Cloning of The Taq Polymerase Gene from Thermus aquaticus into the PUC18 Plasmid

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Cloning of The *Taq* Polymerase Gene from *Thermus aquaticus* into the pUC18 Plasmid

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Abstract:

Taq polymerase is an enzyme derived from the thermophilic bacterium *Thermus aquaticus*, which functions as an important component for DNA replication in that organism. Due to its structural integrity at high temperatures, *Taq* polymerase is highly valued among biologists as an ingredient in PCR. By using only a slight amount of *Taq* polymerase once at the beginning of the reaction, it can allow the added genetic material to multiply exponentially. The purpose of this experiment is extract a portion of the genetic material from *Thermus aquaticus* and place the 2500 bp segment within a pUC18 plasmid. This modified plasmid would then be introduced into DH5α *E.coli,* essentially allowing this bacterium to produce *Taq* polymerase without the stringent requirements to actually grow and harvest enzyme from *Thermus aquaticus*. Though there was amplification of a 2500 bp fragment and several attempts were made to cut and ligate it into pUC18, the supposedly transformed bacterial colonies picked in the end did not yield the desired results.

Introduction:

Polymerase Chain Reaction, or PCR, was discovered by Kary Mullis in 1983 while he was working for the Cetus Corporation, and proved to be an invaluable tool for biologists. The versatility of PCR includes gene cloning, gene expression analysis, DNA sequencing, DNA fingerprinting, and the detection of infectious and genetic diseases (Ferralli *et al.*, 2007). It allows for the rapid amplification of target nucleic acid sequences using three steps. The denaturation cycle is the initial step and causes the DNA templates to melt at high

temperatures and break into single strands. The annealing step then allows the short primers to attach to the free floating single strands, allowing newer and longer strands of DNA to form. The final step is elongation, where the polymerase synthesizes new DNA as the complement of the existing nucleotides, forming strands of DNA identical to the template sample. By constant repetition of these three steps, PCR can yield vast amounts of DNA from a minute original sample (Lo and Chan, 2006). Mullis ended up winning the Nobel Prize in Chemistry for PCR in 1993. Before 1989, however, the task of PCR was slow due to the constant denaturation of the DNA polymerase at the higher temperatures. The DNA polymerase initially used was taken from *E.coli* and experienced degradation whenever the denaturation cycle took place. Scientists needed to continually add new polymerase to replace the depleted stock. The substitution of *Taq* DNA polymerase into the PCR allowed the reaction to go uninterrupted, due to the thermostability of the enzyme (Lo and Chan, 2006). Discovered in Yellowstone National Park, *Thermus aquaticus* is a thermophilic bacterium; an organism that can thrive in high temperatures. Because the polymerase of the bacterium needed to be thermophilic as well due to its environment, it was ideal for PCR. *Science* named it "Molecule of the Year" in 1989, and the *Taq* polymerase market still generates millions of dollars a year due to its need in so many fields. The development and sale of the *Taq* DNA polymerase, however, is not without controversy. The National Parks Service initially saw no revenue come their way as *Taq* DNA polymerase continued to show great commercial value, even though the discovery and the extraction of *Thermus aquaticus* was done in a National Park. Now, all researchers are required to give some portion of revenue back to the Parks Services for the continued use of their environment (Ferralli *et al.*, 2007).

The purpose of this project was to allow the DH5α *E.coli* strain to produce *Taq* polymerase using DNA splicing techniques. Though *Thermus aquaticus* can be grown in normal laboratory settings, the yield of its polymerase is too small for practical uses. Furthermore, there is an increased level of difficulty when trying to grow *Thermus aquaticus* as opposed to DH5α *E.coli. Thermus aquaticus* can only be grown in a complex agar called Castenholz TYE medium at 70°C. The agar consists of multiple chemical elements due to the chemotrophic nature of the bacterium, whereas *E.coli* can be grown in basic media (Engelke *et al.*, 1990). The first part of this project was to grow a sizable amount of bacteria for the experiment. The next part of the project required us to extract the genetic material from the bacterial cells. To make sure that enough DNA material was present, we used PCR to amplify the desired material. Our next step was to cut out the desired 2500 bp segment using Eco RI and Bgl II restriction enzymes. By isolating and replicating the desired band using PCR, we could then splice the fragment into a pUC18 plasmid by cutting it with Eco RI and Bam HI. Then, we could insert the plasmid into the DH5α *E.coli* and select for transformed colonies. We used blue-white selection using ampicillin plates with X-gal; the transformed cells contained ampicillin resistance, which would allow them to grow on the plates unharmed while the ampicillin eliminated other unwanted bacterial contamination. If the bacterial colonies were blue in color, then the cells were not transformed properly and did not contain the plasmid. However, if they possessed a white color and the colonies were somewhat isolated from each other, then they were transformed (Desai and Pfaffle, 1995). The purification steps after obtaining *Taq* polymerase producing *E.coli* will allow us to use heat to exterminate the bacteria and keep the polymerase

intact, which is something that could not be done without the thermophilic nature of the polymerase.

