Building Autonomy into a Cell-Free Protein Producing Gel: Integrating T7 RNA Polymerase

A Thesis

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Master of Engineering

by

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ABSTRACT

Cell-free protein production is a new technology that offers many advantages over conventional cell-based expression techniques, though low yields (in the range of μg/ml) have been a major drawback. With the advent of the p-gel system, a DNA hydrogel consisting of genes as part of the gel scaffolding, yields in the mg/ml range have been demonstrated. As p-gel expression relies on exogenous transcriptional and translational enzymes, cloning these proteins into the p-gel system would increase its autonomy. Here, we attempt to clone T7 RNA polymerase into a vector compatible with the p-gel system. Primers were designed to amplify T7 RNA polymerase from *E. coli* strain BL21 (DE3) as well as attach NcoI and SmaI restriction enzyme sites via PCR. The amplicon was digested and ligated with Roche's pIVEX2.4d, a plasmid that supports cell-free protein expression in *E. coli* lysate with bacteriophage T7 RNA polymerase. The plasmid was then used to transform chemically competent *E. coli* cells. Colonies carrying the plasmid of interest were selected for via colony PCR and submitted for sequencing. Unfortunately, our samples displayed an abundance of mutations that would likely affect T7 RNA polymerase's activity. Nonetheless, with additional work, the plasmid samples produced could be useful in p-gel expression studies.

BIOGRAPHICAL SKETCH

Daniel Baskind received his B.S. in Biological and Environmental Engineering from Cornell University.

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CHAPTER 1

Introduction

The P-gel System

Though well established, cell-based protein expression leaves much to be desired. Through evolution, wild-type cells have optimized the production of their native proteins, transforming metabolites into functional products with stunning efficiency. In coaxing an organism to produce a foreign protein, however, biologists are attempting to fit a square peg into a round hole. Often, proteins require significant modification to achieve even modest expression levels. In order to promote acceptable yields, a precise extracellular environment must be maintained and extensive purification is required to separate a protein of interest from native proteins and structures, resulting in a labor intensive, expensive, and time consuming process. Furthermore, cellular protein production is limited in its scope of expression. Membrane proteins, toxic proteins, proteins that undergo rapid proteolysis, and proteins containing unnatural amino acids are all nearly impossible to express using a cell-based system[1].

Cell-free protein production, with its high degree of customizability, is an interesting alternative to conventional cell-based techniques. The most common approach to cell-free protein expression has been solution phase systems (SPS), in which gene templates are put in solution with cell lysates containing the components required for transcription and translation. Yields with these systems, however, have not extended beyond the µg/ml range[2,3].

Our group has recently developed the p-gel system, a DNA hydrogel made up of genes (linearized plasmids) and branched DNA crosslinkers (X-DNA) that synthesizes protein in the presence of a compatible lysate (Figure 1B)[4,5]. Through the use of compatible sticky ends, these components can be assembled into a gel through a standard ligation reaction (Figure 1A). Unlike SPS, this system's structure offers gene protection for longer production viability as well as a faster turnover rate of expression enzymes through high local gene concentration[5]. The result is a cell-free system with significantly higher yields in the mg/ml range. Furthermore, the p-gel system has demonstrated the ability to generate a wide-spectrum of proteins, though post-translational modification remains a challenge.

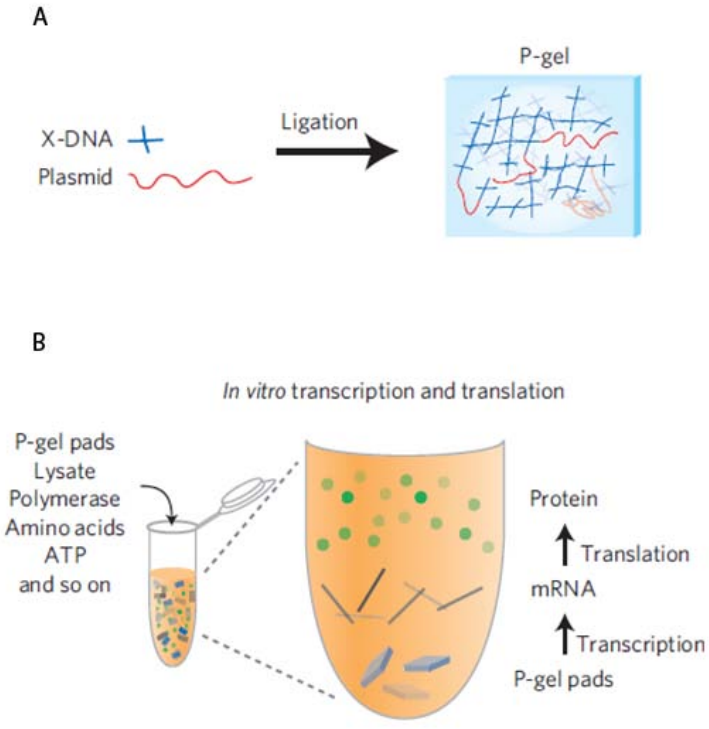

Figure 1: Synthesis and operation of the p-gel. X-DNA and linear plasmids are combined in a ligation reaction to create the p-gel (A). In the presence of a compatible lysate and expression metabolites, protein synthesis occurs (B).[5]

