Comparing Fidelity and Processivity of a *Pfu* Polymerase with a Mutation in His147 Position to Wild Type

Analiese DiConti
Massachusetts Institute of Technology
Department of Biology
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adiconti@mit.edu
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The ability to alter polymerases allows scientists and researchers to find the polymerase that best suites their needs which gives them tighter control over their experiments. The fidelity and processivity of *Pfu* Polymerase with a mutation of Histidine in position 147, located in the unique loop of the exonuclease domain, were studied in order to find if a mutation made protein activity greater or weaker than that of the wild type. Fourteen different mutations were developed and studied between 39 groups via Quikchange® mutagenesis, our group specifically studying a Phenylalanine missense mutation. Once the proteins were expressed in BL21(DE3) *E. coli* and purified via affinity chromatography and gel filtration chromatography, their fidelities were tested using a colorimetric assay and their processivities using various templates to undergo PCR. All fidelity assays were inconclusive due to overdigestion of the plasmids of which's replication was being tested. The H147F mutant was found to have slightly lower processivity than the wild type. It replicated smaller templates equally well, but had more difficulty than the wild type in replicating the 6kb template. Asparagine proved to have the best processivity, replicating all three templates while the wild type could not completely replicate the 6 kb template. The ability of Asparagine and Histidine to bind nucleic acids, most likely due to the electronegative atoms on their side chains, could account for the increased processivity. Further study is recommended in order to ascertain the fidelity of the mutant polymerases. Fidelty is very important in order to ensure the validity of results obtained from experiments involving PCR or other polymerase activities.

DNA polymerases are proteins that replicate DNA. Once the double stranded DNA is denatured or if single stranded DNA is present, DNA polymerase adds individual bases to the 3' end of the denatured template strand. Polymerases can also perform "proof reading" to ensure that the base added is the match of the template strand. If the base is incorrect, the polymerase will cleave the mismatched base and replace it with the correct base. DNA polymerase can be defined by two characteristics: the speed, or processivity, and the accuracy, or fidelity, of the replication process. DNA polymerase with high fidelity and processivity can make many accurate copies of a DNA template.

In the laboratory, the ability to make large quantities of DNA is very important and can be achieved using the technique polymerase chain reaction (PCR). Scientists can alter DNA and then replicate it to amplify expression of the mutated gene or genes using PCR and then study the effects. Because of the high temperatures used in PCR, thermo-stable polymerases must be used such as those isolated from Taq, *Pfu*, and KOD which are archaeabacteria found living in hot springs. Currently, there are only a few types of polymerases available that will not denature under the high temperatures necessary for PCR, limiting scientists in their range of experiments. If scientists can modify a polymerase to suit their needs, they can have tighter control over their experiment.

By studying the crystal structure of the DNA polymerase of *Thermococcus kodakaraensis* (KOD), Hasimoto and his team of researchers designed a mutant polymerase that had higher fidelity than the wild type (2001, as reviewed in the 7.02 Lab Manual). The only change that was made was a substitution of the histidine in position 147 to a lysine (Kuroita et al., 2005). Kuroita et al. looked into the mechanism of proofreading in KOD and concluded that H147 played a role in the polymerase's exonuclease activity. Thus, mutations in this position

could decrease the polymerase's ability to proofread if the substituted amino acid's structure was too different from that of Histidine. For instance, a replacement by Alanine, a hydrophobic hydrocarbon, caused a larger number of errors in the polymerase's replication. But, a replacement by lysine, which is positively charged and basic like Histidine, made exonuclease activity more effective.

In our experiment, we mutated the gene for the polymerase of a homolog of the KOD, *Pyrococcus furiosis* (*Pfu*). Because of the strains' similar structures, each group mutated position His147 in order to compare fidelity and processivity to that of the wild type polymerase. Class data was then compiled to determine which mutants displayed the greatest fidelity and processivity. We tested if, like in KOD, a mutation in this position would make a faster, more accurate mutant. First, we had to create the mutation using site-directed mutagenesis, which randomly mutated a specific region of the *Pfu* gene with contained His147. We then expressed the gene in XL1-Blue *E. coli* cells. Lastly, we tested our enzymes running two assays: one PCR based assay which tested whether our polymerases could replicate DNA and a second which used a forward genetic screen to compare the fidelity of our mutant to that of the wild type.

