Using mutagenesis and molecular cloning techniques to identify key residues within the molecular recognition site of the Glucuronide Membrane Transporter (GusB).

Faculty of Science and Technology

Molecular biology MRes

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16.09.2020

Acknowledgements

First and foremost, I would like to thank my family for being a constant source of strength and support throughout the entirety of this project. I would like to acknowledge Dr Wei-Jun Liang for sharing his expertise and broadening my knowledge of molecular techniques both inside the laboratory. I would also like to thank Qi Zhang, Christine Ciocan, Camilla Domeneghetti and Lydia Doherty for their continuous help and encouragement during long and laborious days in the lab.

Abstract

The glucuronide transport membrane protein (GusB) encoded by the *gusB* gene is essential for the survival of *Escherichia Coli* (*E. coli)* residing in the gut of vertebrate species. This gene, along with four others located on the GUS operon, allows *E. coli* to scavenge carbon from detoxification products produced by the host. These products are known as glucuronides, they are composed of two parts, one is a glycone (glucuronic acid), and the other is an aglycone (various molecules). GusB has shown varying affinity for the substrates that is transports therefore recognising the glycone part very specifically and the agylcone part indiscriminately. However, the binding mechanism used by this protein this is still unknown.

Amino acid residues within GusB must be structurally relevant for molecular recognition of glucuronide molecules; however information relating this is not yet available. Therefore, identifying key residues within the molecular recognition site of GusB became the focus of this research project.

Using *E. coli* as a model organism, site-directed mutagenesis and cloning techniques employed in the laboratory were used to create six residual amino acid changes within the Glucuronide Transporter Protein. These occurred at various positions within the predicted cytoplasmic region of GusB. By substituting amino acids to others with different charges and polarity, it was expected that protein folding mechanisms and the molecular recognition site would be disrupted. Glucuronide transport activity was predicted to change as a result.

Successful cloning to create mutant GusB proteins could not be achieved however, sequence analysis of a previous plasmid pE349 encoding a mutant GusB found an unexpected amino acid mutation at position 218 of the glucuronide membrane transporter. Chromogenic GUS assays showed the change from Asparginine (uncharged) to Lysine (positively charged) caused a reduction in the transport rate of glucuronides. This led to questions as to whether this amino acid change was indeed part of the molecular recognition site. All steps undertaken for mutagenesis and cloning techniques have been detailed and proposals for further research are highlighted. It is hoped that by identifying key amino acid residues, this protein could be manipulated in the future to recognise very specific substrates for use in biosensor and report gene technology.

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Abbreviations

Term	Disambiguation
ATP	Adenosinne Triphosphate
Bp	Base pair
dH_2O	Deionised water
DNA	Deoxynucleic acid
dNTP	Deoxynucleotide triphosphate
EDTA	Ethylenediamine tetra-acetic acid
GFP	Green Fluorescence Protein
Kb	Kilobase
IPTG	Isopropyl β-D-1-thiogalactopyranoside
LB	Luria Bertani
NEB	New England Biolabs
ON	Overnight
PCR	Polymerase chain reaction
pNP	para-nitrophenol
pNPG	4-nitrophenyl-β-D-glucuronic acid; p-nitrophenyl-β-D-glucuronide
RNA	Ribonucleic acid
SAP	Shrimp Alkaline Phosphatase
SDM	Site-Directed Mutagenesis
TAE	Tris acetate EDTA
Tm	Annealing temperature
TM	Transmembrane Helices
Tris	Tris (hydroxyl methyl) aminomethane
X-Gluc	5-bromo-4-chloro-3-indol-b-D-glucuronide

1. Introduction

1.1 An overview of the project

The bacterium *Escherichia coli* is known to inhabit the gut of most endothermic vertebrate species (Denamur et al., 2010). For these organisms to thrive in such environments, they must have a distinct mechanism in place to acquire all the relevant nutrients related to their survival.

Within host species including humans and other mammals, detoxification products known as glucuronides provide a valuable carbon source for the bacteria (Csala et al., 2004; Ishii and Sadowski 2008; Gloux et al., 2010). Everyday exposure to harmful substances is a constant threat to animal species and therefore a reliable defense mechanism must be in place. Glucuronides are formed of two components, the first is the aglycone component; these are substances considered toxic to the organism. These include endogenous metabolic waste, xenobotics, steroids, vitamins, hormones and pharmaceutical drugs (Argikar, 2012). For these to be excreted, they must first become covalently bound to a glucuronic acid also known as a glycone, in turn increasing the polarity of the molecule and the likelihood of elimination (Fujiwara et al., 2018, King et al., 2000, Tephley et al., 1990; Compernolle 1978). However, commensal or symbiotic bacteria inhabiting the gastrointestinal tract are responsible for the alternate fate of these glucuronide molecules.

The *E. coli* bacterium harnesses the ability to transport glucuronides across the cell membrane where they then cleave the molecule in two. The aglycone component is of no use to *E. coli*, this part will be transported back across the bacterial membrane where it is subsequently excreted or re-absorbed by the host in a process known as enterohepatic circulation (Gibson and Skett 2002; Guillemette 2003; Xia et al., 2012). The glycone is used as a source of energy, and the symbiotic relationship between the host and bacteria is established.

This process is essential to *E. coli* however; it would be impossible without a particular set of proteins encoded by the genes of the GUS operon (Hughes et al., 1992, Jain et al., 2006). Operons are described as clusters of genes that are transcribed in a single mRNA or with a single promoter (Alberts et al., 2002; Osbourn and Fields 2009; Alberts et al., 2014). It may seem obvious then that genes transcribing proteins with similar properties or whose products interact are often found and controlled within the same operon. The function of

that particular system can then work more effectively as a result priving beneficial to the organism (Dandekar et al., 1998; Ralston 2008).

A novel example of this comes from the more widely known, Lac Operon. When Glucose is not readily available, *E. coli* is able to metabolise lactose as an alternate source of energy (Griffiths et al., 1999; Becker et al 2013). However, in order to do this they require proteins LacY, LacZ, and LacA. This operon will only be expressed under two conditions; firstly lactose must be present, this is detected by a lac repressor protein found on the operon. Secondly, glucose cannot be present which is detected by another repressor, the catabolic activator protein (Ullmann 2009). If these conditions are met, the inducer allactose will bind to the lac repressor and remove it from the operator region. This in turn allows DNA polymerase to bind to the promoter region and transcription of the desired genes can ensue. CAP is responsible for binding near to the promoter and encourages the binding of DNA polymerase therefore in turn increasing the rate of transcription (Griffiths et al., 1999; Alcantara 2015). Having all of these genes controlled under the same operon, it means that the enzymes and other proteins needed for lactose metabolism are only produced when they are really needed. This ensures that unnecessary energy is not expended by the bacteria (Dean et al., 2008).

Although less widely studied, the Gus Operon is thought to work in a similar way. Within the GUS operon specifically, four genes are present. *gusR* encodes a repressor protein ultimately controlling the transcription of the other functional genes on the operon . Once the desired substrate, in this case a glucuronidated ligand, is present the repressor protein encoded by gusR will temporarily become disassociated from the regulatory region (Hughes et al., 1992; Little et al., 2017). This allows the operator-promoter region to become unobstructed, RNA polymerase can bind and translation of gusA, B and C into their respective proteins will begin ((Novel and Novel 1976, Guo 2014, Little et al., 2017).

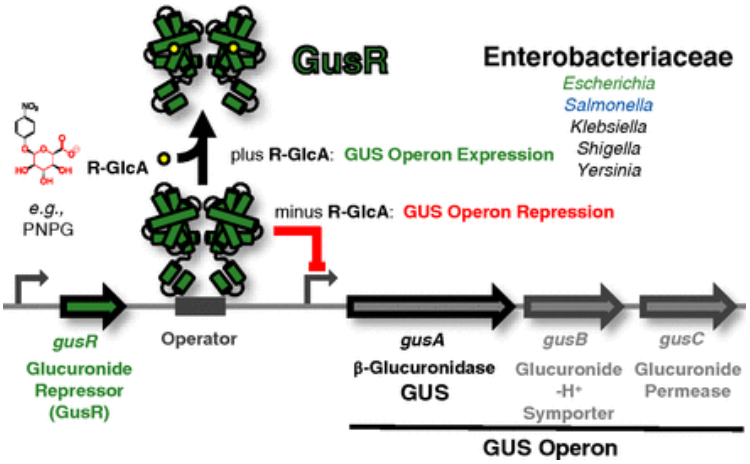


Figure 1-1 Schematic of the GUS operon in Enterobacteriaceae. In the absence of a glucuronide ligand (R-GlcA; yellow), GusR (green) is expected to repress (red) the downstream transcription of the GUS operon proteins GusA, GusB, and GusC (grey) by binding to a specific DNA operator site. In the presence of a glucuronide ligand (e.g., p-nitrophenyl glucuronide), GusR disassociates from the operator to allow GUS operon expression. As shown here, only the Enterobacteriaceae, including several opportunistic bacterial pathogens, contain a GUS operon and GusR (Little et al., 2017).

The *gusA* gene encodes β-D-glucuronidase, this hydrolase is able to cleave a wide variety of glucuronides into its two components, the glycone and aglycone (Gloux et al., 2010). The *gusB* gene encodes a proton-linked glucuronide transporter in the cell membrane (Liang et al., 2005). As for *gusC,* the detailed mechanism of its function remains obscure; however one theory has suggested that it encodes an outer membrane protein aiding GusB with transport (Liang et al., 2005).

Membrane transport systems are diverse, they can act for acquisition of nutrients, excretion of waste or toxic materials, ATP production, nerve signal relay, maintenance of membrane potential, pH regulation, hormone responses, oxidation and reduction of substances (Ludewig and Frommer 2002; Blatt 2004; Otsuka et al., 2005; Claycomb and Tran 2011; Garguad et al., 2011; Watson 2015). The intricate and coordinated way operons can be turned on and off to allow for minimum energy expenditure shows they evolved in a sophisticated manner (Lodish et al., 2002). The proteins found on these operons prove to have sophisticated and intriguing qualities, the broad specificity of GusB, the glucuronide transporter proving to be one of these.

In 1961, Stoebar pioneered a study in which he described the GusB protein to work via active transport. GusB simultaneously transports the selectively bound molecule along with a driving substrate in the same direction across the membrane as seen in Figure 1-2. (Wolfersberg 1994, Liang 1989; Liang 1992; Liang et al., 2005). Within E. coli, an

example of symport transport is seen in which glucuronide uphill transport is coupled with the co-transport of an H^+ ion (Kaback and Wu 1997).

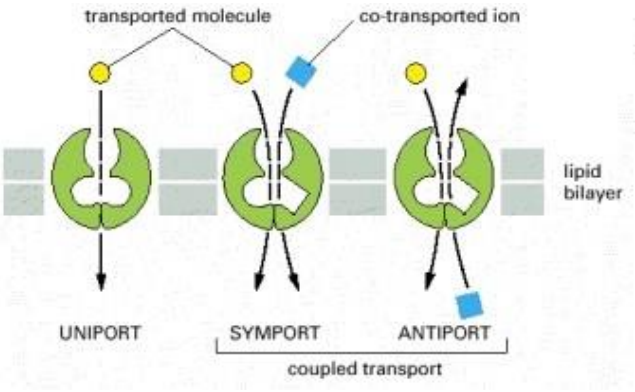


Figure 1-2 Types of transport proteins: Uniporters are the most simple example of transport proteins translocating a singular molecule from one side of the membrane to the other; symporters translocate two substances from one side to the other simultaneously, this is using an ion of some sort (for GusB this is a proton) and antiporters transporta substance one way and a second substance in the opposite direction (Alberts et al., 2002)

Stoebar also presented findings showing that GusB also had differing affinities for some glucuronide molecules as opposed to others (Stoebar, 1961 , Liang et al., 2005). Due to huge variations in aglycone components, questions arise of how this transporter can recognise glucuronides with such diverse properties. Not only this, but why the transport of some substrates is prioritised over others. It would seem logical to assume that the answer lies within the molecular recognition site of the transporter, after all this is where substrates bind and initiate the transport mechanism (Ariga et al., 2007). Structural knowledge is unavailable for the GusB protein although sequence alignments and hydropathy plots created in previous studies by Liang (1987) showed structural similarity in hydrophobic and hydrophilic regions to two other transporter proteins MelB, the Melibiose Carrier protein and LacS, the lactose symport protein (Gunnewijk et al., 1999; Amin et al., 2014). It was suggested then, that this protein might too belong within the Major Facilitator Superfamily (Yan, 2015). Liang (1992) created a model, which depicted the predicted structure of GusB based on the assumption that, like the MFS family, the protein comprised on the 12 TM helices (Figure 1-2) (Yan, 2013). Somewhere amongst these helices, presumably within the outside loop, must lie the substrate binding site which when triggered by a complementary substance will undergo a conformational change allowing transport across the membrane (Madej 2014).

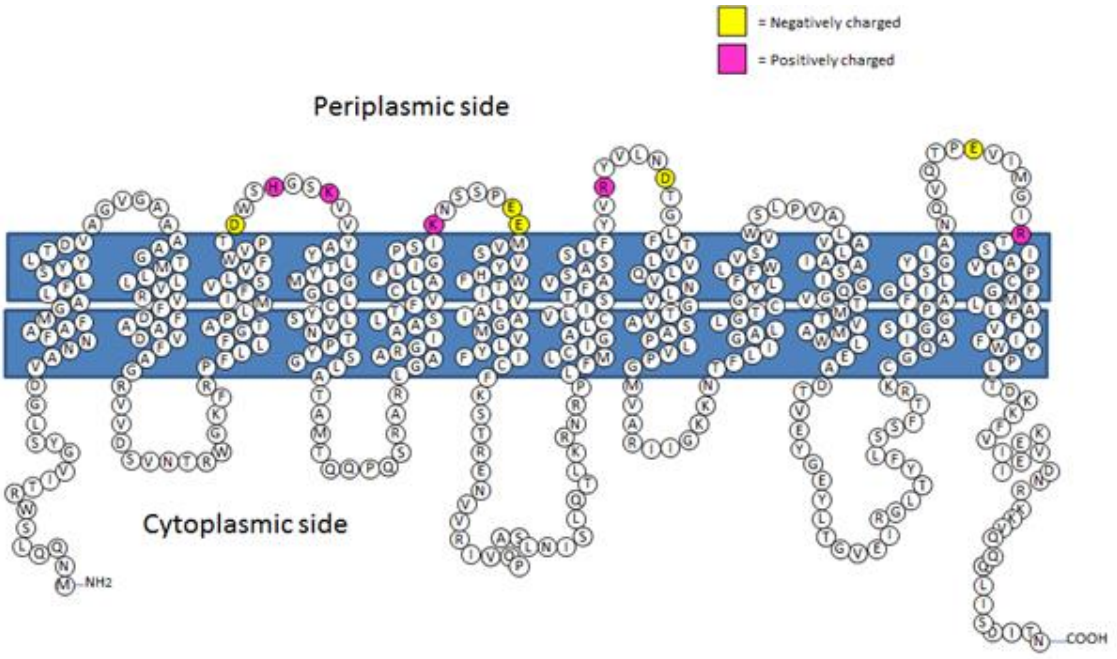


Figure 1-3 Predicted structure of GusB membrane transporter. GusB is show to be within the cell membrane with loops of amino acid residues exposed to the inide and outside of the cell. 12 transmembrane helices can be seen, conforming to the assumption it is part of the MFS. Pink shaded amino acids show positively charged amino acids on the periplasmic side of the membrane and yellow show negatively charged amino acids (adapted from Liang 1992).

It was not until a study by Ishii (2010) was published that any relevant structural information came to light. In the study, Ishii found an elution peak corresponding to a pentameric structure in a number of different elution profiles using size-exclusion High-performance liquid chromatography. He later continued his work and was able to provide two-dimensional electron crystallography images (See figure 1-4). Though this is developmental in regard to structural findings there is still a need for further work to identify the location or structural evidence regarding the molecular recognition site and better understand substrate specificity (Ishii et al., 2013).

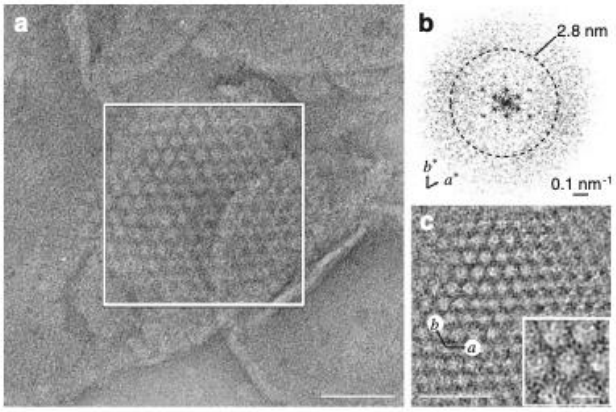


Figure 1-4 Electron micrographic image of 2D crystalline array of GusB. Scale bar = 50 nm. b Computed diffraction pattern of the 2D crystalline array of GusB indicated with a box in (a). The a*- and b*- axis of the hexagonal lattice are indicated. The dotted circle line corresponds to a spacing of 2.8 nm. Diffraction bar is 0.1 nm-1 . c Fourier-filtered image of 2D crystalline array showing the diffraction pattern in (b). All diffraction spots were masked off with Gaussian-shaped circular masks and were inverse Fourier transformed. a = b = 13.75 nm, and c = 120. Scale bar = 50 and 10 nm for the enlarged inset (Ishii et al., 2013)

With the information available taken into consideration, deciphering key structural amino acids residues within the molecular recognition site of GusB became the focus of this project. Mutagenesis allows DNA within an organism to be purposely changed, this allow genes to be mutated therefore leading to a disruption in the final protein structure (Theodorakis, 2008)

Site directed mutagenesis (SDM), one of the most common procedures, can create planned changes in DNA and subsequently in the amino acid sequence allowing specific amino acid to be substituted to another with varying properties such as charge and polarity (Hutchinson et al., 1978; Edelheit, 2009). The ability to deliberately make these changes can help to identify if amino acids are relevant to the molecular recognition site of GusB. Protein and enzymatic activity measurements of normal and mutated proteins can then be monitored and notable changes can be observed using *in vivo* organisms, such as *E. coli* (Storici et al., 2001).

In total, six site-directed mutations were chosen in which specific amino acids were changed to another with differing properties. It is thought that this may cause changes to the transport and function of the protein in comparison to the glucuronide transporter produced by the wild type *gusB* on the pMJB33 plasmid (Bruce and Liang 2014). By relying on molecular cloning techniques, the mutated *gusB* gene was inserted into a

pTTQ18 expression vector, amplified using PCR and then cloned into competent *E. coli* cells (Stark, 1987). pTTQ18 was used as it has a strong *tac* promoter, in addition to this is does not have a naturally occurring *gusB* gene within its sequence.

Should the cloning process be successful, it is possible that this mutation will disrupt the structural integrity of the protein. If the residue proves to be important to either molecular recognition this may cause changes in transport activity indicating a disruption in the binding site and deeming the original amino acid structurally relevant.

A greater understanding of singular genes and proteins in addition to the wider systems they support can allow us to manipulate certain parts in way which may benefit society in the future (Chica, 2015). Protein engineering can be particularly advantageous in the creation of reporter genes, bio-concentrators and biosensors (Van der Meer and Belkin 2010, Gallagher, 2012). However, with structural and functional relationships still not understood these applications for GusB are still a distant prospect. By looking at this protein as a basic structural level, it may uncover the relevance of singular amino acids and internal protein interactions in turn revealing more about the molecular recognition and substrate binding site of GusB glucuronide membrane transporter.

Due to the predicted structure of GusB alignments were conducted with MFS protein XylE (See Appendix 8). Although sequence homology was low, a paper published by Madej et al 2014 recognised mutations at position 27 created a complete loss of Xylose/H+ symport therefore the first mutation seen in Table 1-1 was chosen. In addition to this, current literature outlines the effect that amino acids substituted to another with different charges and polarity can have dramatic effects on protein function and more specifically transport (Zhang et al 2003; Schaefer and Rost, 2012). It is important to recognise that low sequence homology across the MFS is generally low however, structural similarity is common (Vardyet al., 2004; Zhang et al., 2015).

The other mutations chosen in the inner and outer loops were somewhat random, with substitutions being made based on the knowledge that interactions between charges and polarity are important for site structure (Madej and Kaback 2013; Biggin et al., 2016). It would now seem obvious that mutations should have been chosen based on more protein alignments. In addition to this, the focus should have been on residues known to be related to molecular recognition. Or from alignment, amino acid matches with similar charge and polarity with proteins such as MelB, LacS or XylE. This was recognised after on-going training and acquiring of knowledge over the course of this masters project.

The substitution mutations incorporated within the *gusB* gene can be seen in Table 1-1. All plasmid maps can be found in Figure 2-1.

Table 1-1 The six site-directed amino acid mutations of the gusB gene conducted in this study: the name of the mutation is composed of the transmembrane (TM) helices of the substitution mutation as well as the place the amino acid would be found within the gene sequence.

Name of Mutation	Position of mutation on gene	Original Amino Acid Residue	New Amino Acid Residue
pAB-G27D	TM1G27D	Glycine (neutral)	Aspartic Acid (Negatively charged)
pAB-L119C	TM4L119C	Leucine (Hydrophobic)	Cysteine (Neutral)
pAB-H183E	TM6H183E	Histidine (Positively charged)	Glutamic Acid (Negatively Charged)
pAB-N273D	TM8N273D	Asparagine (Polar)	Aspartic Acid (Negatively Charged)
pAB-E362N	TM10E362N	Glutamic Acid (Negatively charged)	Asparagine (Polar)
pAB-R406E	TM12R406E	Arginine (Positively charged)	Glutamic Acid (Negatively charged)

1.2 Aims and Objectives

Aim:

The main aim of this project is to identify key amino acids within the molecular recognition site of the *E. coli* transporter protein GusB.

Objectives:

The first objective of this project is to design oligonucleotide primers for site specific mutations at the correct position on the *gusB* gene; this should be thought of logically and should be designed with consideration to conduct successful PCR reaction. Therefore, annealing temperature, GC content and length should be taken into consideration.

Next, using PCR based site-directed mutagenesis; the six chosen mutations as seen in Table 1-1 should be individually amplified.

Using corresponding molecular cloning techniques, the mutated *gusB* gene will then be cloned into the pTTQ18 vector and finally introduced into competent *E. coli* strains.
Transport based tests (GUS Assay) using chromogenic pPNG will be performed to determine whether amino acid mutations cause changes to transport and possibly disruption to the molecular recognition site. Binding affinity of specific glucuronides will also be assessed.

The clones will be sequenced to see if the desired mutation has been incorporated into the *gusB* gene.

2. Materials and Methods

Throughout this project, procedures relating to cloning and site directed mutagenesis were undertaken in the laboratory spanning over approximately a one-year basis. Lab work was extended from a 6-month to a 12-month period due to a lack of results from cloning procedures and the troubleshooting which followed.

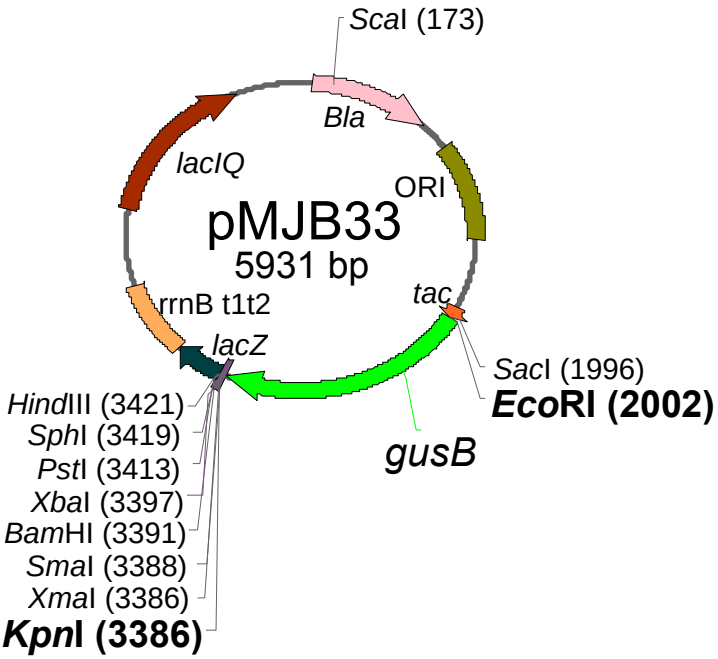
All lab work was undertaken at Bournemouth University under the direction and help of Dr Wei Jun Liang who supervised the project.

It was essential that before any laboratory work could begin both induction and equipment training was completed. In addition to this several forms including risk assessment and COSHH were completed. Due to the use of *E. coli* rather than mammalian or human tissues an ethical checklist was not necessary. All procedures are outlined in the following pages listed in the order they were undertaken, please note these are approximate and some may have been performed on multiple occasions.

A list of plasmid maps for the clones in this study as well as the *E. Coli* strains can be found on the following page and referred back to as required when addressing procedures in the methodology.

Table 2-1 Bacterial Strain Information: The bacterial strains used to make competent cells; *E.coli* 5-alpha and 10-beta strains were ordered from New England Biolabs and were already competent.

Bacterial Strain	Genotype	Additional Strain Information	Where from & CatLog no
MC1061	F– araD139 Δ(ara-leu)7696 galE15 galK16 Δ(lac)X74 rpsL (StrR) hsdR2 (rK– mK+) mcrA mcrB1	- from K12 *E. coli* strain (Mandel 1970) - Transformation Efficiency: >1.0x108 cfu/µg pUC19 DNA	Supervisors own
5-alpha Competent *E. coli* (From NEB)	fhuA2 Δ(argF-lacZ)U169 phoA glnV44 Φ80 Δ(lacZ)M15 gyrA96 recA1 relA1 endA1 thi-1 hsdR17	- Derivative of DH5α. - From K12 *E. coli* strain (Mandel 1970) - Transformation Efficiency: 1 - 5 x 108 cfu/µg pUC19 DNA - T1 phage resistant - endonuclease I (endA1) deficient for high-quality plasmid preparations	New England Biolabs, C2987I
10-beta Competent *E. coli* (From NEB)	Δ(ara-leu) 7697 araD139 fhuA ΔlacX74 galK16 galE15 e14- ϕ80dlacZΔM15 recA1 relA1 endA1 nupG rpsL (StrR) rph spoT1 Δ(mrr-hsdRMS-mcrBC)	- Derivative of MC1061 - From K12 *E. coli* strain (Mandel 1970) - Transformation efficiency: 1–3 x 109 cfu/µg pUC19 DNA -T1 phage resistant - endonuclease I (endA1) deficient for high- quality plasmid preparations	New England Biolabs, C3019I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lacIQ
ORI
pMJB33
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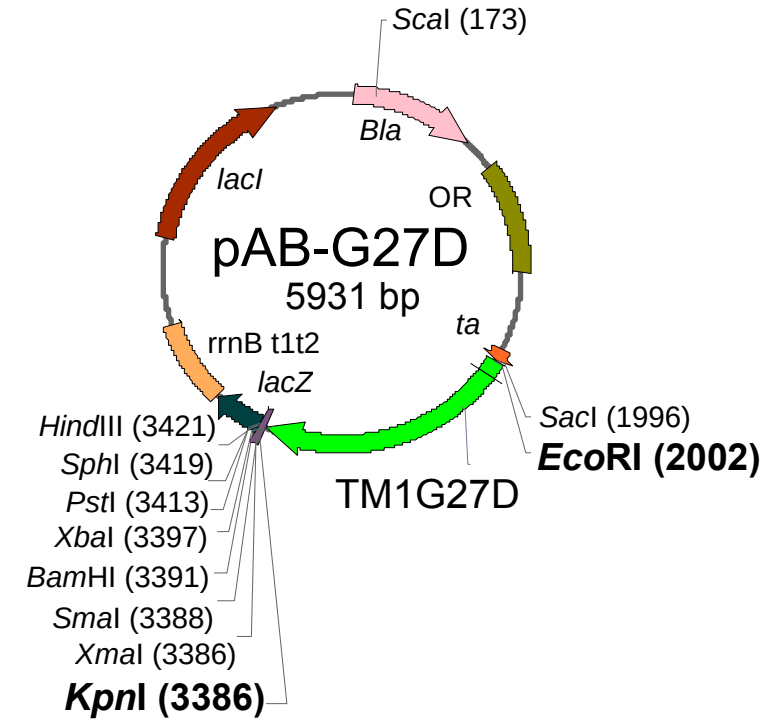
rrnB t1t2
tac
lacZ
SacI (1996)
EcoRI (2002)
gusB
HindIII (3421)
SphI (3419)
PstI (3413)
XbaI (3397)
BamHI (3391)
SmaI (3388)
XmaI (3386)
KpnI (3386)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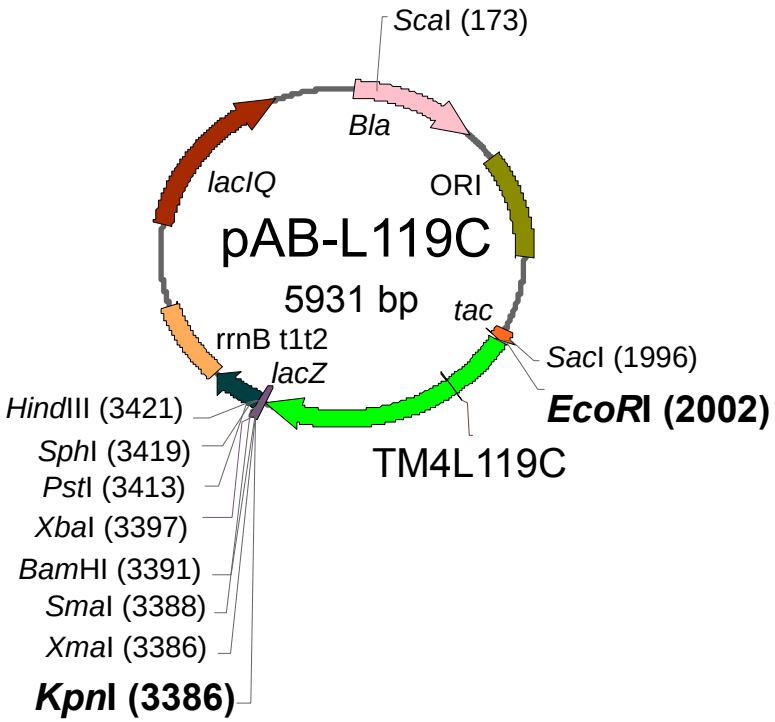
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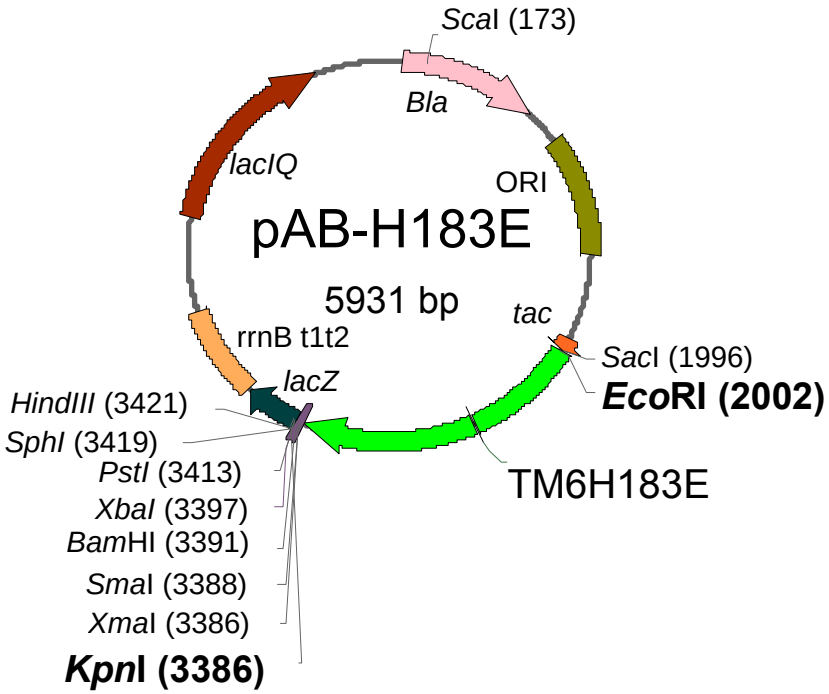
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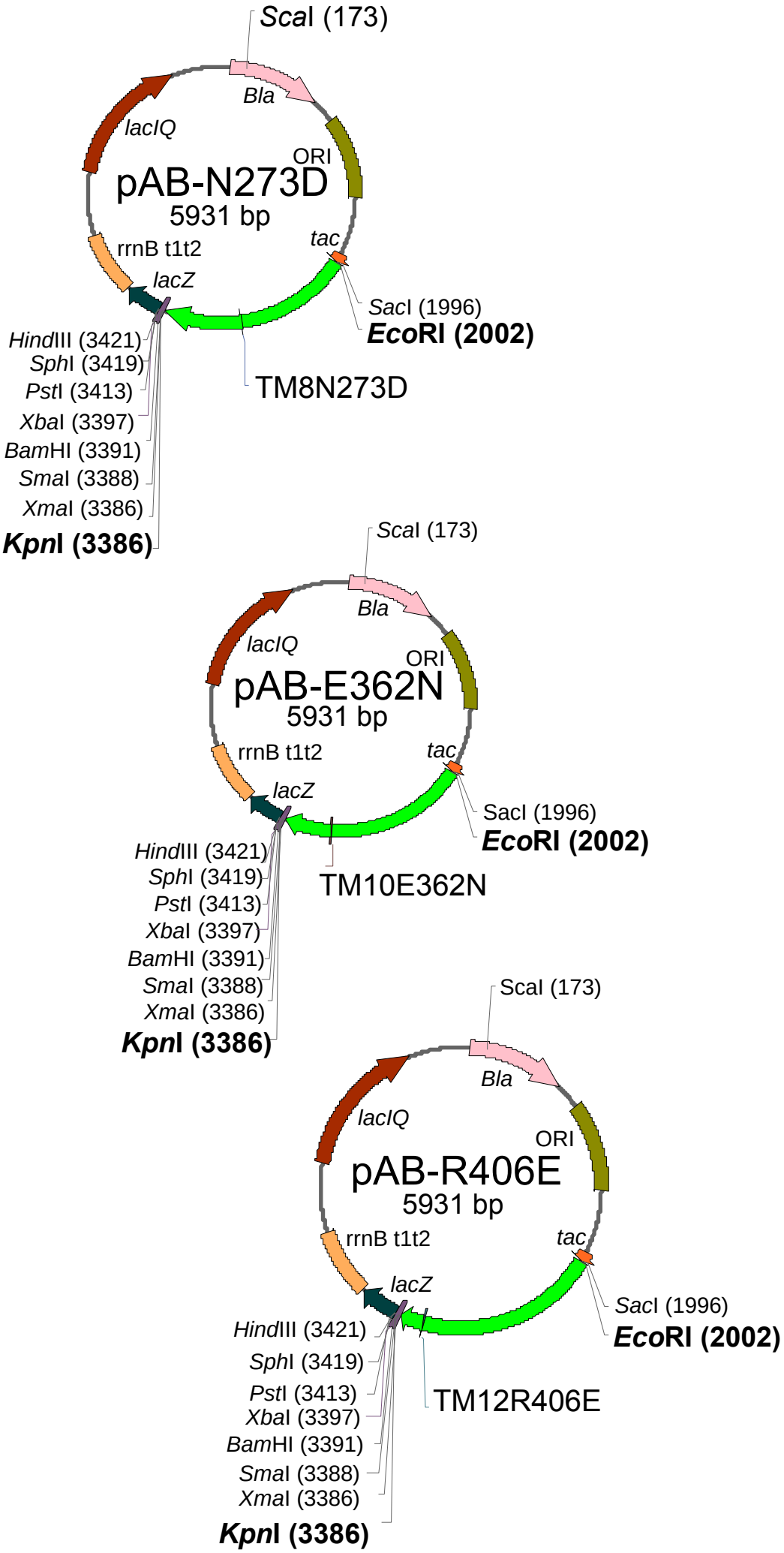
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Figure 2-1 Plasmid Maps of the template plasmid used for PCR amplification and plasmid maps for each plasmid containing site directed mutations created specifically for this project. 1.Wildtype pMJB33 and the plasmids created in this research project containing site directed mutations in the gusB gene: 2. pAB-G27D, 3. pAB-L119C, 4. pAB-H183E, 5. pAB-N273D, 6. pAB-E362N, 7. pAB-R406E. The maps show all restriction sites as well as the tac promoter sequence, origin of replication, antibiotic-resistant marker (Bla) and laclQ.

