Development and validation of reagents for understanding tRNA:ribosome interactions one molecule at a time

Bradley Vincent
MRes Biochemistry

School of Biosciences
University of Kent at Canterbury

Project Supervisors:
Dr Tobias von der Haar & Dr Neil Kad

Abstract

Translation is responsible for the production of all proteins in a cell making it crucial to the survival of all organisms. Translation involves the decoding of an mRNA by ribosomes and tRNAs. Studying tRNA-ribosome interactions in detail is important for modelling protein synthesis, and this has applications in bioprocessing and in understanding gene regulation in diseases.

This project has set out to develop a single molecule technique to image each step of the ribosome:tRNA interaction process. This will enable studying the rate at which translation occurs as well as further define the steps that characterise this process. I have designed and cloned a synthetic DNA sequence which can be used to initiate translation in *in vitro* reactions. By omitting leucine from the translation reaction, ribosomes can be arrested at a specific leucine codon in a state where a six histidine tag protrudes from the ribosomal exit channel. This should enable immobilisation of translating ribosomes on metal-affinity surfaces. The functionality of the synthetic sequence was demonstrated in a reticulocyte lysate system. A complementary detection system that allows localising individual ribosome:mRNA complexes is currently being developed.

Contents

Acknowledgements

I would like to thank the following groups and people:

Dr Tobias von der Haar and Dr Neil Kad – as project supervisors for their fantastic support and expert guidance; without them this project would not have been possible.

All members of the Kent Fungal Group (KFG) – for their support and help during the project and making me feel welcome in the lab.

James P Dowling – a fellow research student who offered guidance on the initial Luciferase assay work.

Abbreviations

DNA: Deoxyribonucleic acid

RNA: Ribonucleic acid
mRNA: Messenger Ribonucleic acid
tRNA: Transfer Ribonucleic acid

IF: Initiation Factor
EF: Elongation Factor

GTP: Guanosine Triphosphate

A/T/G/C: Adenine/Thymine/Guanine/Cytosine

CCD: charge coupled device
CMOS: complementary metal oxide semiconductor

PCR: Polymerase chain reaction

LB: Luria Broth

DTT: Dithiothreitol

(1) Introduction

This project sought to develop new, novel reagents in the pursuit of understanding Translation in a single molecule system. In turn, this would allow research to be conducted on a single molecule level, in real time, in an effort to resolve the questions surrounding the rates of Translation and its control.

In order to progress in this area, it is important that a sound knowledge of Translation and its preceding events is shown, so that when we ultimately focus on this area, it is clear what is being investigated.

One of the steps preceding translation, is transcription. Transcription is the conversion of the double stranded DNA molecule (which contains the required gene) into a single stranded messenger RNA (mRNA) molecule. The DNA is unzipped and RNA Polymerase II will catalyse the formation of mRNA through complimentary base pairing. This mRNA molecule now contains a copy of the gene and must be translated into a protein.

In Eukaryotes, this occurs in distinctly separate steps, where the mRNA will be exported from the nucleus of the cell and to a Ribosome to undergo Translation. However, in Prokaryotes, the process of transcription and translation will occur simultaneously as the genetic material is not kept within a nucleus.

The focus points of this project are the final steps of this process that are completed during Translation and how this system can be affected in a negative way. It is therefore important that an overview of Translation is given in order to understand how it was tested. Translation occurs in 3 distinct stages:

1. Initiation

This step begins with the formation of a complex with the smaller, 30S subunit of the Ribosome. First, three different initiation factors bind to the smaller subunit of the Ribosome (IF1, IF2 and IF3) which along with a tRNA carrying Methionine, bind to the mRNA sequence at the AUG start codon. This complex is now ready to receive the larger 50S subunit of the Ribosome, which once bound, causes the release of the initiation factors[1].

As described before and shown in Figure 1 the ribosome has 3 specific sites: Amino (A), Peptadyl (P) and Exit (E) which are each vital for the rest of the process.

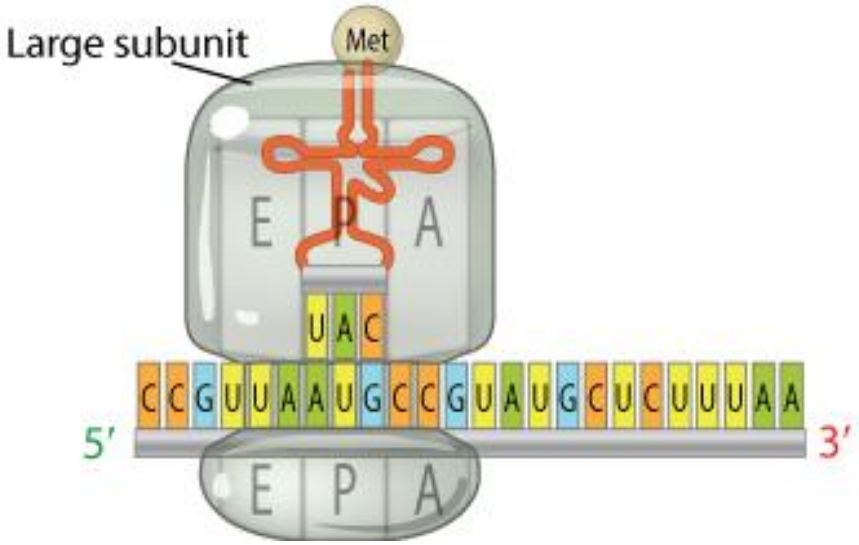

Figure 1. The large ribosomal subunit binds to the small ribosomal subunit to complete the initiation complex.The initiator tRNA molecule, carrying the methionine amino acid that will serve as the first amino acid of the polypeptide chain, is bound to the P site on the ribosome. The A site is aligned with the next codon, which will be bound by the anticodon of the next incoming tRNA1[2].

2. Elongation

Now that translation has begun, the Ribosome has to move along the mRNA in order to read each codon that will form the rest of the protein. This movement occurs in a 5' to 3' direction in a process called translocation. This movement will lead to the next tRNA (in a complex with Elongation factors and GTP as an energy source) entering the A site where GTP is cleaved into GDP and then released along with the Elongation factors for further use. In E.*coli* it is important to note that these Elongation Factors are known as EF-Tu and EF-Ts.

Now that the correct amino acid has arrived in the Ribosome, a peptide bond must be formed with the previous amino acid, via the activity of Peptidyl Transferase. Once this bond has been formed, the Ribosome will translocate once more leading to the tRNA occupying the E site where it is then released to be used again. This process is completed until the STOP codon has been reached[3].

3. Termination

Once any of the three STOP codons (UGA, UUA or UAG) are reached, will cause release factors to bind to the mRNA, removing it from the Ribosome and trigger the deconstruction the Ribosome. The Peptide can now be modified or used where it is needed[4].

Two of the biggest issues that we face in our understanding of Translation are its control and the subsequent ability to define a rate constant. These linked issues are difficult to understand for a multitude of reasons that individually are understood, but in an *in vivo* system are not due to complex interactions such as those between the Ribosome and cognate, near cognate and non-cognate tRNA.

The prevailing thought on Translational control, particularly in the Elongation stage has long been that the degeneracy of the genetic code was insignificant in terms of gene function. As we know, the combination of 64 codons leads to the uptake of the 20 amino acids vital for protein synthesis, however, recently the thought has somewhat changed to the idea that there can be “codon optimality” where synonymous codons are indeed recognised differently[5]. This is highly important as it suggests that we need to fully understand the parameters under which Translational elongation occurs in order to define a rate constant. The crux of Codon optimality is that although a set of codons translate to the same amino acid, there may be a particular codon of that set that is most optimal and hence can have an influence on the overall elongation rate[6].

Additionally, there are some other situations where Translation can be controlled, for example due to a low abundance of aminoacyl-tRNAs corresponding to the correct codon. Recent studies have aimed to expose a deeper understanding of the role of stress in regulating Translation, particular in the Elongation phase. Exposure of cells to hydrogen peroxide, which leads to stress, results in an increase of modified tRNA containing 5-methylcytosine (m5C) which in turn caused the selective translation of mRNA containing the TTG codon.

This stress directly lead to the reduction of activity confirmed by a dual Luciferase and Renilla construct by up to 9.6 times[7], suggesting that in this case the overall rate of Translation would be influenced.

One such area that may be important to further investigate is the role that post-transcriptional modifications play in the coding region of mRNA. This has been demonstrated through N^6-methylation of adenosine (which forms m^6A) which disrupts tRNA selection during translation which in turn will disrupt the elongation step[8]. In tRNA, this modification is vital for its function, more importantly however in mRNA it can inhibit the decoding tRNA[9]. The interaction of one such m^6A codon and the corresponding tRNA has been theorised to closely mirror that of a near cognate tRNA-codon interaction owing to the fact that the position of m^6A within the codon will delay the incorporation of a tRNA. It was found that the location of the modification within the mRNA actually has an effect on the time that it takes for the cognate amino acid to be selected. When the modification appears earlier in the sequence, the delay is significantly larger than when it is further down (up to a 15fold difference for it being at the first codon). In addition to this, the surrounding bases do in fact play a role in mitigating or exacerbating this effect as shown through the translation of different codons (that give the same amino acid). For example, the Cm^6AG glutamine codon showed a 5 fold increase in comparison to the CCm^6A proline codon which only showed a 3 fold increase[10].

It is important to highlight that whilst this modification delays translation, it does not completely disrupt it and prevent it from taking place. For this reason m^6A modifications could be an important regulator of translation as well as other processes such as cell viability. Moreover, regulation of the elongation rate of Translation could actually have an effect on the control of Protein Folding[11].

Complex interactions in Translation such as those described previously can be understood in full detail through the use of single molecule techniques, which will result in the ability to define a rate constant for Translation, which is a vital progression for computational analysis.

Single molecule techniques found their origin in the study of ion channels[12], spawning a large amount of tools that enable us to study an area of interest at the ensemble level allowing us to gain an insight into an entire process in great detail. This is achieved by exploring each individual pathway and providing a probability that this pathway will occur[13]. This is of particular importance as we explore ever more complex processes as we can get an entire view of a Biochemical reaction. As opposed to a normal Biochemical experiment, a single molecule experiment will enable the tracking of individual molecules which in turn can lead to the characterisation of not only the kinetics of the system, but also potential intermediate steps. This is coupled with the ability to not only photograph but also record the process in real time to gain a deeper understanding of our interpretation[14].