Methods

In order to accomplish our goal of mutating the *Pfu* DNA polymerase, we mutated the gene coding for the *Pfu* polymerase, expressed and purified the DNA with the gene and generated a collection of the mutations, and then analyzed the fidelity and processivity of one of our mutations.

Mutagenesis of the gene encoding Pfu polymerase

A QuikChange Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA) was used to introduce mutations into the *Pfu* DNA polymerase gene. This technique used the following degenerate primers to change the DNA sequence:

5' – CCCTCTATNNNGAAGGAGAAGAGTTTGG – 3'
5' – CTCCTTCNNNATAGAGGGTTTCTATATCG – 3'

where the NNN sequence is the random mutation. The top sequence represents the forward primer, and the bottom, the reverse. The following was added to the reaction tube: 10 μL 5X Phusion buffer; 5 μL 10X dNTP mix; 2.5 μL primer mix; 1 μL template DNA (pET-*Pfu*); and 31.5 μL ddH_20. The following PCR program was then run in the thermocycler to replicate the mutations created with the primers:

1) 95 °C for 30 seconds
2) 95 °C for 30 seconds
3) 55 °C for 1 minute
4) 72 °C for 12 minutes
5) Go to step 2 twenty-four times
6) 72 °C for 10 minutes
7) 4 °C overnight

PCR replication and mutant creation was verified using gel electrophoresis on a 0.8% agarose gel with 1X SYBR safe in 1X TAE(40mM Tris, 20mM NaOAc, 1mM EDTA, pH 7.2). A 6X running buffer of 0.25% bromophenol blue and 15% Ficoll in water was used.

After replication, the mutant DNA was purified. First, the Qiagen PCR purification kit (Qiagen, Velno, Netherlands) was used to remove DNA polymerase and other PCR reagents. Then Dpn I restriction enzyme was mixed with the DNA to digest the template DNA to prevent the *E. coli* from taking up the wild type template during transformation.

Cells from the cloning strain of *E. coli* XL1-Blue were transformed with the mutant DNA for amplification. After being mixed with the DNA, the *E. coli* cells were heat shocked at

42°C for 45 seconds and immediately incubated on ice for 2 minutes. SOC medium was then added, and the cells were allowed a growth period of one hour where they were incubated on a roller drum for one hour at 37˚ C. The cells were plated on LB-Amp plates and placed in incubation overnight at 37˚ C. DNA from four of the cell colonies, each with a single mutation, was isolated using the QIAprep Miniprep kit (Qiagen, Velno, Netherlands) according to the manufacturer's instructions. Each of the four mutations was identified using the Applied Biosystems Taq DyeDeoxy Terminator cycle sequencing method with T7 promoter primer (5'-TAATACGACTCACTATACCC-3') and T7 Primer solution, and then one mutant was selected for further study.

Expression and Purification of the mutant Pfu polymerase

Cells from an expression strain of *E. coli* BL21(DE3) were transformed with the mutant DNA to isolate the polymerase. Expression was performed using the pET system (Novagen, Darmstadt, Germany) which places the gene encoding the polymerase under control of the T7*lac*promoter in the pET21b *Pfu* construct. This allows the gene to be expressed only under very specific conditions, namely, in the presence of lactose or a structural homolog like IPTC.

The cells were transformed with the pET-*Pfu* mutants by adding 1 μl of isolated DNA to 50 μl of cells and incubating on ice for 30 minutes. Following incubation, the cells were heat shocked for 20 seconds and then again incubated on ice for 2 minutes. LB medium (270 μl) was added to the tube and allowed to incubate for one hour at 37˚ C on the roller drum. After incubation, the tube was centrifuged and the supernatant removed. The cells were re-suspended in 100 μl of LB medium and then plated on LB-Amp plates.

After an overnight incubation at 37˚ C then storage at 4˚ C until the following laboratory period, the cells were allowed to grow for 4 hours at 37˚ C and then inoculated with 250 ml of

Terrific Broth/Amp. They were then grown for 16 hour at 37˚ C and then harvested. The polymerase gene was then expressed using the Overnight Express Autoinduction System (Novagen, Darmstadt, Germany).

