FUNCTIONAL CHARACTERIZATION OF POL-DUT FUSION ENZYMES FROM *PYROCOCCUS FURIOSUS*

by

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ABSTRACT

FUNCTIONAL CHARACTERIZATION OF POL-DUT FUSION ENZYMES FROM *PYROCOCCUS FURIOSUS*

Asim Kanti Dash

University of Houston-Clear Lake, 2018
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Although polymerase chain reaction is a powerful technique, often polymerizing errors can jeopardize the whole experiment especially when to study a cloned gene product. Long PCR with conventional *Taq* DNA polymerases is one such example, where conversion of dCTP to dUTP can increase the error frequency and dUTP incorporation as well. However, using proofreading polymerases for long PCR, the polymerization rate slows down significantly due to incorporation of dUTP during synthesis followed proofreading. To alleviate this problem, now a day we use dUTPase enzyme in the PCR. To have similar effect, our laboratory has designed two fusion enzyme constructs of both a proofreading polymerase and a dUTPase from *Pyrococcus furiosus*. We are reporting here that both these fusion constructs could remove dUTP from PCR reactions and able to amplify. This project particularly entails successful cloning, expression and purification of a dUTPase enzyme followed by its functional characterization in parallel with the above mentioned fusion enzymes.

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CHAPTER I: OBJECTIVES

High fidelity PCR is a powerful technique to amplify a DNA sequence and is popularly used in gene study. However, incorporation of wrong base pairs (bp) during PCR can jeopardize the whole experiment. One such problem is specifically encountered when long DNA fragment is cloned after amplification with DNA polymerases. We are particularly interested here to overcome this erroneous polymerization during PCR using newly designed recombinant DNA polymerases.

Pfu DNA polymerase (*Pfu*-Pol) is a thermostable enzyme that possesses 3'-5' exonuclease activity, also known as proofreading function, and thereby exhibits high fidelity during long PCR. One drawback of PCR is the accumulation of dUTP due to deamination of dCTP during the repetitive thermocycling steps at high temperature. *Pfu*-Pol can recognize this dUTP as a wrong base when accidentally incorporated. However, the presence of dUTP in the PCR solution slows down the speed of amplification. It is known that the addition of dUTPase in the PCR reaction mixture can covert dUTP to dUMP. Thus, our lab has designed a fusion enzyme in combination of *Pfu*-Pol and dUTPase that will be able to remove dUTP and able to amplify DNA as well. Previously, in our lab we were not able to perform dUTPase assays of these fusion enzymes due to lack of bona fide dUTPase enzyme. Therefore, in this thesis, we have elected for the cloning, purification, and characterization of the *Pfu*-dUTPase so that we could use it as a control in the functional assays for the fusion enzymes.

In this study, we plan to clone the dUTPase gene in a bacterial expression vector employing an In-Fusion Cloning Technology. The gene was expressed in a special bacterial host and the corresponding enzyme was purified using Calmodulin Affinity Chromatography. Next, the enzyme would be used as a control in the functional characterization of the fusion enzymes that we were claiming to have dUTPase activity. Since *P. furiosus* is an archaea and falls in between prokaryote and eukaryote, we wanted to study if the expression of the PCR enzymes in eukaryotes (yeast) would provide more efficiency due to post-translational modifications. In this regard, we have inserted the same constructs from bacterial expression vectors into the yeast expression vectors and looked into their expression and possible enzyme functions.

CHAPTER II: INTRODUCTION

Pfu DNA Polymerase

Thomas D. Brock in 1969 [1] discovered the first thermophilic bacteria, *Thermus aquaticus*, in the hot springs of Yellowstone National Park, Wyoming. This has caused a breakthrough in the field of molecular biology and revolutionized biotechnology. This bacterium is now found to be a member of a special group of gram-negative thermophiles. In 1977, Woese and Fox [2] divided the prokaryotic life into two phylogenetically distinct domains, including 'eubacteria' containing all typical bacteria and 'archaebacteria' representing the group of organisms from diverse and highly specialized niches. Initially, archaebacteria were thought to be ordinary bacteria as they have similar morphological characteristics including size and shape. However, molecular sequence homology implies archaebacteria cannot be considered as ordinary bacteria, rather they are more related to eukaryotes. The enzymes involved in archaebacterial DNA replication, gene transcription and protein translation more closely resemble those of eukaryotic enzymes.

In reality, the enzymes obtained from these organisms are found to be very useful in many sectors as they have inherent thermostability and optimal activity at high temperatures. The first DNA polymerase that was used in PCR was from *Thermus aquaticus*. This can withstand very high, even boiling temperatures, and became the basis for gene cloning study and later used in the human genome project. Lately, there have been new discoveries of many thermophilic and hyperthermophilic enzymes with potential applications in pharmaceuticals, biological research, food industry, biofuels, and so on. For

example, in two-step starch processing, α-amylase from *Pyrococcus furiosus* is used in the liquefaction step since the enzyme is active at a temperature of as high as 105°C that prevents viscosity during gelatinization[3]. Similarly, β-amylase from *Thermoanaerobacterium thermosulfurigens* shows optimum activity at 95°C, which can increase the reaction rate of the saccharification step, resulting in a reduced length of fermentation [3]. Ethanol production from cellulosic materials has always been challenging due to the lack of hyperthermophilic cellulases; however, the combination of endoglucanases and cellobiohydrolases from *Thermotogales* can hydrolyze the embedded lignin in cellulose effectively[4]. In addition, a number of reagents in molecular biology have been obtained from hyperthermophilic organisms such as alkaline phosphatase from *Thermotoga neapolitana* for dephosphorylation, protease S from *P. furiosus* used in protein fragmentation for sequencing, and many of them have been characterized with potential biotechnological importance [5].

More than 100 thermophilic genes have already been cloned and successfully expressed in prokaryotes, e.g., *E. coli*, although archaeal transcription system has also been found similar to the eukaryotic system. Sometimes, low expression has been encountered in bacteria due to the difference in codon usage [6]. High level of expression needs robust promoters like phage T7 promoter. Despite many odds, scientists successfully expressed more than 90% of all thermophilic enzymes in *E. coli* that allowed synthesized enzymes with stability, catalytic, or structural properties similar to the original enzymes.

PCR that was originally invented by Kary Mullis [7] enables researchers to obtain billions of copies of DNA fragments accurately from a minuscule fragment of DNA in the presence of complementary primers specific to the template DNA. Nowadays, it is widely used for various experimental and analytical purposes such as disease detection, genetic analysis, recombinant DNA cloning, functional improvement of proteins through mutation, sequencing and so on. The extensive application of PCR is becoming more approachable due to the discovery of various thermostable polymerase enzymes. Earlier thermostable DNA polymerases did not possess proofreading activities. The DNA polymerase isolated from the hyperthermophilic archaeon, *Pyrococcus furiosus (Pfu)* that grows optimally at 100°C, possess a 3'-5' exonuclease activity [8]. The mostly studied and applicable thermostable enzymes are family A and B amongst seven classified families including A, B, C, D, X, Y and reverse transcriptase [9]. Family A-type DNA polymerases including *Taq*-Pol have higher amplification efficiency than family B-type DNA polymerases (*Pfu*-pol, *TNA1*, *KOD* etc.), while the latter exhibit proofreading activity and thereby higher fidelity [10]. In fact, *Pfu*-Pol exhibits the highest fidelity out of any characterized DNA polymerases to date, with an average error rate approximately 2- to 60-fold lower than other proofreading enzymes and 6- to 100-fold lower than *Taq*-Pol [11]. Since the family B-type DNA polymerases possess 3'-5' exonuclease activity, they demonstrate lower amplification efficiency than A-type polymerases. Amplification efficiency is a crucial parameter in successful routine PCR applications, as it determines the yield of PCR product. The efficiency of product yield depends on target length, GC contents, primer specificity, PCR enzymes, thermocycling conditions and so on [12]. In the past, researchers

used endpoint methods to characterize the amplification efficiency that quantify the intensity of brightness of amplified DNA fragments on an agarose gel to compare with various commercial DNA polymerases. A relevant study reported that using quantitative real-time PCR can compare the efficiency level of various commercial polymerases[12]. *Taq*-Pol and *Pfu*-Pol alone are found to be of no use in amplification of sequence with high fidelity longer than 1 kb; however, some commercially available modified enzymes like *PfuTurbo, PfuUltra* (mutant), Herculase all formulated with dUTPase (from Agilent Technologies, Inc.) could amplify fragments close to ~ 4 kb.

While amplifying a gene or DNA fragment for a study, there is a need for authentic DNA sequence as starting material to avoid any untoward complications during interpreting or analyzing data. Although *Pfu* polymerase possesses 3'-5' exonuclease activity in addition to DNA synthesis, it can still incorporate wrong base during long PCR[13]. The advantage of using *Pfu* polymerase in PCR is its possession of the exonuclease function that ordinarily allows the enzyme to recognize the wrong base if incorporated by mistake and excise the base from its 3' end [14]. This is why the fidelity of *Pfu* polymerase is higher than other polymerases, such as *Taq* polymerase, vent, deep vent, ULTIMA, etc. The PCR fidelity assay found that the error rate of *Pfu* polymerase is 5 and 35-fold lower than deep vent and ULTIMA polymerase, respectively [15]. Some experiments reported that the processivity of DNA polymerase, which is defined as the number of nucleotides that a polymerase can incorporate into DNA during a single template-binding event before dissociating from a DNA template, can be improved through mutational changes. An example of such mutant thermostable DNA polymerase is

Thermococcus onnurineus NA1 (TNA1) with N213D mutation, generated through systemic engineering of Asn210 to Asp215 residues in Exo-II motif [16]. It is noted that Exo-II is one of the three-conserved motifs of the exonuclease domain, responsible for proofreading ability in all family B-type polymerases. This study concluded that the mutant polymerase had been able to increase processivity by 3-fold with a slightly increased error rate than wild-type enzyme. The modified polymerase was very efficient to amplify the human mitochondrial gene up to 16.2 kb in comparison to 2.7 kb by wild-type enzyme. Similar strategy was successfully applied to improve the processivity and proofreading activities of DNA polymerase from *Thermococcus kodakaraensis* KOD1; a point to be noted that the wild type enzyme has strong 3'-5' exonuclease activity. When the exonuclease domain of KOD1 was mutated and mixed to a wild-type enzyme at a particular ratio it improved both proofreading and processivity [17].

Previous studies found that combination of *Pfu* & *Taq* polymerase can reduce the error rate with respect to use only *Taq* polymerase, while this combination has six times higher error rate than *Pfu* polymerase alone. The error rate of *Pfu* polymerase was recorded as 1.3×10^{-6} mutation frequency/bp/duplication compared to 1×10^{-3} by *Taq* polymerase [15]. The polymerization rate of *Pfu*-pol is 550 bp/min compared to 2800 bp/min by *Taq* polymerase, which is a challenging task for rate improvement, although adding extra two minutes per cycle in extension step can overcome this barrier [18]**.** Therefore, *Pfu* polymerase is still a good choice to amplify the gene with correct nucleotides than other polymerases to date [18].

Pfu Deoxyuridine Triphosphatase

Deoxyuridine triphosphatase (dUTPase) from *P. furiosus* is a homotrimeric enzyme encoded by 471 bp long *dut* gene that consists of a short polypeptide of 156 amino acids [19]. Organisms ranging from prokaryotes to eukaryotes, virus, and archaea synthesize dUTPase to catalyze the hydrolysis of dUTP to dUMP to prevent over-accumulation of the former and its concomitant toxicity [20]. It has five motifs, which are conserved in all organisms. Motifs I, II and IV have the highest similarity in the superfamily and can recognize the phosphate site of the dUTP molecule. On the other hand, motif III shows variable sequences compared to eubacterial and eukaryotic dUTPase, which function as an active site of dUTPase binding to the sugar moiety of the substrate (dUTP). While glycine-rich motif V is available in eubacterial and eukaryotic dUTPase, its absence in *Pyrococcus* may act as a factor to survive in extreme temperature [21-23].

There is a unique drawback observed in the PCR reaction that dCTP is hydrolyzed to dUTP at high temperature, which forms a structure similar to dTTP [24]. The chemical structure of uracil and thymine bases are very similar, the only difference is having a methyl group in thymine residue (Fig. 1) [25]. DNA polymerase, for example, *Taq*-Pol cannot recognize this structural difference between dUTP and dTTP; and thereby approaches adding dUTP instead of dTTP. Even, after a few attempts, *Pfu*-Pol cannot prevent the incorrect incorporation of dUTP in the growing DNA strand due to its overaccumulation in the reaction mixture, which leads to instability in PCR product [18].