The overarching goal of using single molecule techniques in investigating translation is that it will enable us to watch the process occur in real time and as such analyse each individual step. Therefore we would be able to study a particular mechanism in great detail and quantify the complete reaction.

While single molecule systems sound very attractive, they are not without their limitations. The most obvious of these is signal; due to the inherent lack of molecules in the system. Therefore it must be concluded that any amplification of this signal has to come from the apparatus that is being used (such as detectors). The entire way that these systems are monitored in real time has had to have been overhauled and improved. CCD and CMOS imaging cameras have been developed to produce images and recordings at a faster frame rate, which allow the system to be analysed far more accurately[15].

In addition to this new fluorophores have been developed that are both more bright and photostable, thus delaying bleaching. An example of these are quantum dots (Qdots) which are both bright and photostable which makes them desirable for use in single molecule systems, allowing you to attach them to a molecule of interest and track them through the system[16-18].

If the parameters of Translation and its control can be fully understood using the aforementioned single molecule experiments, it would be possible to define a rate of Translation which would enable the use computational methods to predict the rate and model of a particular system of interest.

Recent advances in computational analysis have yielded software with the ability to predict the outcome of a known open reading frame (ORF), with it outputting comprehensive data including errors and usage rates (of tRNA and codons) when a Ribosome has finished translation[19]. Such computational models are limited in their scope as they rely on assumptions such as the cognate (or near cognate) tRNAs being the only ones to bind to the Ribosome and as such cannot be used to accurately predict a rate for Translation.

The development of single molecule systems to investigate Translation has been more heavily studied recently with new systems such as a SunTag Fluorescence tagging system being developed specifically to target Translation[21]. This particular system has been used to measure several stages of Translation including initiation and elongation uncovering more heterogeneity among different mRNA molecules[22]. Similarly to the system we have sought to begin developing, it can be applied to the visualisation of mRNA molecules and Translation in living cells.

If we are able to reliably define a rate for Translation using single molecule experiments, it may be possible to shine some light on disease states within the cell associated with abnormalities in Translation. Recent studies have indicated that a form of neurodegeneration in mice can lead to Ribosome stalling as a result of particular mutated codons[20]. In this experiment the prevalence of the AGA codon was indicating a high amount of stalling which would lead to the premature release of misfolded polypeptides found in areas of neurodegeneration[23].

This project sought to develop reagents that could be used in further translational studies. By developing these reagents, further research could be done to shed light on detailed rates of translation as well as its accuracy. Problems that it could help to solve include the accuracy of mRNA translation (tRNA competition, rejection of incorrect tRNA:Amino acid complexes, how the correct tRNA is identified and the rate of uptake of amino acid from the tRNA) as well the identifying the overall rate of translation in a developed single molecule system.

This aim can be achieved by producing a stable DNA plasmid containing the gene for a well-documented and easy to analyse protein. In addition to this, the plasmid will need to contain suitable sequences for analysis and purification, such as a 6x Histidine tag, ensuring that it is a suitable distance from the protein, so that it will be in the exit tunnel of the Ribosome. Moreover, once the Plasmid has been produced, it will need to be verified via sequencing and the proteins activity quantified.

Over the course of this thesis, I will demonstrate the techniques used in the design, refinement and verification of a fully functional Plasmid including cloning, DNA Agarose gel electrophoresis (and staining), DNA extraction/purification and Luciferase assays, ultimately concluding that a viable DNA plasmid has been produced.

(2) Materials and Methodology

1. Initial Design

The initial focus of the project centred on selecting a protein that is well documented and easy to work with. As the aim of the project is to analyse translation, it is important to use a protein that can be easily verified as active and for this purpose, Luciferase was selected. As we are editing an entire Plasmid for this analysis, we would excise the Luciferase gene from a separate Vector that had the gene for ampicillin resistance (PTH727). In addition to this, two spacers[24] were designed (staCFluc_r_Hin and spac_staCFLuc_f – see appendix) which would ensure that the 6x Histidine tag would be suitably exposed when completing translation analysis.

These spacers ensured that the BamHI site would be upstream of the later inserted gene and the HinDIII site would be downstream of it. (see figure 2)

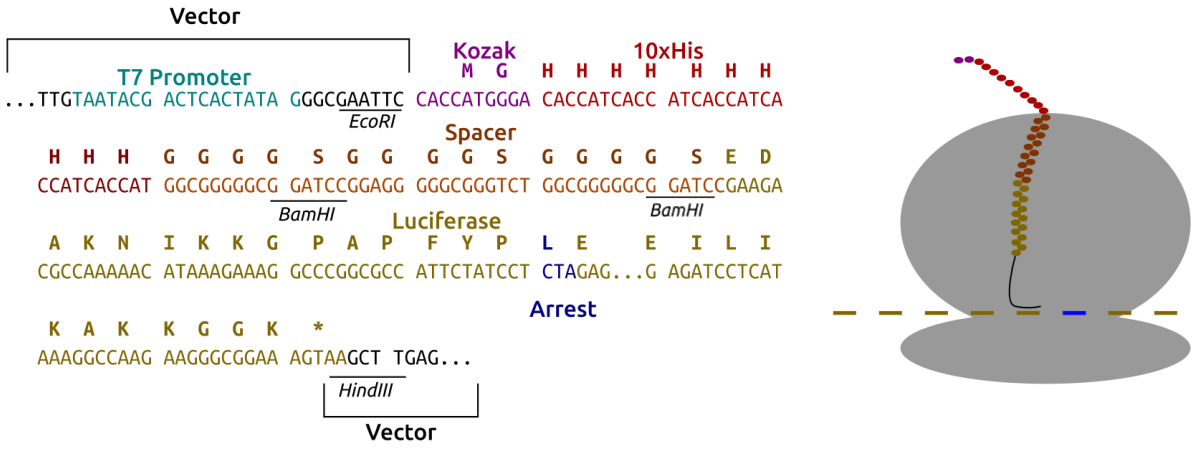

Figure 2. Schematic of the initial design of the Plasmid, focusing on key areas including: T7 Promotor, Kozak Sequence, Histidine tag, Spacers (as described in main body of text) and the Luciferase gene. Highlighted, is the Leucine codon at position 17 of Luciferase; this is the target of arrested translation. In addition to this note the erroneous inclusion of a second BamHI site in the designed spacers

We began the project by selecting the PTH727 plasmid, which had been used in other translational control experiments. This plasmid contained the vital Luciferase gene sequence that was desired to be used as our focus protein. This plasmid was known to have antibiotic resistance to Ampicillin and so any uptake of the plasmid by competent bacterial cells.

1.1 Bacterial Transformation

Initially, a bacterial transformation was used in order to produce cells containing the target Luciferase gene. 50 µl of competent E.coli were added to 1 µl of the plasmid and incubated for 20 minutes on ice. This mixture was then heat shocked at 42°C for 60 seconds and then immediately returned to ice, where 1 ml of LB was added. This was incubated in a shaking incubator for at 37°C for 60 minutes to allow time for the bacterial cells to amplify. 100 µl of the resulting cells were plated onto an LB Ampicillin plate using full aseptic techniques, which was incubated at 37°C, overnight and inspected for colonies.

The resulting colonies were used to inoculate 10 ml of LB ampicillin (one colony per inoculation) to amplify the cells for later purification. Using full aseptic technique, each colony was placed into a sterile aerated growth tube containing 10 ml of LB Ampicillin and incubated at 37°C, overnight in a shaking incubator (8 overnights took place).

These inoculations were then pelleted at 8000rpm for 3 minutes at ambient temperature, where the supernatant was then discarded and the pellets collected for purification.

The resultant pellets contained the Plasmids carrying the Luciferase gene and were purified using a QIAprep Spin Miniprep kit, according to the manufacturer's guidelines.

1.2 PCR Amplification of Luciferase

The purified plasmid containing Luciferase was then used in PCR amplification along with the designed oligonucleotides which would incorporate a spacer sequence upstream of Luciferase (from the BamHI site) as well as a HinDIII site downstream of Luciferase which would be used to incorporate the gene into our target vector.

1 µl of the purified plasmid was mixed with 1 µl each of the primers (spac_staCFLuc_f and staCFLuc_r_Hin) and 25 µl of GoTaq green mastermix, for a total volume of 50 µl (including sterile water). 4 of these samples were produced and entered into a PCR profile to amplify the fragment (sequences in Table 1)

Oligonucleotide	**Sequence (5' – 3')**
spac_staCFLuc_f	GGGGGCG**GATC**CGGAGGGGGCGGGTCTGGCGGGGGCG **GATC**CGAAGACGCCAAAAACATAAAGAAAGGC (69)
staCFLuc_r_Hin	GCGCGCAAGCTTACTTTCCGCCCTTCTTGGCC (32)

Table 1. Table showing the sequence of the primers added to Luciferase to incorporate desired spacers. Numbers in brackets are the total number of bases per sequence listed in 5' to 3' order. Highlighted in bold are two BamHI recognition sequences, the second of which, was erroneously incorporated into the design.

By checking the parameters of the primers, the PCR profile was designed to maximise the yield of the cloned fragment. Full cycles completed were 1x Denaturation (96°C/5 minutes) 30x Elongation (96°C/45 seconds, 50°C/45 seconds and 72°C/1 minute) and 1x Final Extension (72°C/5 minutes) with the samples being held at 5°C thereafter.

1.3 DNA Agarose Gel and Purification

In order to verify that the cloning was successful, a 1% agarose gel was made by mixing 0.5 g with 50 ml of 1% TAE buffer, with 1 µl of Ethidium Bromide being added as a dye for the DNA when visualised under UV light. The resulting PCR samples were run on this gel at 80V for 45 minutes to ensure that full movement had occurred. 25 µl of DNA Hyperladder 1KB were used as a marker with the expectation that the cloned fragment would be around 500 base pairs in size.

With 2 of the trials being successful (see Figure 3 in results section), we could now excise and purify these bands using a GeneJET Gel Extraction Kit as per the manufacturers guidelines. Care was taken to ensure that the gel samples were not exposed to UV light for a prolonged period as to avoid possible base mutations.

Moreover, it was important to ensure that all of the gel containing the correct band was excised, and so this was done with a scalpel, with the gel being re-examined to check for any leftover DNA.

2. Preparation of modified PGEM 3ZF Plasmid

2.1 Target vector Restriction Digest

PGEM 3ZF was selected as the target vector owing to its multiple cloning region as well as suitable restriction enzyme sites that were crucial for the incorporating of extra genetic material.