Purification was achieved by using nickel affinity chromatography. Expression of the polymerase left a 6x His-tag on the C-terminal end of the protein, which allowed purification on a His-trap Fast Flow Ni Sepharose column. First, the *E. coli* cells were lysed by resuspending the cell pellet in 15 mL of lysis buffer containing 30mM Tris-HCl (pH8), 500mM NaCl, 5mM imidazole, 0.1% Triton X-100, 5mM β-mercaptoethanol, and 1% preotease inhibitor cocktail and then adding 150 μL lysozyme. The cells were then incubated on ice for 20 minutes. The cell's chromosomal DNA and RNA were digested by adding 8 μL DNase I, 15 μL $MgCl_2$, and 16 μL RNaseA to the lysate. After incubating on ice for 10 minutes, the mixture was centrifuged for 20 minutes at 12,000 rpm.

After separating the supernatant from the cell pellet, the supernatant and cell pellet (resuspended in pellet buffer containing everything in the lysis buffer excluding the protease cocktail) was passed over the Ni-NTA resin equilibrated with the lysis buffer. Wash buffer, containing the same ingredients as the pellet buffer except 10mM imidizole instead of 5mM, was then passed over the column. Then, as elution buffer containing 30mM Tris-HCl (pH8), 100mM KCl, 150 MMimidizol, 0.1% Triton X-100, and 5 mM β-mercaptoethanol was passed over the column, five 1mL fractions were taken. The protein concentration in the fractions were qualitatively measured with Coomassie Plus reagent and the three fractions with the highest concentration were pooled to make the eluent.

To retain activity, the *Pfu* protein was transferred into a storage buffer containing 40mM Tris-HCl (pH8), 200mM KCl, 2mM DTT, and 0.2mM EDTA using a PD-10 column. After six

.5 ml eluent fractions were collected from the column, once again protein concentration was qualified using Coomassie Plus. The wells with protein present were pooled and then analyzed in a spectrophotometer to record absorbance. The Lambert-Beer Law was then used to calculate the concentration of *Pfu* protein and the protein was stored in a 1:1 mixture (half protein, half glycerol and detergents) of 100% glycerol, Tween-20 and NP-40 (to a final concentration of 0.1% each).

As a control, a 7.5% SDS polyacrylamide gel electrophoresis (SDS PAGE) was run using running buffer containing 25mM Tris-HCl, 192mM glycine, 0.1% sodium dodecyl sulfate (SDS) (pH 8.3). Protein sample buffer (2X) containing 125mM Tris-HCl (pH 6.8), 20% glycerol, 4%SDS, 0.04% Bromophenol Blue, and 10% β-mercaptoethanol was mixed with the samples: cell lysate, cell lysate-pellet, cell lysate supernatant, flow through, wash, eluate, and PD-10. The gel was run at 120 volts and then stained with 30mL Coomassie Blue followed by 30 mL Fast Destain solution (50% methanol, 10% acetic acid) twice and 30 mL of ddH_2O.

Analyis of mutant Pfu polymerase function

The fidelity of the mutant polymerase was compared to that of the wild type by assessing the expression of β-galactosidase in XL1-Blue *E. coli* cells by a colorimetric assay. The wild type *Pfu* was diluted in the storage buffer used in the purification step so both polymerases were present in the same concentration. First, the following reaction mixture was created: 1μL template DNA, 5 μL dNTPs, 5 μL pTS-HindIII primer mix (forward primer: 5'CTT CTA AGC TTT TTA GTG CTT TAC GGC ACC TC3'; reverse primer: 5'GAA GAA AGC TTG TGG CGA GAA AGG AAG GGA AG3'), 5 μL 10X buffer (200mM Tris-HCl (pH 8.8), 100mM KCl, 100mM $(NH_4)_2SO_4$, 1% Triton X-100, 1mg/mL BSA, and 15mM $MgSO_4$), 1 μL *Pfu* polymerase, and 33 μL H20. The mixture underwent PCR on the following program:

1) 98 °C for 30 seconds
2) 98 °C for 10 seconds
3) 56 °C for 20 seconds
4) 72 °C for 4 minutes
5) Go to step 2 twenty-nine times
6) 4 °C overnight

To purify the PCR products, a Qiagen PCR purification kit (Qiagen, Velno, Netherlands) removed all polymerases and PCR reagents. PCR product samples were run on a 0.8% agarose gel just as in the mutagenesis step. The products were then digested by DpnI and HindIII in NEB buffer 2 in the following quantities: 25 μL purified PCR reation, 3 μL 10X NEB buffer 2, 1.2 μL sterile water, 0.4 μL HindIII, and 0.4 μL DpnI. The reactions were incubated overnight at 37°C then purified with the Qiagen PCR purification kit (Qiagen, Velno, Netherlands). Then, T4 ligase (0.5 μL) and 10X T4 ligase buffer (1 μL) were then added to 8.5 μL of cut pTS plasmid. The plasmid mixture was incubated overnight at 16°C.