The uses for the *Taq* DNA polymerase producing *E.coli* range from forensics labs to college campuses; *Taq* DNA polymerase is used frequently here at Albright College as well, mostly in upper level biology courses. Because the patent Cetus had on the production of *Taq* polymerase expired in early 2008, this project could potentially provide Albright College with its own legal source of polymerase (Ferralli *et al*., 2007). Projects ranging from laboratory experiments to independent studies make use of PCR, which require *Taq* polymerase. The creation of the polymerase producing *E.coli* could possibly contribute to the various projects that occur within the biology department. An example is the population genetics studies with field mice done by Dr. Stephen Mech, where PCR is utilized to amplify mice genes. Dr. Gerald Kreider teaches molecular genetics, another instance where certain lab experiments require the use of *Taq* polymerase for amplification of DNA. Studies done with morphologically mutant tobacco plants by Dr. Andrew Samuelsen require the extraction and amplification of the tobacco plant DNA via PCR to compare and contrast genetic differences, requiring the use of *Taq* polymerase. Obtaining our own source of *Taq* polymerase producing *E.coli* could potentially allow us to possess a continuous stock of polymerase, as well as reducing the costs associated with purchasing commercial polymerase.

Materials and Methods:

DNA isolation from *Thermus aquaticus:*

The first step in this project was to grow a large viable sample of *Thermus aquaticus* in order extract enough genetic material for proper DNA isolation. The bacterium samples were purchased commercially from ATCC, and grown in Castenholz TYE media. The media was formed by mixing five parts double strength Castenholz Salts with one part 1% TYE and four parts water. The final pH was adjusted to 7.6 and the bacterium was grown in 100 ml of the liquid media at 70°C in a shaking incubator overnight (Phithero, 1993). 2.0 ml of the sample was preserved in 100% glycerol and stored away in the -20°C freezer to ensure the availability of more *Taq* if needed. The sample was then subjected to genomic prep (Kreider, 2008). The procedure of the genomic prep first required us to centrifuge the liquid culture at 10,000 rpm with the Sorvall SS-34 rotor to isolate the culture pellet. The pellet was then washed with 20 ml of saline and centrifuged again. This step was followed by two incubations, both at 37°C: 15 minutes with one ml of lysozyme and then one hour with 1.2 ml of pronase. The sample was then extracted three times with STE- saturated phenol. Each extraction required 10 ml of phenol to be added and the total sample to be centrifuged at 8,000 rpm for five minutes. The bacterial sample was then extracted twice with 10 ml of Chloroform: isoamyl alcohol in the same manner as the STE- saturated phenol extraction. These steps allowed the degradation of the physical cellular structure in order to allow the extraction of the DNA. The genetic material extracted rested as the aqueous top layer of each of the extractions. Each extraction allowed for the further purification and separation of the genomic DNA from the rest of the precipitate.

The final extraction required the physical pulling of the high molecular weight genomic DNA using a glass rod. Because DNA is naturally negatively charged, it binds to the twirling of the positively charged rod. The sample was then suspended in a solution of 3.0 ml TE buffer overnight to ensure purity (Kreider, 2008).

Another procedure we used to extract DNA was the Wizard Miniprep procedure from Promega (Kreider, 2008). Using six conical tubes with 12.5 ml of culture sample in each, we centrifuged the sample for 10 minutes at 2500 rpm using the IEC centrifuge. After removing all of the excess supernatant and preserving the pellet, the samples were then mixed in again with 400 µl of Cell Resuspension Solution and retransferred to a 1.5 ml microfuge tube. The samples were then treated with 400 µl of Cell Lysis Solution, in order to allow the extraction of the DNA. After that, the samples were treated with 400 µl of Neutralization Solution and centrifuged for 15 minutes at the maximum speed. This was done to ensure that the supernatant, which contained the DNA, was free of any of the white precipitate. The white precipitate was undesired cellular matter that did not contain the desired DNA. To ensure maximum purity, the supernatant was transferred through Miracloth to prevent solid material from passing through. The clean samples were then run through a VacMan Manifold, an apparatus which uses a vacuum to retain the desired DNA sample while extracting out all the unwanted material. Each sample ran through its own individual barrel down a minicolumn, which contained 1.0 ml of resin. The DNA in the minicolumn was then washed once with 2.0 ml of column wash. The minicolumn barrels contained the desired DNA plasmid, and after the samples were dried of all moisture using the centrifuge, they were centrifuged one last time with 50 µl of nuclease-free water which was used to wash and suspend the plasmid in a microfuge tube. The next step was