T7 RNA Polymerase

With this system in its infancy, the potential exists to further increase expression levels. One approach that has shown promise is to replenish the expression enzymes and metabolites by exchanging the lysate[6]. However, with the high expense of lysates, the ability of the p-gel system to produce *its own* expression enzymes is an intriguing possibility. Thus, the incorporation of the T7 RNA polymerase (T7P) came into consideration.

T7P, the chief transcription enzyme from bacteriophage T7, is known for its robust activity and strict promoter specificity[7]. Furthermore, it consists of a single subunit and does not require post-translational modification, making T7P an ideal candidate for expression with the p-gel system.

CHAPTER 2

Materials and Methods

Vector Selection

In order to clone T7P into the p-gel system, it must first be incorporated into a carefully selected plasmid. An appropriate candidate required four key characteristics: 1) compatibility with *Escheria coli* expression enzymes, 2) inclusion of the T7P promoter so that T7P can transcribe its own gene (a positive feedback loop), 3) a specific affinity tag along with a means for removal (i.e. cleavage site), 4) an antibiotic resistance gene for colony selection, and 5) the means to incorporate the T7P gene.

This led to the selection of the Roche RTS pIVEX2.4d plasmid cloning vector. According to Roche[8], this plasmid is designed for use with Roche's RTS cell-free protein expression system, which includes prokaryotic cell lysate. Furthermore, as shown in Figure 2, the plasmid includes a T7P promoter sequence, a factor Xa cleavable N-terminal His_6-tag, an ampicillin resistance gene, and a multiple-cloning site.

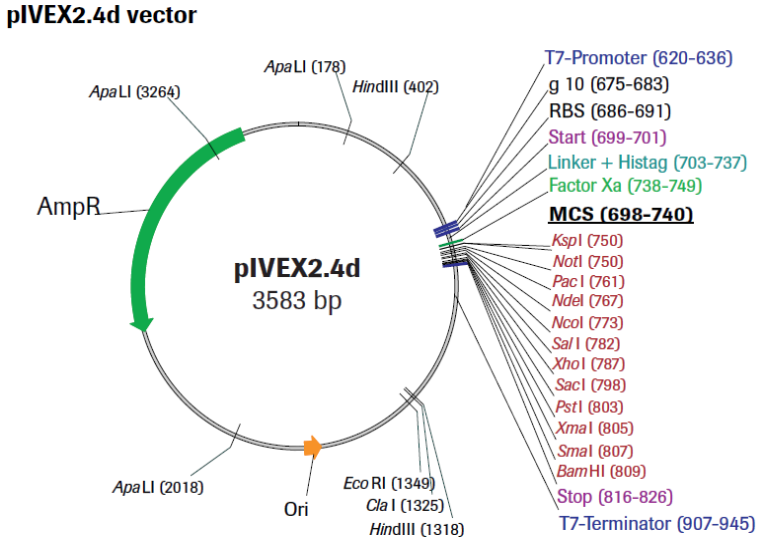

Figure 2: Plasmid map of the pIVEX2.4d vector. The features of this vector include a T7P promoter sequence, a cleavable N-terminal His_6-tag, an ampicillin resistance gene, and a multiple-cloning site.[8]

Primer Design

To produce a T7P sequence suitable for ligation with the plasmid, PCR primers were designed to incorporate suitable restriction sites based on the wild-type T7P sequence[9] (Appendix A1). Roche identifies NcoI and SmaI as the ideal candidates for use with pIVEX2.4d[8]. Accordingly, these restriction sites were placed on the primers to flank the gene and place it in the reading frame established by the T7P promoter included on pIVEX2.4d. Furthermore, the location of NcoI on pIVEX2.4d relative to the N-terminal His_6-tag and Xa cleavage site required that the T7P start codon be included in the restriction site. Unfortunately, the NcoI sequence includes a guanine base following this A-T-G start tag, which differs from the adenine included in the wild-type sequence for T7P. This resulted in a minor missense mutation from asparagine to aspartic acid, which substitutes a carboxyl group for the relatively similar carboxamide functional group. Furthermore, while the N-terminal domain does play a role in binding template DNA, the key amino acids are located further downstream[7], suggesting that this mutation wouldn't significantly affect the activity of T7P.