E. coli XL1-Blue cells were transformed with the pTS vector by allowing the cells to incubate with 5.1μL of β-mercaptoethanol for 10 minutes on ice, and then mixing 1 μL of plasmid to the 50 μL of cells and incubating for 30 minutes. The tubes were heat shocked at 42°C for 45 seconds and incubated on ice for 2 minutes. SOC medium was added (0.45mL) before the cells were incubated on the roller drum for one hour at 37°C and then plated on agar X-gal/Amp and X-gal/Amp/IPTG plates. After overnight incubation at 37°C, the number of blue and white colonies produced by wildtype and mutant polymerase products were compared.

The processivity of the mutant polymerase was tested by the comparison of the ability of the mutant polymerase to amplify three different sized templates (2, 4, and 6 kb) to the wild type. This was accomplished by adding 1 μL of template, 5 μL 10x processivity buffer (200mM Tris-HCl pH 8.5, 100mM KCl, 100mM $(NH_4)_2SO_4$, 1% Triton X-100, 1mg/mL BSA, and 20mM $MgSO_4$), 5 μL dNTPs, 5 μL 10X primer mix (5 μM of pET-Upstream forward primer:

5'ATGCGTCCGGCGTAGA-3' and 5 μM of T7 Terminator reverse primer: 5'GCTAGTTATTGCTCAGCGG-3'), 33 μL water, and 1 μL *Pfu* polymerase and placing each tube in a separate program:

1) 96 °C for 1 minute
2) 96 °C for 15 seconds
3) 55 °C for 20 seconds
4) 72 °C for particular extension time
5) Go to step 2 twenty-nine times
6) 4 °C overnight

Tubes with 2kb template were allowed an extension time of 2 minutes, those with 4kb template, 4 minutes, and those with water or 6kb marker, 6 minutes. Finally, a 0.8% agarose gel (with 1X SYBR-safe in 1X TAE, melted) was run to assess the PCR product lengths.

Results

In order to mutate H147 in the *Pfu* polymerase gene, a QuikChange® was performed. After successful mutation of Histidine to Phenylalanine, two assays were performed in order to compare the fidelity and processivity of the mutant polymerase to those of the wild type.

Fidelity Assay of the H147F mutant

In order to score the fidelity of the Phenylalanine mutant, an assay was devised that tested the polymerase's accuracy in replicating the lacZα gene fragment using a colorimetric assay. A 0.8% agarose gel was run with the PCR products (Figure 1). The Phenylalanine mutant did not replicate as well as the wild type polymerase. The wild type polymerase did not give very thick bands either, but the wild type replicated more than the mutant. After transforming the PCR products into XL1-Blue *E. coli*, expression of β-galactosidase on X-gal/Amp (XA) plates and X-gal/Amp/IPTG (XIA) plates was observed. Colonies that appeared blue meant β-galactosidase was being expressed as the enzyme turns X-gal into blue dye. None of the cells with the

plasmids showed any more than four white colonies and zero blue colonies on either set of plates. The control for DpnI digest (no DNA template) showed a few colonies indicating that the template digest was not 100% efficient. The transformation control, the pUC18 plasmid, showed ~1400 white colonies and 6 blue colonies on the XA plate and ~210 white colonies and ~860 blue colonies on the XIA plate.