The target vector was digested at two important sites, between the forward and reverse primers, using BamHI-HF and EcorI-HF to ensure a correct and full digestion. The sample was prepared by mixing 15µl PGEM 3ZF with 1 µl EACH of BamHI-HF and EcorI-HF, in 4 µl Cutsmart Buffer. A total volume of 40 µl was given with the addition of sterile water. This was incubated for 2 hours at 37^{o}C and then purified to remove the excess enzyme, using a PCR purification kit per the manufacturers guidelines.

2.2 Annealing of oligonucleotides (for insertion)

This would subsequently allow the insertion of more spacers (up_f and up_r) to be added to the target sequence insertion site. In addition to this, they would introduce the Kozak[25] sequence elements up to the BamHI site in the spacers from our first fragment design. The Kozak sequence is highly important as it can have an effect on overall translation elongation rates[26]. (sequences in Table 2)

These 2 oligonucleotides were annealed by mixing 2µl of each Oligonucleotide in 10µl 10x Ligation Buffer for a total volume of 100 µl.
This was then heated to 90°C for 5 minutes and then allowed to cool back to ambient temperature. Once cooled, the annealed oligonucleotides were diluted with 150µl H_2O to their final working concentration.

Oligonucleotide	Sequence (5’ – 3’)
up_f	AATTCCACCATGGGACACCATCACCATCACCATCACCATCACCATGGCGGGGGCG (55)
up_r	GATCCGCCCCCGCCATGGTGATGGTGATGGTGATGGTGATGGTGTCCCATGGTGG (55)

Table 2. Table showing the sequence of the primers added to PGEM 3ZF to incorporate desired spacers and sequences, including the highly important Kozak sequence. Numbers in brackets are the total number of bases per sequence listed in 5’ to 3’ order.

2.3 PGEM 3ZF & Annealed Oligonucleotide Ligation

With each of the separate samples prepared, they would now need to be ligated in order to incorporate the annealed oligonucleotides into the target vector. The ligation was prepared mixing 7µl of restriction digested vector (PGEM-3ZF) with 1 µl of annealed oligonucleotides, 1µl of T4 DNA Ligase and 1µl 10x Ligation buffer for a total volume of 10 µl.

Alongside this, a control ligation was completed without the presence of annealed oligonucleotides, which were replaced with water. This control is of upmost importance as it allows us to compare between the two and if the vector is able to be used in a transformation (post restriction digest) even if the oligonucleotides are not incorporated. This will verify that antibiotic resistance has been conserved as well as check for any possible contamination.

The 2 samples were mixed and incubated in a thermomixer overnight at 18^{o}C in a cold room. After the ligation and subsequent purification of each sample, it was essential to test if the oligonucleotides had in fact been incorporated into the vector. This was done in a similar fashion to the transformations documented in section 1.1, however, in this case all 10 µl of the ligated Plasmid were used in the transformation. An overnight inoculation took place to amplify the bacterial colonies which were pelleted and purified as described in section 1.1 also.

The purified Plasmid was run on a DNA agarose gel as per section 1.3 with the expectation of a large band close to the top of the gel at around 4500 base pairs, which would confirm that all of the spacers had been correctly inserted into the plasmid.

2.4 Modified PGEM 3ZF & Modified Luciferase Ligation

With the presence of a band at around 4500 base pairs, we now needed to ligate the modified luciferase gene into the target vector to complete the construction of the final product.

2.4.1 Modified PGEM 3ZF & Luciferase Restriction Digest

This was begun with a restriction digest of the modified PGEM 3ZF in the same fashion as described in section 2.1; with the exception that HinDIII was used in place of EcorRI to ensure that the Luciferase would be incorporated into the correct section of the plasmid. In addition to this, a double digest of 4 hours took place, to ensure as full a digest as possible had taken place. In order to ensure the correct incorporation of the Luciferase gene, the plasmid containing the gene was also digested with BamHI and HinDIII for 4 hours.

With both constituent parts digested and purified, they were ligated together in the same fashion as described in section 2.4, with the annealed Oligonucleotides being replaced by the digested Luciferase fragment.
In order to verify if a successful Ligation had occurred, the resultant sample was used in a transformation, overnight growth and purified. When run on an Agarose gel, a band of approximately 4700 base pairs was to be expected.

2.5 Sequence analysis

As this 4700 base pair band was present, the samples were excised from the gel as described in section 1.3, purified and prepared for GATC sequencing using M13F and R primers. Sequencing would allow us to check if the construct had been successfully manufactured and if there were any issues.

3 samples were selected for sequencing and 6 total samples would be sent off as the entire target sequence was larger than would be accepted.

Of the successful ligations, samples 1, 4 and 9 were selected, as these appeared to show the greatest chance of success. 5 µl of plasmid was mixed with 5 µl of primer (M13F and M13R) and sent off for analysis.

3. Sequence Analysis and Repair

3.1 Identification of errors

Once the sequencing data was received, multiple checks were used to identify if the construct had any errors and also to check the incorporation of all oligonucleotides. Upon analysis, it was found that there was a flaw in the original construct sequence that had gone unnoticed (see Figure 2 for second BamHI site labelled).

This flaw had resulted in a deletion of vital bases that would need to be reinserted into the construct in order to move on in our experiment. The supporting data and the error itself are discussed in more detail in the results section of this thesis.

3.2 Plasmid repair

The plasmid had to be prepared for the insertion of the new oligonucleotides in a way that would yield a good result. The new repair sequence had to re-insert the missing nucleotides lost as a result of digestion by BamHI. The missing information included vital spacers needed to ensure that the 6x Histidine tag was in the exit tunnel of the Ribosome when translation was arrested.
The plasmid was prepared by a restriction digest as described in section 2.1, however, only BamHI was used. The resulting fragment was purified for repair using PCR.

Initially, an attempt was made to repair the Plasmid using the BV_5 and BV_check Oligonucleotides. Having consulted the parameters of the repair sequences, PCR was completed using the same method described in section 1.2. However, sequence analysis of the resulting Plasmid revealed that the repair sequences were not incorporated correctly into the Plasmid.

Another approach was attempted using the BV_3 repair oligonucleotide and M13F primer to insert the missing section of the sequence. These samples were once more run on an Agarose gel to test if we were successful. This was then sent off for sequencing to test if it was successful.
Sequencing and the subsequent analysis revealed that the repair sequence had been incorporated into the Plasmid and there were enough Codons to ensure that the 6x Histidine tag would be positioned correctly.

4. Initial Testing

4.1 Initial testing (RNA synthesis)

With the construct successfully completed, it was important to now establish its usefulness and if it could still be transcribed and translated to produce Luciferase efficiently. Initial tests were conducted focusing on RNA synthesis solely through in vitro transcription.

Numerous factors would have to be considered for this to produce a viable volume of mRNA in a reliable manner. Issues included the addition of DTT to produce a reducing environment for the reaction[27], the addition of RNAse inhibitor as well as the optimal time to maximise RNA synthesis.

A preliminary test was conducted to assess the viability of in vitro Transcription in this case. The plasmid was linearised by a restriction digest using HinDIII only and purified to remove the enzyme. 10 µl of linearised DNA was mixed with 10 µl T7 RNA Polymerase, 4 µl of 0.5 mM NTP (1 µl of each), 5 µl DTT and 10 µl of 10x buffer, for a total volume of 100 µl (including sterile water). A separate reaction was also conducted with the absence of DTT, which was replaced by water.
The resulting samples were incubated at 37°C for 3 hours before being purified using an RNeasy Plus mini kit, following the manufacturers guidelines.

4.1.1 Urea Gel electrophoresis

In order to test the purified RNA, an Urea must be made as traditional DNA Agarose gels will not work in this case.

For this test, a 6% Urea gel was made by mixing 22g of Urea with 10ml 5x TBE, 7.5 ml Acrylamide/Bisacrylamide 40% (29:1) and sterile water to 50ml. Care was taken to ensure that all of the Urea had dissolved prior to adding 25 µl of 40% APS and 55 µl of TEMED to catalyse the polymerisation of the gel.

The gel was run empty for 30 minutes at 20mA to raise the temperature of the gel system and the wells were also washed with 1x TBE buffer to remove excess Urea.

32 µl of the RNA sample was mixed with 8 µl of fresh buffer (essential that it is free from nucleases) and loaded onto the gel. The gel was run in 1x TBE buffer for 1 hour (until the tracker dye had run) and stained in a solution of ethidium bromide. Upon visualising the gel under UV light, it became obvious that no RNA bands were present on the gel even after re-staining.

Another 2 preliminary trials were attempted (and both failed) and so due to the large number of variables to control, it was decided that a coupled system of transcription and translation would be more suitable for our needs.

5. Further Analysis

5.1 Large scale Bacterial Transformation

Before, any more tests were conducted; we needed to increase our stocks of the Plasmid so that we had a larger supply in reserve.

The bacterial transformation was partly the same as used in section 1.1. 50 µl of competent E.coli were added to 1 µl of the plasmid and incubated for 20 minutes on ice. This mixture was then heat shocked at 42°C for 60 seconds and then immediately returned to ice, where 1 ml of LB was added. This was incubated in a shaking incubator for at 37°C for 60 minutes to allow time for the bacterial cells to amplify. 100 µl of the resulting cells were plated onto an LB Ampicillin plate using full aseptic techniques, which was incubated at 37°C, overnight and inspected for colonies.

The resulting colonies were used to inoculate 50 ml of LB ampicillin (one colony per inoculation) to amplify the cells for later purification. Using full aseptic technique, each colony was placed into a sterile 250 ml conical flask containing 50 ml of LB Ampicillin, which was aerated and incubated at 37°C, overnight in a shaking incubator (2 overnights took place).

These inoculations were then transferred to Falcon tubes and pelleted at 8000rpm for 3 minutes at 5°C, where the supernatant was then discarded and the pellets collected for purification.
The resultant pellets contained the Plasmids carrying the Luciferase gene and were purified using a QIAprep Spin Midiprep kit, according to the manufacturer's guidelines. This larger scale kit yielded a much higher volume, which was frozen to be used throughout the rest of the project.

5.2 Luciferase Assays

Knowing that the Plasmid had been constructed successfully, we now needed to verify the activity of the Luciferase gene. Following the failed RNA synthesis attempts, a T7 Coupled Reticulocyte Lysate System was used which would complete both transcription and translation simultaneously, allowing us to analyse the activity of Luciferase on its substrate.