alcohol purification of the plasmid samples. Each sample was incubated in a -80°C freezer for 20 minutes after 5 µl of 3M sodium acetate and 110 µl of 95% ethanol was added to them. The samples were then centrifuged in a cold room to reduce the solubility of the nucleic acids in the samples. After the removal of the initial dose of 95% ethanol, the samples was treated with 0.5 ml of cold 70% ethanol and centrifuged for 10 minutes at maximum speed. To remove the remaining alcohol after the final purification step, the samples were treated to the SpeedVac for 10 minutes, where temperature and pressure changes caused accelerated evaporation of the remaining alcohol. The samples, now presumably dry, were resuspended in 20 µl of nuclease-free water (Kreider, 2008).

To be certain we obtained the sample, we ran electrophoresis gels to see if we could detect the 2500 bp band characteristic of the *Taq* genomic DNA (Engelke *et al.*, 1990). The gel we normally ran was a 1% agarose gel, where we dissolved 0.3 g of agarose in 30 ml of 1x TBE buffer. The gel was then treated with 30 µl of ethidium bromide to ensure illumination underneath UV light. After the gel solidified, it was immersed in 1x TBE within the electrophoresis unit. All the samples were treated with 5-10 µl of 10x tracking dye to allow us to visually see how far along the gel ran. The samples were placed within their respective wells, with a control and a marker. The marker was used to measure the sizes of each band and consisted of 1 µl Eco RI+ Hind III cut lambda marker, 4 µl orange-blue tracking dye, and 14 µl of nuclease-free water. The gel ran at 85 volts for one hour, which allowed the electrical current to separate the different bands properly with regards to their size (larger bands ran at a slower rate than smaller bands). There were some gels where we excised the 2500 bp band in the

hopes to extract any remaining genetic material (Kreider, 2008). We used the Qiagen Gel Extraction Kit, which allowed us to extract the DNA from the agarose.

PCR Amplification:

The process of DNA isolation demanded purification. The additional purification steps taken resulted in the continual loss of our desired samples. To ensure we had enough of the genomic DNA to cut and ligate with, we had to PCR our samples at various steps of the project (Lo and Chan, 2006). This was also a safety measure to prevent the complete loss of our sample at any point of our project. We followed a specific PCR recipe which seemed to work ideally for *Thermus aquaticus.* 22 µl of nuclease-free water was used as the primary base of the sample. 2 µl of both *Taq* 3' and 5' primers was added to provide the extension of the DNA strands. 15 µl of the genomic DNA itself was used as a template for replication, while 5 µl of thermophilic PCR buffer was used to produce an optimal environment for the reaction to take place. 2.5 µl of nucleotides was added to provide building blocks for the continual replication process. 1.0 µl of magnesium chloride was added as a means to allow template-polymerase interactions to occur (too much magnesium does the opposite). The final component in our PCR recipe was the store bought *Taq* polymerase itself. We experimented with both the bead form and the liquid form. The reaction only requires one wax bead containing the polymerase, or 0.5 µl of the liquid form. The combination of all these elements was placed within the thermocycler, an instrument that regulates the steps vital to PCR. We ran the parameters to do an initial hot start at 94°C for two minutes. The actual cycle was then initiated by one minute at 94°C, one minute at 55°C,

and finally three minutes at 72°C. The protocol was set to run at 40x cycles, and concluded after three hours and 20 minutes (Lo and Chan, 2006).

