicon was visualised on the agarose gel (Fig. 1). Further, the clone was sequenced and BLAST analysis showed sequence similarity with the Taq DNA polymerase of Thermus aquaticus (GenBank: D32013.1).

3.1 Expression and Purification of Taq DNA Polymerase

The Taq DNA polymerase was expressed in E. coli DH5α. The culture was induced with 1 mM IPTG, harvested and the cell disruption was facilitated with lysozyme to begin purification of the soluble protein.

The thermostable nature of Taq DNA polymerase was utilised to eliminate the majority of the cellular proteins, by incubating the cell lysate at 80°C for 1 h. This led to the denaturation and precipitation of the said proteins, thus making it possible for their removal by centrifugation. However, in E. coli there are about 15 other cellular proteins that are known to be more or less thermostable thus, highly likely to be present in the supernatant [1,9]. Hence, ion exchange chromatography was performed to remove these contaminating proteins from the heat-treated supernatant. The isoelectric point (pI) of Taq DNA polymerase is 6.03; hence the soluble fraction had undergone through the anion exchange chromatography in Tris buffer at pH 7.9. At pH 7.9 the net charge of the protein is negative thus these proteins electrostatically bind to the anion exchanger Q-sepharose and are eluted by increasing the concentration of KCl.

A majority of contaminating proteins were eliminated during the anion exchange chromatography but still, some contaminating proteins were present in the eluted fractions of the protein. For further purification, all the fractions were pooled together and desalted for the succeeding purification steps. The desalted protein had undergone through the cation exchange chromatography in S sepharose. HEPES buffer (pH 6.9) and KCl was used for washing the column and the elution of protein with a step gradient method. During the purification, most of the Taq DNA polymerase binds to the matrix electrostatically and most of the co-purified contaminating proteins do not bind to the cation exchanger and goes into the flow through. The bound fraction of Taq DNA polymerase was eluted with a stepwise gradient of KCl and the purity of the eluted protein was observed on SDS-PAGE (Fig. 2c).

Extra precautions had to be taken to remove the residual DNA/RNA/plasmid from the purified Taq DNA polymerase because that might interfere with the diagnostic purpose or bacterial barcoding studies. Hence, the pooled fractions from the S sepharose column were passed through the silica matrix. Any residual nucleic
acid would bind to the silica matrix and the purified protein comes in the flow through. The flow through containing the purified Taq DNA polymerase was dialysed in Taq storage buffer and stored at – 20°C. The purified protein along with the commercial enzyme was compared on a silver stained gel and the purity was examined (Fig. 2d).

The result was analysed on 12% SDS-PAGE and a single band at approximately 94 kDa was observed (Fig. 2c, 2d), there was no trace of non-specific or contaminating protein in purified Taq DNA polymerase.

3.2 Purity Testing

The E. coli cells were lysed with lysozyme to obtain a clear lysate, which was used for the downstream processing. In Taq DNA polymerase preparations, DNA and DNase are the two common impurities that are cumbersome to remove. However, in order to obtain standard grade enzyme, these contaminants should be removed.

The DNA contamination in Taq DNA polymerase may originate from the E. coli genome and the expression plasmid. This DNA can show false positive amplification with the primers designed for the amplification of bacterial genome as well as a common plasmid. It is crucial that higher grade Taq DNA polymerase should not have DNA contamination hence, for the elimination of these contaminants, the cell lysate was treated with DNase enzyme.

The DNase enzyme is also harmful to PCR reactions as it degrades the template DNA and primer before reaching the primary denaturation step; for this reason, it is utterly essential to remove or inactivate the DNase from the Taq DNA polymerase. The thermostable nature of Taq DNA polymerase provides an advantage to precipitate and inactivate almost all the contaminating proteins including DNase at a higher temperature (80°C for 1 hr). The other smaller contaminants were removed by dialysis.

For the ultra-pure preparation of Taq DNA polymerase, an additional purification step was performed with ion-exchange chromatography, to remove the residual contaminating protein and the fragments of small nucleic acid. The ion-exchange chromatography provides an additional protection against residual contamination of DNA and DNase. The theoretical isoelectric point of Taq DNA polymerase is 6.03 whereas DNase

![Fig. 2. Expression and purification of in-house Taq DNA polymerase and comparison with the commercial enzyme. 2a: Lane 1: Prestained protein marker; Lane 2: E. coli cell lysate; 2b: Lane 1: Prestained protein marker; Lane 2: Soluble supernatant fraction after heat treatment; 2c: Lane 1: Prestained protein marker; Lane 2: Purified Taq DNA polymerase after ion exchange chromatography and silica column chromatography; 2d: Lane 1: Prestained protein marker; Lane 2: Commercial Taq DNA polymerase; Lane 3: Purified Taq DNA polymerase](image-url)
and DNA are 5.2 and < 5 respectively. Therefore, these contaminants bound more strongly with anion-exchange resin than Taq DNA polymerase. Finally, these leftover contaminants were removed from Taq DNA polymerase. Therefore, the ultra-grade preparation was doubly safeguarded against DNA and DNase and it is also known as double DNA and DNase free preparation.

The DNA contamination in purified Taq DNA polymerase was tested directly by PCR. The universal bacterial 16S ribosomal DNA and malB gene primers were used for this purpose to detect the bacterial and E. coli genomic DNA contamination in purified Taq DNA polymerase. Similarly, recombinant plasmid contamination was checked by PCR using specific primer for partial Taq DNA polymerase gene.

These three sets of primers enabled to gauge the quality of the purified Taq DNA polymerase in terms of nucleic acid contamination. In all these three cases, amplification was not observed when purified Taq DNA polymerase was used as a source of DNA template (source of contaminating genomic or recombinant plasmid DNA) (Fig. 3a, 3b and 3c). Hence, the purified Taq DNA polymerase was free from DNA contamination.

For the study of DNase activity or DNase contamination of purified Taq DNA polymerase, the assay was performed by incubating it with the plasmid DNA and comparison was done with a commercial enzyme (Fapon Biotech Inc). The amount of plasmid remained the same after overnight incubation with in-house Taq DNA polymerase as well as the commercial enzyme (Fig. 4). The DNase activity was not evident in the purified protein. Again the standard preparation methods met our requirement to produce DNase free Taq DNA polymerase.

3.3 Activity Assay

It is essentially required to know the unit of an enzyme before performing any enzyme assay, because the quantity of an enzyme available or used in an assay is very tough to identify in absolute terms like grams or milligrams, since its purity is usually poor and some part of an enzyme may be in an inactive or partially active state. Most applicable parameters for any enzyme are its functional activity. These activities are generally calculated in terms of enzyme unit (U). For a Taq DNA polymerase 1, unit is defined as the amount of enzyme that incorporates 10 nmol of deoxyribonucleoside triphosphates into the acid-insoluble material in 30 min at 72°C in standard assay conditions.

![Fig. 3. With respect to the DNA contamination, the purity of Taq DNA polymerase enzyme was examined by PCR and compared with commercial Taq DNA polymerase. 3a: PCR amplification with universal bacterial 16S ribosomal DNA primers using purified Taq DNA polymerase. Lane 1: 1kb ladder; Lane 2, 4: Positive control assay; where the bacterial genomic DNA was used as template for purified Taq and commercial Taq DNA polymerase respectively; Lane 3, 5: Purity assay; where purified Taq and commercial Taq DNA polymerase were used as source of DNA template as well as enzyme respectively; 3b: PCR amplification with malB gene-specific primers using purified Taq DNA polymerase. Lane 1: 1kb ladder; Lane 2, 4: Positive control assay; where the bacterial genomic DNA was used as template for purified Taq DNA polymerase and commercial Taq DNA polymerase respectively; Lane 3, 5: Purity assay; where purified Taq DNA polymerase and commercial Taq DNA polymerase were used as source of DNA template as well as enzyme respectively. 3c: PCR amplification with gene-specific primers for Taq DNA polymerase to check plasmid contamination. Lane 1: 1kb ladder; Lane 2 and 3: 1 and 5 µL purified Taq DNA polymerase used as a source of DNA template respectively; Lane 4: Positive control for the assay.](image-url)
Fig. 4. With respect to the DNase contamination, DNase activity assay performed at 37°C for overnight with purified Taq and compared with commercial Taq DNA polymerase. Lane 1: 1 kb ladder; Lane 2: Plasmid only; Lane 3: Plasmid in DNase buffer; Lane 4: Plasmid and DNase enzyme in DNase buffer; Lane 5: Plasmid and purified Taq DNA polymerase enzyme in DNase buffer; Lane 6: Plasmid, commercial Taq DNA polymerase enzyme in DNase buffer.

The most authentic assay for identification of enzyme unit is radiolabelled nucleotide incorporation during PCR amplification. But this method is undesirable due to the usage of radioactivity. There are several alternative methods to identify the enzyme unit and the standard curve preparation method is one of them. Here the standard curve preparation method was used for calculating the enzyme unit.

The assay was carried out to determine the unit of in-house purified Taq DNA polymerase for which standard curve was created using an authentic standard Taq DNA polymerase. For the preparation of standard curve, a different unit of standard Taq DNA polymerase was used in the PCR reaction and the intensity of amplified PCR product was used to create a standard curve. ImageJ software was used to calculate the intensity of amplicon band where peak area represents the intensity of band on the agarose gel. The unit of standard Taq DNA polymerase enzyme vs peak area was used for standard curve preparation. There was a linear increase in peak area against the amount or unit of enzyme taken (Fig. 5b).

In order to determine the unit of the in-house enzyme, a known volume of in-house purified Taq DNA polymerase was used in PCR and the peak area produced against the band intensity of amplified PCR product was extrapolated on the standard curve and finally, enzyme unit of purified Taq DNA polymerase was calculated (Fig. 5b). The standard curve was precise with an R2 value of 0.981. 0.5 µL of the in-house purified enzyme was used in a PCR reaction (Fig. 5a; Lane 9) and the unit was found to be 1.54 U/µL. Thus the purified Taq DNA polymerase is on par with commercial Taq DNA polymerase.

3.4 Validation of In-House Taq DNA Polymerase with Clinical Sample

DNA was isolated from distinct clinical samples and used to test with purified Taq DNA polymerase. Excellent amplification was observed with the target gene in each clinical sample such as Esat6 in M. tuberculosis (Fig. 6a), chuA in uropathogenic E. coli (Fig. 6b) and rpoB in of Acinetobacter spp. (Fig. 6c).

4. DISCUSSION

Taq DNA polymerase is an indispensable enzyme in research fields such as molecular biology and diagnostics. The patent for Taq DNA polymerase has expired and hence many companies and organization are involved in the manufacturing of the enzyme. Different vectors have been used by different investigators to increase the yield of Taq DNA polymerase such as pTTQ18 [3], pUC18 [10], pTZ57R [11], pET 15b [12], pTrc99A [13], pET28b [14]. Similarly, different E. coli expression strains like INV1alphaF' [4], DH1, BL21 (DE3), TOP10 have been used.
There are two major aspects to produce standard grade Taq DNA polymerase commercially, they are yield and quality of the expressed protein. To increase the yield of protein four vital factors need to be taken into consideration namely, the expression strain, vector, concentration of IPTG and induction time period [4]. In our present study, we have transformed the clone in E. coli DH5α strain, induced with 1mM IPTG at 37°C for 12 – 16 h. This combination has increased the expression many folds than the previous reports. At present, the yield of Taq DNA polymerase enzyme was about 45 - 50 mg/L. The purification protocol from previous researchers [3,4,14] found traces of contaminating protein from E. coli. During the crude purification of Taq DNA polymerase, there are chances of cellular protein, DNase and DNA contamination in the purified protein. There are fifteen thermostable cellular proteins that have chances to purify along with Taq DNA polymerase even after heat treatment and these proteins are, outer membrane lipoprotein carrier protein, transcription pausing factor L, maltose-binding proteins, putative EscN protein, FK-506-binding protein, adenylate kinase, ribosomal protein S19, DnaK, galactose glucose binding protein, D-ribose binding protein, trigger factor, GroES etc [1,9]. These proteins may reduce the activity of the enzyme as there may be few inhibitors
The purified Taq polymerase has a fair chance of DNA contamination which might give false positive amplification when primers designed for the amplification of bacterial genome or common plasmid. Hence, it is necessary to get rid of the DNA contamination. Likewise, the Taq polymerase may also be contaminated with DNase enzyme which may not give an accurate result especially in the case of clinical samples, where the template concentration is very low. It may result in a false negative result. Hence, it necessitates the removal of DNase enzyme from the purified protein completely. Thus, in terms of purity and activity, our Taq polymerase is exceptionally good when compared to the other commercial enzyme.

In this study, we have optimised the expression and purification protocol in which the contaminating macromolecules including proteins and nucleic acid were completely removed and finally commercially high-grade enzyme was purified. The in-house purified enzyme has an activity of 1.54 U/µL which is similar to most of the commercial polymerases. The enzyme is also compatible with a few clinical samples which were tested. Thus, the in-house purified Taq DNA polymerase can be used in a wide range of applications. This purified Taq DNA polymerase has proved to be of utmost purity and at par with other commercial enzymes, thus making headway towards its commercialisation as Geneasy Taq DNA polymerase.

5. CONCLUSION

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6. COMPETING INTERESTS

Authors have declared that no competing interests exist.

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