Development of a recombinant *Taq* DNA polymerase enzyme expressed using a synthetic gene and its comparison with a commercial enzyme

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Abstract. *Taq* DNA polymerase is a thermostable enzyme widely used for DNA amplification in the PCR technique. It was initially characterized and isolated from thermophilic bacteria, *Thermus aquaticus*. It was difficult to develop this enzyme using a native host system. Therefore, the development of the recombinant *Taq* DNA polymerase expressed using a synthetic gene is important to improve production efficiency. In this study, we developed the in house *Taq* DNA polymerase recombinant based on a codon-optimized using *E. coli* expression system. We cloned 2685 bp of the *Taq* DNA polymerase gene in the pET151/D-TOPO vector. The gene was synthesized and the expression was analyzed with SDS-PAGE technique which indicated with a 100.9 kDa specific target protein. The concentration and activity of this purified enzyme were 5.17 mg/mL and 4.647 U/ μ L, respectively. The application of this enzyme to the PCR technique showed that this enzyme could amplify the target genes from 200 bp to 3500 bp amplicons with a minimum DNA concentration template 10 ng/ μ L. This assumes that the in house recombinant *Taq* DNA polymerase based on synthetic genes is successfully expressed, purified, and was functional and comparable to the commercial *Taq* polymerase.

Keywords: Codon-optimized, *E. coli* BL21, Ni²⁺-NTA purified system, synthetic *Taq* DNA polymerase

INTRODUCTION

Molecular biology is one of the basic scientific disciplines that has an important role in supporting the development of modern biotechnology. One of the techniques that is used in molecular biology is PCR (polymerase chain reaction). PCR is widely applied for DNA sequencing (Ishino and Ishino, 2014), detection of infectious agents, diagnosis of genetic diseases, genetic identification, fingerprinting, production of genetically modified organisms (GMO),

genetic modification, and production of recombinant proteins (Niimi *et al.*, 2011).

One of the important reagents that is used in PCR is the thermostable DNA polymerase enzyme, which acts as an enzyme that can extend DNA strands in accordance with its template, DNA, or cDNA (complementary DNA). This enzyme is commercially available in the market, but in Indonesia, the procurement of the enzyme still relies on imports, which causes high prices

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and requires a long time to supply. This condition causes the need for domestic research to develop and produce thermostable polymerase enzyme, so that in the future, it can reduce dependence on imported reagents.

Taq DNA polymerase is a thermostable enzyme that was first isolated from thermophilic bacteria, *Thermus aquaticus* (Chien *et al.*, 1976) and successfully applied in vitro to improve PCR test performance (Mullis and Faloona, 1987; Wang *et al.*, 2004). However, the expression of the enzyme in the native host is very low because these thermophilic bacteria only grow at high temperatures. To overcome this problem, *Taq* DNA polymerase gene was expressed in *Escherichia coli* (*E. coli*) (Engelke *et al.*, 1990). The *E. coli* expression system is able to produce recombinant enzymes commercially, but the methods used in the stages of construction, cloning, and purification are quite difficult to do, so the price of the enzymes produced is expensive.

The development of the *Taq* DNA polymerase recombinant in this research used a synthetic gene that encoded the *Taq* DNA polymerase enzyme obtained from GenBank data. The *Taq* DNA polymerase synthetic gene was constructed, and its codon was optimized as a source of DNA template. Genetic engineering construction of *Taq* DNA polymerase sequences with codon optimization was designed to avoid the effect of individual codon usage (codon preferences), which would affect the effectiveness of gene expression, considering that the enzyme will be expressed heterologously using the *E. coli* expression system (Gordeeva *et al.*, 2010). Another advantage of using a synthetic gene is that synthetic gene technology is relatively new and can be designed and modified as needed. In addition, it is also possible to obtain nucleic acid analogues, which cannot be obtained naturally by adding additional nucleotides (Pinheiro *et al.*, 2012).

The purpose of this research is to develop an in house recombinant *Taq* DNA polymerase enzyme using a synthetic gene in the *E. coli* expression system. The production of this *Taq* DNA polymerase recombinant enzyme is expected to reduce dependence on similar products imported, so it will support the advancement of biotechnology in Indonesia.