Processivity Assay of the H147F mutant

In order to score the processivity of the mutant polymerase, an assay was designed to compare the mutant and wild type polyermases' abilities to replicate templates of various sizes. A 0.8% agarose gel was run with the PCR products of the mutant and wild type polymerase and various sizes of template. The negative control (the PCR products with water instead of template DNA) produced no replication. The Phenylalanine mutant polymerase displayed lesser processivity than the wild type. The mutant replicated both the 2kb and 4kb templates entirely but did not produce as many copies as the wild type. Both the wild type and mutant did not replicate as much 6kb template as they did the 2kb and 4kb templates, nor did they replicate the template in its entirety. The wild type polymerase 6kb PCR products showed two bands fairly close together, indicating that the polymerase occasionally fell off the template just before completion. The mutant polymerase 6kb PCR products showed a faint band at 6kb, another fainter band just below it, and a band at about 2kb. It can be concluded that both polymerases had difficulty replicating the 6kb band, but the wild type was more successful. For a list of the class's data of mutants and processivity, refer to Table 1.

QuikChange Mutagenesis

The first step in the experiment was to create the mutant *Pfu* polymerase which was achieved via mutagenesis. After mixing the QuikChange® primers with the pET-*Pfu* template

DNA and placing them in a PCR program, a 0.8% agarose gel was run to confirm that replication occurred. The lack of a band when the gel had completed running shows that the replication was minimal (Figure 3). After DpnI digestion and transformation into XL1-Blue *E. coli* cells, plate with cells that were transformed with only DpnI digested template had a few colonies, proving the DpnI digest effective but not 100% efficeint, while the cells transformed with QuikChange PCR products gave rise to about 4600 colonies. The positive control of the pUC18 plasmid gave rise to around 4000 colonies with an efficiency of $3.8*10^7$ colonies per microgram and the negative control of water as DNA gave rise to no colonies. Therefore, the reactions were not contaminated. MGH Core Sequencing facility sequenced four of the colonies, and none had a mutation in the H147 location. From this point on, another group's H147F mutant was used, which had a codon for Phenylalanine in place of Histidine. The class as a whole produced sequenceable mutations in the H147 position with a frequency of 0.239. See Table 2 for a full list of the class's mutations.

Expression and Purification of H147F Pfu Polymerase

In order to test the mutant polymerase, the mutagenized gene was expressed in BL21(DE3) *E. coli* cells and then purified. After purification of the mutant protein through a His-Trap column and a PD-10 column, a Coomassie Blue test showed protein in all eluate fractions. The absorbance of the mutant protein (at 1/5 dilution with 2x storage buffer) was 0.231. Using Lambert-Beer Law, the concentration was calculated to be 4.31 μM. An SDS PAGE was then performed to assure complete purification of the mutant polymerase (Figure 4). The bands at 91 kDa correlate to the *Pfu* polymerase. There was protein in cell lysate, cell lysate-supernatant, eluate, and PD-10 fractions. The eluate also had another light band which means the protein is not completely pure.

Illustrations

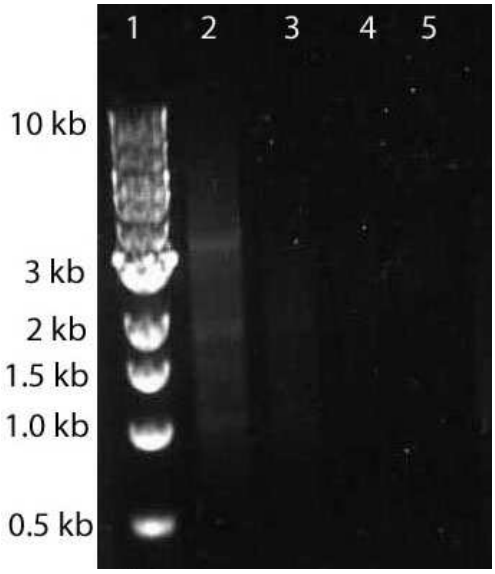


Figure 1 - Fidelity Assay
Agarose gel (0.8%) with lanes filled as follows: 1. 1kb ladder 2. PCR product from wild type *Pfu* polymerase with template, and primers; 3. PCR product from mutant *Pfu* polymerase with template, and primers; 4. PCR product from wild type *Pfu* polymerase with template only; 5. PCR product from mutant *Pfu* polymerase with template only. The lack of any bands shows that the polymerases (both wild type and mutant) did not successfully replicate the template they were given. Photo taken March 6, 2008