Once the primer sequences were selected, they were tested for secondary structure formation resulting from self-complimentarity as well as nonspecific binding on the interior of the T7P sequence using Primer3 v. 0.4.0[10].

Amplification of Gene of Interest

In order to amplify the T7P gene in anticipation of our cloning procedure, *E. coli* strain BL21 (DE3) was used as the genetic source. As the genetic source did not contain the restriction enzyme sites to be used for ligation, a melting point (Tm) was calculated based on bases directly complementary to the genetic source[10]. However, this only accounts for a high efficiency first

passage with the bacterial genome acting as the PCR template. When the amplicon is available as the PCR template, the entire length of the primers is complementary and results in a higher Tm. In order to compensate for this discrepancy, shorter primers (with a Tm similar to the one previously calculated) were included in the PCR reaction[10] (Appendix A2).

To amplify the gene, New England Biolabs Taq 5x Master Mix (N-DNAP) was used with a protocol based on the manufacturer's recommendation. However, due to the inclusion of multiple primer sets, yield optimization was performed with respect to the annealing temperature via gradient PCR (Figure 3). The optimized PCR protocol for N-DNAP is detailed in Appendix B1.

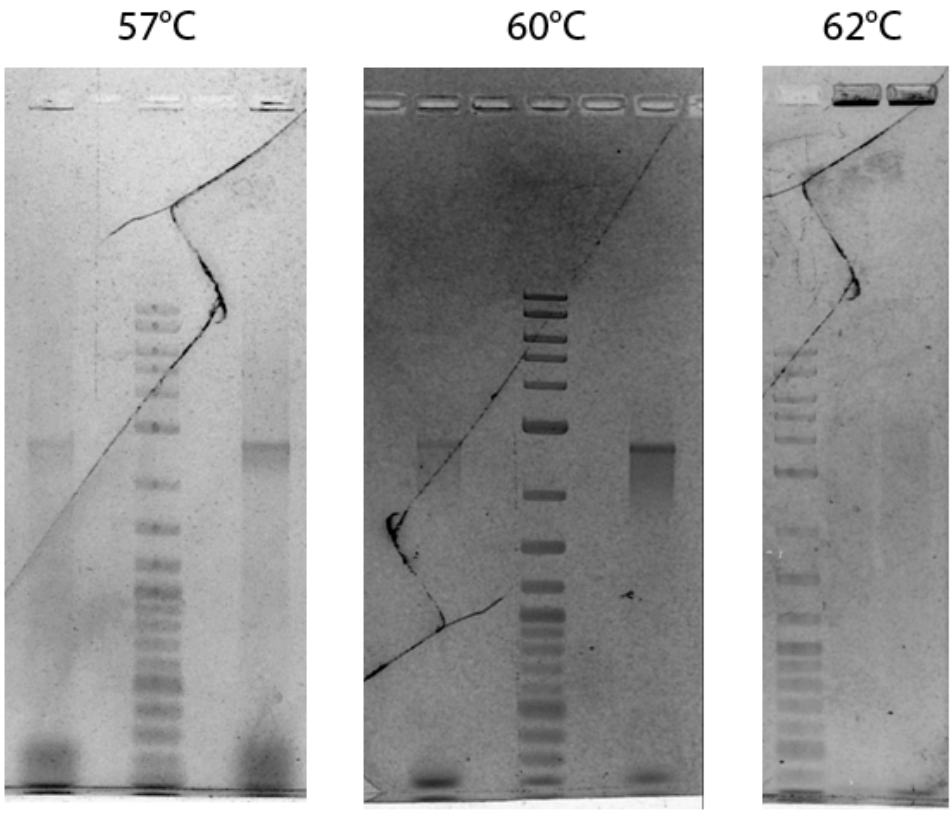

Figure 3: Optimization of the PCR annealing temperature as shown on an electrophoresis gel. A PCR program was written with a temperature gradient of 55°C to 65°C during the annealing step and samples were run in duplicate. Afterwards, the yields were analyzed via gel electrophoresis and three representative annealing temperatures are shown here. 60°C provided the best yields and was chosen for our PCR protocol.

Due to mutation concerns, this procedure was also done using Finnzymes Phusion High-Fidelity DNA polymerase (HF-DNAP). This polymerase boasts an error rate of 4.4×10^{-7}, 5 times lower than that of the N-DNAP. Annealing temperature optimization was done for this procedure as well and the final protocol can be found in Appendix B2. For both N-DNAP and HF-DNAP, an 800 µl reaction was performed. To purify our ~2.6 kb amplicon, the sample was run on a 1% agarose gel (containing 15% GelRed) for 100 mins at 80V before being excised at and extracted via the GenScript® 5M Gel Extraction Kit.