5.2.1 Confirming Luciferase activity

The original (unedited) PGEM 3ZF was used to transform BL21 for the purposes of being a control for this assay, as well as the final edited Plasmid and then grown on an LB AMP plate. These bacterial transformations were completed in the same way as described in section 1.1 up to the inoculation stage, where a 96 well plate containing LB AMP was inoculated 3 times per sample and incubated at 37^{o}C overnight, with one colony being harvested per well and grown in 150µl of LB AMP. In order to prevent any evaporation, the surrounding wells were filled with distilled water.

With the samples prepared, they were now diluted 4 times (120µl of LB AMP mixed with 30µl of the overnight incubation) and incubated for 3 hours for the final growth. In order to conduct the assay itself, the manufacturer's guidelines were followed exactly and once the substrate had been added to the wells, the plate was then placed in a plate reader to find the bioluminescence of the samples. The final data confirmed that the edited construct containing Luciferase was active, due to the samples bio luminescing in the presence of the substrate

5.2.2 Luciferase Assay, with arrested Translation

As the data suggested that the construct could indeed produce Luciferase it was now important to test if translation could be arrested at a particular point. This idea had been built into the construct from initial design and would be tested by omitting Leucine from the amino acid mix used in the test. As this was Leucine was located within Luciferase, (and there were no other Leucine codons before it) translation would be arrested and the substrate would no longer be correctly digested (as Luciferase would not be produced).

To test this fully, a control was set up containing the full amino acid mixture ensuring that Luciferase could be produced and the substrate digested. Measurement was once again taken via Bioluminescence, with the expectation being to compare arrested translation with the control. To ensure reliability, the test was completed in triplicate with a t-test being used to interpret the data.

As shown in Figure 6 we can see an average of 162 for the –Leucine trial which is comparable with the background bioluminescence of the test. Therefore, this suggests that translation has been arrested, due to a lack of Bioluminescence when compared to the +Leucine trial which had an average reading of 571.6.
However, further controls are needed to unequivocally prove this hypothesis and are further discussed in the Results and Discussion sections.

(3) Results

PCR Amplification of Luciferase

The initial focus of the project was to gather samples of the desired Luciferase gene (in the PTH 727 plasmid) which could be further used in the construction of our final Plasmid.
By editing the Plasmid, we could insert vital spacer sequences that would ensure that the 6x Histidine tag would protrude from the Ribosomal exit tunnel correctly.
The design of the spac_staCFLuc_f oligonucleotide was actually flawed in the beginning and this was highlighted in Table x in section 1.2. When looking closely at the sequence, we can see the presence of two BamHI recognition sequences, characterised by the motif GATC.

…G**GATC**CGGAGGGGGCGGGTCTGGCGGGGGCG**GATC**C…

The incorporation of this second site would ultimately lead to the deletion of part of this spacer which would result in a shorter sequence. While this oversight is unfortunate, it was fortunately, not catastrophic and would later be repaired as discussed in the Plasmid Repair section.

When these spacers were initially incorporated into the Plasmid via PCR, the expectation was to see a strong band of around 500 base pairs. This would indicate the successful uptake of the spacers by the Plasmid which could then be digested to release the crucial edited Luciferase fragment.

In order to check for the incorporation of the spacers, a DNA Agarose Gel was used along with DNA markers indicating a Base pair size. In this case, DNA HyperLadder I was used as this contained two lower bands of 400 and 600 base pairs.
With the gel run and visualised under UV light, it was apparent that trials 2 and 3 were the most successful, with trial 4 showing a smaller band. In addition to this, trial 1 showed no band at all and was considered to be negative.

Highlighted in red on Figure 3 are the two successful trials which were excised and purified for further use in the project. Smaller bands are also visible on the bottom of the gel which were attributed to the smaller spacers.

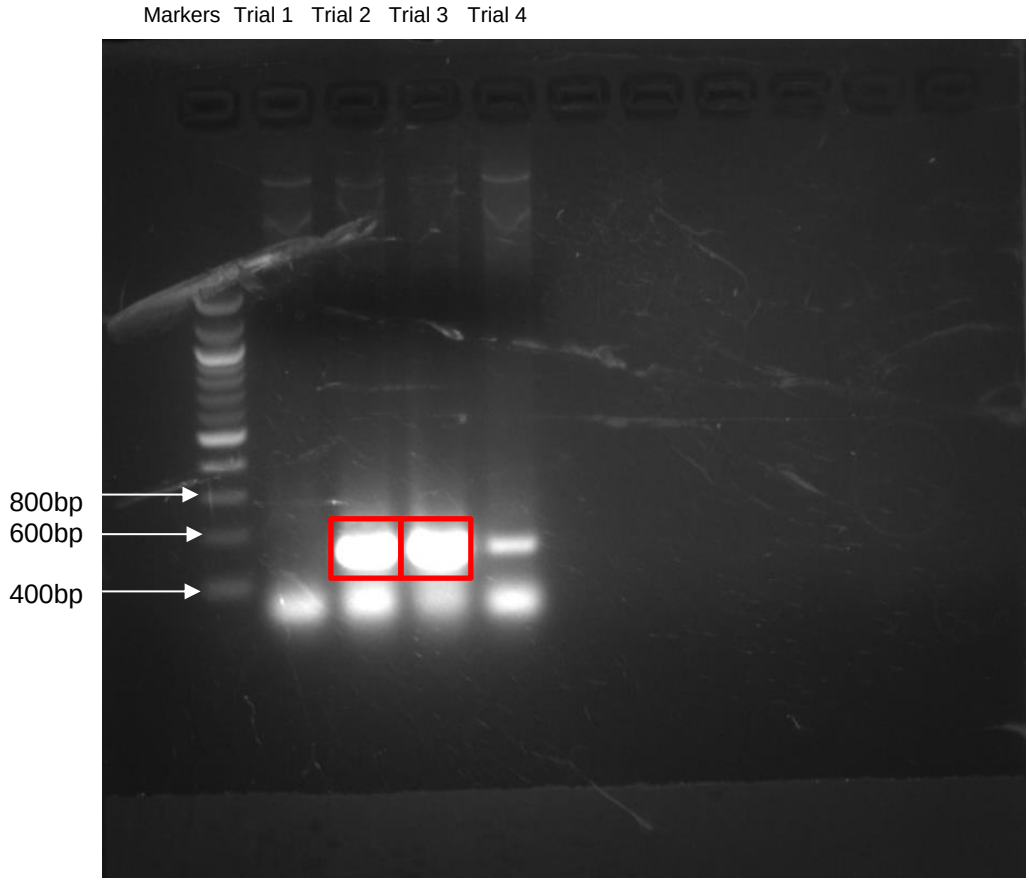

Figure 3. Ethidium Bromide stain of a DNA Agarose Gel for modified Luciferase Gene (including spacers). Samples were prepared via PCR where they contained GoTaq master mix including green dye. Full cycles completed were 1x Denaturation (96^{o}C/5 minutes) 30x Elongation (96^{o}C/45 seconds, 50^{o}C/45 seconds and 72^{o}C/1 minute) and 1x Final Extension (72^{o}C/5 minutes) with the samples being held at 5^{o}C thereafter. Each 50µl sample was loaded into a well on a 1% Agarose gel which was run at 80V for 45 minutes. Our sample was expected to be ~500bp in size and two successful samples are highlighted in the red boxes. The gel was visualised under UV light and the two successful trials were excised and purified for further use.

PGEM 3ZF & Annealed Oligonucleotide Ligation

With the confirmation that the Luciferase containing Plasmid had been successfully edited, we moved onto preparing PGEM 3ZF for the insertion of the Luciferase fragment.
This began with a restriction digest of the Plasmid to prepare for the insertion of more oligonucleotides, which would also incorporate the Kozak and His tag sequences. The oligonucleotides would ultimately reside upstream of the BamHI site inserted before the Luciferase gene.
This set of oligonucleotides were inserted via a Ligation of our target vector and the annealed oligonucleotides; moreover, this step had to be completed prior to the insertion of the Luciferase sequence.

In order to verify the insertion of these oligonucleotides, the resulting Ligation was used in a bacterial transformation, which was purified and tested on a DNA Agarose gel. DNA HyperLadder I was used once as this contained the length of bands necessary to compare against our samples.
With the gel run and visualised under UV light, it was apparent that all trials were successful as a band of approximately 4500 base pairs was shown.

Shown on figure 4 are the markers at 4000 and 5000 bp with trial 1 highlighted in red for comparison. Visualisation of the gel appeared to show the trials were successful and thus the Ligation was carried forward for further modifications.

Figure 4. Ethidium Bromide stain of a DNA Agarose Gel for modified PGEM-3ZF vector (including up_f and up_r spacers). Samples were prepared via digestion of the BamHI and EcorI sites at 37^{o}C for 2 hours, where they were then ligated with the annealed oligonucleotides of up_f and up_r at 18^{o}C, overnight. Samples were then used to transform competent cells, grown and purified. Each 50µl sample was loaded into a well on a 1% Agarose gel which was run at 80V for 45 minutes. Our sample was expected to be ~4700bp in size. The gel was visualised under UV light, with all trials appearing to be successful and so two successful trials were excised and purified for further use.

Modified PGEM 3ZF & Modified Luciferase Ligation

With the presence of a band at 4500 bp, the next step was to ligate the modified luciferase gene into the target vector to complete the construction of the final Plasmid.

In order to prepare the modified PGEM 3ZF for the insertion the Luciferase gene, a BamHI & HinDIII restriction digest took place so that the gene would be positioned perfectly.
This point is where the deletion of spacers in Luciferase took place. The second BamHI site in the modified Luciferase was also digested leading to the erroneous deletion of 30 bases, an amount that would go unnoticed on a subsequent DNA Agarose gel.

With the Ligation of the two constituent parts complete, the final Plasmid was used in a transformation and run on a DNA Agarose gel, with the expectation of seeing a band at just under 5000 base pairs.

DNA HyperLadder I was used once as this contained the length of bands necessary to compare against our samples. With the gel run and visualised under UV light, it was apparent that all but one of trials were successful as a band of approximately 5000 base pairs was shown (see figure 5).

Important to note is the presence of smaller bands further down the gel which were theorised to be a result of the erroneous digestion of the sample by the second BamHI site.

With the presence of the larger 5000 base pair bands, trials 1, 4 and 9 (highlighted in red) were selected for sequence analysis which would later reveal the extent of the deletion caused by the extra BamHI site.