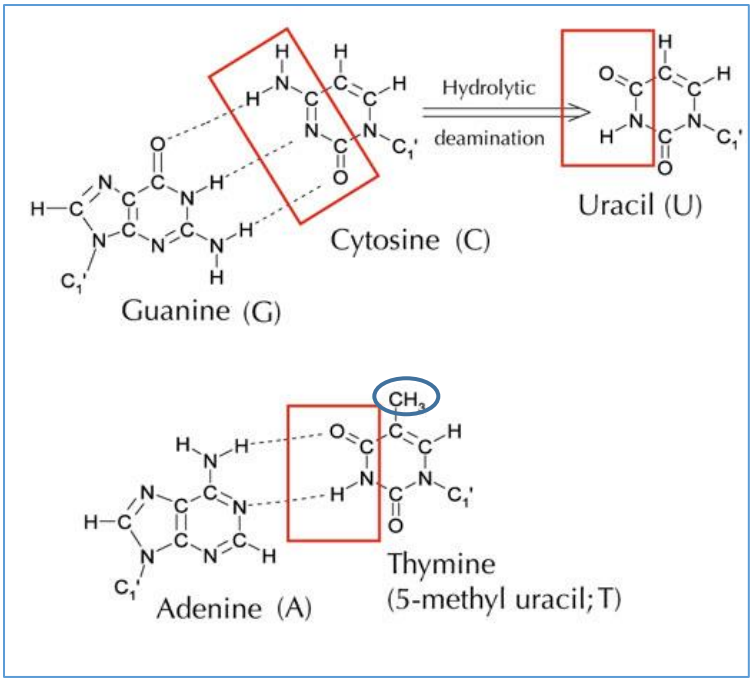

Figure 1: Deamination of Cytosine in Longer Thermocycling.

In *in vivo* condition, before entering into transcription stage, organisms frequently excise the double stranded DNA to eliminate the incorporated dUTP by uracil DNA

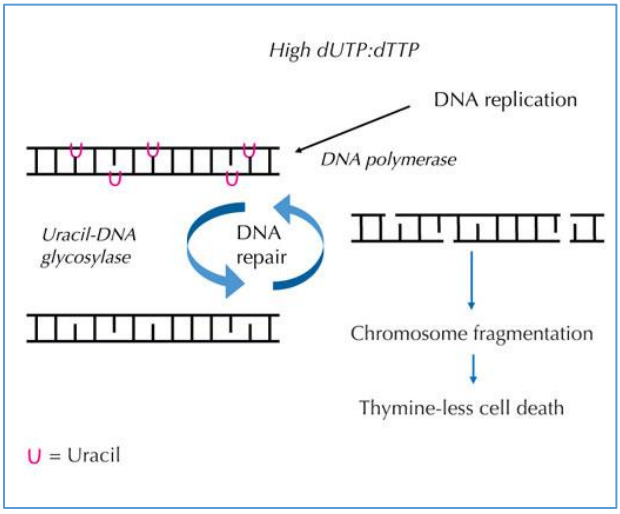

Figure 2: *In vivo* Deamination by dCTP Deaminase. Uracil-DNA glycosylase repairs the mechanism by eliminating uracil nucleotide.

glycosylase. After several excisions, the repairing mechanism cannot continue the process due to overaccumulation of dUTP, resulting in thymine-less cell death (Fig. 2) [26, 27].

Prospects of Recombinant DNA Polymerase

The replicative DNA polymerases incorporate thousands of nucleotides per second into newly synthesized strand without dissociating from primer-template DNA. In contrast, non-replicative polymerases resolute the gaps created during replication, recombination and in DNA repairings. The natural replicative polymerases are likely to be more efficient in *in vivo* condition than *in vitro*. Therefore, replicative polymerases from possible sources could be engineered for *in vitro* use having huge biotechnological importance. The goal of many studies was to identify the novel replication factors directly associated with complex replication mechanism that would contribute the improvement of polymerization. To improve the polymerase activity with high efficiency, researches were not only bound to discover new polymerases but also develop the existing polymerases through recombinant DNA technology. Previous studies reported that typical small peptide molecules adjacent to polymerase would not interfere the polymerization efficiency, rather increase the processivity of slower enzymes [28]. A chimeric polymerase enzyme can be designed through the appropriate in-frame ligation of supplementary sequences and therefore require profound knowledge of structure and function of each domain. While there is a considerable sequence diversity amongst various DNA polymerases, still exhibit similarity in three-dimensional structure. In general, polymerases look vaguely like a right hand, with fingers, palm and thumb subdomains. The catalytic domain is located in the palm, fingers join the incoming nucleotides into growing strand and thumb hold the newly synthesized

DNA [29]. A study reported that deletion of eight amino acids from thumb domain in the Klenow fragment reduces the binding affinity more than 100 folds and greatly affects the processivity [30]. On the other hand, fused fragment supports the polymerase affinity to template DNA through non-specific binding.

A study reported the advantage of incorporating thioredoxin binding domain (TBD) sequences from phage T3 DNA polymerase into the thumb position of both wild type and 5'-3' exonuclease-deficient *Taq* Polymerases [28]. Both the resulting recombinant proteins showed ~15% distinguishable activity compared to wild type *Taq* polymerase. Even in the presence of thioredoxin, the performance of Exo$^-$ *Taq*-Pol/TBD in the PCR increased >30 folds compared to the wild type enzyme.

Processivity is a major hindrance for many thermostable polymerase enzymes. However, many polymerases possess the sliding clamp, for example in eukaryotes, which tether the polymerase to DNA, and therefore prevent the dissociation from template DNA. To increase the processivity and fidelity, a novel study revealed the significance of replication factors required for polymerase enzyme. Through yeast-two-hybrid assay, thirty different proteins were identified as interacting replication factor that contributed to the polymerase processivity. An important example is the proliferating cell nuclear antigen (PCNA) found in *Archaeoglobus fulgidus*, which tethers other proteins to DNA [31]. After careful sequence analysis, they constructed a chimeric enzyme by fusing PCNA sequence into *Taq* DNA polymerase. This strategy enhanced the processivity significantly and allowed longer amplification up to 5 kb as compared to 1.5 - 2 kb by *Taq* polymerase alone [31]. A similar study reported that fusing a different combination of helix-hairpin-helix

(HhH) motifs into either amino- or carboxyl-terminus of five different DNA polymerases including *Taq* and *Pfu* can improve the polymerase function[32].

The deletion of ExoII domain from *Taq* polymerase elucidated the lacking of processivity, suggesting the role of this domain in binding the DNA double helix. A study reported that the replacement of exonuclease factor by a prominent processivity factor 'Sso7d' from archaebacteria *Sulfobolus solfataricus (Sso)* can improve the processivity of *Taq* and *Pfu* polymerases, without affecting the polymerase activity and stability [33]. Even the mutation in Sso7d enhances the performance of chimeric protein. The engineered *Taq* polymerase with Ssco7d that lacks ExoII and full-length *Taq* with same domain exhibited similar PCR activity for the amplification product up to 5 kb of less extension time compared to regular *Taq* polymerase. It was noted that the *Taq* polymerase missing ExoII was unable to amplify the target. Interestingly, *Pfu*-Pol with the chimeric domain in C-terminus reduced the enzyme's low processivity and enhanced the product length up to 15 kb [33].

In the present study, we are reporting an engineering strategy to obtain a fusion enzyme of a DNA polymerase and a dUTPase from *P. furiosus*. The hypothesis behind fusing these enzymes that retain functionality is based on the relative smaller size of the dUTPase that is 18 kDa only. We think fusing this smaller enzyme on either side of a relatively very large *Pfu* polymerase would not interfere with the polymerization function. It is also predictable that this dUTPase enzyme would also remain active if the joining

between the two enzymes contains some amino acids like glycine, which can provide more conformational flexibility.

CHAPTER III: METHODOLOGY

3.1. MATERIALS

3.1.1. Plasmids Source

The bacterial expression plasmid, pCAL-Kc, was purchased from STRATAGENE® Inc. For expression purposes in yeast, the pYES2 plasmid DNA was a kind gift from Dr. Ariza, Biotechnology Program at University of Houston-Clear-Lake.

3.1.2. Restriction Enzymes, PCR Supplies and Other Reagents

All restriction enzymes, reaction buffers, and Q5® high fidelity DNA polymerase were purchased from New England Biolabs® Inc. The 2X GoTaq® Green Master Mix was purchased from Promega Inc. PPiLight™ inorganic pyrophosphate assay kit was obtained from Lonza, Inc.

3.1.3. Culture Media Preparation

A. SOC media

In 90 mL of dH_2O, 2 g tryptone, 0.5 g of Difco yeast extract and 0.05 g of NaCl were mixed along with 1 mL of 250 mM KCl. The final volume was made up to 100 mL and then autoclaved at 15 psi for 20 minutes at 120°C. At the falling temperature, 0.5 mL of 2 M $MgCl_2$ and 2 mL of glucose (1.8 g/10 mL) were added to the media.

B. LB media and agar with preferred antibiotics

The LB broth was prepared by the addition of 10 g tryptone, 5 g yeast extract, and 5 g NaCl to 900 mL dH_2O. For LB agar plates, 18 g of bacto-agar (Enova, Inc.) was added extra to the above recipe. The final volume was made up to 1 L by adding dH_2O and then autoclaved for 20 minutes at 15 psi and 120°C. Once the temperature of the flask was between 55 and 60 ^{0}C, the antibiotics Ampicillin or Kanamycin were added according to the desired final concentration. Immediately, about 25 to 30 mL of agar media was poured on to petri dishes (100 mm × 15 mm) and let solidified in the laminar flow hood.

C. Terrific Broth (TB)

About 50.8 g of ready to use terrific broth (Fisher Scientific, Inc.) was dissolved in 900 mL dH_2O and then 4 mL of glycerol was added to the solution. The final volume was made up to 1 L with additional dH_2O and then autoclaved for 20 minutes at 15 psi and 120°C.

D. YPDA media

To prepare 1 L of YPDA broth media, 20 g of Difco peptone and 10 g of yeast extract were added to 900 mL dH_2O. The adenine hemi-sulfate was added to a final conc. of 0.003% and then the final volume was made up to 1 L. The media was autoclaved for 20 minutes at 15 psi and 120°C. Then it was allowed to cool to ~ 55˚C and then we added dextrose to a final conc. of 2%. For the YPDA plates, bacto-agar (20 g/L) was added to the above recipe before autoclave.

E. Selective media

We prepared Synthetic Dropout (SD) media where only uracil was omitted for auxotrophic selection. To prepare 1 L of SD-Ura broth, 26.7 g of minimal SD base (Clontech, Inc.) and 0.77 g of uracil dropout supplement were added to 900 mL milli-Q H_2O. To prepare SD-Ura plates, we added 20 grams of bacto-agar to 26.7 g of SD base. After adding all components, the final volume was made up to 1 L and then autoclaved at 15 psi for 20 minutes at 120°C.

3.1.4. In-Fusion Cloning Primers List

We designed In-Fusion primers following Clontech's online In-Fusion primer design tool and then synthesized from IDT Inc. The list of primer sequences containing restriction sites are shown below.

Table 1. Primers Sequences (restriction sites in italic bold) and Corresponding Restriction Enzymes

Primer	Sequence	Restriction Enzyme
UIFP	5'-AGGAGATATA***CCATGG***CTAGCATGCT-3'	NcoI
DIRP	5'-TTCCATCGTCGCTT***GGTACC***GAGTTT-3'	KpnI
IF-Pol-Hin-FWD	5'-AGGGAATATT***AAGCTT***ATGATTTTAGATGTGGATT AC-3'	HindIII
IF-Pol-Xba-REV	5'-GATGCGGCCC***TCTAGA***C***TCTAGA***CTAGTGGTGGTG GTGGTGGTGGGATTTTTTAATGTTAAGC-3'	XbaI
IF-DUT-Hin-FWD	5'-AGGGAATATT***AAGCTT***ATGCTACTTCCAGAC-3'	HindIII
DUT-Rev-XbaI	5'-GATGCGGCCC***TCTAGA***C***TCTAGA***CTAGTGGTGGTG GTGGTGGTGGAGTTTCTTTC-3'	XbaI

3.1.5. Bioneer Buffer (10 X)

The buffer for *Pfu* polymerase-based PCR reaction was prepared by the following protocol, originally designed by Bioneer, Inc.