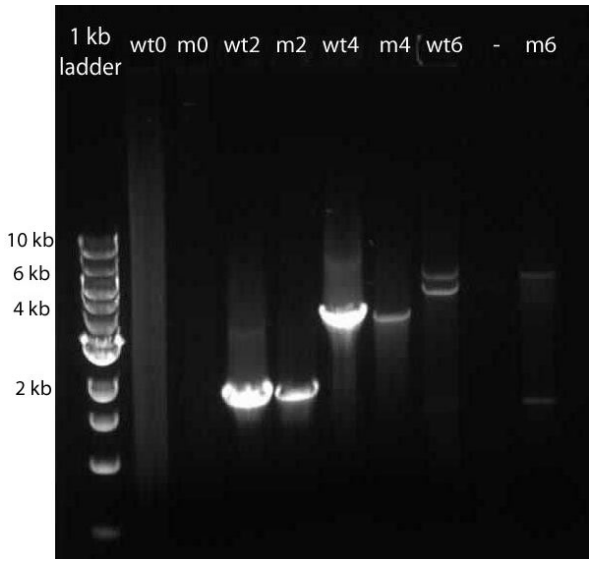


Figure 2 - Processivity Assay
Agarose gel (0.8%): lanes are marked as follows: Polymerase: wt indicates wild type polymerase was added, m indicates mutant. Template: the number corresponds to template length in kb (with 0kb meaning water was added in lieu of template). Bands of equal brightness and distance show comparable processivity. The wild type was more processive than the H147 mutant, shown by line thickness and the replication of the 6kb template. The wild type gave 5kb and 6kb products while the mutant gave a 6kb and 2kb products. Photo taken March 13, 2008

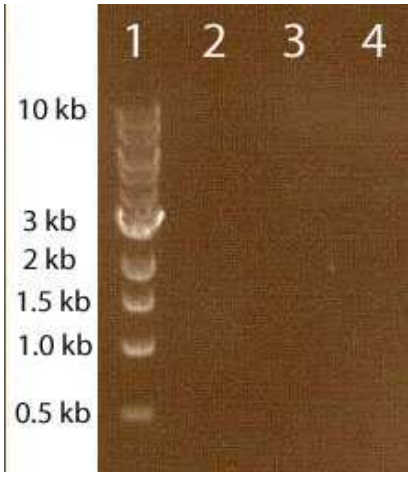


Figure 3 – PCR amplification of QuikChange® Mutagenesis products
Both primers and template are present in lane 2, only template is present in lane 3, and only primers in lane 4. No bands are visible on the gel indicating unsuccessful amplification. If it had been successful, a band at ~8kb in lane 2 would have appeared. Photo taken February 12, 2008.

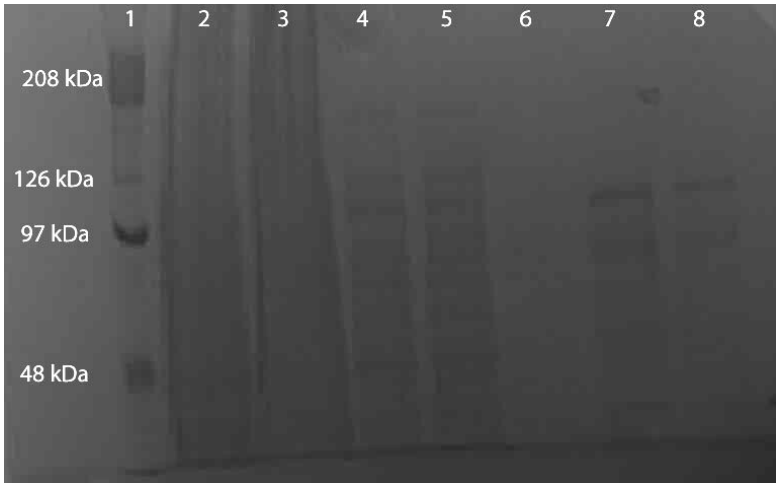


Figure 4 - SDS PAGE Purification Analysis
The lanes contained the following: 1-Molecular weight marker; 2-cell lysate; 3-cell lysate-pellet; 4-cell lysate-supernatant; 5-flow through; 6-wash; 7-eluate; 8-PD-10. Bands as ~91kDa correspond to the *Pfu* polymerase. Darker band corresponds to more protein, so the total amount of protein decreases in each lane. Multiple light bands in lanes 7 and 8 show impurities. Photo taken March 4, 2008.