Cutting and ligation of DNA:

After enough genomic DNA was obtained, the next part of the project involved cutting the desired 2500 bp out of the genomic DNA and placing it into the cut section of the pUC18 plasmid (Desai and Pfaffle, 1995). To do this, we first had to cut the DNA with Eco RI and Bgl II restriction enzymes. The amplified genomic DNA contains Eco RI and Bgl II sites that were built into the primers. The recipe we used called for 8.6 µl of nuclease- free water, 4 µl of Buffer D to allow for an optimal condition for the enzyme to cut, 0.4 µl of acetylated BSA, 23 µl of the DNA sample itself, and 1.0 µl of Eco RI and Bgl II each. This was incubated overnight at 37°C to ensure proper cutting. At the same time, the cutting of the pUC18 plasmid was done using Eco RI and Bam HI because there is a Bgl II site within the pUC18 plasmid that prevents the use of Bgl II. The recipe called for 15 µl of nuclease-free water, 10 µl of the plasmid itself, 3 µl of 10x Buffer for the reaction to take place in, and 1 µl of Eco R1 and Bam HI each. We were able to use Bgl II for *Taq* DNA and Bam HI for the plasmid because they have compatible ends. Before the ligation between the *Taq* fragment and the cut pUC18 plasmid could take place, we had to remove all of the existing enzymes from each sample to prevent further cutting in later steps (Kreider, 2008). The samples were "cleaned" using the Micropore- EZ enzyme removal columns. Centrifuging the samples through the columns removed all traces of enzymes. One percent agarose quantification gels were then done to see if we obtained the right sized band with regards to the DNA fragment and the plasmid. The ligation between the DNA fragment and the

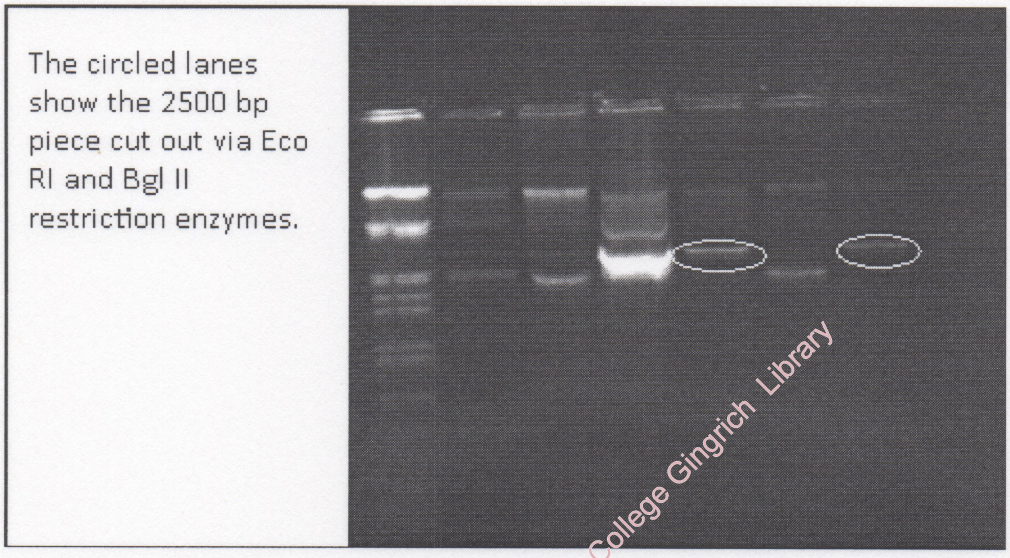
plasmid was an overnight process and consisted of 22 µl of nuclease-free water, 2 µl pUC18 cut plasmid, 20 µl of cut *Taq* DNA fragment, 1 µl of T4 DNA ligase for recombination, and 5 µl of 10x ligase buffer to provide an optimal environment for the ligase to work properly (Phithero, 1993).

Blue-white selection:

After the ligation, the modified pUC18 plasmid needed to be inserted into viable DH5α *E.coli* (Kreider, 2008). Before this step, however, we prepared some Amp-Blue selection plates using agar from Invitrogen. 200 ml of the agar was produced and distributed evenly among 10 petri dishes. This would allow visual selection of transformed colonies. The production of transformed cells first involved a 1:5 dilution of the modified plasmid with TE buffer to form a final volume of 5.0 µl. The samples were then combined with a tube of 20 µl cells/tube of DH5α *E.coli*. The samples were left on ice for 30 minutes, and then heat shocked in a 42°C water bath for exactly 40 seconds. The samples were then put on ice again for an additional five minutes. 100 µl of S.O.C medium was added to each of the tubes, which then was placed on a platform shaker to be incubated at 37°C for one hour. Along with the control, each tube was then divided into a 20 µl and a 100 µl portion, and each portion was applied to one of the Amp-Blue selection plates to be incubated overnight at 37°C. The different portions of the same samples allowed us to visually see the differences between the concentrations. The next day we visually picked off the colonies that were been fully transformed. The blue colonies were transformed only by the plasmid alone, and were undesirable. The white colonies that we sought were bacteria that were transformed by cells containing both the plasmid and the insert. Bacteria