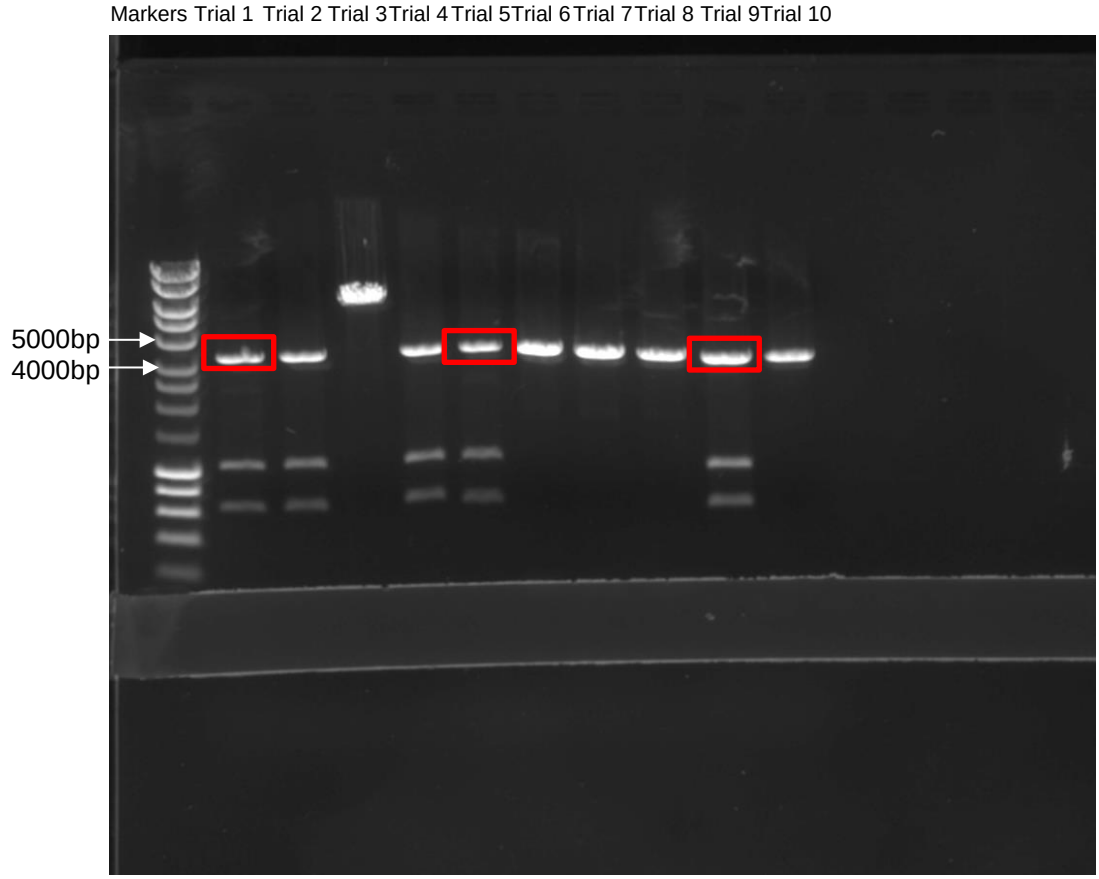

Figure 5. Ethidium Bromide stain of a DNA Agarose Gel for modified PGEM-3ZF vector containing all spacers and Luciferase gene. Samples were prepared via digestion of the BamHI and HinDIII sites at 37^{o}C for 4 hours, where they were then ligated with the modified Luciferase gene at 18^{o}C, overnight. Samples were then used to transform competent cells, grown and purified. Each 50µl sample was loaded into a well on a 1% Agarose gel which was run at 80V for 45 minutes. Our sample was expected to be ~5000bp in size and all but one trial appeared to be successful. The gel was visualised under UV light, with trials 1, 4 and 9 selected for sequencing (highlighted in red).

Plasmid Repair

Having identified the issue with the Plasmid which lead to a deletion of 30 bases (See figure 6), we designed a new set of spacers which would be re inserted to position the 6x Histidine tag (when translated) correctly.

When designing the original sequence, two BamHI sites had been erroneously inserted into the construct meaning that some of the new bases added in were being excised again when digested. While this was an unfortunate error, it was fairly straight forward to repair the construct by designing new oligonucleotides that contained the missing information (see table 3).

Oligonucleotide	**Sequence (5' – 3')**
BV_5	GATCTGGAGGGGGCGGGTCTGGCGGGGGCG (30)
BV_3	GATCCGCCCCCGCCAGACCCGCCCCCTCCA (30)
BV_check	GAGATGTGACGAACGTGTACATCG (24)

Table 3. Table showing the sequence of the primers added to the incomplete Plasmid. These sequences would be used to re insert spacers into the Plasmid so that the 6x Histidine tag was a suitably placed in the Ribosomal exit tunnel. Numbers in brackets are the total number of bases per sequence listed in 5' to 3' order.

In retrospect, an initial sequence study of the designed oligonucleotides should have been undertaken as this would have identified this kind of problem and prevented it from occurring. However as stated before, this was fortunately only a small issue and it was easily fixed.

The Plasmid was digested by BamHI and submitted to a standard PCR amplification cycle in an attempt to clone in the correct Oligonucleotides. However this was unsuccessful on multiple samples so a different approach was considered.

As the error happened at the beginning of the Plasmid, we could fortunately attempt to clone in the fragment using the M13F primer as well as the BV_3 oligonucleotide.

Subsequent DNA Agarose gel analysis revealed that this had indeed been successful (see figure7) as all 7 trials showed a strong band at around 5000 base pairs. An example of this is highlighted in Red on the figure.

Note the presence of some bands at the top and bottom of the gel which were theorised to be heavy and light primer dimers, causing contamination.

Similarly to the previous attempts, we could not be completely sure that the Plasmid was fully repaired until a sequence analysis was completed. By doing this, we could show a map of the Plasmid, including all of the relevant sites (see figure 8).

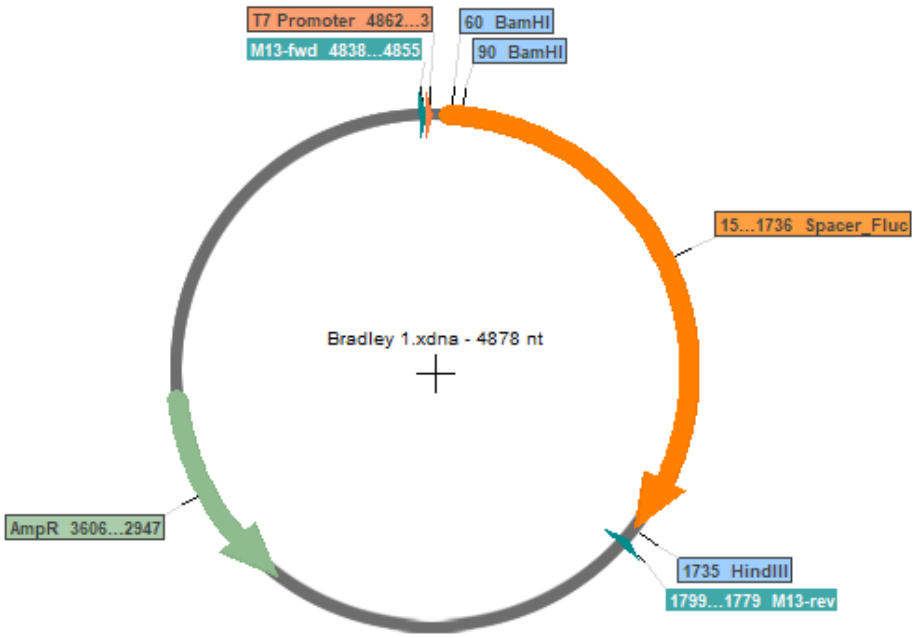

Figure 6. Plasmid map of the incomplete Vector, highlighting the errors discussed. Note that this INCLUDES the spacers that were deleted, to show where in the Plasmid the deletion occurred.

A key feature highlighted are the two BamHI sites, one of which was erroneously inserted at position 60 and 90. In the final construct, the second of these sites was no longer present.

A total length of 4878 nucleotides is shown.