Amplification of Vector

From a previous experiment, a highly stable *E. coli* clone (Appendix C) containing pIVEX2.4d was available. Furthermore, the plasmid carried the gene for renilla luciferase, an approximately 1 kilobase insert. As the gene was inserted using the same set of sticky ends as were intended for T7P in this experiment, a successful digestion would provide the desired vector for ligation with T7P. To reanimate the frozen stock, scrapings were used to inoculate LB media (100 µg/ml) and incubated at 37°C on a 200 rpm shake platform overnight. The plasmid was then purified through the GenScript® Quickclean 5M Miniprep Kit.

Plasmid Preparation

After amplifying our vector and insert, digestion reactions were prepared. New England Biolabs High-Fidelity NcoI and SmaI were chosen for the double digestion as they display high efficiency in the same buffer with minimal star activity across a large temperature range. However, the optimal reaction temperatures were different, so the reaction was completed in two steps. In separate 20µl reactions for the vector and the insert, both enzymes were included at a

concentration of 10 U/µg of DNA substrate. The reaction was then incubated for 5 hours at 25°C followed by 5 hours at 37°C, the ideal reaction temperatures for SmaI and NcoI, respectively. To purify our products, the digestion solutions were run on a 1% agarose gel (containing 15% GelRed) for 100 mins at 80V before being excised and extracted via the GenScript® 5M Gel Extraction Kit as before (Figure 4).

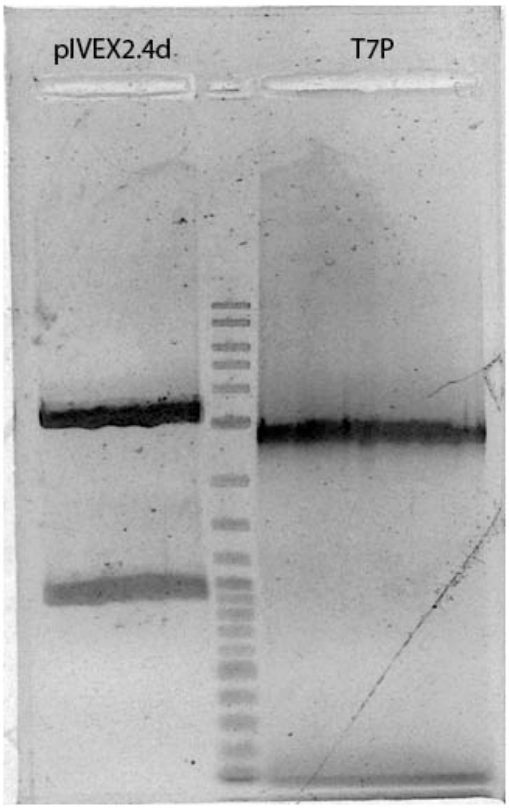

Figure 4: Double digestion of the pIVEX2.4d and T7 RNA polymerase as shown on an electrophoresis gel. The DNA bands are consistent with the expected size of our vector (~3.5 kb) and insert (~2.7 kb). The lower band in the left lane is ~1 kb, the expected size of the insert (renilla luciferase) removed from the circular plasmid substrate.

In order to prepare our plasmid for transformation, several 10 µl ligation reactions were prepared with a spectrum of insert-to-vector molecular ratios ranging from 10:1 to 1:10. In each case, a minimum of 100 ng of each DNA segment was incubated with 1U of Lucigen T4 ligase

and buffer at 25°C for 2 hours. The efficiency of the ligation reaction was determined through comparison to the control, a ligation reaction without the insert (Figure 5).

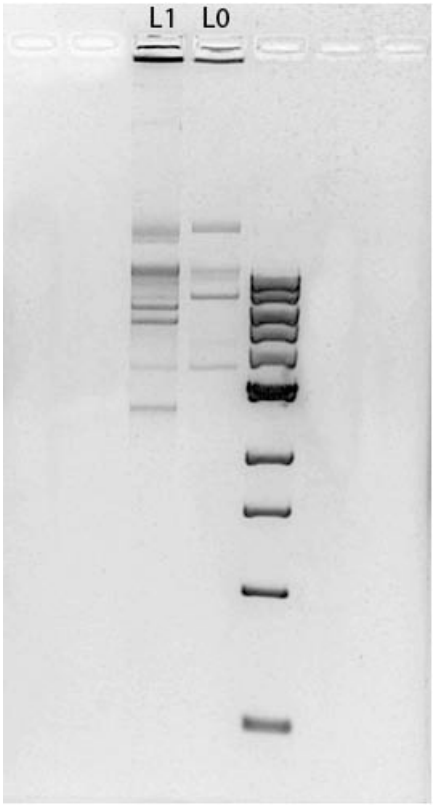

Figure 5: Ligation efficiency compared to a control on an electrophoresis gel. The control (L0) is a ligation with only the vector, while the test ligation reaction (L1) includes both the vector and insert. The clear difference in bands between the two lanes demonstrates the efficiency of our ligation reaction. The multiple bands in lane L0 (and their appearance in L1) suggest that vector dimers form readily and need to be eliminated through selection by colony PCR.

Transformation

Invitrogen TOP10 chemically competent cells were chosen as the transformants. Similar to *E. coli* strain DH10B, TOP10's genotype (Appendix D) makes it highly stable with a low mutation rate. DNA recombination is prevented by knocking out recA (recombinase), while the removal of endA (endonuclease) improves plasmid preparations by eliminating non-specific digestion by Endonuclease 1. Furthermore, this strain doesn't feature T7P in its genome (i.e. non-DE3), eliminating the risk of a false positive resulting from colony PCR.

To prepare for the transformation, T7 ligase was inactivated by incubating the ligation mixture at 70°C for 15 minutes to improve transformation efficiency. To cool the ligation mixture and thaw the frozen competent cells, both were placed on ice for 5 minutes. Then, 5 µl of the ligation mixture was added to 50 µl of competent cells and incubated on ice for an additional 5 minutes. To heat shock the cells, they were placed in a 42°C water bath for 30 seconds before being returned to the ice for another 2 minutes. Then, 50 µl of the transformation mixture was spread on LB agar plates containing ampicillin (100 µg/ml) and incubated at 37°C overnight to allow for colony growth (Figure 6).

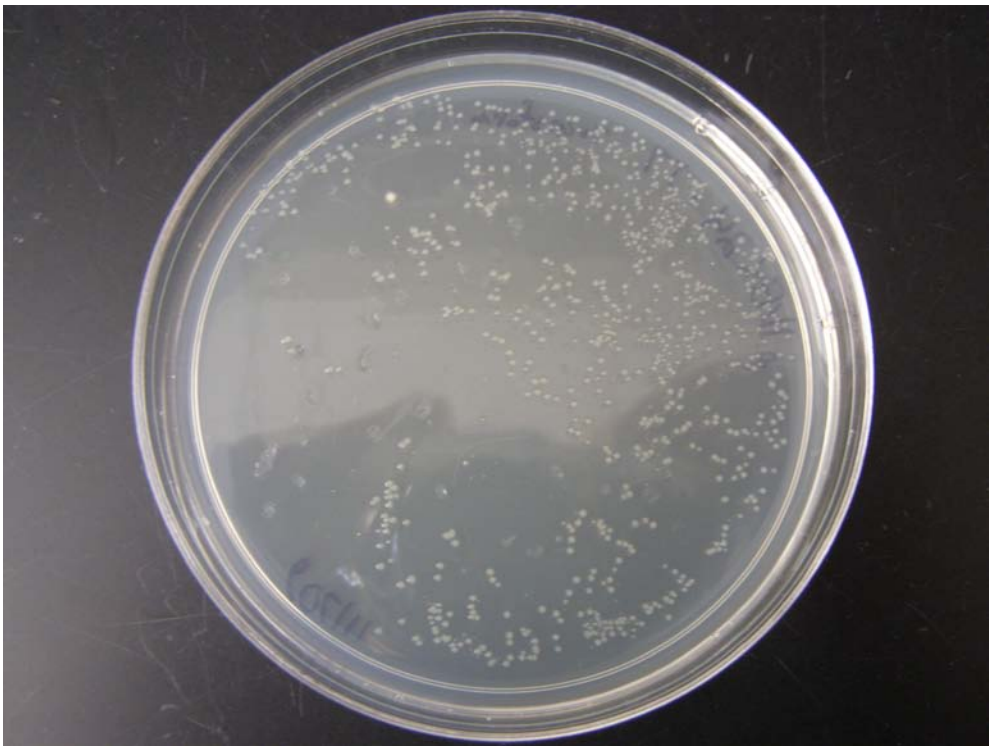

Figure 6: Transformed *E. coli* colonies. After transforming TOP10 chemically competent cells, the transformation mixture was spread on an LB agar plate containing ampicillin (100 µg/ml) and incubated at 37°C overnight. The colonies that developed are shown here.

Selection

As our restriction enzymes produced palindromic sticky ends, the ligation reactions had the potential to produce dimers of either the vector or the insert. As only the vector provides ampicillin resistance, antibiotic selection eliminated the possibility of a colony carrying an insert dimer. However, antibiotic selection alone could not eliminate colonies carrying a vector dimer. Instead, colony PCR was required to select for colonies carrying the plasmid of interest.

In order to produce genetically homogenous colonies, colonies were chosen from the transformation plates and streaked for isolation on new LB agar plates (100 µg/ml) as shown in Figure 7.

Figure 7: Genetically homogenous colonies produced by streaking for isolation. A colony was taken from the transformation plate and streaked on a new LB agar plate containing ampicillin (100 µg/ml) in a diffuse pattern to generate isolated colonies. The plate was then incubated at 37°C overnight and the colonies that developed are shown here.

Through a procedure similar to that used to amplify our gene of interest (Appendix D), colony PCR reactions were performed and run on 1% agarose gel (containing 15% GelRed) for 100 mins at 80V (Figure 8). Colonies that produced a DNA band of the correct size (~2.7 kb) were used to inoculate 50 ml of inoculate LB media (100 μg/ml) and incubated at 37°C on a 200 rpm shake platform overnight. The plasmids were then purified through the GenScript® 5M Gel Extraction Kit as before.

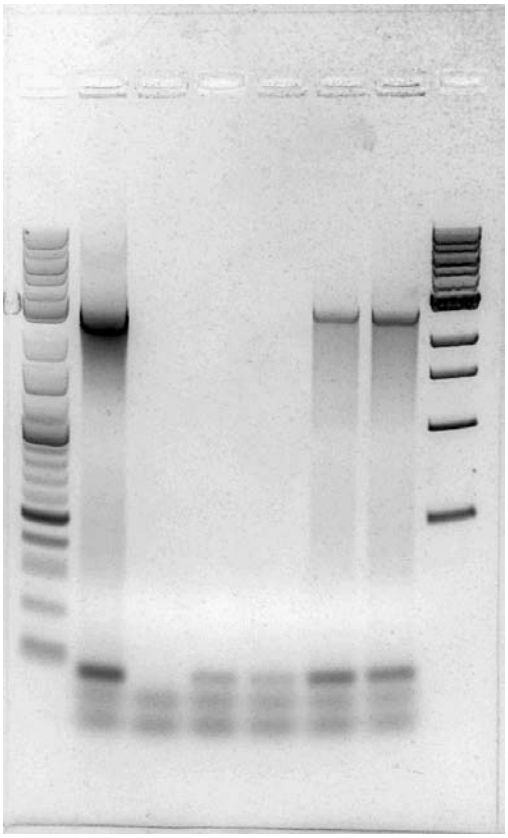

Figure 8: Colony PCR for isolated transformant colonies as shown on an electrophoresis gel. Given that pIVEX2.4d provides ampicillin resistance, transformants carrying vector dimers as well as those carrying the plasmid of interest would grow our LB agar plates containing ampicillin (100 μg/ml). The appearance of a ~2.6 kb band indicates the presence of our gene of interest, implying the corresponding colony carries the plasmid of interest.

Sequencing

In order to check the integrity of our gene of interest, plasmid samples were submitted to the Cornell University Life Sciences Core Laboratories Center for sequencing with the Applied Biosystems Automated 3730 DNA Analyzer. With a confidence interval of 50-500 bp following a sequencing primer, 6 primers were required to accurately sequence the entire T7P gene (Figure 9). In accordance with the Biotechnology Resource Center DNA Sequencing Handbook, primers were designed to be 17-25 nucleotides long and have a Tm of 55-65°C. Once the primer sequences were selected, they were tested for secondary structure formation resulting from self-complimentarity as well as nonspecific binding to our plasmid[10].

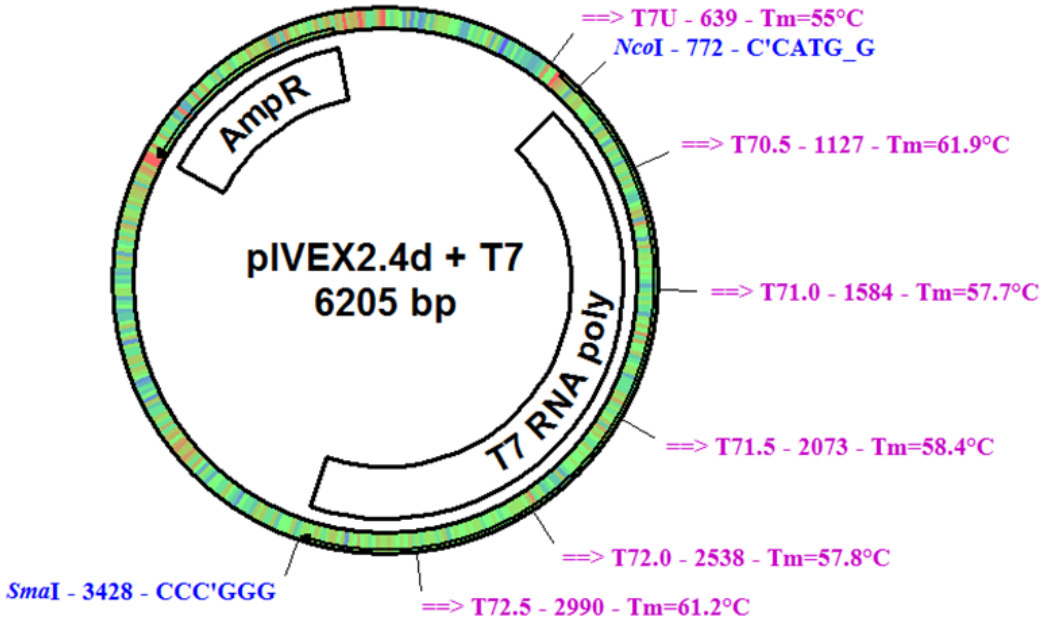

Figure 9: Map of the plasmid of interest with 6 sequencing primers. The primers were spaced to assure that the entire length of the gene of interest would be sequenced within the confidence interval of the Applied Biosystems Automated 3730 DNA Analyzer. The locations of the restriction sites used to insert the T7 RNA polymerase gene as well as the primers melting temperatures are displayed in the figure.

Once the DNA Analyzer results were available, they were assembled into a consensus sequence and checked against the T7P sequence as described in the literature[9].

CHAPTER 3

Results and Discussion

Mutations

Both the N-DNAP and HF-DNAP techniques generated multiple colonies containing our gene of interest. However, upon sequencing it was found that none of the samples displayed complete homogeneity to the published sequence, displaying multiple mutations in each submitted sample. The most common error was a point mutation that changed a single nucleotide (i.e. adenine substituting for guanine). In a few cases, these mutations were silent due to a synonymous substitution that wouldn't alter the amino acid sequence. More commonly however, a missense mutation would occur, changing the codon to code for a different amino acid. The ramifications of these mutations are difficult to anticipate, but may impact the expression or activity of T7P. In the case of a nonsense mutation, the nucleotide substitution resulted in a stop codon, which would trigger the early termination of the peptide during translation, most likely rendering the protein useless.

Another family of mutations that appeared in our results was frameshift mutations: the addition or subtraction of a single nucleotide, shifting all of the downstream codons out of the proper reading frame. Consequently, the amino acid sequence following the mutation site is completely altered, resulting in a distinctly different peptide.

Source of Mutation

There are a number of different areas of our procedure that could have contributed to the high mutation rate seen in our final product. The first to consider is the genetic source,

BL21(DE3). As our genetic source was an isolated BL21(DE3) colony, genetic homogeneity among genomes is assumed. Thus, if mutations were present in this genetically homogenous population, we would expect our clones to exhibit at least some of the same mutations. Instead, mutation locations and types were highly varied, making the bacterial genome an unlikely source of the problem.

The amplification of our insert via PCR is another potential source of mutation. While the use of HF-DNAP produced fewer mutation sites per sample than N-DNAP (2.4 ± 1.0 versus 7.2 ± 2.9) over a very small sample size, it seems very unlikely that this degree of mutation is due to PCR alone. With the 4.4×10^{-7} error rate of the Finnzymes DNA polymerase, a 2.7 kb amplicon should encounter an error every 875 duplications, approximately. This would generate an overwhelmingly homogenous mutation-free population, though a few mutants are likely. The fact that all of our samples showed multiple mutations without exception suggests there are other factors to consider.

Our competent cells could also generate mutations during their multiplication. However, the TOP10 genotype is consistent with industry standards for stable cloning and expression, making this an unlikely source of mutation as well.

CHAPTER 4

Conclusions and Outlook

Without a clear indication of the mutation source, the issue deserves further investigation. One way to accomplish this would be to sequence the insert at each stage of the procedure – namely the genetic source and amplified gene of interest. Nonetheless, with additional work, our results could be useful in p-gel expression studies.

Viability Testing

While all of our samples did have several mutations, a few candidates avoided major mutations (i.e. nonsense and frameshift mutations). It's plausible that candidates with few missense mutations could preserve their activity nonetheless, but viability tests would be necessary to draw this conclusion. As the pIVEX2.4d vector is intended for use with Roche's cell-free RTS *E. coli* system, this solution phase system could be used to produce the T7P mutant. As this expression system uses T7P in its transcriptional process, we would need to separate the expressed product from that introduced in the lysate. Fortunately, the N-terminal His_6-tag would allow for the affinity purification of our product and could be removed via factor Xa cleavage. Once purified, and with a compatible DNA template (e.g. the plasmid produced above), the activity levels of our T7P mutant could be determined through quantitative real-time reverse-transcription PCR and comparison to a control.

Site-directed Mutagenesis

Alternatively, a mutation-free plasmid could be generated through site-directed mutagenesis. By using the wild-type sequence at the location of the mutation as a primer, PCR would generate a new plasmid with the mutation corrected. While the generated plasmid will contain a nick, this is be corrected by the competent cell (albeit with a lower transformation efficiency).[11]

Predictions

While cloning T7 RNA polymerase into the p-gel is an exciting prospect in terms of increasing its autonomy, expressing other transcriptional and translational machinery requires significant post-translational modification, which, to date, has not been demonstrated in the p-gel.

Beyond the incorporation of expression enzymes into the p-gel, the various extensions of this project hold the potential to make significant strides in synthetic biology. Oscillatory networks of transcriptional regulators, such as the repressilator, have been constructed within living cells, however the abundance of interferences posed by the cells' intrinsic activity result in a high-degree of noise[12]. With the relatively simple framework of the p-gel, these interferences would be significantly reduced. As the body of work concerning the expression kinetics of the p-gel increase, designing finely-tuned biological circuits is a distinct possibility.

APPENDIX

Appendix A: PCR Primers

A1: "Long" primers

Primer 1 (Forward)

5′-CTGTCCATGGACACGATTAACATCGCTAAGAAC-3′

ATGAACACGATTAACATCGCTAAGAACGACTTCTCTGAC…

Asp → Asn

Tm = 62.7C (53.1C)

Primer 2 (Reverse)

5′-ATATACCCGGGTTACGCGAACGCGAAGTCCGACTCTAAG-3′

TTACGCGAACGCGAAGTCCGACTCTAAGATGTC…

Tm = 67.7C (53.6C)

The inclusion of the NcoI restriction site (green) and SmaI restriction site (red) are shown above. While the melting temperatures of the two primers are distinctly different, only the complementary region needs to be considered. The relevant melting temperatures are shown above in parentheses. Also, note the missense mutation induced by the use of the NcoI site, coding for aspartic acid instead of asparagine.

A2: "Short" primers

Primer 1 (Forward)

5′-CTGTCCATGGACACGATTAACATCGCTA-3′

CTGTCCATGAACACGATTAACATCGCTAAGAACGACTTCTCTGAC…

Asp → Asn

Tm = (52.9C)

Primer 2 (Reverse)

5′-ATATACCCGGGTTACGCGAACGCGAAGTC-3′

ATATACCCGGGTTACGCGAACGCGAAGTCCGACTCTAAGATGTC…

Tm = (53.2C)

As the shorter primers were designed with consideration to the amplicons, the entire length of these primers were considered in melting temperature calculation.

Appendix B: PCR Protocols

B1: New England Biolabs Taq 5x Master Mix

Reagents

Component	Concentration
Long Forward Primer	0.2 µM
Short Forward Primer	0.5 µM
Long Reverse Primer	0.2 µM
Short Reverse Primer	0.5 µM
Taq 5x Master Mix	1x
E. coli strain BL21(DE3)	0.1 µl per 50 µl reaction
Nuclease-free water	Quantum suffice

Protocol

Phase	Temperature	Time	
Initial denaturation	95°C	5 min	
Denaturation	95°C	1 min	30 cycles
Annealing	60°C	30 sec	
Extension	72°C	2 min	
Final Extension	72°C	5 min	

B2: Finnzymes Phusion High-Fidelity DNA Polymerase

Reagents

Component	Concentration
Long Forward Primer	0.2 µM
Short Forward Primer	0.5 µM
Long Reverse Primer	0.2 µM
Short Reverse Primer	0.5 µM
5x Phusion® HF Buffer	1x
dATP	200 µM
dTTP	200 µM
dGTP	200 µM
dCTP	200 µM
E. coli strain BL21(DE3)	0.1 µl per 50 µl reaction
Nuclease-free water	Quantum suffice

Protocol

Phase	Temperature	Time	
Initial denaturation	98°C	5 min	
Denaturation	98°C	30 sec	20 cycles
Annealing	64°C	30 sec	
Extension	72°C	2 min	
Final Extension	72°C	5 min	

Appendix C: **E. coli** ***Strain TOP10 Genotype***

F– mcrA Δ(mrr-hsdRMS-mcrBC) Φ80lacZΔM15 ΔlacX74 recA1 araD139 Δ(ara leu) 7697 galU galK rpsL (Str^R) endA1 nupG

Appendix D: Colony PCR Protocol

Reagents

Component	Concentration
Short Forward Primer	0.2 µM
Short Reverse Primer	0.2 µM
Taq 5x Master Mix	1x
Transformant colony	0.1 µl per 50 µl reaction
Nuclease-free water	Quantum suffice

Protocol

Phase	Temperature	Time	
Initial denaturation	95°C	5 min	
Denaturation	95°C	1 min	30 cycles
Annealing	60°C	30 sec	
Extension	72°C	2 min	
Final Extension	72°C	5 min	

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