that do not contain either are theoretically susceptible to the ampicillin in the agar, while those with the plasmid inserted within them possess ampicillin resistance (Kreider, 2008). The desired colonies were picked off with sterile wooden sticks and transferred in 20 ml of LB liquid agar to allow further growth. The agar was also treated with 20 µl of ampicillin to ensure only the desired bacteria would grow. After the bacteria had grown in a 37°C incubator overnight, they were subjected to the Miniprep Wizard procedure in order to obtain purified genetic material. This material was then tested with a 1% gel to see if the bacterium actually contained the plasmid and the DNA fragment (Phithero, 1993).

Results:



Thermus aquaticus was grown successfully in the Castenholz media at 70°C. The concentration of the *Taq* DNA, after multiple extractions and amplifications, was measured to be an average of 7.5 ng/µl, which was possible after the DNA was cut successfully with Eco RI and Bgl II restriction enzymes. After the ligation process, the blue-white X-gal plates yielded eight white colonies, which were then grown separately in AmpBlue plates. The grown samples

did not contain any further traces of the modified plasmid when examined within a one percent electrophoresis gel.

Discussion:

The farthest point we reached in this project was the blue-white selection of the bacterial colonies. There could have been multiple reasons to why the selected bacterial samples did not contain both the desired plasmid and the DNA fragment. When inoculating the transformed DH5α *E.coli* on the Amp-Blue petri dishes, we were looking for the white colonies that were supposedly transformed properly. Besides full transformation, colonies can also appear to be white if their plates have been incubated too long and they developed ampicillin resistance. If that was the case, then the colonies we selected may have been contamination instead of our desired product (Desai and Pfaffle, 1995). Another possible reason that prevented us from achieving our desired results could have been errors in any of the various PCR protocols that were done (Engelke *et al.*, 1990). As the project progressed further, we needed to PCR our samples to compensate for their progressive loss. Initially, we did not receive viable results using the previous parameters of the thermocycler. Only by increasing the cycles up from 35x to 40x did we see results. The materials used in the PCR could have also led to the loss of our product. PCR samples are dosage sensitive, and require the precise proportion of viable ingredients for optimum results. Older materials can lead to the degradation of the DNA instead of its amplification, while an unbalanced proportion of the materials can lead to inefficient amplification. Too little nucleotide can restrict the building of DNA strands, while a high level of magnesium chloride can inhibit the process altogether. Due to the molecular scale

of this project, results can only be confirmed using gels. Unsuccessful PCR can produce inaccurate results on the gel which may have led us to discard previous samples (Engelke *et al.*, 1990).

Another possible reason for the lack of transformation in the bacteria could have been the lack of activity of the enzymes in both the *Taq* DNA and the pUC18 digests (Desai and Pfaffle, 1995). Both of the samples may have been cut at one point in the experiment, but there is always a chance for religation; the fragment cut out could have reinserted itself back to its original location at some point in the experiment, or the two cut ends could have possibly ligate together. The size similarity of the plasmid cut and the DNA fragment could have been another factor which prevented transformation. Because the 2500 bp DNA fragment fits almost exactly in the space in pUC18 cut by Eco RI and Bam HI, there was a possibility that the fragment did not get a chance to fit in properly. This is why the ligation experiment calls for the concentration of the DNA fragment to be about 20x more than the pUC18 plasmid, to increase the chances of ligation in the proper place. The miscalculation of the DNA: plasmid concentration ratio could have been the reason why the ligation might have failed (Desai and Pfaffle, 1995).

Because the scale of this project is on a molecular level, many aspects can result in the loss of material. Even the PCR followed by gel elution for the right band can leave much of the desired product behind. The reasoning behind this is to ensure as much purity as possible. The progression of the project only results in more of the initial product being lost. The only method of product restoration is either PCR or gel eluting the remaining sample to leave out as little as

possible. The scale of the project demands extreme care and precision when taking part in the multiple steps of this project. The only way to ensure you have the desired product at each step of the project is to run a quantification electrophoresis gel, which demands 5 µl of product each time. With the sample being depleted and restored at various steps of the project, it is impossible to see with the naked eye if the final product resembles the initial starting material.

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