Table 1. H147 mutants and their replication of 2kb, 4kb, and 6kb templates

Mutant	2kb	4kb	6kb
H147F	+	+	-
H147Y	+	+	-
H147V	+	+	-
H147S	+	+	+
	+	+	-
	+	-	-
H147W	+	+	+
	-	+	+
	-	+	-
H147A	+	+	+
	+	+	-
	+	+	-
	+	+	-
	+	+	-
	+	-	-
H147N	+	+	+
	+	+	+
	+	+	+
	+	+	-
	+	+	-
H147I	+	+	+
	+	+	-
H147R	+	+	-
	+	-	-
	+	-	-
	-	-	-
H147C	+	+	-
	+	+	-
	+	+	-
	+	+	-
H147L	+	+	-
H147T	+	+	-
	+	+	-
	+	-	-
H147K	+	+	-
	+	+	-
	+	-	-
H147G	+	+	-
	+	+	-

Most groups saw a 2kb and 4kb band for wild type polymerase. Only 4 groups observed a 6kb band for wt polymerase.

Table 2. Full Mutagenesis Results of Class

Amino Acid	Total Number of AA	Codon	Total # of Codon
Ala	7	GCA GCC GCG	3 2 2
Arg	2	AGA	2
Asn	4	AAC	4
Asp	1	GAC	1
Cys	4	TGC TGT	3 1
Glu	1	GAA	1
Gly	2	GGG GGT	1 1
Ile	3	ATA ATC ATT	1 1 1
Leu	1	TTG	1
Lys	2	AAA AAG	1 1
Phe	1	TTC	1
Ser	6	AGC AGT TCC TCT	3 1 1 1
Stop	3	TAG TGA	2 1
Thr	2	ACA ACC	1 1
Trp	1	TGG	1
Tyr	2	TAC	2
Val	2	GTC GTG	1 1
His	108	CAC	108
Unable to Sequence	32		
Total number of samples	184		

In 184 samples, the above results were obtained from the QuikChange® Mutagenesis. Almost 60% of samples did not show any mutagenesis and almost 20% of samples could not be sequenced.

Discussion

The discovery of new faster and more accurate polymerases will be very useful in the laboratory, as scientists can have tighter control over their experiments. Scientists can only do as much as laboratory conditions will allow, and having a more accurate polymerase will take away a "contaminating factor" from their work. Also, if fidelity and processivity can be altered, scientists can find a polymerase or other mutated molecule that specifically suits their needs in the lab, which will make scientific hypotheses easier to pursue. The aim of this study was to discover whether a change in one amino acid residue, H147, would affect the activity of *Pfu* polymerase. For the Phenylalanine mutant, processivity was slightly worse than the wild type, and the fidelity was inconclusive.

In the fidelity assay, none of the ligand mixtures produced more that four white colonies and no blue colonies, which means no more than four colonies took up the plasmids replicated by the polymerases, and all of the plasmids taken up had functional lacI genes. The most probable explanation is that the mixtures were most likely over digested with the restriction enzymes, which did not allow the template to re-ligate so it could not be taken up by the *E. coli*. There was a large number of white colonies on the IPTG plates with the pUC18 transformation control, but all blue colonies were expected, since pUC18 has the lac operon and IPTG caused constitutive expression of β-galactosidase. This means that X-gal was not added to the plates in proper quantities, so β-galactosidase expression could not be properly quantified. The few blue colonies on the pUC18 plates without IPTG showed that the lac operon system is "leaky" and some β-galactosidase could be expressed despite the lac repressor being active.

For the ligand mixtures, plates with IPTG were expected to display constitutive expression of β-galactosidase if the lacZα was replicated. Plates without IPTG were expected to

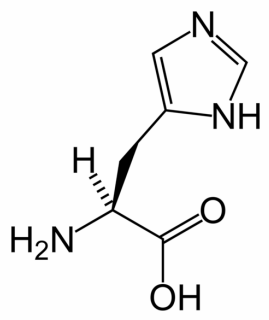
display constitutive expression of β-galactosidase if the lacI gene was not properly replicated and uninducible expression if it was. In order to make a proper quantification, 100 colonies were needed. Since only 4 were cultured from each of the ligands, no conclusion can be made about polymerase fidelity.