Table 2. Buffer Reagents for *Pfu* DNA polymerase

Components	Stock Conc.	Final Conc.	Volume Added
Tris-HCl, pH 9.0	1 M	20 mM	20 µL
KCL	3 M	50 mM	17 µL
$MgCl_2$	1 M	2.5 mM	2.5 µL
BSA	10 mg/mL	10 µg/mL	1 µL
ddH_2O	X	X	334.5 µL
Glycerol	80 %	50 %	625 µL

3.1.6. Buffers for Calmodulin Affinity Chromatography (STRATAGENE Affinity® Manual)

A. $CaCl_2$ binding buffer

Table 3. Preparation of $CaCl_2$ Binding Buffer from Stock Solutions

Name of Components	Stock Conc.	Final Conc.	Volume Added
Tris-HCL (pH 8.0)	1 M	50 mM	5 mL
NaCl	4.5 M	150 mM	3.3 mL
β-mercaptoethanol	14.3 M	10 mM	70 µL
Magnesium Acetate	1 M	1.0 mM	100 µL
Imidazole	1 M	1.0 mM	100 µL
$CaCl_2$	1M	2 mM	200 µL
ddH_2O	x	x	91.23 mL

B. Lysis buffer for bacterial crude lysate preparation

Table 4. Cell Lysis Buffer

Components	Stock Conc.	Final Conc.	Volume Added
$CaCl_2$ binding buffer	x	x	24.70 mL
β-mercaptoethanol	14.3 M	10 mM	17.5 µL
PMSF	100 mM	1 mM	250 µL
Imidazole	1 M	1 mM	25 µL
Lysozyme	10 mg/mL	200 µg/mL	80 µL

C. Column Wash Buffer

Table 5. Column Wash Buffer

Components	Stock Conc.	Final Conc.	Volume Added
$CaCl_2$ binding buffer	x	x	49.916 mL
β-mercaptoethanol	14.3 M	10 mM	34 µL
Imidazole	1 M	1 mM	50 µL

D. Protein Elution Buffer (150 mM)

Table 6. NaCl (150 mM) Elution Buffer

Components	Stock Conc.	Final Conc.	Volume Added
Tris-HCl	1 M	50 mM	500 µL
β-mercaptoethanol	14.3 M	10 mM	7 µL
EGTA	1 M	2 mM	20 µL
NaCl	4.5 M	150 mM	333 µL
Milli-Q H_2O	X	X	9.14 mL

3.1.7. Enzyme Storage Buffer

Table 7. Enzyme Storage Buffer

Components	Stock Conc.	Final Conc.	Volume Added
Tris-HCl	1 M	20 mM	100 µL
DDT	100 mM	1 mM	50 µL
EDTA	500 mM	0.1 mM	1 µL
KCl	3 M	100 mM	167 µL
Nonidet-P40	100 %	0.1 %	5 µL
Tween-20	100%	0.1 %	5 µL
Glycerol	80 %	50 %	3.125 mL
Milli-Q H_2O	X	X	1.55 mL

3.1.8. Recipes for SDS-PAGE Gel Formulation and Running Buffer

A. Resolving gel (10%) for 12 mL

ddH_2O -	4.9 mL
30% Acrylamide	3.96 mL
Gel Buffer (1.5M; pH-8.8)	3.0 mL
10% SDS	0.12 mL
TEMED	6 µL
10% APS	60 µL

B. Stacking gel (4%) for 10 mL

ddH_2O -	6.1 mL
30% Acrylamide-	1.3 mL
Gel Buffer (0.5M; pH-6.8)	2.5 mL
10% SDS	0.1 mL
TEMED	50 µL
10% APS	10 µL

C. 4 X SDS-PAGE sample loading buffer

62.5 mM Tris-HCl, pH 6.8

2% SDS

25 % Glycerol

0.01 % Bromophenol Blue

5% β-mercaptoethanol

D. 10 X SDS-PAGE Gel Running buffer (1L)

3.03 g Tris base

24 g Glycine

1.0 g SDS

1000 mL ddH_2O

3.1.9. Western Blotting Buffers

All buffers were stored at 4˚C.

A. Transfer buffer (1 L)

Table 8. Transfer Buffer

Components	Volume
10 X Tris-Glycine buffer (Bio-Rad)	100 mL
Methanol	200 mL
Milli-Q H_2O	700 mL

B. Blocking buffer (100 mL)

Table 9. Blocking Buffer

Components	Volume
1x PBS buffer	100 mL
Non-fat dry milk	5 g

C. Washing buffer (1 L)

Table 10. Washing Buffer

Components	Volume
1x PBS buffer	1000 mL
Tween 20	0.5 mL

D. PBS buffer (1 L)

Table 11. PBS Buffer (pH 7.4)

Components	Quantity
NaCl	8.5 g
Na_2HPO_4	1.4 g
NaH_2PO_4	0.2 g
Milli-Q H_2O	1000 mL

3.2. METHODS

3.2.1. Primers Design for Amplification of *Pfu dUTPase*

The dUTPase (DUT) encoding gene '*dut*' was amplified by PCR using *Pfu* genomic DNA as a template, obtained from ATCC® (American Type Culture Collection). The primers including UIFP and DIRP, were designed in accordance with In-Fusion® HD Cloning Kit User Manual. We included the restriction sites of *Nco*I and *Kpn*I in forward and reverse primers, respectively, so that the corresponding sites in the vector plasmid pCAL-kc could be exploited for insertion. Our lab had already cloned the open reading frame (ORF) of *Pfu* DNA polymerase in pCAL-kc with *Nco*I and *Kpn*I restriction sites in the upstream and downstream. In addition, fusion constructs with dUTPase were generated. Two different orientations based upon the cloning positions of DUT with respect to *Pfu* polymerase are shown here (Fig.3).

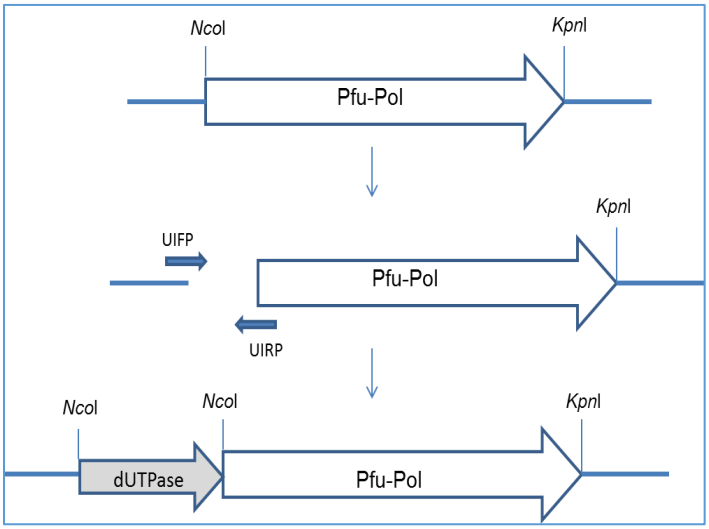

Figure 3: Strategy of Fusion Gene Construction in pCAL-kc and Positions of the Primers for *dut* Insertion.

The *dUTPase* at *Nco*I site, i.e., upstream of *Pfu* DNA polymerase ORF, was named as Pol-N. Similarly, when inserted at the *Kpn*I site, i.e., downstream of pol gene, we named it as Pol-K (Fig. 4). There is an online In-Fusion primer design tool at the TaKaRa Clontech's website. We designed our primers using the tool that converted the gene specific primers into In-Fusion primers. It is to be noted that In-Fusion primers maintain at least 15 bp homology from the 5'-ends that is complementary to the vector, while the 3'-ends of the primers remain gene specificity (Fig. 5). Overall, primers were designed carefully so that typical features are maintained that includes GC contents, primer annealing, and melting temperature. We also checked for any possible hairpin structure formation in the primer sequence.

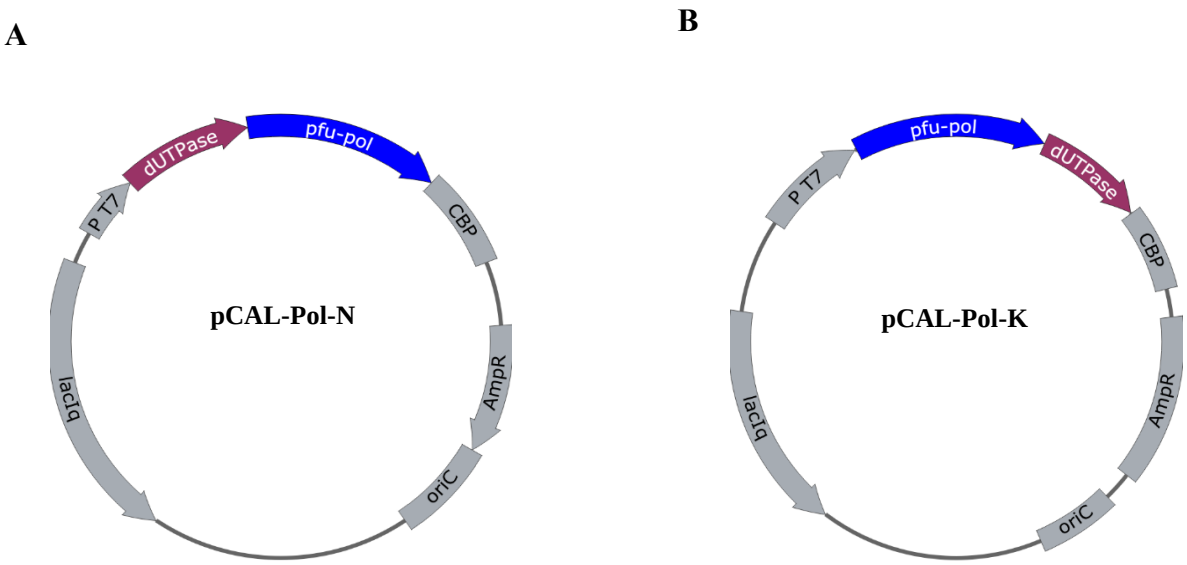

Figure 4: Vector Map of Two Different Fusion Constructs. A) Pol-N: dUTPase located in N-terminal of *Pfu*-Pol, B) Pol-K: dUTPase located in C-terminal of *Pfu*-Pol

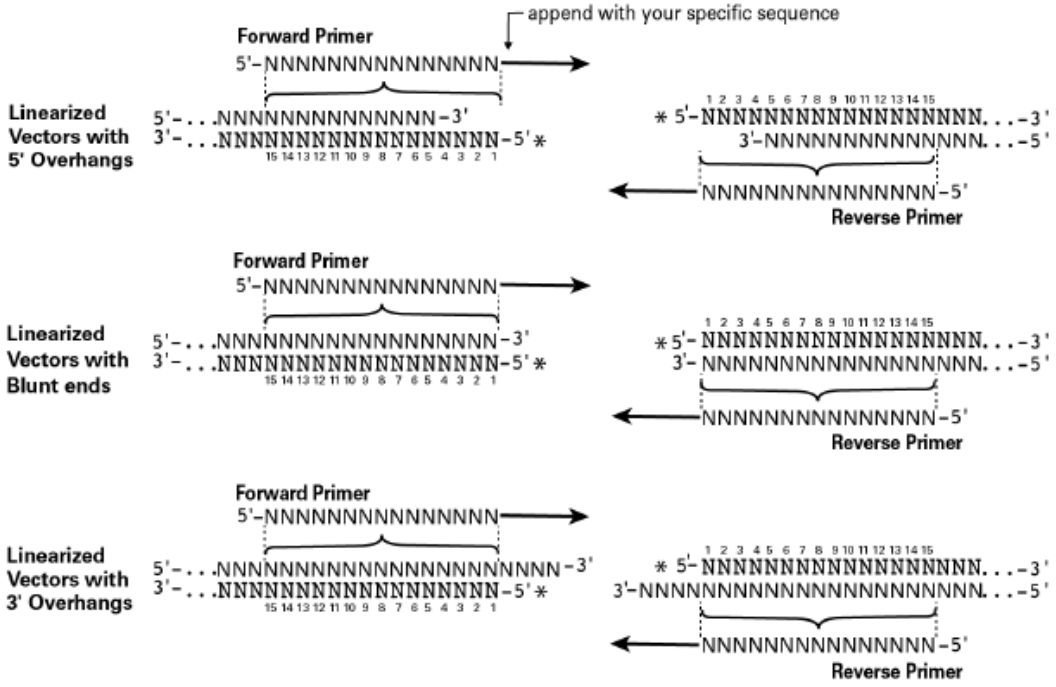

Figure 5: Guidelines for Universal Primer Design.

3.2.2 PCR Amplification of dUTPase

All PCR reaction components including nuclease-free water and a ready-to-use 2X GoTaq® Green Master Mix (Promega, Inc.), were assembled in a tube placed onto ice. GoTaq® Green Master Mix has bacterially derived *Taq* polymerase, dNTPs, $MgCl_2$ in a reaction buffer with optimized pH at 8.5. The advantage of using Green Master Mix is that it contains two dyes (blue and yellow) for monitoring the progress of DNA bands during electrophoresis. Pre-made PCR master mix was aliquoted into 0.2 mL PCR tubes, and then gene specific primers and template DNA were added into the corresponding tubes (Table 12). Bio-Rad Thermocycler T100 was employed for the PCR. The thermocycling condition was set up as shown in Table 13.