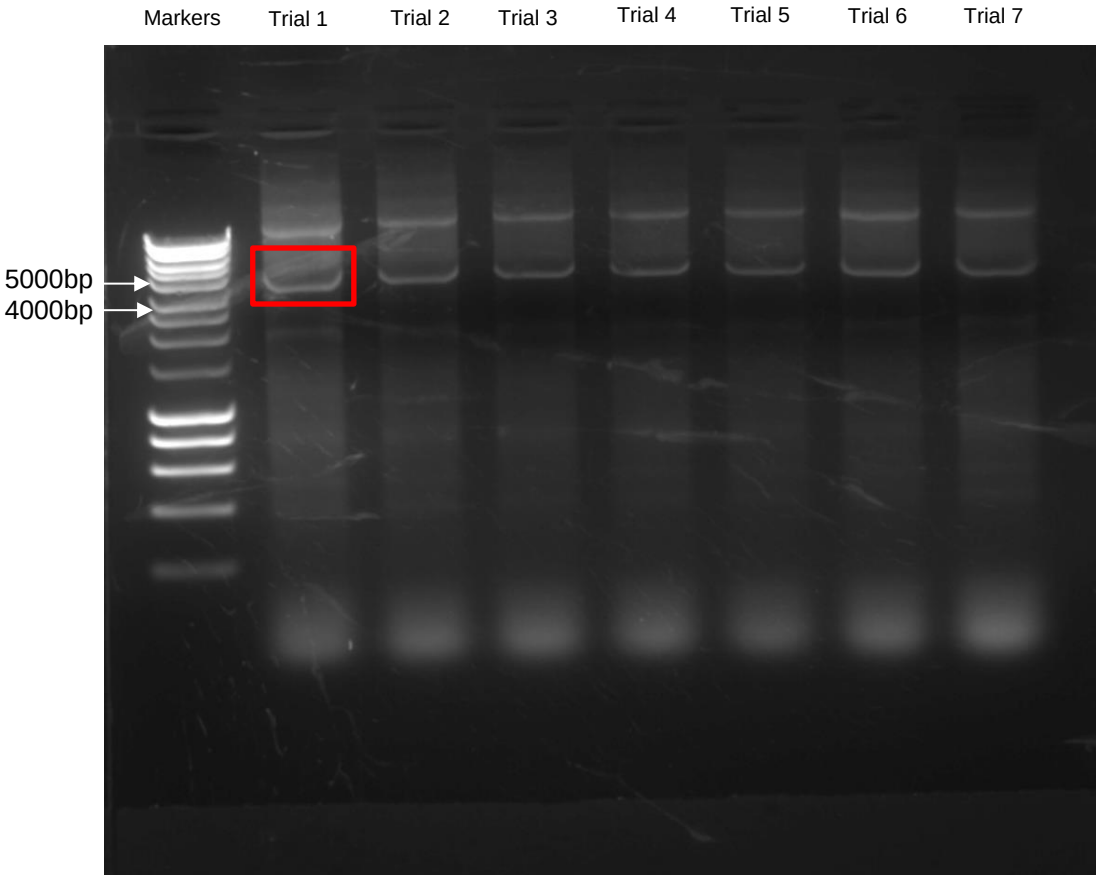

Figure 7. Ethidium Bromide stain of a DNA Agarose Gel for modified PGEM-3ZF vector containing all spacers and Luciferase gene. This is the completed repair of the construct, to re-insert missing bases incorrectly removed through erroneous BAMHI insertion. Samples were prepared via digestion of the BamHI site at 37^{o}C for 4 hours, where they were then used in a PCR amplification with M13F Primer and BV_3. Full cycles completed were 1x Denaturation (96^{o}C/5 minutes) 30x Elongation (96^{o}C/45 seconds, 50^{o}C/45 seconds and 72^{o}C/1 minute) and 1x Final Extension (72^{o}C/5 minutes) with the samples being held at 5^{o}C thereafter.

Each 50µl sample was loaded into a well on a 1% Agarose gel which was run at 80V for 45 minutes. Our sample was expected to be ~5000bp in size and all trials appeared to be successful. The gel was visualised under UV light, with trial 1 (red box) being excised, purified and sent off for sequencing.

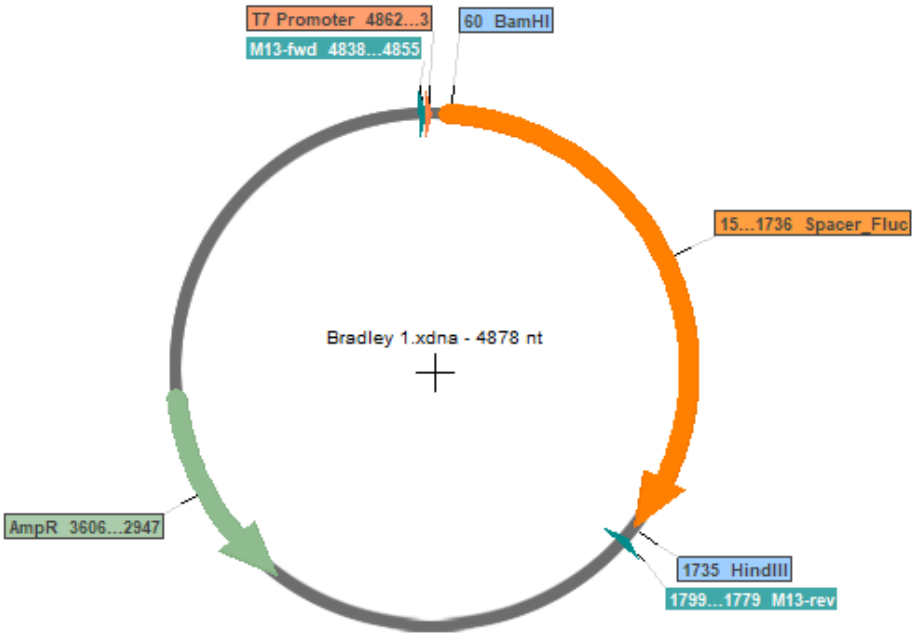

Figure 8. Plasmid map of the fully complete Vector, preliminarily named “Bradley 1” highlighting key features of the Plasmid.

All key features that have been necessary to build this Plasmid are shown, including the T7 promoter, Ampicillin resistance gene, M13F & R primers and all Restriction enzyme sites.

A total length of 4878 nucleotides is shown, achieved with the re insertion of new spacers to reach the same length as needed.

Luciferase Assays (and RNA synthesis)

As discussed in section 4.1 of materials and methods, RNA synthesis was attempted in vitro but no discernible data was able to be collected. In total, 4 trials were attempted and the samples collected were tested on a Nanodrop to ascertain their concentrations.

Trial 1: 0.23 µg/ml
Trial 2: 0.22 µg/ml
Trial 3: 0.17 µg/ml
Trial 4: 0.14 µg/ml

As shown, these concentrations are exceedingly low and with this data, it was decided that a coupled reticulocyte lysate system would be used instead.

Initially, the activity of the Luciferase gene needed to be confirmed. By measuring Luminescence, we could ascertain that the Luciferase gene was active and that it was actively degrading Luciferin to produce light as a by-product. As shown in Figure 9 an average Bioluminescence of 15.66 was given for the control Plasmid (without Luciferase) showing that the substrate was not degraded. Conversely, an average of 370 was given for the “Bradley 1” Plasmid, showing that the gene was active and that with the substrate being degraded, light was being emitted.

To test this fully, a control was set up containing the full amino acid mixture ensuring that Luciferase could be produced and the substrate digested. Measurement was once again taken via Bioluminescence, with the expectation being to compare arrested translation with the control. To ensure reliability, the test was completed in triplicate with a t-test being used to interpret the data and a bar graph to visualise the data (Figure 9).

Data processing revealed that the T-Statistic of this triplicate was -33.97 and the P Value was 0.000432636. Note that the negative of T-Statistic two tail was -4.03, with the T-Statistic being significantly less than that. This analysis allowed us to conclude that the data sets were independent of one another, that is the Plasmid is active, producing Luciferase and this did not happen by chance.

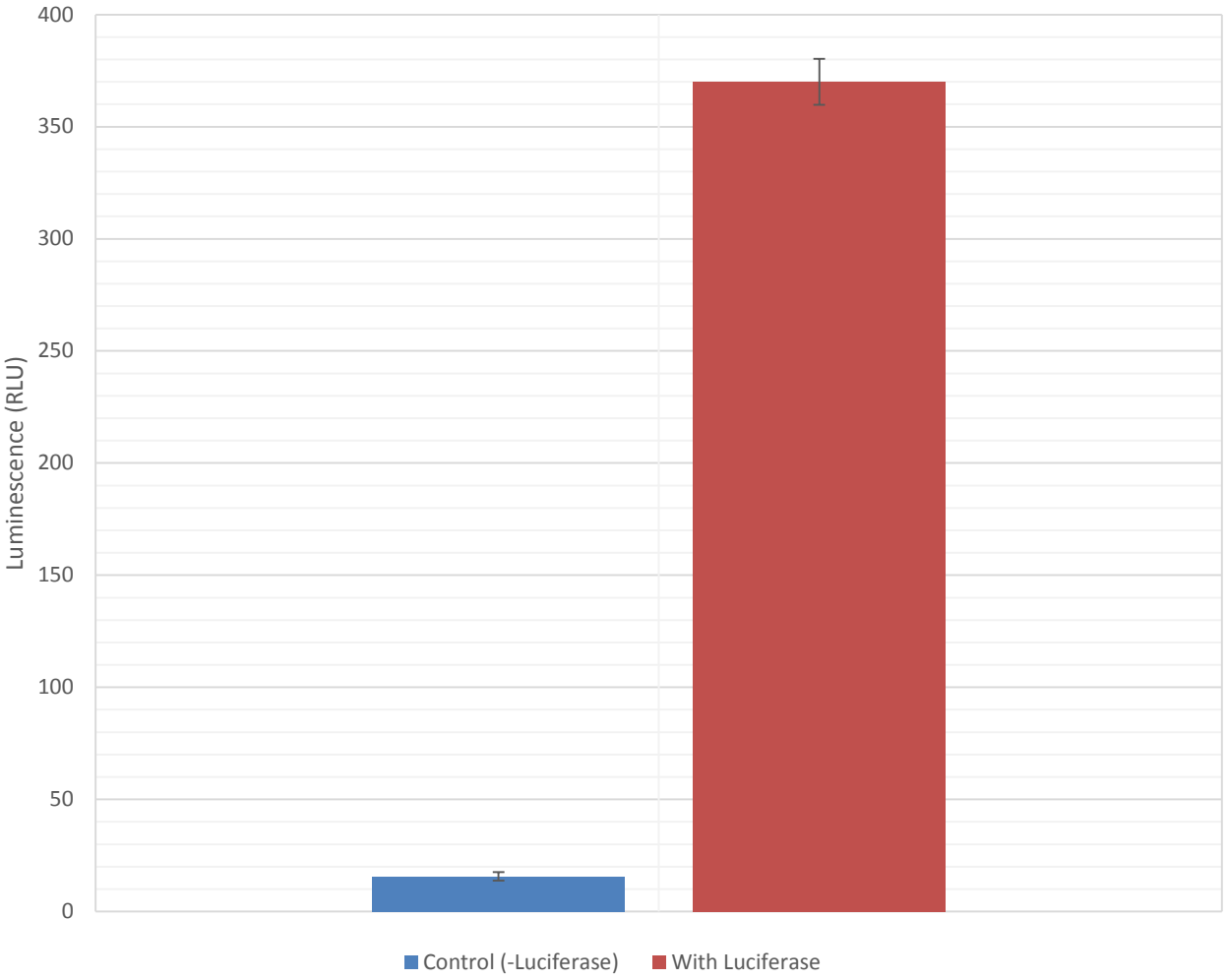

Figure 9. Bar graph representation of Luciferase assay on modified PGEM 3ZF and unmodified PGEM 3ZF (without Luciferase gene) to compare the activity of Luciferase on its substrate. Measurement was completed in the form of Bioluminescence which would be present with the degradation of substrate by Luciferase. The graph indicates that without the Luciferase gene present, there is no Bioluminescence above background readings (Blue) whereas the modified vector shows an average reading of 370 (Red). Error bars have been included in the representation of the data to indicate the precision of data collected. This data was collected to ensure that the modified construct contained active Luciferase, which would be further used in the project.

The table of raw data shows the control and Plasmid trials highlighted in red, with the control being that of Row B 2 – 4.