To ensure template was replicated by the polymerase, a PCR was run. The Phenylalanine mutant's PCR products did not display as bright of bands on the agarose gel as the wild type did, which could mean that either the mutant did not replicate as quickly as the wild type or that the concentrations were not equal due to improper purification. The latter theory is evidenced by the SDS PAGE that was run in the expression and purification step. PAGE can display protein impurities but not nucleic acids. This means DNA contamination could have occurred and given a falsely high absorbance measurement. This would cause the calculation of concentration of the mutant protein to be inflated and less protein to be used in the experiment, and thus less replication.

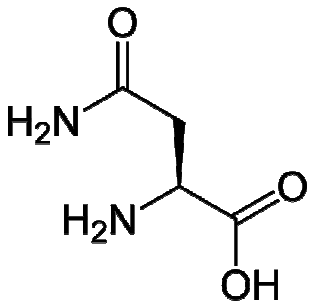
In the processivity assay, both polymerases replicated the 2kb and 4kb templates completely, but the mutant polymerase did not create as many copies as the wild type, as illustrated by the relative brightness of the bands in Figure 2, this can once again be attributed to speed of replication or to a lower concentration of mutant polymerase.

Neither the wild type nor the mutant polymerase displayed perfect processivity of the 6 kb template, but the wild type was more successful. The inability to completely replicate the template could be due to an extension time that was too short, causing the polymerase to fall off before it could complete replication. The wild type polymerase could get to 5kb before it fell off, while the mutant could only complete 2kb, possibly meaning that the mutant polymerase replicated slower than the wild type.

The class results show that Asparagine mutants were the most successful in replicating 6kb template, most likely due to structural similarities between the amino acids, show below:



Histidine



Asparagine

Both side chains contain nitrogen and are very electronegative, which is useful in hydrogen bonding, the same bonds used by nucleic acids. Kuroita et al. established that H147 was useful in exonuclease activity (2005). This would then make sense that amino acids with electronegative atoms increase the polymerase's ability to stay on the DNA for long periods of time since the amino acid can bond bases to be added in replacement of a mismatched base.

In the QuikChange® mutagenesis, no mutant *Pfu* genes were produced, most likely because no mutant DNA was picked up by the *E. coli* cells. This is evidenced by the presence of colonies on the DpnI control plate and the equal number of colonies on the pUC18 plate and the mutant plate. Cells do not transform nicked DNA as well as they do circular DNA, so it was expected there would be more colonies on the pUC18 plate than the QuikChange® PCR product plate, since the pUC18 vector is circular and the QuikChange® products were nicked. Since the number of colonies on the plates were equal, one can assume the DNA taken up on the mutant DNA plate was circular, and thus not the QuikChange® products. The DNA taken up would have been the original template which was not digested by the DpnI. The DpnI was proved to be not completely effectively by the presence of colonies on the plate with cells transformed with

only template and DpnI. Since no mutant genes were transformed, the sequencing results of four of the colonies all lacked a mutation in the H147 position. This same issue occurred in almost 60% of the class's samples. In order to prevent this, the DpnI should be allowed to digest the DNA for a longer period of time to ensure complete digestion of the parental template.

The class mutations gave rise to 17 of the 20 amino acids. Glutamine, Proline, and Methionine were absent. Glutamine and Proline's only codons have a C in the first position. The primers that were used did not contain a C in the first position of position 147 to ensure that Histidine was not accidentally reproduced. Arginine, the other amino acid encoded with a C in the first position of the codon, has other codons, which explains its appearance in the samples. Methionine most likely didn't appear due to mere chance.

The goal of this experiment was to compare the processivity and fidelity of the H147 mutant *Pfu* polymerases, specifically H147F, to the wild type. Class data was then compiled to determine which amino acids suited as sufficient replacements for this specific Histidine which was thought to play a role in polymerase exonuclease activity. Although Kuroita et al. found that a Lysine replacement created a polymerase with higher fidelity of KOD (2005), this experiment showed that a Lysine replacement had at most comparable processivity to the wild type. Because the fidelity results were inconclusive, it cannot be determined whether the H147K mutant created more accurately copied PCR products as it did in KOD.

This experiment should be performed again in order to determine which amino acid replacement produces the most accurate DNA copies. Fidelity is very important in DNA replication, because without accuracy, one can never be sure of the products of a PCR, and this could lead to many complications in an experiment.

Work Cited

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