Table 12. PCR Set Up Using Promega GoTaq® Green Master Mix (GoTaq® DNA polymerase, 400 µM dNTPs, 3 mM $MgCl_2$, and 2X Green GoTaq® reaction buffer, pH 8.5)

Order	Component	Volume (µL)	Final Conc.
1	Nuclease free water	13	X
2	Master mix, 2X	15	1X
3	UIFP (10µM)	0.5	0.17µM
4	DIRP(10µM)	0.5	0.17µM
5	*Pfu* genomic DNA (10 ng/µL)	1	30 pg
	Total volume	30	

Table 13. Cycling Conditions of dUTPase Amplification

Cycle	Steps	dUTPase	
		Temp (˚C)	Time
1	Initial Denaturation	94	3 min
30	Denaturation	94	45 secs
	Annealing	58	30 secs
	Extension	72	30 secs
1	Hold	4	∞

3.2.3. Amplification of *Pfu*-pol, dUTPase, Pol-N, and Pol-K for Cloning into Yeast Vector pYES2

The target genes (*Pfu*-Pol & dUTPase) and their fusions (Pol-K & Pol-N) were amplified for insertion into yeast expression plasmid pYES2, which is also called a ‘shuttle vector’ as it can also propagate through bacteria. In this case, the recombinant plasmids (pCAL-pol, pCAL-DUT, pCAL-Pol-K & pCAL-Pol-N) previously isolated from *E. coli*

were used as a template for subsequent PCR amplification. The primers were designed similarly as discussed above while changing the restriction sites to *Hin*dIII in forward primer and X*ba*I in reverse primer with 15 bp overhangs that are complementary to the double digested pYES2 vector ends with *Hin*dIII and *Xba*I.

3.2.4. Restriction Digestion of pCAL-Kc and pYES2

Since the primers were designed for In-Fusion cloning purposes, the PCR products remain 15 bp homology, which requires complementary sticky ends for complete ligation. This was achieved by double digestion of pCAL-Kc and pYES2 using *Nco*I & *Kpn*I and *Hin*dIII & *Xba*I that cut at the unique restriction sites, respectively. The double digestion

Table 14. Restriction Digestion of pCAL-Kc and pYES2

Vectors	DNA vol.(µL)	10X reaction buffer (NEB) (µL)	10X BSA (µL)	NcoI+KpnI (µL)	HindIII+XbaI (µL)	ddH_2O (µL)
pCAL-Kc (105 ng/µL)	10	3	3	1 µL of each	-	12
pYES2 (69 ng/ µL)	15	3	3	-	1 µL of each	7

at the *Nco*I and *Kpn*I sites of pCAL-Kc removed the kemptide and thrombin sequences as both located between the digestion sites, whereas the inserts were incorporated into the same reading frame as the Calmodulin Binding Peptide (CBP). Similarly, pYES2 also generated 5´-overhangs after digestion with *Hin*dIII and *Xba*I that complementary to the 5´-ends of primers. Reaction mixtures followed the recommended protocols, which includes 1 µg of DNA and 1 µL of each restriction enzyme in a final volume of 30 µL

(Table 14). Incubation was done at 37˚C for one hour. The digested products were analyzed by 1% agarose gel and the corresponding DNA bands were cut out with a clean blade for use with the PCR-clean-up kit.

3.2.5. Agarose Gel Electrophoresis

To prepare a 1% agarose gel, 0.5 g of agarose powder was added to 50 mL of 1X TAE (Tris, Actetic acid, EDTA) buffer in a 250 mL Erlenmeyer flask. The mixture was boiled in microwave oven until the agarose completely dissolved (usually takes about 90 sec). Then, once the temperature cools down to around 60^0C, 2.5 µL of ethidium bromide (10 mg/mL stock) was carefully added to the solution to a final conc. of 0.5 µg/mL. After swirl mixing, the gel solution was poured into the casting tray with a 10-well comb. Once the gel gets solidified that usually takes around 25 minutes the comb was removed and adequate amount of 1X TAE buffer was poured into the gel chamber so that it covers the top of the gel not more than 5 mm.

Before running the gel, about 10 µL of 1 kb reference ladder (Thermo Fisher GeneRuler®) was loaded into the well as the mass reference and amplicons size, followed by experimental samples containing loading dye. The gel was run at 120 V for ~40 minutes until the bromophenol blue dye reaches $3/4^{th}$ of the gel. After that, the gel was visualized under UV using Fotodyne® transilluminator.

3.2.6. DNA Extraction from Gel

GenCatch™ Advanced Gel Extraction kit (Epoch Life Science) was used to retrieve the PCR product or linearized plasmid DNA from the gel with maximum purity. First, a gel slice is obtained based on the UV image that corresponds to our interested DNA size. The weight of the gel slice in an Eppendorf/microcentrifuge tube was determined by subtracting the initial weight of tube. Next, 3 volumes of GEX buffer were added to 1 volume of gel slice. The mixture tube was incubated in water bath at 55˚C for 5 to 10 minutes and inverted every 1 to 2 minutes until completely dissolved. A *GenCatch*™ extraction column was placed onto a collection tube and the gel mixture was transferred into the column, this was followed by centrifugation at 5000 rpm for 30 secs. The flow-through was discarded. After that, 0.5 mL of WN wash buffer was added into the column and centrifuged under the same condition. The flow through was discarded, and the same volume of WS buffer was loaded while the centrifugation was run for 60 secs at the same speed. The column was centrifuged again for 3 minutes at 12,000 rpm to remove any ethanol residue that may affect the purity of DNA. The sample column was transferred into a new 1.5 mL microcentrifuge tube for eluting the sample with 15-30 µL of elution buffer loaded at the center of the column and then allowed two minutes incubation at room temperature. The following centrifugation was done at 12,000 rpm for 60 secs and the eluted DNA was stored at -20°C.

3.2.7. DNA Quantification by Spectrophotometer

The concentration and purity of gel purified DNA and the digested plasmid were measured using NanoDrop 2000 spectrophotometer (Thermo Scientific). As a blank, 1 µL of nuclease-free water or elution buffer was used. The machine measures the DNA concentration at wavelengths of 260 and 280 and provides 260/280 ratio to indicate the purity of the samples.

3.2.8. In-Fusion Ligation

In-Fusion HD Eco-dry cloning kits containing In-Fusion enzymes is the cornerstone of In-Fusion cloning technology (Clontech In-Fusion®HD EcoDry User manual PT51565-1). In this ligation technique, the enzyme allows the ligation of PCR generated target DNA sequences into the linearized vector by recognizing 15 bp overlap at their ends (**Fig. 6**).

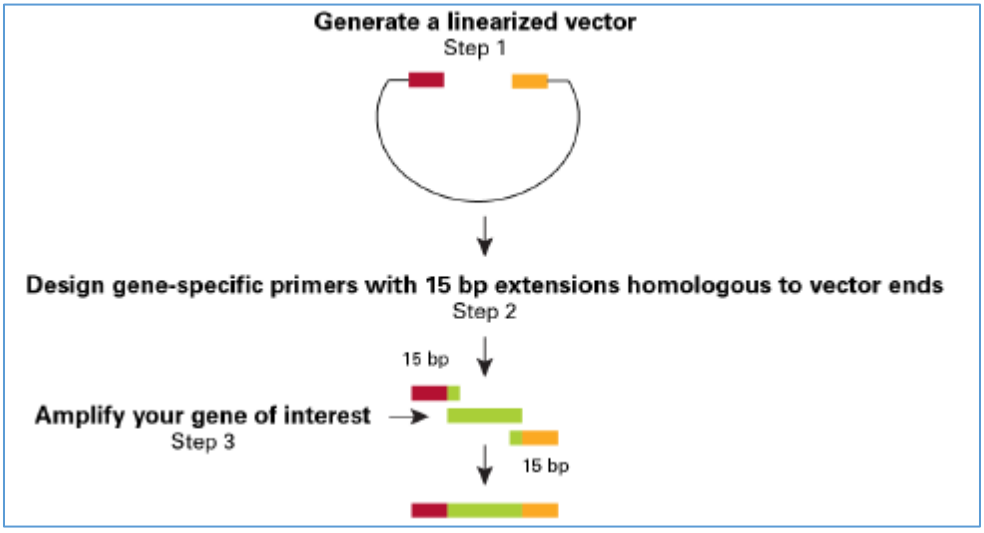

Figure 6: Overview of the In-Fusion Protocol.

This 15 bp overhangs in both ends were engineered by the designed primers as discussed previously. The ligation reaction mixture was placed in a small tube provided by manufacturer containing reaction materials in a lyophilized form, in which, 8 µL of vector

and 2 µL of PCR product were added based on the recommended concentrations for In-Fusion ligation. One reaction tube was set up as a negative control, in which the PCR product was replaced with sterile water. All of the reaction tubes were placed in a thermocycler for 15 minutes incubation at 37°C, followed by 15 minutes at 50°C.

3.2.9. Transformation into Bacteria

Plasmid transformation into Steller or DH5α cells was done by chemical transformation method in which the cells were treated with divalent cations such as $CaCl_2$ for enhancing competency. A brief heat shock was applied to create temporary pores that allow exogenous DNA to pass through cell membranes. Prior to transformation, competent cells were taken out from -80°C and thawed on ice. The prelabeled 1.5 mL of microcentrifuge tubes were chilled on ice containing no more than 100 ng of DNA in 5 µL for a 100 µL reaction mixture. The reaction tubes were incubated in ice for 30 minutes, followed by heat incubation for 60 seconds in a 42°C water bath. Cells were immediately transferred onto the ice for 2 minutes. About 400 µL of SOC medium at room temperature was added to the cells and incubated with continuous shaking for 1 hour at 37°C, which allows cell recovery and generating antibiotic resistance protein encoded in the plasmid backbone. The tubes were centrifuged at 3,000 rpm for 1 minute to collect the cells at the bottom, and ~400 µL of supernatant was discarded from the upper portion. The cell pellet was resuspended well and spread onto LB agar plates containing the appropriate antibiotic using sterile glass beads. The plates were incubated at 37°C overnight.

3.2.10. Transformation into Yeast

In LiAc mediated yeast transformation, a single colony (YNN281) with 2-3 mm in diameter was inoculated into 1 mL of YPDA and vortexed vigorously for 5 minutes to remove any clumps. Then, it was transferred into a flask containing 25 mL of YPDA and incubated at 30°C for 16-18 hours under shaking at 250 rpm. On the next day, the OD_{600} was checked before inoculating 6 mL of overnight culture to a flask containing 60 mL of YPDA. The diluted culture was checked again and more overnight culture was added if necessary to reach the OD_{600} of 0.2-0.3. Again, the cultures were incubated under the same conditions till the OD_{600} reach 0.5-0.6. The cultures were then divided into two 50 mL falcon tubes and harvested at 1000 × g for 5 min at room temperature. The supernatant was discarded and the pellets were resuspended in 20 mL of sterile water. The cells were pooled into one tube and centrifuged at 1000 × g for 5 minutes. The supernatant was removed and the cell pellets were resuspended in 1.5 mL of freshly prepared sterile 1X TE/1X LiAc. Prior to adding competent yeast cells, 0.1 µg of plasmid DNA and 100 µg of carrier DNA were mixed in 1.5 mL microcentrifuge tubes. Then, 100 µL of LiAc treated competent cells were added into each tube and mixed by spontaneous vortexing. In the following step, 600 µL of sterile PEG/LiAc solution was pipetted to facilitate the transformation during incubation at 30°C for 30 minutes under shaking at 200 rpm. After incubation, 70 µL of DMSO was added into each tube and mixed 5-6 times by gentle inversion without vortexing. The preferred method of heat shock was incubating in a water bath at 42°C for 15 minutes, followed by transferring onto ice for 2 minutes. A brief spin down at 14,000 rpm was applied to discard the supernatant and the pellets were resuspended in 500 µL of

sterile 1X TE. From the resuspended mixture, 100 µL of each sample was plated on the pre-warmed SD-Ura plate that would allow the growth of only transformants. All of the plates were incubated at 30°C till the colonies appeared.

3.2.11. Colony PCR of Bacterial and Yeast Transformants

A successful bacterial transformation was verified by performing Colony PCR using individual colonies as template DNA (4-8 colonies were used individually). A PCR master mix was prepared first containing 2X GoTaq® Green Master Mix, and corresponding primers. Then, 15 µL each of the master mix was distributed into each 0.2 mL PCR tube. An individual colony was taken with a toothpick, streaked onto a selective plate (e.g. LB-Amp) and the same toothpick was mixed into the PCR reaction mixture. The streaked plate was placed at 37°C for overnight incubation.

However, the colony PCR for the transformed yeast cells requires extra steps in order to lyse the cell wall. The colonies were treated with 40 µL of TE buffer containing 2% Triton-X-100 and were vigorously shaken for 1-2 minutes after a pinch of glass beads were added. Then, the tubes were centrifuged for 2 minutes at high speed. The supernatant was transferred into a new tube from which 2 µL of supernatant was added to each 15 µL reaction mixture as discussed above.

3.2.12. Plasmid DNA Isolation and Sequencing

A transformed colony from each selective plate was inoculated in 5 mL LB media containing 100 µg/mL of ampicillin and incubated for overnight shaking at 37°C. On the following day, cells were harvested by centrifuging at 5000 rpm for 2-3 minutes in a bench top centrifuge. For small-scale plasmid isolation, we employed GenElute kit from Sigma-

Aldrich, Inc. The rescued plasmids were eluted in nuclease-free water. For DNA sequencing, we sent our samples to the Lone Star (LS) Labs, Inc. (http//: www.lslabs.com) located at the Texas Medical Center, Houston.