	1	2	3	4	5	6	7	8	9	10	11	12
A	17	18	19	18	17	19	15	16	28	49	28	16
B	19	18	12	17	14	16	13	27	376	384	350	14
C	18	20	14	9	17	17	18	13	11	14	16	13
D	20	22	24	13	12	19	12	16	19	16	22	12
E	16	14	20	12	12	14	19	14	16	11	18	14
F	17	12	17	20	19	18	12	15	16	13	11	13
G	20	19	13	10	13	18	15	13	17	13	16	14
H	11	14	13	8	20	17	16	11	12	16	11	16

Table 4. Table showing the raw data of the Luciferase assay on modified PGEM 3ZF and unmodified PGEM 3ZF (without Luciferase gene). Highlighted in red are the data points where the reaction occurred. All other cells recorded the luminescence for purposes of comparison. All data is in Relative Light units (RLU). The average Bioluminescence for the control was 15.66 RLU and the average Bioluminescence for the Plasmid was 370 RLU.

Using the data described in Table 4, the Standard deviation for the control was shown to be 3.21 and the Standard deviation for the Plasmid was 17.78.
This indicates that the spread of the data is low and can be considered more reliable as the data lies close to the average.

These figures were also used to calculate the standard error used in the visual representation of the error bars (all done electronically).
In this experiment, Relative Light Units are given as there is not a specific unit for the Bioluminescence of Luciferase. The detection of light is as a result of the proportional expression of the Luciferase gene which shows that the higher the detection, the more the gene is being expressed.
With this data analysis, we concluded that the Luciferase gene in the Plasmid was active. We could reliably state this conclusion thanks to a significant disparity between the T-Statistic and the negative of T-Statistic 2 tail. Moreover, the P value being so low showed that this was highly unlikely to have happened by chance.
To test this conclusion fully, as well as begin testing if Translation could be arrested, a further experiment was conducted, this time with and without the presence of

Leucine. A control was set up containing the full amino acid mixture ensuring that Luciferase could be produced and the substrate digested. Measurement was once again taken via Bioluminescence, with the expectation being to compare arrested translation with the control which did contain Leucine, therefore allowing Translation to take place and Luciferin to be broken down.

To ensure reliability, the test was once again completed in triplicate with a T-test being used to interpret the data and a bar graph to visualise the data (10).

Data processing revealed that the T-Statistic of this triplicate was -9.15 and the P Value was 0.000396. Note that the negative of T-Statistic two tail was -2.776445105, with the T-Statistic being less than that. Moreover, the P-value being so low confirmed that this could not have happened by chance.

This analysis allowed us to conclude that the data sets were independent of one another, showing that without the presence of Leucine, Translation could potentially be arrested. Conversely, the control confirmed once more that Translation could occur in this system as shown by the high readings for Luminescence. It is important to realise that this does not unequivocally prove that cell death has not occurred and further experimentation is needed to prove this, as discussed later.

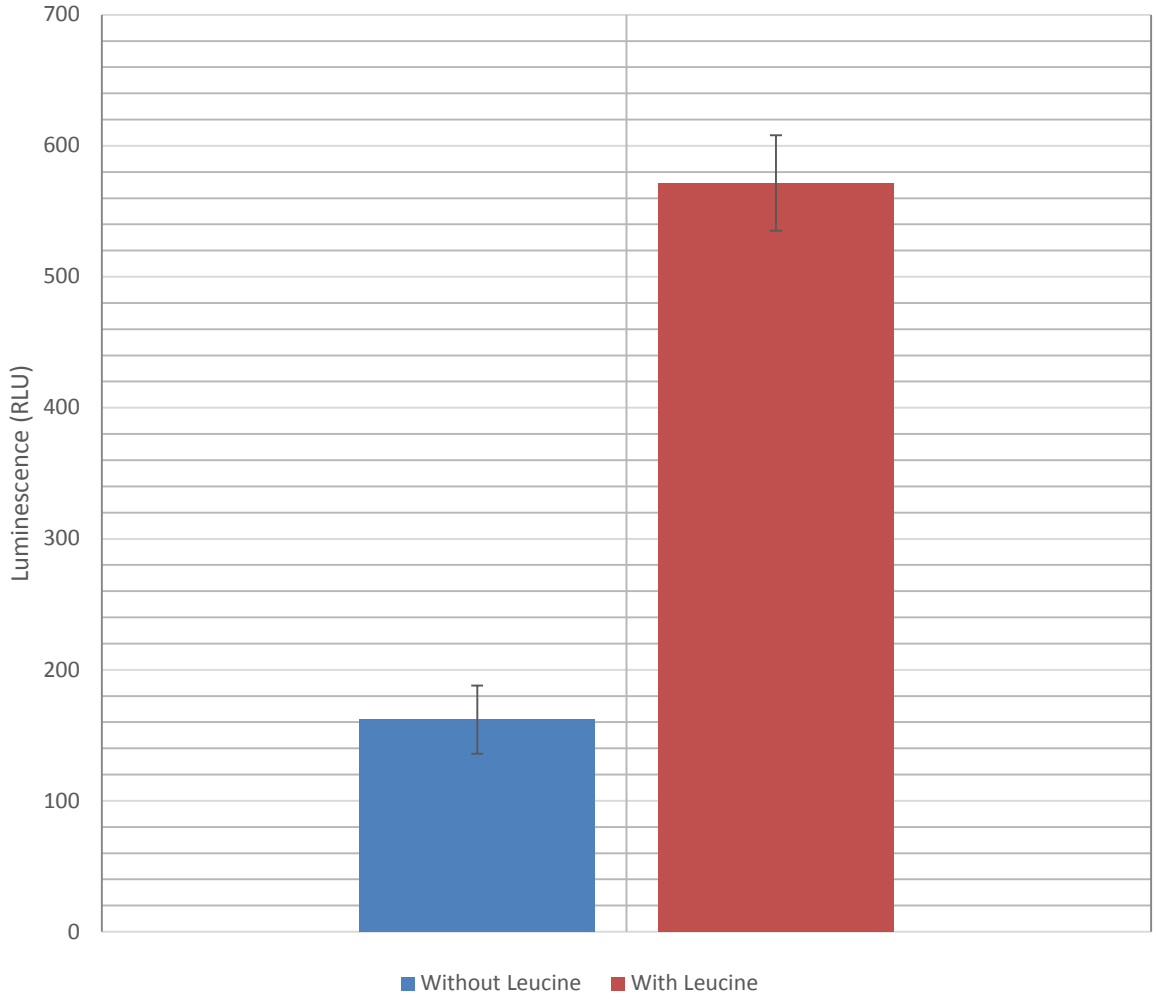

Figure 10. Bar graph representation of Luciferase assay on modified PGEM 3ZF (containing Luciferase) with and without the presence of Leucine in the amino acid mix, to compare the activity of Luciferase on its substrate. Measurement was completed in the form of Bioluminescence which would be present with the degradation of substrate by Luciferase. This was completed in triplicate with a standard deviation being calculated as well as conducting a t-test.

The control indicated that without Leucine being present, translation would not occur, leading to the substrate not being digested and thus no discernible Bioluminescence was detected above background (Blue).

The trials that did contain Leucine indicated that translation was occurring as an average Bioluminescence of 571.6 was detected as the substrate was indeed being digested (Red).

The table of raw data shows the control and Plasmid trials highlighted in red, with the control being that of Row B 9 – 11.

	1	2	3	4	5	6	7	8	9	10	11	12
A	96	139	142	133	131	117	119	109	150	155	136	106
B	150	213	127	146	115	113	108	149	622	592	501	108
C	116	116	124	122	120	132	128	119	112	139	105	132
D	124	157	109	126	107	133	92	133	107	120	109	129
E	118	123	148	122	133	103	112	109	141	117	125	79
F	127	116	115	123	101	103	118	111	136	111	98	109
G	117	120	104	97	124	106	114	123	110	105	114	99
H	138	106	110	158	110	103	108	117	95	117	110	98

Table 5. Table showing raw data of Luciferase assay on modified PGEM 3ZF (containing Luciferase) with and without the presence of Leucine in the amino acid mix. Highlighted in red are the data points where the reaction occurred. All other cells recorded the luminescence for purposes of comparison. All data is in Relative Light units (RLU). The average Bioluminescence for the control was 571.66 RLU and the average Bioluminescence for the Plasmid was 162 RLU.

Using the data from Table 5, the Standard deviation for the control was shown to be 63.01 and the Standard deviation for the Plasmid was 45.18. It must be noted that there is a higher spread between the data points in this experiment however it was still far within an acceptable range. Additionally, the background readings indicated by the Control are shown to be higher across the entire experiment which was a result of the use of a different piece of equipment due to the previous one being unavailable.

These figures were once again used to calculate the standard error used in the visual representation of the error bars (all done electronically).
With this data analysis, we concluded that Translation was being arrested without the presence of Leucine. We could reliably state this conclusion thanks to a significant disparity between the T-Statistic and the negative of T-Statistic 2 tail. Moreover, the P value being so low showed that this was highly unlikely to have happened by chance.

(4) Discussion

In order to progress our understanding of translation, this project sought to begin development of new reagents to be used in single molecule systems. The most important of these reagents, was a stable plasmid, whose parameters are easily characterised. A well characterised Plasmid expressing a heavily documented protein, in this case Luciferase, would enable us to harness the power of single molecule experiments to ascertain a rate of Translation that could potentially be applied to existing computational models. While we have successfully created a stable Plasmid capable of expressing Luciferase, which has been verified by means of a Luciferase assay, we have fallen short of unequivocally establishing that Translation can be arrested in this system and as such further work will be needed to verify this.

The initial design of the plasmid centred on choosing a well characterised protein that is easy to use and investigate. For this purpose, Luciferase was chosen, which along with the designed spacers, would have a Leucine codon at position 17, meaning that it would be a sufficient distance from the 6x Histidine complex vital for analysis. In this model, the Leucine codon would be our arrest codon, where translation is arrested, allowing further analysis of the interactions between mRNA, Ribosomes and tRNA:Amino acid complexes. By having this codon a sufficient distance from the Histidine tag, we could purify our mRNA:ribosome complex to give pure active Ribosome fractions.
Vitally, however, with translation being arrested, we could insert fluorescently tagged tRNA:Leucine and monitor the system, in real time, using single molecule techniques.