MATERIALS AND METHODS

Synthetic gene constructions

The target gene for synthetic recombinant *Taq* DNA polymerase enzyme obtained from Genbank data as a reference with access code J04639.1, encodes *Thermus aquaticus* DNA polymerase (PolI) gene (Lawyer *et al.*, 1989). The gene sequences then were “codon optimized” (GeneArt, Life Technologies). The sequences of optimized codon *Taq* DNA polymerase were synthesized by GeneArt® Gene Synthetics, Life Technologies (USA) in the form of a double-stranded DNA, which has been inserted into the pET151 / D-TOPO plasmid.

Protein expression

The protein expression followed the modified procedure from the manufacturer (Invitrogen, USA), as 5 µL (50 ng) of the synthetic gene in pET151/D-TOPO was directly transformed in one vial of *E. coli* BL21 (DE3). The vial containing the transformant was incubated for 30 minutes on ice and continued with heat shock reaction at 42°C for 30 seconds. Then, 250 µL SOC medium was added and continued with incubation in the incubator shaker with 150 rpm at 37°C for 30 minutes. After that, the transformant was inoculated into 10 mL of LB medium containing 100 µg/mL ampicillin and was grown at 37°C overnight. Then, the inoculum was transferred into 200 mL LB medium containing 100 µg/mL ampicillin and was grown for 3 hours at 37°C. IPTG (*Isopropyl-β-Dthiogalactopyranoside*) with final concentration of 10 mM was added to the medium and incubated at 37°C for 1 until 3 hours with shaking at 150 rpm and collected every hour to obtain the optimum expression. After induction for 3 hours, the cells were harvested by centrifugation at 4000 xg for 20 minutes at 4°C. The supernatant was removed, and the pellet containing the recombinant protein was resuspended with Bug Buster protein extraction solution (1 gram pellet in 5 mL solution) (Novagen, USA) and homogenized. The solution was shaken while incubated on ice for 1 hour. After that, the solution was centrifuged at 16000 xg for 20 minutes at 4°C, and supernatant as a clear lysate was collected for further protein

purification process. The collected inoculum and supernatant were analyzed with SDS-PAGE.

Protein purification

The purification of clear lysate was conducted using the immobilized metal affinity chromatography (IMAC) technique following the modified from Probond (Invitrogen, USA). The column was prepared with 2 mL of the resin containing Ni^{2+} -NTA particle into a filtration column and the resin allowed to settle completely by gravity (5–10 minutes). After that, about 6 mL native binding buffer at final pH 8.0 (250 mM NaH_2PO_4 , 2.5 M NaCl, 10 mM Imidazole, pH 6.0) were added into the column, and the resin was resuspended by alternately inverting and gently tapping then allowing the resin to settle completely by gravity, and then the supernatant was removed. About 8 mL clear lysates were added into the filtration column and incubated on ice for one hour with shaking, and flow-through was collected. The column was then washed with washing buffer at final pH 8.0 (250 mM NaH_2PO_4 , 2.5 M NaCl, 20 mM Imidazole, pH 6.0) and repeated four times. The resin was settled by gravity, and the supernatant containing unbound proteins was removed. Finally, the histidine-tagged proteins were eluted with 8 mL native elution buffer at final pH 8.0 (250 mM NaH_2PO_4 , 2.5 M NaCl, 250 mM Imidazole, pH 6.0), and supernatant was collected for dialysis processing.

The dialysis process was performed using 14000 MWCO (molecular weight cut off) cellophane membrane against 50 mM Tris-HCl pH 8.5 for one night. The enzyme was concentrated using PEG (polyethylene glycol) 6000 until obtaining one-tenth of the initial volume. The protein concentration was quantitatively measured using nanodrop C1000 (Thermofisher, USA) at 280 nm wavelength. The analysis of *Taq* DNA polymerase enzyme recombinant expression was performed using SDS-PAGE technique.

Polymerase activity assay

The enzyme activity assay was performed using a modified EvaEZ Fluorometric Polymerase Activity Assay kit (Biotium, US), following the procedure of Suppan *et al.* (2015) as the reaction mixture contained 10 μL 2xEvaEZ fluorometric

polymerase activity mix, 9 μL sterile dH_2O , and 1 μL polymerase enzyme with unknown concentration. The reaction was run on the realtime PCR (iQ5, Biorad) at the isothermal temperature of 72°C for 45 minutes and the RFU (relative fluorescent unit) value at the end-point condition was measured. A reaction profile of fluorometric polymerase activity assay was plotted with X-axis as time in minutes and Y-axis as fluorescence reading. Then, the slope of the curve was determined, and it represented the initial rate of fluorescence change (fluorescence unit/minutes). The specific activity of the sample was estimated by comparing the initial rate of fluorescence change of recombinant protein with the reaction curve of the standard commercial *Taq* DNA polymerase (Platinum *Taq* DNA polymerase, Invitrogen). When the DNA polymerase is active, the primer is extended to form a double-stranded product that binds the EvaGreen dye, with the resulting increase in fluorescence. The rate of increase shows a positive correlation with the polymerase activity (Tveit and Kristensen, 2001).

Application in PCR

The target gene used as DNA template for PCR amplification was SRV-2 provirus gene (Saepuloh *et al.*, 2009). The 25 μL PCR reaction mixture (PCR mix) contained 1 μL primary F and R with 10 pmol/ μL on each primer, 5 μL 5x PCR buffer (200 mM Tris HCl pH 8.8, 100 mM $(\text{NH}_4)_2\text{SO}_4$, 100 mM KCl, 20 mM MgSO_4 , 1 mg/ml BSA, 1% Triton-X), 1 μL 10 mM dNTPs, 2 μL 50 mM MgCl_2 , 1 μL *Taq* DNA polymerase enzyme recombinant, 12 μL dH_2O free nuclease, and 2 μL DNA SRV-2/A549 as template DNA. As a control, we used the commercial *Taq* DNA polymerase enzyme (Platinum *Taq* DNA polymerase, Invitrogen). The PCR amplification was conducted in the PCR machine (Veriti thermal cycler, Applied Biosystems, USA) with pre-denaturation at 94°C for 3 minutes, denaturation at 94°C for 30 seconds, annealing at 55°C for 30 seconds, and extension at 72°C for 2 minutes. This process was repeated 35 cycles and ended with the post-PCR process at 72°C for 7 minutes. The PCR amplification product was observed on 1% agarose gel using the Sybr safe in TAE 1x buffer at 100 volts for 45 minutes and visualized using Geldoc machine (Biorad).

The sensitivity test was performed by serial dilution of the provirus SRV-2 DNA template at the concentrations from 100 µg/µL to 1 ng/µL using DNA SRV-2/A549 as template DNA and Env 5737 as a forward primer and -5943 as a

reverse primer. Meanwhile, the processivity assay was performed to analyze the ability of *Taq* DNA polymerase recombinant to amplify the target gene at : 206 bp, 506 bp, 1971 bp, 2501 bp to 3531 bp (Table 1).

Table 1. Primers sequences for processivity assay.

Primer names	Sequences	Amplicons (bp)
SRV1/3 5737 U19 (Forward)	5'-CCAGATGGCTACCAGAACGAY-3'	
SRV-2 5943L (Reverse)	5' AGGGCTTACCGTGTGTG-3'	206
SRV-2 6243L (Reverse)	5'-CAAACCTGTGGGGACATTGCTA-3'	506
SRV-2 7708R (Reverse)	5' GAAACTGCGCCTGTCT-3'	1971
SeRV 441F (Forward)	5' GCCGACAGTTAAAGTGAAAG-3'	
SeRV 2942R (Reverse)	5' TGCTTTGTCCAATTCCTCTT-3'	2501
SeRV 3972R (Reverse)	5' TCTACGGATAGTTGCTTTTGA-3'	3531

RESULTS AND DISCUSSION

Gene construction

The total of 2685 bp nucleotides sequences the *Thermus aquaticus* DNA polymerase gene obtained from GenBank (J04639.1) was used as a reference for the *Taq* DNA polymerase synthetic gene template. This codon was optimized to obtain the optimum gene expression level in heterologous expression system. Codon optimization is used to avoid the effect of codon bias, which refers to the fact that the frequency of codons usage that encodes certain amino acids varies in different organisms (Brown, 2002). Therefore, this codon optimization correlates with the gene expression level.

Based on GeneOptimizer (GeneArt, USA) bioinformatic program the nucleotides sequences obtained a codon adaptation index (CAI) value of 0.98, which indicated that the codon usage was well adapted to the bias of *E. coli* as the host expression system. This optimized gene should therefore allow high and stable expression rate in this expression system. CAI measures the similarity between the codon usage of a gene and the codon usage of a reference group of genes (Sharp and Li, 1987). Its values range from 0 (when the codon usage of a sequence and that of the reference set are very different) to 1 (when both codon usages are the same). This index is the most effective of all codon bias measures for predicting gene expression levels (Henry *et al.*, 2007; Puigbò *et al.*, 2007).

Nucleotide sequences before and after codon optimized were aligned using the *Clustal Omega* (<https://www.ebi.ac.uk>) and resulted in the identity matrix value of 72.18%. This means that there was 27.82% nucleotide difference between the native *Taq* DNA polymerase sequences and the optimized one. The percentage value of the identity matrix refers to the quantitative similarity measurement between two DNA sequences, amino acids, or other (Quick and Sikela 2020). The optimized nucleotides were translated into amino acids using the *blastx* program (<https://blast.ncbi.nlm.nih.gov/Blast.cgi>) and produced 894 amino acids encoding *Taq* DNA polymerase enzyme.

The codon optimized *Taq* DNA polymerase sequences was synthesized and inserted into the pET151/D-TOPO expression vector. The vector contained T7 promoters and several other insertion genes, such as polyhistidine coding genes (6xHis), TEV recognition site, and V5 epitope at N-terminal position. The open reading frame (ORF) area encoding the *Taq* DNA polymerase recombinant enzyme at the nucleotide position of 396 bp until 3080 bp was in frame with initial ATG of the vector. The total of 2685 bp nucleotides containing UAA stop codon were inserted to the sequences due to the N-terminal position of the vector.

Protein expression

The *Taq* DNA polymerase synthetic gene in pET151/D-TOPO vector has been successfully expressed in *E. coli* BL21 (DE3). This strain has

specially designed for heterologous gene using a T7 promoter and has lysogen lambda DE3 which carries the T7 RNA polymerase gene under the control of the LacUV5 promoter. Analysis of *Taq* DNA polymerase recombinant protein expression using the SDS PAGE technique

showed a 100 kDa specific protein band (Figure 1). This result was in accordance with the protein molecular weight calculation using Compute/Mw program (web.expasy.org) which resulted in 100.9 kDa.

Figure 1. *Taq* DNA polymerase recombinant protein expression observed by Coomassie blue staining shown with 100 kDa protein target band. The level of protein expression after IPTG induction is indicated by the intensity of the band. M : broad range protein marker, 0-3 : hours of induction.

The protein expression level of recombinant *Taq* DNA polymerase enzyme was induced using IPTG through activation of T7 RNA polymerase related gene (Pluthero, 1993). The SDS PAGE analysis indicated that IPTG enhances the expression of *Taq* DNA polymerase recombinant proteins that is qualitatively observed from the intensity of the bands (Figure 1). The optimum expression is obtained after the induction with IPTG for 3 hours. The optimization of induction time length had a substantial effect on the protein recombinant yield. Addition of IPTG induces the lacUV5 promoter to produce T7 RNA polymerase, which in turn initiates high-level expression of the target gene in the plasmid (Studier and Moffattf, 1986).

The coding sequence of the T7 RNA polymerase is inserted into the bacterial chromosome under the control of the inducible lac UV5 operon and is transcribed by the endogenous *E. coli* polymerase. The lac repressor protein (LacI) regulates access to the T7 RNA polymerase coding sequence by binding to the lac UV5 operon. Protein expression induction is triggered by the addition of the inducer such as IPTG, which is a structural non-metabolizable

analogue of allolactose. The T7 RNA polymerase produced after induction specifically transcribes the coding sequence of the protein of interest that is inserted into the expression plasmid under the control of the T7 promoter (Studier and Moffattf, 1986; Studier *et al.*, 1990).

***Ni*²⁺-NTA affinity chromatography enzyme purification**

In this study, *Taq* recombinant DNA polymerase protein was produced in soluble protein and did not form an inclusion body. Therefore, the cells could be lysed in native condition with native buffer without being denatured. This condition is useful to maintain enzyme activity, biological substances, and simplifies the protein purification process. The recombinant *Taq* DNA polymerase enzyme was purified using the immobilized metal affinity chromatography technique containing Ni²⁺-NTA (Nickel-Nitrilotriacetate) resin to eliminate non-specific proteins. This resin was used to bind the polyhistidine polypeptide (6xHis) encoded by the 6xHis gene in the pET151/D-TOPO vector. The elution was performed using a buffer system containing imidazole at 250 mM. Imidazole will compete with 6xHistag to bind the

resin containing Ni^{2+} metal ion resulting in the eluates as purified recombinant *Taq* DNA polymerase enzyme. This purification results were analyzed using the SDS PAGE technique which showed a 100.9 kDa band in the elution fraction.

This purification technique had successfully minimized the nonspecific proteins target and resulted in 5.17 mg/mL of the purified recombinant *Taq* DNA polymerase enzyme (Figure 2).

Figure 2. Analysis of Ni^{2+} -NTA affinity chromatography to the *Taq* DNA polymerase recombinant enzyme using SDS-PAGE with Coomassie blue staining technique. M: broad range protein marker; Lane 1 : crude extract, Lane 2 : flow through of the lysate after binding to resin fraction, Lane 3 : washing with 20 mM imidazole and Lane 4 : elution fraction which was indicated with 100.9 kDa specific protein targets.

Figure 3. Enzyme sensitivity measurement of purified *Taq* DNA polymerase recombinant against the target DNA using various concentrations of template (3a) compared with the commercial *Taq* DNA polymerase enzyme (3b). Lane M = 100 bp DNA marker; Lane 1= 100 $\mu\text{g}/\mu\text{L}$; Lane 2= 10 $\mu\text{g}/\mu\text{L}$; Lane 3=1 $\mu\text{g}/\mu\text{L}$; Lane 4=100 $\text{ng}/\mu\text{L}$; Lane 5=10 $\text{ng}/\mu\text{L}$ and Lane 6=1 $\text{ng}/\mu\text{L}$.

Figure 4. Processivity measurement of purified *Taq* DNA polymerase recombinant enzyme (1 uL each reaction or 4.647 unit) (a) to amplify a variety of provirus SRV-2 target gene size compared with commercial *Taq* DNA polymerase enzyme (b) long sized amplified gene target.

Application of Taq DNA polymerase recombinant enzyme

The PCR technique was applied to the purified recombinant *Taq* DNA polymerase enzyme to determine the enzyme activity in the amplified target gene. In this research, the enzyme activity was measured based on the commercial *Taq* DNA polymerase with known activity enzyme unit. The polymerase enzyme activity unit was 4.647 unit/ μ L obtained from the measurements based on the relative fluorescent units (RFU) at the end-point conditions.

In this research, we also measured sensitivity and processivity of purified recombinant *Taq* DNA polymerase. The sensitivity assay was performed to determine the minimum concentration of the DNA template that could be amplified by this *Taq* DNA polymerase recombinant. The sensitivity of this *Taq* DNA polymerase recombinant was 10 ng/ μ L DNA concentration that could be amplified using primer *Env* 5737 - 5943 SRV-2 provirus target gene that resulted in 206 bp amplicon. This sensitivity assay was also compared to the commercial *Taq* DNA polymerase as a reference (Figure 3).

The processivity assay proved the efficacy of the polymerase to synthesize the target gene

according to the DNA template. This assay was measured to assess the amplification range of target genes at the same DNA template concentration, in this case 100 μ g/ μ L. Our purified *Taq* DNA polymerase enzyme successfully amplified the variety of SRV-2 provirus genes targets at the amplicons 206 bp, 506 bp, 1971 bp, 2778 bp until 3531 bp and compared with commercial *Taq* DNA polymerase enzyme (Figure 4).

According to sensitivity and processivity results of this purified *Taq* DNA polymerase recombinant enzyme, it is indicated that this enzyme was possible to be applied to the PCR technique extensively on various gene targets. The availability of this in house *Taq* DNA polymerase recombinant enzyme has been expected to be applied in the molecular biology research, especially in the PCR or RT-PCR technique.

CONCLUSION

The optimized codon of 2685 bp *Taq* DNA polymerase synthetic gene cloned in pET151/D-TOPO vector was successfully expressed in *E. coli*

BL21 (DE3). The SDS-PAGE analysis of *Taq* DNA polymerase gene expression result was demonstrated by the presence of a specific protein band at 100.9 kDa. The Ni²⁺-NTA purification technique successfully minimized the non-target proteins and resulted in 5.17 mg/mL purified protein. The application of this enzyme in the PCR technique indicated that, the recombinant enzyme could amplify the various target genes of provirus SRV-2 at 10 µg/µL minimum concentration with a variety of amplicons ranging from 200 bp–3500 bp in size. In general, this preliminary study has successfully developed a *Taq* DNA polymerase recombinant enzyme using optimized synthetic codon.

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REFERENCES

- Brown, T. A. 2002. Genomes. 2nd ed. Oxford: Wiley-Liss.
- Chien, A., David, B. E., & Trela, J. M. 1976. Deoxyribonucleic acid polymerase from the extreme thermophile *Thermus aquaticus*. *Journal of Bacteriology* 127(3): 1550–1557.
- Engelke, D. R., Krikos, A., Bruck, M. E., & Ginsburg, D. 1990. Purification of *Thermus aquaticus* DNA polymerase expressed in *Escherichia coli*. *Analytical Biochemistry* 191: 396–400.
- Gordeeva, T. L., Borschevskaya, L. N., & Sineoky, S. P. 2010. Improved PCR-based gene synthesis method and its application to the *Citrobacter freundii* phytase gene codon modification. *Journal of Microbiological Methods* 81: 147–152.
- Henry, I., Sharp, P. M., Sheffield, E. R. S. 2007. Predicting gene expression level from codon usage bias. *Molecular Biology and Evolution* 24(1): 10–12.
- Ishino, S. & Ishino, Y. 2014. DNA polymerases as useful reagents for biotechnology - The history of developmental research in the field. *Frontiers in Microbiology* 5(465): 1–9.
- Lawyer, F. C., Stoffel, S., Saiki, R. K., Myambo, K., Drummond, R., & Gelfand D. H. 1989. Isolation, characterization, and expression in *Escherichia coli* of the DNA polymerase gene from *Thermus aquaticus*. *Journal of Biological Chemistry* 264(11): 6427–6437.
- Mullis, K. & Faloona, F. 1987. Specific synthesis of DNA in vitro via a polymerase-catalyzed chain reaction. *Methods in Enzymology* 155: 335–350.
- Niimi, H., Mori, M., Tabata, H., Minami, H., Ueno, T., Hayashi, S., & Kitajima, I. 2011. A novel eukaryote-made thermostable DNA polymerase which is free from bacterial DNA contamination. *Journal of Clinical Microbiology* 49(9): 3316–3320.
- Pinheiro, V. B., Taylor, A. I., Cozens, C., Abramov, M., Zhang, S., Chaput, J. C., Wengel, J., Marleen, R., Peak Chew, S. Y., McLaughlin, S. H., et al. 2012. Synthetic genetic polymers capable of heredity and evolution. *Science* 336(6079): 341–344.
- Pluthero, F. G. 1993. Rapid purification of high-activity *Taq* DNA polymerase. *Nucleic Acids Research* 21(20): 4850–4851.
- Puigbò, P., Guzmán, E., Romeu, A., & Garcia-Vallvé, S. 2007. OPTIMIZER: A web server for optimizing the codon usage of DNA sequences. *Nucleic Acids Research* 35: 126–131.
- Quick, V. S. & Sikela, J. 2020. Percent identity of genomic DNA and amino acid sequences. *Center for Academic Research and Training in Anthropogeny*: 3–5. <https://carta.anthropogeny.org/moca/topics/percent-identity-genomic-dna-and-amino-acid-sequences>.
- Saeputloh, U., Iskandriati, D., Sadikin, M., & Pamungkas, J. 2009. Expression of simian retrovirus type D serotype 2 envelope in insect cell using baculovirus expression vector system. *Microbiology Indonesia* 3(2): 91–95.
- Sharp, P. M. & Li, W. 1987. The codon adaptation index - a measure of directional synonymous codon usage bias, and its potential applications. *Nucleic Acids Research* 15(3): 1281–1295.
- Studier, F. W. & Moffatt, B. A. 1986. Use of bacteriophage T7 RNA polymerase to direct selective high-level expression of cloned genes. *Journal of Molecular Biology* 189: 113–130.
- Studier, F. W., Rosenberg, A. H., & Dubendorff, J. W. 1990. Use of T7 RNA polymerase to direct expression of cloned genes. *Methods in Enzymology* 185: 60–89.
- Suppan, M., Shamsuddin, S., Ismail, A., & Balakrishnan, V. 2015. PCR-based gene synthesis, cloning, expression, purification and characterization of *Bst* DNA polymerase in *E. coli* cells. *Current Synthetic and Systems Biology* 3(3): 1–6.
- Tveit, H. & Kristensen, T. 2001. Fluorescence-based DNA polymerase assay. *Analytical Biochemistry* 298: 96–98.
- Wang, Y., Prosen, D. E., Mei, L., Sullivan, J. C., Finney, M., & Vander Horn, P. B. 2004. A novel strategy to engineer DNA polymerases for enhanced processivity and improved performance *in vitro*. *Nucleic Acids Research* 32(3): 1197–1207.