3.2.13. Protein Expression and Preparation of Cell Lysate

Recombinant plasmids were transformed into BL21-Gold (DE3) strain for high-level protein expression and non-proteolytic cleavage as the expected end result. Transformation steps were followed as discussed above. To begin a starter culture for recombinant protein expression, a single colony of each type of clone including *Pfu*-Pol, dUTPase, Pol-K & Pol-N was inoculated into 5 mL LB broth containing 5 µL of ampicillin and allowed to grow overnight at 37°C. After nearly 14 hours incubation, the culture was transferred into 250 mL of TB broth containing 125 µL of ampicillin (50 µg/mL) and allowed to grow additional 2-3 hours to reach an OD_{600} ~ 0.5. At the desired OD_{600}, 312.5 µL of isopropyl β-D-1-thiogalactopyranoside (IPTG) was added to reach a final conc. of 1 mM, which induced the protein expression level at its maximum. The culture flasks were incubated again for 5-6 hours at room temperature with gentle shaking. The cells were harvested at 2700 × g for 10 minutes at 4°C and the media was then drained off. The cell pellet was either used for crude lysate preparation or stored at -80°C.

3.2.14. Affinity Column Chromatography

Bacterial cell pellets (from 100 mL culture) were resuspended in 3 mL $CaCl_2$ binding buffer, then 60 µL of lysozyme (10 mg/mL) was added to the resuspended solutions and the mixture was rotated for 15 minutes at room temperature. The sonication was performed

to disrupt the cells at low speed for 4 × 30 secs each followed by one-minute interval of cooling on ice. After that, 3µL of each NP-40 and Tween-20 was added to reach a final conc. of 0.1% that facilitated the cell wall disruption process more effectively, and also the non-target proteins were deactivated selectively through heat incubation at 75°C for one hour. The samples were chilled on ice for 2 minutes and centrifuged at 15,000 × g for 15-20 minutes at 4°C.

The resulting supernatant known as crude lysate was transferred into 15 mL tubes containing 500 µL of equilibrated Calmodulin resin and then placed for overnight rotation at 4°C, which allowed affinity interaction between target proteins and Calmodulin resin.

Each overnight incubated lysate slurry was loaded into Bio-Rad Poly-Prep® chromatography columns that generated resin-column bed. The first collection of flow through was passed again through the column for maximum resin binding. The wash buffer ($CaCl_2$ binding buffer with imidazole and β-mercaptoethanol) to wash away non-bound molecules was used at a recommended volume of 10 times of column bed volume. The samples were eluted with 600 µL elution buffer (250 mM NaCl and β-mercaptoethanol) in two fractions 300 µL each.

3.2.15. SDS-PAGE

The molecular weights (MW) of interested proteins were estimated by SDS-PAGE analysis. Two hand casted polyacrylamide gels were prepared using discontinuous buffer system including a large-pore stacking gel (4%) on top of a small-pore resolving gel (10%) for better resolution. TEMED and APS that trigger the polymerization of monomeric

acrylamide was added to the solution just before pouring polyacrylamide monomer solution in the casting cassette. The resolving gel mix was poured up to the mark that left ~ 1 cm space below the top of the inner plate. A layer of butanol was added on top of the resolving gel to prevent air exposure that interferes with polymerization. After ~30 minutes when the gel was solidified, butanol was removed by slanting the gel and washing with water followed by soaking with a tissue paper. Then the stacking gel mix was poured on the top of the resolving gel and 10-teeth comb was placed in the cassette. After another 30 minutes to allow for solidification, the comb was pulled straight up slowly and gently. The gel clamps were placed in the electrophoresis apparatus, and both buffer chambers were filled with 1X SDS running buffer, according to the instructions by the manufacturer.

In sample loading, 20 µL of each flow through, wash, elution-1 and elution-2 was mixed in 4X SDS sample loading buffer, and then boiled at 95°C for 5 minutes just before loading into the wells. About 10 µL of Precision Plus Protein™ All Blue Standards (Bio-Rad) was loaded as molecular weight marker. The electric current was applied at constant voltage (~ 200V), until the front dye reached the very bottom of the gel. After a complete run, the gel was carefully separated from the cassette and the gel was transferred in Milli-Q H_2O. The gel was washed 3 times for 5 minutes each with gentle shaking.

3.2.16 Gel Code Blue Staining

Gel code blue staining was performed according to manufacturer's protocol. In short, the gel after SDS-PAGE run was rinsed in 200 mL ddH_2O for three times to remove SDS from the gel as it interferes staining. Then the gel was placed in a tray with sufficient

amount of GelCode™ Blue Safe Protein Stain (Thermo Scientific, Inc.) and gently shaken for an hour. The gel then rinsed again with ample water three times until the protein bands brightens up.

3.2.17. Western Blotting

Western blot was performed to specify the proteins onto nitrocellulose membrane through semi blot technique. In this method, the SDS-PAGE gel was equilibrated first into transfer buffer three times for 5 minutes each with gentle shaking. The transferred gel and the nitrocellulose membrane were sandwiched horizontally between the pre-soaked blotting pads in the electrophoretic transfer apparatus (Trans-Blot® SD Semi-Dry Cell, Bio-Rad). The cathode plate was placed carefully onto the stack and electrolyzed for 30 minutes at 15 V. The blotted membrane was transferred in Milli-Q water to remove any gel particles and the membrane was stored at 4°C until proceed to the next step.

The membrane was submerged in 20 mL blocking buffer under continuous shaking for one hour followed by rinsing in 20 mL Milli-Q water for 5 min at least twice. To prepare a primary antibody solution, 2.5 µL of mouse anti-CBP monoclonal antibody (0.5 µg/µL, GeneScript) was added to the 10 mL blocking buffer to reach a final ratio of 1:4000 of primary antibody in wash buffer, according to the manual. The primary antibody solution covered the surface of the membrane which was then incubated for one hour at RT. To remove any non-specifically bound antibodies, the membrane was washed four times for 5-10 minutes each in 20 mL washing buffer under gentle shaking. For the secondary antibody, alkaline phosphatase conjugated anti-mouse IgG antibody was diluted, applied

and incubated similar to the treatment with the primary antibody. After four times washing in wash buffer, the membrane was made contacting in 5 mL chromogenic substrate solution (Chromogen, Invitrogen^{TM}) until the bands of interest had reached the desired intensity. The membrane was washed with ddH_2O for 2 min and the bands were then analyzed.

3.2.18. Protein Quantification Method

The total protein was quantified using *RC DC* protein assay kit from Bio-Rad, which is based on the Lowery assay with the improvement of compatibility for reducing agent as well as detergent. A reference standard of known protein such as bovine serum albumin (BSA-10 mg/mL) was established first, and then compared to determine the unknown concentrations. Therefore, a series of dilutions ranging from 0.1 mg/mL to 1.5 mg/mL, were prepared using the same buffer containing the sample proteins (Table 15). Reagent A was prepared by mixing 50 parts of *DC* Reagent A with 1 part of *DC* Reagent S for use in a later step, according to the assay protocol. The total volume of Reagent A was calculated by the following formula.

(# Standards + # unknowns) × (# replicates) × (volume of Reagent A per sample) = total volume of Reagent A

First, 25 µL of each sample protein and standard was pipetted into clean, dry microcentrifuge tubes, and then 125 µL of *RC* Reagent I was added, vortexed and incubated for 1 minute at room temperature. After that, 125 µL of *RC* Reagent II was added in every tube; again vortexed, and then centrifuged at 15,000× g for 5-10 minutes. The supernatant was drained completely off the tubes by inverting on the clean absorbent tissue papers. Pre-made Reagent A (127 µL) was added to each sample and standard, vortexed well and incubated until the pellet completely dissolved. In the last step, 1 mL of *DC* Reagent B was added to each tube, vortexed again and incubated for 15 minutes at room temperature. After 15 minutes of incubation, 200 µL from each tube was pipetted into 96 well transparent plate. The absorbance was measured at 700 nm using microplate reader (Tecan Infinite 200® Pro)

Table 15. Diluted Albumin (BSA) Standard Solutions

Vial	Volume of Diluent (µL)	Volume and Source of BSA (µL)	Final BSA Concentrations (µg/mL)
A	170	30	1,500
B	100	100 of vial A dilution	750
C	100	100 of vial B dilution	375
D	100	100 of vial C dilution	187.5
E	100	100 of vial D dilution	93.75
F	100	0	0 (Blank)

3.2.19. Exploitation of *Taq* Pol Based PCR to Determine dUTPase Activity

A routine PCR was carried out with or without added dUTP to investigate the dUTPase function, and the fusion enzymes. Here we have added equimolar amount of dUTP nucleotides in lieu of dTTP, which should prevent the formation of PCR product during amplification since the dUTPase will show its catalytic activity on dUTP. For the dUTPase activity assay, a master mix was made for no storage, storage, dUTPase, which was dispensed into PCR tubes to a final volume of 20 µL (Table 16).

Table 16. PCR Components for dUTPase Activity Assay

Name of Components	Volume (µL)	Master Mix
5X Go Taq Buffer	4	18
ddH_2O	11	54
dNTPs/dNTPs+dUTP/dUTP (10 mM)	0.5	2.25
Template (pCAL-DUT, 15.6 ng/µL)	1	4.5
UIFP (10 µM)	1	4.5
DIRP (10 µM)	1	4.5
Go Taq Polymerase	0.5	2.25
dUTPase	1	-
Total volume	20	

3.2.20. Exploitation of *Pfu*-Pol Based PCR Assay to Determine dUTPase Activity

It is a known fact that unlike *Taq* polymerase *Pfu* polymerase cannot incorporate dUTP as proofreading entity would not allow. Therefore, we modified the PCR assay a little as compared to section 3.2.19. Here, in addition to all dNTPs, we mixed dUTP in the

PCR assay. We compared *Pfu*-Pol and other fusion enzymes if they have dUTPase activity. If they do, then in presence of dUTP there should be a PCR product.

3.2.21. Inorganic Pyrophosphate (PPi) Assay

The inorganic pyrophosphates (PPi) generated in reaction mixtures as a by-product of dUTP conversion, were detected using PPiLight™ inorganic pyrophosphate assay (Lonza, Inc.) (Fig. 7). A standard reference curve was the pinpoint basis to conclude that the intensity of light was directly proportional to the amount of PPi present in the reaction. To prepare the standard, a serial dilution was made in a range from 0.313 µM to 10 µM using a 1mM stock of pyrophosphate. In addition, the dUTP molecules in 10X Bioneer buffer were incubated for one hour at 85°C in the presence of affinity purified dUTPase, Pol-K, and Pol-N in a 20 µL reaction mixture. For the assay, 5 µL of each reaction sample was diluted in sterile water to a final volume of 40 µL.

Next, 40 µL of each sample and standard were pipetted into Nunc Luminescence 96 well micro plate, and then 20 µL each of converting and detection reagents was added and incubated for 15 minutes covered with aluminum foil. The relative light units (RLUs) produced in the reaction mixture were measured using TopCount® NTX™ Microplate Scintillation and Luminescence Counter (Packard BioScience Co.) in Prof. Mill's Lab.

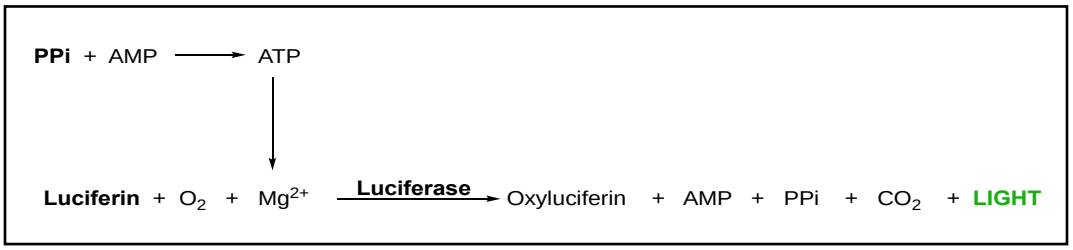

Figure 7: Bioluminescent Reaction. In the presence of PPi the detection reagent catalyzes the conversion of AMP to ATP. The assay uses luciferase, which produces light from the newly formed ATP and luciferin.

CHAPTER IV: RESULTS

4.1. In-Fusion Cloning of *dUTPase* in pCAL-Kc

4.1.1. Amplification of dUTPase cDNA Using In-Fusion Primers

The first goal was to insert the dUTPase encoding '*dut'* gene into pCAL-Kc expression vector so that the derived enzyme could be used as a known control, especially to compare the catalytic role of tagged dUTPase in the fusion proteins [34]. Therefore, the entire dUTPase coding region was amplified using gene-specific primers containing *Nco*I in forward primer (UIFP) and *Kpn*I in reverse primer (DIRP). The expected product size of around 471 base pairs was noticed in lane 3 of agarose gel electrophoresis (Fig. 8). It should be pointed out that the negative control at which DNA template was replaced by water failed to produce any PCR product (lane 2, **Fig. 8**), reflecting the specific amplification of dUTPase gene was achieved without any contamination in PCR reagents.

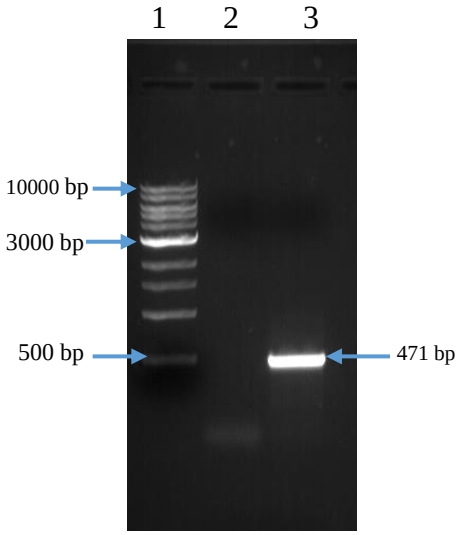

Figure 8: PCR Amplification of *'dut'* Using UIFP and DIRP Primers. Products were analyzed by 1% agarose gel. Lane 1: 1 kb DNA ladder (GeneRuler), Lane 2: Negative control (NC), Lane 3: PCR product of '*dut*' gene

4.1.2. Restriction Digestion of pCAL-Kc Using *Kpn*I and *Nco*I

The plasmid, pCAL-Kc, was linearized by two restriction endonuclease digestion in order to ligate dUTPase insert in an orientation specific manner. Since the restriction sites including *Nco*I and *Kpn*I positioned closely in the cloning vector, no detectable size difference was observed after a single and double digestions that resulted in bands of approximate 5.8 kb size as displayed in Fig. 9 (compare lanes 3 and 4 to lane 6). Additionally, an uncut plasmid was also run in parallel to compare linearized plasmids with the supercoiled version (lane 2).

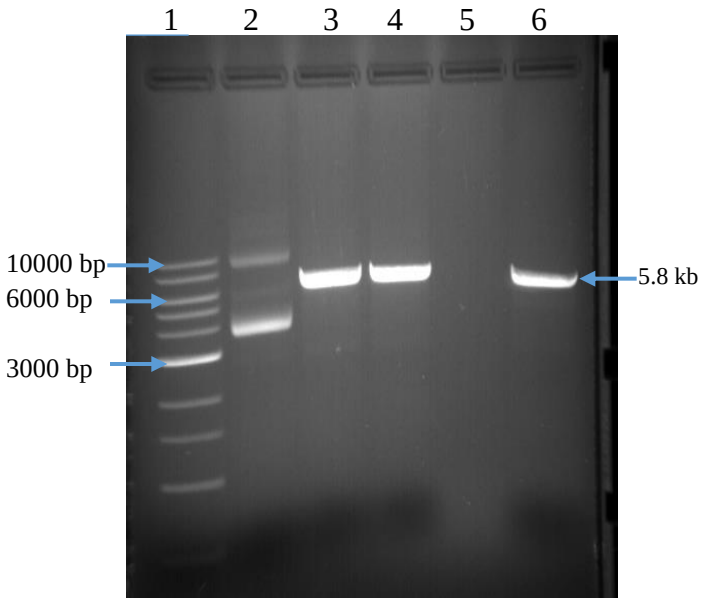

Figure 9: Restriction Digestion of pCAL-Kc Using *Kpn*I and *Nco*I. The digested products were verified by 1% agarose gel. Lane 1: 1 kb DNA ladder (GeneRuler); Lane 2: Uncut plasmid; Lane 3: Digestion by *Kpn*I; Lane 4: Digestion by *Nco*I*;* Lane 5: no sample; Lane 6: Double digestion with *Kpn*I and *Nco*I.

4.1.3. Screening of Bacterial Clones Containing pCAL-DUT by Colony PCR

Whether the amplified PCR product of dUTPase was successfully ligated into the pCAL-Kc vector, we analyzed the bacterial transformants by colony PCR (Fig. 10). Eight individual transformants were taken for the assay. The sizes of all amplicons derived from colony PCR except for lane 8 matched the size of control '*dut'* gene (471 bp, lane 2).

These positive colonies were then used for plasmid isolation using Sigma GenElute Miniprep Kit, and the concentrations were checked by Nanodrop spectrophotometer. The average DNA concentration was found to be around 100 ng/µL.

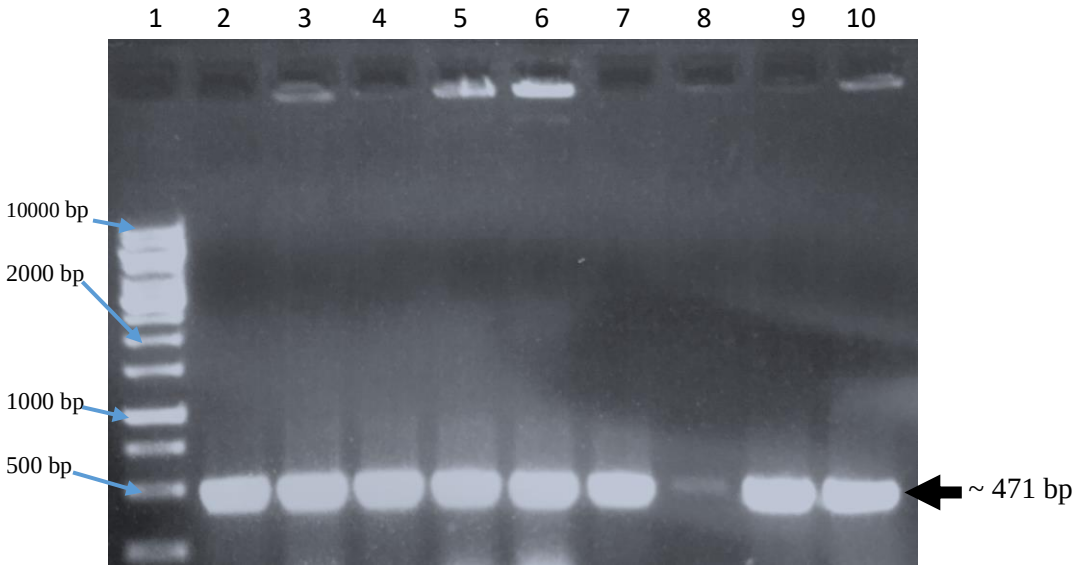

Figure 10: Colony PCR of dUTPase Using 2X GoTaq® Green Master Mix. PCR products were verified by 1% agarose gel. Lane 1: 1 kb ladder (GeneRuler), Lane 2: Positive control for dUTPase, Lane 3-10: PCR samples using various colonies as template DNA

4.1.4. Screening of Positive Bacterial Clones by Restriction Digestion

Besides colony screening, we also confirmed the presence of DUT insert in the isolated plasmids by the double digestion with *Nco*I and *Kpn*I. As shown in Fig. 11, the agarose gel electrophoresis of digested samples in at least one clone showed the 471 insert, which matched the size of dUTPase (lane 4).

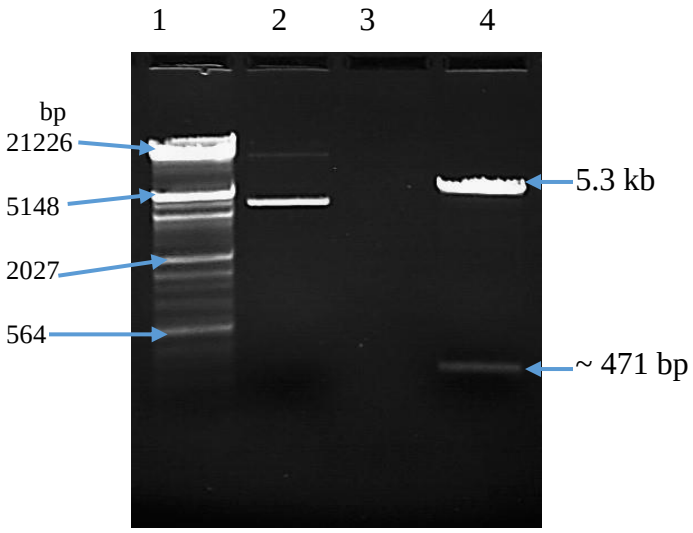

Figure 11: Restriction Digestion of pCAL-DUT. Digested products were analyzed on 1% agarose gel. Lane 1: λ DNA ladder, Lane 2: Uncut pCAL-DUT, Lane 4: double digestion of pCAL-DUT using *Nco*I and *Kpn*I

4.2. Screening of Yeast Clones Containing Pol, dUTPase and Pol-K Recombinant Constructs by Colony PCR

The amplified targets including *Pfu*-pol, dUTPase, and Pol-K were ligated into pYES2 vector and then the recombinant plasmids were transformed into YNN281. An underlying evidence of ligation and the presence of inserts in precise location were confirmed by colony PCR as well as restriction digestion. In Fig. 12, the size of each

amplicon in lanes 2-5 is identical and consistent to the length of *Pfu*-pol gene. In addition, the amplicons in next four lanes are found to have relatively larger size, indicating the successful fusion of *Pfu*-pol and dUTPase, known as Pol-K. Similarly, an expected size of dUTPase was spotted at the bottom of agarose gel.

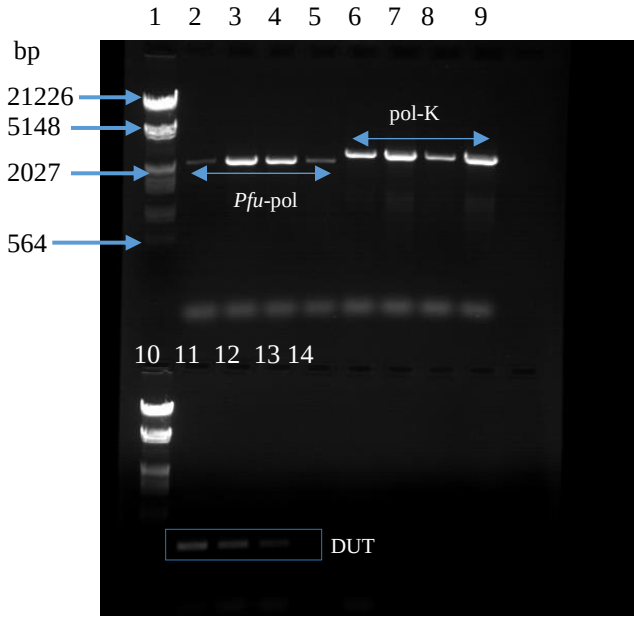

Figure 12: Colony PCR of Yeast Transformants Containing *Pfu*-pol, dUTPase, and Pol-K. PCR was set up using 2X GoTaq® Green Master mix and corresponding primers; and visualized by 1% agarose gel. Lane 1& 10: λ DNA ladder, Lane 2-5: *Pfu*-pol, Lane 6-9: Pol-K, Lane 11-14: dUTPase

4.3. Screening of Yeast Clones Containing Pol, dUTPase and Pol-K Recombinant Constructs by Restriction Digestion

Further analysis was done by double digestion of bacterially propagated recombinant yeast plasmids using *Hind*III and *Xba*I. The resulting fragments were analyzed based on the expected number of digestions occurred in both ORF and inserts ends. *Pfu*-pol is a 2,327 bp long gene that typically contains *Hind*III (1,919 bp) and *Xba*I (2,184 bp) restriction sites in the coding sequence, which give two additional bands upon the splitting

of intact *Pfu*-pol. As shown in Fig. 13, while both *Pfu*-pol and Pol-K generated four fragments after double digestions, the third fragments from these two genes were not related in size due to the presence of dUTPase in Pol-K that enlarged the fragment size. In case of recombinant dUTPase, only a small fragment was seen, which meets the expected size of dUTPase (lane 3, Fig. 13).

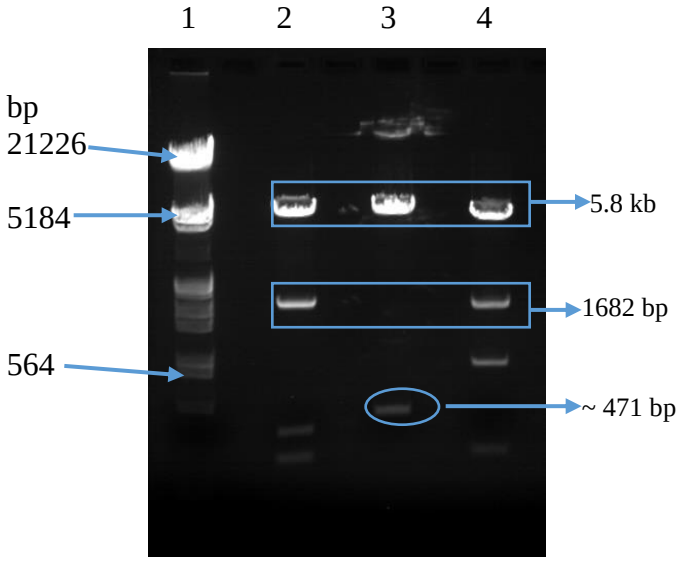

Figure 13: Insert Verification in Yeast by Restriction Digestion of *Pfu*-pol, dUTPase, and Pol-K. Fragments were analyzed on 1% agarose gel. Lane 1: λ DNA ladder, Lane 2: *Pfu*-pol (*Hind*III+*Xba*I), Lane 3: dUTPase (*Hind*III+*Xba*I), Lane 4: Pol-K (*Hind*III+*Xba*I)

4.4. Purity Determination of Affinity Purified *Pfu*-Pol, dUTPase, Pol-K, and Pol-N

The SDS-PAGE analysis determines the molecular weights (MW) of unknown proteins by comparing to pre-stained protein standards. We ran SDS-PAGE of our affinity-purified fractions to determine approximate sizes of the expressed proteins. The crude cell lysate, flow-through, wash, and eluted samples were run in parallel with some variations. It should be noted that we also purified wild type and fusion of *Pfu*-pol during this

experiment and ran them here for comparative analysis. In Figure 14A, the flow-through (lanes 5 & 10) of both *Pfu*-pol and dUTPase had some non-bound proteins; however, there were no contaminating proteins observed in the wash fractions (lanes 4 & 9), indicating apparent absence of any contaminating proteins in the column. The MW of native *Pfu*-pol is ~90 kDa, corresponding to the sharp bands in lanes 2 & 3 between 75 and 100 kDa (Fig. 14A). In contrast, dUTPase fractions in lanes 7 & 8 were not detected, which may have been resulted from a very low concentration of dUTPase in the sample to be stained. When we repeated this analysis after concentrating the sample fractions and noticed a band with an approximate size of 18 kDa (lane 3, Fig. 14B).