In order to reach this stage several steps were taken to ensure that a stable and functional DNA Plasmid was designed and created. Although an initial oversight had led to the erroneous deletion of the spacers, this was quickly noticed and rectified through the insertion of the missing sequence using PCR cloning techniques.
With the Luciferase gene being present in our Plasmid, it was important to measure its activity as a baseline for all future attempts at translation. This was achieved using a coupled Rabbit reticulocyte system that would complete both transcription

and translation simultaneously. In addition to this, the kits used allowed us to tailor the amino acid mixtures available to omit Leucine completely.

Initial testing confirmed that against unedited PGEM 3ZF, our construct was in working order, able to produce Luciferase under regular conditions. The degradation of the Luciferin substrate emits light[28] as one of its products and this was measured in the form of Bioluminescence, which was indeed identified in trials with the Luciferase containing Plasmid.

Further analysis would show us that the removal of Leucine from the amino acid mixture could potentially halt translation. While this is assumed an obvious consequence of the action, it is none the less paramount that this was investigated as it formed one of the pillars of future analysis. However, it is important to note that this needs to be fully confirmed through further testing. In order to establish that Translation has in fact, arrested and that cell death has not occurred, we must test to see if Translation can be continued if Leucine is placed back into the system. At this stage, it cannot be unequivocally confirmed that only Translation has arrested, merely that the removal of Leucine prevents further Translation from occurring.

With the data being quantified by means of a t-test, we could see the reliability of concluding that the removal of Leucine would halt translation in this initial test. T-stat was shown to be -9.15 and a P value of 0.000396 was given. These values together not only indicated that the hypothesis could be accepted but also that this was statistically unlikely to have happened by chance.

This is not enough to be certain that cell death has not occurred. In order to verify that Translation can be resumed, Leucine must be placed back into the system and analysed for the emittance of light (an indicator that Translation has occurred). Moreover, an additional test that can be attempted is a purification of the system to allow for an SDS PAGE analysis to verify a partial production of protein. Subsequent Western blotting would be possible due to the exposed Histidine tag on the protein and the expectation would be to see a band of around 6kDa. This is doubly important as it would also confirm that at the arrested stage of Translation, the His tag can be used for purification to gather Ribosome fractions.

If we can validate that Translation is arrested and can be restarted, we will be able to then move onto single molecule study of this system, which will help to yield data on the kinetics of Translation in further detail. One such recent experiment established the effect that tRNA amino acylation exerts on the rate of Translation. The development of a model allowed the prediction of the way that heterologous genes can influence expression and thereby Translation at the cellular level.

Other research has indicated that it is also possible to deduce in vivo Translation rates from in vitro gathered data. The developed computational model uses data gathered on the time take for each individual step to estimate the rate of protein synthesis based on existing models[29]. This new computational model was compared against independent sets of in vivo data on protein synthesis which found it to be highly accurate[30-32]. This data is significant to this project as it will enable us to compare our rate of translation against existing data and evaluate its significance. Moreover, by characterising each step in greater detail, we could further expand these models to be able to move closer to defining a rate for other Translation processes more accurately.

Future work

With the plasmid being confirmed as producing our desired Protein, Luciferase, a strong base has been set up from which further experimentation can take place. Further progress must be made in order to verify that Translation has arrested and that Ribosomes can be purified from the arrested solution.

The first port of call will be to rigorously test the Vectors ability to resume Translation after it has been arrested and confirm that cell death has not occurred. This is a straightforward process and can be achieved by adding 1µl of the amino acid mix that contains Leucine (the omitted amino acid) as well as another 25µl of the substrate and analysing the plate. If Translation has successfully restarted, there should be a clear signal of Bioluminescence detected. Subsequent purification and Western Blotting should reveal a Protein of approximately 61kDA in size which would be the fully Translated Luciferase.

Furthermore, it could be important that a reliable protocol is established to produce a large volume of pure mRNA from this Plasmid. This would help to save time and money as this can be done relatively cheaply in comparison to the coupled system used for initial analysis. However, it is important to note that there are a variety of parameters that would need to be evaluated and noted, including (but not limited to) the concentration of nucleoside triphosphates (NTP), the addition of DTT (to stabilise sulfhydryl groups) and the addition of RNAse inhibitor (and subsequent purification to remove this). If these parameters can be established easily, the RNA can be identified on a Urea gel, using cDNA as a marker.

Moreover, in order to visualise the arrested mRNA:Ribosome complex, a light source would need to be established and inserted into the system. This could be achieved through the insertion of fluorescent amino acids into the system, through PCR, which could result in the creation of a scaffold to which the complex could attach. Furthermore, the light source is essential for all other single molecule analysis and so this work must be completed before furthering our work.

In order to purify the arrested mRNA:Ribosome:Protein complex, the Histidine tag will be used. As the Histidine tag is sufficiently before the first Leucine codon in Luciferase, it will have already been translated. Thus, the Histidine tag will be protruding from the Ribosomal exit tunnel, enabling purification to take place. This could take the form of Ni-NTA chromatography. By using a Nickle charged affinity resin, we can bind the HIS tag to the column meaning that the Ribosomes can be eluted, as well as the mRNA in separate fractions. Crucially, this form of purification can be done in both native and denaturing conditions, meaning that a more reliable protocol could be established.

If a model can be established using the combination of single molecule experiments and computational modelling, we will be able to predict a rate of Translation as well as identify rate limiting steps and their impact on the process. This development could be vital in enhancing our understanding of genetic diseases such as Neurodegeneration or Fragile X Syndrome[33].

References

1. Crick, F. On protein synthesis. Symposia of the Society for Experimental Biology 12, 138–163 (1958)
2. Clancy et al, Translation: DNA to mRNA to Protein. Nature (2008)
3. Clancy et al, Translation: DNA to mRNA to Protein. Nature (2008)
4. Trobro S et al, Mechanism of the Translation Termination Reaction on the Ribosome. Biochemistry, 11296–11303 (2009)
5. Dos Reis M et al, Solving the riddle of codon usage preferences: a test for translational selection. (2004)
6. Presnyak V et al, Codon Optimality Is a Major Determinant of mRNA Stability. Cell, VOLUME 160, ISSUE 6, P1111-1124, (2015)
7. Clement T.Y. Chan et al, Reprogramming of tRNA modifications controls the oxidative stress response by codon-biased translation of proteins. Nature Article number: 937 (2012)
8. Choi et al, N6-methyladenosine in mRNA disrupts tRNA selection and translation-elongation dynamics, Nature Structural & Molecular Biology volume 23, pages 110–115 (2016)
9. Choi et al, N6-methyladenosine in mRNA disrupts tRNA selection and translation-elongation dynamics, Nature Structural & Molecular Biology volume 23, pages 110–115 (2016)
10. Johansson, M., Zhang, J. & Ehrenberg, M. Genetic code translation displays a linear trade-off between efficiency and accuracy of tRNA selection. Sci.USA 109, 131–136 (2012)
11. Kim, S.J. et al. Translational tuning optimizes nascent protein folding in cells. Science 348, 444–448 (2015).
12. Neher E, Sakmann B, Single-channel currents recorded from membrane of denervated frog muscle fibres. Nature.260, 799-802 (1976)
13. Hughes et al, Single molecule techniques in DNA repair: A primer, DNA Repair Volume 20, 2-13 (2014)
14. Ritort F, Single-molecule experiments in biological physics: Methods and applications. J. Phys.: Condens. Matter 18, R531–R583 (2006)
15. S. Saurabh, S. Maji, M.P. Bruchez, Evaluation of sCMOS cameras for detection and localization of single Cy5 molecules, Optics Express 20 7338–7349 (2012).

16. W.C.W. Chan, S. Nie, Quantum dot bioconjugates for ultrasensitive noniso-topic detection, Science 281 (1998) 2016–2018.
17. M.P. Bruchez, Quantum dots find their stride in single molecule tracking, Current Opinion in Chemical Biology 15 775–780 (2011)
18. Hughes et al, Single molecule techniques in DNA repair: A primer, DNA Repair Volume 20, 2-13 (2014)
19. Chu et al, A novel and versatile computational tool to model translation. Bioinformatics, Volume 28, Issue 2, 292–293 (2012)
20. Ishimura R et al, RNA function. Ribosome stalling induced by mutation of a CNS-specific tRNA causes neurodegeneration. Science.345(6195):455-9 (2014)
21. Tanenbaum ME et al, A protein-tagging system for signal amplification in gene expression and fluorescence imaging. Cell, 159, 635-646 (2014)
22. Yan X et al, Dynamics of Translation of Single mRNA Molecules In Vivo. Cell, 165, 976-989 (2016)
23. Ishimura R et al, RNA function. Ribosome stalling induced by mutation of a CNS-specific tRNA causes neurodegeneration. Science.345(6195):455-9 (2014)
24. Chu et al, Translation elongation can control translation initiation on eukaryotic mRNAs, The EMBO Journal 33, 21-34 (2014)
25. Kozak M, An analysis of 5'-noncoding sequences from 699 vertebrate messenger RNAs. Nucleic Acids Research, Volume 15,8125–8148 (1987).
26. Acevedo et al, Changes in global translation elongation or initiation rates shape the proteome via the Kozak sequence. Scientific Reportsvolume 8, Article number: 4018 (2018)
27. Chamberlin M et al, Characterization of T7-specific Ribonucleic Acid Polymerase. Journal of Biological Chemistry 248, 2235-2244 (1972)
28. Cole H et al, The ATP pool in Escherichia coli. I. Measurement of the pool using a modified luciferase assay. BBA Bioenergetics 143, 445-453 (1967)
29. Rudorf S et al, Deducing the Kinetics of Protein Synthesis In Vivo from the Transition Rates Measured In Vitro. PLOS Computatinal Biology (2014)
30. Curran J & Yarus M, Rates of aminoacyl-tRNA selection at 29 sense codons in vivo. Journal of Molecular Biology, Volume 209, Issue 1, 65-77 (1989)

31. Sørensen M & Pederson S, Absolute in vivo translation rates of individual codons in Escherichia coli: The two glutamic acid codons GAA and GAG are translated with a threefold difference in rate. Journal of Molecular Biology, Volume 222 Issue 2, 265-280 (1991)
32. Kramer E & Farabaugh J, The frequency of translational misreading errors in E. coli is largely determined by tRNA competition. RNA, Issue 13, 87-96 (2007)
33. Richter J, Pausing on Polyribosomes: Make Way for Elongation in Translational Control. Cell VOLUME 163, ISSUE 2, P292-300 (2015)

Declaration

'No part of this thesis has been submitted in support of an application for any degree or other qualification of the University of Kent, or any other University or Institution of learning'.

Signed:

Date: