ISOLATION AND CHARACTERIZATION OF TAQ DNA POLYMERASE AND OPTIMIZATION AND VALIDATION OF NEWLY DESIGNED THERMAL CYCLERS

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Approval of the thesis

ISOLATION AND CHARACTERIZATION OF TAQ DNA POLYMERASE AND OPTIMIZATION AND VALIDATION OF NEWLY DESIGNED THERMAL CYCLERS

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ABSTRACT

ISOLATION AND CHARACTERIZATION OF TAQ DNA POLYMERASE AND OPTIMIZATION AND VALIDATION OF NEWLY DESIGNED THERMAL CYCLERS

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February 2011, 118 pages

Amplification of target DNA *in vitro* via polymerase chain reaction (PCR) is a widely used scientific technique in molecular biology. This method relies on repeated heating and cooling cycles of the DNA and enzyme mixture, resulting with the enzymatic replication of the DNA. A heat stable Taq DNA polymerase and a thermal cycler that enables repeated heating/cooling cycles are the two key components of the PCR. In this study we have produced a high activity Taq DNA polymerase and used this enzyme to validate and optimize two newly developed thermal cyclers- a conventional and a capillary thermal cycler.

Taq DNA polymerase gene was amplified from *Thermus aquaticus* DNA, was cloned and overexpressed using Gateway® recombination cloning technology.

Highly active Taq DNA polymerase enzyme was purified from *E.coli* and its activity was tested by PCR, using different sources of DNA. Our results showed that the enzyme activity of the produced Taq DNA polymerase was not significantly different from the commercial available Taq DNA polymerase. To further characterize the purified enzyme, endonuclease and nicking activities

were also tested to be absent. The fidelity of the purified Taq DNA polymerase was also tested and found to be the same as the commercially available Taq polymerases.

In this study, in addition to the production of a Taq polymerase, optimization studies for two new thermal cyclers, a conventional and a capillary, was also carried out. The conventional thermal cycler was found to be as efficient as the commercially available thermal cyclers in the 95% confidence interval. The capillary thermal cycler was tested as a proof of concept and our results showed that it works less efficiently due to the insufficient insulation and capillary tubes being longer than the capillary tube holder.

Keywords: Taq DNA polymerase; thermal cycler; PCR; Gateway cloning system

ÖZ

TAQ DNA POLİMERAZ ENZİMİNİN İZOLASYONU VE KARAKTERİZASYONU VE YENİ TASARLANAN PZR CİHAZLARININ OPTİMİZASYONU VE VALİDASYONU

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Polimeraz Zincir Reaksiyonu (PZR), hedef nükleik asit dizisinin canlı organizma içinde bulunmadan, labaratuvar koşulları altında çoğaltılmasına yarayan moleküler biyolojik yöntemlerin kilometre taşlarından biridir. Yöntem, DNA solüsyonunun, DNA polimeraz enzimi varlığında tekrarlı ısıtılıp-soğutulmasına, sonuç itibariyle de çoğaltılmasına dayanmaktadır. Yüksek sıcaklığa dayanıklı Taq DNA polimeraz enzimi ile farklı sıcaklık dalgalanmalarını sağlayan thermal cycler cihazı bu işlemin en önemli iki elemanıdır. Bu çalışmada, yüksek verimli Taq DNA polimeraz enzimi üretilmiş ve üretilen bu enzim konvansiyonel ve kapiler olmak üzere iki farklı thermal cycler cihazının optimizasyon çalısmalarında kullanılmıştır.

Taq DNA polimeraz geni, *Thermus aquaticus* DNA çoğaltılarak, Gateway® rekombinasyon klonlama teknolojisi ile klonlanmış ve eksprasyonu sağlanmıştır.

Yüksek aktiviteye sahip Taq DNA polimeraz enzimi *Escherischia coli* bakterisinden izole edilmiş ve aktivitesi farklı kaynaklardan elde edilmiş DNA'ların PZR ile çoğaltılmasıyla gösterilmiştir. Elde edilen sonuçlara göre,

labaratuvar koşullarında izole ettiğimiz Taq DNA polimeraz'ın aktivitesiyle ticari muadillerinin aktivitesi çok büyük oranda benzerlik göstermektedir. Elde edilen enzimin ileri tanımlanmasında, endonükleaz ve nicking aktivitelerinin olmadığı gösterilmiştir. Bu bağlamda, üretilen DNA polimeraz enziminin fidelitesi aslına uygunluğu bakımından diğer ticari enzimlerle benzerlik gösterdiği belirlenmiştir.

Bu çalışmada, Taq polimeraz enzim üretimiyle birlikte konvensiyonel ve kapiler olmak üzere iki adet yeni thermal cycler cihazının optimizasyon çalışmaları da gerçekleştirilmiştir. Konvensiyonel thermal cycler'in % 95'lik güvenilirlik aralığında değerlendirildiğinde ticari muadilleri kadar verimli olduğu gözlemlenmiştir. Kapiler thermal cycler cihazının ise yetersiz izolasyon koşulları ve kapileri cihaz uyuşmazlığı nedenlerinden dolayı daha az verimli olduğu söylenebilir.

Anahtar kelimeler: Taq DNA polimeraz; thermal cycler; PZR; Gateway rekombinasyon klonlama teknolojisi

To my family Elif, Ayla & Nazmi YILDIZ,

&

Sinan ÖZER

for their support, encourgement & endless love…

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Ammonium per sulfate	APS
Cetyltrimethylammonium bromide	CTAB
Dimethyl sulfoxide	DMSO
Ethylenediaminetetraacetic acid	EDTA
Isopropyl β-D-1-thiogalactopyranoside	IPTG
Phenylmethanesulfonylfluoride	PMSF
Sodium Dodecyl Sulfate	SDS
Tris-Acetate-EDTA	TAE

CHAPTER 1

INTRODUCTION

1.1 DNA Polymerase

DNA polymerase, the enzyme that synthesizes new DNA strands, was discovered in 1955, and was firstly purified in 1956 from *Escherichia coli* (Kornberg et al., 1956). Kornberg synthesized DNA from different sources with the purified DNA polymerase in 1957. After the discovery of the first DNA polymerase, biochemical, thermodynamic and structural studies were performed to characterize its function (Brautigam and Steitz, 1998; Eom et al., 1996; Joyce and Steitz, 1987; Kim et al., 1995; Korolev et al., 1995). Following these experiments, new DNA polymerases were found.

More than 50 different DNA polymerases have been identified since 1956. These studies showed that DNA polymerases have different characteristics and structural differences. Depending on their amino acid sequences and three dimensional structure, DNA polymerases are classified in 7 families; the family A (Ito and Braithwaite, 1991), the family B (Ito and Braithwaite, 1991), the family C (Ito and Braithwaite, 1991), the family D (Ito and Braithwaite, 1991), the family X (Filee et al., 2002), the family Y DNA polymerase (Yang, 2005), the family RT (Rothwell and Waksman, 2005).

Although there are grouped into several families, DNA polymerases have several fundamental features. DNA polymerases synthesize the new strand in the direction of 5’ to 3’, which is antiparallel with respect to the template strand and this is called as 5’-3’ polymerase activity. DNA polymerase requires a free 3’-

OH group to add 2- deoxyribonucleotide 5 triphosphate (dNTPs) into the growing chain (Hübscher et al., 2010). The strand that provides the free 3' OH group is called the primer and the mechanism is described as the RNA priming (Brutlag et al., 1971). The primer that is composed of RNA is synthesized by a distinct type of a single- strand dependent RNA polymerase (Rowen and Kornberg, 1978) (Griep, 1995).

DNA polymerase also requires Mg^{+2} ions as a cofactor and Mg^{+2} ions are crucial for the interaction of DNA polymerase with the template and the primer (Fisher and Korn, 1981).

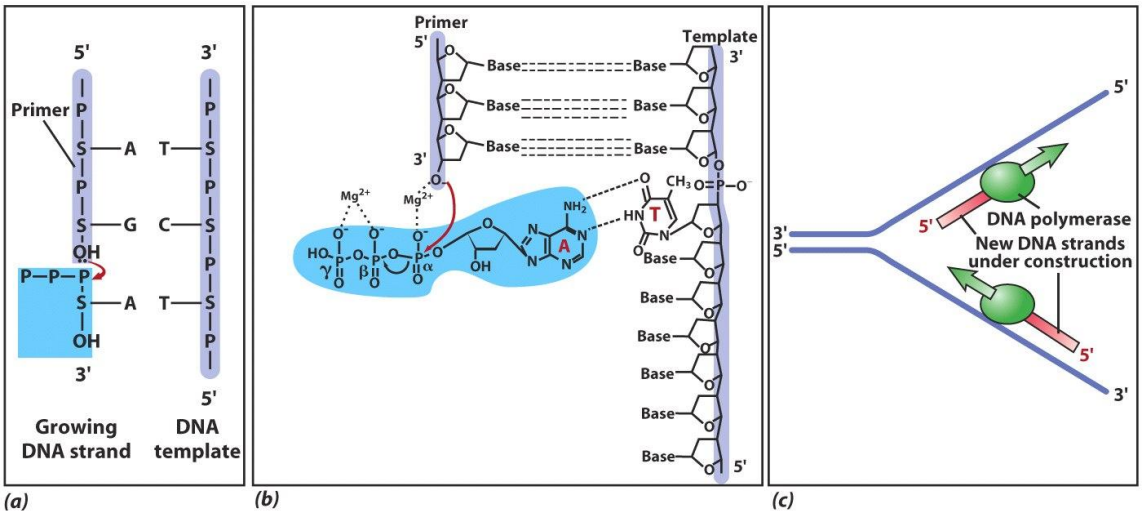


Figure 13-8 Cell and Molecular Biology, 4/e (© 2005 John Wiley & Sons)

Figure 1.1 Schematic representation of the incorporation of nucleotides onto the 3' end of a growing strand by a DNA polymerase (Karp, 2004)

To synthesize a new strand of DNA; DNA polymerase requires single-stranded DNA. First, DNA helicase unwinds double-stranded DNA using ATP to break hydrogen bond and newly formed single stranded DNA is kept separated by DNA binding proteins. Later, the primase constructs primer. Finally, DNA polymerase synthesizes the new strand using the template strand in the 5' to 3' direction. Because of DNA polymerase nature, while the one strand of the template DNA is continuously synthesized (leading strand), the other strand is discontinuously synthesized (lagging strand). In the lagging strand, small DNA segments are constructed and these small segments are called Okazaki fragments. Then, primers of Okazaki fragments are removed and these gaps are filled by the action of the DNA polymerase. Finally, DNA ligase seals the strand (Hübscher et al., 2010).

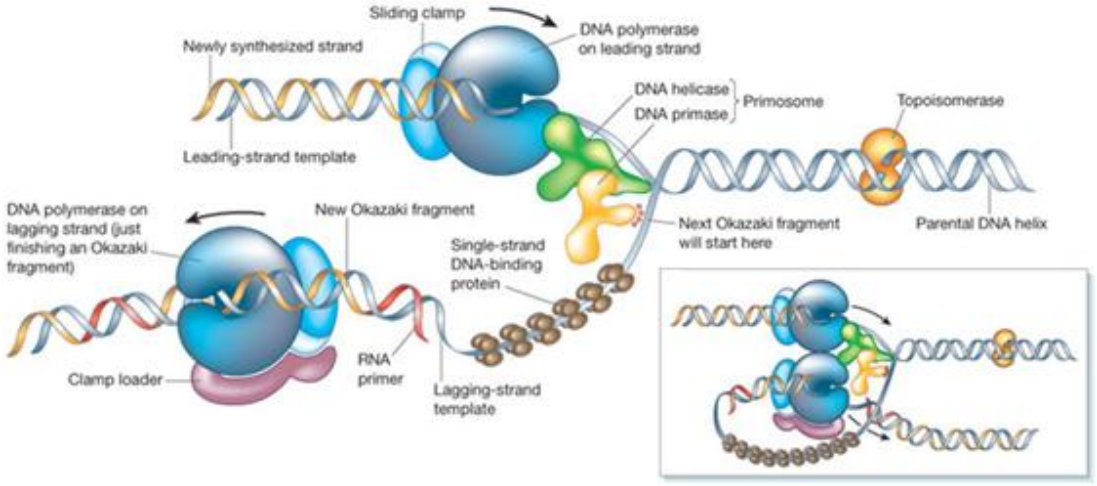


Figure 1.2 Schematic representation of DNA synthesis by DNA polymerase (Alberts, 2003)

In addition to 5'-3' polymerase activities, DNA polymerases can also have different activities: 5'-3' exonuclease activity that corrects the wrong nucleotides in the same direction of synthesis and 3'-5' exonuclease activity that allows it to proofread newly synthesized DNA (Karp, 2004; Klug et al., 2006).

1.2 Thermostable DNA Polymerase

DNA polymerase I from *Escherichia coli* was used first to amplify desired DNA template. Nevertheless, since DNA polymerase I is heat labile, it was required to be added after the DNA denaturation step while DNA existed as two single strands, and annealing of the primer to DNA template step in the each cycle. Addition of the DNA polymerase in each cycle was very difficult, time consuming and prone to error (Saiki et al., 1988).

Finding of the thermostable DNA polymerases leads the development of the PCR techniques as we know of today. Since, thermostable DNA polymerases are heat stable, there is no need to add thermostable DNA polymerase after each cycle. The first isolated thermostable DNA polymerase was *Taq* DNA polymerase from *Thermus aquaticus* (Chien et al., 1976). Thereafter, many DNA polymerases from *Thermus* strains such as *Top* (Kim et al., 1998), *Tfi* (Jung et al., 1997), other thermophilic bacteria strain such as *Pfu* (Lundberg et al., 1991), *Vent* (Cariello et al., 1991) were isolated and utilized for amplification.

1.3 Taq DNA Polymerase

1.3.1 *Thermus aquaticus*

Brock and his colleagues isolated *Thermus aquaticus* from a thermal spring in Yellowstone National Park in 1969. Although *T.aquaticus* is a gram-negative, nonsporulating, nonmotile rod, it was observed that its colony morphology is affected by the growth temperature and the growth stage of the culture, that the

organism can also form the long filamentous. While the growth temperature of *T.aquaticus* is between 40 ^{o}C- 79 ^{o}C, the optimum temperature is 70 ^{o}C. The organisms have a yellow cellular pigment and they are obligatory aerobic. The optimum pH for the growth of organism is between 7.5- 7.8. An artificial growth medium should consist of several sugars, organic acids, nitrogen source and minerals. *T.aquaticus* does not require vitamins and amino acids. The GC content of *T.aquaticus* DNA is between 65.4%-67.4% (Brock and Freeze, 1969).

1.3.2 Structure of Taq DNA Polymerase

The three–dimensional structures of a number of DNA polymerase enzymes are available (Joyce and Steitz, 1987; Kim et al., 1995). Klenow fragment of DNA polymerase I from *E.coli* was first structured by protease treatment. Klenow fragment has the 5'-3' polymerase and 3'-5'exonuclease activity, while it lacks 5'-3' exonuclease (Klenow and Hennings.I, 1970). This structure is named as right hand. Domains of the Klenow DNA polymerase are known as the fingers, the palm, and the thumb (Li et al., 1998; Ollis et al., 1985).

Taq DNA polymerase has both 5'-3' polymerase and 5'-3' exonuclease activities. Although Taq DNA polymerase has similar sequences of 3'-5' exonuclease region of *E.coli*, it lost the this activity (Tindall and Kunkel, 1988). The structure, shape and domains of Taq DNA polymerase are similar to DNA polymerase Klenow fragment.

Taq DNA polymerase and DNA polymerase I from *E.coli* have a sequence identity of 38% depending on amino acid sequence alignment (Lawyer et al., 1989). The structural alignment of Taq DNA polymerase and *E.coli* DNA polymerase I, on the other hand, revealed a similarity of 32%, while the similarities of 5'-3' exonuclease domain (residues 1 – 291), nonfunctional 3'-5'exonuclase domain (residues 292 - 423) and 5'-3' polymerase (residues 424 -

832) are 32%, 14% and 49%, respectively (Kim et al., 1995; Korolev et al., 1995; Li and Waksman, 2001; Villbrandt et al., 1997).

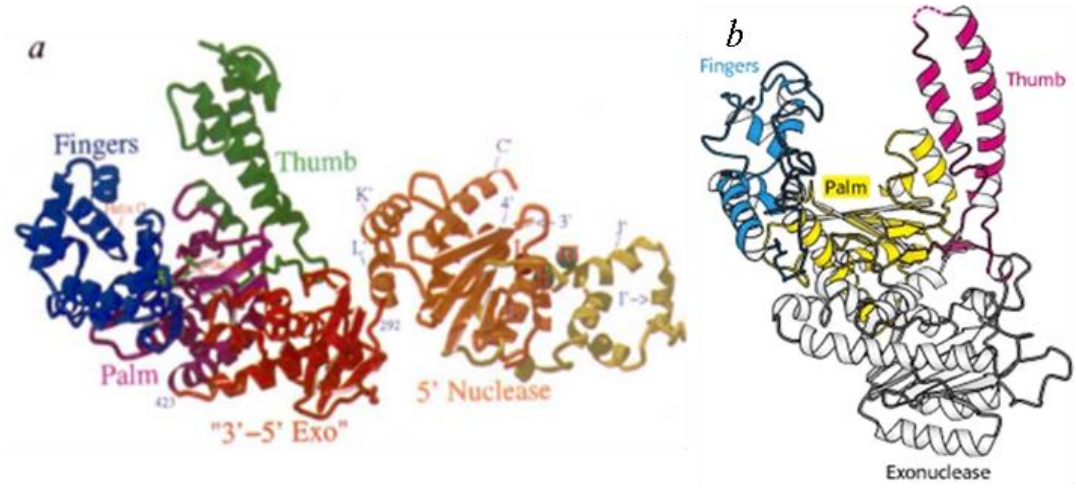


Figure 1.3 Schematic representation of the structure of DNA polymerases **a)** Taq DNA polymerase (Kim et al., 1995) **b)** DNA polymerase I (JM et al., 2002).

Taq DNA polymerase contains four times more hydrogen bonds compared to the DNA polymerase from *E.coli* and therefore thermally more stable. In addition to this, the ratio of leucine to isoleucine and arginine to lysine are higher in the Taq DNA polymerase 4.4 and 1.3 fold, respectively, which may result from the higher GC content. Moreover, two salt bridges between subdomains in the polymerase domain become hydrophobic. These changes are not affected the function of Taq DNA polymerase (Kim et al., 1995).

1.3.3 Characterization of Taq DNA Polymerase

Taq DNA polymerase belongs the Family A similar to DNA polymerase I from *E.coli* (Ito and Braithwaite, 1991). *Thermus aquaticus* DNA polymerase is a single polypeptide with 832 amino acids and a molecular weight of 94 kDa (Lawyer et al., 1989).

The fidelity of the Taq DNA polymerase is affected by pH, temperature, salt concentration and concentration of metallic ions (Tindall and Kunkel, 1988). pI value of Taq polymerase is 6.4 (Oktem et al., 2007). While Taq DNA polymerase works at a pH between 7 and 8, the optimum pH is 7.8. Taq DNA polymerase requires divalent cations such as Mg^{+2} and Mn^{+2} like other DNA polymerases as a cofactor. Most favorable condition of the Taq DNA polymerase is in the 1-2 mM Mg^{+2} concentration range. When considering Mn^{+2} effects on Taq DNA polymerase, it is partial and optimum in the 2 mM. According to studies, there is no effect of calcium ion on Taq DNA polymerase. The addition of the salt such as KCl and NaCl also affects the activity of Taq DNA polymerase. The optimal concentration of NaCl is 40 mM, whereas that of KCl is 55-60 mM. The activity of Taq DNA polymerase is inhibited above 100 mM of either KCl or NaCl (Chien et al., 1976; Lawyer et al., 1993; Tindall and Kunkel, 1988). The optimum temperature for Taq DNA polymerase is between 70- 80 ^{o}C. It is shelf life at 97.5 ^{o}C for 9 min (Lawyer et al., 1993). The error rate of base substitution of Taq DNA polymerase at 70 ^{o}C is 1/9000 bp (Tindall and Kunkel, 1988).

1.4 Prokaryotic Expression System

Expression systems are very crucial for the production of high level recombinant proteins. Selection of a suitable expression system relies on many aspects; for example cell growth characteristics, expression levels, post translational modification and the desired protein's biological activity (Goeddel, 1990;

Hodgson, 1993). Bacterial (Shatzman and Rosenberg, 1987), yeast (Hitzeman et al., 1981), insect (Kitts and Possee, 1992) and mammalian (Kaufman, 1990) expression systems are widely used today. Although bacterial expression systems have some drawbacks such as the accumulation of insoluble proteins due to the large amount of expression (inclusion bodies), the presence of toxic cell wall or the lack of eukaryotic enzymes for the post translational modification, bacterial expression systems, especially *E.coli*, are a very valuable in the recombinant protein production. Popularity of *E.coli* in the bacterial expression system is due to knowledge about its genetics and physiology, availability of its genome sequences, high growth rate with ability of expression heterologous proteins in simple and inexpensive media, large scale production and easy purification of recombinant protein due to recombinant fusion proteins (Baneyx, 1999; Casadaban et al., 1983; Jonasson et al., 2002; Makrides, 1996; Schumann and Ferreira, 2004; Shatzman and Rosenberg, 1987).

In the prokaryotic expression systems, plasmids contain a tightly regulated promoter, a positive selection region such as an antibiotic resistance gene, species specific origin of replication and sometimes a region that encodes for a recombinant fusion protein, a multiple cloning region which includes several restriction enzymes cut sites side by side. In Table 1.1, some promoters are used for recombinant proteins production in prokaryotic expression system.

Table 1.1 Promoters used for the high expression level of genes in prokaryotic expression systems (Makrides, 1996)

Promoter (source)	Regulation	Induction
lac (*E. coli*)	*lacI*, *lacI*^{q}	IPTG
	lacI(Ts),[a] *lacI*^{q}(Ts)[a]	Thermal
	lacI(Ts)[b]	Thermal
trp (*E. coli*)		Trp starvation, indole acrylic acid
lpp (*E. coli*)		IPTG, lactose[c]
phoA (*E. coli*)	*phoB* (positive), *phoR* (negative)	Phosphate starvation
recA (*E. coli*)	*lexA*	Nalidixic acid
araBAD (*E. coli*)	araC	L-Arabinose
proU (*E. coli*)		Osmolarity
cst-1 (*E. coli*)		Glucose starvation
tetA (*E. coli*)		Tetracycline
cadA (*E. coli*)	*cadR*	pH
nar (*E. coli*)	*fnr* (FNR, NARL)	Anaerobic conditions, nitrate ion
tac, hybrid (*E. coli*)	*lacI*, *lacI*^{q}	IPTG
	lacI^q	Thermal
trc, hybrid (*E. coli*)	*lacI*, *lacI*^{q}	IPTG
	lacI(Ts),[a] *lacI*^{q}(Ts)[a]	Thermal
lpp-lac, hybrid (*E. coli*)	*lacI*	IPTG
P_{syn}, synthetic (*E. coli*)	*lacI*, *lacI*^{q}	IPTG
Starvation promoters (*E. coli*)		
p_L (λ)	λ*c*I*ts*857	Thermal
p_L-9G-50, mutant (λ)		Reduced temperature (<20°C)
cspA (*E. coli*)		Reduced temperature (<20°C)
p_R, p_L, tandem (λ)	λ*c*I*ts*857	Thermal
T7 (T7)	λ*c*I*ts*857	Thermal
T7-*lac* operator (T7)	*lacI*^{q}	IPTG
λp_L, p_{T7}, tandem (λ, T7)	λ*c*I*ts*857, *lacI*^{q}	Thermal, IPTG
T3-*lac* operator (T3)	*lacI*^{q}	IPTG
T5-*lac* operator (T5)	*lacI*^{q}, *lacI*	IPTG
T4 gene 32 (T4)		T4 infection
nprM-lac operator (*Bacillus* spp.)	*lacI*^{q}	IPTG
VHb (*Vitreoscilla* spp.)		Oxygen, cAMP-CAP[e]
Protein A (*Staphylococcus aureus*)		

The common promoter utilized in prokaryotic expression system is the lac promoter. This system is inducible and therefore overproduction of protein that may otherwise cause toxic effect or retardation of bacterial growth is prevented (Dong et al., 1997; Hoffman and Rinas, 2004). Lac operon is regulated with an inducer that leads to expression of the genes in lac operon. The lac promoter is induced by lactose or by a synthetic inducer isopropylthiogalactoside (IPTG). If lactose is absent in the medium, repressor binds to lac operator located in the downstream of the lac promoter and prevents the transcription of β-galactosidase that is necessitated for the degradation of lactose. If lactose is present in the

medium as a sole carbon source, then lactose binds to repressor preventing its interaction with the lac operator. Transcription takes places and recombinant gene is also transcript, as the recombinant gene is located downstream of the lac promoter. When β-galactosidase gene is transcripted, recombinant gene is also transcripted. In the lac promoter system, IPTG is used as the inducer as IPTG cannot be hydrolyzed by β- galactosidase.

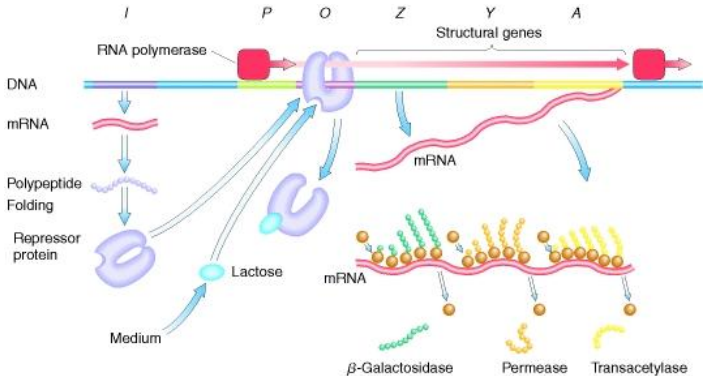


Figure 1.4 Schematic representation of lac operon in prokaryotes expression system (Griffiths A.J.F., 1999)

1.5 Thermal Cycler

Once Taq DNA polymerase has been isolated (Chien et al., 1976), the idea of *in vitro* DNA amplification was conceived by Karry Mullis in the mid-‘80s (Mullis, 1987, 1990). This method *in vitro* DNA amplification is called polymerase chain reaction (PCR) (Saiki et al., 1985). After that, by the discovery of PCR in 1993, Karry Mullis was given the Nobel Prize in Chemistry.

PCR is a very practical technique used for the amplification of a specific region of DNA *in vitro*. In PCR, there are three basic steps; DNA denaturation, primer annealing and new strand extension. During the denaturation step, double stranded template DNA becomes single stranded by breaking hydrogen bonds between bases. To break hydrogen bonds, the high temperature such as 94- 95 ^{o}C is required. Following the denaturation step, primer, a short single stranded DNA and complementary with template, is annealed to ssDNA in the annealing step. Annealing temperature is important for the optimization of PCR to form hydrogen bond between the template DNA and the primer. In the extension step, DNA polymerase adds free 3'-OH group to add 2-deoxyribonucleotide 5 triphosphate (dNTPs) into the growing chain at 72 ^{o}C. These steps are repeated for 30 -40 cycles to obtain enough number of DNA copy.

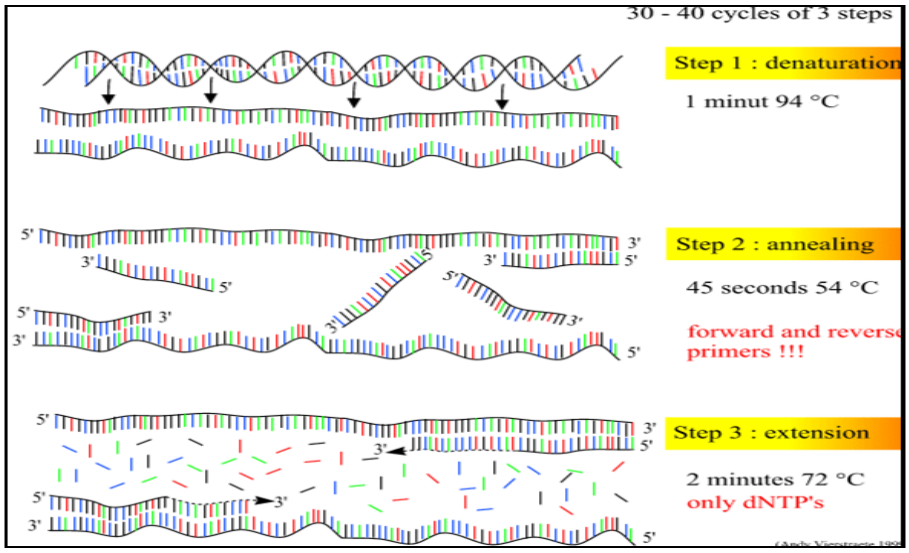


Figure 1.5 Schematic representation of polymerase chain reaction (PCR) (http://users.ugent.be/~avierstr/principles/pcr.html)

The temperature cycles in the PCR was first done in a water bath or heat block. The first automatic PCR machine, thermal cycler, was developed by changing temperature repeatedly in the heating and cooling system. The system was hold up to 24 samples. The computer program in the thermal cycler automatically controls the temperature cycles in each step within cycles. The temperature of each step in the cycle, the duration of steps and cycle number can be programmable and programmed PCR reaction automatically is carried out. The first thermal cycler had two waterbaths, 37 ^{o}C and 96 ^{o}C connected with sample tubes, valves and associated plumbing. Temperature of water can exchange very fast by pumping (Weier and Gray, 1988).

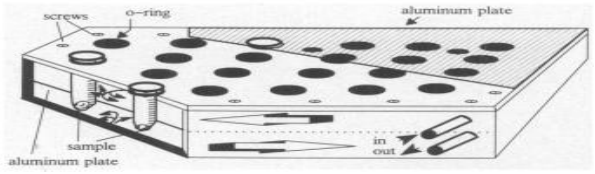


Figure 1.6 Schematic representation of sample holder of the first thermal cycler (Weier and Gray, 1988)

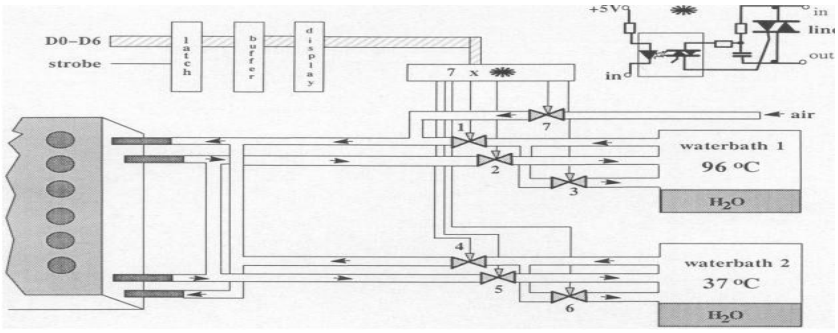


Figure 1.7 Schematic representation of the whole system of thermal system (Weier and Gray, 1988)

After the first commercially available thermal cycler, technology used for the construction of thermal cyclers has been dramatically changed. In the first thermal cycler, heating and cooling system was based on resistive heaters and a compressor, respectively. This system was not used at temperatures below 15 ^{o}C (Bierschenk et al., 1995).

Later, thermoelectrical heat pumps, Peltier units, were begun to use instead of the external compressor and water. In the first application of thermoelectrical pumps, thermal cycler cannot resist the temperature changes. Nevertheless, by the development of thermoelectric elements, the problem was solved (Bierschenk et al., 1995).

In Peltier heat pumps system, heat transfer from sample tube can be 1 ^{o}C/seconds within 0-100 ^{o}C depending on the characteristics of the Peltier system used. The principles of Peltier elements depend on the differences of voltage between two materials. When applying positive voltage, sample vials are cooled. After reaching low temperature, the polarity of voltage is changed and the vial is heated up to 100 ^{o}C. Cooling performance of Peltier system depends on the heat sink which decreases the temperature by fluid medium such as air, water (Aivazov et al., 1997; Bierschenk et al., 1995; Shafai and Brett, 1996). After the construction of the thermal cycler with Peltier system, gradient block in thermal cycler which allows variety of temperatures in various well of the block was developed to overcome the problem of the adjusting annealing temperature. For different primer sets, annealing temperature is optimized for obtaining desired PCR product. The time that is required for optimization is decreased with the invention of gradient block (Danssaert et al., 1996).

In the past, to avoid of the condensation of water in the PCR reaction mixture, oil was used to cover reaction mixture. In the modern thermal cyclers, in addition to Peltier system, a heated lid, is also used to prevent condensation of

the PCR mixture (Jones, 1993). In Figure 1.8, the schematic representation of thermal cycler that was heated and cooled by Peltier system was shown.

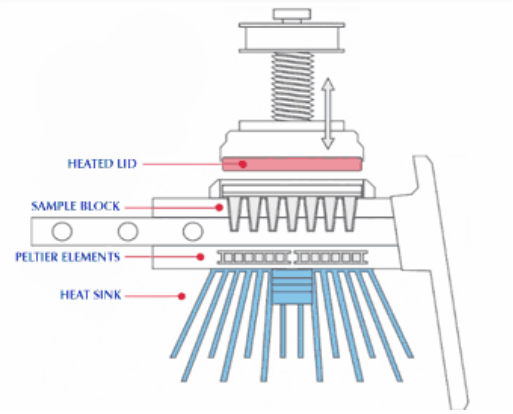


Figure 1.8 Schematic representation of thermal cycler that is cooled by Peltier system (http://www.dharmacon.com/uploadedImages/Home/Products/PCR_Research_Products/PCR_Instruments/Piko_Thermal_Cycler/HEATPUMP.gif)

In 1992, Higuchi and collogues was studied the analysis of PCR kinetics by staining double strand DNA with ethidium bromide (Higuchi et al., 1992). Higuchi and collogues observed amplification of DNA in thermal cycler in 1993. Ethidium bromide is in use for staining double stranded DNA which irradiated by UV light. This was observed by CCD camera. The amount of fluorescence intensity increases with the increasing amount of DNA with ethidium bromide by cycle. Higuchi and collogues plotted the graph of intensity value of DNA versus cycle number. By this experiment, the first real-time PCR machine was constructed (Higuchi et al., 1993). This real-time PCR machine has been developed and there are different types available. Real-time PCR method is very fast, precise method (Raghavan et al., 2006). Moreover, it is used as a

quantification method of gene expression and construction of melting curve. Instead of the usage of ethidium bromide, today labeled hybridization probe such as TaqMan probe, Molecular Beacons, labeled PCR primer or SYBR green are used as a fluorescent dye of DNA (VanGuilder et al., 2008).

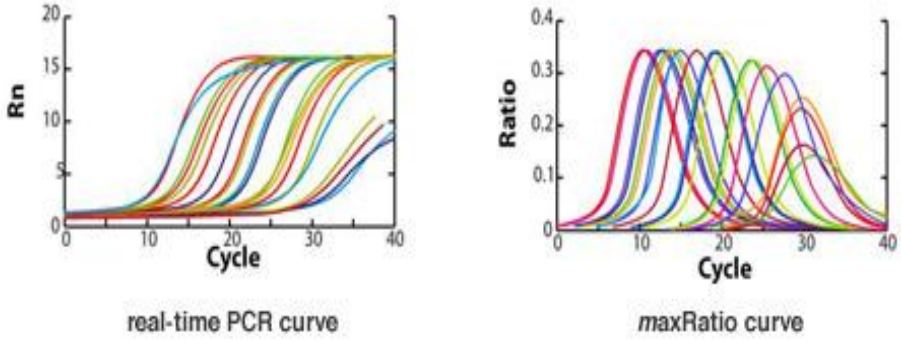


Figure 1.9 Representation of real time PCR result. The first one shows the fluorescence intensity versus cycle number. The second graph shows the melting curve of the sample (http://www.abbottmolecular.com/us/technologies/real-time-pcr/maxratio-data-analysis.html).

1.6 Aim of This Study

In this study, it is described that the cloning of the Taq DNA polymerase gene from *Thermus aquaticus* in the Gateway Expression System. In the Gateway Expression system, pCR®8/GW/TOPO® TA cloning vector was in use as an entry vector and pDEST14™ used as an expression vector. Induction method and lysis method was optimized to obtain maximum amount of active Taq DNA polymerase enzyme. Enzyme activity of this protein was determined using of different source of DNA, bacteria, plasmid, human, and different Tm of the

primer by comparing other commercial available Taq DNA polymerase enzyme. Some results were sequenced for obtaining the misincorporation rate of isolated DNA polymerase enzyme. The nicking activity and restriction endonuclease activity of produced Taq DNA polymerase enzyme was tested. In addition to this, newly developed two thermal cyclers were tested by different source of DNA, bacterial, plasmid, human primer by comparing other available thermal cycler.

CHAPTER 2

MATERIALS AND METHODS

2.1 Materials

2.1.1 Chemicals

In this study all chemicals were purchased from AppliChem Chemical Company, Sigma-Aldrich Chemical Company, and Merck Chemical Company. Each solution was prepared with distilled water in experiments.

2.1.2 Synthetic Oligonucleotides

All oligonucleotides were purchased from the company Integrated DNA Technologies (Coralville, IA, USA) in lyophilized form.

2.1.3 Bacterial Strain

Thermus aquaticus YT1 purchased from American Type Culture Collection (ATCC, catalog number 25104).

E.coli strain TOP10 bacteria were used for preparation of plasmid and cloning. Competent *E.coli* was gently provided by Tufan Öz and Hamdi Kamçı, from Biotechnology Department, METU, Ankara, Turkey.

2.1.4 Expression Plasmid Vector

pCR®8/GW/TOPO® vector was purchased from Invitrogen's pCR®8/GW/TOPO® TA Cloning® Kit for cloning (catalog no: K2500-20).

pDEST™14 expression vector was purchased from Invitrogen's Gateway® pDEST™14 Vector (catalog no: 11801-016).

2.2 Methods

2.2.1 DNA Isolation Methods

Plasmid DNA isolation process was performed using GeneJet Plasmid Miniprep Kit of Fermentas (catalog no: K0503). With respect to the kit protocol, a single colony from plate was selected and inoculated into LB medium added. It was incubated 12-16 hrs at 37°C. It was then transferred to centrifuge tube and centrifuged at 6800 xg for 2 min at RT. Supernatant was discarded and pellet was resuspended with resuspension solution (250 µl) by pipetting. Lysis solution (250 µl) was put and mixed thoroughly by inverting the tube 4-6 times. The neutralization solution (350 µl) was added and mixed immediately and thoroughly by inverting the tube 4-6 times. The mixture was centrifuged at 12000 xg for 5 min. The supernatant was transferred into the supplied GeneJET™ spin column using pipetting and was centrifuged at 12000 xg for 1 minute. Then the flow through was removed and the column was placed into the same collection tube. 500 µl of wash solution was washed and centrifuged at 12000 xg for 60 sec. The flow through was removed and the column was placed into the same collection tube. The washing step was done again. Then the column is centrifuged at 12000 xg for 1 min and transferred into a microcentrifuge tube. 30 µl of sterile distilled water was added into spin column and incubated for 2 min at RT. Finally, the spin column was centrifuged at 12000 xg for 2 minutes and the plasmid DNA at -20 °C.

2.2.1.2 Bacterial Genomic DNA Isolation

2.2.1.2.1 CTAB Method for Bacterial Genomic DNA Isolation

The genomic DNA was isolated from *Thermus aquaticus* with CTAB Bacterial DNA isolation method (Meade et al., 1982; Silhavy et al., 1984).

Thermus aquaticus was inoculated into the *Thermus* 162 Medium for 2 days at 70 ^{o}C. The grown bacteria culture was put into the centrifuge tube and centrifuged for 2 min at 13000 rpm.

The supernatant was removed and pellet was resuspended in 567 µl TE buffer by pipetting. Then, 10% SDS (30 µl) and 20 mg/mL proteinase K (3 µl) were put. The solution was mixed thoroughly and incubated at 37 ^{o}C for 1 hour. 5 M NaCl (10 µl) is added into the mixture and mix thoroughly. Then 80 µl of CTAB/NaCl solution is added into the mixture. After mixing solution thoroughly, it is incubated for 10 min at 65 ^{o}C. Then an approximately equal volume (0.7 to 0.8 ml) of chloroform/isoamyl alcohol (24:1) was put in and then mixed thoroughly. It was centrifuged at 14000 rpm for 5 min and upper phase was taken into a new centrifuge tube. One volume phenol/chloroform/isoamyl alcohol (25:24:1) was put into the centrifuge tube and mixed thoroughly. The mixture was centrifuged at 14000 rpm for 5 min and then the upper phase was taken into a new centrifuge tube. 0.6 volume of isopropanol (0.42 to 0.48 mL) was put and then mixed the tube. The tube was centrifuged at 14000 rpm for 5 min and then the pellet was washed with 70% cold ethanol. After washing step, it is removed by centrifugation at 14000 rpm for 5 min. Supernatant was removed and pellet was dried in the hood at RT. Finally, pellet was dissolved with 50 µl nuclease free water and stored at +4 ^{o}C.

2.2.1.2.2 Bacterial DNA Isolation with Heating

Thermus aquaticus was inoculated into the *Thermus* 162 Medium for 2 days at 70 ^{o}C. 1.5 ml incubated *Thermus aquaticus* bacteria culture was put into 2 ml eppendorf tubes. 5 eppendorf tubes were put into 100 ^{o}C waterbath for each; 10 minutes, 20 minutes, 30 minutes, 90 minutes time period, correspondingly. From each tube, 250 µl sample was taken into new tubes and the remaining samples was centrifuged at 13000 xg for 5 min. The supernatant of each sample was taken into the new centrifuge tubes.

2.2.1.2 3 Bacterial DNA Isolation with NANObiz DNA Isolation Kit

The genomic DNA was isolated from *Thermus aquaticus* using NANObiz DNA isolation kit, Turkey.

Thermus aquaticus was inoculated into the *Thermus* 162 Medium at 70 ^{o}C for 2 days. The grown bacteria culture was put into the tube and centrifuged at 14000 rpm for 3 min.

Pellet was resuspended by 300 µl lysis and neutralization buffer. After that, 3 µl 200 µl/mL proteinase K was added and mixed by pipetting. The mixture was incubated at 50 ^{o}C for 15 min. Washing buffer I was added into incubated mixture and then was mixed by pipetting. Mixture was loaded into the column and centrifuged at 10000 rpm for 1 min. After removing the flow through from collection tube, 700 µl washing buffer II was added into spin column. The column was centrifuged at 10000 rpm for 1 min and then discarded the flow through from collection tube. The washing and centrifugation steps were repeated. After that, centrifugation at 10000 rpm for 2 min was carried out. The spin column was placed into a new centrifuge tube. 50 µl of nuclease free water was put into the spin column and it was incubated 2 min at RT. Finally, it was centrifuged at 10000 rpm for 1 min and store bacterial DNA at +4 ^{o}C.

2.2.2 Cloning of Taq DNA Polymerase Gene by GATEWAY Cloning System

2.2.2.1 PCR Conditions for Taq DNA Polymerase Gene

Depending on the known Taq DNA polymerase gene sequence (GenBank Accession No: J04639, Appendix A), the precise primers for Taq DNA polymerase gene were ordered. Sense PCR primer sequence was 5'-CACCATGAGGGGGATGCTGCCC-3' and antisense PCR primer sequence was 5'-TCACTCCTTGGCGGAGAGC-3'.

The PCR reaction was carried out by Herculase II Fusion Taq DNA polymerase. It was purchased from the Agilent Technologies Company. Taq DNA polymerase gene PCR product of 2502 bp was obtained.

Optimized PCR conditions for Taq DNA polymerase gene is listed as follows:

Table 2.1 Optimized conditions of PCR to amplify Taq DNA polymerase gene

Ingredients	Final Concentration
dH_2O	
Reaction Buffer	1X
DMSO	6%
Sense primer	0.2 µM
Antisense primer	0.2 µM
dNTP	0.25 mM
T. aquaticus genomic DNA	50 ng/µl
Herculase polymerase	5U

Table 2.2 PCR cycling conditions to amplify Taq DNA polymerase gene

Steps	Taq DNA polymerase gene PCR Parameters
Denaturation	94°C 5 minutes
Amplification	94°C 15 seconds
	56°C 45 seconds
	72°C 2 minutes
Number of cycles	35
Final extension	72°C 5 minutes

2.2.2.2 Elution of Taq DNA Polymerase Gene PCR Product from Agarose Gel

Taq DNA polymerase gene PCR product was loaded into agarose gel and was eluted from agarose gel by QIAGEN gel extraction kit. With respect to the kit protocol, the DNA fragment from agarose gel was cut. It was weight and 100 mg of excised gel considered as 100 µl and 3 volumes of Buffer QG was added to 1 volume of gel. The mixture was incubated at 50 °C till it dissolved. 1 volume isopropanol was added into the sample and was loaded to QIAquick spin column in collection tube. It was centrifuged at 14000 rpm for 1 min and removed flow through. 0.5 mL of Buffer QG was put into the column and centrifuged at 14000 rpm for 1 min. The flow through was removed and it was centrifuged at 14000 rpm for 1 min. Column was placed into centrifuge tube. Finally, 50 µl of sterile distilled water was loaded and centrifuged it for 1 min at 14000 rpm.

2.2.2.3 Cloning of Amplified Taq DNA Polymerase Gene into Entry Vector

Amplified Taq DNA polymerase gene was cloned into the pCR®8/GW/TOPO® TA cloning vector (entry vector) by kit protocol.

With respect to the kit protocol, 4 µl gel eluted Taq DNA polymerase PCR product, 1 µl salt solution and 1 µl pCR®8/GW/TOPO® TA cloning vector were put into the 0.5 ml PCR tubes and mixed gently. The mixture was incubated at RT for 30 minutes. Following that, it was put into the ice and preceded to transformation.

6 µl TOPO cloning reaction was added into a vial of chemically competent *Escherichia coli* cells and mixed gently. The mixture was incubated on ice for 30 min. *E.coli* cells were heat shock at 42 ^{o}C for 60 seconds without shaking and the tube was transferred immediately on ice. 250 µl S.O.C medium was added into the tube and the tube was incubated at 37 ^{o}C for 1 hour with shaking. The bacteria were spreaded on LB agar plate containing 100 µg/ml spectinomycin, and incubated overnight at 37 °C.

2.2.2.4 Colony PCR of Entry Vector

To confirm cloning of Taq DNA polymerase gene in a correct direction into entry vector, colony PCR was done. Two sets of primers were used. In one PCR reaction, sense primer was universal M13F (-20), 5'-GTAAAACGACGGCCAG-3′ and antisense primer was specific to internal side of Taq DNA polymerase gene, 5'-GACTCGTCCCCGGTCAGGGC-3'. In the second PCR reaction, sense primer was specific to internal side of Taq DNA polymerase gene, 5'-ACTTCGGGGTCCTCTACGGC-3' and antisense primer was universal M13R, 5'-AACAGCTATGACCATG-3'. The PCR products were analyzed by agarose electrophoresis.

The grown bacteria on LB agar containing 100 µg/ ml spectinomycin are used as a template and replica plate was done for each used bacteria.

Optimized PCR conditions for 25 µl colony PCR of Taq DNA polymerase gene is listed as follows:

Table 2.3 Optimized conditions of colony PCR of Entry vector

Ingredients	Final Concentration
dH_2O	
Reaction Buffer	1X
$MgCl_2$	1.5 mM
Sense primer	0.4 µM
Antisense primer	0.4 µM
dNTP	0.4 mM
Fermentas Taq polymerase	1.25U

Table 2.4 Colony PCR cycling conditions of Entry vector

Steps	Entry Vector PCR Parameters
Denaturation	94°C 5 min
Amplification	94°C 30 sec
	54°C 45 sec
	72°C 3 min
Number of cycles	35
Final extension	72°C 5 min

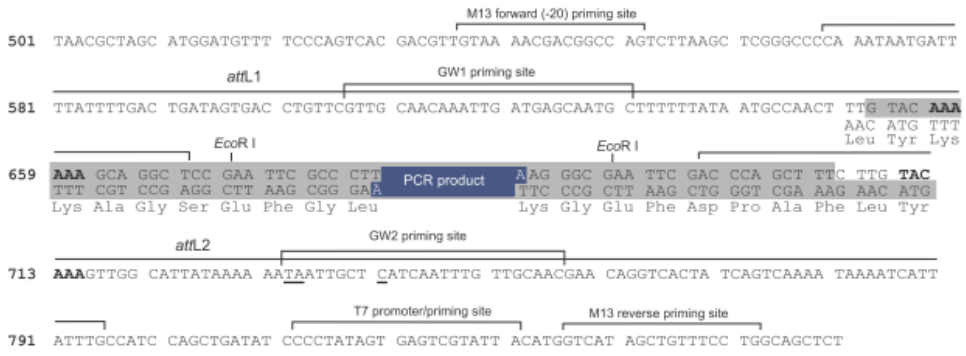


Figure 2.1 Representation of PCR product for TOPO® Cloning into entry vector

The map of pCR®8/GW/TOPO® TA cloning (entry) vector and this vector containing Taq polymerase gene was shown in the Appendix B.

The band size of first reaction of colony PCR of TOPO cloning vector was 696 bp and the band size of second reaction of colony PCR of TOPO cloning vector was 661 bp.

2.2.2.5. Sequencing of Amplified Taq DNA polymerase Gene

After confirming cloning of Taq DNA polymerase gene in a correct direction into cloning vector, the correct form of plasmid was sent to MC lab to be sequenced with different primer that was selected using known sequencing of Taq DNA polymerase gene (Appendix A).

2.2.2.6 LR Recombination Reaction

After confirming the sequence of the cloned entry vector containing Taq DNA polymerase gene, this gene containing vector was isolated from Top10 strain of *E.coli* using plasmid isolation kit.

With respect to the kit protocol, 0.6 µl pDEST14 vector, 0.6 µl entry vector containing Taq DNA polymerase gene, 3 µl TE buffer, 1 µl Gateway LR Clonase II enzyme mix were put into the 0.2 ml PCR tubes and mixed gently. The mixture was incubated at 25 ^{o}C for 1 hour. Following that, the mixture was placed into the ice and 0.6 µl proteinase K was put into the incubated mixture. The new mixture was incubated at 37 ^{o}C for 10 min and then was preceded to transformation.

5.8 µl LR recombination reaction mixture was put into a vial of competent *Escherichia coli* cells and mixed gently. The mixture was incubated on ice for 30 min. The *E.coli* cells were heat shock at 42 ^{o}C for 60 sec without shaking and the

tube was transferred immediately on ice. 250 µl S.O.C medium was added into the tube and the tube was incubated at 37 ^{o}C for 1 hour with shaking. The bacteria were spreaded on LB agar plate containing 100 µg/ml spectinomycin, and incubated overnight at 37 °C.

2.2.2.7 Colony PCR of Expression Vector

To confirm cloning of Taq DNA polymerase gene in a correct direction into entry vector, colony PCR was done. Two sets of primers were used. In one PCR reaction, sense primer was universal T7 sequencing primer, 5'-TAATACGACTCACTATAGGG-3' and antisense primer was specific to internal side of Taq DNA polymerase gene, 5'-GACTCGTCCCC GGTCAGGGC-3'. In the second PCR reaction, sense primer was specific to internal side of Taq DNA polymerase gene, 5'-ACTTCGGGGTCCTCTACGGC-3' and antisense primer was universal T7 terminator primer 5'-GCTAGTTATTG CTCAGCGG-3'.

The grown bacteria on LB agar containing 100 µg/ml ampicilin are used as a template and replica plate was done for each used bacteria.

Table 2.5 Optimized conditions of colony PCR of Expression vector

Ingredients	Final Concentration
dH_2O	
Reaction Buffer	1X
$MgCl_2$	1.5 mM
Sense primer	0.4 µM
Antisense primer	0.4 µM
dNTP	0.4 mM
Fermentas Taq polymerase	1.25U

Table 2.6 Colony PCR cycling conditions of Expression vector

Steps	Expression Vector PCR Parameters
Denaturation	94°C 5 min
Amplification	94°C 30 sec
	54°C 45 secs
	72°C 3 min
Number of cycles	35
Final extension	72°C 5 min

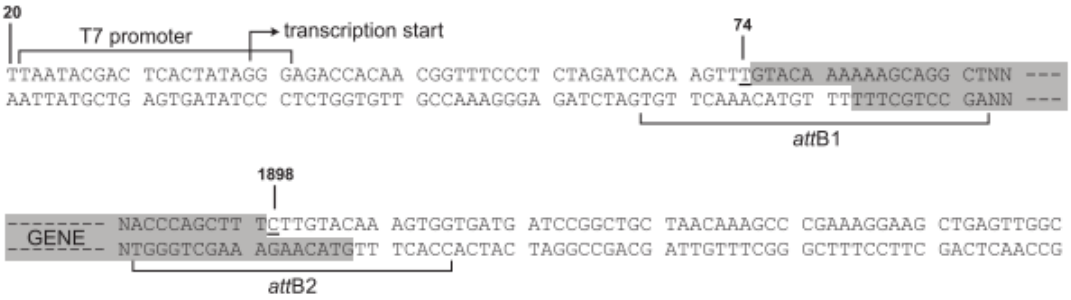


Figure 2.2 Representation of recombination region of the expression clone resulting from pDEST™14 entry clone

The map of pDEST™14 cloning vector and the pDEST™14 cloning vector containing Taq polymerase gene was shown in the Appendix B.

The band size of first reaction of colony PCR of TOPO cloning vector was 622 bp and the band size of second reaction of colony PCR of TOPO cloning vector was 613 bp.

2.2.3 Determination of *E.coli* Growth

Growth curve was determined by indirect measurement, turbidity method (Koch and Crandall, 1968; Madigan and Martinko, 2006). *E.coli* bacteria containing Taq DNA polymerase gene plasmid were spreaded on LB agar plate and incubated overnight at 37 °C. Then grown one colony was selected and inoculated into LB medium and incubated over night at 37 ^{o}C as a first culture. Following that, 50 µl was taken from first culture and was inoculated into 50 ml LB medium containing 100 µg/ml ampicilin. This culture was incubated at 37 ^{o}C with 130 rpm shaking and every 30 min the sample was taken and the absorbance of bacterial turbidity at 600 nm was measured in the Shimadzu UVmini 1240. LB medium was used as a blank solution. Dilution of sample was done by LB medium when $OD_{600\ nm}$ measurement of turbidity exceeds 1.

2.2.4 Determination of Bacteria Induction Method

The bacteria were spreaded from glycerol stock to plate and incubated overnight at 37 ^{o}C. After that one colony was taken and then inoculated into the LB medium. It was incubated overnight at 37 ^{o}C.

50 µl preculture bacteria was inoculated into 10 mL LB medium. Four different induction methods were tried to obtain the highest amount of Taq DNA polymerase enzyme. First one was that the inoculated bacteria were incubated until $OD_{600\ nm}$ was equal to 0.2 and then 0.5 mM IPTG was added. After that the bacteria were incubated for 18 hours at 37 ^{o}C. The second method was that the inoculated bacteria were incubated until $OD_{600\ nm}$ was equal to 0.4 and then 0.5 mM IPTG was added. Following that, these bacteria were incubated for 18 hours at 28 ^{o}C. The third method was that the inoculated bacteria were incubated until $OD_{600\ nm}$ was equal to 0.8 and then 0.5 mM IPTG was added. After the bacteria was incubated for 18 hours at 37 ^{o}C. The fourth method was that the inoculated bacteria were incubated until $OD_{600\ nm}$ was equal to 0.4 and then 0.5 mM IPTG

was added. After the bacteria was incubated for 3 hours at 37 ^{o}C. The fourth bacteria were put into -80 ^{o}C to continue the rest of procedure with other methods.

After these steps, the same procedure was applied to all methods. The bacteria were centrifuged at 10000 xg for 10 min at 4 ^{o}C for each tube. After that, pellet was resuspended in 1 ml Buffer A. Then they were centrifuged at 10000 xg for 10 min at 4 ^{o}C. The supernatant was discarded and pellet was resuspended in 250 µl Buffer A with lysozyme (final concentration 4 mg/mL). These mixtures were incubated at RT for 15 min and then were added 250 µl Buffer B. The mixtures were incubated at 75 ^{o}C for 30 min. After that the tubes were centrifuged at 14000 xg for 20 min at 4 ^{o}C and supernatants were collected and Taq DNA polymerase enzyme amount was determined.

2.2.5 Determination of Lysis Method of *E.coli*

The bacteria were spreaded from glycerol stock to plate and incubated overnight at 37 ^{o}C. After that one colony was taken and then inoculated into the LB medium. It was incubated overnight at 37 ^{o}C. Preculture bacteria were inoculated into 1L LB medium. The inoculated bacteria were incubated until $OD_{600\ nm}$ was equal to 0.2 and then 0.5 mM IPTG was added. After, the bacteria were incubated for 18 hours at 37 ^{o}C. The bacteria were centrifuged at 10000 xg for 10 min at 4 ^{o}C for each tube. After that, supernatant was removed and pellet was resuspended in 100 ml Buffer A. Then they were centrifuged at 10000 xg for 10 min at 4 ^{o}C.

After that, two different methods were applied each culture. In one method, pellet was resuspended in 25 ml Buffer A with lysozyme (final concentration 4 mg/mL). In the second one, pellet was resuspended in 50 ml Buffer A and then high pressure homogenizer was applied at +4 °C 35000 psi (2.41 kbar) for 4 times.

These mixtures were incubated at RT for 15 min and then were put in 25 ml Buffer B. The mixtures were incubated at 75 ^{o}C for 30 min. After that they were centrifuged at 14000 xg for 20 min at 4 ^{o}C and the supernatants were collected and the enzyme amount was determined.

2.2.6 Taq DNA Polymerase Enzyme Purification

Taq DNA polymerase protein was purified using ammonium sulfate precipitation (Jakoby, 1971). Firstly, 21% ammonium sulfate of the supernatant was added into the supernatant. Then this mixture was centrifuged at 20000 xg for 20 min at 4^{o}C and collected supernatant. 16% ammonium sulfate was added into the supernatant to obtain the concentration of 37% ammonium sulfate in the mixture. Following this step, it was centrifuged at 34000 xg for 30 min at 4 ^{o}C and finally pellet was resuspended by 4 ml Taq storage buffer.

After ammonium sulfate precipitation, Vivaspin 500 µl Mw 50000 Dalton spin concentrator (Sartorius stedim biotech) was used to be purified Taq DNA polymerase further. Two methods were compared. In the first method, Taq DNA polymerase enzyme was diluted 10^{-1} by distilled water and loaded into the column. It was centrifuged at 12000 xg for 20 min. The flow through was removed and the column was washed with 100 µl distilled water. It was centrifuged at 12000 xg for 20 min and flow through was discarded. This washing step was repeated 3 times. Finally, Taq DNA polymerase enzyme was taken by pipetting of 100 µl Taq storage buffer without disturbing gel. In the second method, dilution and washing step was done by Taq storage buffer.

2.2.7 Taq DNA Polymerase Characterization

2.2.7.1. Determination of Protein Amount

The protein amount was decided by Bradford method (Bradford, 1976). During determination of protein amount, 5X Bradford reagents was diluted to 1X and then filtered again. To prepare standard curve, bovine serum albumin with 10, 20, 40, 60, 100, and 160 µg/ml concentrations were used. To determine protein amount, 480 µl distilled water and 20 µl samples was put into the 5 ml of 1X Bradford reagent. After incubation of 10 minutes, the absorbance of the sample at 595 nm was measured by Shimadzu UVmini 1240. The mixture of 500 µl distilled water and 5 ml 1X Bradford reagent was used as a blank solution. Protein concentration was calculated by the following formula.

Equation 2.1 The equation of protein concentration calculation

$$\text{Protein Concentration (mg/ml)} = \frac{\text{OD}_{595\text{ nm}}}{\text{Slope of Standard Curve}} \times \text{Dilution Factor}$$

2.2.7.2 Determination of Taq DNA Polymerase Enzyme with SDS-PAGE

Determination and purity of the protein are observed by SDS polyacrylamide gel electrophoresis, in a discontinuous buffer system as described by Laemmli (Laemmli, 1970) using 4% stacking gel and 12% separating gel. The separating and stacking gel solutions were prepared just before use as given in Table 2.5 in the given order.

Table 2.7 Components of separating and stacking gel solutions

Constituents	**Seperating Gel (12%)**	**Stacking Gel (4%)**
Gel solution (ml)	2	0.415
Distilled water (ml)	1.65	1.7
Seperating gel buffer (ml)	1.25	-
Stacking gel buffer (ml)	-	0.315
10% SDS (ml)	0.05	0.01
10% APS(ml)	0.035	0.01
TEMED (ml)	0.0035	0.0025

Vertical polyacrylamide slab gel electrophoresis was performed using omniPAGE mini vertical electrophoresis unit (Cleaver Scientific Ltd., UK). The sample unit that was required for pouring gel was consisted of two glass plates that had 1 mm spacer in the form of thin strips to separate the plates. The module was placed in soft silicone seals and pressure bars.

Firstly, the separating gel solution was poured between two glass plates without any air bubbles until the desired height of gel was obtained and immediately the gel was covered with a layer of isopropanol to obtain smooth gel surface. After polymerization of separating gel solution, isopropanol was removed completely. Then the stacking gel solution was poured between two glass plates without any air bubbles till the glass plates were covered with gel and immediately comb was inserted into stacking gel solution. Following polymerization, comb was removed and wells and gel casting was filled with 1X gel running buffer.

After preparation of gel, samples were diluted with 4X sample buffer. 1 volume of sample was mixed with 3 volume of sample buffer and heated at 90 ^{o}C for 10 min. The unstained protein molecular weight marker, purchased from Fermentas,

was heated at 90 ^{o}C for 5 min. The heated samples and ladder were loaded into the different wells. After loading the sample, the electrophoresis unit was connected to the power supply, Biolab, and electrophoresis was run at 10 mA in stacking gel and at 15 mA in separating gel.

After running of the gel, visualization of the polyacrylamide gel was done by silver staining method (Blum et al., 1987). Firstly, gel was removed from glass plate and then put into the fixing solution for one hour with vigorously shaking. After that, fixing solution was removed and washing solution was pour into the gel for 20 minutes. This step was repeated 3 times with shaking. This solution was removed and pretreatment solution was poured and incubated for 1 minute with shaking. Pretreatment solution was poured off and gel was washed with distilled water for 20 seconds and 2 times. It was impregnated by silver nitrate solution by 20 min with shaking. Then gel was washed with 2 times distilled water for 20 seconds. Till the color was developed, it was incubated in the development solution. The gel was washed with stop solution for 2 min and the stored in the stop solution.

2.2.7.3 Determination of Taq Polymarase Enzyme Activity and Fidelity

Different source of DNA, bacteria, plant, human, and different Tm of the primer was used for determination of enzyme activity by comparing with commercially available Taq DNA polymerase (Fermentas Taq DNA polymerase). After PCR reaction, for fidelity test, the some PCR product was sent to REFGEN for finding error rate of the Taq DNA polymerase.

2.2.7.4 Determination of Taq DNA Polymerase Enzyme Unit

Plasmid DNA was used for determination of unit of enzyme in the size of 270 bp. Unsaturated amplified DNA product band intensity was compared by

commercially available Taq DNA polymerase (Fermentas Taq DNA polymerase) and determined the unit of the enzyme.

2.2.7.5 Determination of Restriction Endonuclease and Nicking Activity

1 µg genomic DNA and sample was incubated at 65 ^{o}C for 16 hours to find out the restriction endonuclease activity. As a control, TaqI Restriction Endonuclease, purchased from Fermentas, (catalog number: ER0671), was used.

1 µg plasmid, sample, $MgCl_2$ and Taq reaction buffer with KCl was incubated at 65^{o}C for 4 hours to find out the nicking activity

2.2.8 Thermal Cycler Experiments

Different source of DNA, bacteria, plant, human, and different Tm of the primer was used for determination of newly developed thermal cycler activity by comparing commercially available thermal cycler (Biolab thermal cycler).

2.2.9 Agarose Gel Electrophoresis for PCR Product

The PCR products were analyzed by electrophoresis on 1% agarose gel buffered with 1X TAE buffer and stained with 10% ethidium bromide. Agar in 1X TAE buffer was melted by the microwave. It was poured into the electrophoresis gel tray and comb was placed. After that comb was removed and electrophoresis gel tank was filled by 1X TAE buffer.

The GeneRuler™ 1 kb and 50 bp DNA Ladder, purchased from MBI Fermentas, was used to determine PCR product size. DNA ladder was mixed with 6X loading buffer, purchased from MBI Fermentas, and water in the order of ratio 1:1:4. The PCR product was prepared by mixing 6X loading buffer at a final concentration of 1X. After that the prepared DNA ladder mixture and PCR

product mixture was loaded into the well of the agarose gel. Power supply was adjusted to 100 V and the gel was run about for 30 minutes. The bands are visualized under UV light instrument. Vilber – Lourment Infinity 100 was used as a UV light source.

2.2.9 UV Imaging

Electrophoretic analysis was done by UV illuminating instrument. In this study, UV light source wasVilber- Lourment Infinity 1000. Bands were visualized and format of images were JPEG format.

2.2.10 Band Intensity Analysis

Experiments were done in duplicates. Net fluorescence intensity values were found by removing background intensity from band intensity using Image J software (Abramoff et al., 2004). Figure 2.3 depicts the analysis and scanning process.

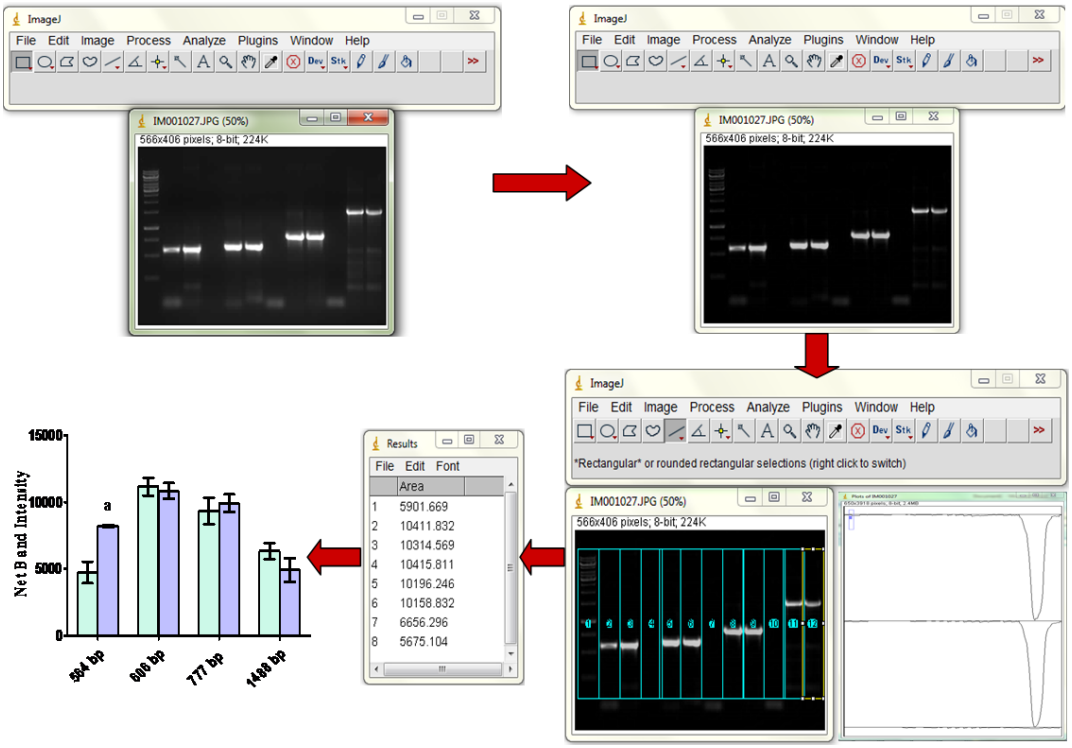


Figure 2.3 Schematic representation of analysis of the band density

2.2.11 Statistical Analysis

The GraphPad prism software was utilized in order to perform statistical analysis of the color intensity. The mean and the standard error of the means of the replicas were calculated. The variances in mean values were calculated in unpaired t-test analysis at 95% confidence interval.

CHAPTER 3

RESULTS AND DISCUSSION

In this study, *Thermus aquaticus* DNA polymerase was overexpressed and purified from *Escherichia coli* and this enzyme was optimized to be used in two new thermal cycler systems that have been developed.

Recombinant Taq DNA polymerase gene was cloned from *Thermus aquaticus* and transformed into the *Escherichia coli* using Gateway expression system. Conditions for the expression and purification have been optimized to harvest best yield. Purified Taq polymerase has been characterized and compared with the commercially available DNA polymerases. In the second part of this study, purified Taq DNA polymerase was used to optimize two new thermal cycler machines; one conventional and one capillary.

3.1 *Thermus aquaticus* DNA Isolation

Three different methods have been used to isolate *Thermus aquaticus* genomic DNA as previously explained in the "Material and Methods".

First, the simple heating method was utilized for genomic DNA isolation from *Thermus aquaticus*. Later, we tried the commonly used CTAB method; however we could not obtain any yield with this method. Since, *Thermus aquaticus* DNA was bound to pellet in the centrifugation step in the CTAB method and heating method after centrifugation. Since *Thermophiles* DNA has histone –like proteins and polyamines that is specific to *Thermophiles* on DNA. Histone like proteins winds DNA and prevents degradation of double-stranded DNA. Polyamines

bound to DNA and may cause aggregation or conformation changes of DNA. In addition to these, DNA gyrase leads to positive supercoiling of closed circular DNA and this conformation of DNA lead to resist denaturation. These structures, histone like proteins, polyamines and DNA gyrase may lead DNA to bind pellet. *Thermus aquaticus* DNA was not suitable for PCR amplification due to presence of protein on DNA (Grosjean and Oshima, 2007; Madigan and Oren, 1999; Terui et al., 2005).

At this stage we successfully isolated *Thermus aquaticus* genomic DNA with the column purification method. This DNA was successfully obtained enough DNA for further steps and suitable enough for further PCR amplification.

3.2 Cloning of *Thermus aquaticus* DNA Polymerase Gene

3.2.1 Amplification of Taq DNA polymerase Gene

Thermus aquaticus genome DNA was isolated using NANObiz DNA Isolation Kit as it was mentioned. Depending on the known *Thermus aquaticus* DNA polymerase gene sequence (GenBank Accession No: J04639.1) shown in the Appendix A, the specific primers for the Taq DNA polymerase gene were designed and used for the amplification from genomic DNA of *Thermus aquaticus*. Taq DNA polymerase gene was amplified by Herculase enzyme (Agilent Technologies). The error rate of Herculase enzyme, 1/10000 is lower than the Taq DNA polymerase, 1/9000, so it was used in the amplification of Taq DNA polymerase gene. After the amplification, PCR products were isolated from the agarose gel for further processes (Figure 3.1).

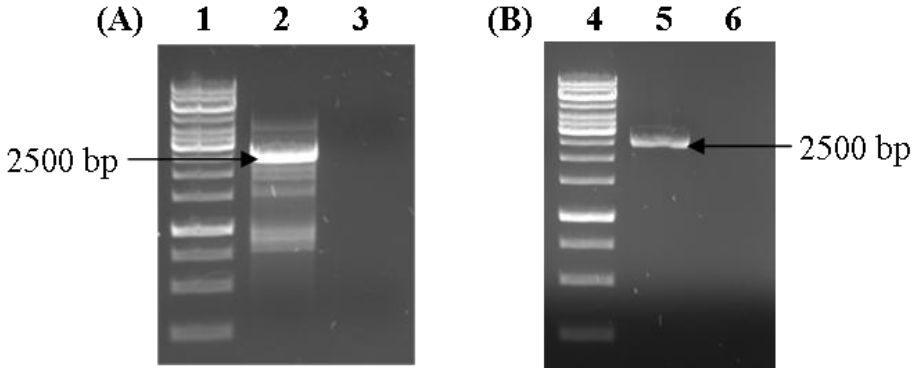


Figure 3.1 Electrophoretic analyze of amplification of Taq DNA polymerase gene from *Thermus aquaticus* in 1% Agarose gel electrophoresis. **(A)** The amplification of Taq DNA polymerase gene from *Thermus aquaticus* before gel elution **(B)** The amplification of Taq DNA polymerase gene in gel electrophoresis after gel elution **Lane 1, 4:** DNA Ladder (1 kb), **Lane 2, 5**: Taq DNA polymerase gene PCR product, **Lane 3, 6:** Negative control of PCR

3.2.2. Confirmation of Taq DNA Polymerase Gene Insertion into the Entry Vector

Cloning of Taq DNA polymerase gene into entry vector was based on 3' deoxythymidine (T) residues of cloning vector and 3' deoxyadenosine (A) residues of 3' ends of Taq DNA polymerase gene which was added into Taq DNA polymerase gene PCR product by commercial Herculase polymerase. These ends allow Taq DNA polymerase gene to ligate cloning vector.

To confirm cloning of Taq DNA polymerase gene into the cloning vector, colony PCR was done. Depending on the Figure 3.2, 3rd and 5th colonies, gave the positive results so these colonies were selected and isolated to be sequenced (Figure 3.3).

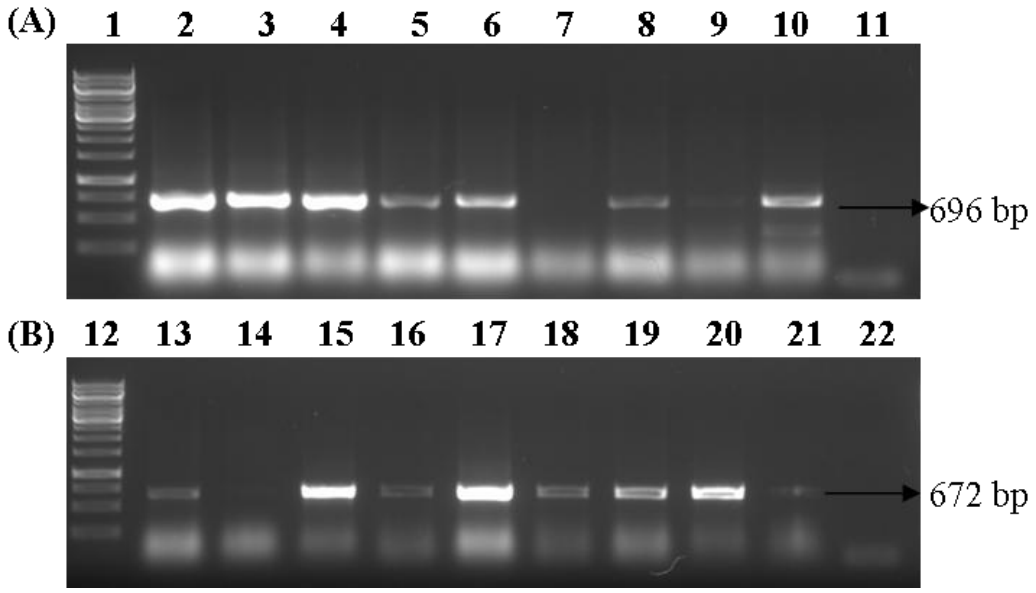


Figure 3.2 Electrophoretic analysis of amplification of entry vector containing Taq DNA polymerase gene by colony PCR in 1% Agarose gel electrophoresis. **(A)** The amplification of entry vector containing Taq DNA polymerase gene with M13 forward primer and Taq DNA polymerase gene from 549 bp reverse primer **(B)** The amplification of entry vector containing Taq DNA polymerase gene with Taq DNA polymerase gene from 1996 bp forward primer and M13 reverse primer **Lane 1, 12:** DNA Ladder (1 kb) **Lane 2, 13:** 1st colony **Lane 3, 14:** 2nd colony **Lane 4,15:** 3rd colony **Lane 5, 16:** 4th colony **Lane 6, 17:** 5th colony **Lane 7, 18:** 6th colony **Lane 8, 19:** 7th colony **Lane 9, 20:** 8th colony **Lane 10, 21:** 9th colony**, Lane 11, 22:** Negative control of this experiment

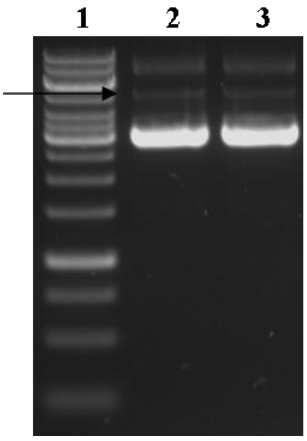


Figure 3.3 Electrophoretic analysis of plasmid isolation of 3rd and 5th selected colonies in 1% Agarose gel electrophoresis **Lane 1:** DNA Ladder (1 kb) **Lane 2:** 3rd colony **Lane 3:** 5th colony

Two colonies were picked and examined for the correctness of the inserted Taq DNA polymerase gene sequence. While 1 insertion has 2 base pair mutations, other was 100% identical to the published Taq DNA polymerase gene sequence. In the mutated insertion, mutations were at the 497th and 737th bases and alanine amino acid had been changed into valine amino acid. Therefore, the second insertion was used in the further steps.

3.2.3 Confirmation of Insertion of Taq DNA polymerase Gene into the Expression Vector

Entry vector has attL1 and attL2 regions and pDEST14 vector, has attR1 and attR2 region. These regions were recombined by the LR Clonase enzyme mix mediates containing recombination proteins Integrase, Excision, and the *E. coli*-encoded protein Integration Host Factor (Landy, 1989).

In the LR reaction, integrative recombination and excision recombination takes place. In the integrative recombination, the one attL region of TOPO cloning vector and one attR region of expression vector are cut and rebound in the existence of Integrase and Integration Host Factor (Landy, 1989). While Integrase, having type I topoisomerase, can cut and resealing of attL and attR sites of DNA by a covalent lnt-DNA intermediate lack of any high-energy cofactors (Craig and Nash, 1983; Landy, 1989; Langegustafson and Nash, 1984), Integration Host Factor binds DNA minor groove based on the patterns of protection against modification by dimethyl sulfate (DMS) and of the sugar-phosphate backbone by hydroxyl radicals (Craig and Nash, 1984; Drlica and Rouviereyaniv, 1987; Landy, 1989). In this reaction, attP (viral site) and attB (bacterial sites) are generated. In the excisive recombination, att B and attP are generated from attL and attR region and the viral chromosome excised by Excision protein and Integration Host factor. Excision protein promotes efficient recombination between attL and attR by helping Integrase, Integration Host Factor and salt (Abremski and Gottesman, 1981; Bushman et al., 1985; Landy, 1989) (Figure 3.4).

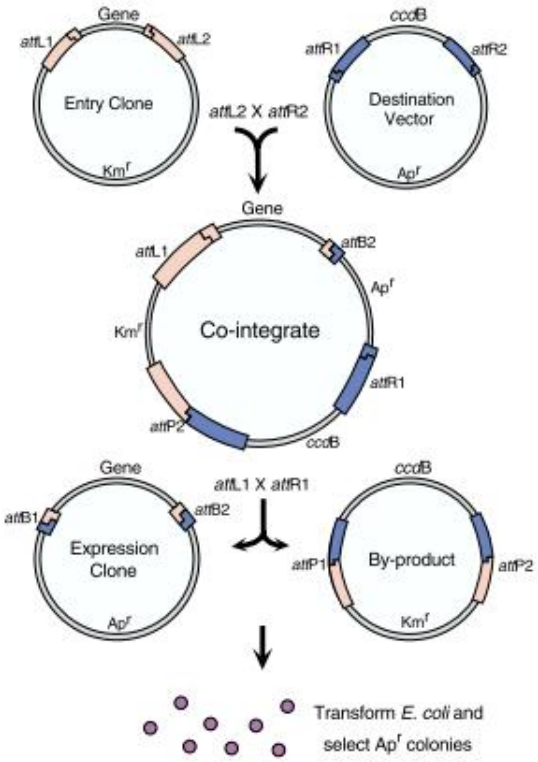


Figure 3.4 Schematic representation of GATEWAY Cloning Technology as an operating system for cloning and subcloning DNA from instruction manual of GATEWAY™ Cloning Technology.

After LR reaction, insertion of the Taq DNA polymerase gene into the expression vector was checked and verified by colony PCR method.

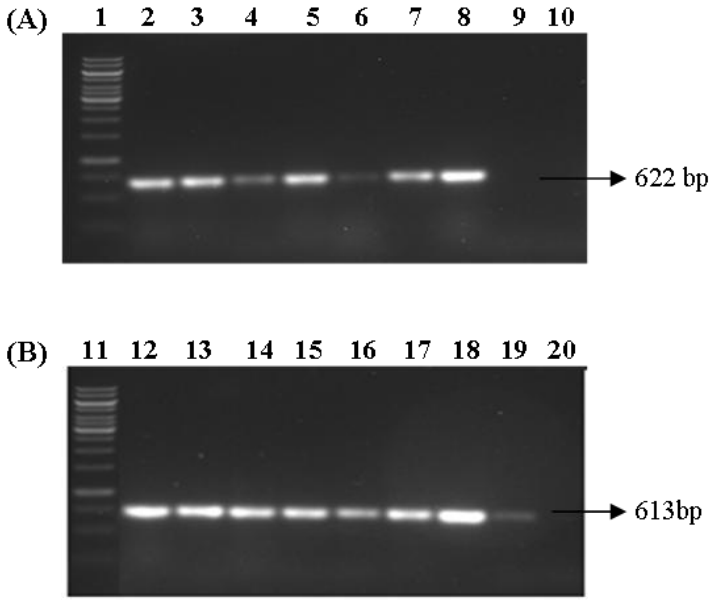


Figure 3.5 Electrophoretic analysis of amplification of pDEST14^{TM} vector containing Taq DNA polymerase gene by colony PCR in 1% Agarose gel electrophoresis **(A)** The amplification of pDEST14^{TM} vector containing Taq DNA polymerase gene with T7 promoter primer and Taq DNA polymerase gene from 549 bp reverse primer **(B)** The amplification of pDEST14^{TM} cloning vector containing Taq DNA polymerase gene with Taq DNA polymerase gene from 1996 bp forward primer and T7 terminator primer **Lane 1, 11:** DNA Ladder (1 kb) **Lane 2, 12:** 1st colony **Lane 3, 13:** 2nd colony **Lane 4, 14:** 3rd colony **Lane 5, 15:** 4th colony **Lane 6, 16:** 5th colony **Lane 7, 17:** 6th colony **Lane 8, 18:** 7th colony **Lane 9, 19:** 8th colony **Lane 10, 20:** negative control of this experiment

All colonies gave positive results (Figure 3.5). Since, in pDEST14^{TM} vector ccdB protein are present and ccdB protein gene, exchanged from pDEST vector to entry vector is toxic and it is allow negative selection (Bushman et al., 1985; Landy, 1989; Makrides, 1996). Due to the toxic effect of the ccdB protein, after LR reaction, bacteria that carry the nonrecombinant pDEST14^{TM} vector, cannot survive. If bacteria have mutation or have mutated then, they can survive. In

addition to this, pDEST14^{TM} vector has antibiotic resistance gene that is required for positive selection of the recombinant protein. pDEST14^{TM} and entry vector has different selection, ampicilin and spectinomycin, respectively.

3.3 Growth Curve Analysis of *E.coli*

Proper overexpression of a recombinant protein in *E. coli* heavily depends on the physiological state of the organism. Therefore, determination of induction time Taq DNA polymerase enzyme is crucial to get the maximum yield, but also enzymes in proper condition.

Growth of bacterial population has four phases in the batch culture, enclosed system; lag phase, exponential phase, stationary phase and death phase. In lag phase, biomass of bacteria raises instead of the number of the bacteria due to adjustment to new conditions just after inoculations. In the exponential phase, a constant growth rate is attained and exponential increase in the cell density is observed when presence of plentiful nutrient. The rate of growth depends on the environmental conditions such as medium composition, temperature. In the stationary phase, growth of bacteria is limited due to scarce of nutrient and deposition of waste product. There is no net increase in cell number. In death phase, bacterial die due to depletion of nutrient (Madigan and Martinko, 2006).

In this study, the growth rate of recombinant *E.coli* was determined by the turbidity measurement method, indirect measurement of microbial growth. This measurement based on the spectroscopic method. In spectroscopic measurement, light is sent through the sample and while some light can pass through photocell, some light cannot due to scattering light from bacteria. Photocell measures the unscattered light and gives measurement in optical density or photometer units. The number of unscattered light decrease with increasing the density of bacteria. This method is useful because of being quick and easy to perform. However, this method also has some limitation. The stationary phase is not observed as a linear

and the death phase is not observed. Since the death cells also affect turbidity (Madigan and Martinko, 2006).

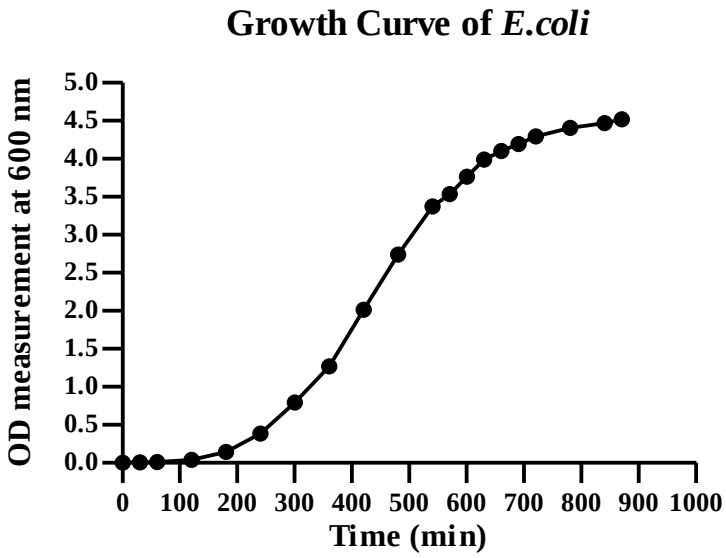


Figure 3.6 Representation of growth curve of recombinant *E.coli* that containing plasmid with Taq DNA polymerase gene

In this study, batch system was used. Three phases as described before, log phase, lag phase and stationary phase, were observed (Figure 3.6). As stated previously, stationary phase was not linear because of the turbidity of the death cell. Lag phase was selected to produce high Taq DNA polymerase.

3.4 Confirmation of Taq DNA polymerase Enzyme Characterization

Isolation and purification optimization of Taq DNA polymerase enzyme was determined by SDS-PAGE gel electrophoresis method. In this study, SDS-PAGE

gel was visualized by silver staining method. Since, this staining method, 1-10 ng /band, is more sensitive than Coomassie Blue R—250 staining, 50-100 ng/band (Blum et al., 1987; Rabilloud, 1992; Switzer et al., 1979).

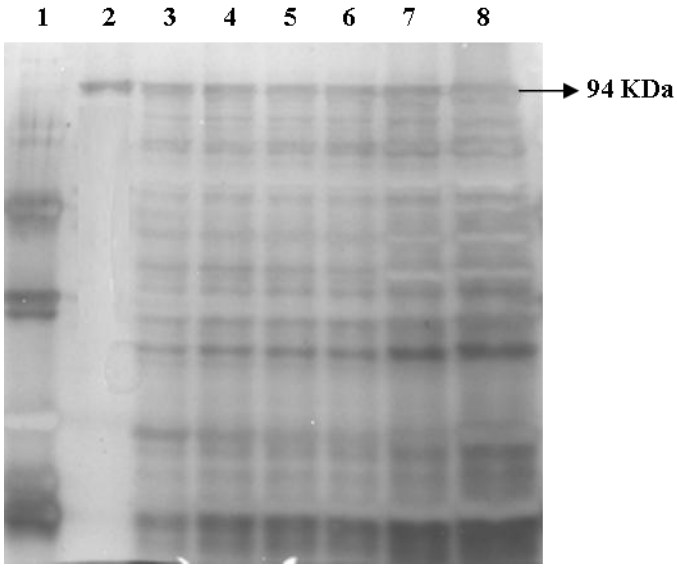


Figure 3.7 SDS-PAGE analyses for determination of total protein amount in the silver staining **Lane 1:** Protein molecular weight ladder, **Lane 2:** 50 Unit commercial Taq DNA polymerase, **Lane 3** 1 µg total protein, **Lane 4:** 2 µg total protein **Lane 5:** 3 µg total protein **Lane 6:** 4 µg total protein **Lane 7:** 5 µg total protein **Lane 8:** 20 µg total protein

Firstly, the amount of total protein that was loaded into well of SDS-PAGE gel electrophoresis was optimized to be visualized easily. Isolated total protein amount was decided by Bradford method as it was mentioned in the Material and Methods. Then 1 µg, 2 µg, 3 µg, 4 µg, 5 µg and 20 µg of total protein were applied into the wells of slab gel and it was visualized by silver staining method.

Depending on the Figure 3.7, 20 µg total proteins were too much for silver staining. In this study, 1 µg total protein was selected to be loaded into the gel because there is not too much difference between 2 µg, 3 µg, 4 µg and 5 µg total proteins.

3.4.1 Confirmation of Bacterial Induction Method

For recombinant protein expression, regulation of protein expression is important. In this study, Taq DNA polymerase gene expression was regulated by inducible system, lac operon (Klug et al., 2006; Lawyer et al., 1989). Based on previous studies, the amount of IPTG was 0.5 mM (Grimm and Arbuthnot, 1995; Roayaei and Galehdari, 2008; Yang et al., 2008).

In this study, 4 different induction procedures were tried to obtain maximum amount of active Taq DNA polymerase enzyme. IPTG was added into the culture medium when $OD_{600\ nm}$ was 0.2 (late of the log phase), 0.4 (beginning of the lag phase), 0.8, and 0.9 (before mid-lag phase) at 37 ^{o}C. Then different induction time period such as 20 hours, 18 hours, 3 hours and temperature such as 37 ^{o}C and 28 ^{o}C was tried. Different induction method was crucial for obtaining active Taq polymese enzyme. Since, when *E.coli* produce recombinant protein at high rate, sometimes they tend to be in a stress conditions and unfavorable folding and aggregation of protein is occurred. This structure is called inclusion bodies (Sorensen and Mortensen, 2005; van den Berg et al., 1999; Villaverde and Carrio, 2003). To decrease aggregation of recombinant protein, some parameters such as temperature, expression rate, target protein engineering is controlled (Jonasson et al., 2002). In addition to inclusion bodies formation, recombinant protein production may prevent the growth of bacteria and bacteria cannot reach enough concentration due to high energy requirement of recombinant protein synthesis (Dong et al., 1997; Hoffman and Rinas, 2004). Four different conditions were tried. As inferred from Figure 3.6, all conditions gave similar result so the 1st condition, IPTG added into the culture medium when $OD_{600\ nm}$

was 0.2 which is the end of the log phase and then 20 hours incubation was selected to prevent inclusion bodies formation (Figure 3.8).

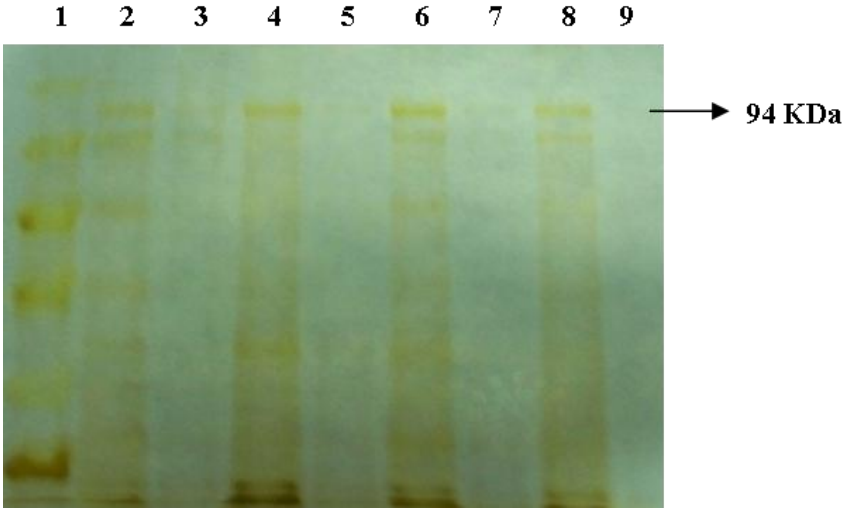


Figure 3.8 SDS-PAGE analysis for determination Taq DNA polymerase gene expression method **Lane 1:** Protein molecular weight ladder **Lane 2:** Addition IPTG when $OD_{600\ nm}$: 0.2 then incubation at 37^{o}C for 18 hours. **Lane 3:** 1/10 dilution of Lane 2, **Lane 4:** Addition IPTG when $OD_{600\ nm}$: 0.4 then incubation at 28^{o}C for 18 hours, **Lane 5:** 1/10 dilution of Lane 4 **Lane 6:** Addition IPTG when $OD_{600\ nm}$: 0.8 then incubation at 37^{o}C for 18 hours, **Lane 7:** 1/10 dilution of Lane 6, **Lane 8:** Addition IPTG when $OD_{600\ nm}$: 0.9 then incubation at 37^{o}C for 3 hours **Lane 9:** 1/10 dilution of Lane 8

3.4.2 Selection of the Lysis Method of *E.coli*

After selection of the induction method, next we optimized the lysis method of as it was also a crucial step to obtain high amount of active protein. Two different methods, chemical treatment and physical treatment were tried.

Chemical treatment was the lysozyme treatment. Lysozyme breaks β-1,4–glycosidic bonds between N-acetylmuramic acid and N-acetylglucosamine in pepdidoglycan (Madigan and Martinko, 2006). Lysozyme treatment in the *E.coli*, Gram negative cell is less effective than the Gram positive cell owing to the presence of an outer membrane and low portion of peptidoglycan. Nevertheless, lysozyme with EDTA and Tris buffer pH: 8 can lyse *E.coli* bacteria (Repaske, 1956, 1958). Nevertheless, dextrose, also used in the lysis buffer, avoids lysis of bacteria. In the presence of dextrose *E.coli* is present as a protoplast form. After heating process, protoplast form of *E.coli* is burst (Mahler and Fraser, 1956).

The second method was high pressure homogenizer, physical treatment to lyse *E.coli*. The principle of the high pressure homogenizer is that the sample is transferred from high pressured chamber to low pressured chamber. Firstly, intracellular pressure of sample increases in the high pressured chamber. Then when sample transferred into the low pressured chamber very quickly, sample cannot adjust the internal pressure of the cell so it is burst and release the intracellular contents. Generation of pressure depends on the type of the high pressure homogenizer (Bailey and Meagher, 1997; Brookman, 1974; Sauer et al., 1989). In this study, high-pressure cell disruption system developed by Constant Systems Ltd (Daventry, UK) was used. Depending on Constant Systems Ltd., the maximum pressure used for *Escherichia coli* is 35 kpsi (2.41 kbar). For this reason, in this study, 35 kpsi was used for disruption of *E.coli*.

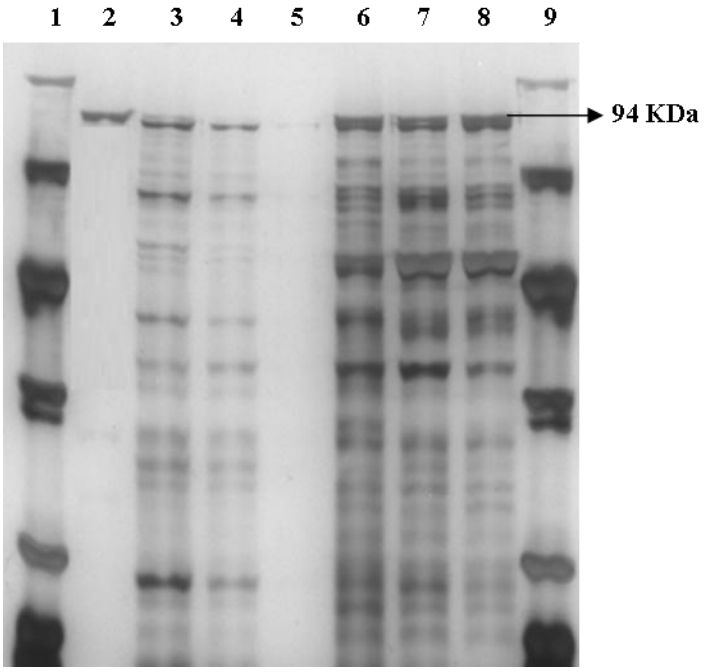


Figure 3.9 SDS-PAGE analyses for lysis method of *E.coli* for isolation of Taq DNA polymerase enzyme 1 µg total protein was loaded into the well. **Lane 1, 9:** Protein molecular weight ladder, **Lane 2:** 50 Unit commercial Taq DNA polymerase **Lane 3-5:** Lysozyme as a lysis method of *E.coli* **Lane 6-8:** High Pressure Homogenizer as a lysis method

In the Figure 3.9, two methods were compared for bacterial lysis. While when bacteria were lysed by high pressure homogenizer the total protein amount of crude extract is 3.30 mg/ml, the total protein is 3.45 mg/ml when bacteria were lysed by lysozyme. High pressure homogenizer method yields more Taq DNA polymerase enzyme than lysozyme enzyme treatment. Moreover, while *E.coli* was disrupted by lysozyme, in the lane 5, Taq DNA polymerase enzyme was not obtained. Since lysozyme may not work enough to lyse cell. In the lysozyme treatment, enzyme stability, time manner and cost for large scale may cause problem. High Pressure Homogenizer was used for further experiments.

3.4.3 Confirmation of Taq DNA Polymerase Enzyme Purification

Determination of the lysis method was followed by ammonium sulfate precipitation. In ammonium sulfate precipitation method, proteins are separated depending on the differences of solubility in the salt solution. In the ammonium sulfate precipitation, firstly low salt concentration is used. Following this, salt concentration is increased with decrease the solubility of protein. This process is called salting out. By this method, some proteins depending on the solubility difference are eliminated desired protein (Nelson and Cox, 2005). Depending on previous studies (Lawyer et al., 1993; Lawyer et al., 1989; Roayaei and Galehdari, 2008), 16% and 37% ammonium sulfate was used in this study. While, Taq DNA polymerase enzyme was soluble in the 16% ammonium sulfate, it was insoluble in the 37% ammonium sulfate.

After ammonium sulfate precipitation, the purity of Taq DNA polymerase enzyme was not enough. Therefore, Vivaspin 500µl spin concentrator was used. The pore size of Vivaspin 500 µl spin concentrator column was 50000 Dalton. Using Vivaspin 500 µl spin concentrator column, one protein was not removed from Taq DNA polymerase protein. By further purification, two methods were tried. In the Figure 3.10 the method efficiency was shown.

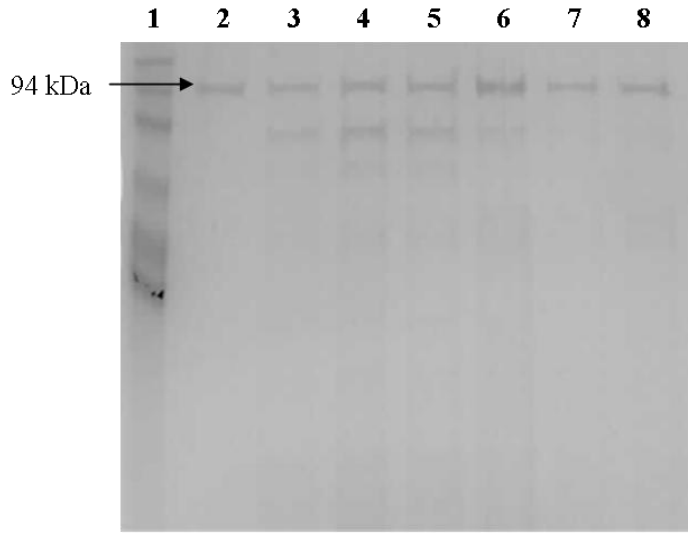


Figure 3.10 SDS-PAGE analysis of Taq DNA polymerase enzyme purification by Vivaspin 500 µl Mw 50000 Dalton spin concentrator (Sartorius stedim biotech) **Lane 1:** Protein molecular weight marker **Lane 2:** 50 unit commercial Taq DNA polymerase **Lane 3-5:** Taq DNA polymerase enzyme was diluted and washed by distilled water **Lane 6-8:** Taq DNA polymerase enzyme was diluted and washed by Taq storage buffer.

In the second method, Taq DNA polymerase enzyme was obtained as a pure form than in the first method (Figure 3.10). Taq DNA polymerase was obtained as a pure form, in the two methods; the amount of Taq DNA polymerase enzyme is same with commercial Taq DNA polymerase enzyme.

3.4.4 Confirmation of Taq DNA Polymerase Enzyme Activity

Produced Taq DNA polymerase enzyme activity was tested with different DNA template and to produce different amplification products. Produced Taq DNA polymerase enzyme was compared with the commercially available Taq DNA polymerase. Genomic DNA from different organisms has different features.

Plasmid DNA, that is small (1-1000 kilobase pairs), circular and in solution bacterial DNA was found as a separated from each other. Amplification of plasmid DNA is much easier than genomic DNA. The second source is bacterial DNA (0.6 Mbp to 10 Mbp) that was used for testing enzyme activity. Most bacterial DNA is circular similar to plasmid DNA but it is more complex than plasmid DNA due to the changing of GC content of DNA. In addition to this, some bacterial DNA has histone like protein that is bound to DNA tightly and bacterial DNA has some modification (Galperin, 2007; Madigan and Martinko, 2006). For this reason, the amplification is more difficult than plasmid DNA. The third source used in the enzyme activity of Taq DNA polymerase is human genome, linear and is nearly 3 billion base pair. Histone protein is bound to the human genome to reduce the size. It has highly repetitive region and the GC content is highly. Moreover, human DNA has some modification such as methylation, acetylation (Karp, 2004; Makalowski, 2001). For this reason, amplification of human DNA can be considered more difficult than plasmid and bacterial DNA.

First, plasmid DNA was used as a template for the produced Taq DNA polymerase to test enzyme activity was compared to the commercially available Taq DNA polymerase. In Figure 3.11, amplification of plasmid DNA by the commercially available Taq polymerase and produced Taq DNA polymerase with different length, 270 bp, 471 bp, 618 bp and 1197 bp was shown. As based on Figure 3.12, the difference of net band intensity between plasmid DNA amplification of the commercially available Taq polymerase and produced Taq DNA polymerase was not observed for all different length.

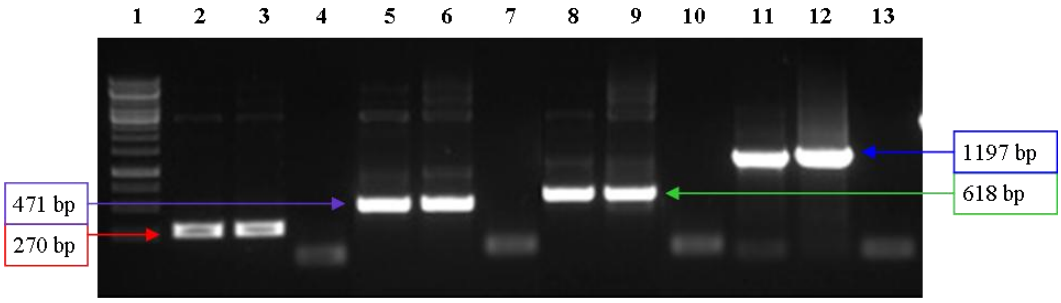


Figure 3.11 Electrophoretic analysis of the amplification of plasmid DNA by commercial and produced Taq DNA polymerase enzyme and different size primer, 270 bp, 471 bp, 618 bp and 1197 bp primer respectively in 1% Agarose gel electrophoresis **Lane 1:** DNA Ladder (1 kb) **Lane 2, 5, 8, 11**: The amplification by commercial Taq DNA polymerase **Lane 3, 6, 9, 12:** The amplification by produced Taq DNA polymerase **Lane 4, 7, 10, 13** Negative control of experiment

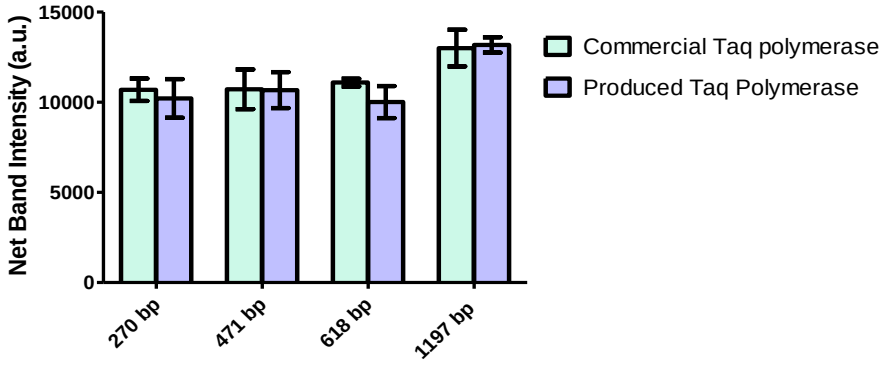


Figure 3.12 The intensity analysis of amplification of plasmid DNA by commercial and produced Taq DNA polymerase enzyme and different size primer, 270 bp, 471 bp, 618 bp and 1197 bp primer respectively. The histogram bars represent the average band intensity of the spots. The net intensity value could be seen on section 2.2.10. Standard error of mean could be seen as error bars.

To further test of the enzyme activity, bacterial genome was used next. The amplification of bacterial genome with different primer, 564 bp, 606 bp, 777 bp and 1488 bp, was shown in the Figure 3.13. Experiments results indicate that there is no significant difference between commercial and produced Taq DNA polymerase in the band intensity of 606 bp, 777 bp and 1488 bp. However, in the amplification of bacterial DNA in the size of 564 bp, there is a significant difference in the band intensity. Based on the result, produced Taq DNA polymerase has higher activity than commercial Taq DNA polymerase in the 95% confidence interval (Figure 3.14).

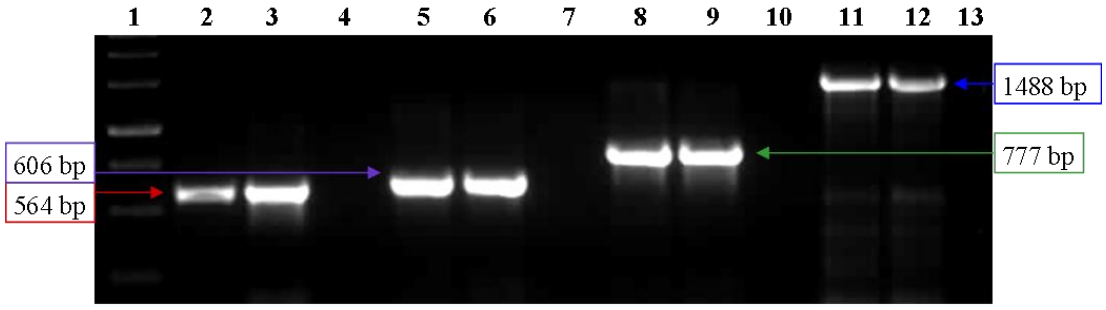


Figure 3.13 Electrophoretic analysis of amplification of bacterial genome by commercial and produced Taq DNA polymerase enzyme and different size primer, 564 bp, 606 bp, 777 bp and 1488 bp primer in 1% Agarose gel electrophoresis, respectively **Lane 1:** DNA Ladder (1 kb) **Lane 2, 5, 8, 11:** The amplification by commercial Taq DNA polymerase **Lane 3, 6, 9, 12:** The amplification by produced Taq DNA polymerase **Lane 4, 7, 10, 13:** Negative control of experiment

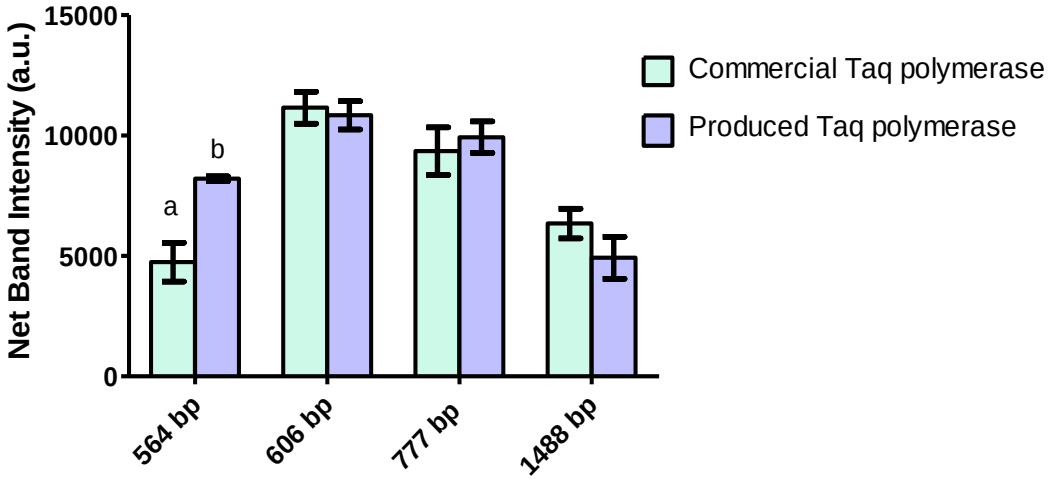


Figure 3.14 The intensity analysis of amplification of bacterial DNA by commercial and produced Taq DNA polymerase enzyme and different size prime 564 bp, 606 bp, 777 bp and 1488 bp primer, respectively. The histogram bars represent the average band intensity of the spots. The net intensity value could be seen on section 2.2.10. Standard error of mean could be seen as error bars. Significant difference at $p<0.05$ is indicated by letter.

As previously mentioned, human DNA was also used to compare enzyme activity of produced and commercial Taq DNA polymerase. As shown in Figure 3.15 and Figure 3.16, the amplification of human DNA was demonstrated. The net band intensity was indicated that the enzyme activity of produced and commercial Taq DNA polymerase was not different in the 95% confidence interval when they amplified human genome in the size of 115 bp, 472 bp, 644 bp, 1000 bp (Figure 3.17).

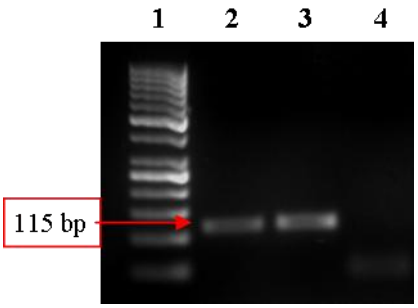


Figure 3.15 Electrophoretic analysis of amplification of human genome by commercial and produced Taq DNA polymerase enzyme in the size of 115 bp primer in 2.5% Agarose gel electrophoresis **Lane 1:** DNA Ladder (50 bp) **Lane 2:** The amplification by produced Taq DNA polymerase **Lane 3**: The amplification by commercial Taq DNA polymerase **Lane 4:** Negative control of experiment

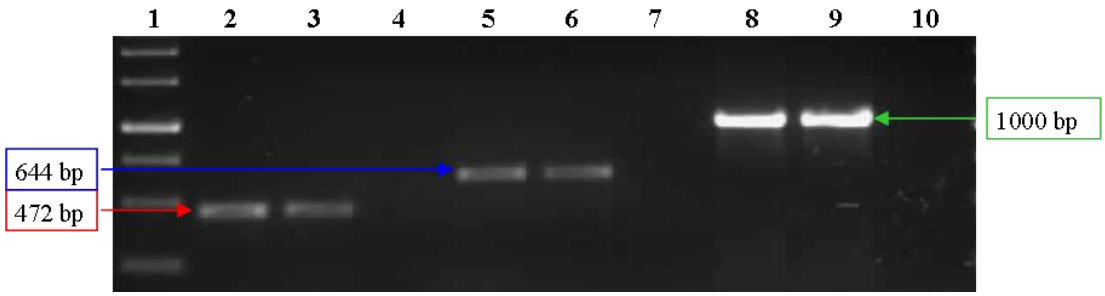


Figure 3.16 Electrophoretic analysis of amplification of human genome by commercial and produced Taq DNA polymerase enzyme. Human DNA was amplified with different size, 472 bp, 644 bp and 1000 bp in 1% Agarose gel electrophoresis, respectively **Lane 1:** DNA Ladder (1 kb) **Lane 2, 5, 8:** The amplification by produced Taq DNA polymerase **Lane 3, 6, 9:** The amplification by commercial Taq DNA polymerase **Lane 4, 7, 10:** Negative control of experiment

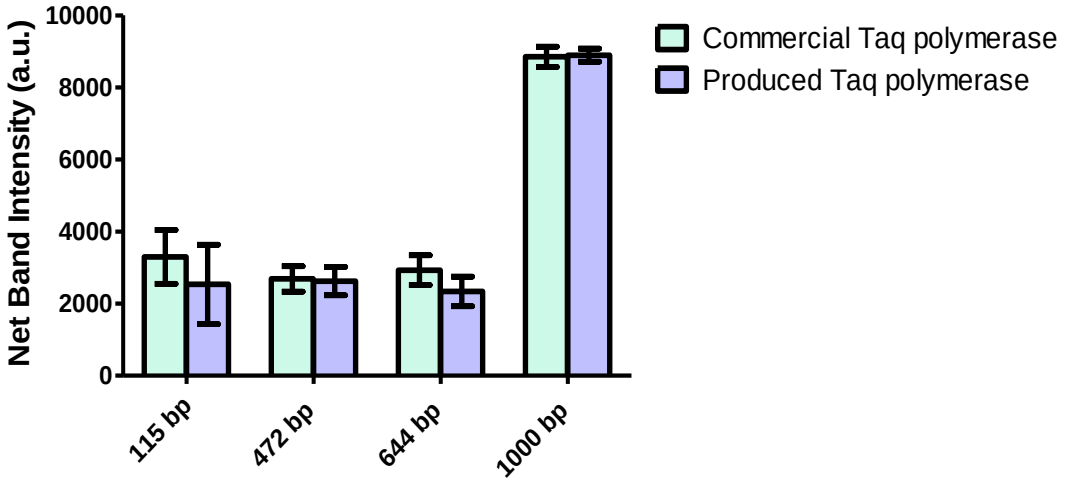


Figure 3.17 The intensity analysis of amplification of human DNA by commercial and produced Taq DNA polymerase enzyme and different size prime 115 bp, 472 bp, 644 bp and 1000 bp primer respectively. The histogram bars represent the average band intensity of the spots. The net intensity value could be seen on section 2.2.10. Standard error of mean could be seen as error bars.

These results, altogether, indicated that the enzyme activity of produced Taq DNA polymerase is same with the commercial available one.

3.4.5 Determination of Taq DNA Polymerase Unit

One unit of Taq DNA polymerase is the enzyme catalyzes the incorporation of 10 nmol of deoxyribonucleotides into a polynucleotide in 30 min at 70 °C. As based on previous studies, enzyme activity determines using radioactively labeled dTTP (Lawyer et al., 1993; Lawyer et al., 1989). However, radioactively labeled dTTP was not used to determine Taq DNA polymerase unit. Instead of this, unsaturated amplification of plasmid DNA was compared with commercial and purified Taq DNA polymerase (Figure 3.18). Based on result, the unit of

purified Taq DNA polymerase was same with commercial one when produced Taq DNA polymerase was diluted 1/10 ratio (Figure 3.19).

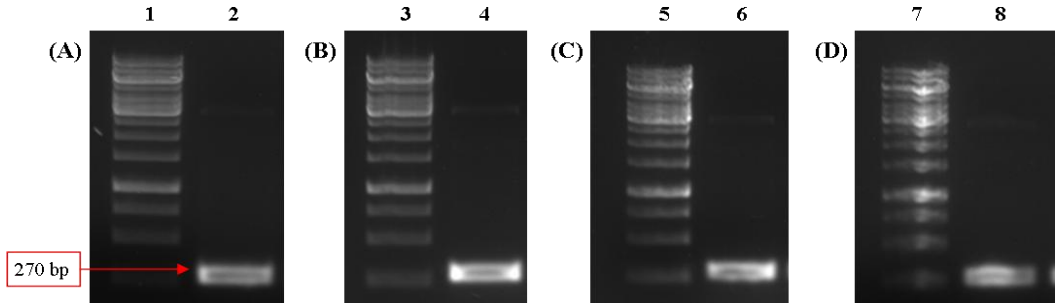


Figure 3.18 Electrophoretic analysis of determination of Taq DNA polymerase unit by commercial and produced Taq DNA polymerase enzyme, 1/5 dilution and 1/10 dilution of purified Taq DNA polymerase in 1% Agarose gel electrophoresis **Lane 1, 3, 5, 7:** DNA Ladder (1 kb) **Lane 2:** The amplification by commercial Taq DNA polymerase **Lane 4:** The amplification by purified Taq DNA polymerase **Lane 6:** The amplification by 1/5 purified Taq DNA polymerase **Lane 8:** The amplification by 1/10 purified Taq DNA polymerase

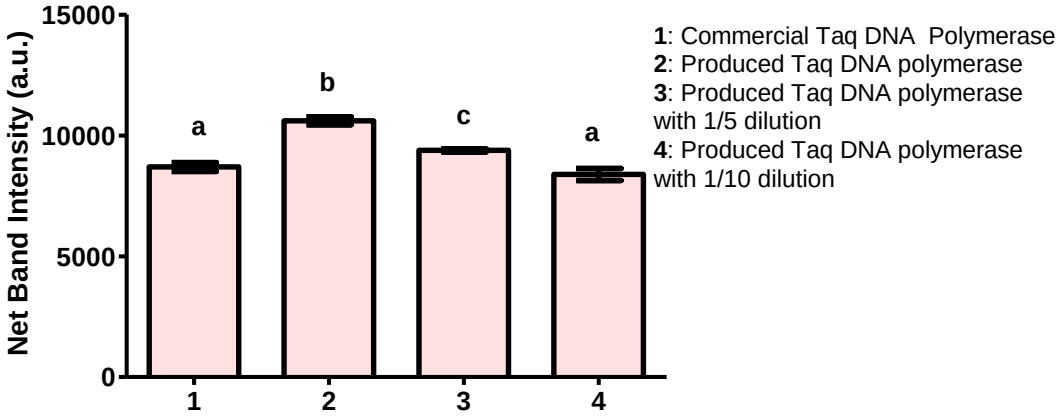


Figure 3.19 The intensity analysis of determination of Taq DNA polymerase unit. The histogram bars represent the average band intensity of the spots. The net intensity value could be seen on section 2.2.10. Standard error of mean could be seen as error bars. Significant difference at $p<0.05$ is indicated by different letter.

3.4.6 Determination of Effect of Purification on Enzyme Activity

Isolated Taq DNA polymerase was purified with Vivaspin 500 µl Mw 50000 Dalton spin concentrator. To understand of the effect of purification on enzyme activity, unsaturated amplification of plasmid DNA was compared with commercial Taq DNA polymerase, 1/10 diluted produced Taq DNA polymerase and purified Taq DNA polymerase. Purified Taq DNA polymerase was compared with 1/10 diluted Taq DNA polymerase (Figure 3.20). Since while isolated Taq DNA polymerase was purifying, it was diluted in 1/10 ratio. Based on Figure 3.21, purification of Taq DNA polymerase significantly affected Taq DNA polymerase enzyme activity due to the treatment of purification in the 95% confidence interval.

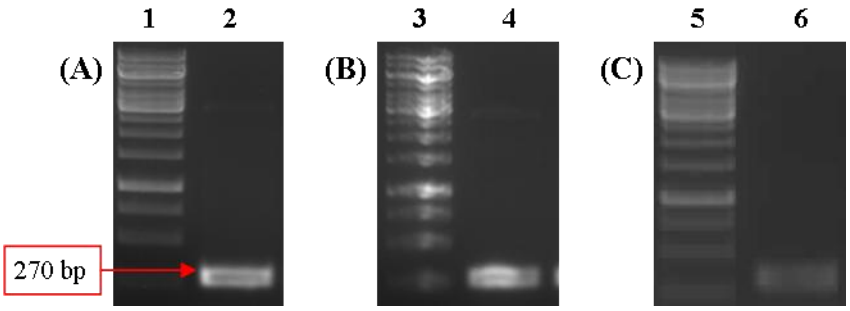


Figure 3.20 Electrophoretic analysis of determination of effect of purification on enzyme activity in 1% Agarose gel electrophoresis **Lane 1, 3, 5:** DNA Ladder (1 kb) **Lane 2:** The amplification by commercial Taq DNA polymerase **Lane 4:** The amplification by 1/10 diluted Taq DNA polymerase **Lane 6:** The amplification by purified Taq DNA polymerase

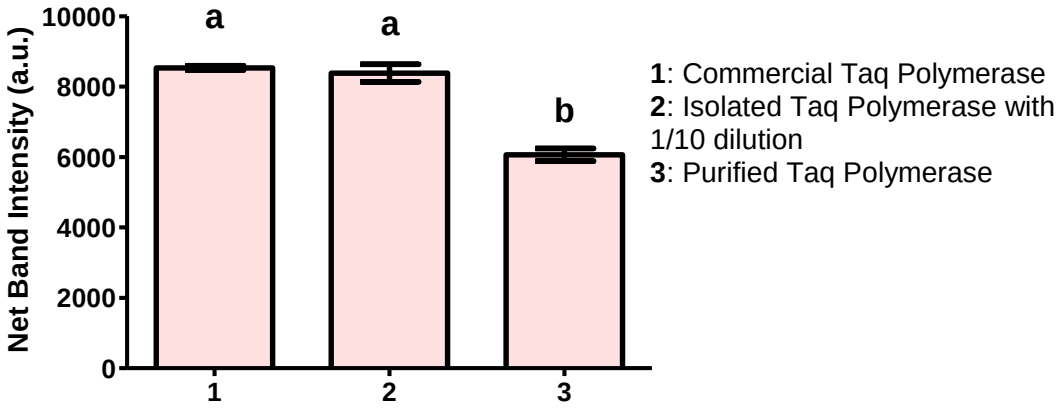


Figure 3.21 The intensity analysis of determination of effect of purification on enzyme activity. The histogram bars represent the average band intensity of the spots. The net intensity value could be seen on section 2.2.10. Standard error of mean could be seen as error bars. Significant difference at $p<0.05$ is indicated by different letter.

3.4.7 Determination of Taq DNA polymerase Fidelity

Various PCR products were sequenced to determine the error rate of produced Taq DNA polymerase and the error rates were compared with commercial Taq DNA polymerase. Depending on the chromatogram results, not shown in the thesis, commercial and produced Taq DNA polymerase error rate were found within the acceptable range of Taq DNA polymerase error rate.

Table 3.1 The fidelity of Taq DNA polymerase enzyme

PCR product size	**Commercial Taq DNA Polymerase Misincorporation Rate**	**Produced Taq DNA Polymerase Misincorporation Rate**
250 bp	0.5%	0.5%
564 bp	0.2%	0.2%
777 bp	0.3%	0%
1488 bp	0.6%	0.5%

3.4.8 Confirmation of Taq DNA polymerase enzyme restriction endonuclease and nicking activity

Produced Taq DNA polymerase was tested by restriction endonuclease and nicking activity. Restriction endonuclease enzyme can recognize the specific sequences of DNA and cleave DNA from recognition sequence. The presence of restriction endonuclease enzyme affects the PCR activity and leads to incorrect or wrong amplification of DNA. Therefore, produced Taq DNA polymerase was tested for presence of Taq I restriction endonuclease enzyme that cuts DNA from

5’T-CGA 3’ sites. Restriction endonuclase activity was compared with commercial Taq DNA polymerase and Taq I restriction enzyme. As inferred from Figure 3.22, in purified and commercial Taq DNA polymerase there was no restriction endonuclease activity (Chan et al., 2011).

In the nicking activity, DNA was nicked. For this reason, there can be no amplification or wrong amplification. For this reason, DNA was tested about the presence of nicking activity. There was no nicking activity in the produced and commercial Taq DNA polymerase (Figure 3.23).

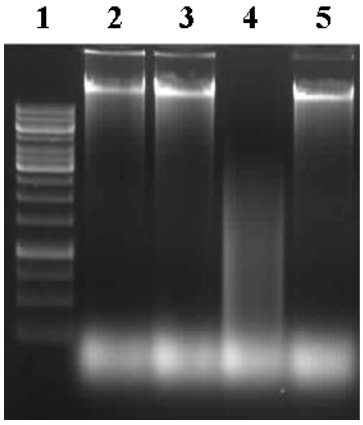


Figure 3.22 Electrophoretic analysis of restriction endonuclease activity in 1% Agarose gel electrophoresis **Lane 1**: DNA Ladder (1 kb) **Lane 2:** The restriction endonuclease activity by commercial Taq DNA polymerase **Lane 3:** The restriction endonuclease activity by produced Taq DNA polymerase **Lane 4:** Taq I restriction endonuclease activity **Lane 5:** negative control of restriction endonuclase activity

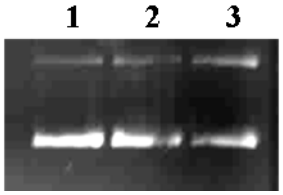


Figure 3.23 Electrophoretic analysis of nicking activity in 1% Agarose gel electrophoresis **Lane 1:** Nicking activity by commercial Taq DNA polymerase **Lane 2:** Nicking activity by produced Taq DNA polymerase **Lane 3:** Negative control of nicking activity

3.5 Efficiency Determination of Newly Developed Thermal Cycler

In this study, two newly developed thermal cyclers were tested for conditions and validation purposes. The first thermal cycler was conventional thermal cycler. The second thermal cycler was a capillary thermal cycler.

3.5.1 Determination of Conventional Thermal Cycler Efficiency

In the study, the optimization and validation of the newly designed thermal cycler was performed. This conventional PCR machine and all its control cards and embedded chip software were designed at NANObiz Ltd. Co., only peltier units were obtained commercially. The most important issues were to reach the desired temperature and precisely stay at that temperature for the desired. After testing the temperature and time step, the first prototype of the conventional thermal cycler was developed (Figure 3.24).

Following of that, the first prototype of the conventional thermal cycler was tested and it was amplified plasmid DNA with the length of 617 bp. This result

was shown in the Figure 3.25. However, thermal cycler needed to approve the working conditions to obtain good result.

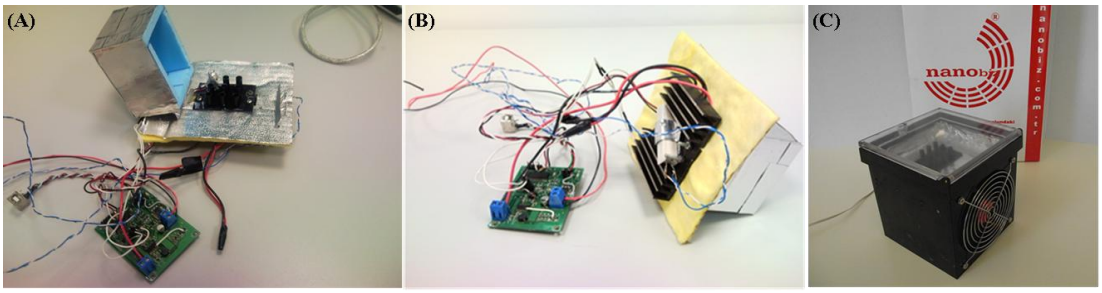


Figure 3.24 The first prototype of newly developed conventional thermal cycler

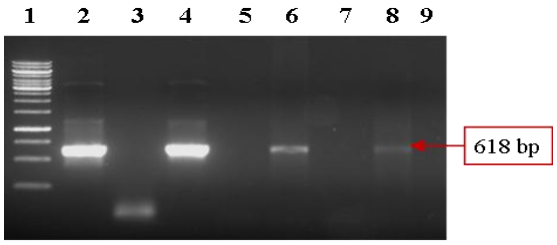


Figure 3.25 Electrophoretic analysis of the first prototype of conventional thermal cycler in 1% Agarose gel electrophoresis by plasmid DNA was amplified with 618 bp length of size. **Lane 1:** DNA Ladder (1 kb) **Lane 2:** The amplification was done in the commercial thermal cycler by commercial Taq DNA polymerase **Lane 4:** The amplification was obtained in the commercial thermal cycler by produced Taq DNA polymerase **Lane 6:** The amplification was done in new conventional thermal cycler by commercial Taq DNA polymerase **Lane 8:** The amplification was done in new conventional thermal cycler by commercial Taq DNA polymerase **Lane 3, 5, 7, 9:** Negative control of experiment

The first prototype of thermal cycler had some problems related with the heating unit and its control cards. These were solved and the second prototype was constructed (Figure 3.26). The ramped rate of the second prototype conventional thermal cycler was 0.4-0.5^{o}C and the deviation of the temperature was ± 0.1-0.2 ˚C. The second prototype was more efficient than the first one. The second prototype was tested using different DNA source, plasmid DNA, bacterial DNA and human DNA.

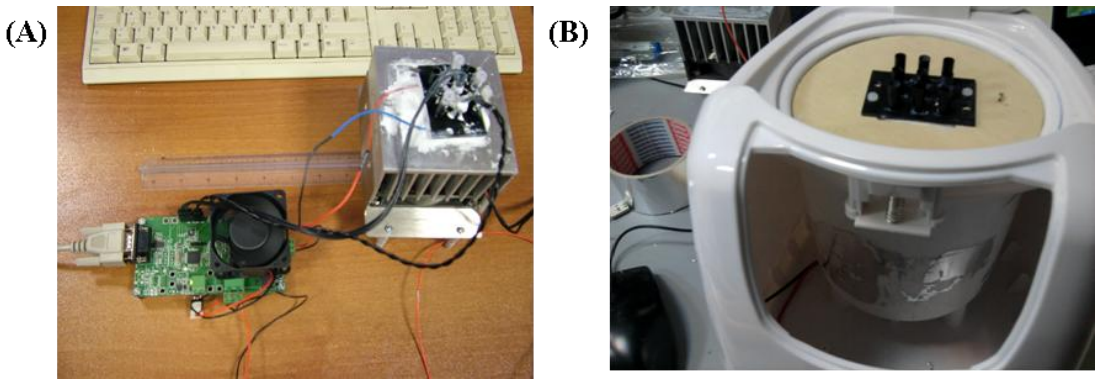


Figure 3.26 The second prototype of newly developed conventional thermal cycler

Developed conventional thermal cycler is directly connected to the computer. For this reason, software was developed and using these software thermal cycles were adjusted. The screenshots of the developed software can be seen in Figure 3.27. Moreover, the logarithm was developed to adjust desired temperature in polymerase chain reaction.

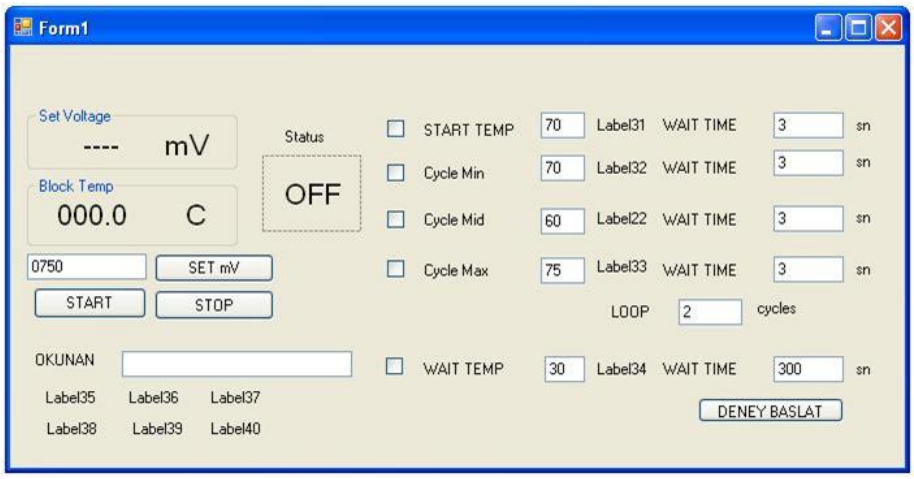


Figure 3.27 The software of thermal cycler for computer

The second prototype of thermal cycler was tested using the plasmid DNA and by comparing commercial thermal cycler. In addition to this, in commercial conventional thermal cycler, the produced Taq DNA polymerase was also used.

The amplification size of plasmid DNA was 270 bp, 471 bp and 618 bp. Based on the electrophoretic analysis (Figure 3.28) and band intensity analysis (Figure 3.29), differences in amplification of plasmid DNA between the second prototype and commercial conventional thermal cycler was not significantly in the 95% confidence interval.

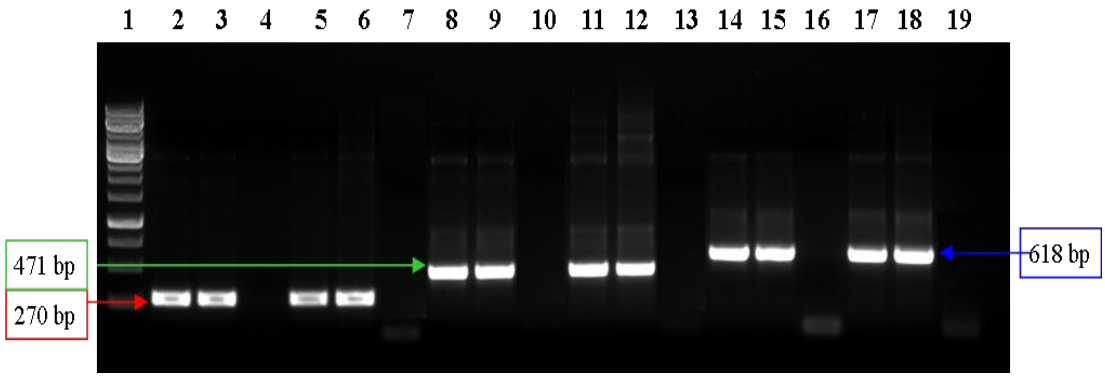
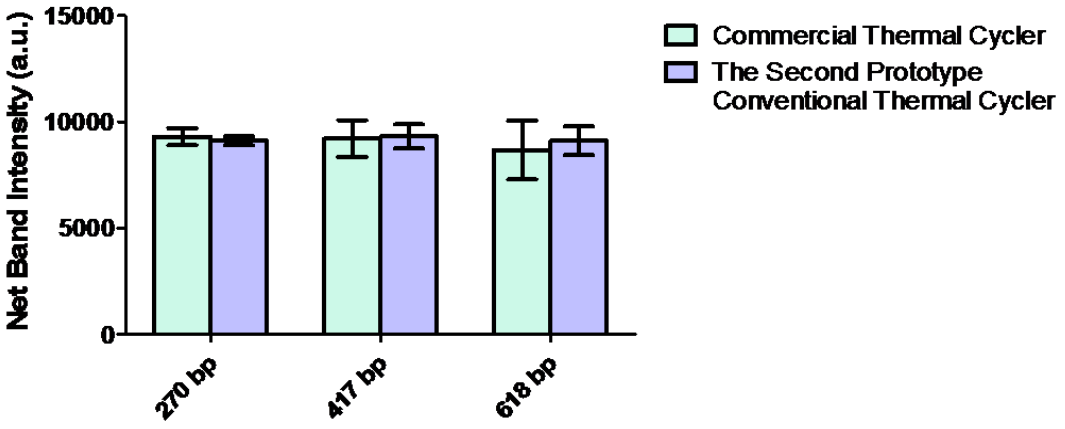


Figure 3.28 Electrophoretic analysis of the second prototype of conventional thermal cycler in 1% Agarose gel electrophoresis. Plasmid DNA was amplified with 270 bp, 4711 bp, 618 bp length of size, respectively. **Lane 1:** DNA Ladder (1 kb) **Lane 2, 8, 14:** The amplification was done in the commercial thermal cycler by commercial Taq DNA polymerase **Lane 3, 9, 15:** The amplification was done in the second prototype conventional thermal cycler by commercial Taq DNA polymerase **Lane 5, 11, 17:** The amplification was obtained in the commercial thermal cycler by commercial Taq DNA polymerase **Lane 6, 12, 18:** The amplification was done in the second prototype conventional thermal cycler by produced Taq DNA polymerase **Lane 4, 7, 10, 13, 16, 19:** Negative control of this experiment

(A) Commercial Taq DNA Polymerase



(B) Produced Taq DNA Polymerase

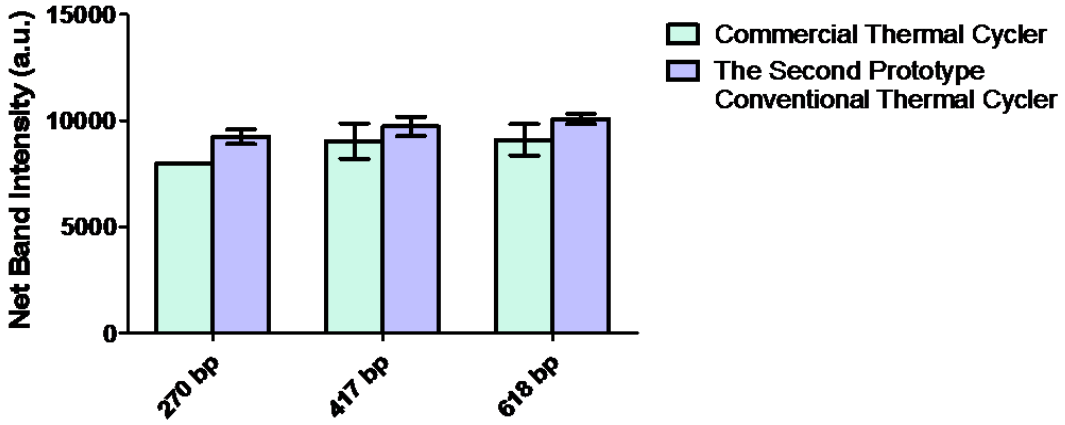


Figure 3.29 The intensity analysis of the second prototype of conventional thermal cycler and commercial thermal cycler. **(A)** The amplification of plasmid DNA by commercial Taq DNA polymerase with length of 270 bp, 471 bp, 618 bp was represented, respectively **(B)** The amplification of plasmid DNA by produced Taq DNA polymerase with length of 270 bp, 471 bp, 618 bp was represented, respectively. The histogram bars represent the average band intensity of the spots. The net intensity value could be seen on section 2.2.10. Standard error of mean could be seen as error bars.

The second thermal cycler prototype was also tested using the bacterial DNA with the amplification size, 606 bp and 777 bp. Derived from the electrophoretic analysis (Figure 3.30) and band intensity analysis (Figure 3.31), differences in the amplification of plasmid DNA between the second prototype conventional thermal cycler and commercial thermal cycler was not significantly in the 95% confidence interval.

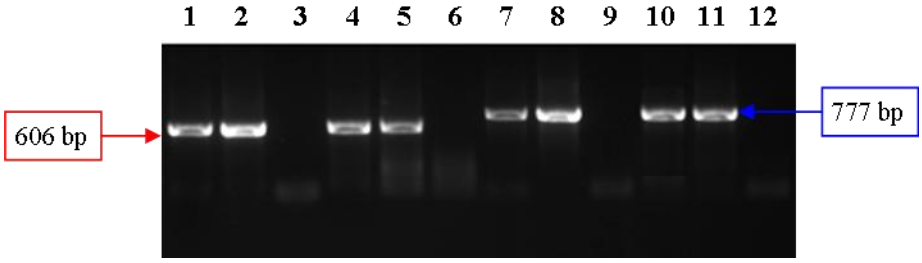


Figure 3.30 Electrophoretic analysis of the second prototype of conventional thermal cycler in 1% Agarose gel electrophoresis. Bacterial DNA was amplified with 606 bp and 777 bp length of size, respectively. **Lane 1, 7:** The amplification was done in the commercial thermal cycler by commercial Taq DNA polymerase **Lane 2, 8:** The amplification was done in the second prototype conventional thermal cycler by commercial Taq DNA polymerase **Lane 4, 10:** The amplification was obtained in the commercial thermal cycler by produced Taq DNA polymerase **Lane 5, 11:** The amplification was done in the second prototype conventional thermal cycler by produced Taq DNA polymerase **Lane 3, 6, 9, 12:** Negative control of experiment

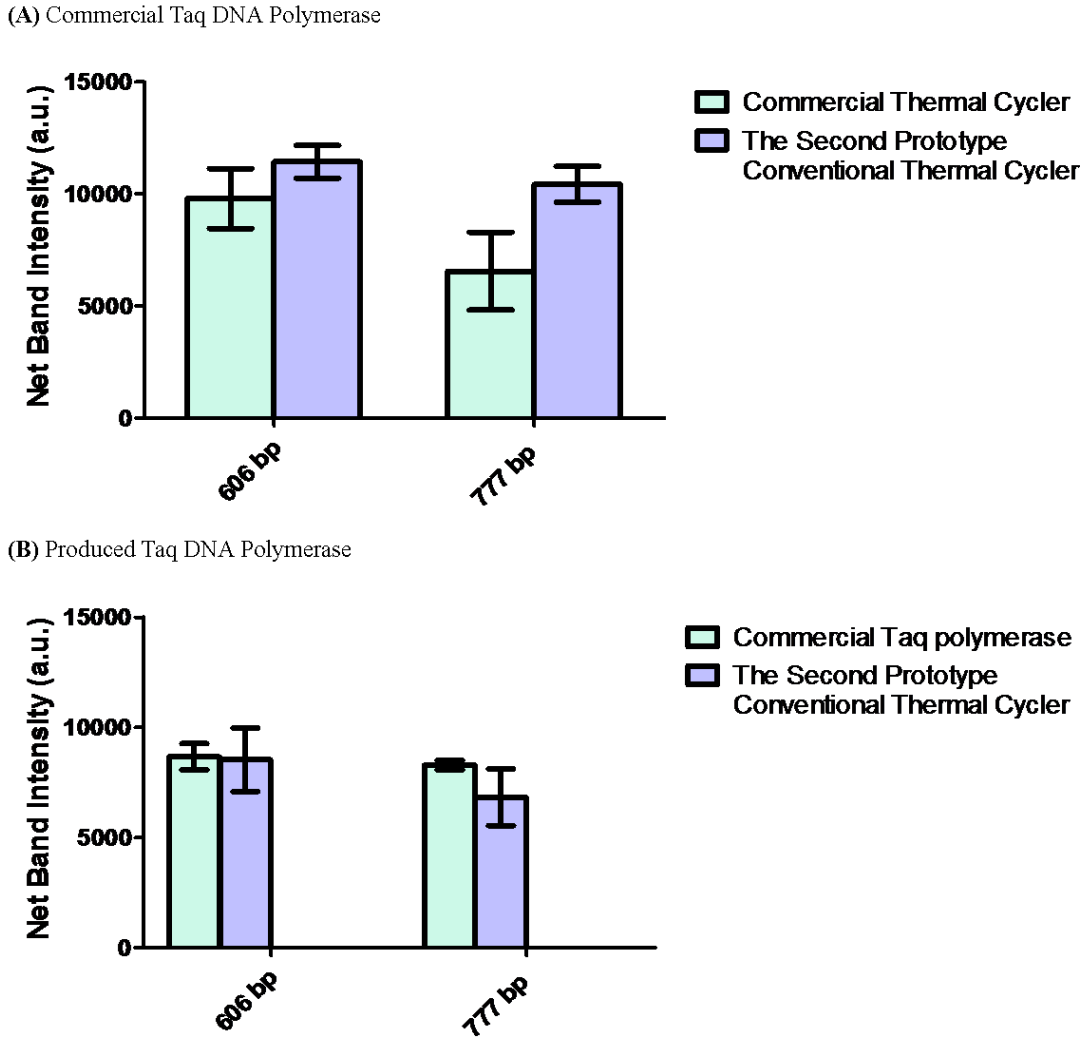


Figure 3.31 The intensity analysis of the second prototype of conventional thermal cycler and commercial thermal cycler. **(A)** Amplification of bacterial DNA by commercial Taq DNA polymerase with length of 606 bp, 777 bp was represented; respectively **(B)** Amplification of bacterial DNA by produced Taq DNA polymerase with length of 606 bp, 777 bp was represented; respectively. The histogram bars represent the average band intensity of the spots. The net intensity value could be seen on section 2.2.10. Standard error of mean could be seen as error bars.

The second conventional thermal cycler prototype was also tested using the human DNA with the amplification size, 115 bp, 472 bp and 644 bp (Figure 3.32, Figure 3.33). Derived from the band intensity analysis (Figure 3.34), differences in amplification of plasmid DNA between the second prototype of conventional thermal cycler and commercial thermal cycler was not significantly in the 95% confidence interval in the usage of produced Taq DNA polymerase. However, there was a significant difference between the second prototype of conventional thermal cycler and commercial thermal cycler in the usage of commercial Taq DNA polymerase. Since, logarithm program was not adjusted very well to obtain desired annealing temperature. When compared produced and commercial Taq DNA polymerase, amplification efficiency of produced Taq DNA polymerase is higher than commercial one. Since, produced Taq DNA polymerase may have more tolerance to change temperature than commercial Taq DNA polymerase.

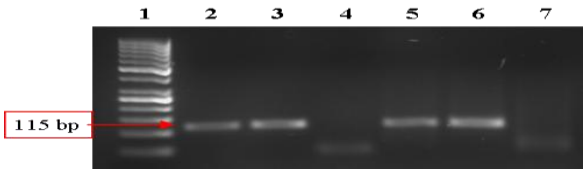


Figure 3.32 Electrophoretic analysis of the second prototype of conventional thermal cycler in 1% Agarose gel electrophoresis Human DNA was amplified with 115 bp length of size. **Lane 1:** DNA Ladder (50 bp) **Lane 2:** The amplification was done in the commercial thermal cycler by commercial Taq DNA polymerase **Lane 3:** The amplification was done in the second prototype of conventional thermal cycler by commercial Taq DNA polymerase **Lane 5:** The amplification was obtained in the commercial thermal cycler by produced Taq DNA polymerase **Lane 6:** The amplification was done in the second prototype of conventional thermal cycler by produced Taq DNA polymerase **Lane 4, 7:** Negative control of experiment

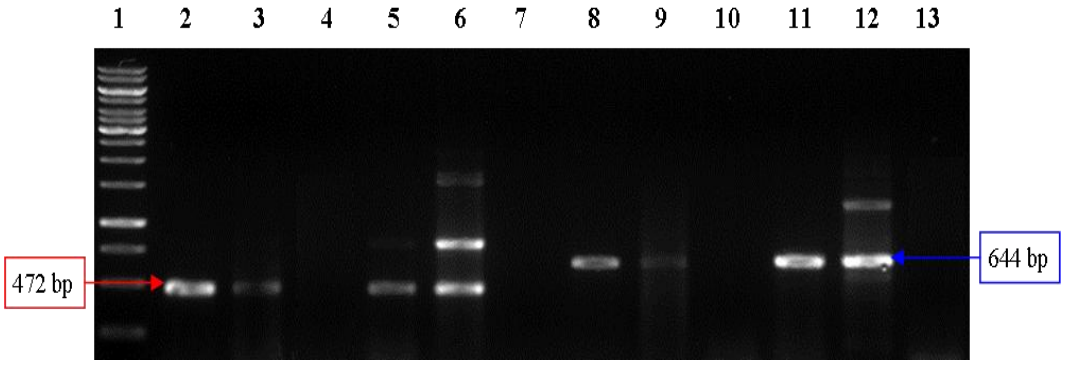


Figure 3.33 Electrophoretic analysis of the second prototype of conventional thermal cycler in 1% Agarose gel electrophoresis. Human DNA was amplified with 472 bp, 644 bp length of size. **Lane 1:** DNA Ladder (1 kb) **Lane 2, 8:** The amplification was done in the commercial thermal cycler by commercial Taq DNA polymerase **Lane 3, 9:** The amplification was done in the second prototype of conventional thermal cycler by commercial Taq DNA polymerase **Lane 5, 11:** The amplification was obtained in the commercial thermal cycler by produced Taq DNA polymerase **Lane 6, 12:** The amplification was done in the second prototype of conventional thermal cycler by produced Taq DNA polymerase **Lane 4, 7, 10, 13:** Negative control of experiment

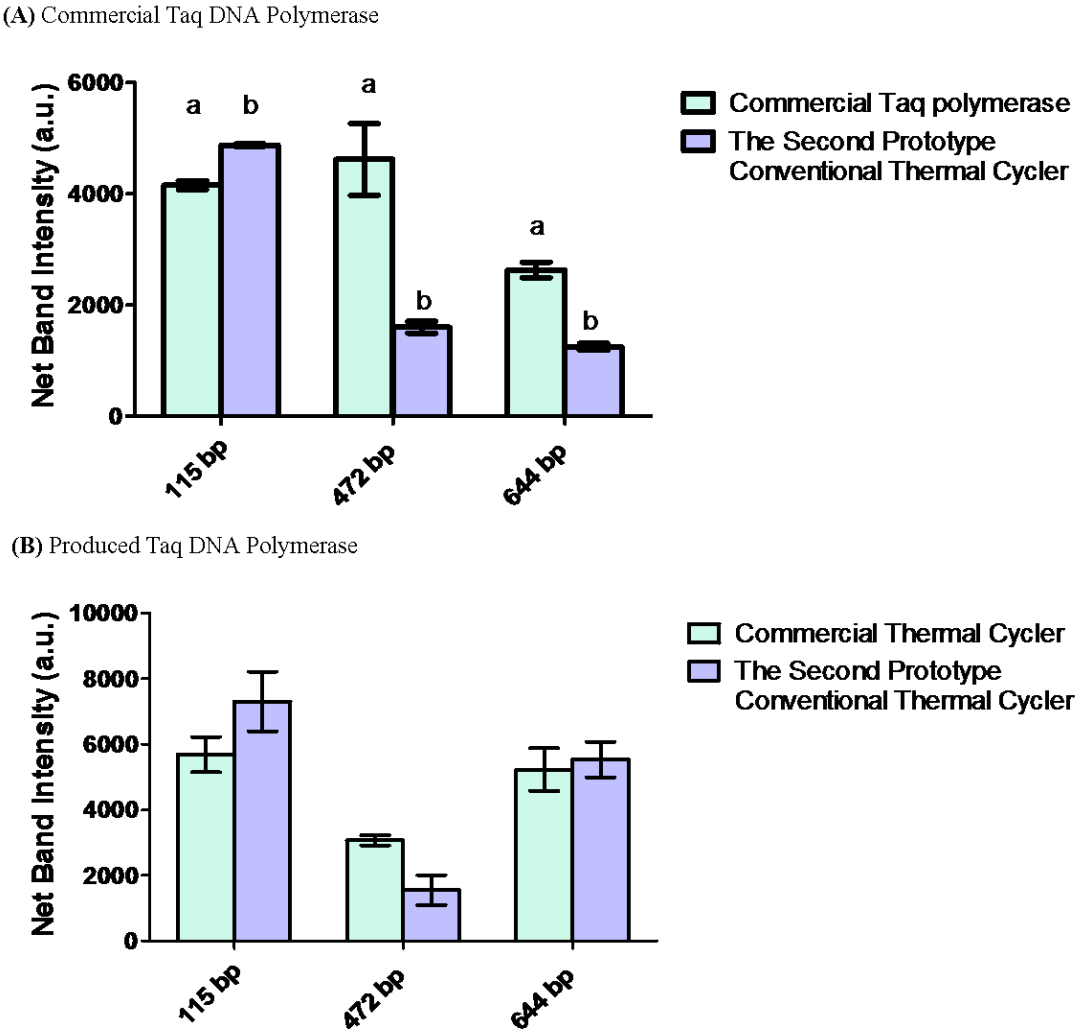


Figure 3.34 The intensity analysis of the second prototype of conventional thermal cycler and commercial thermal cycler. **(A)** The amplification of human DNA by commercial Taq DNA polymerase with length of 115, 472 bp, 644 bp was represented, respectively **(B)** The amplification of human DNA by produced Taq DNA polymerase with length of 115 bp, 472 bp, 644 bp was represented respectively. The histogram bars represent the average band intensity of the spots. The net intensity value could be seen on section 2.2.10. Standard error of mean could be seen as error bars.

3.5.2 Determination of the Capillary Thermal Cycler Efficiency

In this study, the same technology was used in the capillary thermal cycler. The one difference was that the sample holder was for glass capillary tubes. The second difference was that instead of usage of heating and cooling of one peltier system, three peltier systems were used for different temperature and liquid was rotate by tubing in these peltier system based on the principle of PCR method. For this reason, this system is much faster than the conventional thermal cycler. However, there was one problem, PCR mixture was boiling in denaturation temperature of PCR method, 94 ^{o}C. This problem was solved with clamp. The ends of capillary tubes were clamped and liquid cannot evaporate due to lack of space.

Figure 3.35 The prototype of capillary thermal cycler

The prototype of capillary thermal cycler (Figure 3.35) was tested using the plasmid DNA and bacterial DNA. Due to lack of commercial capillary thermal cycler, the comparison was done by conventional thermal cycler. While the amplification size of plasmid DNA was 471 bp and 618 bp (Figure 3.36, Figure 3.37), the amplification size of bacterial DNA was 564 bp and 644 bp (Figure 3.38, Figure 3.39). Based on the electrophoretic analysis and band intensity analysis, the efficiency of capillary thermal was lower than the conventional thermal cycler. Since, there were two problems in this system. The one problem was that capillary tubes was longer than the capillary tube holder so some mixture was not amplified very well. In addition to this problem, due to presence of unsolved problem of capillary thermal cycler, the insulation was not efficient so temperature of block was affected from environmental temperature. Although this system was working, the efficiency is lower than commercial available thermal cyclers.

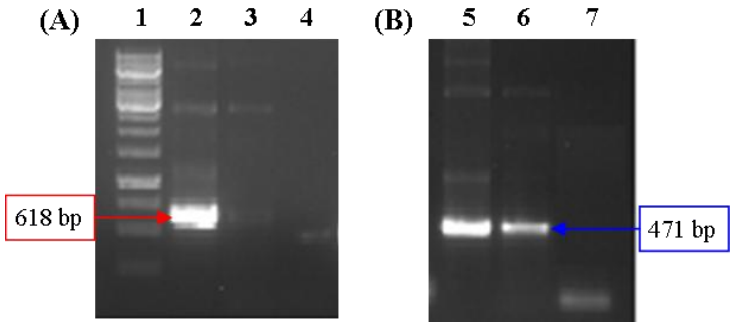


Figure 3.36 Electrophoretic analysis of prototype of capillary thermal cycler in 1% Agarose gel electrophoresis Plasmid DNA was amplified with 618 bp and 471 bp length of size, respectively. **Lane 1:** DNA Ladder (1 kb) **Line 2, 5:** The amplification was done in the commercial conventional thermal cycler by commercial Taq DNA polymerase **Line 3, 6:** The amplification was done in prototype of capillary thermal cycle by commercial Taq DNA polymerase **Line 4, 7:** Negative control of experiment

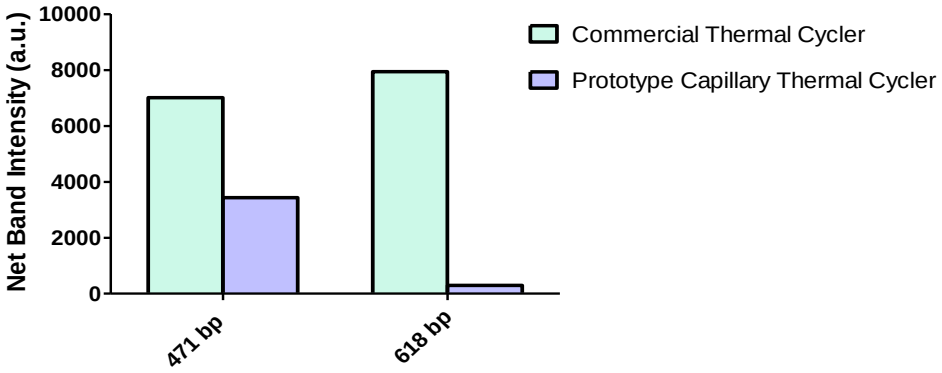


Figure 3.37 The intensity analysis of the prototype of capillary thermal cycler and commercial thermal cycler. The amplification of plasmid DNA by commercial Taq DNA polymerase with length of 471 bp, 618 bp was represented, respectively. The histogram bars represent the average band intensity of the spots. The net intensity value could be seen on section 2.2.10.

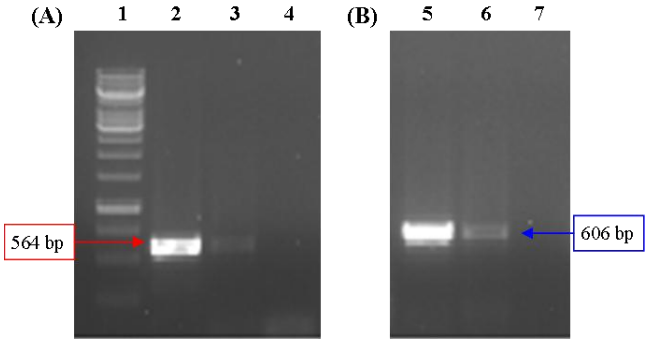


Figure 3.38 Electrophoretic analysis of prototype of capillary thermal cycler in 1% Agarose gel electrophoresis. Bacterial DNA was amplified with 564 bp and 606 bp length of size, respectively. **Line 1:** DNA Ladder (1 kb) **Line 2, 5:** The amplification was done in the commercial thermal cycler by commercial Taq DNA polymerase **Line 3, 6:** The amplification was done in prototype of capillary thermal cycler by commercial Taq DNA polymerase **Line 4, 7:** Negative control of experiment

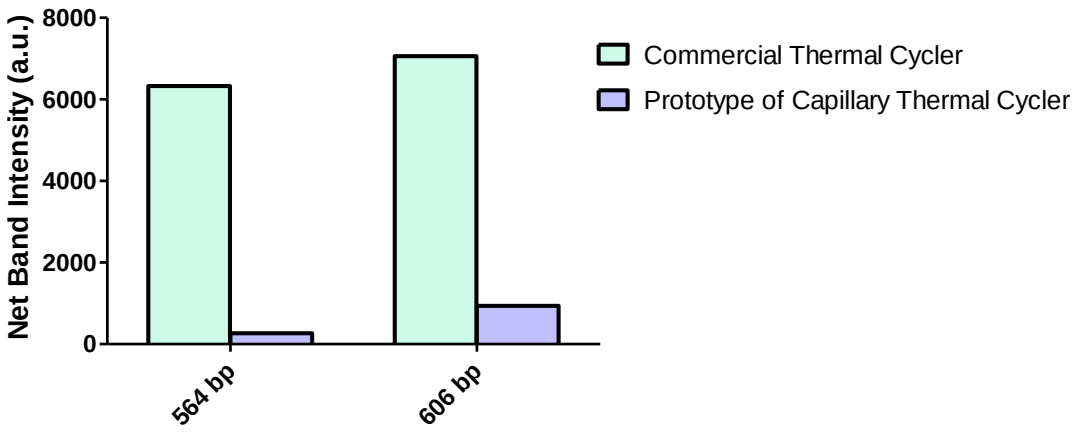


Figure 3.39 The net band intensity analysis of the prototype of capillary thermal cycler and commercially available thermal cycler. The amplification of bacterial DNA by commercial Taq DNA polymerase with length of 564 bp, 606 bp was represented, respectively. The histogram bars represent the average band intensity of the spots. The net intensity value could be seen on section 2.2.10.

CHAPTER 4

CONCLUSION

In this study, high activity Taq DNA polymerase was cloned, purified and characterized for its enzymatic activities. Two new thermal cyclers, conventional and capillary were also optimized and validated to be used for the polymerase chain reaction.

Thermus aquaticus genomic DNA, isolated from *Thermus aquaticus* using NANObiz DNA Isolation Kit, was used for the amplification of Taq DNA polymerase gene and cloned in *Escherichia coli* using Gateway® recombination cloning technology. Optimization studies were performed to obtain activate Taq DNA polymerase enzyme at a high yield.

The purified Taq DNA polymerase enzyme activity was confirmed by amplifying several DNA target fragments from different sources, plasmid DNA, bacterial DNA and human DNA. This Taq DNA polymerase was compared with another commercially available Taq DNA polymerase using band intensity value as the comparison criterion. Enzyme activity of produced Taq DNA polymerase was found to be not significantly different from commercial Taq DNA polymerase for all DNA sources in the 95% confidence interval Endonuclease and nicking activities were also tested and could not be observed. The fidelity of the purified Taq DNA polymerase was also checked and the misincorporation rate was found to be within the acceptable range. Our results have shown that the purified Taq DNA polymerase has the same efficiency and characteristics of the commercially available Taq DNA polymerase.

Two thermal cyclers, conventional and capillary thermal cycler, were also tested and optimized for their efficiency. These thermal cyclers have been developed by NANObiz Ltd. Co. and their specifications were adjusted in accordance with the results of this study. Results obtained using the first prototype of the conventional thermal cycler was utilized to design the specifications of the second prototype. The second prototype, as a result, was as efficient as a commercially available conventional thermal cycler. The capillary thermal cycler was tested for PCR as a proof of concept and shown that it works under the conditions tested. Obtained results will be used for the second prototype of the capillary thermal cycler to be used in a microfluidics device.

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APPENDIX A

TAQ DNA POLYMERASE GENE SEQUENCES

```
   1  ATGAGGGGGA TGCTGCCCCT CTTTGAGCCC AAGGGCCGGG TCCTCCTGGT
  51  GGACGGCCAC CACCTGGCCT ACCGCACCTT CCACGCCCTG AAGGGCCTCA
 101  CCACCAGCCG GGGGGAGCCG GTGCAGGCGG TCTACGGCTT CGCCAAGAGC
 151  CTCCTCAAGG CCCTCAAGGA GGACGGGGAC GCGGTGATCG TGGTCTTTGA
 201  CGCCAAGGCC CCCTCCTTCC GCCACGAGGC CTACGGGGGG TACAAGGCGG
 251  GCCGGGCCCC CACGCCGGAG GACTTTCCCC GGCAACTCGC CCTCATCAAG
 301  GAGCTGGTGG ACCTCCTGGG GCTGGCGCGC CTCGAGGTCC CGGGCTACGA
 351  GGCGGACGAC GTCCTGGCCA GCCTGGCCAA GAAGGCGGAA AAGGAGGGCT
 401  ACGAGGTCCG CATCCTCACC GCCGACAAAG ACCTTTACCA GCTCCTTTCC
 451  GACCGCATCC ACGTCCTCCA CCCCGAGGGG TACCTCATCA CCCCGGCCTG
 501  GCTTTGGGAA AAGTACGGCC TGAGGCCCGA CCAGTGGGCC GACTACCGGG
 551  CCCTGACCGG GGACGAGTCC GACAACCTTC CCGGGGTCAA GGGCATCGGG
 601  GAGAAGACGG CGAGGAAGCT TCTGGAGGAG TGGGGGAGCC TGGAAGCCCT
 651  CCTCAAGAAC CTGGACCGGC TGAAGCCCGC CATCCGGGAG AAGATCCTGG
 701  CCCACATGGA CGATCTGAAG CTCTCCTGGG ACCTGGCCAA GGTGCGCACC
 751  GACCTGCCCC TGGAGGTGGA CTTCGCCAAA AGGCGGGAGC CCGACCGGGA
 801  GAGGCTTAGG GCCTTTCTGG AGAGGCTTGA GTTTGGCAGC CTCCTCCACG
 851  AGTTCGGCCT TCTGGAAAGC CCCAAGGCCC TGGAGGAGGC CCCCTGGCCC
 901  CCGCCGGAAG GGGCCTTCGT GGGCTTTGTG CTTTCCCGCA AGGAGCCCAT
 951  GTGGGCCGAT CTTCTGGCCC TGGCCGCCGC CAGGGGGGGC CGGGTCCACC
1001  GGGCCCCCGA GCCTTATAAA GCCCTCAGGG ACCTGAAGGA GGCGCGGGGG
1051  CTTCTCGCCA AAGACCTGAG CGTTCTGGCC CTGAGGGAAG GCCTTGGCCT
1101  CCCGCCCGGC GACGACCCCA TGCTCCTCGC CTACCTCCTG GACCCTTCCA
1151  ACACCACCCC CGAGGGGGTG GCCCGGCGCT ACGGCGGGGA GTGGACGGAG
1201  GAGGCGGGGG AGCGGGCCGC CCTTTCCGAG AGGCTCTTCG CCAACCTGTG
1251  GGGGAGGCTT GAGGGGGAGG AGAGGCTCCT TTGGCTTTAC CGGGAGGTGG
1301  AGAGGCCCCT TTCCGCTGTC CTGGCCCACA TGGAGGCCAC GGGGGTGCGC
1351  CTGGACGTGG CCTATCTCAG GGCCTTGTCC CTGGAGGTGG CCGAGGAGAT
1401  CGCCCGCCTC GAGGCCGAGG TCTTCCGCCT GGCCGGCCAC CCCTTCAACC
1451  TCAACTCCCG GGACCAGCTG GAAAGGGTCC TCTTTGACGA GCTAGGGCTT
1501  CCCGCCATCG GCAAGACGGA GAAGACCGGC AAGCGCTCCA CCAGCGCCGC
1551  CGTCCTGGAG GCCCTCCGCG AGGCCCACCC CATCGTGGAG AAGATCCTGC
1601  AGTACCGGGA GCTCACCAAG CTGAAGAGCA CCTACATTGA CCCCTTGCCG
1651  GACCTCATCC ACCCCAGGAC GGGCCGCCTC CACACCCGCT TCAACCAGAC
```

1701 GGCCACGGCC ACGGGCAGGC TAAGTAGCTC CGATCCCAAC CTCCAGAACA
1751 TCCCCGTCCG CACCCCGCTT GGGCAGAGGA TCCGCCGGGC CTTCATCGCC
1801 GAGGAGGGGT GGCTATTGGT GGCCCTGGAC TATAGCCAGA TAGAGCTCAG
1851 GGTGCTGGCC CACCTCTCCG GCGACGAGAA CCTGATCCGG GTCTTCCAGG
1901 AGGGGCGGGA CATCCACACG GAGACCGCCA GCTGGATGTT CGGCGTCCCC
1951 CGGGAGGCCG TGGACCCCCT GATGCGCCGG GCGGCCAAGA CCATCAACTT
2001 CGGGGTCCTC TACGGCATGT CGGCCCACCG CCTCTCCCAG GAGCTAGCCA
2051 TCCCTTACGA GGAGGCCCAG GCCTTCATTG AGCGCTACTT TCAGAGCTTC
2101 CCCAAGGTGC GGGCCTGGAT TGAGAAGACC CTGGAGGAGG GCAGGAGGCG
2151 GGGGTACGTG GAGACCCTCT TCGGCCGCCG CCGCTACGTG CCAGACCTAG
2201 AGGCCCGGGT GAAGAGCGTG CGGGAGGCGG CCGAGCGCAT GGCCTTCAAC
2251 ATGCCCGTCC AGGGCACCGC CGCCGACCTC ATGAAGCTGG CTATGGTGAA
2301 GCTCTTCCCC AGGCTGGAGG AAATGGGGGC CAGGATGCTC CTTCAGGTCC
2351 ACGACGAGCT GGTCCTCGAG GCCCCAAAAG AGAGGGCGGA GGCCGTGGCC
2401 CGGCTGGCCA AGGAGGTCAT GGAGGGGGTG TATCCCCTGG CCGTGCCCCT
2451 GGAGGTGGAG GTGGGGATAG GGGAGGACTG GCTCTCCGCC AAGGAGTGA

APPENDIX B

PLASMID MAPS

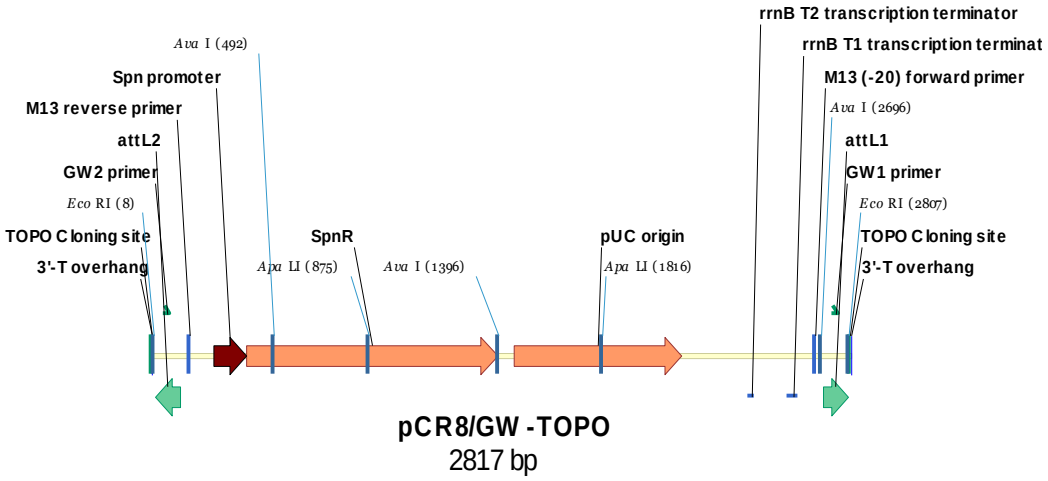

Figure B.1 The plasmid map of pCR®8/GW/TOPO® TA cloning vector

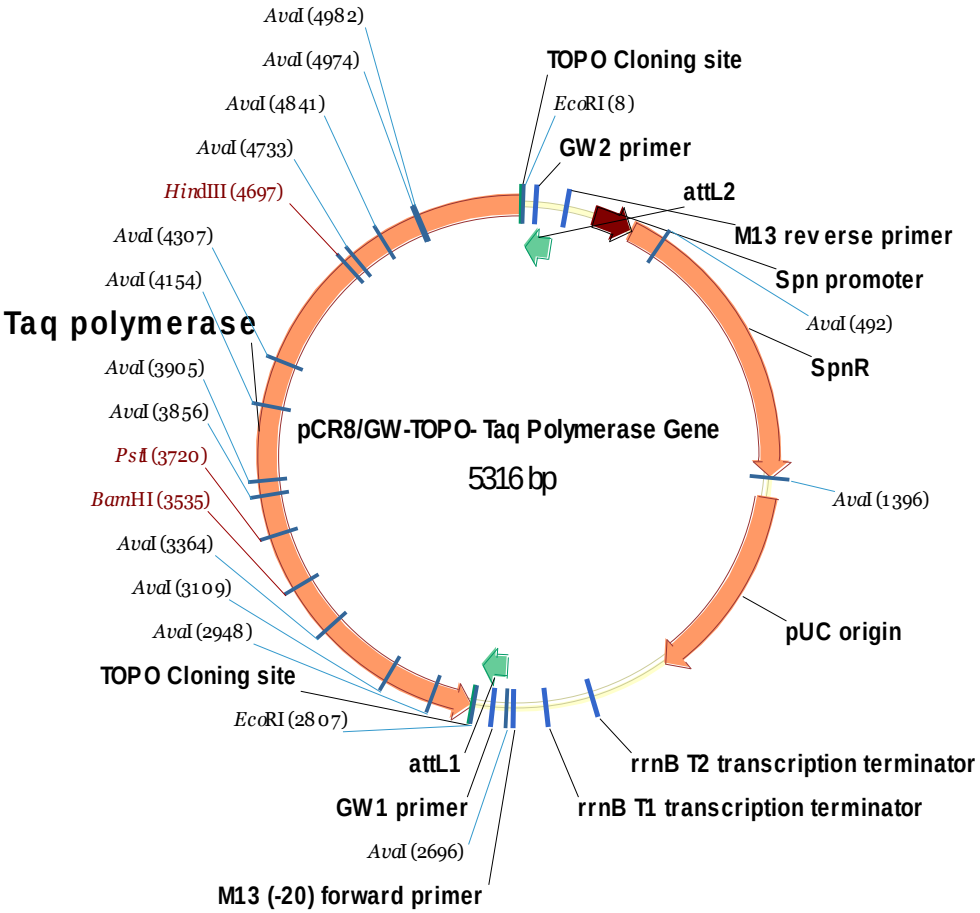

Figure B.2 The plasmid map of pCR®8/GW/TOPO® TA vector with Taq polymerase gene

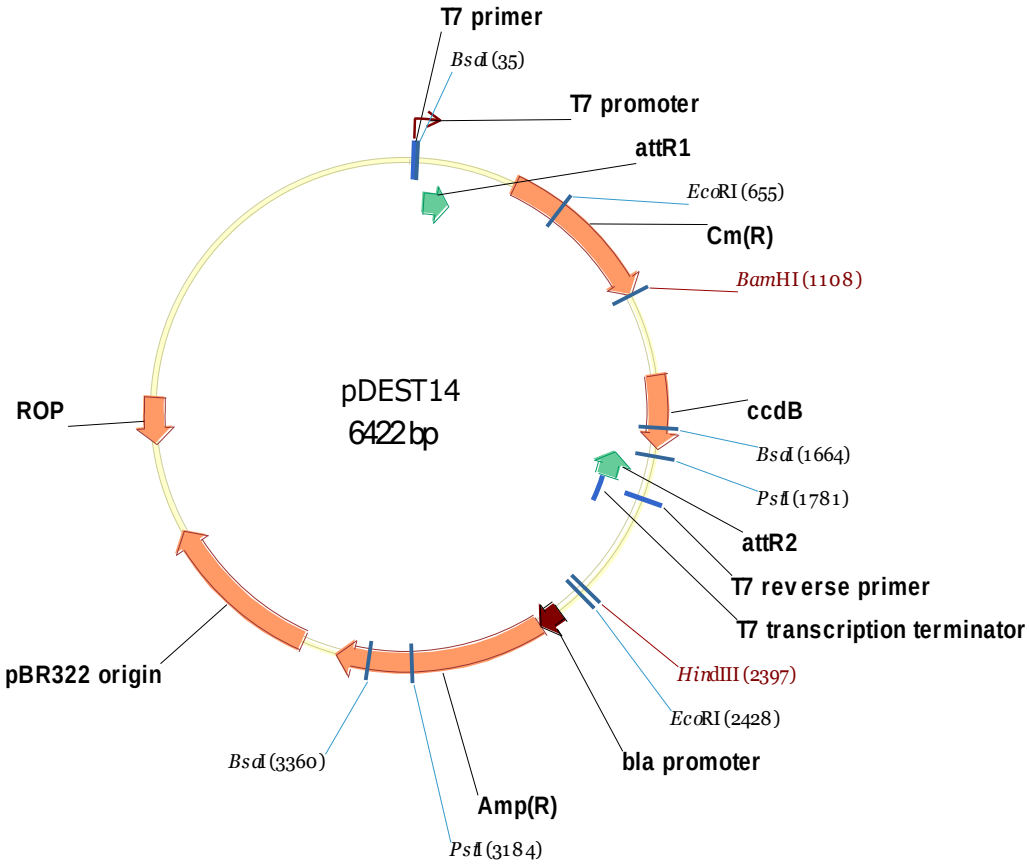

Figure B.3 The plasmid map of pDEST14 vector

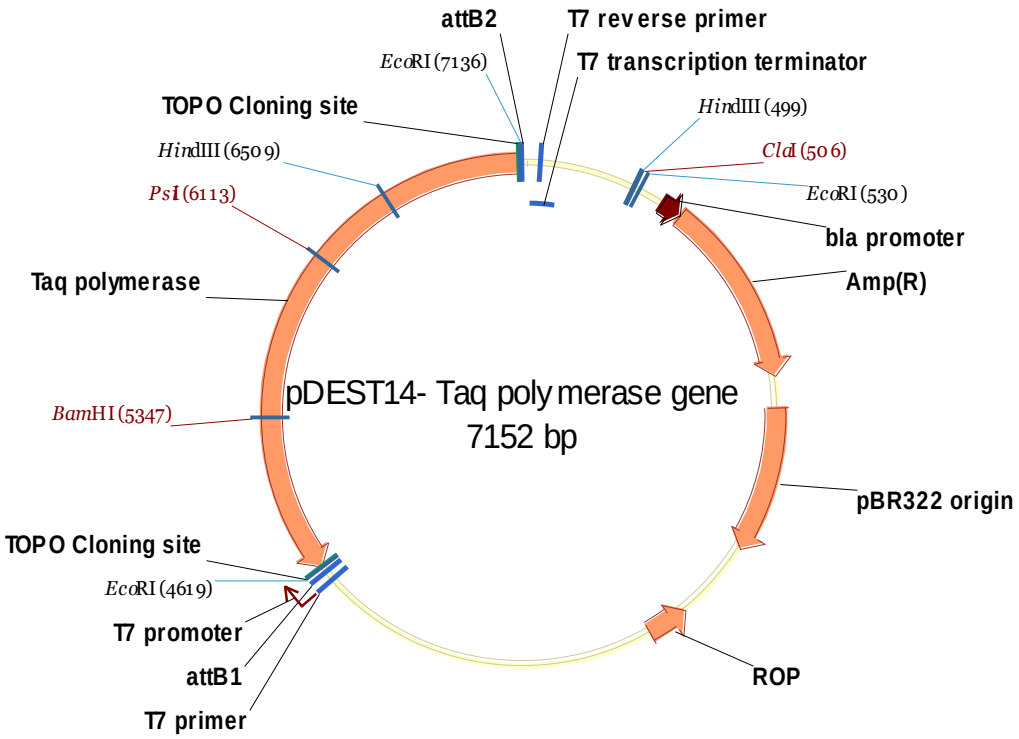

Figure B.4 The plasmid map of pDEST14 vector with Taq polymerase gene

APPENDIX C

COMPOSITION OF BACTERIAL CULTURE MEDIA

Thermus 162 Media

Table C.1 Composition of *Thermus* 162 Media

Component	**Amount of component (1000 ml)**
Yeast extract	1 g
Tryptone	1 g
Nitrilotriacetic acid	100 mg
$CaSO_4$ x 2 H_2O	40 mg
$MgCl_2$ x 6 H_2O	200 mg
0.01 M Fe citrate	0.5 ml
Trace element solution (Table C.3)	0.5 ml
Phosphate buffer (Table C.2)	100 ml

Table C.2 Composition of Phosphate Buffer pH:7.2

Component	**Amount of component (100 mL)**
KH_2PO_4	0.544 g
Na_2HPO_4 x 12 H_2O	4.3 g

Table C.3 Composition of Trace Element Solution

Component	**Amount of component (1000 mL)**
$H_2 SO_4$	0.5 mL
$MnSO_4$ x H_2O	2.28 g
$ZnSO_4$ x 7 H_2O	0.5 g
H_3BO_3	0.5 g
$CuSO_4$ x 5 H_2O	25 mg
Na_2MoO_4 x 2 H_2O	25 mg
$CoCl_2$ x 6 H_2O	45 mg

LB Media

Table C.4 Composition of LB Media pH 7

Component	**Amount of component (1000 mL)**
Yeast Extract	5 g
Tryptone	10 g
NaCl	10 g
Agar	15 g

APPENDIX D

PREPARATION OF BUFFER

D.1. BUFFERS FOR DNA ISOLATION METHODS

D.1.1 TE Buffer

(10 mM Tris HCl, 1 mM EDTA)

1.211 g Tris-HCl and 0.29224 g EDTA was dissolved in 1000 mL of distilled H_2O.and pH is adjusted to 8.0. Solution was sterilized by filter sterilization and stored at room temperature.

D.1.2 10% SDS

10 g SDS was dissolved in 100 mL of distilled H_2O. Solution was sterilized by filter sterilization (filter size: 0.4 μm) and stored at room temperature.

D.1.3 5 M NaCl

29.22 g NaCl was dissolved in 100 mL of distilled H_2O and stored at room temperature.

D.1.4 CTAB/ NaCl

(10% CTAB in 0.7 M NaCl)

4.1 g NaCl was dissolved in 80 ml water and 10 g CTAB was slowly added while heating and stirring. Solution volume was completed to 100 mL.

D.1.5 Chloroform / Isoamyl alcohol

240 mL Chloroform and 10 mL Isoamyl alcohol was mixed and stored at 4^{o}C.

D.2. BUFFERS FOR BRADFORD, SDS-PAGE AND STAINING METHODS

D.2.1 5X Bradford Reagent

It was prepared by dissolving 500 mg of Coomassie Brilliant Blue G250 in 250 ml of 95% ethanol. Then 85% (w/v) phosphoric acid was added into the mixture and the solution was diluted to 1L by distilled water. Finally, the solution was filtered and kept at the refrigerator.

D.2.2 Gel Solution

(30% Acrylamide / Bisacrylamide)

58.4g acrylamide and 1.6 N'N'- bis-methylene-acrylamide was dissolved in 200 mL of distilled water. It was stored at 4^{o}C in the dark.

D.2.3 Separating Gel Buffer

(1.5 M Tris-HCl pH 8.8)

54.45 g Tris-base was dissolved in 300 mL of distilled water and pH was adjusted to 8.8.

D.2.4 Stacking Gel Buffer

(0.5 M Tris-HCl pH: 6.8)

30 g Tris-base was dissolved in 300 mL of distilled water and pH was adjusted to 6.8.

D.2.5 10% Ammonium per Sulfate

0.06 g ammonium per sulfate was dissolved in 600 µL distilled water and it was prepared freshly.

D.2.6 5X stock running buffer

(25 mM Tris, 192 mM glycine, 0.1% SDS, pH :8.3)

15 g Tris-base and 72 g glycine were dissolved in 1000 mL distilled water and it was stored at 4^{o}C.

D.2.7 1X stock running buffer

600 mL 5X stock running buffer was diluted with 2400 mL distilled water to 1X stock running buffer. 3 g SDS was added into the solution.

D.2.8 Silver Staining Fixing Solution

150 mL methanol, 36 mL Acetic acid and 150 µL 37% formaldehyde were mixed and completed into the 300 mL distilled water.

D.2.9 Silver Staining Washing Solution

(50% Ethanol)

600 mL ethanol was mixed with 600 mL distilled water. It was stored at room temperature.

D.2.10 Silver Staining Pretreatment Solution

0.08 g sodium thiosulfate was dissolved in 400 ml distilled water and stored at room temperature in dark solution.

D.2.11 Silver Staining Silver Nitrate Solution

0.8 g silver nitrate was dissolved in 400 mL distilled water and 300 µL formaldehyde was added into the solution. It was stored at room temperature in dark place.

D.2.12 Silver Staining Developing Solution

9 g potassium carbonate was dissolved in 300 mL distilled water and 8 mL silver staining pretreatment solution (D.2.10) and 300 µL 37% formaldehyde was added into the solution. It was completed to 400 mL by distilled water.

D.2.13 Silver Staining Stop Solution

200 mL methanol, 48 mL acetic acid , 153 mL distilled water was mixed and stored at room temperature.

D.4. BUFFERS FOR TAQ DNA POLYMERASE ISOLATION METHOD

D.4.1 0.84 M IPTG

2 g IPTG was dissolved into the 10 mL distilled water and sterilized with lter sterilization (filter size: 0.4 µm) and stored at-20 ^{o}C.

D.4.2 Buffer A

(50 mM Tris pH 7.9, 50 mM Dextrose, 1 mM EDTA)

10 mL 1M Tris (pH:7.9), 3.6 mL 50% dextrose, 0.5 mL 0.5 M EDTA , 186 mL distilled water was mixed and autoclaved. It was stored at room temperature.

D.4.3 Buffer B

(10 mM Tris pH7.9, 50 mM KCl , 1 mM EDTA, 1 mM PMSF, 0.5% Tween 20, 0.5% Nonidet P40)

1mL 1M Tris pH:7.9, 5 mL 1M KCl, 200 µL 0.5 M EDTA, 10 µL 100 mM PMSF, 250 µL Tween 20, 250 µL Nonided P40 and 93.3 mL was mixed. It was stored at 4^{o}C in the dark.

D.4.4 Taq Storage Buffer

(20 mM Tris pH:7.9, 100 mM KCl, 0.1 mM EDTA, 50 % Glycerol, 1 mM DTT, 0.5% Nonidet P40, 0.5% Tween20)

0.2 mL 1M Tris pH:7.9, 1 mL 1M KCl, 2 µL 0.5 M EDTA, 25 mL 80% glycerol, 50 µL Tween 20, 50 µL Nonided P40 and 2.45 mL was mixed. It was stored at 4^{o}C in the dark.

50 X TAE (Tris-Acetate) BUFFER

242 g Tris-base, 57.1 mL Glacial acetic acid 100 mL 0.5 M EDTA (pH: 8.0) were mixed in 1L of distilled H_2O.

APPENDIX E

PRIMER SEQUENCES

Primers sequences for Plasmid DNA (417 bp)

Antisense primer: 5'GAATTAGGATCCATGGCTTCAAAGCGTATTCTTAAAGAG
Sense primer: 5'AGTCCAAAGCTTCTATCCCATAGCATACTTTTGAGTCCAA

Primers sequences for Plasmid DNA (618 bp)

Antisense primer: 5’CTTTCGGATCCATGGCGAGCTCCGATACCGAACGCA
Sense primer: 5'TGGATTAAGCTTTTACAGGTGTGTCCTCAGCTTTATCTTCAGA

Primers sequences for Plasmid DNA (1197 bp)

Antisense primer: 5’ GGAGGGCTACGAGGTCCGCA 3’
Sense primer: 5’ CCCGGTACTGCAGGATCTTC 3’

Primers sequences for Bacterial DNA (564 bp)

Antisense primer: 5' CCATGGATGATGTCTGATTTCGCAC 3’
Sense primer: 5' AGATCTTTATCGGCCCAGGCTCT 3’

Primers sequences for Bacterial DNA (606 bp)

Antisense primer: 5' GGATCCATGCAAGCCAAAACGTTC 3’
Sense primer: 5' AGATCTTCAGGGGTAGACGACCG 3’

Primers sequences for Bacterial DNA (777 bp)

Antisense primer: 5' GGATCCATGAAACGCATCGCCAT 3’
Sense primer: 5' AGATCTTTACTGGATCTTGGCCTGT 3’

Primers sequences for Bacterial DNA (1488 bp)

Antisense primer: 5' CCATGGATGAAAGCAATTACAGTGTCG 3’
Sense primer: 5' AGATCTCTATTTGCTGGCGGGCTG 3’

Primers sequences for Human DNA (115 bp)

Antisense primer: 5' TATGACAACGAATTTGGCTAC 3’
Sense primer: 5' TCTCTCTTCCTCTTGTGCTCT 3’

Primers sequences for Human DNA (472 bp)

Antisense primer: 5' TGCCTTCTTGCCTCTTGTCT 3’
Sense primer: 5' TTGATTTTGGAGGGATCTCG 3’

Primers sequences for Human DNA (644 bp)

Antisense primer: 5' TGCCTTCTTGCCTCTTGTCT 3’
Sense primer: 5' CTGCAAATGAGCCTACAGCA 3

APPENDIX F

PCR CONDITIONS

Table F.1 Optimized Conditions of Plasmid DNA (417- 618 bp)

Ingredients	Stock Concentration	Amount	Final Concentration
dH_2O	-	14.75	
Reaction Buffer	10 X	2.5 µl	1X
$MgCl_2$	25 mM	1.5 µl	1.5 mM
Sense primer	5 µM	1.5 µl	0.3 µM
Antisense primer	5 µM	1.5 µl	0.3 µM
dNTP	2 mM	2.5 µl	0.2 mM
Plasmid DNA	100 ng/ µl	0.5 µl	50 ng
Fermentas Taq polymerase	5U/µl	0.25 µl	1.25U

Table F.2 PCR Conditions of Plasmid DNA (417- 618 bp)

Steps	Plasmid DNA PCR Parameters
Denaturation	94^oC 5 minutes
Amplification	94^oC 30 seconds
	61^oC 30 seconds
	72^oC 30 seconds
Number of cycles	35
Final extension	72^oC 5 minutes

Table F.3 Optimized Conditions of Plasmid DNA (1197 bp)

Ingredients	Stock Concentration	Amount	Final Concentration
dH_2O	-	11.25	
Reaction Buffer	10 X	2.5 µl	1X
$MgCl_2$	25 mM	1.5 µl	1.5 mM
Sense primer	5 µM	2 µl	0.4 µM
Antisense primer	5 µM	2 µl	0.4 µM
dNTP	2 mM	5 µl	0.4 mM
Plasmid DNA	100 ng/ µl	0.5 µl	50 ng
Fermentas Taq polymerase	5U/µl	0.25 µl	1.25U

Table F.4 PCR Conditions of Plasmid DNA (1197 bp)

Steps	Plasmid DNA PCR Parameters
Denaturation	94^oC 5 minutes
Amplification	94^oC 30 seconds
	54^oC 45 seconds
	72^oC 3 minutes
Number of cycles	35
Final extension	72^oC 5 minutes

Table F.5 Optimized Conditions of Bacterial DNA (564- 606- 777 bp)

Ingredients	Stock Concentration	Amount	Final Concentration
dH_2O	-	14.0 µl	
Reaction Buffer	10 X	2.5 µl	1X
$MgCl_2$	25 mM	2.5 µl	2.5 mM
Sense primer	10 µM	1 µl	0.4 µM
Antisense primer	10 µM	1 µl	0.4 µM
dNTP	2 mM	2.5 µl	0.2 mM
Bacterial DNA	100 ng/ µl	1 µl	100 ng
Fermentas Taq polymerase	5U/µl	0.5 µl	2.5 U

Table F.6 PCR Conditions of Bacterial DNA (564- 606- 777 bp)

Steps	Bacterial DNA PCR Parameters
Denaturation	94°C 5 minutes
Amplification	94°C 1 minute
	58°C 1 minute
	72°C 1 minute
Number of cycles	40
Final extension	72°C 5 minutes

Table F.7 Optimized Conditions of Bacterial DNA (1488 bp)

Ingredients	Stock Concentration	Amount	Final Concentration
dH_2O	-	12.75 µL	
Reaction Buffer	10 X	2.5 µL	1X
$MgCl_2$	25 mM	2.5 µL	2.5 mM
Sense primer	10 µM	1 µL	0.4 µM
Antisense primer	10 µM	1 µL	0.4 µM
dNTP	2 mM	2.5 µL	0.2 mM
DMSO		1.25 µL	5%
Bacterial DNA	100 ng/ µl	1 µL	100 ng/µL
Fermentas Taq polymerase	5U/µl	0.5 µl	2.5 U

Table F.8 PCR Conditions of Bacterial DNA (1488 bp)

Steps	Bacterial DNA PCR Parameters
Denaturation	95°C 5 minutes
Amplification	95°C 30 seconds
	73°C 30 seconds
	72°C 100 seconds
Number of cycles	9
Amplification	95°C 30 seconds
	63°C 30 seconds
	72°C 2 minute
Number of cycles	29
Final extension	72°C 5 minutes

Table F.9 Optimized Conditions of Human DNA (115 bp)

Ingredients	Stock Concentration	Amount	Final Concentration
dH_2O	-	14.25 µl	
Reaction Buffer	10 X	2.5 µl	1X
$MgCl_2$	25 mM	1.5 µl	1.5 mM
Sense primer	5 µM	1.5 µl	0.3 µM
Antisense primer	5 µM	1.5 µl	0.3 µM
dNTP	2 mM	2.5 µl	0.2 mM
Human DNA	100 ng/ µl	1 µl	100 ng
Fermentas Taq polymerase	5U/µl	0.25 µl	1.25 U

Table F.10 PCR Conditions of Human DNA (115 bp)

Steps	Human DNA PCR Parameters
Denaturation	94°C 5 minutes
Amplification	94°C 30 seconds
	54°C 30 seconds
	72°C 30 seconds
Number of cycles	35
Final extension	72°C 5 minutes

Table F.11 Optimized Conditions of Human DNA (472- 644-1000 bp)

Ingredients	Stock Concentration	Amount	Final Concentration
dH_2O	-	13.75 µl	
Reaction Buffer	10 X	2.5 µl	1X
$MgCl_2$	25 mM	2 µl	2 mM
Sense primer	5 µM	1.5 µl	0.3 µM
Antisense primer	5 µM	1.5 µl	0.3 µM
dNTP	2 mM	2.5 µl	0.2 mM
Human DNA	100 ng/ µl	1 µl	100 ng
Fermentas Taq polymerase	5U/µl	0.25 µl	1.25 U

Table F.12 PCR Conditions of Human DNA (472-644-1000 bp)

Steps	Human DNA PCR Parameters
Denaturation	94°C 5 minutes
Amplification	94°C 30 seconds
	55°C 30 seconds
	72°C 30 seconds
Number of cycles	35
Final extension	72°C 5 minutes

APPENDIX G

TABULATED VALUES OF NET FLUORESCENCE INTENSITIES

Table G.1 Mean values and SEM for Figure 3.12

Enzyme Activity Test with Plasmid DNA	**Net Fluorescence Intensity (a.u.)**	
	Commercial Taq DNA polymerase	**Produced Taq DNA polymerase**
270 bp	10689.960 ± 620.351	10213.620 ± 1069.517
471 bp	10719.150 ± 1097.617	10673.610 ± 995.1278
618 bp	11093.270 ± 212.646	10010.030 ± 890.0396
1197 bp	13005.200 ± 1019.770	13179.670 ± 431.2246

Table G.2 Mean values and SEM for Figure 3.14

Enzyme Activity Test with Bacterial DNA	**Net Fluorescence Intensity (a.u.)**	
	Commercial Taq DNA polymerase	**Produced Taq DNA polymerase**
564 bp	4742.959 ± 808.8249	8207.348 ± 98.05042
606 bp	11157.250 ± 661.8585	10841.950 ± 589.2118
777 bp	9354.595 ± 987.3682	9932.286 ± 657.344
1488 bp	6345.705 ± 618.0447	4924.932 ± 870.0507

Table G.3 Mean values and SEM for Figure 3.17

Enzyme Activity Test with Human DNA	**Net Fluorescence Intensity (a.u.)**	
	Commercial Taq DNA polymerase	**Produced Taq DNA polymerase**
115 bp	3295.955 ± 745.9336	2534.131 ± 1102.218
472 bp	2687.749 ± 354.1064	2623.098 ± 393.2025
644 bp	2929.604 ± 416.3077	2335.396 ± 410.3626
1000 bp	8849.395 ± 277.9692	8893.611 ± 181.7782

Table G.4 Mean values and SEM for Figure 3.19

Unit Determination	**Net Fluorescence Intensity (a.u.)**
Commercial Taq DNA Polymerase	8697.576 ± 183.564
Produced Taq DNA polymerase	10606.380 ± 161.4864
Produced Taq DNA polymerase with 1/5 dilution	9379.977 ± 58.58831
Produced Taq DNA polymerase with 1/10 dilution	8384.944 ± 251.6396

Table G.5 Mean values and SEM for Figure 3.21

Unit Determination	**Net Fluorescence Intensity (a.u.)**
Commercial Taq DNA Polymerase	8530.909 ± 61.61338
IsolateTaq DNA polymerase with 1/10 dilution	8384.944 ± 251.6396
Purified Taq DNA Polymerase	6064.259 ± 182.2873

Table G.6 Mean values and SEM for Figure 3.29

Efficiency Test with Plasmid DNA & Commercial Taq DNA Polymerase	**Net Fluorescence Intensity (a.u.)**	
	Commercial Thermal Cycler	**Second Prototype of Thermal Cycler**
270 bp	9302.797 ± 398.150	9106.735 ± 206.505
471 bp	9215.943 ± 862.794	9320.377 ± 561.702
618 bp	8672.691 ±1382.094	9115.563 ± 773.723

Table G.7 Mean values and SEM for Figure 3.29

Efficiency Test with Plasmid DNA & Produced Taq DNA Polymerase	**Net Fluorescence Intensity (a.u.)**	
	Commercial Thermal Cycler	**Second Prototype of Thermal Cycler**
270 bp	7989.720 ± 12.620	9246.629± 342.861
471 bp	9032.049 ± 833.904	9727.089 ± 454.020
618 bp	9098.542 ± 736.143	10078.080 ± 237.077

Table G.8 Mean values and SEM for Figure 3.31

Efficiency Test with Bacterial DNA & Commercial Taq DNA Polymerase	**Net Fluorescence Intensity (a.u.)**	
	Commercial Thermal Cycler	**Second Prototype of Thermal Cycler**
606 bp	9801.54 ± 1332.73	11433.930 ± 729.298
777 bp	6558.94 ± 1731.21	10436.590 ± 798.056

Table G.9 Mean values and SEM for Figure 3.31

Efficiency Test with Bacterial DNA & Produced Taq DNA Polymerase	**Net Fluorescence Intensity (a.u.)**	
	Commercial Thermal Cycler	**Second Prototype of Thermal Cycler**
606 bp	8668.06 ± 593.06	8531.178 ± 1441.954
777 bp	8286.34 ± 217.09	6826.795 ± 1287.374

Table G.10 Mean values and SEM for Figure 3.34

Efficiency Test with Human DNA & Commercial Taq DNA Polymerase	**Net Fluorescence Intensity (a.u.)**	
	Commercial Thermal Cycler	**Second Prototype of Thermal Cycler**
115 bp	4156.382 ± 80.446	4877.086 ± 24.452
472 bp	4618.288 ± 645.088	1600.178 ± 105.119
644bp	2630.982 ± 140.672	1247.137 ± 62.673

Table G.11 Mean values and SEM for Figure 3.34

Efficiency Test with Human DNA & Produced Taq DNA Polymerase	**Net Fluorescence Intensity (a.u.)**	
	Commercial Thermal Cycler	**Second Prototype of Thermal Cycler**
115 bp	5684.395 ± 563.421	7305.588 ± 910.010
472 bp	3071.399 ± 156.581	1552.039 ± 453.031
644 bp	5229.917 ± 655.196	5530.614 ± 539.887

Table G.12 Mean values and SEM for Figure 3.37

Efficiency Test with Plasmid DNA & Commercial Taq DNA Polymerase	**Net Fluorescence Intensity (a.u.)**	
	Commercial Thermal Cycler	**Prototype of Capillary Thermal Cycler**
471 bp	5222.276	3436.482
618 bp	4116.781	294.584

Table G.13 Mean values and SEM for Figure 3.39

Efficiency Test with Bacterial DNA & Commercial Taq DNA Polymerase	**Net Fluorescence Intensity (a.u.)**	
	Commercial Thermal Cycler	**Prototype of Capillary Thermal Cycler**
564 bp	3295.581	265.078
606 bp	4001.241	939.460