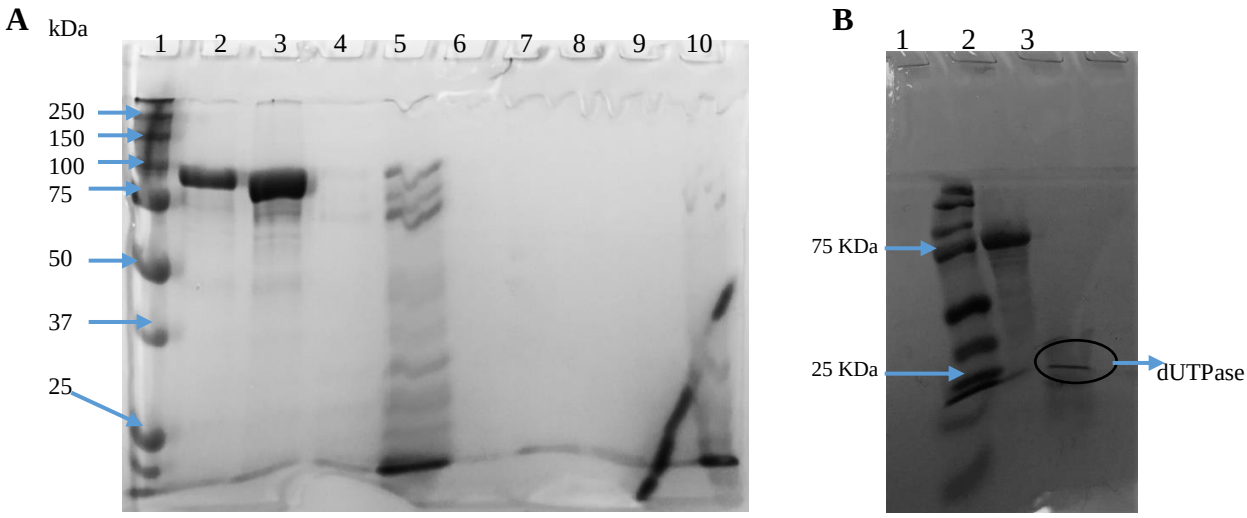

Figure 14. SDS-PAGE Analysis of Affinity Purified Samples. **A)** 10% SDS-PAGE analysis of *Pfu*-pol and dUTPase. Lane 1: Precision Plus Protein™ All Blue standards (Bio-Rad), Lane 2: *Pfu*-pol (E1), Lane 3: *Pfu*-pol (E2), Lane 4: wash (*Pfu*-pol), Lane 5: flow through (*Pfu*-pol), Lane 6: No sample, Lane 7: dUTPase (E1), Lane 8: dUTPase (E2), Lane 9: wash (dUTPase), Lane 10: flow through (dUTPase). **B)** Concentrated dUTPase analyzed in 10% resolving gel only. Lane 1: Precision plus dual color (Bio-Rad), Lane 3: dUTPase protein

We ran another SDS-PAGE for analysis of the fusion proteins (Pol-K and Pol-N) by 10% resolving gel. The theoretical MW of fusion proteins including Pol-K and Pol-N would be 108 kDa (90 kDa + 18 kDa), since both are the combination of *Pfu* polymerase and dUTPase. Thus, the expected size of bands in lanes 4 & 5 (Fig. 15A) can be characterized as Pol-K since the bands were spotted between 100 and 150 kDa marker bands. Similarly, Pol-N was also determined in lane 9 of Fig. 15B around the same location.

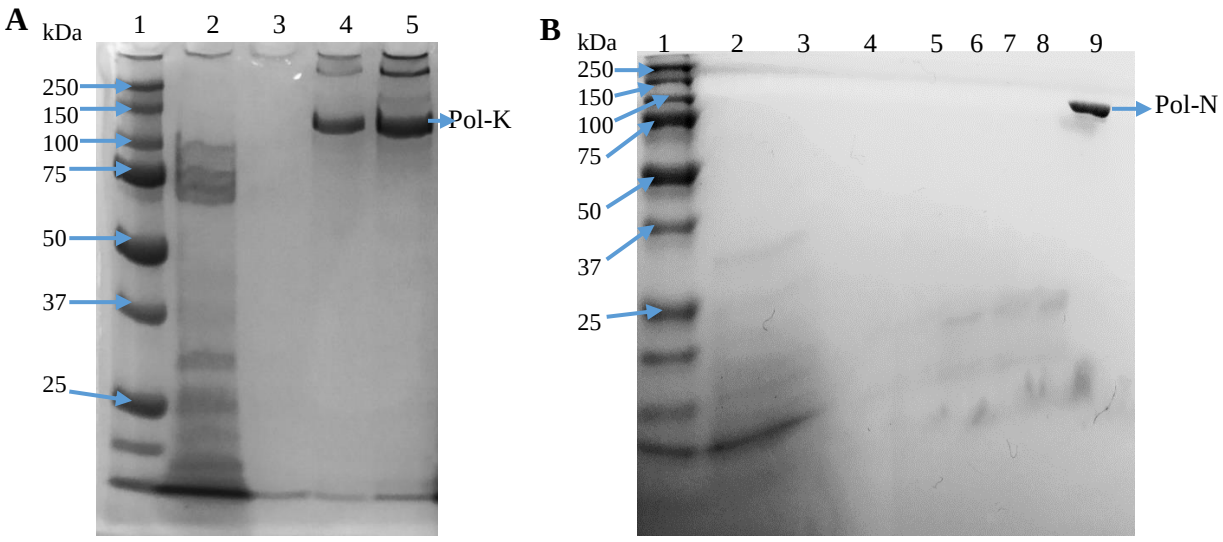

Figure 15: SDS-PAGE Analysis of Pol-K and Pol-N in 10% resolving gel. **A)** Lane 1: Precision Plus Protein™ All Blue standards (Bio-Rad), Lane 2: flow through (Pol-K), Lane 3: wash (Pol-K), Lane 4: Pol-K(E1), Lane 5: Pol-K(E2). **B)** Lane 1: Precision Plus Protein™ All Blue standards (Bio-Rad) flow through (Pol-N), Lane 9: Pol-N(E1)

4.5. dUTPase Specification by Western Blot

We opted for identification of dUTPase expression by an indirect Western blotting assay. Since the dUTPase was tagged to the CBP, an anti-CBP antibody from mouse could be used in the Westerns. As shown in Fig. 16, although there were some non-specific bands observed in flow-through lane (lane 3) but there was no band detected in the wash sample

(lane 4). All bands in lanes 5 through 8 can be recognized as CBP tagged-dUTPase, since the protein size is approximate 22 kDa that matches monomeric dUTPase (18 kDa) conjugated to CBP tag (4 kDa).

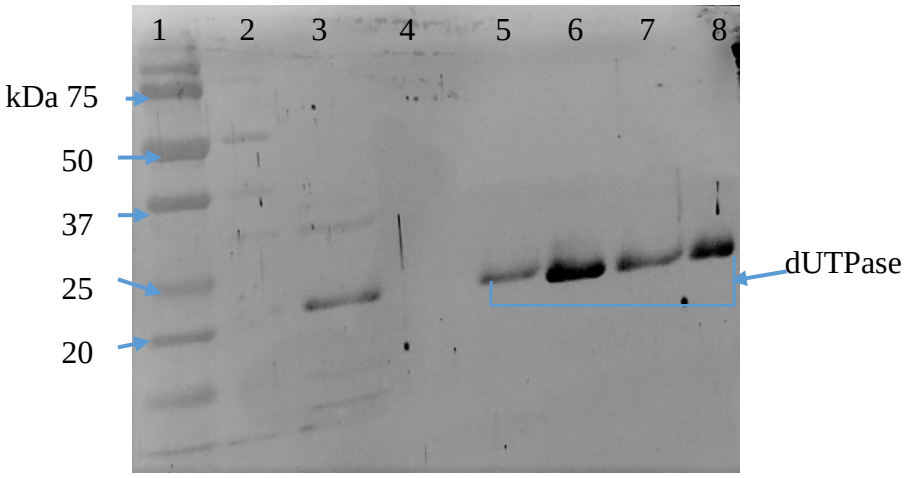

Figure 16: Western Blotting of dUTPase (DUT) Using Mouse Anti-CBP Monoclonal Antibody. Lane 1: Precision Plus Dual Color protein marker (Bio-Rad®), Lane 3: FT, Lane 4: Wash, Lane 5: Non-concentrated DUT-S1, Lane 6: Concentrated DUT-S1, Lane 7: Non-concentrated DUT-S2, Lane 8: Concentrated DUT-S2

4.6. Removal dUTP by dUTPase Enzyme in a *Taq*-Pol Based PCR

Taq DNA polymerase can incorporate dUTP in place of dTTP, i.e., they cannot differentiate between the two; this could be due to their lack of 3'-5' exonuclease activity. Therefore, if we could remove the dUTP from the PCR this would prevent polymerization. We performed *Taq*-PCR with or without dUTPase in the presence of dUTP (Fig. 17). In control sample (lane 2), the Green Master Mix provides dTTP as one of the dNTPs, although in rest of the samples dTTP was replaced by dUTP (lanes 3-6, Fig.17). All amplicons observed in the gel were expected to be the amplified products of '*dut'* gene,

since the product size closely matched to a reference band of 500 bp**.** In contrast, upon providing dUTPase to the reaction mixture (lanes 5 & 6, Fig. 17), the *Taq* DNA polymerase could not amplify the gene of interest, suggesting our purified dUTPase was able to catalyze the conversion of dUTP into dUMP and inorganic pyrophosphates.

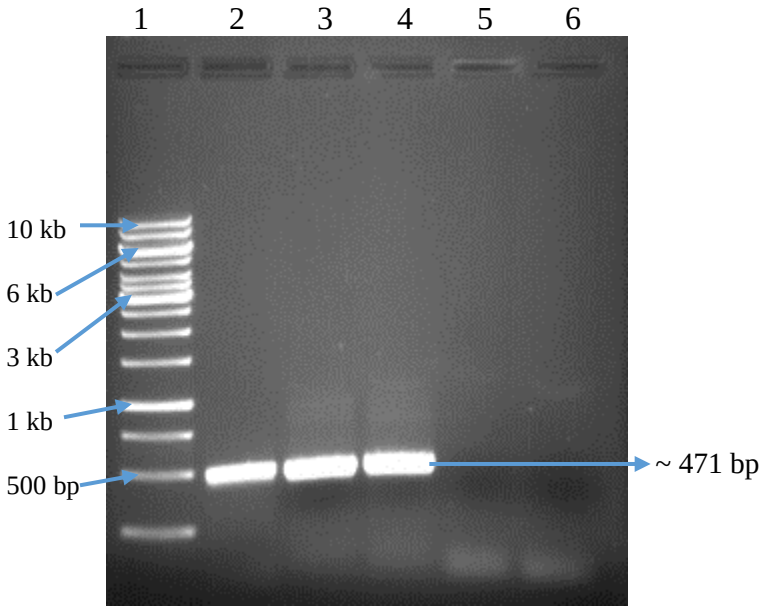

Figure 17: Functional Analysis of dUTPase in the Presence and Absence of dUTP Residue. PCR amplicons were verified by 1% agarose gel. Lane 1: 1 kb Marker (GeneRuler), Lane 2: positive control (regular dNTPs), Lane 3: No Storage (similar to +Ve but present dUTP), Lane 4: storage (protein buffer), Lane 5: dUTPase-S1, Lane 6: dUTPase-S2

4.7. The dUTPase Function Analysis in Fusion Enzymes Using PCR Assay

DNA polymerase function and DUT activity of newly purified proteins were tested by subjecting them in PCR with or without dUTP residue. It is to be noted that presence of dUTP nucleotide interferes with *Pfu*-Pol based amplification. In this PCR assay, the enzymatic functions of *Pfu* DNA polymerase, Pol-K, and Pol-N were compared (Fig. 18).

We also investigated whether the dUTPase in fusion protein had any interfering effect on *Pfu* DNA polymerase during extension. As expected, no product was formed when dUTP was introduced in *Pfu* polymerase-based PCR reaction (lane 2). However, *Pfu*-pol showed its natural polymerase activity when the dUTP was not added to the dNTPs (lane 3). A similar effect was observed in PCR mixture when dUTPase was added with or without dUTP in the reaction (lanes 4 and 5). This also indicated dUTPase enzyme did not interfere *Pfu*-Pol function when separately added. The fusion protein, Pol-K, demonstrated the polymerase function in similar condition (lanes 6 & 7) indicating its functional dUTPase entity. However, we observed weaker functions for Pol-N (lanes 8 & 9).

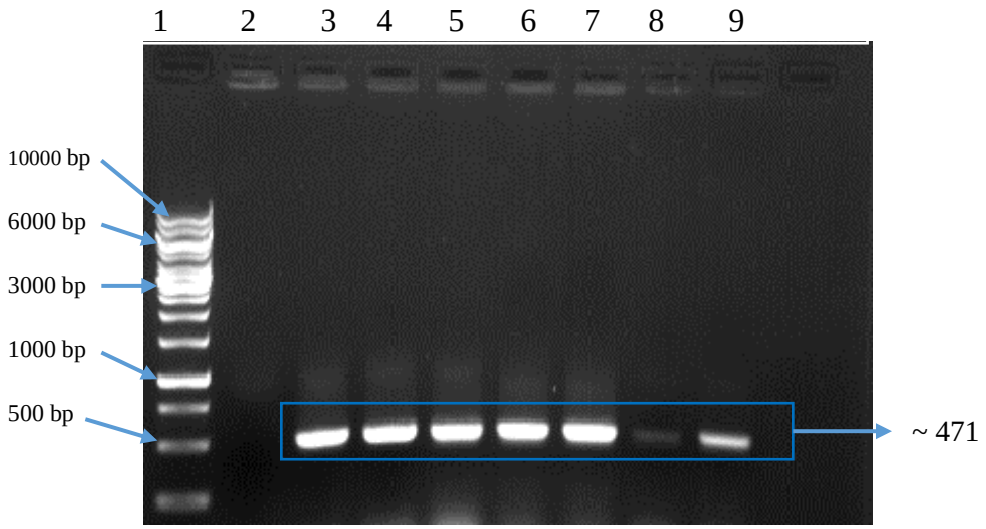

Figure 18: Enzymatic Function of Affinity Purified Fusion Proteins. PCR products were visualized on 1% agarose gel. Lane 1: 1 kb Marker (GeneRuler), Lane2: *Pfu*-pol(+dUTP), Lane 3: *Pfu*-pol (No dUTP), Lane 4: *Pfu*-pol+dUTPase (+dUTP), Lane 5: *Pfu*-pol +dUTPase (No dUTP), Lane 6: Pol-K (No dUTP), Lane 7: Pol-K(+dUTP), Lane 8: Pol-N (No dUTP), and Lane 8: Pol-N (+dUTP).

4.8. Quantitative Analysis of Inorganic Pyrophosphate Formation During dUTP Catalysis by dUTPase

Inorganic pyrophosphate (PPi) assay further confirmed the catalytic function of dUTPase that catalyzes the dUTP to dUMP and PPi as a byproduct. The amount of byproduct produced in the reaction mixture was related to the concentration of enzyme. This was monitored by luminometer that measured the light absorbed by PPi. As shown in Fig. 19, gradual increase of dUTPase amount positively related to the RLU (relative light unit) values, indicating the enzyme's significant catalytic activity; however, higher concentrations of sample showed less RLUs indicating reaction interference by contaminants.

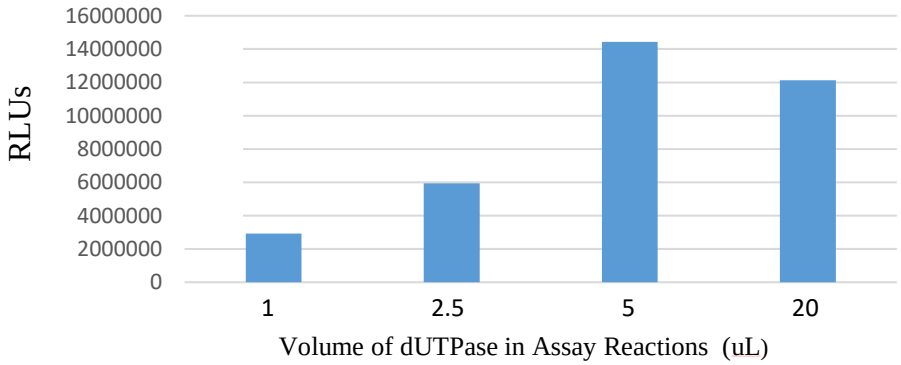

Figure 19: Quantification of PPi Molecules from Various dUTPase Reactions

By the same PPi Light assay, we have analyzed all purified proteins including *Pfu* polymerase, dUTPase, Pol-K, and Pol-N (Fig. 20). Our controls showed very negligible amount of RLUs, for example, storage buffer and *Pfu*-Pol used in the assay. We also observed significant dUTPase activity in Pol-K and Pol-N samples as compared to negative controls.

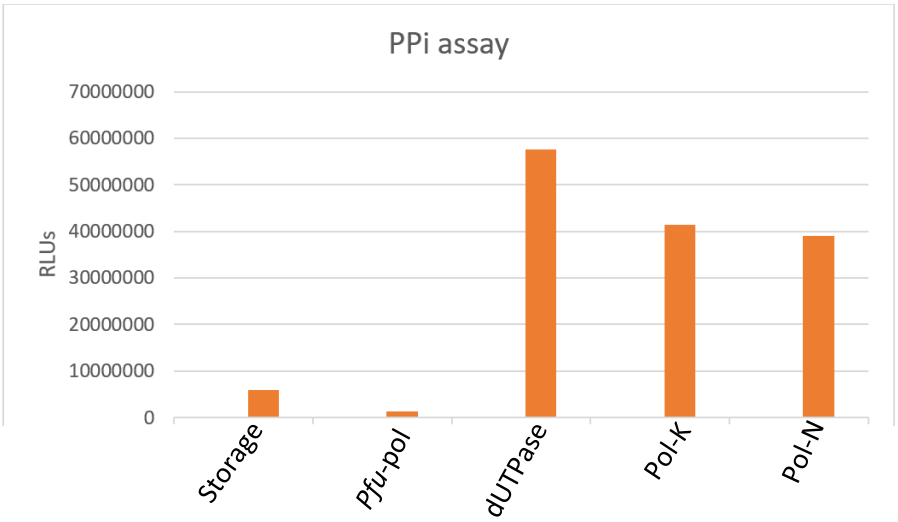

Figure 20: Catalytic Function Analysis of the Fusion Proteins (Pol-K & Pol-N) Using Luminometer

CHAPTER V: DISCUSSION

dUTPase is a ubiquitous enzyme that exists in all forms of life including prokaryotes, eukaryotes, protozoa, viruses and mammalian cell [35, 36]. However, the intracellular level varies from species to species since all free-living organisms are not indispensably required to express the protein. The dUTPase is critical in thermophiles or hyperthermophiles organisms, especially for archaebacteria, that prevents the misincorporation of dUTP during replication, and eases the continuous process of proofreading of DNA polymerase. Thereby, archaeal dUTPase is a good choice for the prevention of dUTP inhibitor as encountered in the long PCR reaction. Previously, biotechnologists separately added the dUTPase enzyme in PCR reactions containing either *Taq* pol or *Pfu*-pol and obtained better PCR yield while maintaining high fidelity [12, 34].

In our experiment, for purification convenience we have successfully cloned the dUTPase gene in frame with CBP in the bacterial expression plasmid pCAL-Kc. It is to be noted, pCAL-Kc belongs to Agilent Technologies protein purification kit that employs a special *E. coli* strain, BL21(DE3), that allows for T7 inducible expression system[37]. In this regard, In-Fusion cloning strategy allowed us direct recombination between the ends of inserts and vector DNA ends. This was very useful in terms of time saving and efficiency of ligation. We confirmed correct insertion of the PCR products into the plasmids by colony PCR, restriction digestion analysis, and Sanger DNA sequencing. The sequence was BLAST searched in NCBI website and almost 100% alignment obtained (not shown).

We claim to have purified the dUTPase successfully since 22 kDa protein was visualized by SDS-PAGE, which agrees to the reported size of 18 kDa for DUT [34] and

additional 4 kDa for CBP[38]. Furthermore, to ascertain the presence of tagged CBP, we performed western blotting using mouse anti-CBP monoclonal antibody.

This study was also aimed to elucidate the enzymatic function of previously cloned *Pfu*-pol and fusion constructs including Pol-K and Pol-N. SDS-PAGE revealed an apparent molecular weight of *Pfu*-pol about 90 kDa, corresponding to a reported size of hyperthermophilic archaeon TNA1[39]. Moreover, the theoretical size of both Pol-K and Pol-N matched to the combined size of entire *Pfu*-pol and dUTPase, suggesting the synthesis of chimeric protein in bacteria was successful.

The inhibitory function of dUTPase was demonstrated by intentionally adding dUTP by replacing of dTTP in PCR with *Taq* Pol. As a result, no detectable bands were observed from *Taq*-pol based PCR containing dUTPase and dUTP. This effect was further analyzed by inorganic pyrophosphate assay since the role of dUTPase is to hydrolyze dUTP into dUMP and pyrophosphate (PPi). In this assay, luciferase catalyzes luciferin into oxyluciferin, PPi, and light in presence of ATP (see Fig. 7). The intensity of light emitted is directly proportional to the amount of pyrophosphate produced in the reaction mixture. From this assay, it was confirmed that increasing the concentration of dUTPase positively related to the byproduct (PPi) formation, which was inferred by the amount of signal light (RLUs) recorded in luminometer.

In this study, we have successfully shown the characteristic activities of polymerase and dUTPase of the newly synthesized fusion proteins (Fig. 21). To address the effect of dUTPase conjugated to *Pfu*-pol, dUTP was added in a PCR mixture as a potent polymerase (*Pfu*-pol) inhibitor. The presence of resulting PCR product was found to be similar to the

one created by *Pfu*-pol alone. Since the fusion protein (Pol-K) produced a similar PCR product with or without dUTP, we can conclude that the dUMP obtained from dUTP hydrolysis had no negative effect on PCR, which is in good agreement to a previous study [34]. We noticed variable PCR efficiency of Pol-N compared to Pol-K based on band intensity in the presence of dUTP. However, there are at least some activity of both enzymatic domains as deduced from repeating experiments (not shown). We think the probable reason behind this may be due to pipetting error so that somehow the enzyme was not added accurately. Another alternative view could be the positional effect, i.e., dUTPase at N- terminal of *Pfu*-Pol may interfere with each other's function.

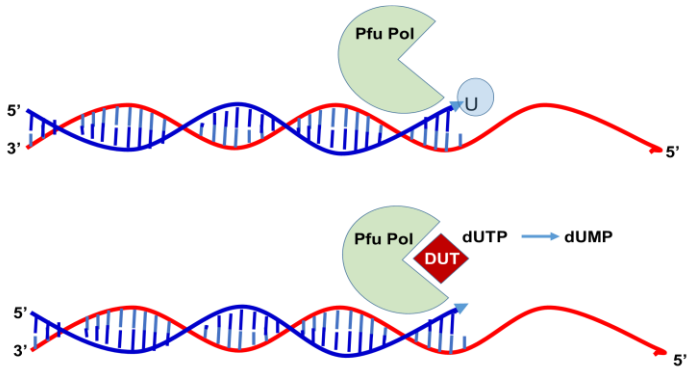

Figure 21: dUTP Conversion by Fusion Enzyme

There are some optimizations and modifications needed to perform a long PCR in the future as we have observed these fusion enzymes are not very efficient producing more than 1 kb gene sequence (data not shown). For comparative enzyme analysis, we will have to quantify the enzyme concentrations so that enzyme kinetics could be determined. To do so a very large-scale protein purification can help. However, we performed a quantitative

PCR to observe the amplification efficiency between commercial *Taq* polymerase and fusion protein made in our laboratory.

To analyze eukaryotic-host (yeast) based enzyme function, we successfully inserted these genes into yeast expression vector plasmids. However, we were not able to show enzyme expression in *S. cerevisiae* after repeating the experiment twice. Therefore, further optimization in induction and lysis steps showed be considered to overcome this.

In future, the improvement of polymerase efficiency can also be examined by the mutational changes in the DNA sequences or by inserting other kinds of amino acids in between two domains. We may employ NMR or X-ray crystallography studies of the fusion proteins to improve the constructs.

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