2.1 Preparation of LB Media and LB Agar for culturing bacteria cells.

LB media and Agar plates were needed throughout. They act as a nutrient base which enables bacteria cultures (*E. coli* in this study) to grow, this may be either in a liquid culture or agar plates on which chosen samples are streaked.

The following constituents were added into a 1 litre (L) DURAN bottle:

600 ml of Distilled water

10 g Tryptone (amino acid mix)

5 g NaCl

10 g Yeast Extract

Once the correct solution had been made up, the appropriate amount of distilled water was added to make the final volume 1 L, the glass container was placed into the autoclave making sure that this was put on the 'liquid media' setting. Another separate 1 L solution of LB media was made intended for agar plates therefore 15 g Agar was added before autoclaving the solution for around 20 minutes, a metal stirring rod was added and put onto a metal plate in order to mix the solution prior to autoclaving. For experiments that required antibiotic-containing media or plates (crucial for this project as clones with contain anti-biotic resistance gene), ampicillin was added to the solution AFTER the sterilisation using the autoclave due to the sensitivity at high temperature. In this case ampicillin was added at a concentration of 100μg/ml.

For plates:

Firstly, it is essential to keep any bottle containing LB media sealed not in use to avoid contamination. A Bunsen burner was lit and placed on the blue flame- once the bottle was opened the rim and the lid of the bottle were heated for sterilisation purposed (Sanders 2012). LB media was poured onto the plate, it was essential not to touch the lid of the bottle nor plate to avoid contamination.

The plates were left to cool and set, they were then labelled appropriately with the date and initials e.g. LB media, 12/03/2016, AM. If the LB plates contained ampicillin this was

included in the name and marks were left on the side of the plate to make this clear. They were kept in refrigerator until needed at other points during the project. When microorganisms need to be grown and harvested, they can be taken from pre-prepared *E. coli* samples and inoculated onto plates of liquid media. This can then be heated to promote bacteria growth.

2.2 Plasmid DNA extraction:

Plasmid DNA extraction (using Quicklyse Mini-prep kit from Qiagen) allows the isolation of plasmid DNA from bacteria strains using the alkaline lysis treatment (Ehrt and Schnappinger, 2003). In this project pTTQ18 was used, this plasmid vector has been designed to have high levels of regulated gene expression levels in addition to a strong promoter (Stark 1987; Surade et al., 2006).

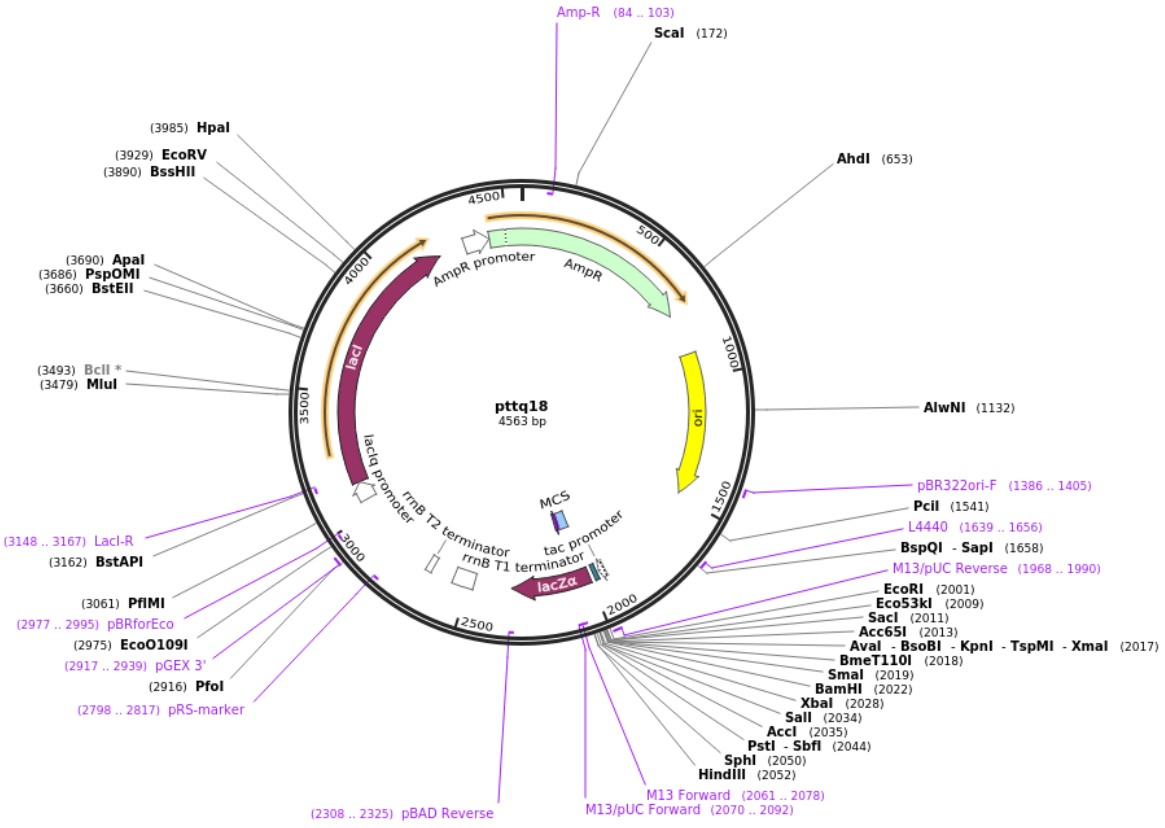

Figure 2-2 The pTTQ18 plasmid map this map shows the the lac promoter, lacI and lacZ gene, the ampicillin resistance gene, and the origin of replication. All restriction sites are also shown including EcoRI and KpnI which were the restriction enzymes used for this project (Stark et al., 1987)

1ml of bacterial culture was pipetted into separate 1.5 ml microcentrifuge tubes. These were clearly labelled to avoid any mix up throughout the process. Each sample was then placed into the centrifuge at 13,000rpm for 3 minutes to precipitate the bacteria. The liquid was poured away; any remanence of liquid in the tube was discarded. 250 µl of Buffer P1 was then added to each tube containing the DNA sample; the cells were re-suspended using the vortex mixer. Once mixed adequately, all samples were left to stand for 2 minutes, 250 µl of Buffer P2 was then added and the tubes were inverted 6 times. This was followed by the addition of 350 µl Buffer N3; tubes were shaken and then suspended upside down for 5-10 seconds.

The samples were then centrifuged for 10 minutes at 13,000 rpm, 800 µl of the supernatant from each sample was transferred into separate spin columns using a pipette and centrifuged for a further minute. The aqueous flow through was discarded. 0.5 ml of Buffer PB was added into each spin column and centrifuged for 1 minute, the remaining liquid was discarded. Next the DNA left in the column was washed using 750 µl of PE buffer; this was centrifuged again for a further minute. An additional 350 µl of PE buffer was added and centrifuged for 1 minute. The liquid was again discarded then centrifuged once more to remove any residual buffer. All samples were put into the incubator (37˚C) for a few minutes to remove any ethanol left from the PE buffer, residual ethanol could inhibit subsequent enzymatic reactions. Once removed from the incubator the mini-column now containing just the purified DNA was placed into a new 1.5 Microcentrifuge tube where 50 µl of Buffer EB was added and centrifuged for 1 minute to re-suspend the DNA within the solution. The filter was then removed, and it was made sure that the lid of the microcentrifuge was shut properly. All samples were then stored at -20°C for future use. Gel electrophoresis was then conducted to identify pTTQ18 according to size (4563 bp).

2.3 Oligonucleotide Primer Design for the mutant *gusB* genes

Oligonucleotide primers for specific PCR amplification were designed carefully all based on the intended DNA template, a wild type pMJB33 clone containing the *gusB* gene (See appendix 3)(Bruce and Liang 2014). The primers included site-specific mutations to create an amino acid change at chosen points within the sequence. According to Dieffenbach et al., (1993) there are a number of general rules that should be taken into consideration. Primers should be 18-24 nucleotides in length, at this length they are adequate for specificity but short enough to bind to the template at the annealing temperature. The GC content should be around 40-60% to keep the melting temperature between around 52-

58°C as primers with a melting point lower than this result in non-specific binding (Lorenz 2012). For flanking primers restriction sites for appropriate restriction enzymes had to be included with random nucleotides added at either end to allow cutting. Corresponding primers were made to have a T^m within 5˚C of each other so both primers will anneal to the template within the same reaction (Singh and Kumar 2001).

Primers for this project are seen below:

Table 2-2. The forward and reverse mutagenic primers from 5'-3' as designed for this project. Nucleotides highlighted in red show where the substitution mutation needs to be made. The melting temperature (T_m) for forward and reverse primers are shown in the last column. Yellow and blue highlighted parts of the sequence represent nucleotides not included in the *gusB* sequence; these are added for efficient restriction digest later stages of cloning (Feeney et al., 2014). Green highlighted sections show the open reading frame of *gusB*.

Name of primer (Forward and Reverse)	Primer Sequence	T_m for primers (˚C)
TM1G27DF	5'- TTCGCAATGACGGCGCTCTT-3'	65.3
TM1G27DR	5'- AAGAGCGCCGTCATTGCGAA-3'	
TM4L119CF	5'-ATGGGCTGTGGGCTTTGCTAC-3'	67.8
TM4L119R	5'- GTAGCAAAGCCCACAGCCCAT -3'	
TM6H183EF	5'-TCGGTATACGAGTTCTGGACA-3'	63.9
TM6H183ER	5'-TGTCCAGAACTCGTATACCGA-3	
TM8N273DF	5'-GTGCAAGACCTGGTTGGTA-3'	62.6
TM8N273DR	5'-TACCAACCAGGTCTTGCAC-3'	

TM10E362NF	5’-GGCGCTGAGCGCTGATAC-3’	66.3
TM10E362NR	5’-GTATCAGCGCTCAGCGCC-3’	
TM12R406EF	5’-GGCATCGAGACATCAATTG-3’	60.4
TM12R406ER	5’- CAATTGATGTCTCGATGCC -3’	
ER1gusBF (based on pMJB33 clone)	5’-AATAAGAATTCATGAATCAACAACTCTCCTG-3’	-
KpIgusBR (based on pMJB33 clone)	5’-TTGGTACCTTAATTAGTGATATCGCTGATTAAT-3’	-

```
atgaatcaacaactctcctggcgcaccatcgtcggctacagcctcggtgacgtcgccaat
 M  N  Q  Q  L  S  W  R  T  I  V  G  Y  S  L  G  D  V  A  N
aacttcgccttcgcaatgggggcgctcttcctgttgagttactacaccgacgtcgctggc
 N  F  A  F  A  M  G  A  L  F  L  L  S  Y  Y  T  D  V  A  G
gtcggtgccgctgcggcgggcaccatgctgttactggtgcgggtattcgatgccttcgcc
 V  G  A  A  A  A  G  T  M  L  L  L  V  R  V  F  D  A  F  A
gacgtctttgccggacgagtggtggacagtgtgaatacccgctggggaaaattccgcccg
 D  V  F  A  G  R  V  V  D  S  V  N  T  R  W  G  K  F  R  P
tttttactcttcggtactgcgccgttaatgatcttcagcgtgctggtattctgggtgccg
 F  L  L  F  G  T  A  P  L  M  I  F  S  V  L  V  F  W  V  P
accgactggagccatggtagcaaagtggtgtatgcatatttgacctacatgggcctcggg
 T  D  W  S  H  G  S  K  V  V  Y  A  Y  L  T  Y  M  G  L  G
ctttgctacagcctggtgaatattccttatggttcacttgctaccgcgatgacccaacaa
 L  C  Y  S  L  V  N  I  P  Y  G  S  L  A  T  A  M  T  Q  Q
ccacaatcccgcgcccgtctgggcgcggctcgtgggattgccgcttcattgacctttgtc
 P  Q  S  R  A  R  L  G  A  A  R  G  I  A  A  S  L  T  F  V
tgcctggcatttctgataggaccgagcattaagaactccagcccggaagagatggtgtcg
 C  L  A  F  L  I  G  P  S  I  K  N  S  S  P  E  E  M  V  S
gtataccatttctggacaattgtgctggcgattgccggaatggtgctttacttcatctgc
 V  Y  H  F  W  T  I  V  L  A  I  A  G  M  V  L  Y  F  I  C
ttcaaatcgacgcgtgagaatgtggtacgtatcgttgcgcagccgtcattgaatatcagt
 F  K  S  T  R  E  N  V  V  R  I  V  A  Q  P  S  L  N  I  S
ctgcaaaccctgaaacggaatcgcccgctgtttatgttgtgcatcggtgcgctgtgtgtg
 L  Q  T  L  K  R  N  R  P  L  F  M  L  C  I  G  A  L  C  V
ctgatttcgacctttgcggtcagcgcctcgtcgttgttctacgtgcgctatgtgttaaat
```

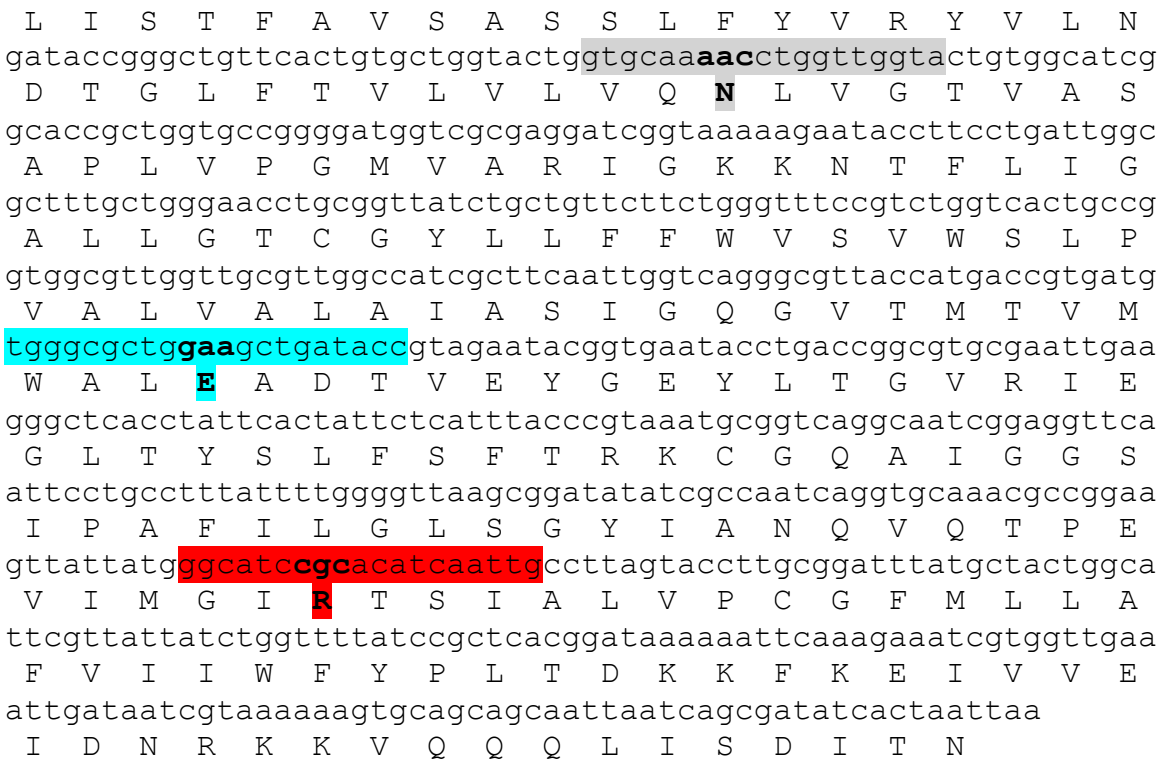

Figure 2-3 The GusB Nucleotide and Protein sequence. Highlighted section show where each set of forward and reverse primers will bind to the sequence. The highlighted amino acid underneath the nucleotide sequence identifies the location where the substitution mutation will occur. **Yellow**: TM1G27D **Pink:** TM4L119C **Green:** TM6H183E **Grey:** TM8N273D **Turquoise:** TM10E362N **Red**: TM12R406E. Forward primers will attach to the template DNA fragment at the start of the highlighted sequence for each mutant and copy until the stop codon on the gene. This will happen in reverse for the reverse primers therefore creating fragments with different molecular weights.

2.4 Diluting Primers

After designing the primers, the proposed sequences were ordered from Eurofins (www.eurofins.co.uk), these were rehydrated with TE buffer to a concentration of 100µM and stored at –20˚C until further use. The buffer should have a pH of 8 to stop DNases from reacting with other substrates (Bansal 2013).

2.5 DNA template and vector preparation

For this project *gusB* was the gene of interest, therefore a DNA template containing this gene must be prepared prior to use in the later stages of cloning. It was also essential to prepare the expression vector pTTQ18 as this would be needed towards the end of the cloning process (Stark, 1987).

pMJB33 and pTTQ18 samples had been stored at -80°C to ensure no enzymatic reactions occurred and degradation of samples was prevented.

E. coli cultures containing the plasmids were streaked onto agar plates using a sterile metal loop. All the plates contained ampicillin in order to prevent the growth of bacteria that did not contain the gene of interest.

It is important to note that plasmid vectors containing the gene of interest also included an antibiotic resistance gene; this meant bacterial strains that had taken up the plasmid could still be harvested on these plates, even in the presence of ampicillin.

NOTE: the metal loop used to streak cells was sterilised before and after streaking to avoid contamination (Sanders 2012).

The plates streaked with bacteria were placed upside down into an incubator at 37°C and left overnight in order avoid loss of moisture. This can also help to stop unwanted contaminants or condensation falling from the lids onto the agar plates (Kercher 1995).

The following morning the plated samples were checked for signs of bacterial growth. Bacteria colonies were present and strains could be inoculated into liquid LB media.

All samples were put into a shaking incubator at 250rpm at 37°C then left overnight. The following morning checks were made to see if cells had grown, if the mixture was cloudy this had been successful and plasmid DNA extraction using the QIAGEN mini-prep kit could go ahead (see Section 2.2).

2.6 Concentration determination of plasmid DNA

Concentration of DNA and protein was measured at A_{260}nm/A_{280nm} with using a Nanodrop2000™. The Nano-drop measures the absorbance of UV radiation by DNA to determine the purity of the sample. A_{260} values represent the concentration of nucleotides and A_{280} values represent the concentration of purified protein. Assessing the concentration of DNA it is possible to calculate the optimum primer dilution for PCR to be calculated. This increases the chances of successful PCR results. In this study pMJB33 was used as this plasmid already contained a functioning GUS operon.

For primer DNA, concentrations of 50l were optimum with A_{260} readings of between 0.2-0.8. The A_{260}/A_{280} ratio should be between 1.8-2 in order to be within optimal purity range. For any results that exceeded this, dilutions were made, and samples were measured again until they met optimum absorbency levels.

The sample with the highest purity could then be used for primary PCR.

2.7 dNTPs Preparation

To amplify DNA, a dNTP solution containing four nucleotide bases was needed containing dATP (adenine), dCTP (cysteine), dGTP (guanine) and dTTP (thymine). The stock solution should have a final concentration of 10 mM. The stock solution can be stored in a freezer and aliquoted when needed in PCR reactions. The stock should be diluted accordingly prior to carrying out any further reactions to the optimum concentration of 2mM.

Table 2-3. dNTP recipe for 100mM stock solution containing sodium salts: dATP (adenine), dCTP (cysteine), dGTP (guanine) and dTTP (thymine) are all needed for amplification reactions.

Component	Amount (µl)	Concentration
dATP	25	100 mM
dGTP	25	100 mM
dCTP	25	100 mM
dTCP	25	100 mM
Distilled H_2O	150	n/a

2.8 Amplification of DNA using the Polymerase Chain Reaction (PCR)

The polymerase chain reaction is a technique invented by Kary Mullis (1983). The main objective of PCR is to copy a specific DNA sequence and then amplify this to produce thousands or sometimes even millions more. It is essential for the next steps in the cloning process and in eventually obtaining mutated GusB proteins. Before beginning the procedure, all components were placed on ice.

Table 2-4 PCR Amplification Reaction Mix. Constituents needed for a 50 µl reaction solution to carry out PCR amplification: Volume and concentration for each have been given.

Constituent	Amount needed (µl)	Final Concentration
Distilled H_2O (dH_20)	32.5	-
5X Green GoTaq® Flexi Reaction Buffer (Promega Ltd)	10	-
25 mM $MgCl_2$	2	1 mM
1 mM dNTP	1	20 µM
10 µM Primer DNA (Forward)	1	0.2 µM
10 µM Primer DNA (Reverse)	1	0.2 µM
Plasmid DNA (pMJB33)	2	27.1 ng
GoTaq DNA Polymerase (Promega Ltd)	0.5	5 u/µl

Primers listed in section 2.2 required a further 1 in 10 dilution for the PCR reaction. It made sense to make up a bulk batch for future use therefore 10µl of each primer was aliquoted to the corresponding 0.5ml Microcentrifuge tube, 90 µl of distilled H2O was then added and mixed thoroughly with a pipette. This meant that the primer solution was readily available at the correct concentration of 0.2 µM.

Twelve 0.2 ml PCR tubes were labelled according to sample name, 32.5 µl of distilled H_2O was added to all tubes. Next 10 µl of 5X Flexi buffer was added, this increases sample density, which helps it to sink into gel wells during electrophoresis. 2 µl of 25 mM $MgCl_2$ and 1 µl of 1 mM dNTP was also added to all tubes. These components can be made into a master mix before allocating into separate aliquots for each PCR reaction.

Each specifically designed primer with the desired mutation (as seen in Table 2-2) was added to the reaction mix. Each primer, at this stage, was added to a singular and separate reaction to the others therefore meaning there were 12 different PCR tubes containing differing primers in each. Depending on the nature of the primer, a corresponding forward (*EcoRI*) or backwards primer was added (*KpnI*).

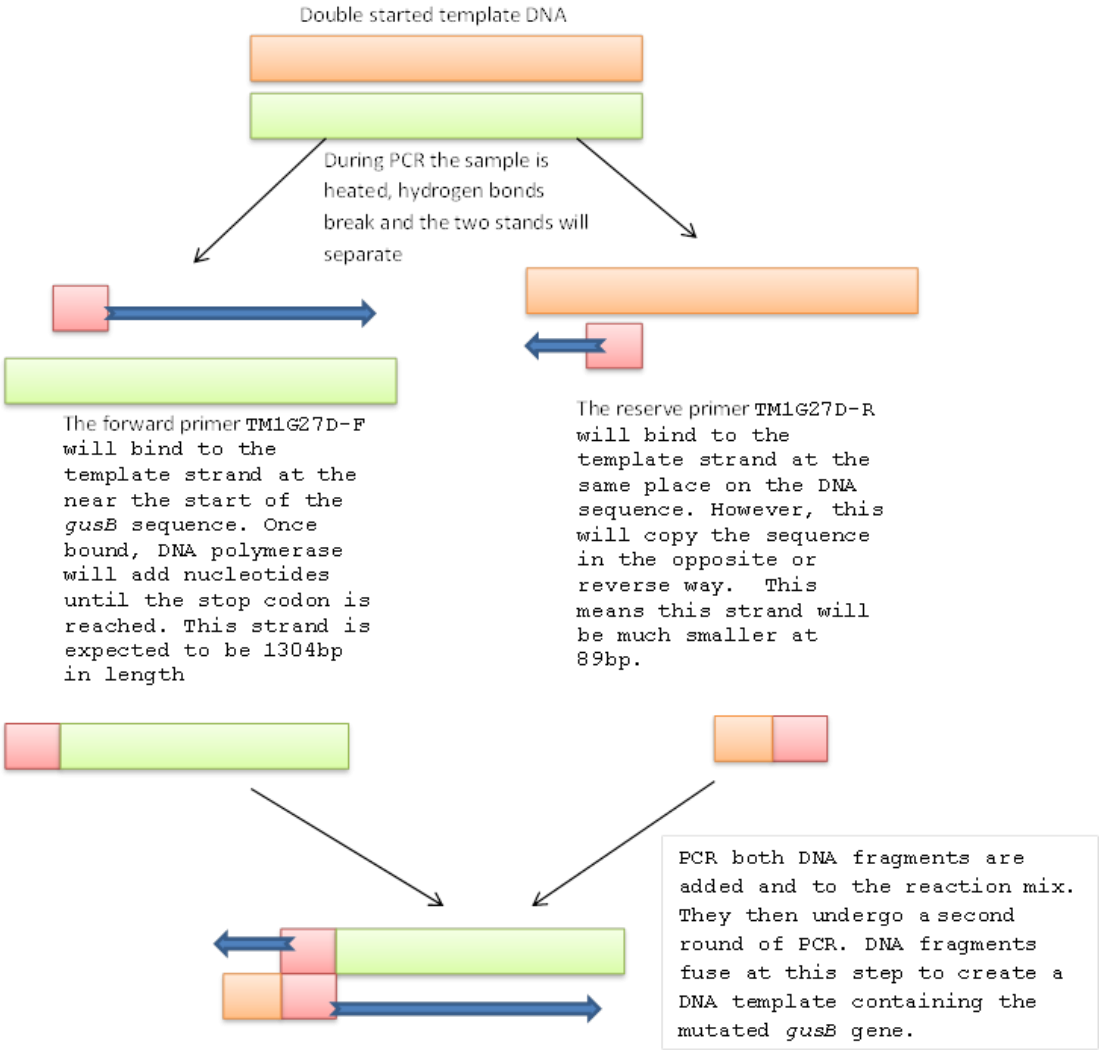

Figure 2-4 A diagram to show the overlap extension of DNA fragments in PCR amplification. Two DNA fragments are amplified with primers containing an overlapping sequence. One fragment is amplified first a 5' primer and the other fragment is with a 3' primer. After amplification of both the DNA fragments is mixed, PCR is performed with primers to produce fused DNA. Primer annealing allows an overlap extension reaction, resulting in fusion of the two fragments containing the desired mutation.

The template DNA (pMJB33) was aliquoted to all PCR tubes and the solution was gently mixed using a pipette. Finally, GoTaq polymerase was added and all tubes which were immediately placed on ice. The temperature needed for annealing in a PCR reaction depends on the T_m of the primers, because each primer has a different annealing temperature a gradient PCR programme is beneficial (Fukui and Ishii, 2001). This allows each sample to work at the optimum annealing temperatures which often reduces the chance of non-specific amplification.

The samples were placed into the Peqstar Thermocycler PCR machine. All relevant conditions used for PCR amplification can be seen in Table 2-4. Once removed all samples were immediately stored in the freezer until gel electrophoresis was performed.

Table 2-5. Recommended PCR thermal cycling conditions when using the GoTaq© DNA Polymerase in PCR amplification- As recommended by Promega™

Step	**Temperature (C)**	**Time (minutes)**	**Number of Cycles**
Initial Denaturation	95	2	1
Denaturation	95	1	30
Annealing	58-62*	1	30
Extension	72	1	30
Final Extension	72	5	1
Soak	4	Indefinite	1

* This differed depending on the T_m of primers designed for this project as seen in Table 2-2.

2.9 Fusion of gene fragments using fusion PCR

After the PCR products were 'cleaned up' using the QIAquick PCR Purification Kit (catalogue number 28104). Oligonucleotide primers were diluted 10 times in Tris EDTA ($T_{10}E_{1)}$ buffer, because the DNA concentration within the samples from primary PCR was too high. For fusion PCR to be successful both mutated forward and reverse DNA primers must be fused together in order to produce a single double stranded fragment containing the desired mutation (as created using primer design).

The PCR reaction included a temperature gradient at which the primer melting temperature of both forward and reverse primers were taken into consideration, a temperature that would allow for the binding of both primers to the template DNA.

The PCR reaction was as follows (See following page):

Table 2-6. **Constituents needed for PCR amplification intended for fusion of gene fragments**. This applies to the amount of each component needed to make up a 50µl solution volume and final concentration of each is stated below.

Constituent	Amount needed for 50 µl reaction (µl)	Final Concentration
Distilled H_2O (dH_20)	32.5	-
5X Green GoTaq® Flexi Reaction Buffer (Promega Ltd)	10	-
25 mM $MgCl_2$	2	1 mM
1 mM dNTPs	1	20 µM
EcoRI forward flanking primer	1	0.2 µM
KpnI Reserve flanking primer	1	0.2 µM
Forward primer (samples 1-6)	2	30-50ng
Reverse primer (samples 1-6)	2	30-50ng
GoTaq DNA Polymerase (Promega Ltd)	0.5	5 u/µl

2.10 Preparing electrophoresis buffer

Tris-acetate-EDTA (TAE) is a commonly used buffer for agarose electrophoresis where DNA can be separated according to the size of the fragment. A concentrated stock solution (50x TAE) was made prior to performing Gel Electrophoresis.

2.11 Gel Electrophoresis

The purpose of gel electrophoresis is to separate DNA fragments according to their molecular size. A corresponding molecular weight ‘ladder’ was used as means of determining the size of each product on the gel. This allows identification of products yielded during PCR amplification, based on their expected size and molecular weight. An electric field is applied the tank containing the gel, negatively charged DNA will then

migrate through the agarose gel towards the positive electrode. If the size of the DNA fragment is larger it will migrate at a slower rate and vice versa (Lee et al., 2012).

2.11.1 Electrophoresis gel preparation

40 ml of 1X TAE (Tris-Acetate-EDTA) buffer was measured in a measuring cylinder and transferred to a flask. 0.6 g of agarose was weighed and added into the flask containing the 1X TAE. This was swirled gently to mix then placed in the microwave until the agarose had dissolved fully. The mixture was left to cool for a few minutes then 0.4 ml of Sybr safe cell stain™ was added, the flask was swirled until the stain was evenly distributed throughout the solution. This made the desired 1.5% agarose gel.

2.11.2 Loading the samples for electrophoresis

The 1 kilo base (kb) ladder (0.05 µg/µl) from New England Biolabs was loaded into the first well of the gel, located on the far-left hand side. 1 µl of green flexi buffer was allocated to a micro-centrifuge tube; 4 µl of the PCR product was then added and mixed thoroughly using a pipette. The dye contains both blue and yellow dyes which separate during electrophoresis, so the migration can be tracked. The 5 µl sample is then added into separate wells; the exact location of each sample is noted.. These steps were repeated for the separate PCR products.

2.11.3 Electrophoresis

The Bio-Rad power pack for Electrophoresis was left to run for 40 minutes. Bubbles should appear at negatively charged electrode closest to the wells as DNA travels towards the positive electrode.

2.11.4 Visualising the gel with a blue light box

The gel was placed onto the surface of the blue light box getting rid of any air bubbles between the surface and the gel. The orange cover was placed onto of the trans illuminator box covering the gel, then turned on to produce the UV light. All lights were turned off in the room and a dark curtain was been pulled so the Fluorescent bands (DNA) could be assessed.

2.12 DNA Purification using QIAquick PCR purification kit (Qiagen)

After primary PCR it was advantageous to clean the solution of all other constituents such as the $MgCl_2$, dNTP's as well as non-specific amplification products. This ensures that in next stage of cloning desired DNA products from the first reaction will be purer increasing the chance of successful gene fusion. DNA purification was done using the QIAquick PCR purification kit (Qiagen).

The final solution should contain pure DNA fragments from the PCR amplification process; this can be checked again using gel electrophoresis.

2.13 Purification of PCR products using Sepharose

Amplification of the DNA proved difficult. This could have been due to low concentration of DNA after purification of PCR products produced in the first round. It is also possible small fragments of the poly-linker were still present in the solution after purification using the QIAquick PCR Purification kit™. An alternative method using a Sepharose (separation pharmacia agarose) spin column was conducted as a means of troubleshooting with the hope that this would later result in fusion of PCR products. Sterile glass beads were used prior to this step to prevent the leaking of Sepharose and other products.

The PCR solution was then passed through a beaded form of agarose and washed using the 10mM Tris-EDTA Na_2, pH 8.0 solution ($T_{10}E_1$).

A 0.5 ml centrifuge tube was used which acted as the spin column, first the lid was removed, and a small hole was created in the bottom using a sterile safety pin. This was then placed into a larger 1.5 ml tube. Cutting the end from one of the sterilised pipette tips, a pipette was used to take up 50µl of the glass beads, being careful not to pick up too much of the solution the beads were deposited in the spin column.

500-600 µl of Sepharose was added and then set to spin at 8000 rpm in the centrifuge for 2 minutes, the flow through was discarded and then the sample was centrifuged again to remove any remaining solution and ensure the beads formed a compact layer.

One sample from PCR amplification was added into the spin column and set to spin at 8000 rpm for 2 minutes to allow the purified DNA to bind the membrane- this was repeated for each sample and due to the risk of contamination the beads were washed thoroughly. The column was replaced and new pipette tips were used. Finally elution

buffer is added to the column, centrifuged forcing the purified DNA sample to be rinsed through into the column.

2.14 Removal of the plasmid DNA template

DpnI treatment aims to digest methylated template DNA (Mierzejewska et al., 2014). Any PCR products should not be methylated at the restriction site therefore any products such as the original plasmid DNA will be removed from the sample during this step. This mean helps to reduce the number of parental colonies during transformation resulting in higher transformation efficiency.

To the remaining solution from DNA purification, 5.5 µl of cutsmart buffer was added followed by 1 µl of DpnI. This was incubated at 37˚C for 1 to 2 hours to ensure digestion of template DNA occurred.

2.15 Preparation of gel for gel extraction

Gel extraction involves manually extracted the desired DNA fragment from an agarose gel after electrophoresis. A larger gel and comb were needed; this allows an increased volume of the PCR sample to be used and ensures a high yield of the desired product is available for extraction.

All other steps from this point followed the previous electrophoresis method in section 2.8.

2.16 Purification of DNA fragments of the *gusB* gene using the gel extraction method

By using gel extraction, it is possible to isolate DNA fragments. Once electrophoresis has been performed, desirable PCR products can be identified by their molecular weight using the 1kb ladder. This then allows intact pieces of the gel to be extracted and used in the steps of the cloning process. For this the QIAquick DNA Gel Extraction Kit (catalogue number 28704) was used.

The gel was placed on a trans-illuminator where the band representing the DNA fragments were illuminated, a picture was then taken to document their position. Each band was cut from the gel using a scalpel; this had to be precise to prevent excess agarose being present in the sample. It was imperative to clean the scalpel immediately after use to avoid any contamination.

Sterilised micro-centrifuge tubes were labelled accordingly with the sample name to avoid mix up. Using electronic weighing scales an empty tube was weighed and recorded. The

fragment was then placed into the corresponding tube and the size of the gel fragment could be determined. This process was repeated for each gel fragment. Next 3 volumes of a solubilising-binding buffer (buffer QG) was added to 1 volume of gel (100 mg~100 µl).

All samples were incubated at 50˚C for 10 minutes; every 2-3 minutes the samples were suspended using a vortex to dissolve the gel. Once dissolved fully the mixture showed a yellow colour, if the solution was an orange/red, sodium acetate (pH5) was added to decrease the pH as a higher pH can decrease DNA yield. 1 gel volume of isopropanol was needed per sample, all together 878 µl is needed, this can then be allocated accordingly into the separate micro-centrifuge tubes containing the DNA fragments, once added they were mixed using a pipette. 2 ml of each sample was transferred into separate, labelled spins columns containing a collection tube; these were then put into a centrifuge to spin at 13,000 rpm for 1 minute making sure it was balanced accordingly. Any flow through was discarded and placed back into the column, 500 µl the binding buffer (QG) was added and centrifuged for a further minute at 13,000 rpm. Flow through was discarded and 750 µl of PE wash buffer was added and left to stand for 2-5 minutes. All samples were centrifuged for 1 minute at 13,000 rpm, again flow through was discarded and samples centrifuged for a further 1 minute to remove any residual buffer. The spin column was added to a clean micro-centrifuge tube, 50 µl of elution buffer (EB) was added and centrifuged for 1 minute, after they were put into the incubator for a few minutes to remove any ethanol then stored in freezer for future use in the cloning process.

2.17 Preparation of DNA ends for sub cloning

Both plasmid vector pTTQ18 and fused products had to be digested via restriction digestion. *KpnI* and *EcoRI* Enzymes corresponding to restriction sites, which do not cut within the *gusB* gene, were used.

The following process was followed for this:

Before starting the following components were place on ice:

10X NEBuffer 2, 10 mg/ml BSA, *KpnI*, *EcoRI*, Plasmid expression vector DNA (pTTQ18), Purified DNA product and Distilled water.

Sterilised micro-centrifuge tubes were labelled accordingly with sample name/number. 38 µl of distilled water was added to a sterile micro-centrifuge tube using a pipette. 5 µl 10X NEBuffer, 10 mg/ml BSA were then added to all tubes. 5 µl of plasmid vector pTTQ18 was added to a correctly labelled tube and 5 µl of each sample was added to correctly labelled tubes. 1 µl of *EcoRI* was added to one of the micro-centrifuge tubes containing the

pTTQ18 plasmid and 1 µl of *KpnI* was added to the other tube also containing pTTQ18. *KpnI* and *EcoRI* were added to all purified DNA fragments, as these are able to undergo a double digest. These were then incubated at 37°C for 2-3 hours, all samples for double digest were left for 4-5 hours. .

Electrophoresis was performed for all samples to check a cut has been made, if the process was successful purified DNA samples were stored in the freezer. The second restriction enzymes were then added to the samples and incubated for an additional 2-3 hours.

2.18 DNA purification to remove enzymes used in restriction digest

DNA Phenol Chloroform Extraction using phenol–chloroform–isoamyl alcohol (Phe/Chl/IAA, 25:24:1) (Sigma CAS number: 136112-00-0) has to be conducted, this is because heat it is not possible to de-active the *KpnI* enzyme using the heat

Phenol-choroform-isoamyl alcohol mixture (phenol) (pH 8) was added to samples (100 µl to samples labelled 1-6*;* 300 µl to samples labelled *Kpn1 and EcoRI*). This chemical is an irritant, so it is vital this process was conducting using a fume cupboard.

Two visible layers should be seen in the bottle of phenol, however if the glass is too dark or this is not possible, around 300 µl of water was added into a micro-centrifuge tube followed by 300 µl of phenol, if the phenol sinks to the bottom and forms a separate layer layer this shows that the chemical is ok to use. After the phenol had been added to the samples they were separately vortexed in order to mix the solution. A white precipitate was visible (proteins) however at pH 7.8-8.3 phenol can remove RNA and protein. This meant the remaining solution contained DNA only. Each sample was then centrifuged at 10,000 rpm for 10 minutes, meanwhile a new set of clean, sterilised micro-centrifuge tubes were labelled for each sample. The clear aqueous layer on top of the solution (this is the purified DNA) was transferred to a micro-centrifuge tube; the process was then repeated for each sample.

This procedure must be done in an extremely precise manner to prevent contamination and to ensure proteins are not present in the final sample, this could affect PCR assay (Mirmomeni et al., 2010).

2.19 DNA Precipitation using ethanol

The steps of ethanol precipitation aim to purify and concentrate the DNA that has been recovered during gel extraction and the phenol treatment process (Becker et al., 1996).

Firstly 220 µl of Ethanol was added to samples 1-6 containing 100µl solutions, 640 µl was added to pTTQ18 samples labelled '*KpnI* and *EcoRI*' containing 300µl solutions.

Samples were left in the freezer at -20°C overnight.

All samples were centrifuged at 13,000 rpm for 10 minutes at 4˚C. Ethanol was poured out without disturbing the DNA; this was then covered with tin foil and left at room temperature overnight to dry the DNA precipitate. 100 µl of binding buffer (PB) was added to samples 1-6 and 200 µl to pTTQ18 Samples '*KpnI* and *EcoRI*'. These were left at room temperature for 20 minutes. Each sample was mixed slowly by pipetting then transferred to labelled spin columns. The method for PCR purification using the QIAquick PCR purification kit™ was used to obtain 30 µl of each concentrated sample (Section 2.9). Agarose Gel Electrophoresis was then performed on an agar gel to see if the DNA was present (Section 2.11)

2.20 Dephosphorylation of 5' end of DNA

In the cloning process this step, Shrimp Alkaline Phosphatase (SAP) Treatment is used to dephosphorylate the 5' ends of the DNA, this will stop the plasmid DNA from re-ligating (Nilsen et al., 2001). It is essential that it remains linearised; uptake of the mutated DNA fragment by the plasmid is required in the next step which would be impossible should re-ligation occur. This step therefore reduces background colonies in future steps.

For each 30 µl sample 3.5 µl of 10X cutsmart buffer and 1 µl of SAP was added, these were put into the incubator at 37˚C for 30-60 minutes. To inactivate the SAP enzyme the samples were then incubated 65˚C for 5 minutes on a heat block.

2.21 Ligation of desired DNA fragments

Ligation allows the vector DNA (pTTQ18)(See Figure 2-2) to join with the PCR products containing the mutated gene (New England Biolabs 2017).

Once this step was complete, the ligation reaction was set up using controls and left overnight at 16°C. The next morning the mixture was then heat shocked at 65°C for 10 minutes in order to denature the T4 ligase.

Table 2-7 Ligation mix containing different ratios of vector to insert DNA - shown in red. '*Control 1' shows uncut pTTQ18 (will produce colonies if conditions are optimal for the reaction) as a positive control to test transformation efficiency in the next step of cloning '**Control 2' shows cut pTTQ18 used as a negative control in the next step; this should produce no colonies. L1 refers to the reaction mix with a vector ratio of 1:1, L2 refers to the reaction mix with a vector ratio of 1:2 and L3 refers to the reaction mix with a vector ratio of 1:3.

	Volume (µl)	Volume (µl)	Volume (µl)	Volume (µl)	Volume (µl)
Components	Control 1*	Control 2**	L1 (samples 1-6) (1:1)	L2 (samples 1-6) (1:2)	L3 (samples 1-6) (1:3)
Vector	1	1	1	1	1
Insert	-	-	1	2	3
10X Ligase Buffer	2	2	2	2	2
T4 Ligase	-	1	1	1	1
H2O	17	16	15	14	13

2.22 Making Competent Cells

Although some competent DH5α *E. coli* cells DH10β were brought from New England Biolabs, another strain MC1061 obtained from my supervisor Wei-Jun Liang's previous work was used, this needed to be made competent before use using a calcium chloride procedure (see Table 2-1 for relevant information on strains).

MC1061 is a parental NO2947 *E. coli* strain previously used successfully by my supervisor Wei-Jun Liang for inner membrane protein research due to its ability to tolerate high level of expression. MC1061 has a functional *gusA* however; the *gusB* is non-functional due to a mutation at the 100th codon from Proline to Leucine. This would decrease the chances of background activity from *E. coli* strains and transport seen would likely be the results of MC1061 successfully taking up the pTTQ18 plasmid containing the mutated *gusB.* In addition to this it has an IPTG-inducible *tac* promoter, therefore this strain will used for transport tests should transformation be successful.

The other strains were used due to their high transformation efficiency; this was needed due to such low concentrations of DNA being present after ligation meaning super competent cells were required. If bacteria colonies were harvested from these cells, the

plasmids could then be extracted using the mini-prep procedure and transformed into MC1061 cells for transport tests and future protein work.

E. coli MC1061 was inoculated in LB media overnight placed in the orbital shaker at a temperature of 37°C in order to promote growth. The MC1061 culture was inoculated into fresh liquid media and was set to shake vigorously until the cells reached A_{600} = 0.2-0.3 also known as log phase. Once log phase was reached the culture was chilled on ice, 1 ml of cells were then transferred to micro-centrifuge tubes and put into the centrifuge for 2 minutes at 10,000 rpm at a temperature of 4°C. The supernatant was discarded and the cells were re-suspended in 0.5ml of ice cold, sterile calcium chloride buffer (50 mM $CaCl_2$, 10mM Tris-HCl, pH 8.0).

All suspended samples were left on ice for 15 minutes, next they were centrifuged at 10,000 rpm for 1 minute at room temperature. The supernatant was discarded and the cells were re-suspended in 1/15 of the original volume (66 µl) of ice cold, calcium chloride buffer solution.

2.23 Transformation

This step allows the plasmid harbouring the *gusB* gene to be taken up by the competent *E. coli* cells; antibiotic selection was used to increase the chances of non-specific transformation. A positive control containing pTTQ18 and pMJB33 was also set up during the experiment to test their transformation efficiency.

5-10 µl of plasmid DNA was added to 200 µl of competent cells kept on ice; all samples were placed back on ice for 30 minutes. Next, samples were heat shocked at 42°C for 2 minutes on a heating block to encourage uptake of DNA, then returned to ice box immediately for 1-2 minutes. 330 µl of LB media was added to the samples, all micro centrifuge tubes were placed in a flask making sure they were tightly shut and placed in the orbital shaker at 37°C to at 250 rpm for 1 hour. Ampicillin containing plates were dried in the incubator during this time, once dry 200 µl of the cells was pipetted onto plates and spread using an L shaped spreader. The plates were then be placed upside down into an incubator and left to grow over night at 37°C.

2.24 Assay to test transport of the Glucuronide Transporter Protein (GusB)

2.24.1 Pilot test: time course

This assay helps to determine the time it takes for both *gusA* and *gusB* to be induced, allowing transport of pNPG and cleavage to occur. This test also allows the determination

of how long it is before background GusA activity occurs when pNPG is added. Prior to this experiment, overnight cultures of different E. coli strains were set up in LB media containing ampicillin (100 μl/ml). For each separate strain one colony was added into a flask (flasks promote better cell growth than falcon tubes) containing LB media. All the above steps were conducted using a flame to reduce the risk of contamination.

Flasks were placed into the orbital shaker at 250 rpm and at 37˚C for around 16 hours.

With all successfully grown strains, 200μl of the sample was added into 20ml of LB containing ampicillin. The samples were then placed back into the orbital shaker at 37˚C for 3 hours. IPTG (1 mM IPTG final concentration) was added to just one of each of the samples and set to shake for one hour in the orbital shaker. For each sample, 0.5 ml was added into a 1.5 ml micro-centrifuge tube containing LB and 0.5 ml into a 1.5 ml micro-centrifuge tube containing Sodium Carbonate ($NaCO_3$, 1 M) that were put straight onto ice.

p-nitrophenyl-β-D-glucuronide (pNPG) was added to all remaining samples. At the following times 0.5 ml of each sample was taken and pipetted into a micro-centrifuge tube containing LB and 0.5 ml into a micro-centrifuge tube containing $NaCO_3$ and put on ice:

2 minutes

5 minutes

10 minutes

15 minutes

20 minutes

After time course had ended, a $NaCO_3$ blank was added to a cuvette and put into the blank slot on the absorbency spectrophotometer. All samples containing $NaCO_3$ were first put into the centrifuge at 13,000 rpm for 1 minute to precipitate DNA cells at the bottom of the tube. All samples from the centrifuge were removed and using the supernatant all samples were measured at A_{405}.

Once measure the DNA precipitate was re-suspended in LB media using a vortex. Once re-suspended the samples were pipetted one by one into cuvettes and measured at A600. The blank in the spectrometer was changed from $NaCO_3$ and instead LB was used. All samples were then re-inoculated, and a culture was left to grow overnight for mini-prep the next day.

2.24.2 Transport test using differing p-nitrophenyl-β-D glucuronide (pNPG) concentrations.

To work out the K_m V_{max} values and to determine binding affinity for the mutated *gusB* transport tests using different concentrations of pNPG concentrations were tested using spectrophotometry. OD_{600} was used to measure bacteria concentration and OD_{405} measures cleaved pNP from pNPG concentration.

Cells from plates containing pTTQ18, pMJB33 and pE349A plasmids respectively were inoculated in 15 ml of LB media containing ampicillin; they were put into the orbital shaker at 37°C for 16 hours to promote cell growth.

If the cells have grown adequately overnight, the culture was re-inoculated in 20 ml of LB with a 1 in 30 dilution. There were two samples set up for each strain at this point, to one of each IPTG was added at a 1mM concentration and these were placed back into the orbital shaker under the same conditions for a further 3 hours.

Once cells have grown in the media and protein expression has been induced by IPTG, six different falcon tubes should be assigned to each sample with pNPG concentrations of 0.25, 0.5, 0.75, 1 and 1.5 mM. pNPG was then aliquoted to each sample with a final volume of 3ml.

Once the pNPG was added, all samples were left for 15 minutes. 500 µl of each sample was added into 500µl of 1M Sodium Carbonate ($NaCO_2$), this will lower the pH and stop the GusB from cleaving the pNPG. Another 500 µl of each sample was added into 500 µl of LB media. All were put into the centrifuge for 2 minutes to precipitate the DNA.

The supernatant from all samples containing $NaCO_3$ was transferred to cuvettes and PNP concentration was measured at 405nm using the Shimadzu UV-1800 Spectrophotometer. For samples intended to measure the bacterial concentration using, the flow through containing LB was discarded, and making sure the DNA was not removed. 1 ml of fresh LB was then added, the cells and they were suspended using the vortex. All samples were transferred to cuvettes and this was measured at 600 nm.

2.24.3 p-nitrophenyl-β-D-glucuronide (pNPG) test for standard curve

A p-nitrophenyl-β-D-glucuronide (pNPG) test needed to be set up to allow for comparison with samples from the transport tests. When pNPG is cleaved into PNP it creates a chromogenic response. The *E. Coli* strain (MC1061) used to transform these clones did not contain functional GusB, therefore cleavage by GusA should means the plasmid containing

the mutant GusB has likely been taken up. Transport results for functionality can then be compared to the Wildtype and negative control (pTTQ18).

pNPG was diluted to at the following concentrations: 10, 20, 50, 100, 200 and 500 nM into 1 ml LB solutions. These were all left for 15 minutes. 500µl of each was then added to 1M $NaCO_3$, Samples were transferred to cuvettes and measured at 405 nm.

3. Results

The overall aim of this project intended to find residues within the GusB protein that were significant to molecular recognition. To achieve this aim, success at each stage of the cloning section is imperative. Oligonucleotide primers were designed to introduce site specific mutations into the GusB sequence, PCR was then used to amplify DNA products containing the mutations. Restriction digest was completed to cut the insert and plasmid DNA (pTTQ18) so the amplified DNA fragment could then be inserted into the plasmid during ligation. Finally, the plasmid be introduced into competent *E. coli* cells in the transformation stage and cells containing the modified plasmid can be harvested. If transformation is successful within the MC1061 E. coli strain, this will allow transport assays to be conducted and after mini prep clones can be sent for sequencing to confirm if mutation within the sequence was successful.

This section will detail all the results obtained throughout the cloning process. Variations in procedures at each step were introduced to optimise the results for each procedure.

3.1 Amplification of DNA fragments using the Polymerase Chain Reaction (PCR)

In order to generate mutants, a forward and reverse primer must first be amplified in this step of the cloning process. Successful amplification of DNA in lanes 2, 3, 4, 7 are seen below in Figure 3-1. Both forward and reverse DNA fragments for the mutation based at position L119C of the *gusB* gene (identified by the green arrows) were amplified and therefore can continue to the next stage of cloning.

Table 3-1. Expected molecular weight (bp) of mutated DNA fragments using designed forward and reverse primers after PCR amplification. All results could be compared to this in order to see if the bands corresponded to the expected weight in order to see in specific amplification has occurred. This can be seen on a DNA ladder when samples are run on agarose gel electrophoresis.

Name of Mutation	Expected Molecular Weight of Forward primer (bp)	Expected Molecular Weight of Reverse Primer (bp)
TM1G27D	1304	89
TM4L119C	1026	369
TM6H183E	839	558
TM8N273D	558	839
TM10E362N	352	1040
TM12R406E	165	1228

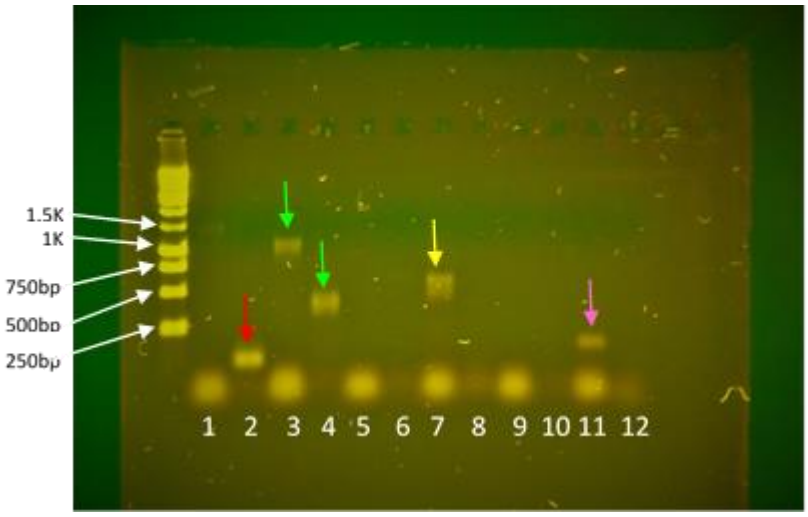

Figure 3-1 Electrophoresis results showing products yielded from primary PCR, the arrows represent bands for different DNA fragments. Results can be seen for samples loaded in lanes 2 (Red arrow=Tm1G27DR), 3 (Green=TM4L119CF), 4 (Green=TM4L119CR), 7 (Yellow=TM8N273DF) and 11 (Pink= TM12R406EF). Samples in lane 3 and 4 are the only PCR products representing corresponding forward and reverse DNA Fragments. Gel: 1.2% agarose, 70V, 40 minutes.

As a means of troubleshooting to achieve amplification for other mutagenic primers, different concentrations of pMJB33 plasmid DNA were tested (1 pM, 10 pM, 100 pM, 1 nM, 2.4 nM) to see which produced the clearest band, this would show the optimum concentration of template DNA and would be used in primary PCR amplification. This

fragment was was amplified using primer ER1gusBF amd KpIgusBR as seem in Table 2-2. Lane 3 shows smearing and multiple bands which could mean contamination of the samples or non-specific binding. The results showed that pMJB33 template DNA with a concentration of 10 pM was most suitable (as seen in Figure 3-2).

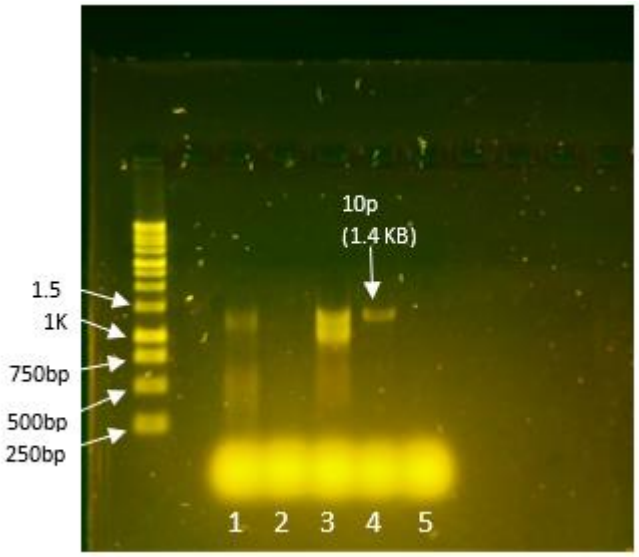

Figure 3-2. PCR amplification for pMJB33 at differing concentrations. Plasmid template DNA can be seen in the following: Lane 1=2.4nM 2=1nM 3=100pM 4=10pM 5=1pM. The molecular weight of *gusB* is around 1.4kB therefore it is expected that the desired products will be seen at 1.4kB. The clearest band can be seen using 10pM concentrations of template DNA in the PCR reactions mix.
Gel: 1.2% agarose, 70V, 40 minutes.

After making the changes above and also increasing the concentration of primer DNA to 0.4 µM, amplification of other the other mutated DNA fragments plus the initial PCR products was achieved. Although the dNTP concentration was too high, the amplified forward and reverse mutagenic primers for the following samples could be used in fusion PCR: pAB-G27D (lanes 1, 2) pAB-L119C (lanes 3, 4) pAB-H183E (lanes 5, 6) pAB-N273D (lanes 7, 8).

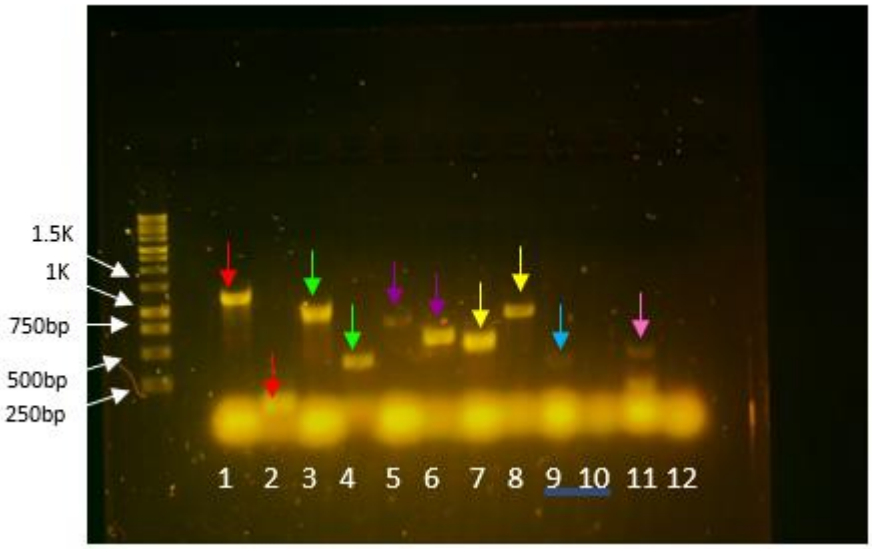

Figure 3-3 Third attempt at primary PCR amplification, lanes represent: 1=TM1F, 2=TM1R (shown by red arrows), 3=TM4F, 4=TM4R (shown by green arrows), 5=TM6F, 6=TM6R (shown by purple arrows) 7=TM8F, 8=TM8R (shown by yellow arrows). The last 4 lanes are TM10F (blue arrow) and R and TM12 F (pink arrow) and R, the lack of results may be due to conditions not being optimal or an issue with the reaction mix. These samples were not used in the next stage, fusion PCR. Gel: 1.2% agarose, 70V, 40 minutes.

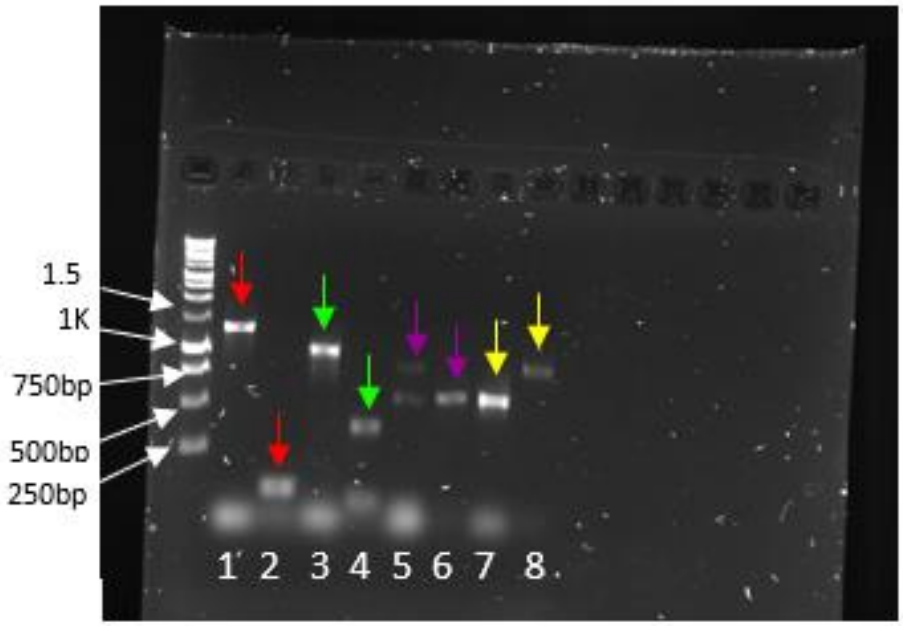

Figure 3-4 Second set of amplification results for primary PCR to yield more stock of products for the next stage in the cloning process (as above) lanes represent: 1=TM1F, 2=TM1R (shown by red arrows), 3=TM4F, 4=TM4R (shown by green arrows), 5=TM6F, 6=TM6R (shown by purple arrows) 7=TM8F, 8=TM8R (shown by yellow arrows). Gel: 1.2% agarose, 70V, 40 minutes.

3.2 Amplification of fused gene fragments using fusion PCR

Before fusion of DNA fragments is attempted, primary PCR products must first be treated with the enzymes DpnI, this will target any unwanted methylated DNA within the reaction mix. PCR products do not have methylated DNA and therefore will not be affected, this will increase the purity of the sample (Chen and Janes 2002). The template plasmid originates from the *Dam+ E. coli* strain MC1061; this means they have methylated adenines in any GATC sequences on the plasmid. When the PCR products are digested with DpnI, only the non-mutated and methylated templates will be eliminated meaning the desired mutated plasmids only are left in the solution increasing purity (Palmer and Marinus 1994). In fusion PCR 5' forward and reverse *gusB* primers as well as 3' forward and reverse primers were added to the solution. For each, desired mutagenic fragments were added also added to the reaction mix along with corresponding flanking primers, this meant that the whole of the double stranded DNA fragment encoding each site-directed mutation would be amplified.

After primary PCR products were purified using Sepharose (Section 2.13), corresponding DNA fragments were added to the reaction mix for the second round of PCR amplification. Fusion of the DNA fragments can be seen in lane 1 at 1.4 kb which is the size of the *gusB* gene. The bands on the gel in lane 2(TM4L119C), 3(TM6H183E) and 4(TM8N273D) show non-specific amplification. Smears above such as the one seen in lane 3 may indicate too much $MgCl_{2;}$ smears below the products may indicate too much plasmid DNA was added to the reaction mix. The non-specific bands observed may be due to inaccurate annealing temperatures they may be too low therefore gradient PCR could produce more specific results.

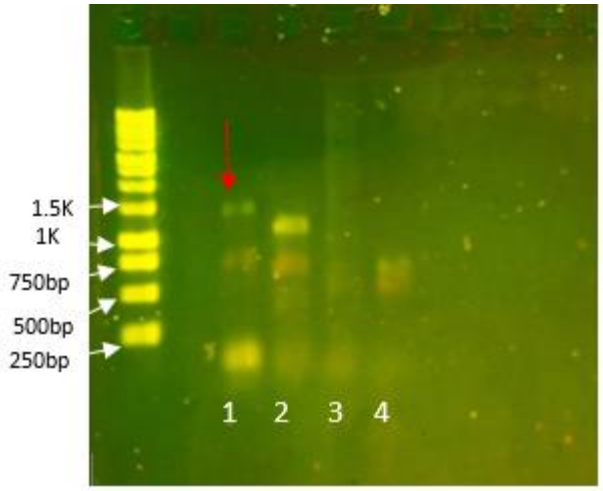

Figure 3-5. Second attempt (2nd step) PCR amplification to achieve gene fusion of sample fragments, Fused PCR products can be seen in lane 1 which is the expected size of the gusB gene (1.4 KB). This fragment could is expected to contain mutation TM1G27D (represented by the red arrow). Non-specific amplification can be seen in lanes 2-4. The multiple bands may be due to the annealing temperature being too low resulting in non-specific binding of primers to the target DNS sequence. Gel: 1.2% agarose, 70V, 40 minutes.

After multiple attempts resulted in non-specific amplification, primer sequences were checked to see if they correctly encoded *gusB* as well as checking that the 5' and 3' forward and reverse primers of each were complementary. This should have been checked prior to beginning the experiments; however this was a learning point in this project. These concluded sequences were as expected and troubleshooting of fusion PCR continued. The annealing temperature of both forward and reverse primers differed for each and therefore a gradient PCR was set up, this enabled the fragments to react at the optimum temperature. Also, primer which were non-diluted and 5X diluted primers were used for PCR in order to optimise primer concentration and conditions for PCR.

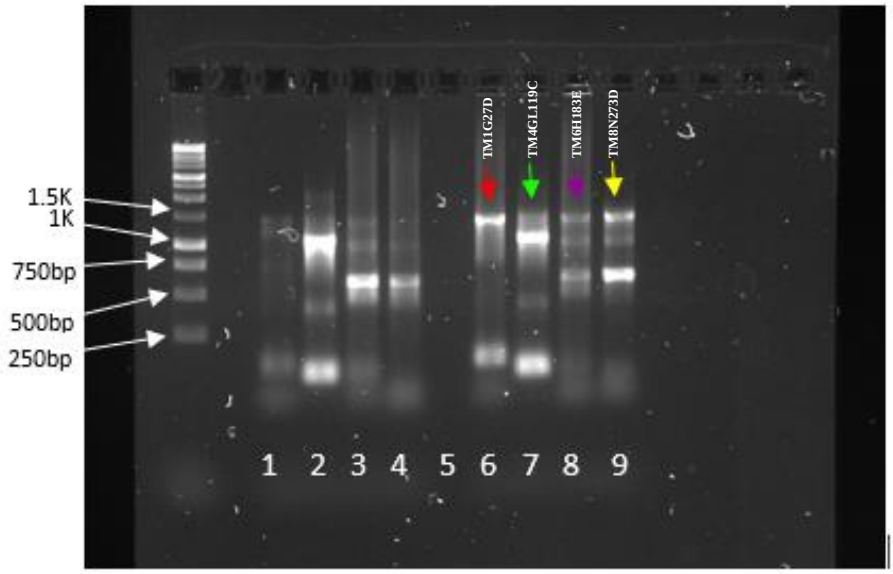

Figure 3-6 Fourth attempt 2nd stage PCR amplification: Lanes 1-4 show PCR fusion attempts with concentrations of 1mM template DNA. Non-specific amplification of primary products can be seen however some fusions has occurred which are all expected to be 1.4kB. The right size products can be seen in lanes 6 (red TM1G27DF+R), 7 (green TM4L119CF+R), 8(purple TM6H183EF+R) and 9 (yellow TM8N273DF+R) show primers with concentrations of 0.2 µM. Fusion occurred for all four products; non-specific amplification still occurred however fusion bands are clearer. Smears above show too much $MgCl_2$ was added and the amplification of non-specific products and smears below show primers should be more diluted. Gel: 1.2% agarose, 70V, 40 minutes

Fusion PCR using primers with a concentration of 0.2 µM show successful amplification of products using gradient PCR temperatures and conditions required for each set of primer (see Figure 3-6). The conditions were not optimal, however the bands seen do represent fused products. Using results from this experiment in addition to products previously yielded using primer concentration of 0.1µM (see in Figure 3-7) gel extraction could be performed. This allows isolation of fused DNA fragments (1.4 kb) from unwanted products in the solution. All products or bands with a molecular weight of 1.4 kb were presumed to be the mutagenic versions of the *gusB* gene, sequencing results could determine this in the final stages (PCR results for amplification can be seen in Figure 3-8).

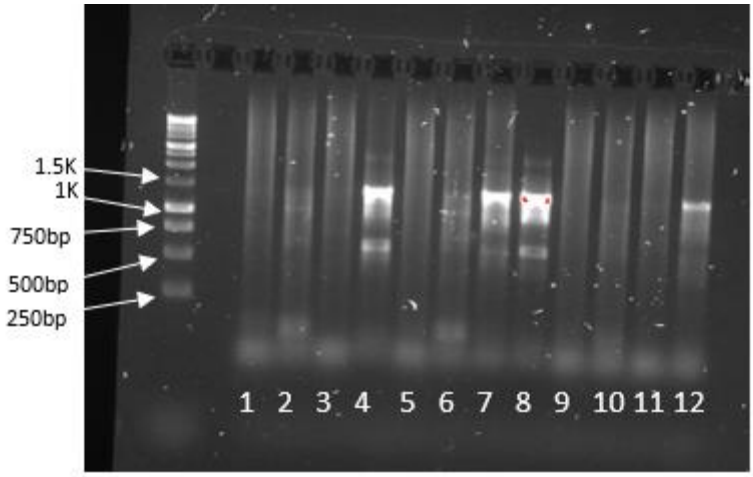

Figure 3-7. Fifth attempt at 2nd step PCR amplification: lanes 1-4 (1. TM1G27D, 2. TM4L119CF, 3. TM6H183E, 4.TM8N273D) show 1/8 diluted primers with a concentration of 0.125 µM; lanes 5-8 (5. TM1G27D, 6. TM4L119CF, 7. TM6H183E, 8.TM8N273D) show 1/10 diluted primers with a concentration of 0.1 µM and 9-12 (9. TM1G27D, 10. TM4L119CF, 11. TM6H183E, 12.TM8N273D) show 1/12 diluted primers with a concentration of 0.08 µM. Fusion occurred in lanes 4, 7, 8 and 12. Smears still indicate primers were too concentrated and $MgCl_2$ concentration was too high.

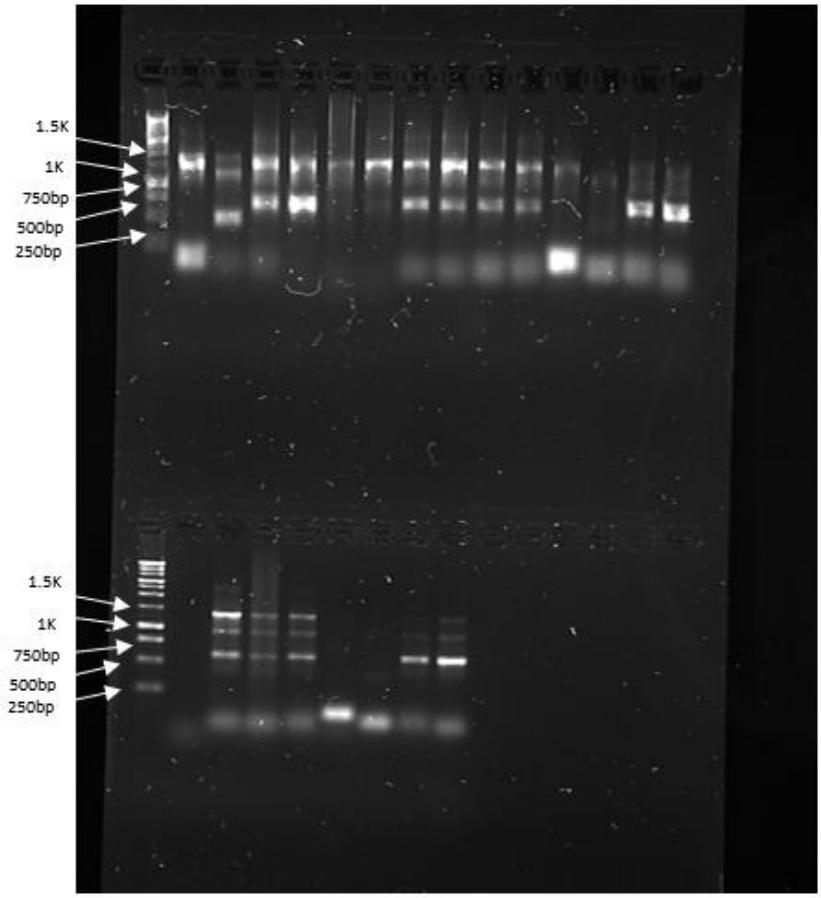

Figure 3-8. Amplification of already fused PCR products using 0.2 µM and 0.1 µM concentrations of predicted mutagenic DNA fragments. Successfully amplified PCR

products underwent a second round of PCR amplification to yield as much product as possible for the next stages of the cloning process. DNA concentrations of 0.2 (top gel) and 0.1 µM (bottom gel) were used in the reaction mix, increased annealing temperatures were also used for all reactions. Due to human error, some lanes show no bands . TM1G27D 0.2 µM= lane 2, 6 and10 for the top gel and TM1G27D 0.1 µM= lane 3 on the bottom gel. TM4L119C 0.2 µM= lane 3, 7 and 11 on the top gel and TM4L119C 0.1 µM= lane 4 and 5 on the bottom gel. TM6H183E 0.2 µM= lane 4, 8 and 12 on the top gel and TM6H183E 0.1 µM= lan9e 6 and 7 (no results) on the bottom gel. TM8N273D 0.2 µM= lane 5, 9, 13, 14 & 15 (no product amplified in lanes 13, 14 and 15) on top gel. TM8N273D 0.1 µM= lane 8 and 9 on the bottom gel. Please note: Lane 1 contains the ladder on both gels.

3.3 Purification of gene fragments using the gel extraction method

After successful fusion was achieved agarose gel extraction was carried out as a means of DNA purification as seen in Section 2.14. Purification using the Qiagen PCR Purification kit was previously carried out however this provided little success and with the presence of primary products still in the reaction mix gel extraction was necessary.

After fused products were extracted using the gel extraction method, electrophoresis was conducted to see how much DNA had been recovered. Bands showing fused products at 1.4kb could only be detected for two samples as seen in Figure 3-9, this showed that a lot of the DNA from fusion PCR had been lost in the extraction and purification process. This was also confirmed in concentration determination; Nanodrop readings seen in Table 3-2 showed less than 1 ng/µl was present for each of the samples which indicated why the bands were not visible on the gel. For the next stages of the cloning process 5 ng/µl of DNA is required, therefore by using increased volumes of DNA, cloning could continue. Bands were visible for samples possibly containing mutations TM1G27D and TM8N237D. These became the priority for future steps as it was assumed these could yield the best results.

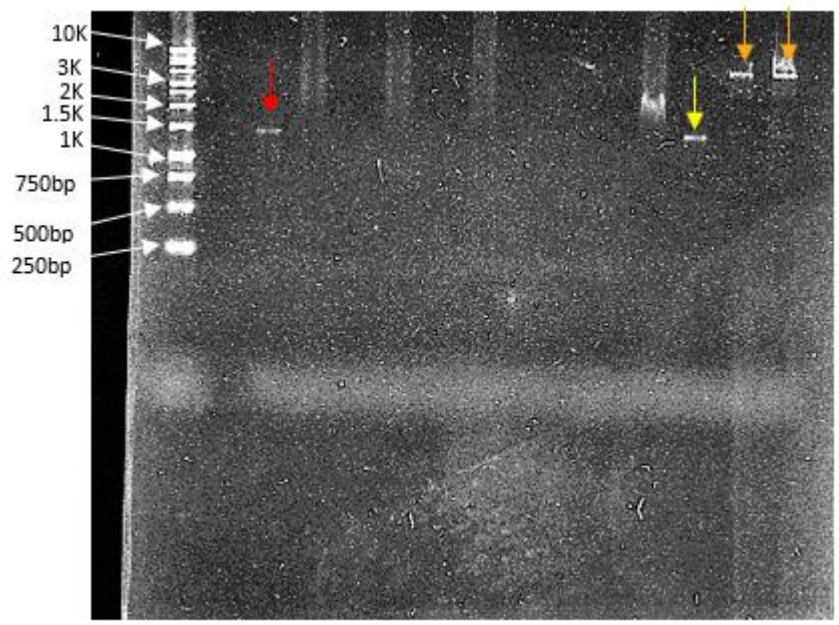

Figure 3-9. Purified products after gel extraction treatment: bands can be seen in lane 1 and lane 12 showing fused *gusB* products (the red arrow identifies TM1G27D and the yellow arrows identify TM8N237D). Lane 13 shows PTTQ18 cut with *KpnI* and lane 14 shows PTTQ18 cut with *EcrI (*orange arrows). The other lanes also contain samples however, after purification DNA concentration may be too low to see bands.

Table 3-2 DNA concentration of samples 1-6 using Nano-Drop 2000 technology after purification by the agarose gel extraction method, samples were tested to find out DNA concentration. This was to find out how much was needed for the ligation mix in the next stage of the cloning process.

Sample	Concentration of DNA (ng/µl)	Approximate amount needed for ligation (µl)
1: pAB-G27D	0.6	9
2: pAB-L119C	0.1	50
3: pAB-H183E (1)	0.2	25
4: pAB-N273D (1)	0.9	6
5 pAB-H183E (2)	0.2	25
6: pAB-N273D (2)	0.7	8

3.4 Restriction Digest, Ligation and Transformation of clones

Once purification of PCR products has been conducted both the mutagenic DNA fragment and plasmid expression vector pTTQ18 (map can be seen in figure 2-2) needs to be digested with restriction enzymes so the mutated *gusB* gene can later be cloned into the

expression vector. The PCR products underwent double digest with both with *Kpn*I and *EcoR*I for 3 hours. pTTQ18 was first treated with *Kpn*I then *EcoR*I (both high fidelity enzymes). Most enzymes can be inactivated via incubation at around 65-80°C for 20 minutes. However, this does not apply for *Kpn*I therefore pTTQ18 and the DNA fragments had to be treated with phenol and purified using ethanol precipitation. Ligation was then completed.

After the above steps were taken transformation was attempted using procedures to make competent *E. coli* MC1061 cells (see Section 2.22) and using already competent commercial *E. coli* cells: DH10-β and DH5-α (Table 2-1). Two controls were used, a positive control: undigested pTTQ18 as a negative control: and non-ligated pTTQ18 cut by restriction enzymes and dephosphorylated. The first attempt did produce any results.

Purified DNA fragments and the expression vector pTTQ18 were re-ligated and the correct positive control was used to test transformation efficiency. However, once again, transformation was not successful, and the controls were not effective which may indicate issues with the plates themselves. This led to beliefs that the concentration of ampicillin used for the plates may have been too high. New plates were made 1/1000 dilution of 100 mg/ml ampicillin. As a means of troubleshooting both pTTQ18 and pMJB33 were transformed with competent 5-aplha and 10-beta *E.coli* cells. A table showing details for both plasmids are found below.

Table 3-3. Template DNA (pMJB33) and plasmid vector (pTTQ18) used in this project. Both were used as positive controls in transformation. Following information regarding genotype can be found below:

Plasmid name	Plasmid Information	Source
pTTQ18	> pTTQ18 Expression vector; *Ptac bla* *tac* promoter, *rrnB* transcription terminator, polylinker and *lacZ* alpha fragment from pUC18, and *lacIQ* gene (See figure 2-2 for plasmid map) (Stark, 1987)	Based on pUC18 vector from *E. coli*.
pMJB33	>WLI69590_03_CP2 (pMJB33_100R_I23 (See figure 2-1 for plasmid map) (Bruce and Liang 2014)	Wildtype plasmid containing only functioning *gusB* from *E. coli*

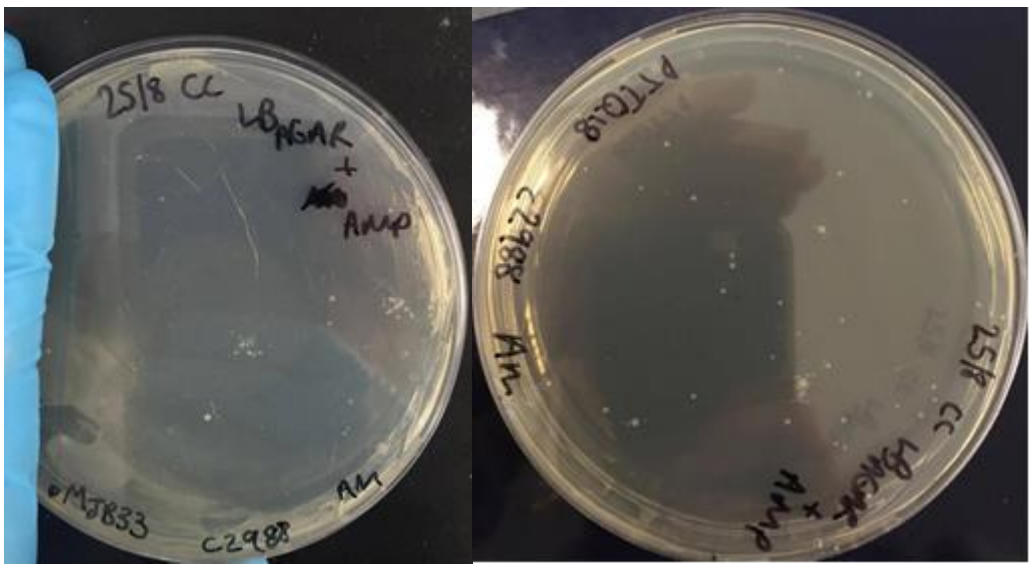

Figure 3-10. Transformation of pMJB33 and pTTQ18 in competent DH5α *E. coli* cells From New England Biolabs. Single colonies of *E. coli* bacteria can be seen on both plates which indicated that successful transformation occurred for samples pMJB33 and pTTQ18. Transformation efficiency is considered low as colonies are not substantial**.**

The results showed that DH5-α *E. Coli* cells were competent as single colonies can be seen, therefore the problem lies elsewhere. Transformation efficiency was still very low. In this case the most logical cause for failure to transform the mutated clones would be that ligation did not work, this is likely as there was only a small concentration of DNA after purification. In addition to this, if any of the ends were left nicked and the plasmid had not circularised, then no colonies would be seen (Welsh et al, 2016). The 10-beta cells on the other hand showed no colonies for pTTQ18 or pMJB33 positive controls, these cells were previously used by other students and the stock was old which could mean degradation or contamination had occurred before use in this project.

3.5 Sequencing of clones

Samples of the E. coli colonies potentially harbouring pTTQ18 with mutated *gusB* were sent off as well as purified products from successful PCR products. Unfortunately, the qualities of the samples were very poor deeming them unsuitable for sequencing purposed. A previous *gusB* clone named pE349A, 349 referring to the position of the point mutation, was sent for re-sequencing due to unexpected transport activity. Sequencing results showed a second mutation at position 218 changing the Asparagine amino acid to Lysine.

3.6 GUS Assay

Although transformants of clones designed for this project were unsuccessful, previous clones obtained by Dr Wei-Jun Liang and past students in the lab were tested against each other using GUS assay transport tests (Little et al, 2017). In addition to this negative control: pTTQ18 which does not contain the GUS operon and a positive control: wildtype *E. coli* strain pMJB33 which has a functional *gusB* gene were used. By doing these transport assays, it is possible to assess whether any of the site directed mutations are structurally relevant to the molecular recognition site within the protein. Changes to transport compared to the wildtype (pMJB33) would suggest it is.
All clones were transformed into MC1061 *E. Coli* strains with abolished *gusB* activity therefore; very little background activity should be seen.
A_{405} results show the absorbency of the sample against the blank and A_{600} measures the cell count. Results are below:

Table 3-4. Cell Count and Absorbency test measured using absorbency at A_{405} and cell count at A_{600} for six samples. pTTQ18 does not contain *gusB* and therefore is the negative control. pMJB33 does contain a functioning *gusB* and is the positive control. All other strains are mutated strains from past student projects working on the *gusB* gene. Transport of glucuronide substrates for all clones had decreased compared to the wildtype. This was expected however, the decrease in absorbency for pR210G contradicted past results.

Sample name	Absorbency (A_{405})	Cell count (A_{600})
pTTQ18	0.04	0.53
pMJB33	0.77	0.59
pP100A	0.07	0.44
pE349A	0.06	0.38
pR210G	0.29	3.46

3.6.1 p-nitrophenyl-β-D-glucuronide (pNPG) transport test

When iPTG and pNPG wer added to LB containing clones, the gus gene would be transcribed and both transport by GusB and cleaving by GusA would occur producing a chromogenic response (a yellow colour). A more vivid yellow will indicate a higher concentration of cleaved pNPG in the solution and the absorbency value will also increase. All clones showed a lower absorbency than the wildtype plasmid pMJB33, which may indicate that the transporter protein is not working as efficiently for the mutants in comparison to the natural isolate as seen in Table 3-4. Some of these results were unexpected since pR210G, a *gusB* mutant plasmid, had previously produced results showing increased rate of transport when tested with X-Gluc however, as seen in the table when tested using pNPG the transport had decreased by over half. pP100A, also a mutated GusB plasmid showed transport rate significantly lower than pMJB33 and activity seemed to completely diminish in comparison to the wildtype. In previous unpublished tests this mutation resulted in a significant increase in the activity therefore results obtained here were questionable. After repeats, similar findings were observed therefore, all plasmids from previous *gusB* projects were sequenced. pR210G and pP100A sequencing showed the samples used only contained vector DNA and therefore were not true transformants. With *gusB* not present within vector DNA, this explained the low absorbency for the pP100A plasmid. The reading for pR210G may have been because of background activity.

A new mutation changing Asparagine to Lysine at position 218 of the *gusB* gene in pE349A (See Appendix 5) could possibly have links to the molecular recognition site, with this second mutation, the tests showed that barely any pNPG could be transported across

the membrane despite having the *gusB* gene which creates the presumption that the residue mutated could be essential for molecular recognition or binding.

3.6.2 GUS Assay time course: pilot experiment

Just taking the A_{405} and A_{600} readings was not enough to identify if the molecular recognition site of any of the proteins had been changed or if transport had been affected. A time course was set up in which the reaction was stopped at a number of time points from 0 minutes to 20 minutes to determine at which time point transport was most efficient (see figure 3-11). It was found that transport between 10-15 minutes was optimum. This allowed the next part of the assay to be conducted, which would then determine the binding affinity for each different sample. To determine the optimum time the calculation below was done.

Figure 3-11. A graph to show the transport activity of different samples containing a mutagenic GusB at five time intervals. The most dramatic differences in transport activity occur at the 10 and 15-minute mark therefore this should be how long samples are left, this represents the peak time taken for both GusA and GusB to be transcribed and for pNPG to be transported into the cell and cleaved. pMJB33 from the unpublished work of Bruce and Liang was used as a positive control as this contains the wildtype *gusB*. pTTQ18 was used as a negative control and would be expected to show the least activity due to no functional *gusB*. The other clones represent those from the unpublished work of Qi Wei Yao (2015). This will be used in the next stage of the GUS assay.

The results show that the most dramatic changes in transport occur from the 10 to the 15 minutes mark, this will be used for the next step in the GUS assay.

3.6.3 pNP concentration test for standard curve

Results from pNPG transport tests showed that an increase in pNPG transport also created an increase in absorbency (A_{405}). Using the equation as seen below, linear regression was calculated using the data points. This will allow comparison of A_{405} Absorbency readings from this data set and for pTTQ18, pMJB33 and pE349A results in Section 3.6.4 so that the concentration of the samples can be determined.

$$\frac{A_{405}}{A_{600} \times 0.68 \text{ (dry mass of cells)}}$$

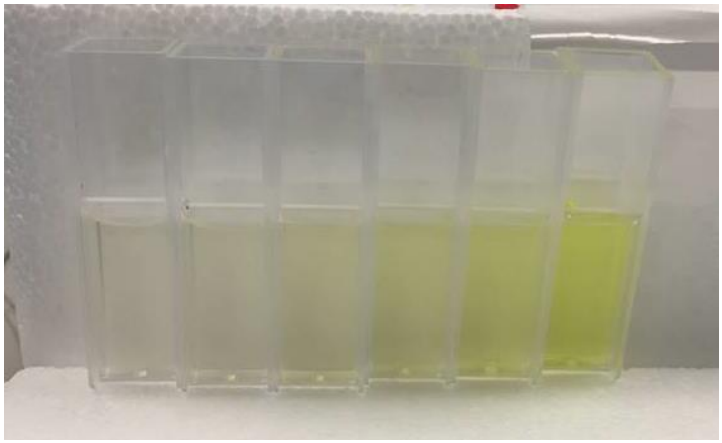

Figure 3-12 Samples containing different concentrations of pNP for standard curve. The samples range from left to right show: 10 nM, 20 nM, 50 nM, 100 nM, 200 nM and 500 nM of pNP. pNPG substrates are transported by GusB across the cell membrane and are cleaved by GusA. This produced a chromogenic response, the yellow colour becomes more vivid as the concentration of pNP increases.

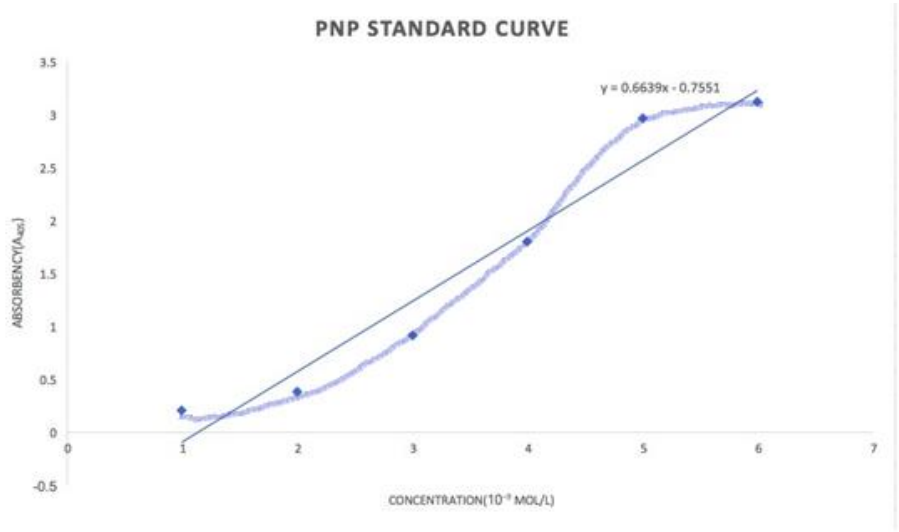

Figure 3-13 pNP standard curve graph showing linear regression. As concentration (1=10 nM, 2=20 nM, 3= 50 nM, 4= 100 nM, 5=500 nM) increases absorbency (A405) also increases. A405 is the measure of pNP therefore this reflects the concentration of the cleaved glucuronide substrate. The equation for the line of best fit is y= 0.6639x – 0.7551

3.6.4 GUS Assay: optimal pNPG concentration determination

After 3 hours of induction by IPTG (for all + samples), pNPG was added to each sample at concentrations of 0.25 mM, 0.5 mM, 0.75mM, 1 mM and 1.5 mM. This was the left for 15 minutes, this was the time at which transport was found to be most efficient in the pilot study (Section 3.6.2). After converting the concentration of pNPG to nM using the standard curve graph the results showed that the wild type pMJB33+, which was used as a positive control, had the most efficient binding efficiency and rate of transport showing highest absorbency readings. The pTTQ18 results indicated very little transport had occurred, small amounts of background activity was seen shown by a low absorbency (A_{405}) reading. pE349A showed even less transport efficiency than pTTQ18 despite the presence of *gusB* in the genotype.

The first peak highlighted on Figure 3-18 could possibly show the Vmax for GusB binding which means GusB becomes saturated at 0.25-0.3 nM. After this point it is thought that the transporter had already reached its full capacity therefore adding more substrate could cause adverse effects. The second peak and rise in absorbency may be due to the cell breaking down and therefore becoming more permeable due to a very high concentration

of pNPG. GusA would still cleave the substrate however this is not representative of the transport of GusB.

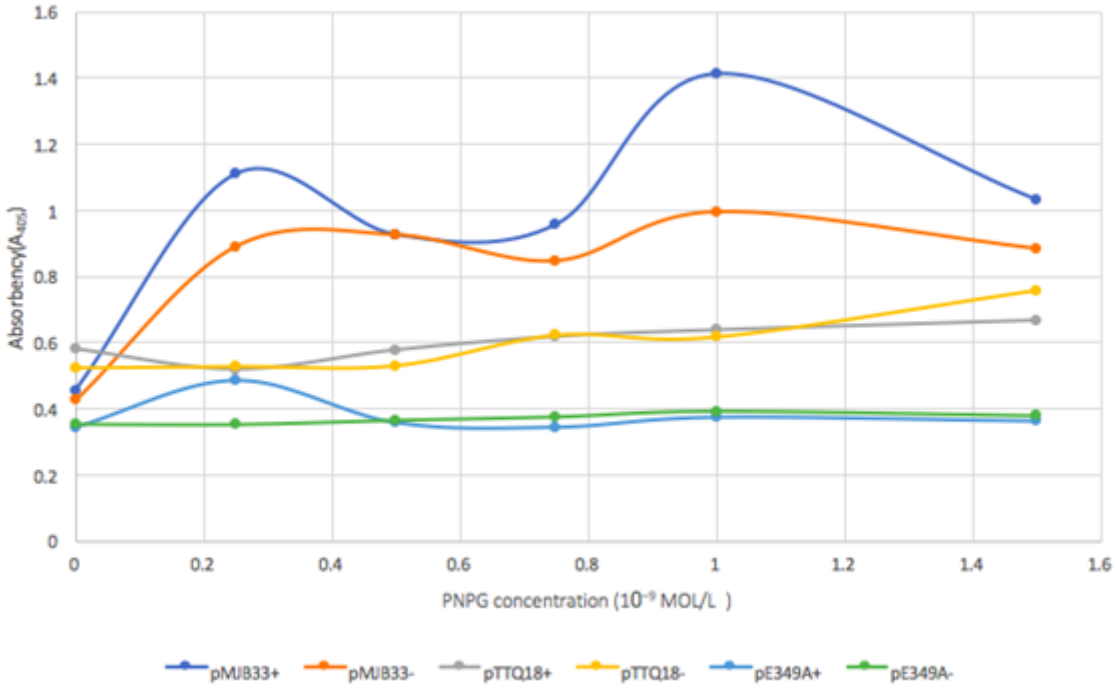

Figure 3-14. A graph showing the absorbency of samples pMJB33 +/-, pTTQ18 +/- and pE349 +/- with differing pNP concentrations. For all '+ samples' pNPG was added to the reaction mix. All '– samples' were used as a negative control as a means of comparison, these contained no pNPG within the reaction mix. Coloured lines represent different samples as seen in the key. pMJB33+ containing the wildtype GusB shows the highest absorbency readings meaning it had the highest concentration of pNP substrate after induction. pE349A+ on the other hand showed very little absorbency.

4. Discussion

4.1 Findings presented in this study

Substrate specificity for the glucuronide membrane transport protein, encoded by *gusB*, presents an interesting subject due to its ability to recognise such a broad variety of substrates. Not only this, but binding affinity for some glucuronide substrates have been found to be higher for some in comparison to others (Stoebar 1961; Liang, et al., 2005). The answer is thought to be related to the structure of the molecular recognition site.

Some structural evidence related to the GusB protein has been published by Liang et al (1992 & 2005) predicting it to be made up of 12 trans-membrane helices such as those within the Major Facilitator Superfamily (MFS), however nothing which is able to identify the location or transport mechanism of the molecular recognition site. By looking at the protein at a primary structural level, key amino acid residues and interactions between these could be investigated. TM1G27D s was chosen after reading a paper by Madej et al 2014, alignment was conducted against GusB showing a 19.8% identity (See appendix 8). The paper suggested the amino acid at position 27 was potentially important in substrate binding therefore this residue was changed for GusB.

The other point mutations were chosen at random, GusB has relatively low sequence identity to those MFS transporters whose structures are known however, with the knowledge gained in this project more educated choices could have been made. For example, published evidence relating to binding and molecular recognition sites for MFS protein such as MelB and LacS should have been researched thoroughly. Using alignment, similar residues could then have been changed in GusB.

On the other hand, regardless of sequence homology and categorisation all proteins function differently and the amino acids that were substituted could have indeed been relevant for molecular recognition.

The introduction of these six mutations was attempted using primer design and molecular cloning processes, in which amino acids with varying properties would be substituted for another. Each one of these mutated DNA fragments was then amplified using the PCR amplification method. The forward and reverse fragments were then fused and amplified once again in the second stage of PCR. DNA products for mutations at position 27 and 273 on the *gusB* gene were still present after purification and therefore the steps for cloning continued.

Other studies have been conducted focussing on the effect of site directed mutagenesis on proteins within the MFS (Bannam et al., 2004; Lewinson et al., 2006; Holyoake and Sansom 2007). One particular study by Antala et al., (2015) aimed to use SDM to decipher whether mutations affect the kinetics or the selectivity of ZIP protein, the human zinc transporter (hZIP4) and in turn whether this had any effects on the transport mechanism. Like GusB, hZIP4's structure is predicted to have 12 trans-membrane helices and too has been linked to the MFS. Findings from this study showed an amino acid change from Histidine to Alanine at positions 379, 507 and 536 altered the K_m, V_{max} and also the substrate specificity. It also was suggested that different amino acid interactions were responsible for specificity of different substrates. In fact, within ZIP member proteins, residues which make up the coordination site are variable and amino acid differences within the site were thought to contribute to differing cation specificity amongst the ZIP transporters (Antala and Dempski., 2012; Dempski, 2012)

.

Another study by Lee et al., (2015) also focussed on a protein categorised within the MFS, Synaptic vesicle glycoprotein 2A (SV2A). In order to understand the transport mechanism and binding characteristics of the protein in regard to racetam drug substrates, site directed mutagenesis was again conducted. It was found that a mutation at position 670 in the protein resulted in a complete loss of binding and therefore transports ceased deeming this structurally relevant for substrate binding and recognition however despite a 15% shared identity, position 670 shows as a gap in the sequence at this position (See appendix 9). They identified this position using sequence conservation analysis and molecular dynamic modelling which enabled them to suggest further residues that could line the binding pocket, these were later confirmed experimentally.

It would have been logical then to conduct more alignments with other MFS proteins and create changes at equivalent positions within the GusB sequence.

Still, these studies prove that even one site-specific mutation can have drastic effects on the recognition, binding affinity and transport of specific substrates (Alegre et al., 2016). It was expected then, that creating mutations in GusB might also produce similar results. Differences in binding affinity and transport of glucuronide substrates once amino acid substitutions were introduced were therefore predicted if the cloning process had been successful. Using the PyMOL software by Schrödinger an up to date model was made which predicts the 3D structure of GusB, this included a key to show where substitutions were made (See Appendix 11).

Although the mutated DNA fragments designed for this project could not be transformed into *E. coli* strains. A plasmid containing a mutation in position 349 of the *gusB* gene produced interesting result. In an unpublished study by Qi Wei Yao (2015) supervised by Dr Wei-Jun Liang, transport tests using chromogenic X-gluc showed that the transport activity remained like that of the wildtype plasmid pMJB33 containing a fully functioning *gusB*. However, when different transport tests were done in this study activity had almost completely diminished. This raised concerns that contamination may have occurred or, this may not be the correct sample. The pE349A plasmid was sent off for sequencing; the *gusB* gene sequence still existed on the plasmid and included the mutation at the expected position. However, this wasn't the only change to be observed, a mutation had also occurred at position 218 changing Asparagine (uncharged) to Lysine (charged) (See Appendix 5).

Looking at alignment with XylE, the equivalent position of the 218 residue does not show homology and current literature does not reveal this to be particularly important for molecular recognition. The MelB alignment which showed a 27% identity with GusB does not show any sequence homology at this exact spot however; the three following amino acids in the sequence are identical (See Appendix 10). The literature and studies relating to the binding site of MelB however do not yet identify these to be actively important in molecular recognition or the binding site (Ethayathulla et al., 2014). However, this could be studied more closely in future work. In addition to this the sequence alignment conducted against SV2A showed sequence homology at position 218, although like MelB no current research names this as an important residue for molecular recognition this again should be studied further.

Additionally, in the research by Qi Wei Yao (2015), transport activity in another of his clone's pR210G also showed a drastic reduction in transport for X-Gluc. This was in line with results obtained in this project from pNPG Gus Assay (see Table 3-4, Figure 3-11). It was concluded in Qi's research that the R210G mutation have created a change to the molecular recognition site, especially due to there being charged amino acids in the surrounding area. This would explain the changes in transport. The mutation found at position 218 is extremely close to this position.

If Qi's assumptions were correct this mutation would explain the changes in transport activity during GUS assay. Such a dramatic change in transport after the inclusion of a second mutation at position 218 could reveal that this amino acid too is structurally

relevant to the molecular recognition site, and to the transport of p-Nitro-Phenol-Glucuronide substrates. It could also be possible that having two mutations at position 349 and 218 simultaneously within the *gusB* gene caused too much disturbance in the final structure of the translated protein and this upset molecular recognition and in turn transport activity.

Looking at literature published for other MFS proteins similar to GusB, such as XylE, MelB and LacS, it does not appear that there are any relevant residues at this exact location however further alignments could help map this mutation to see if a relationship could be seen.

Another thing to consider is that for proteins to work effectively it is important that they are translocated to the correct location within the cell. During or after protein translation has occurred within the cytosol, the protein must be translocated to their desired position in order to function effectively (Mori and Ito 2001). This is achieved due to the presence of signal sequences within the amino acid chain which act as directions to their final destination (Schatz and Dobberstein, 1996; Driessen and Nouwen 2008). It is possible then that the introduction of mutations to the amino acid sequence could result in proteins not reaching the correct position within the cell (Wickner and Schekman 2005).. For this project in particular, it is possible that the lack of transport in the assays could be due to disruption to the signal peptides rather than the molecular recognition site of GusB. This would mean that the glucuronide transporter may not have reached the cell membrane and therefore the transport of glucuronides would have been impossible (Rapoport 2007).

Because of time constraints, further investigation as well as transport test repeats using positive and negative controls could not be carried out therefore it is suggested that this is repeated in the future.

4.2 Difficulties faced during the project

Throughout the project several procedural based issues arose. This was especially true for PCR. Repetition and multiple troubleshooting attempts had to be conducted for failed experiments. Optimisation for PCR conditions is extremely important, therefore in order to resolve common problems (non-specific amplification, too high concentrations of DNA, primer and $MgCl_2$ and optimal annealing and melting temperatures) PCR had to be done repetitively until the desired DNA fragments were amplified (Lorenz 2012).

Later on in the project, it appeared issues with ligation had occurred due to no colonies being present on plates for mutated cells. However, positive controls did show colonies after transformation (See Figure 3-10). This may have bene due to incompatible DNA ends, damaged DNA or contamination or incomplete digestion, suggestions on how this could be identified will be covered in Section 4.4 (NG & Sarkar 2012).

This was costly in terms of time and often halted the cloning process for long periods. Theoretically the methods used for cloning in this project should be simple, however adaptations of these needed to be made on numerous occasions specifically during DNA amplification. This meant the initial time frame was compromised and the later stages of cloning suffered as a result. In hindsight, contingency plans should have been put into place to account for this.

4.3 Other problems faced

Technical malfunctions with vital machinery caused huge problems throughout this study. This resulted in the cloning process being delayed.

The PCR machine being used was unknowingly malfunctioning, during cycles the power would cut off and the machine would start again which meant many products were non-specific and repeats of the procedure were necessary. This meant that the all PCR amplification methods that had undergone troubleshooting needed to be repeated.

Other issues halted the cloning process which had not been considered prior to starting the project. Long delays for chemical orders meant there were long periods between procedures. In addition to this other vital equipment including the autoclave, vital for sterilisation, were out of use. This contributed to the lack of testable clones being produced. Even though extra time was given these factors proved to be a massive hurdle

4.4 Methodology based problems

4.4.1 Issues with PCR

4.4.1.1 Amplification of primary PCR products: Problems and Troubleshooting

Many processes carried out in the lab were repetitive however; this was particularly problematic for primary and fusion PCR. Although in theory the procedure should be simple, achieving optimum conditions to produce the desired DNA fragments proved to be more difficult than expected due to the sensitivity of this procedure (Garibyan & Avashia, 2013).

Fused products were achieved for four of the samples visibly showing *gusB* DNA fragments at 1.4kb which is the expected size of the *gusB* gene. However, this was after numerous troubleshooting attempts.

DNA amplification began using the PCR protocol in GUS protocols (Gallagher 2012) was followed containing a mix of flanking primer and mutagenic primers to produce six separate *gusB* mutants. After testing the amplification using gel electrophoresis, it showed that DNA amplification attempts were unsuccessful. The smearing of bands seen the bottom of the gel showed too much DNTP was added, this was then diluted in the next reaction from 20 µM to 15 µM. The absence of bands representing the mutated DNA fragments may be due to low concentrations of primer or plasmid template in the reaction mixture (Roux 2009). To obtain better results for all products, each component was added to the samples separately rather than by creating a master mix. This ensures the optimum concentration of each substance is present in the final reaction mix. Primers were re-diluted from stock samples again to a concentration of 0.2 µM. After these steps were taken PCR was successful for some of the samples.

As a means of troubleshooting to achieve amplification for other DNA fragments from the remaining mutagenic primers, a new stock of dNTP was made to rule out the possibility of contamination or degradation of old stock. Also, plasmid DNA (pMJB33) was extracted and a fresh stock prepared. As some of the samples showed no result, primers were again re-diluted, to concentrations of 0.4 µM and all were mixed well prior to a third attempt at PCR using a pipette. This ensured the DNA was eluted within the solution. Changing the template DNA (pMJB33) concentration to the optimum of 10 pM also improved results (Section 3.1, Figure 3-3)

4.4.1.2 Amplification of fused PCR products: Problems and Troubleshooting

Fusion PCR came with several problems due to technical issues with the PCR machine as well as difficulties in creating the optimum conditions for amplification of fused DNA fragments. The first time 2nd step PCR was conducted no results were obtained at all. The reaction was conducted at 60˚C which may have been too high for some of the primers to anneal to the template seen as annealing temperature should be around 5˚C below T_m (See Table 2-2).

In the next attempt, smears above products indicate that the $MgCl_2$ concentration was too high therefore this was reduced from 1 mM to 0.8 mM. Smears present below the products

may indicate the concentration of DNA products from primary PCR was also too high (Owczarzy et al., 2008; Roux, 2009). This was reduced to 0.2 µM. The non-specific bands observed may be due to inaccurate annealing temperatures therefore gradient PCR using different temperatures was conducted with the aim of producing more specific fusions (Lorenz 2012). In addition to this purification of all DNA fragments from primary PCR was also repeated using sepharose.

Troubleshooting was a lengthy process for second-step PCR, with concentrations being changed for each constituent many times. However, eventually fused DNA fragments were obtained when 0.2 µM of primer was used. When products were visualised the observations made, such as smearing, indicated the reaction mix still had volume and concentration issues. This again was related to and high concentrations of $MgCl_2$ (Ruiz-Villalba et al., 2017).

Non-specific primer binding can be a frequent problem and can obscure PCR results. Due to the exponential nature of PCR, if primers bind to a non-specific site on the template DNA strand, this will be amplified in the following cycles and millions of copies of this fragment will be produced (Apte and Daniel, 2009). If this goes un-noticed it can lead to false positive results and the later stages of cloning will suffer because of this.

To avoid this, the following steps can increase specificity of products: primers can be put into NCBI Blast along with the template strand to identify any other binding sites in the sequence; the amount of PCR cycles should be reduced; increase the annealing temperature as primers bind non-specifically as lower temperatures; lower the concentration of both primer and MgCl; Reduce the extension and annealing cycle times; Make sure that primer, template DNA, dNTP solutions and all other reagents are not contaminated; use the Touchdown PCR method starting at a higher temperature then lowering each cycle until the annealing temperature is reached (Hyndman and Mitsuhashi, 2003; Ruiz-Villalba et al., 2011; Schoenbrunner et al., 2017; New England Biolabs, 2020). In addition to the inclusion of a positive to test if the primers are binding well and a negative control will show in any contamination is present.

Troubleshooting could have continued for PCR to achieve optimum results with clearer more specific bands however, time restraints meant that the next stages of cloning were required. Extraction of expected DNA fragments with a molecular weight of 1.4 kb were obtained using the gel extraction method and the practical work pushed ahead.

4.4.2 Ligation and Transformation: Problems and Troubleshooting

The final step in the cloning process, transformation, was the most problematic as none of the cloned samples showed any single colonies however the positive controls (uncut pTTQ18 and pMJB33) did. Still, transformation efficiency was relatively low and although it appeared the plates and competent cells were not the reason for lack of transformation this may have been a limiting factor (Liu et al., 2014; Nagamani et al., 2019). Problems with ligation could too be the cause of this failure, lack of DNA seen on the gel after restriction digest and phenol extraction may implicate a lack of DNA needed for this process even though only 5-10 ng is recommended for ligation reactions (Cranenburgh 2004). A higher concentration of DNA should have been used to begin with and although the problem had potentially been identified time did not allow for the experiment to be repeated especially with small quantities of sample left to perform ligation.

4.5 What would be done differently if given the chance

Although some of the aims established at the beginning of this project were met, the main objective was not achieved. This raises questions regarding what could have been done differently in order to obtain the desired results.

First and foremost, all mutations other than TM1R27D were randomly selected. In hindsight, this is not logical and more educated decisions could have been made. By doing alignments with similar MFS proteins such as LacS, MelB and FucP, amino acids with known functional relevance could have been targeted. Also, by applying current knowledge detailing important residues involved in molecular recognition, alignments would help to map which residues on the GusB protein are equivalent (Damián-Almazo and Saab-Rincón 2013). Mutations should have been made at these sites, if changes in transport were seen this can be compared to the other proteins.

With the lack of experience at the start of the project, it seems the number of site directed mutations first established was too ambitious for the time frame. Six pairs of primers could have been reduced meaning less time was spent trying to achieve results in the early stages of the cloning process. For example, troubleshooting of PCR was attempted many times to try and yield results for each set of primers. It would have been more beneficial to the project if the successful attempts became the focus, this would have allowed the cloning

process to continue and all steps could have been completed including sequencing and meaningful transport tests.

A more thought out contingency plan relating to each step of the cloning process would also have been beneficial especially for time management and efficiency purposes. This may have included a rule that after two attempts were made using the same methodology, this should be tweaked logically based on the prior results. Alternate methods could have also been researched and tested at certain points rather than continuous trial and error of the same procedure.

In addition to this, each step of the cloning process is extremely sensitive; it would then seem advantageous to include both positive and negative controls at every point. A positive control is a control which is expected to work under the conditions that you are using in the experiment, if this does not work it indicates it is something wrong with the way the experiment is being conducted. If the positive control does achieve results but the test subject does not, then it shows the conditions are not ideal for this specific reaction and therefore this can be manipulated (Tubbs and Stoler 2009).

For instance, in PCR reactions, if they positive control fails it can indicate something is wrong with the master mix, the temperature setting is unfavourable or annealing and extension times are not right for amplification of these samples. If, however a band is seen for this reaction but not for the other samples when visualized using electrophoresis, it could show a lack of or excess template (Banasik et al., 2016).

A negative control should not give any results and it not expected to work, it is most commonly used to check for contamination within the cloning process (Sambrook and Russell 2001). For example, this could be bacteria colonies which have not been modified to harbour antibiotic resistance genes growing on agar plates containing Ampicillin during transformation (Davies and Davies, 2010; Choi, Ro and Yi, 2019). In specific reactions results being seen for the negative control can also relate to other issues. For example, in PCR it can also be used to check for multiple binding sites of the primers (Banasik et al., 2016). These issues can be extremely detrimental to the entire process therefore using controls can help to narrow down the problem, this should have been fully utilized at every step.

Due to failure in the later stages of cloning the following should have been done, if time allowed. Firstly, after restriction digest gel electrophoresis should have carried out with digested and undigested samples (Szeberényi 2013). The uncut plasmid will be supercoiled and should appear smaller whereas the cut plasmid will be bigger as it is no longer

supercoiled (Stowell and Tweedie 2006). This can be done with the insert too. Once successful restriction digest is confirmed ligation can go ahead. Positive and negative controls at this stage are imperative as it can help rule out issues in with transformation. For example, linear plasmid with no ligase to control the presence of uncut vector, linear plasmid with ligase to control the presence of re-circularized vector; uncut plasmid to check the competency of cells and a ligation with buffer to check contamination (Carson et al., 2012; Carson et al., 2019).

It is important to identify that too much time was focused on conducting transport assays on clones from other GusB projects, rather than on the success of my own. However, by including these clones in the scope on my work I was able to gain knowledge of how to conduct procedures I might have not reached with my own project. In addition to this, a previously undiscovered mutation in the pE429A clone was found after sequencing. Once this was found, rather than conducting Gus assay using pNPG and iPTG, it would have been beneficial to test transport using X-Gluc tests. This was the method used in the original research project and would therefore allow better comparisons to be made to determine if the amino acid at position 218 was important for transport within the GusB protein.

4.6 Recommendations for future work

4.6.1 Recommendation for immediate progression of this study

It would be logical to continue the cloning process for successfully fused DNA fragments obtained in this project. By repeating PCR amplification steps for successfully fused products, a higher volume of sample can be obtained to increase the chances of successful ligation. This in turn will increase the likelihood of transformation of desired DNA plasmids. Once transformants are produced and single colonies have been observed, extraction of plasmid DNA through mini-prep should follow. All samples should then be sequenced to determine whether the expected mutation was created within the sequence of *gusB* if this was successfully incorporated into the genome of the pTTQ18 plasmid.

If nucleotide and protein sequences show that site directed mutagenesis was successful, transport activity should be assessed using transport tests in which iPTG is used to induce both the GusA and GusB on the plasmid, the pNPG substrate is then added. If the glucuronide transporter has been transcribed the substrate will be transported into the cell and GusA will cleave this. In turn a chromogenic response will occur. OD_{600} will then be

measured for bacteria concentration and OD_{405} for pNPG concentration. Positive and negative controls should be used as seen in section 2.24.2.

Regarding the pE349A plasmid, transport activity tests using GUS assay should be repeated. This would provide further evidence that perhaps the mutation at position 349 and 218 directly affects the transport mechanism of the GusB protein. It is important to remember that proteins within the MFS have a 12 TM helical structure comprising of two 6 helix domains in which amino acids interact through charges and hydrogen bonding. Within the XylE protein specific amino acid residues within different domains have identified in molecular recognition functionality. Hydrogen bonds between the following residues were thought to form the binding site: (TM5), Gln288/Gln289/Asn294 (TM7), Trp392 (TM10) and Gln415 (TM11) making up eight hydrogen bonds, then Phe24 (TM1), Tyr298 (TM7) and Trp416 (TM11) form the sugar binding pocket. Mutations in these residues caused diminished function therefore a changed from a neutral amino acid to one with a positive charge could definitely effect these interactions (Wisedchaisri et al., 2014). Looking at the alignments for XylE and GusB there are some matches and amino acids with similar properties at these sites so it would be beneficial to investigate this further in addition to the pE349A/N218K mutant discovered in this project.

As previously discovered by Stoebar (1961), the Glucuronide Transport Membrane protein has varying binding affinity for glucuronide substrates. When repeating transport activity tests, different glucuronides could also be used in addition to pNPG. The level of transport activity in addition to binding affinity for certain substrates could then be assessed using colorimetric analysis. Comparisons of the pE349A/D218 and pMJB33 proteins should then be made. The addition of a poly-histidine tag would allow purification of the mutated GusB protein (Hengen 1995; Mohanty and Wiener 2004).

4.6.2 Future prospects should the molecular recognition site of GusB be located

4.6.2.1 GusB as a potential Biosensor

Professor Leland Clark Jr constructed the first biosensor in 1956. He studied how different analytes could be measured in the body using intelligent electrochemical sensors in the form of enzyme transducers to do so. This was demonstrated by an experiment where glucose oxidase was captured by an oxygen electrode with the use of a dialysis membrane (Clark and Lyons 1962). This opened the window of using biosensors for medical

purposes; the measurement of glucose levels within the blood could be used as indication of diabetes and allows for self-monitoring of the condition (Bartlett and Whitaker 1987; Yoo and Lee 2010; Fung Ang et al., 2015). From this point a range of different biosensors were constructed using this basic principle as a model. It was not until later that more advanced biosensors were introduced. By manipulating enzymes, nucleic acids, cell receptors, antibodies and intact cells different types of responses could be measured quantitatively using transducers such as electrochemical, optical, and thermometric (Turner and Malhotra 2003; Setford and Newman 2005). GusB transport assays including GUS substrates X-Gluc which produces a blue precipitate after hydrolysis by the GusA; and pNPG which produces a yellow colour when cleaved are efficient at providing fast visible chromogenic responses (Fedorenko 2011., Little et al., 2017) If the molecular recognition site could be manipulated to only transport specific molecules the GUS system could provide an instant visual response indicating the presence of the molecule in question

It is possible that future GusB applications could involve making changes to the specificity of the binding site to recognise only specific substrates, discriminating certain glucuronides but transporting others creating a controlled and reliable identification system. However, first the position of the molecular recognition site needs to be located

Identifying key amino acids within the molecular recognition can have numerous advantages. Should the molecular recognition site be located and if the amino acids that make up this structure are found, mutations could allow for sensitivity and specificity to be manipulated resulting in the recognition of particular glucuronide substrates (Chung Kim and Joon Kang 2008, Harris et al., 2017). If expressed together with GusA to cleave substrates and perhaps a fluorescent marker, this could be a useful tool in the detection of a whole range of chemical substances especially taking into consideration the variability of glucuronide molecules.

By making these changes at nanoscale biosensors can be created. Biological biosensors are described as devices that identify the presence or concentration of an analyte, including biomolecules, biological structures and microorganisms (Mehrotra 2016).

Using modified bacteria strains, which already encode the GusA glucuronidase protein plus GusB with a modified recognition site, a fluorescent marker or other visual marker would need to be expressed in conjunction with GusB to detect certain metabolites quickly and easily. One example could be the detection of analytes within urine. This could be useful to detect substances in drug testing, to detect changes in hormone levels relating to disease and also can access dietary habits. The molecular recognition site only allowing the

binding of transport of one substrate means that anything cleaved by GusA should be the metabolite of interest; this could then be measured via the colorimetric response.

The applications for biosensors are great with medical uses; food, military and plant sciences making up just a few. One major use is for monitoring harmful substances present in the environment such as pesticides (Mehrotra 2016). Large scale use of pesticides for controlling unwanted organisms such as insects, weeds and bacteria is now a huge part of agriculture and although is beneficial to farmers in terms of productivity this can have detrimental effects on organisms and the surrounding area (Aktar et al., 2009; Verma and Bhardwaj 2015). The ability for these chemicals to spread through systems including the soil, area and water sources means that the poisonous nature of some pesticides will cause huge problems to organisms that ingest the substance and therefore a better system for detection is necessary (McEwen and Stephenson 1979; Aktar et al., 2009).

The use of Gus system would mean detection would specific. In addition to this the process would be non-invasive, fast and the added advantage is it would not necessarily need to be conducted within a laboratory setting.

4.6.2.2 GusB as a potential reporter gene

The *gusA* gene of *E. coli* is currently used as an enzymatic reporter gene in various fields. Reporter genes allow the expression and the promoter activity to be assessed (Karimi et al., 2009). One use of *gusA* is to test if bacteria are present in liquids by using chromogenic β-glucuronides such as p-Nitro-Phenol-Glucuronide, if present this will be cleaved to produce pNP and glucuronic acid which produces a chromogenic response (Kim et al., 2005; Palchette and Mascini, 2008). In addition to this *gusA* has been widely used within the plant industry in transgenic plants due to its absence in these organisms. This gene is inserted into plants and then tested using chromogenic studies such as X-Gluc (Jefferson et al., 1986; Tsomlexoglou et al., 2002). Tissues containing the gene will show a blue colour, which has helped with regards to promoter analysis of naturally occurring gene and with gene expression (Hull and Devic, 1995; Tehryung et al., 1999; Myronovskyi et al., 2011). Current GUS systems do however have limitations; a number of biochemical and molecular influences can affect expression of the GUS genes resulting in quantification of results being questioned due to inhibitors of Gus enzymes. It was found inhibitor molecules were common within plant cells, with variable affinity towards the *E.*

coli enzyme (Fior and Gerola, 2009). This would surely be a downfall of this current system.

Protein engineering of reporter genes is becoming increasingly common way to tackle inconclusive or unreliable results. One example being the Green Fluorescence Protein (GFP) from the jellyfish species *Aequorea Victoria* (Soboleski et al., 2005). A cloned version of this protein produces a visible fluorescent light when expressed without the need for other cofactors (Chalfie 1994). The discovery of GFP was pivotal in the molecular cloning and biological because of its easily detectable properties. It can be utilised as a reporter gene and screening tool to study gene expression, transfection and aids our understanding of many organisms and the systematics of biological systems (Chalfie 1994; Filmeier et al., 2000; Soboleski et al., 2005). Since the discovery of GFP in the 1960's, it has been found that the protein generally overexpressed comparative to endogenous proteins, and the GFP may also cause disruptions in protein function (Michaelson and Phillips 2006). However, to combat such problems scientists have engineered the protein using mutagenesis techniques to alter a number of characteristics including the excitation and emission spectra, this allows for distinct markers to observe a number of biological events occurring at one time (Hanson and Köhler 2001; Kobayashi et al., 2008). As well as this structural and functional relationships can be visualised in vitro through illumination (Heim and Tsien 1996).

If both *gusA* and *gusB* could be used together as a reporter system to test expression, in vivo studies may well be conducted. By including the glucuronide transporter as a secondary reporter under the same promoter as GusA, substrates could be transported and cleaved to produce a colorimetric response in the cells or tissue. This would only occur if the gene of interest were expressed in the first place of course. This would mean that extracting cells and tissues for in vitro studies of expression might not be necessary.

In addition to this site directed mutagenesis within GusB could mean specificity dramatically increased, other biological influences affecting the GUS system would be minimal and therefore current limitations might be reduced. Whether this is a valuable tool in the future depends on the continuation of study into the structure, function and molecular recognition of the GusB protein.

5. Conclusion

Prior to undertaking this study, research regarding the molecular recognition site of GusB was scarce and therefore the decision to identify key amino acids relating to this was made. By creating mutations in the g*usB* gene amino acids would be changed in the final structure of the GusB protein. It was suspected that this would disrupt with the molecular recognition site causing changes in substrate affinity and binding. This would be achieved using molecular cloning techniques.

Despite persisting efforts and troubleshooting of procedures, the process was unsuccessful. Fused DNA fragments, thought to encode a mutated *gusB*, were achieved through PCR amplification. When expressed these were hoped to produce mutations at position 27 and 273. The cloning procedures that followed were conducted however; results could not be obtained and the desired products were not produced. In order to prove the mutations were successful, transformation and sequencing should be conducted. If these steps can confirm a site directed mutation has been achieved, transport tests via GUS assay can then detect whether substrate affinity and molecular recognition has been affected.

This project however, did uncover new information regarded the mutated GusB protein pE349A. When sent for sequencing, the expected mutation was not just found at position 349 but also a substitution from an uncharged Asparagine to a positively charged Lysine was incorporated into the sequence of *gusB* at position 218. GUS assay transport tests showed diminished transport compared to the pMJB33 protein with a functional GusB. It is advised that X-Gluc test are conducted to allow comparative results with previous research. In addition to this more sequence alignments to proteins within the MFS should be conducted to see if equivalent positions within these proteins have known links to the molecular recognition site or binding pockets.

6. References

Akkermans, A.D.L., Wilson, K.J., Sessitch, A., Corbo, J., Giller, K.E., Jefferson, R.A., 1995. B-glucuronidase (GUS) transposons for ecological and genetic studies of rhizobia and other Gram-negative bacteria. *Microbiology*. 141, 1691-1705.

Aktar, W., Sengupta, D., Chowdhury, A., 2009. Impact of pesticides use in agriculture: their benefits and hazards. I*nterdisciplinary toxicology.* 2(1), 1-12.

Alberts, B., Bray, D., Hopkin, K., Johnson, A., Lewis, J., Raff, M., Roberts, K., Walter, P., 2014. *Essential Cell Biology*. 4th Edition. New York: Garland Science.

Alberts, B., Johnson, A., Lewis, J., Raff, M., Roberts, K., Walker, P., 2002. Ion Channels and the Electrical Properties of Membranes. *Molecular Biology of the Cell* [online]. 4th Edition. New York: Garland Science.

Alegre, K., Paul, S., Labarbuta, P. and Law, C., 2016. Insight into determinants of substrate binding and transport in a multidrug efflux protein. *Scientific Reports*, 6(1).

Antala, S. and Dempski, R., 2012. The Human ZIP4 Transporter Has Two Distinct Binding Affinities and Mediates Transport of Multiple Transition Metals. *Biochemistry*, 51(5), pp.963-973.

Antala, S., Ovchinnikov, S., Kamisetty, H., Baker, D., Dempski, R.E., Computation and Functional Studies Provide a Model for the Structure of the Zinc Tansporter hZIP4. *Journal of Biological Chemistry*. 290, 17796-17805.

Apte, A. and Daniel, S., 2009. PCR Primer Design. Cold Spring Harbor Protocols, 2009(3).

Argikar, U.A., Unusual Glucuronides. *Drug Metabolism and Disposition*.40(7), 1239-1251.

Ariga, K., Hill, J.P., Endo, H., 2007. Developments in Molecular Recognition and Sensing at Interfaces. *International Journal of Molecular Sciences*. 8(8), 864-883.

Banasik, M., Stanisławska-Sachadyn, A. and Sachadyn, P., 2016. A simple modification of PCR thermal profile applied to evade persisting contamination. *Journal of Applied Genetics*, 57(3), pp.409-415.

Bannam, T., Johanesen, P., Salvado, C., Pidot, S., Farrow, K. and Rood, J., 2004. The Clostridium perfringens TetA(P) efflux protein contains a functional variant of the Motif A region found in major facilitator superfamily transport proteins. *Microbiology*, 150(1), pp.127-134.

Bansal, M.P., Molecular Biology and Biotechnology: basic experimental protocols. 1st Edition. India: The Energy and Resources Institute (TERI).

Bartlett P.N., Whitaker R.G., 1987. Strategies for the development of amperometric enzyme electrodes. *Biosensors*.3, 359–379

Becker, J.M., Caldwell, G.A., Zachgo, E.A., 1996. Biotechnology: A Laboratory Course. Second Edition. San Diego: Academic Press Inc.

Bini, E., Honarbakhsh, M., Ruhl, I., Sannino, D., Villafane, AA. 2012. Development of a thermostable β-glucuronidase-based reporter system for monitoring gene expression in hyperthermophiles. *Biotechnology and Bioengineering*, 109(7), 1881-1886

Blumenthal, T., Operons in eukaryotes. *Briefings in functional genomics and proteomics*. 3(3), 199-211.

Bruce, MJ. 2014. Cloning the E.coli EC12414 gusB gene in E. coli NEB 5-alpha. BSc Dissertation. Bournemouth University.

Carson, S., Miller, H., Witherow, D. and Srougi, M., 2019. *Molecular Biology Techniques*. 4th ed. London: Elsivier, pp.247-248.

Chalfie, M., Tu, Y., Euskirchen, G., Ward, W.W., Prasher, D.C., 1994. Green Fluorescent Protein as a marker for gene expression. *Science*. 363(5148). 802-805.

Chen and Janes, 2002. PCR Cloning Protocols, Second Edition. New Jersey: Humana Press Inc.

Chica, R.A., 2015. Protein Engineering in the 21st Century. *Protein Science*. 24(4), 431-433.

Choi, S., Ro, H. and Yi, H., 2019. *DNA Cloning: A Hands-On Approach*. 1st ed. Netherlands: Springer Nature.

Chung Kim, D., Joon Kang, D, 2008. Molecular Recognition and Specific Interactions for Biosensing Applications. *Sensors*. 8, 6605-6641

Claycomb, J.R., Tran, J.Q.P., 2011. Introductory Biophysics: Perspectives on the Living State. 1st Edition. Sudbury: Jones and Bartlett Publishers.

Compernolle, F., Van Hees, G., Blanckaert, N., Heirwegh, K.P., 1978. Glucuronic acid conjugates of bilirubin-IXα in normal bile compared with post-obstructive bile. Transformation of the 1-O-acylglucuronide into 2-, 3-, and 4-O-acylglucuronides. *Biochemical Journal*. 171(1). 185-201.

Cranenburgh, R., 2004. An equation for calculating the volumetric ratios required in a ligation reaction. *Applied Microbiology and Biotechnology*, 65(2).

Csala, M., Staines, A.G., Banhegyi, G., Mandl, J., Coughtrie, M.W., Burchll, B., 2004. Evidence for multiple glucuronide transporters in rat liver microsomes. *Biochemical Pharmacology*. 68(7), 1353-1362.

Damián-Almazo, J. and Saab-Rinco, G., 2013. Site-Directed Mutagenesis as Applied to Biocatalysts. *Genetic Manipulation of DNA and Protein - Examples from Current Research*,

Dandekar, T., Snel, B., Huynen, M., Bork, P., 1998. Conservation of gene order: a fingerprint of proteins that physically interact. *Trends in Biochemical Science*, 23(9), 324-328. .

Davies, J. and Davies, D., 2010. Origins and Evolution of Antibiotic Resistance. *Microbiology and Molecular Biology Reviews*, 74(3), pp.417-433.

Dempski, R., 2012. The Cation Selectivity of the ZIP Transporters. *Metal Transporters*, pp.221-245.

Dieffenbach, C.W., Lowe, T.M.J., Dveksler, G.S., 1993. General Concepts for PCR Primer Design. *Genome Research*. 3, 530-537.

Driessen, A. and Nouwen, N., 2008. Protein Translocation Across the Bacterial Cytoplasmic Membrane. *Annual Review of Biochemistry*, 77(1), pp.643-667.

Edelheit, O., Hanukoglu, A., Hanukoglu, I., 2009. Simple and efficient site-directed mutagenesis using two single-primer reactions in parallel to generate mutants for protein structure-function studies. *BMC biotechnology*. 9(61).

Ehrt, S. and Schnappinger, D., n.d. Isolation of Plasmids from E. coli by Alkaline Lysis. *E. coli Plasmid Vectors*, pp.75-78.

Ethayathulla, A., Yousef, M., Amin, A., Leblanc, G., Kaback, H. and Guan, L., 2014. Structure-based mechanism for Na+/melibiose symport by MelB. *Nature Communications*, 5(1).

Eurofinsgenomics.eu. 2016. *PCR Primer Design Tool*. [online] Available at: <https://www.eurofinsgenomics.eu/en/ecom/tools/pcr-primer-design/> [Accessed 9 January 2016].

Feeney, M., Lopilato, J. and Murphy, K., 2014. Designing PCR Primers Painlessly †. *Journal of Microbiology & Biology Education*, 15(1), pp.28-29.

Feilmeier, B.J., Iseminger, G., Schroeder, D., Webber, H., Phillips, G.J., 2000. Green fluorescent protein functions as a reporter for protein localization in Escherichia coli. *Journal of Bacteriology*. 182(14), 4068-4076.

Fujiwara, R., Yoda, E., Tukey, R.H., 2018. Species differences in drug glucuronidation: Humanized UDP-glucuronosyltransferase 1 mice and their application for predicting drug

glucuronidation and drug-induced toxicity in humans. *Drug Metabolism Pharmokinetics*. 33(1), 9-16.

Fung Ang, L., Yee Por, L., Fei Yam, M., 2015. Development of an Amperometric-Based Glucose Biosensor to Measure the Glucose Content of Fruit. *PLOS one* [online]. 10(3), e0111859.

Gallagher, S.R (2012). *GUS Protocols: Using the GUS Gene as a Reporter of Gene Expression*. San Diego: Academic Press, Inc. 7-219.

Garibyan, L. and Avashia, N., 2013. Polymerase Chain Reaction. *Journal of Investigative Dermatology*, 133(3), pp.1-4.

Gibson, G.G., Skett, P., 2001. *Introduction to Drug Metabolism*. 3rd Edition. Cheltenham: Nelson Thornes Publishers.

Gloux, K., Berteau, O., El Oumami, H., Béguet, F., Leclerc, M., Doré, J., 2010. A metagenomic β-glucuronidase uncovers a core adaptive function of the human intestinal microbiome. *Proceedings of the National Academy of Sciences of the United States of America*, 108(1), 4539-4546..

Guillemette, C., 2003. Pharmacogenomics of human UDP-glucuronosyltransferase enzymes. *The Pharmacogenomics Journal.* 3(3), 136-158.

Guo, J., 2014. Transcription: the epicentre of gene expression. *Journal of Zhejiang University Science B.* 15(5), 409-411.

Harris, N.J., Findlay, H.E., Sanders, M.R., Kedzierski, M., Santos, A.D., Booth, P.J., 2017. Comparative stability of Major Facilitator Superfamily transport proteins. *European Biophysics Journal* [online], 249(1), 1-9.

He, Y., Chen, Y., Alexander, P., Bryan, P.N., Orban, J., 2008. NMR structures of two designed proteins with high sequence identity but different fold and function. *PNAS.* 105(38), 14412-14417.

Heim, R., Tsien, R.Y., 1996. Engineering green fluorescent protein for improved brightness, longer wavelengths and fluorescence resonance energy transfer. *Current Biology*. 6(2), 178-182.

Hengen, P.N., 1995. Purification of His-Tag fusion proteins from Escherichia coli. *Trends in Biochemical Sciences*, 20(7), pp.285-286.

Holyoake, J. and Sansom, M., 2007. Conformational Change in an MFS Protein: MD Simulations of LacY. *Structure*, 15(7), pp.873-884.

Hutchison, C.A., Phillips, S., Edgell, M.H., Gillam, S., Jahnke, P., Smith, M., 1978. Mutagenesis at a specific position in a DNA sequence. *The journal of biological chemistry*. 253(18), 6551-6560.

Hyndman, D. and Mitsuhashi, M., n.d. PCR Primer Design. *PCR Protocols*, pp.81-88.

Ishii, K. and Fukui, M., 2001. Optimization of Annealing Temperature To Reduce Bias Caused by a Primer Mismatch in Multitemplate PCR. *Applied and Environmental Microbiology*, 67(8), pp.3753-3755.

Ishii, N. 2010. Investigation on Stability of Transporter Protein, Glucuronide Transporter from Escherichia coli. *Journal of Membrane Biology*, 235(2), 63-72.

Ishii, S., Sadowsky, M.J., 2008. Escherichia coli in the Environment: Implications for Water Quality and Human Health. *Microbes and Environments*, 23(2), 101-108. .

Jefferson, R. A., Burgess, S.M., &and Hirsh D. (1986). β-Glucuronidase from Escherichia coli as a gene fusion marker. *Proceedings of the National Academy of Sciences of the U S A.* 83 (22), 8447-8451.

Kaback, H.R., Wu, J., 1997. From membrane to molecule to the third amino acid from the left with a membrane transport protein. *Quarterly Reviews of Biophysics,* 30(4), 333-364..

Karimi, M., Goldie, L., Cruickshank, M., Moses, E. and Abraham, L., 2009. A critical assessment of the factors affecting reporter gene assays for promoter SNP function: a reassessment of −308 TNF polymorphism function using a novel integrated reporter system. *European Journal of Human Genetics*, 17(11), pp.1454-1462.

Kim, K.W., Franceschi, V.R., Davin, L.B., Lewis, N.G., 2006. B-Glucuronidase as Reporter Gene: Advantages and Limitations. *Arabidopsis Protocols*. New Jersey: Humana Press. .

King, C.D., Rios, G.R., Green, M.D., Tephly, T.R., 2000. EDP-glucuronsyltransferases. *Current Drug Metabolism*. 1(2), 143-161.

Kobayashi, T., Morone, N., Kashiyama, T., Oyamada, H., Kurebayashi, N., Murayam, T., 2008. Engineering a Novel Multifunctional Green Fluorescent Protein Tag for a Wide Variety of Protein Research. *PLOS one* [online]. 3(12), e3822.

Lee, J., Daniels, V., Sands, A.A., Lebon, F., Shi, J., Biggin., 2015. Exploring the Interaction of SV2A with Racetams Using Homology Modelling, Molecular Dynamics and Site-Directed Mutagenesis. *PLOS One.* 10(2), e0116589

Lee, P.Y., Costumbrado, J., Hsu, C.Y., Kim, Y.H., 2012. Agarose gel electrophoresis for the separation of DNA fragments. *Journal of Visualized Experiments.* 20(62), 3923.

Lewinson, O., Adler, J., Sigal, N. and Bibi, E., 2006. Promiscuity in multidrug recognition and transport: the bacterial MFS Mdr transporters. *Molecular Microbiology*, 61(2), pp.277-284.

Liang, W. J. 1992. The glucuronide transport system of Escherichia coli. Ph.D. thesis. University of Cambridge, Cambridge, England.

Liang, W.J., 1989. Studies on the glucuronide operon of *Escherichia coli*. M.Phil. thesis. University of Cambridge, Cambridge, United Kingdom.

Liang, W.J., Wilson, K.J., Xie, H., Knol, J., Suzuki, S., Rutherford, N.G., Henderson, P.J.F., Jefferson, R.A. (2005). The gusBC Genes of Escherichia coli Encode a Glucuronide Transport System. *Journal of Bacteriology*. 187 (7), 2377-2385

Little, M., Pellock, S., Walton, W., Tripathy, A. and Redinbo, M., 2017. Structural basis for the regulation of β-glucuronidase expression by human gut Enterobacteriaceae. *Proceedings of the National Academy of Sciences*, 115(2), pp.E152-E161.

Liu, Y., Zheng, H., Zhan, G., Qin, W., Tian, L. and Li, W., 2014. Establishment of an efficient transformation protocol and its application in marine-derived Bacillus strain. *Science China Life Sciences*, 57(6), pp.627-635.

Lodish, H., Berk, A., Zipursky, S.L., Matsudaia, P., Baltimore, D., Darnell, J., 2002. Overview of Membrane Transport Proteins. *Molecular Cell Biology* [online]. 4th Edition. New York: W. H. Freeman.

Lorenz, T., 2012. Polymerase Chain Reaction: Basic Protocol Plus Troubleshooting and Optimization Strategies. *Journal of Visualized Experiments*, (63).

Ludewig, U., Frommer, W.B., 2002. Genes and Proteins for Solute Transport and Sensing. *The Arabidopsis Book [online], 1, e0092.*

Madej, G.M, 2014. Function, Structure, and Evolution of the Major Facilitator Superfamily: The LacY Manifesto. *Advances in Biology* [online]. Article 523591, 20 pages

Mandel, M., Higa, A., 1970. Calcium-dependent bacteriophage DNA infection. *Journal of Molecular Biology*. 53(1), 159-162

Maramorosch, K., Koprowski, H., 1971. Methods in Virology, Volume 5. New York: Academic Press.

McEwen, F.L., Stephenson, G.R., 1979. *The use and significance of pesticides in the environment*. New York: John Wiley &and Sons.

.

Mehrotra, P. 2016. Biosensors and their application- A review. *Journal of Oral Biology and Craniofacial Research*. 6(2), 153-159.

Michaelson, D., Phillips, M., 2006. The Use of GFP to Localize Rho GTPases in Living Cells. *Methods in enzymology*. 406, 296-315.

Mierzejewska, K., Siwek, W., Czapinska, H., Kaus-Drobek, M., Radlinska, M., Skowronek, K., Bujnicki, J., Dadlez, M. and Bochtler, M., 2014. Structural basis of the methylation specificity of R.DpnI. *Nucleic Acids Research*, 42(13), pp.8745-8754.

Mirmomeni, M.H., Sajjadi Majd, S. Sisakhtnezhadm, S., Doranegard, F., 2010. Comparison of the Three Methods for DNA Extraction from Paraffin-Embedded Tissues. *Journal of Biological Sciences*, 10, 261-266

Mohanty, A.K. and Wiener, M.C., 2004. Membrane protein expression and production: effects of polyhistidine tag length and position. *Protein Expression and Purification*, 33(2), pp.311-325.

Mori, H. and Ito, K., 2001. The Sec protein-translocation pathway. *Trends in Microbiology*, 9(10), pp.494-500.

Mullis, K.B., Saiki, R.K., Gelfand, D.H., Stoffel, S., Scharf, S.J., Higuchi, R., Horn, G.T., Erlich, H.A., 1988. Primer-directed enzymatic amplification of DNA with a thermostable DNA polymerase. *Science*, 29(239), 487-491.

Nagamani, G., Alex, S., Soni, K., Anith, K., Viji, M. and Kiran, A., 2019. A novel approach for increasing transformation efficiency in E. coli DH5α cells using silver nanoparticles. *3 Biotech*, 9(3).

New England Biolabs, 2017. Troubleshooting Transformation Reactions [online]. Ipswich: NEB.

New England Biolabs, 2017. *Cloning Ligation* [online]. Ipswich: NEB.

New England Biolabs, 2017. *Transformation* [online]. Ipswich: NEB.

Ng, D. and Sarkar, C., 2012. Model-guided ligation strategy for optimal assembly of DNA libraries. *Protein Engineering Design and Selection*, 25(10), pp.669-678..

Nilsen, I.W., Øverbø, K., Olsen R.L., 2001. Thermolabile alkaline phosphatase from Northern shrimp (Pandalus borealis): protein and cDNA sequence analyses. *Comparitive biochemistry and physiology. Part B, Biochemistry &and Molecular biology*. 129(4), 853-861.

.

Novel, M., Novel, G., 1976. Regulation of β-glucuronidase synthesis in *Escherichia coli* K-12: Constitutive mutations specifically derepressed for uidA expression. *Journal of Bacteriology*. 127, 406-417.

Osbourn, A.E., Fields, B., 2009. Operons. *Cellular and Molecular Life Sciences*, 66(23), 3755-3775.

Otsuka, M., Matsumoto, T., Morimoto, R., Arioka, S., Omote, H., Moriyama, Y., 2005. A human transporter protein that mediates the final excretion step for

toxic organic cations. *Proceedings of the National Academy of Sciences of the United States of America*. 102(50), 17923-17928.

Owczarzy, R., Moreira, B., You, Y., Behlke, M. and Walder, J., 2008. Predicting Stability of DNA Duplexes in Solutions Containing Magnesium and Monovalent Cations. *Biochemistry*, 47(19), pp.5336-5353.

Palchette, I, Mascini, M., 2008. Microbial Metabolism-based Biosensor. *Principles of Bacterial Detection: Biosensors, Recognition Receptors and Microsystems*. New York: Springer.

PyMOL Molecular Graphics System, Version 1.2r3pre, Schrödinger, LLC

Ralston, A., 2009. Operons and Prokaryotic Gene Regulation. *Nature Education*. 1(1), 216.

Rapoport, T., 2007. Protein translocation across the eukaryotic endoplasmic reticulum and bacterial plasma membranes. *Nature*, 450(7170), pp.663-669.

Roux, K., 2009. Optimization and Troubleshooting in PCR. *Cold Spring Harbor Protocols*, 2009(4), pp.pdb.ip66-pdb.ip66.

Ruiz-Villalba, A., van Pelt-Verkuil, E., Gunst, Q., Ruijter, J. and van den Hoff, M., 2017. Amplification of nonspecific products in quantitative polymerase chain reactions (qPCR). *Biomolecular Detection and Quantification*, 14, pp.7-18.

Sambrook, J. and Russell, D., 2001. *Molecular Cloning*. Cold Spring Harbor: CSHL press.

Sanders, E.R., 2012. Aseptic Laboratory Techniques: Plating Methods. *Journal of Visualized Experiments* [online]. 63, 3064.

Schaefer, C. and Rost, B., 2012. Predict impact of single amino acid change upon protein structure. *BMC Genomics*, 13(Suppl 4), p.S4.

Schatz, G. and Dobberstein, B., 1996. Common Principles of Protein Translocation Across Membranes. *Science*, 271(5255), pp.1519-1526.

Schoenbrunner, N., Gupta, A., Young, K. and Will, S., 2017. Covalent modification of primers improves PCR amplification specificity and yield. *Biology Methods and Protocols*, 2(1).

Setford, S.J., Newman, J.D., 2005. Enzyme Biosensors. *Methods in Biotechnology: Microbial Enzymes and Biotransformations*. Volume 17. Totowa: Humana Press Inc.

Soboleski, M., Oaks, J. and Halford, W., 2005. Green fluorescent protein is a quantitative reporter of gene expression in individual eukaryotic cells. *The FASEB Journal*, 19(3), pp.1-442.

Stark, M.J., 1987. Multicopy expression vectors carrying the lac repressor gene for regulated high-level expression of genes in Escherichia coli. *Gene*, 51(2-3), 255-267.

Stoeber, F. (1961). Sur la biosynthése induite de la β-glucuronidase chez Escherichia coli. *Comptes rendus hebdomadaires des séances de l'Académie des sciences*. 244 (7), 950-952.

Stoeber, F. 1961. Etudes des proprietes et de la biosynthese de la glucuronidase et de la glucuronide-permease chez Escherichia coli. *Theses de Docteur es Sciences*. University of Paris, Paris, France. .

Storici, F., Lewis, K.L., Resnick, M.A., 2001. In vivo site-directed mutagenesis using oligonucleotides. *Nature Biotechnology*. 19, 773-776.

Szeberényi, J., 2013. Problem-solving test: Digestion of a plasmid with restriction endonucleases. *Biochemistry and Molecular Biology Education*, 41(3), pp.189-190.

Tephly, T.R., Burchell, B., 1990. UDP-glucuronosyltransferases: a family of detoxifying enzymes. *Trends in pharmacological sciences*. 11(7), 276-279.

Tubbs, R. and Stoler, M., 2009. *Cell And Tissue Based Molecular Pathology*. Philadelphia: Churchill Livingstone/Elsevier.

Turner, A.P.F., Malhorta, B.D., 2003. *Advances in Biosensors*. Volume 5. Amsterdam: Elsevier Science B.V.

Tweedie, J. and Stowell, K., 2005. Quantification of DNA by agarose gel electrophoresis and analysis of the topoisomers of plasmid and M13 DNA following treatment with a restriction endonuclease or DNA topoisomerase I. *Biochemistry and Molecular Biology Education*, 33(1), pp.28-33.

Van der Meer, J.R., Belkin, S., 2010. Where microbiology meets microengineering: design and applications of reporter bacteria. *Nature Reviews: Microbiology*. 8(7). 511-522.

Vardy, E., Arkin, I., Gottschalk, K., Kaback, H. and Schuldiner, S., 2004. Structural conservation in the major facilitator superfamily as revealed by comparative modeling. *Protein Science*, 13(7), pp.1832-1840.

Verma, N., Bhardwaj, A. Biosensor Technology for Pesticides- A review. *Applied Biochemistry and* Biotechnology. 175(6), 3093-3119.

Wickner, W. and Schekman, R., 2005. Protein Translocation Across Biological Membranes. *Science*, 310(5753), pp.1452-1456..

Wilson, K.J., Hughes, S.G., Jefferson, R.A., 1992. The Escherichia coli gus operon, induction and expression of the gus operon in E. coliE.

coli and the occurrence and use of GUS in other bacteria. *GUS Protocols, Using the GUS Gene as a Reporter of Gene Expression*. New York: Academic Press.

Wisedchaisri, G., Park, M., Iadanza, M., Zheng, H. and Gonen, T., 2014. Proton-coupled sugar transport in the prototypical major facilitator superfamily protein XylE. *Nature Communications*, 5(1).

Wolfersberg, M.G., 1994. Uniporters, symporters and antiporters. *The Journal of experimental biology* .196, 5-6.

Wu, W., Zhang, H., Welsh, M. and Kaufman, P., 2011. *Gene Biotechnology*. Boca Raton: CRC Press.

Xia, B., Zhou, Q., Zheng, Z., Hu, M., Liu, Z., 2012. A Novel Local Recycling Mechanism That Enhances Enteric Bioavailability of Flavonoids and Prolongs Their Residence Time in the Gut. *Molecular pharmaceuticals*. 9(11), 3246-3258.

Yan, N., 2013. Structural advances for the major facilitator superfamily (MFS) transporters. *Trends in Biochemical Sciences*, 38(3), 151-159.

Yao, Q., 2015. Site-Directed Mutagenesis of the Cytoplasmic Domain of Glucuronide Transporter from Escherirhcia coli K12. BSc Dissertation. Bournemouth University

Zhang, H., Links, P., Ngsee, J., Tran, K., Cui, Z., Ko, K. and Yao, Z., 2003. Localization of Low Density Lipoprotein Receptor-related Protein 1 to Caveolae in 3T3-L1 Adipocytes in Response to Insulin Treatment. *Journal of Biological Chemistry*, 279(3), pp.2221-2230.

Zhang, X., Zhao, Y., Heng, J. and Jiang, D., 2015. Energy coupling mechanisms of MFS transporters. *Protein Science*, 24(10), pp.1560-1579.Akkermans, A.D.L., Wilson, K.J., Sessitch, A., Corbo, J., Giller, K.E., Jefferson, R.A., 1995. B-glucuronidase (GUS) transposons for ecological and genetic studies of rhizobia and other Gram-negative bacteria. *Microbiology*. 141, 1691-1705.

7. Appendices

1. GusB nucleotide sequence; GusB amino acid sequence and SNP primer design

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Start primer for GUSB

5′-CAAACAATGAATCAACAACTCTCCTGGCGC-‘3

5′-GTTTGTTACTTAGTTGTTGAGTGGACCGCG-‘3

End primer for GUSB

5′-TTAATCAGCGATATCACTAATTAATATTCA-3′

5′-AATTAGTCGCTATAGTGATTAATTATAAGT-3′

Primers for mutagenesis

1.

TM1G27DF
5′-TTCGCAATGACGGCGCTCTT-3′

TM1G27DR
5′-AAGAGCGCCGTCATTGCGAA-3′

Annealing Tm=65.3

TM10E362NF
5′-GGCGCTGAGCGCTGATAC-3′

TM10E362NR
5′-GTATCAGCGCTCAGCGCC-3′

TM6H183EF
5′-TCGGTATACGAGTTCTGGACA-3′

TM6H183ER
5′-TGTCCAGAACTCGTATACCGA-3

TM8N273DF
5′-GTGCAAGACCTGGTTGGTA-3′

TM8N273DR
5′-TACCAACCAGGTCTTGCAC-3′

TM12R406EF
5′-GGCATCGAGACATCAATTG-3′

TM12R406ER
5′- CAATTGATGTCTCGATGCC -3′

TM4L119CF
5′-ATGGGCTGTGGGCTTTGCTAC-3′

TM4L119CR
5′- GTAGCAAAGCCCACAGCCCAT -3′

ER1gusBF (based on pMJB33 clone)
5′-aataagaattcatgaatcaacaactctcctg-3′

KpIgusBR (based on pMJB33 clone)
5′-ttGGTACCTTAATTAGTGATATCGCTGATTAAT-3′

2. Tm recommendation for primers

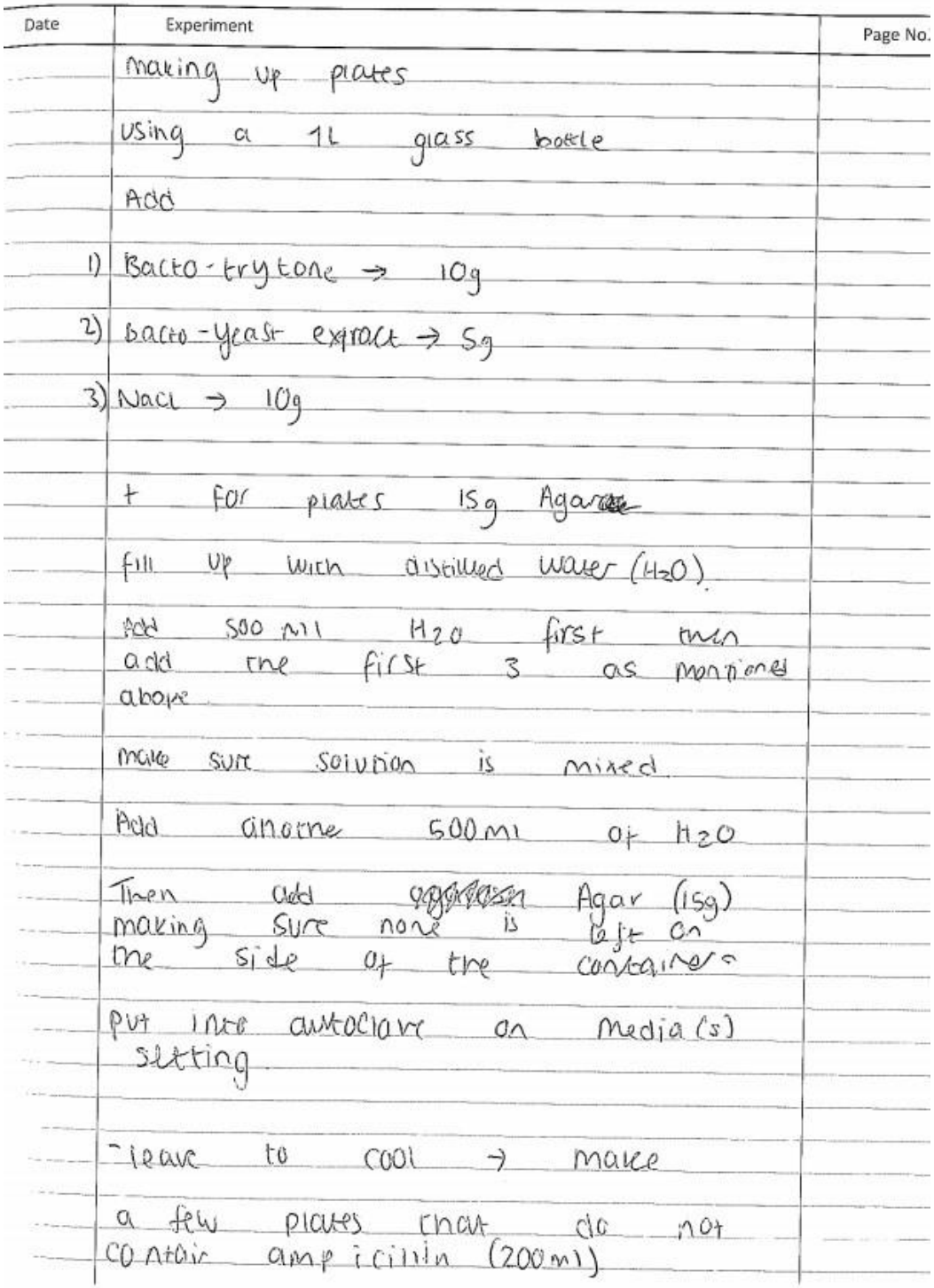
Tm for PCR reaction (GIBCO-BRL)

CG	18-mer	19-mer	20-mer	21-mer	22-mer	23-mer	24-mer	25-mer	26-mer	27-mer	28-mer	29-mer	30-mer	31-mer	32-mer	33-mer
1	46.3	48.1	49.8	51.3	52.7	53.9	55.1	56.1	57.1	58.0	58.9	59.6	60.4	61.0	61.7	62.3
2	48.6	50.3	51.9	53.3	54.5	55.7	56.8	57.8	58.7	59.5	60.3	61.1	61.7	62.4	63.0	63.5
3	50.8	52.4	53.9	55.2	56.4	57.5	58.5	59.4	60.3	61.1	61.8	62.5	63.1	63.7	64.3	64.8
4	53.1	54.6	56.0	57.2	58.3	59.3	60.2	61.1	61.8	62.6	63.3	63.9	64.5	65.0	65.5	66.0
5	55.4	56.8	58.0	59.1	60.1	61.1	61.9	62.7	63.4	64.1	64.7	65.3	65.8	66.3	66.8	67.3
6	57.7	58.9	60.1	61.1	62.0	62.8	63.6	64.3	65.0	65.6	66.2	66.7	67.2	67.7	68.1	68.5
7	59.9	61.1	62.1	63.0	63.9	64.6	65.3	66.0	66.6	67.1	67.6	68.1	68.6	69.0	69.4	69.7
8	62.2	63.2	64.2	65.0	65.7	66.4	67.0	67.6	68.2	68.6	69.1	69.5	69.9	70.3	70.7	71.0
9	64.5	65.4	66.2	66.9	67.6	68.2	68.8	69.3	69.7	70.2	70.6	70.9	71.3	71.6	71.9	72.2
10	66.8	67.6	68.3	68.9	69.5	70.0	70.5	70.9	71.3	71.7	72.0	72.4	72.7	73.0	73.2	73.5
11	69.1	69.7	70.3	70.8	71.3	71.8	72.2	72.5	72.9	73.2	73.5	73.8	74.0	74.3	74.5	74.7
12	71.3	71.9	72.4	72.8	73.2	73.5	73.9	74.2	74.5	74.7	75.0	75.2	75.4	75.6	75.8	76.0
13	73.6	74.0	74.4	74.7	75.0	75.3	75.6	75.8	76.0	76.2	76.4	76.6	76.8	76.9	77.1	77.2
14	75.9	76.2	76.5	76.7	76.9	77.1	77.3	77.5	77.6	77.8	77.9	78.0	78.1	78.2	78.3	78.4
15	78.2	78.3	78.5	78.6	78.8	78.9	79.0	79.1	79.2	79.3	79.4	79.4	79.5	79.6	79.6	79.7

Tm = 81.5 + 41 x CG% - 675/mers Annealing Temperature = Tm – 5 degree C

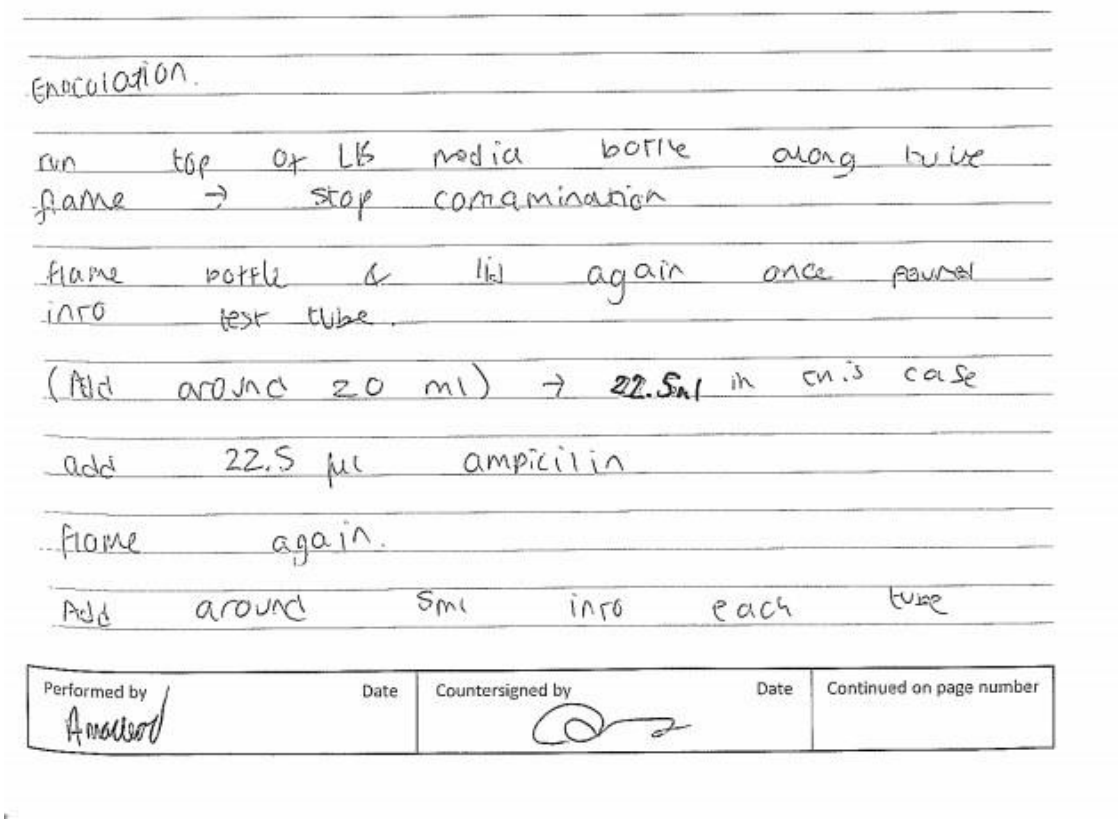
3. **Examples of procedures carried out in the lab:** Please note this is a sample, not all pages are included.

Making of LB media and plates



Date	Experiment	Page No.
	making up plates	
	using a 1L glass bottle	
	Add	
1)	Bacto-trytone → 10g	
2)	bacto-yeast extract → 5g	
3)	NaCl → 10g	
	+ for plates 15g Agar	
	fill up with distilled water (H_2O)	
	Add 500 ml H_2O first then add the first 3 as mentioned above	
	make sure solution is mixed	
	Add another 500 ml of H_2O	
	Then add Agar (15g) making sure none is left on the side of the container	
	put into autoclave on Media (s) setting	
	leave to cool → make	
	a few plates that do not contain amp icillin (200 ml)	

Inoculating of strains



Enoculation.

run top of LB media bottle along bunsen flame → stop contamination

flame bottle & lid again once poured into test tube.

(Add around 20 ml) → 22.5ml in this case

add 22.5 µl ampicillin

flame again.

Add around 5ml into each tube

Performed by	Date	Countersigned by	Date	Continued on page number
Amadeo				

Avoid contact as much as possible.

& flame at each point.

flame the ring & leave to cool but do not move.

PMJB 33 → add 1 small colony to media.

make sure when adding to tube it is slanted to avoid any contaminant falling in.

Same for pTTQ18

keep colony's upside down & put back into fridge

→ put test tubes into incubator & set to shake to promote cell growth

(37°C at 250 rpm)

Performed by	Date	Countersigned by	Date	Continued on page number
Amacleod				

Continued from page number

mini-prep • Get ice box before you begin!

follow steps as before.

• make sure P2 buffer has 0.2 N NaOH and 1% SDS (detergent). SDS will need to be incubated in winter as this will crystalise.

Add 1ml ON culture to labelled eppendorf

MJ633, R210G, P100A, D69V, E349A, PTTG18)

place into centrifuge for 3 mins to pellet culture.

pour liquid away & pipette any remaining liquid. using 100 µl pipette.

Add 250 µl of P1 buffer, vortex to resuspend cells vortex side of eppendorf → not too vigourously)
→ this seperates cells

leave to stand for a few minutes.
Add 250 µl of P2 & invert 6 times
Add 350 µl of N3 (make sure this has been left on ice). Immediately shake & vortex upside down for a few seconds.

Put on ice for 30mins

Centrifuge for 10 mins at 13,000 rpm.

Performed by	Date	Countersigned by	Date	Continued on page number
Amacleod				

Continued from page number

pipette. 800 µl of supernatent into 2.0 spin Column & put into centrifuge for 1 min.

→ discard supernatent

Add buffer PB (0.5ml) centrifuge for 1min

→ discard supernatent

Add 750 µl PE buffer & centrifuge for 1min.

Add another 250µl & centrifuge for 1min
→ discard supernatent & centrifuge for a furthur 60 secs to remove residual liquid.

Put into incubator (37°C) to get rid of any ethanol.

Add 50µl of EB then centrifuge for 1min.

Remove filter.

DNA labelled S110116 DNA in freezer Bg (LIEBHERR) top drawer, pink rack.

Performed by	Date	Countersigned by	Date	Continued on page number
Amacleod				

Continued from page number | Page number 64

Nanodrop

Toshiba laptop → DNALAB (password)

Clean both metal bits on top & bottom.

We use thermo science nanodrop 2000 spectrometer

Add 1µl H20 to foward & backward lens
- this is the blank.

Put the arm down & click ~~measure~~ blank

Lift arm up & clean from lens with tissue

Qi PTTQ18 sample → Add 1µl & click measure.

A260	A280	260/280	Conc.
3.152	1.579	1.88	157.6 ng/µl

↓ too high needs to → dilute sample & re-measure.
below 0.8 & above 0.2

We do a 1in 10 dilution → mix thoroughly

The best is 0.5/0.6 A260

The diluted sample was 0.24 → this is slightly too low so we will do a 1 in 5 dilution.

A260	A280	260/280	Conc
0.351	0.188	1.87	17.6

Performed by: A. macleod | Date | Countersigned by | Date | Continued on page number

Continued from page number | Page number 65

MJB33 gusB pcr

A260	A280	A260/280	conc.
0.864	0.461	1.88	43.4
0.387	0.208	1.86	19.3

2:1 dilution

A260/280 (show purity) should be between 1.8 - 2.0

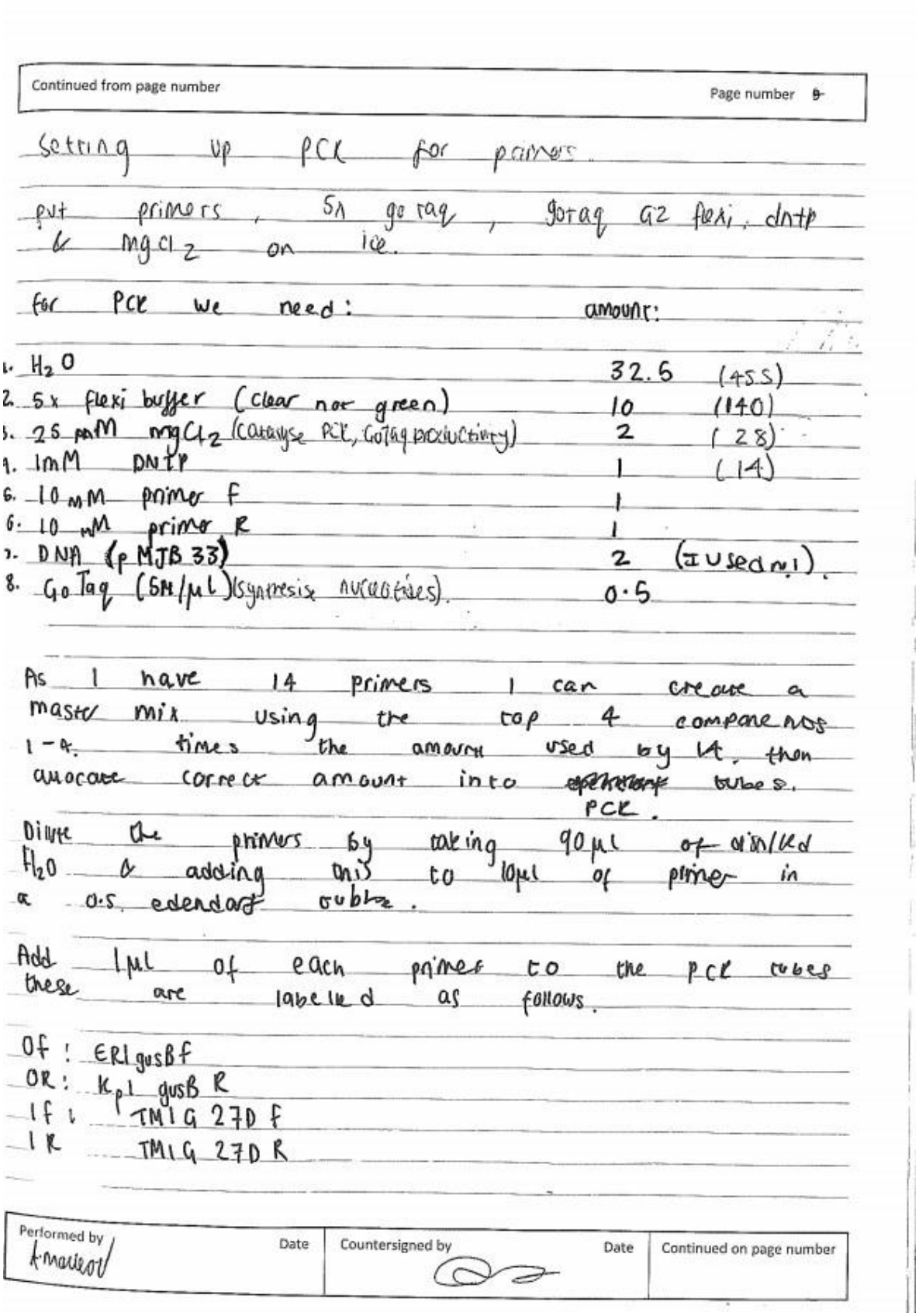
When we do nanodrop we measure optimal density NOT Absorbance

↳ we measure the light that passes through the substance (DNA).

so 0.387 × 2 = 0.774

Performed by: A. macleod | Date | Countersigned by | Date | Continued on page number

Primary PCR amplification and Troubleshooting



Continued from page number — Page number 9

Setting up PCR for primers.

put primers, 5x go taq, gotaq G2 flexi, dntp & $MgCl_2$ on ice.

For PCR we need:	amount:	
1. H_2O	32.5	(455)
2. 5x flexi buffer (clear not green)	10	(140)
3. 25 mM $MgCl_2$ (Catalyse PCR, GoTaq productivity)	2	(28)
4. 1mM DNTP	1	(14)
6. 10 mM primer F	1	
6. 10 mM primer R	1	
7. DNA (pMJB 33)	2	(I used ng)
8. Go Taq (5M/µL) (synthesis nucleotides)	0.5	

As I have 14 primers I can create a master mix using the top 4 components 1-4. times the amount used by 14, then allocate correct amount into ~~eppendorf~~ tubes. PCR.

Dilute the primers by taking 90µl of distilled H_2O & adding this to 10µl of primer in a 0.5 eddendorf tube.

Add 1µl of each primer to the PCR tubes these are labelled as follows.

OF: ERI gusB f
OR: Kpl gusB R
1F: TM1G 27D F
1R: TM1G 27D R

Performed by k mallon | Date | Countersigned by | Date | Continued on page number

4F: TM4L119CF
4R: TM4L119CR
6F: TM6
6R: TM6
8F: TM8
8R: TM8
10F: TM10
10R: TM10
12F: TM12
12R: TM12

PMJB33 DNA 78.1ng/ml = we need to take 6µl DNA + 10µl H2O = 16µl

* Before adding ~~Taq~~ Tag mix solution with a 50µl pipette.

Add 2µl of DNA to each pcr tube.

here.

Add taq polymerase (GoTaq) (5u/µl) to each tube & place straight on ice.

Insert all tubes in PCK machine & run for 2 hours. (peqlab)

Remove & incubate.

Performed by	Date	Countersigned by	Date	Continued on page number
A mackay				

24/04/17

PCR was done again but with more diluted DNTP + primers.

→ All measurements were the same as before.

I did gradient PCR according to the annealing Tm of my primers to try + get a better result.

25/04/17

Gel electrophoresis → gel made + run @ 70V for 40 minutes

Bay		
	1	1kB
	2	/
	3	1f
	4	1R
	5	4f
	6	4R
	7	6f
	8	6R
	9	8f
	10	8R
	11	10f
	12	10R
	13	12f
	14	12R

Performed by	Date	Countersigned by	Date	Continued on page number
A.Mallard				

25/4/17

The gel showed an additional set of lines at the top of my gel, this may be due too the concentration of my plasmid DNA being to high. Judging from past experience, I made a 2.5 pg. Conc. solution to use instead as the optimum is 1pg - 1ng.

The gel was also smeared so I will slightly reduce the amount of $MgCl_2$ used.

H_2O	33 µl
5 x flexi	10 µl
$MgCl_2$	1.5 µl
DNTP	1 µl
F primer	1 µl
R primer	1 µl
Plasmid DNA (mJ653)	2 µl
GoTaq	0.5 µl
	= 50 µl.

Gel electrophoresis.

Bay		
1		1kB (newly made up. previous was not made correctly)
2		/
3		1f
4		1R
5		4f
6		4R
7		6f
8		6R
9		8f
10		8R

Performed by	Date	Countersigned by	Date	Continued on page number
AMallard				

Troubleshoot PCR

Although I achieved good bands from the previous experiment, the DNTP concentration was too high therefore I am going to do the same experiment with decreased DNTP & increased primer conc.

H_2O	~~33~~ 33
5X flexi	10
$MgCl_2$	2
DNTP	0.5
F prime	1.5
R prime	1.5
MJB33 (2.5N, 1N, 100P, 10P, 1P)	1
GB taq	0.5

- The first time I ran this no band was achieved however I felt this may be due to human error (illness)

The second time I received good results

Bay

1	1KB	
2	/	
3	2.4	N
4	1	N
5	100	P
6	10	P → This band was best
7	1	P

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A. Macleod				

Electrophoresis

Continued from page number | Page number 11

Electrophoresis. (if TAE only 50 x 20ml Tae + 980ml H2O)

Tris
1mM stock
40 mM

$\frac{1000}{40} = 25$ $\frac{500}{25} = 20\,\mu L$

per 40 ml Tae - 1.5 % agarose - 0.6 agarose
Add 4 µl SyBr safe DNA gel stain:

1. Add 0.6 agarose to flask
2. 40ml Tae (1x) added.
3. microwave 30 seconds + swirl
4. if not clear stir & microwave more.
5. wait until it is cool to touch
6. Add 0.4 µl SyBr safe gel stain.
7. pour into tray + add comb
8. leave to set
9. remove gel from base & put the set gel onto a gel tank. (reservoir).
10. Remove comb
    - Mine still had gel in wells so we flushed with a pipette.

11. Add 1 µl of loading onto parafilm & then add 4 µl of each sample to this & mix with pipette.
12. load each sample (5µl) into seperate wells of the gel.

Performed by	Date	Countersigned by	Date	Continued on page number
L.macleod				

DPN1 treatment

Continued from page number

Page number 69

DPN1 treatment of MJB33 gusB.

10µl of sample 1.

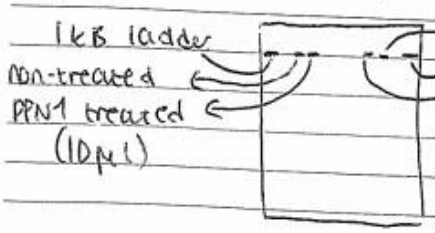
add 0.5 µl dPN1 → incubate for 37°C.

10µl each on gel.

- Take 10µl of PCR product Sample 1 & add to seperate. eppendorf.
- Add 0.5 µl of DPN1 to remaining solution
- leave for around 1hr at 37°C in incubator.
- Create a 1.5 agarose gel
- Add 10µl of each sample to a gel.

→ Why are we doing this?

We need to see in dPN1 cuts in the midde of gusB.



- Did not cut un-methylated PCR product → this is good.

Performed by	Date	Countersigned by	Date	Continued on page number
A. MacLeod				

Continued from page number

PCR for fusion

follow steps for previous pcr however instead of genomic DNA I am using:

TM6 F + R → 2 µl
TM8 F + R → 2 µl
TM12 F + R → 2 µl

These are my purified samples which worked during electrophoresis.

Electrophoresis

Bay 1 → loading dye

Bay 3 → TM6 (5µl)
Bay 4 → TM8 (5µl)
Bay 5 → Tm12 (5µl).

My samples did not work so I am going to run PCR again, increasing the magnesium concentration by 1µl & decreasing the temperature.

So run PCR as before for the fusion however with the following changes:

31.5 µl H2O
3 µl MgCl

+ run cycle at 56°C instead of 60°C.

Performed by	Date	Countersigned by	Date	Continued on page number
Amacleod				

Continued from page number

Ecr1	H_2O	38 µl
Kpn1	10 x Buffer (NE)	5µl
	10 mg/ml BSA	1µl
	plasmid DNA	6µl
	Ecr 1	1µl

Incubate at 37°C for 2 hrs → run gel.

then add 1µl KPN1.

Performed by	Date	Countersigned by	Date	Continued on page number
A.macleod				

Continued from page number | Page number 118

PCR fusion w. differing concentration of primers.

H_2O	32.5
5x flexi	10
$mgCl_2$	2
DNTP (1mM)	1
f primer	2 ; > ① no dilution ② 1/5
R primer	2 ;
GoTaq	0.5

@ 62°C

Electrophoresis

Bay		
1		
2		
3	1	①
4	4	①
6	6	①
6	8	①
7		
8	1	②
9	4	②
10	6	②
11	8	②

fusion for all 1/5 products HOWEVER.. $MgCl_2$ could be reduced + primers should be diluted more

Performed by Amalleod | Date | Countersigned by | Date | Continued on page number

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PCR fusion was done again however with the following changes.

H_2O	31		
5x flexi	10		
$mgCl_2$	1.5		
DNTP (1mM)	1	①	②
f	2	(1/20)	(1/50)
R	2		
(F)	1		
(R)	1		
GoTaq	0.5		

@ 62°C

Electrophoresis

Bay		
1	14B	
2	/	
3	1	①
4	4	①
5	6	①
6	8	①
7		
8	1	②
9	4	②
10	6	②
11	8	②

→ No results → primers too diluted.

Performed by Amalleod | Date | Countersigned by | Date | Continued on page number

PCR fusion troubleshoot

		①	②	③
H_2O	31			
5x flexi	10			
$MgCl_2$	1.5			
DNTP	1			
F	2	1/8	1/10	1/12
R	2			
(E)	1			
(R)	1			
GoTaq	0.5			

@ 62.0°C w. gradient.

DNA purification

Continued from page number | Page number 41

DNA Purification

take 1µL from PCR products.

Add 5X (5µL) of Buffer PB (ethanol already added)

Place in spin column & 2ml collection tube.

Centrifuge for 60 secs to bind DNA.

Add 750 µl PE & centrifuge for 60 secs.

↳ discard flow through.

Centrifuge again to remove residual buffer.

Transfer to clean 1.5ml MC. tube

Add 30 µl EB (for increased DNA conc.) let stand for 1 min then centrifuge. 60 secs.

Follow steps on pg. 39 after this:

(refer to this for steps & issues)

Performed by	Date	Countersigned by	Date	Continued on page number
Amacleod				

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Electrophoresis → for gel extraction

Make up a large gel using appropriate amounts of components based on volume of the container
(10.2 x 15 x 0.7) = 105ml

TAE : 105 ml
Agarose : 1· g
Sybrsafe : 10.5 µl
- Add Tae + agarose into a 150ml flask
- microwave to dissolve agarose + leave to cool
- pipette 10.5µl sybrsafe
- pour gel into container with 15 prong comb.

Performed by	Date	Countersigned by	Date	Continued on page number
Amalled				

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Gel extraction of fused products.

1) zero an eppendorf on scales

2) cut the first band on the gel with a scalpel → wear protective goggles + leave band above cut to be photographed afterwards.

3). Wipe scalpel and repeat the above step for each different sample.

4) place each sample into seperate labelled eppendorf + re-weigh the tubes → record weight.

① Tm1 8/6 ② → 80mg
② Tm4a 8/6 ② → (small) 90mg
③ Tm6 8/6 ② → 100mg
④ Tm8 8/6 ② → 50mg
⑤ Tm6 15/6 ② → 115mg
6 Tm8 15/6 ② → 80mg

saved in lab cold fridge.

Performed by	Date	Countersigned by	Date	Continued on page number
Amalled				

el extraction continued

more of the same samples were ran via electrophoresis + cut from the gel in the same way as before.

) find the weight on new extracts I weighed :ach eppendorf containing fragments from yesterday hen took the weight of previous fragment to ind out the weight of the eppendorf.

zero scales with both in then re-weigh with ew extract.

) ~~Beginner~~ 103 mg
:) 40 mg
.) 60 mg
t) ~~80~~ mg
5) 40 mg
6) 80 mg

So final weight =

1) Tm1 8/6 = 183mg
2) Tm4 8/6 = 130 mg
3) Tm6 8/6 = 160 mg
4) Tm5 8/6 = 90 mg
5) Tm6 ② 15/6 = 155 mg
6) Tm8 ⑤ 15/6 = 160 mg

We need 3 volumes Buffer QG to 1 volume gel (100mg ~ 100µl)

So amount of QG for each sample:

1) 549 µl
2) 390 µl
3) 480 µl
4) 270 µl
5) 465 µl
6) 480 µl

Incubate at 50°C for 10 minutes → vortex every 2-3 mins to help dissolve the gel

Once dissolved check mixture is a yellow colour (-if not add 10µl 3M sodium acetate pH 5 to turn yellow)

Add 1 gel volume of isopropanol to sample + mix

So all together we need 870 µl

Allocate correct amount (see page before) into each separate eppendorf → mix

place in 2ml collection tube + centrifuge for 1 min → balance accordingly.
-discard flow through. + place pace into column

Add 500µl QG (for sequencing) + centrifuge for 1 minute → discard flowthrough.

Add 750µl PE buffer + let stand for 2-5 mins

Centrifuge for 1min → discard flow through then centrifuge for another minute to get rid of.

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Restriction digest. → To test if DPN1 is working correctly

Buffer 5x PCR	10µl
MJB33 non-PCR product	10 µl
DPN1	0·5 µl
H_2O	29.5µl

Before adding DPN1, take 10 µl of solution that has not been treated with enzyme
↳ use as comparison on gel

Also take 10µl of sample 2 PCR product & put into sep. eppendorf for same purpose.

Add 0·5 µl of DPN1 to sample 2 PCR solution left over.

Create gel & leave sample 2 and non PCR MJB33 with added DPN1 in incubator for 1-2 hrs at 37 °C.

load samples onto gel for electrophoresis

Bay 1 → 1kB
2 → NA
3 → sample 2 → no DPN1
4 → sample 2 → DPN1 treatment.
5 → MJB33 → no DPN1
6 → MJB33 → DPN1 treatment.

Performed by	Date	Countersigned by	Date	Continued on page number
A. macleod				

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If DPN1 cuts the methylated MJB33 & not sample 2 → this is good and shows enzyme activity is fucntioning correctly.

Performed by	Date	Countersigned by	Date	Continued on page number
A·Macleod				

Phenol Extraction and Ethanol Precipitation

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phenyl extraction CN Sigma 7761-100ml

Due to the inability to heatshock my enzyme phenyl extraction will be d

Using the fume cupboard allocate the following amount of Phenyl to samples

1	100 µl	You are supposed to see 2 layers in the bottle of phenyl however the glass was too dark, therefore we added 300µl of H_2O to an eppendorf and 50µl of water → the phenyl sunk to the bottom + water stayed on top → this is good !!
2	100 µl	
3	100 µl	
4	100 µl	
5	100 µl	
6	100 µl	
PGEL	300 µl	
PKPol	300 µl	

Vortex to mix layers → will see white precipitate (proteins) this chemical at pH 7.8-8.3 will get rid of RNA + protein but keep DNA.

Centrifuge at 10,000 rpm for 10 minutes.

Label a new set of eppendorf's for each sample.

Pipette (100µl pipette) the clear aqueous layer on top of solution → this is the purified DNA.

Transfer to clean sterilised eppendorf's

Add the same amount of phenyl as before, vortex + centrifuge again for 10 mins @ 10,000 rpm.

Remove clear aqueous solution → make sure NO Phenyl IS ADDED !!

Performed by	Date	Countersigned by	Date	Continued on page number
A. MacLeod				

Continued from page number | Page number 132

Add 220µl Absolute Ethanol to 100µl samples + 640µl to the 300µl samples
(70% DNA precipitates at this percentage).

freeze in labcold until Monday

make sure all phenol eppendorfs are poured into falcon tubes + both are stored in the fume cupboard as this is very toxic.

Centrifuge for 10 mins at 4°C.

pour out ethanol without disturbing → pellet cannot be seen as is too small.

Continue with DNA purification.

Performed by	Date	Countersigned by	Date	Continued on page numbe
A Macleod				

Shrimp Alkaline Phosphatase (SAP) Treatment

Continued from page number

Page number 134

Alkaline Phosphatase treatment

30 µl DNA.

3.5 µl cusmart buffer (10 x)

1 µl SAP

Heat + incubate at 37°C for 30-60 mins

Then to inactivate heat to 65°C for 5 mins.

Performed by A Mallon

Date

Countersigned by

Date

Continued on page number

Continued from page number

Page number 135

nanodrop conc. for samples for Ligation

1	0.6	→	10µl
2	0.1	→	50µl
3	-0.2	→	100µl
4	0.9	→	6 µl
5	-0.2	→	100µl
6	0.7	→	8 µl

Performed by A Mallon

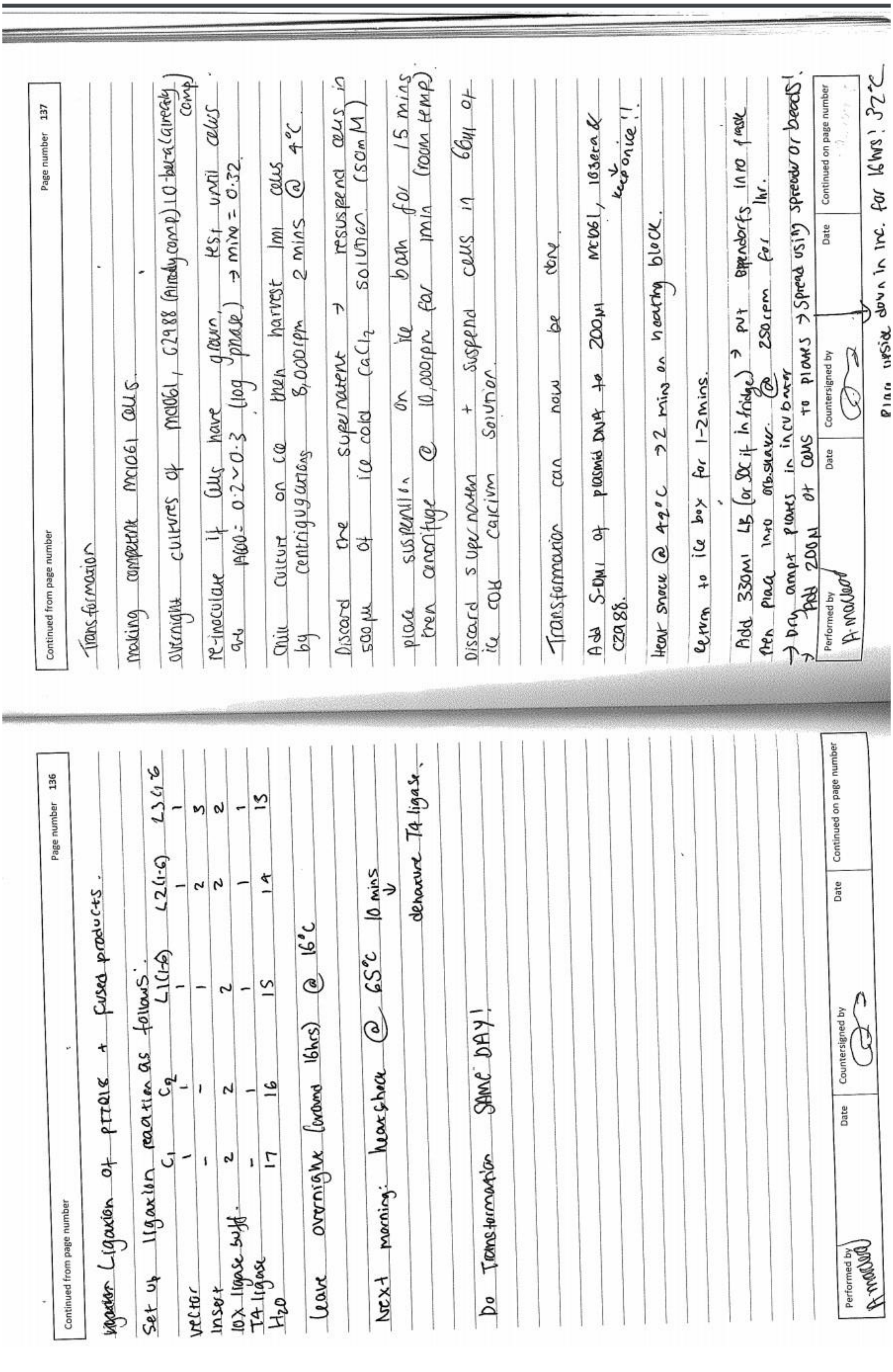
Date

Countersigned by

Date

Continued on page number

Ligation and Transformation



Continued from page number   Page number 136

~~ligation~~ Ligation of pTTQ18 + fused products.

Set up ligation reaction as follows:

	C1	C2	L1(1-6)	L2(1-6)	L3(1-6)
vector	1	1	1	1	1
insert	–	–	1	2	3
10x ligase buff.	2	2	2	2	2
T4 ligase	–	1	1	1	1
H_2O	17	16	15	14	13

Leave overnight (around 16hrs) @ 16°C

Next morning: heatshock @ 65°C 10 mins → denature T4 ligase.

Do Transformation SAME DAY!

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Transformation

making competent MC1061 cells.

overnight cultures of MC1061, C2988 (Already comp) 10-beta (already comp)

re-inoculate if cells have grown, test until cells are A600: 0.2~0.3 (log phase) → mine = 0.32.

Chill culture on ice then harvest 1ml cells by centrifugations 8,000rpm 2 mins @ 4°C.

Discard the supernatent → resuspend cells in 500μl of ice cold $CaCl_2$ solution (50mM)

place suspension on ice bath for 15 mins then centrifuge @ 10,000rpm for 1min (room temp).

Discard supernatent + suspend cells in 66μl of ice cold calcium solution.

Transformation can now be done.

Add 5-10μl of plasmid DNA to 200μl MC1061, 10beta & C2988. keep on ice!!

Heat shock @ 42°C → 2 mins on heating block.

return to ice box for 1-2mins.

Add 330μl LB (or SOC if in fridge) → put eppendorfs into flask then place into orb.shaker @ 250rpm for 1hr.

→ Dry amp+ plates in incubator

→ Add 200μl of cells to plates → Spread using spreader or beads!

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Place upside down in inc. for 16hrs! 37°C

Gel extraction for higher yield of DNA

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Gel extraction - for more DNA!
Repeat steps as before ☺
sample

1 - 1
2 - 4
3 - 6
4 - 8
5 - 8
6 /

New weights/ measurements

Eppendorf weight before — After

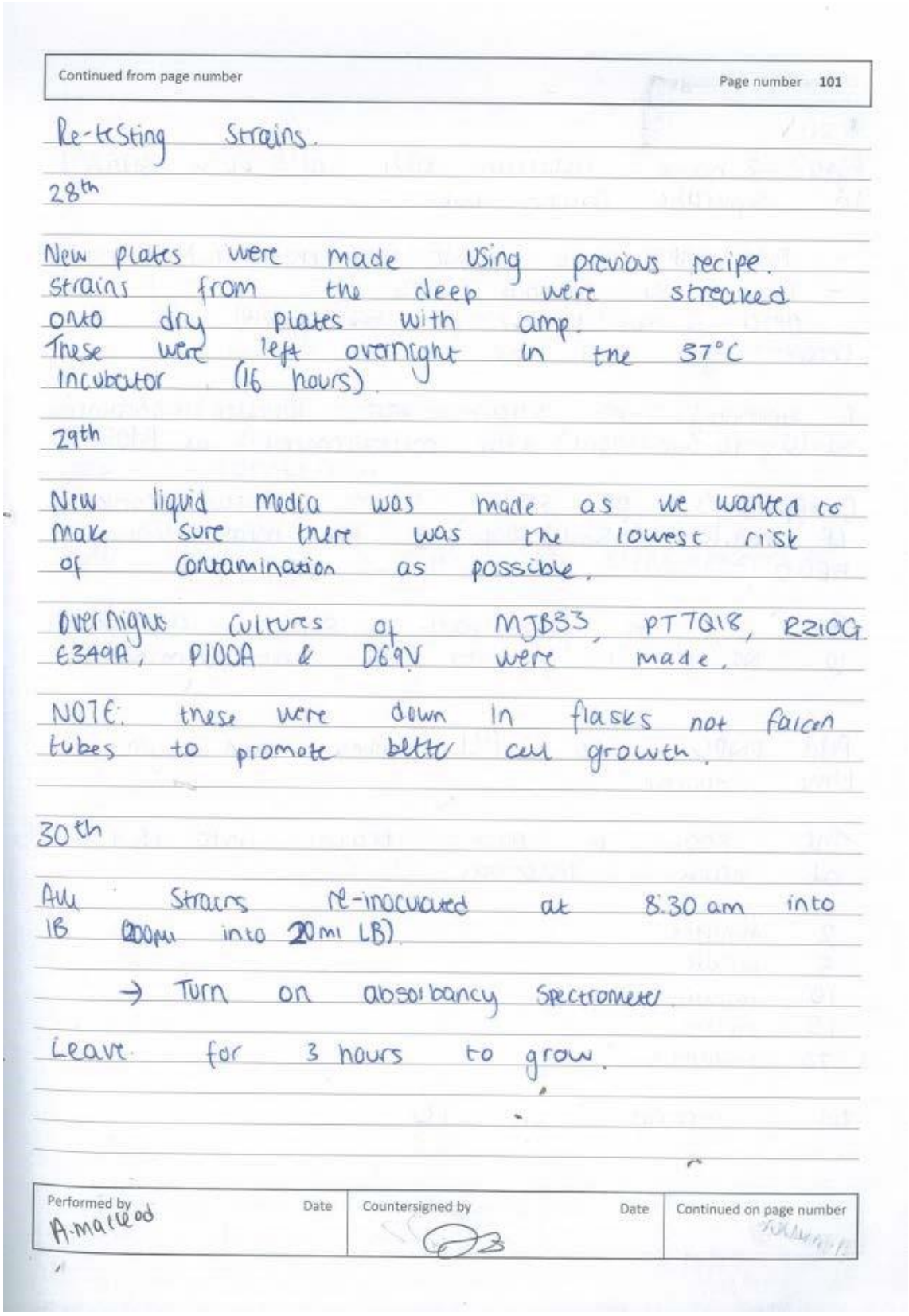
1 = 0.996g — 0.054 g (54mg)
4 = — 0.096 g (96 mg)
6 = — 0.116 g (116mg
8, = — 0.126 g (126mg)
8₂ = — 0.118 g (118mg)

QG

1 = 54mg = 162µl
4 = 96mg = 288µl
6 = 116mg = 348µl
8, = 126mg = 378µl
8₂ = 118mg = 354µl

Performed by A. macleod — Date — Countersigned by — Date — Continued on page number

Gus Assay Transport tests: Time Course, pNPG concentration and pNP



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Re-testing Strains.

28th

New plates were made using previous recipe. Strains from the deep were streaked onto dry plates with amp. These were left overnight in the 37°C incubator (16 hours).

29th

New liquid media was made as we wanted to make sure there was the lowest risk of contamination as possible.

Overnight cultures of MJB33, PTTQ18, R210G E349A, P100A & D69V were made.

NOTE: these were down in flasks not falcon tubes to promote better cell growth.

30th

All Strains re-inoculated at 8.30 am into 1B (200µl into 20ml LB).

→ Turn on absorbancy spectrometer.

Leave for 3 hours to grow.

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After 3 hours allocate 10ml of each strain in separate falcon tubes.

- Add IPTG to ONE of these (1mM IPTG final)
- Leave one without IPTG
(IPTG is an inducer for the operon)

Leave for 1 hour.

6 eppendorfs per strain were labelled containing $NaCO_3$ (0.5ml | 500µl) → for measurement at A405

6 eppendorfs per stain were labelled containing LB media (0.5ml | 500µl) → for measurement at A600.

Take 1 ml from each strain add 1/2ml to $NaCO_3$ + 1/2 to LB at 0' minutes

Add PNPG to ALL tubes and begin time course.

Take 500ml of each strain into LB + $NaCO_3$ at each interval:

2 minutes
5 minutes
10 minutes
15 minutes
20 minutes

Put Straight on ice.

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Measure all LB samples at A600 first.

Measure all samples with $NaCO_3$
↳ Spin first for 2minutes at 13,000 rpm and use supernatent.

Re-inoculate all samples ON by adding 20µl in 2ml for mini prep tomorrow.

After mini-prep run gel electrophoresis!

Performed by: A Markeas | Date | Countersigned by | Date | Continued on page number

Results from pg. 101-103.

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A600	0'	2'	5'	10'	15'	20'
MJB33 +	0.57	0.56	0.55	0.55	0.58	0.57
MJB33 -	0.57	0.58	0.57	0.55	0.56	0.57
TTQ18 +	0.33	?	0.35	0.33	0.33	0.32
TTQ18 -	0.35	0.34	0.34	0.33	0.34	0.33
210G +	0.57	0.59	0.56	0.55	0.54	0.55
210G -	0.58	0.59	0.57	0.58	0.56	0.56
100A +	0.38	0.39	0.39	0.40	0.38	0.41
100A -	0.42	0.42	0.42	0.46	0.41	0.43
69V +	0.63	0.35	0.64	0.62	0.63	0.63
69V -	0.64	0.63	0.63	0.62	0.65	0.63
349A +	0.34	0.45	0.33	0.35	0.34	0.34
349A -	0.39	0.65	0.37	0.39	0.39	0.40

* E349+ had 1.5mi PTTQ15- had 0.5 → PTTQ18 may have mixed with e39+ sample at 2 minutes interval
→ DO not count three results marked.
→ majority running at 2 mins.

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A450	0'	2'	5'	10'	15'	20'	A600/A50
MJB33 +	0.02	0.11	0.45	0.58	0.67	0.72	
MJB33 -	0.08	0.12	0.30	0.51	0.61	0.64	
PTTQ18 +	0.01	0.03	0.06	0.10	0.14	0.18	
PTTQ18 -	0.01	0.05	0.09	0.10	0.16	0.17	
R210 G +	0.05	0.05	0.08	0.13	0.15	0.22	
R210 G -	0.04	0.05	0.08	0.12	0.14	0.18	
P100A +	0.01	0.07	0.09	0.16	0.18	0.21	
P100A -	0.02	0.09	0.11	0.17	0.18	0.24	
DG9 V +	0.05	0.07	0.05	0.08	0.09	0.13	
DG9 V -	0.06	0.07	0.04	0.06	0.11	0.13	
E349A +	0.06	0.02	0.05	0.07	0.06	0.09	
E349 V -	0.01	0.03	0.03	0.06	0.10	0.09	

Performed by A.macleod | Date | Countersigned by | Date | Continued on page number

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Gus addasy → PNPG conc. test

Innoculate colony from plates into 150ml LB → leave to grow overnight. (6 strains)

After 16 hours, reinoculate 1/50 and leave to grow for 4hours → Add IPTG to one/two strains (one induced one not)

Add pNPG to 1ml of each strain +/- IPTG at following concentrations:

- 0
- 0.25
- 0.5
- 0.75
- 0
- 1.25
- 1.5

Leave for 15 minutes then ~~xxxxxxx~~ ASC 500ml to NaCO3

↳ spin down & measure supernatent @ A405

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A.macleod				

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Gus Assay → PNP concentration.

Inoculate ON cultures for pMJB33, pTTQ18 & pE349A overnight (16hrs)

Re-inoculate 1/30 → 3 strains, 2 each x 20ml

(with IPTG) (without IPTG)

(200ml of 100mM)

Leave to grow for 3 hrs.

In the meantime dilute PNP at 10, 20, 50, 100, 200 & 500nm

↳ measure each at 405 (3 per conc)

This will be used for Standard curve to test samples against.

	20	50	100
1)	1)	1)	1)
2)	2)	2)	2)
3)	3)	3)	3)

200	500
1)	1)
2)	2)
3)	3)

Next page for results

Performed by	Date	Countersigned by	Date	Continued on page number
A.macleod				

(10) 1) 0.2183
2) 0.1995
3) 0.2218

(20) 1) 0.3713
2) 0.3897
3) 0.3792

(50) 1) 0.9256
2) 0.9245
3) 0.9058

(100) 1) 1.7890
2) 1.8205
3) 1.7975

(200) 1) 2.9896
2) 2.9491
3) 2.9882

(500) 1) 3.0704
2) 3.1641
3) 3.1390

ONce other strains have grown for 3/3/2 hours add pNpG 1mM conc. to all samples. T measure at A405 + A600 after 15 minutes

500µl $NaCO_3$ (A405

500µl LB (A600)

Results (A405)

	75µl	100µl	150µl	225µl	300µl	450µl → for pNp 1mM
	0mM	0.25mM	0.5mM	0.75mM	1mM	1.5mM
MJB33 +				→ Discoum		0.0688
1	0.0560	0.1593	0.1867	0.005	0.0850	0.0865
2	0.0520	0.1225	0.0766	0.0817	0.0693	0.1051
3	0.0193	0.1057	0.1264	0.0767	0.0899	0.0412
MJB33 -						
1	0.8329	0.0652	0.0864	0.0896	0.0642	0.0785
2	0.0524	0.0495	0.0791	0.0605	0.0920	0.0580
3	0.0470	0.0553	0.073	0.0667	0.0847	
PTTQ18 +	0.1101					
1		0.3897	0.5363	0.8944	0.4914	0.7771
2	0.2711	0.3923	0.5994	0.5013	0.5050	0.3937
3	0.2013	0.3642	0.4100	0.7593	0.4274	0.6250
PTTQ18 -						
1	0.3564	0.5140	0.5955	0.2683	0.2741	0.2815
2	0.3725	0.2074	0.2961	0.2742	0.2816	0.2921
3	0.5170	0.2065	0.2657	0.2846	0.3268	0.3076

A600

	0	0.25	0.5	0.75	1	1.5
MJB33 +						
1	0.6287	0.4016	0.3614	0.4280	0.3413	0.6520
2	0.4872	0.4441	0.5493	0.5844	0.5280	0.3221
mjB33 +						
1	0.6440	0.4026	0.6340	0.4186	0.4222	0.3802
2	0.4017	0.4096	0.3689	0.5708	0.3880	0.5110
PTTA18 +				0.5281		
1	0.3531	0.4918	0.5365	0.5286	0.5235	0.3119
2	0.5674	0.5810	0.5687	0.5420	0.5130	0.4859
PTTQ18 +						
1	0.2291	0.5810	0.5620	0.5579	0.5414	0.5141
2	0.5949	0.5766	0.5674	0.5619	0.5284	0.5278
E349 +						
1	0.7074	0.6790	0.6812	0.6655	0.6673	0.6617
2	0.7195	0.6648	0.6836	0.7019	0.6303	0.6395
E349 −						
1	0.6939	0.6952	0.6730	0.6742	0.6684	0.6582
2	0.6785	0.6772	0.6819	0.6685	0.6076	0.5799

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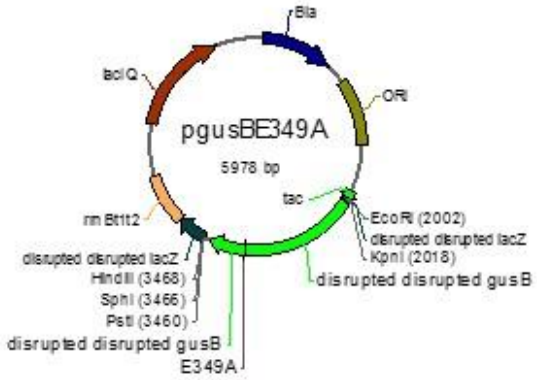
A. McLeod				

A405

	0	0.25	0.5	0.75	1	1.5
MJB33 +						
1	0.2400	0.3801	0.4220	0.4445	0.4379	0.4363
2	0.2472	0.3910	0.4420	0.4348	0.4480	0.4461
MJB33 +						
1	0.2425	0.4666	0.4603	0.4732	0.6117	0.4730
2	0.2461	0.4571	0.4921	0.4981	0.5611	0.4688
PTTQ18 +						
1	0.2424	0.2886	0.2896	0.3004	0.3044	0.2988
2	0.2507	0.2908	0.3104	0.3816	0.3111	0.3210
PTTQ18 +						
1	0.2421	0.2903	0.3253	0.3447	0.3559	0.3498
2	0.2430	0.3250	0.3431	0.3650	0.3440	0.3634
E349A +						
1	0.2390	0.3881	0.2515	0.2531	0.2453	0.2326
2	0.2623	0.2528	0.2480	0.2487	0.2516	0.2501
E349A −						
1	0.2483	0.2449	0.2495	0.2404	0.2408	0.2419
2	0.2477	0.2504	0.2596	0.2756	0.2711	0.2381

Performed by Amacad | Date | Countersigned by | Date | Continued on page number

5. pE349A sequence and plasmid map



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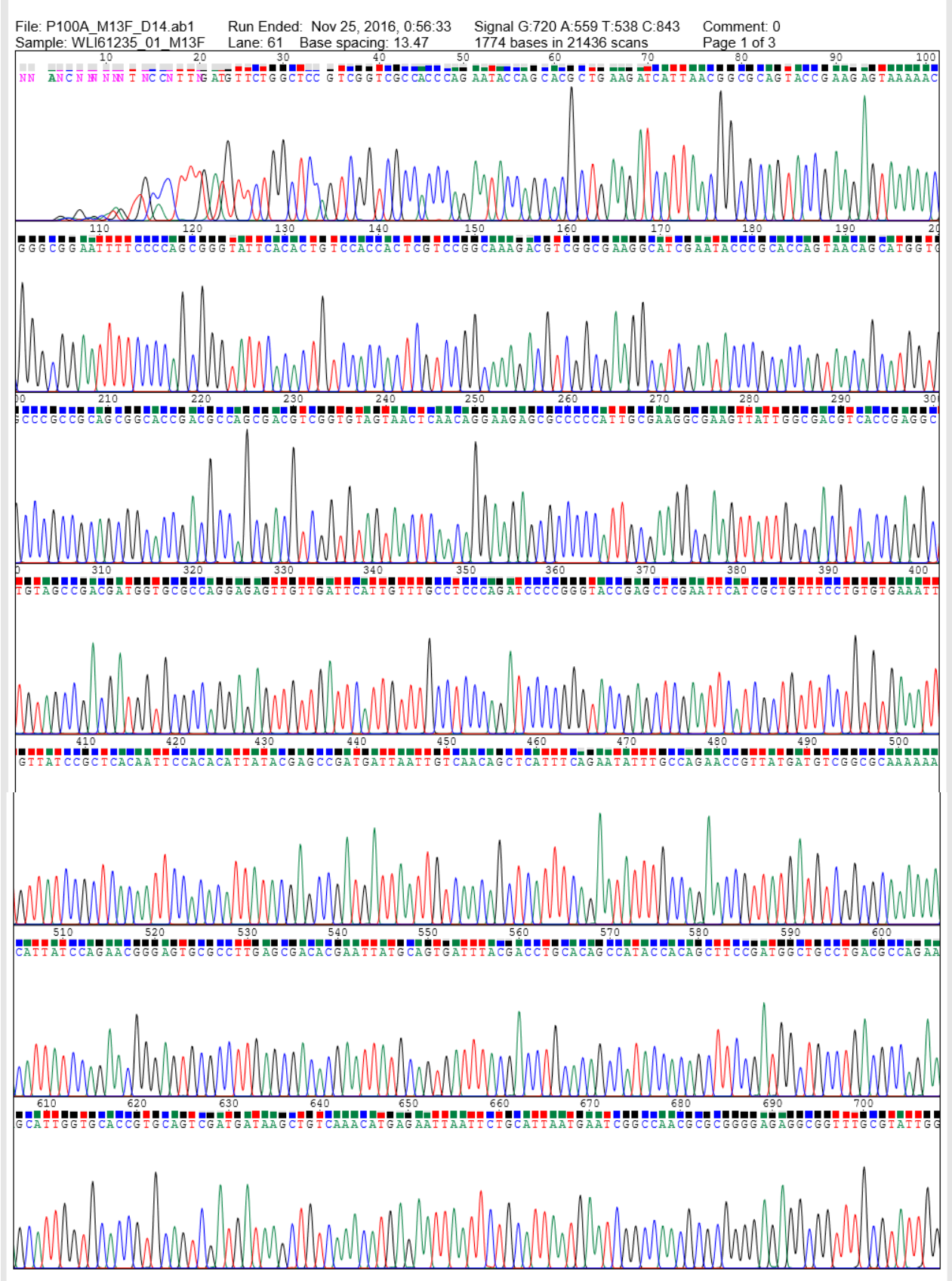
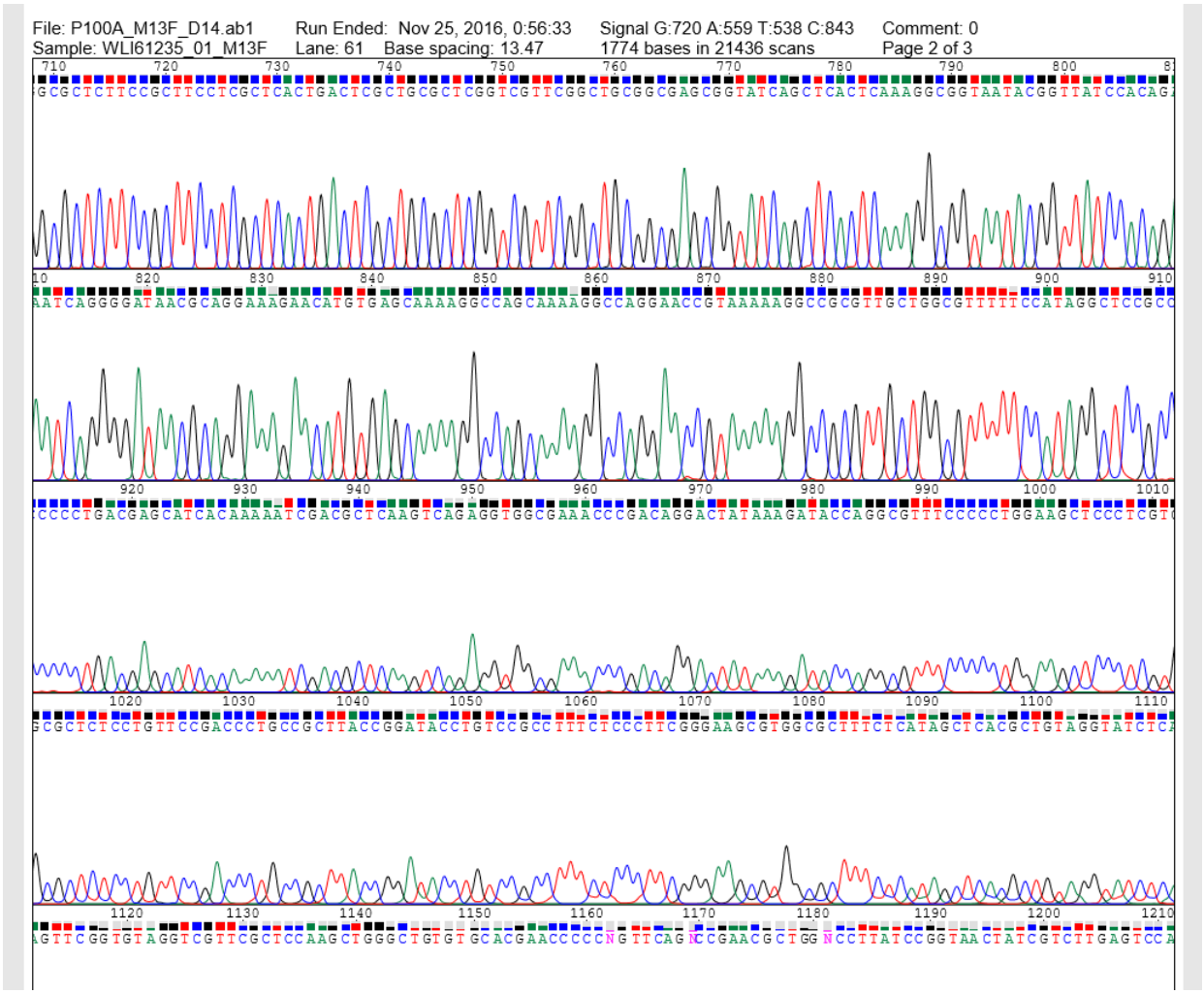
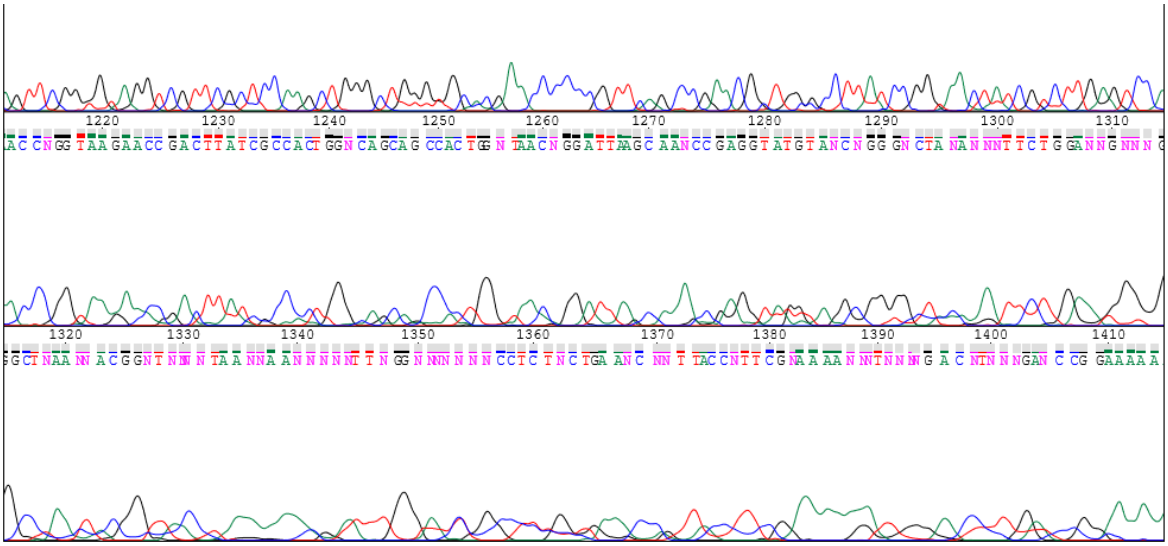
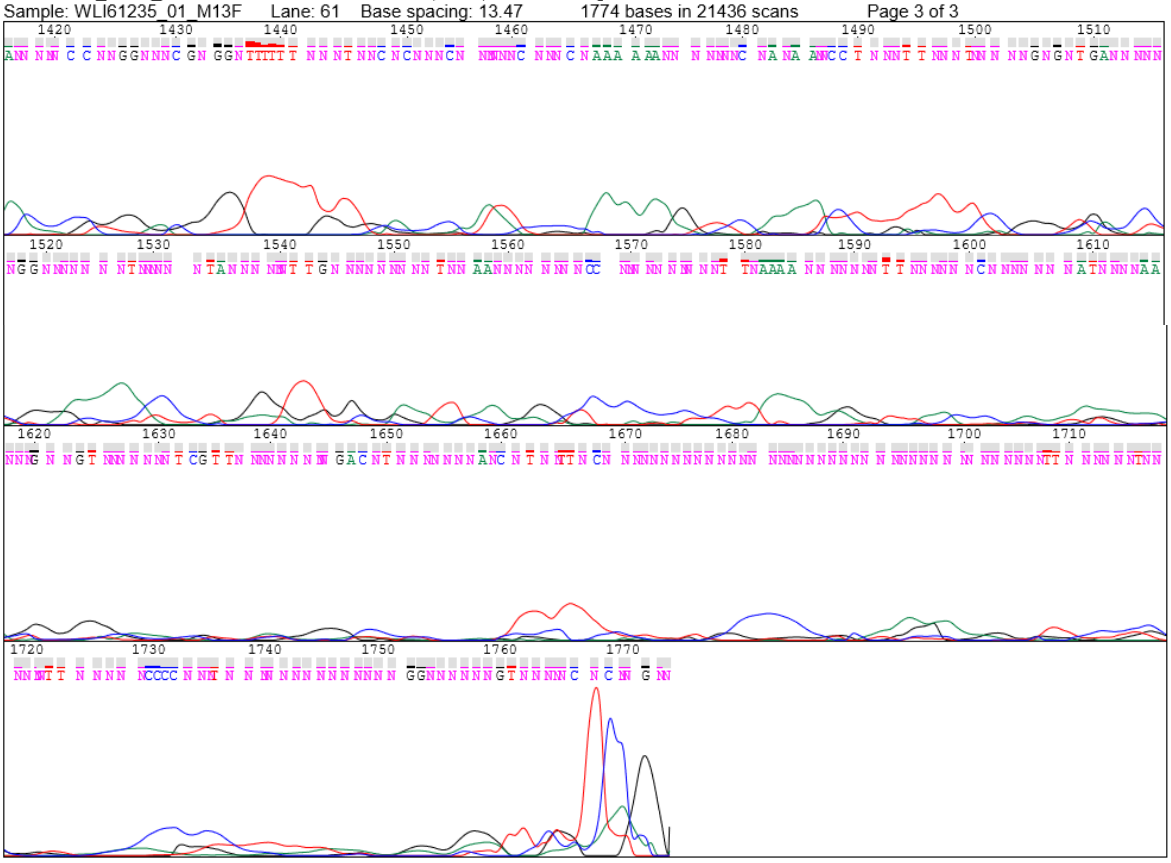
6. pE349A Sequencing Results




File: P100A_M13F_D14.ab1 Run Ended: Nov 25, 2016, 0:56:33 Signal G:720 A:559 T:538 C:843 Comment: 0
Sample: WLI61235_01_M13F Lane: 61 Base spacing: 13.47 1774 bases in 21436 scans Page 2 of 3




File: P100A_M13F_D14.ab1 Run Ended: Nov 25, 2016, 0:56:33 Signal G:720 A:559 T:538 C:843 Comment: 0
Sample: WLI61235_01_M13F Lane: 61 Base spacing: 13.47 1774 bases in 21436 scans Page 3 of 3

7. Evaluative Supplement

First and foremost, the most rewarding part of undertaking this investigation was the process of taking a subject which was new to me and being able to develop a vast understanding of all aspects concerned with the topic. When I first started this project, my knowledge surrounding the *gusB* gene and the GUS operon was minimal. I understood the basics of the system due to past biomolecular studies however, the depth of understanding needed for this was extensive and a lot of reading, questions and hard work was put in to achieve the results in the project and to obtain the knowledge surrounding the subject area. This was ongoing and at every single point for desk-based research was needed to understand the practical side of the project. This however was not the most difficult part for me, it was in fact the lab work which proved to be the most challenging.

When the project began molecular cloning techniques and mutagenesis were not skills that I possessed and although I was familiar with the theory I had not previously conducted and designed my own experiments. Behind each lab procedure carried out is a basic protocol which needed to be shaped and moulded to fit with what I was trying to achieve, although in theory what I was trying to do was easy in practice getting experiments exactly right and getting any sort of result was much harder than anticipated. Often the lab work became a trial and error, educated guesses as to why certain experiments would not work were constructed based on reading troubleshoot guides, journals, books and articles and discussions with my supervisor and fellow students. Each step in the cloning process threw up new challenges which sometimes created knock backs and made me feel dis-heartened because the work being put in was not giving back the results I needed.

It came to my attention that in fact conducting science experiments of this kind was sensitive and the smallest issue could bring adverse effects the results I obtained. The inability for me to complete the cloning process on the last step of this project brought me great distain and a sense of under achieving as this was my ultimate goal with the project and my samples could not be checked to see if they were in fact clones or related to the molecular recognition site of GusB. I did not achieve the initial goal set out however the wealth of knowledge I gained both in lab and in regard to the subject matter is not comparable to the knowledge I had at the beginning.

Another setback was ill health during the project which at times meant lab work could not be completed, along with this hold ups due to chemical order processing and machine and equipment repairs created false results and also a dis-jointed journey throughout the cloning process. Some of the issues also can attributed to my own personal struggles, I often try and work alone trying to Figure things out for myself. This lead to repeated experiments and lot of unsuccessful attempts, if discussed properly with my supervisor and other students at the time this may have reduced the number of mistakes made. Although weekly plans were put in place better time management could have ensured that enough time was given for each step taking into consideration any additional work that may need to be done including trouble shooting, this would then give better direction for

the project rather than unrealistic expectation which came from a lack of experience in the lab.

Besides the difficulties faced in this project I felt passionate about the subject and the more I learnt the more I respected the work being done in the field of molecular biology. The practice is difficult, uncertain and at times tedious however it is through this practice that we can uncover the basic building blocks and processes responsible for driving life itself. The desire to understand the intricacies of this subject seemed natural for me especially when it related to me and everyone around me. Frustration from being unable to obtain results drives me even more to carry on learning and trying to uncover more information about *gusB,* I have invested a lot of time into the gene which sparks intrigue and questions about this gene still which are still left unanswered.

Apart from all of the technical skills I obtained, my supervisor also suggested I try to hone my writing skills however, after years of bad habits and being taught incorrectly this was hard for me. By comparing articles, learning to engage the reader, creating a story and setting themes in my writing I was able to improve my work. This is not just necessary for writing a thesis but will also be transferable in other areas of my life.

Doing this Master of Research project has made me realise that science is what I want to pursue in life, it has the ability to drive you to distraction but when results are achieved the feeling of accomplishment completely outweighs the negatives. Science, especially molecular biology is on the forefront of technology. Having finished this project, I have now started a job within Pharmacological research on Phase I drugs. I hope to use all I have learnt during my postgraduate study to aid my success in this industry.

Bournemouth University *COSHH Assessment Form: Transormation of* E. coli

1. Assessor:	**2. Assessment Date:**	**3. Assessment Review:**		
I. Green	2/2/11	Next Review Date: 2/2/12 11/8/12 15/10/13 4/10/14 11/11/15 27/05/16	Reviewed on: Date: 11/8/11 15/10/12 4/10/13 11/11/14 27/05/15	Reviewed By: I. Green I. Green I. Green I. Green E. Franklin
4. Summary of process or method (or make specific reference to written protocol to be used) :				
Mixing media and culturing bacteria				
5. Key Activity/Task (in relation to exposure potential e.g. mixing, filling, spraying, etc.):		**6. People who <u>could</u> come to harm** (number & roles e.g. students)		
Weighing powders and handling microorganisms		Staff and students, up to 3 members of staff and 69 students		
7. Duration of Exposure (minutes, hours and how often):		**8. Location and Conditions of Use** (e.g. lab, room, temp etc.)		
4 hours, repeated 3 times for staff, once for students		Christchurch House and Dorset house labs		

	9. Hazardous ingredients: (copy form/add more rows as req'd)	**10. Quantities Used**	**11. Workplace Exposure Limit (WEL)**	**12. Risk/safety phrases**	**13. Actual Potential Route of Exposure** (E.g. by inhalation)	**14.Datasheet Attached? Y/N**
A	E. coli JM 109 cultures	100 ml	N/A	Non-pathogenic. No risk listed on MSDS	Eye/skin contact, swallowing, inhalation	Y
B	LB agar	500 g	N/A	Not listed as hazardous	Eye/skin contact, swallowing, inhalation	Y
C	Tris	1 l		Not a hazardous substance or mixture according to Regulation (EC) No. 1272/2008. This substance is not classified as	Eye/skin contact, swallowing, inhalation	Y

				dangerous according to Directive 67/548/EEC.		
D	HCl (1M)	1 L	1 PPM	**Hazard Statements:** H290- May be corrosive to metals H315- Causes skin irritation H319- Causes serious eye irritation H335- May cause respiratory irritation **Precautionary Statements:** P261- Avoid breathing dust/fume/gas/mist/vapours/spray P305+P351+338- IF IN EYES: Rinse cautiously with water for several minutes. Remove contact lenses, if present and easy to do. Continue rinsing.	Eye/skin contact, swallowing, inhalation	Y
E	$CaCl_2$	500 g	N/A	**Hazard Statements:** H319- Causes serious eye irritation **Precautionary Statements:** P305+P351+338- IF IN EYES: Rinse cautiously with water for several minutes. Remove contact lenses, if present and easy to do. Continue rinsing.	Eye/skin contact, swallowing, inhalation	Y
F	Amipcillin	2ml 100mg/ml	N/A	**Hazard Statements:** H317- May cause an allergic skin reaction. H334- May cause allergy or asthma symptoms or breathing difficulties if inhaled. **Precautionary Statements:** P261- Avoid breathing dust/ fume/ gas/ mist/ vapours/ spray. P280- Wear protective gloves. P342 +P311- If experiencing respiratory symptoms: Call a POISON CENTER or doctor/physician.	Eye/skin contact, swallowing, inhalation	Y
15. Control Measures						

A) *E. coli* JM 109

1) Have less hazardous alternatives

These have been considered, but none found

2) Engineering controls

Provide appropriate exhaust ventilation at places where dust is formed when using powders.

3) Personal protective equipment
Lab coats, safety spectacles and nitrile gloves must be worn to protect skin and eyes.

4) Other measures

i) E. coli JM 109 is non-pathogenic and has been assigned to group 1 as defined by Schedule 3 of the Control of Substances Hazardous to Health Regulation (2002). There is no foreseeable risk that the planned genetic transformation will affect the pathogenicity of the organism.

ii) Every effort must be made to contain E. coli JM 109 in the lab areas. All materials coming into contact with the organism must be disposed of as biohazardous waste in a biohazard bin. Suitable sterilising action (e.g. flaming, soaking in a suitable biocide such as bleach or autoclaving) must be undertaken before material exposed to genetically modified E. coli is binned. Bins should be hermetically sealed and disposal through a commercial provider arranged as soon as possible. These measures are to ensure both health and safety and to ensure that no genetically modified microorganism is accidentally released in to the environment!

iii) Clean down all working surfaces must be cleaned with a biocide (Biocleanse) after the work has been completed.

5) Storage

Cultures should be stored in Dorset house labs, or if long term storage is required, the -70°C freezer in Dorset house can be used.

B) Other material

1) Have less hazardous alternatives

These have been considered, but none found

2) Engineering controls

Provide appropriate exhaust ventilation at places where dust is formed when using powders.

3) Personal protective equipment
Lab coats, safety spectacles and nitrile gloves must be worn to protect skin and eyes.

4) Other measures

Clean down all working surfaces must be cleaned with a biocide (Biocleanse) after the work has been completed.

5) Storage

Store in cool place. Keep container tightly closed in a dry and well-ventilated place. Containers which are opened must be carefully resealed and kept upright to prevent leakage.

Now mark in the letters from the list of 'Hazardous Ingredients' above to indicate potential danger:

16. Indication of Danger				**17. Route of Exposure**		**18. Chemical State**		**19. Flammability**		**20. Volatility**		**21. Dust rating**	
Very Toxic		Irritant	D	Inhalation	ALL	Solid	B C E	Flammable		Low	A B C E	Low	A D
Toxic		Sensitiser		Skin Contact	ALL	liquid	A B C D E	Highly flammable		Medium	D	Medium	B C E
Corrosive	D	Carcinogen		Eye Contact	ALL	Gas/vapour	D	Extremely flammable		High		High	
Harmful	F	Mutagenic		Swallowing	ALL			Oxidising					
Biological Agent		Toxic to reproduction		Injection				Explosive					

22. First Aid Procedures (as advised from Material Safety Data Sheet)

If inhaled	**If skin contact**	**If eye contact**	**If swallowed**	**If injected**
If breathed in, move person into fresh air. If not breathing, give artificial respiration. Consult a physician.	Wash off with soap and plenty of water. Consult a physician.	Rinse thoroughly with plenty of water for at least 15 minutes and consult a physician.	Never give anything by mouth to an unconscious person. Rinse mouth with water. Consult a physician.	Consult a physiciian

23. Spillage Procedures: ⟶	**Following the control measures in section 15:**

24. Disposal Arrangements	**Solids:** Pick up and arrange disposal without creating dust. Sweep up and shovel. Keep in suitable, closed containers for disposal. **Liquids:** Soak up with inert material and dispose of as hazardous waste. Keep in suitable, closed containers for disposal **Microbial material:** Thoroughly spray area with biocide (Bioclense) and mop up thoroughly with inert material (e.g. blue towelling). Dispose of material in a biohazard bin.			
Collection	**Swill down sink**	**Evaporation**	**In normal waste**	**Other**
All microbial waste must be sent for commercial disposal, i.e. place in a biohazard bin.	Run to waste with plenty of water			
25. Are the risks adequately controlled? (Write 'Yes' or 'No'):	Yes			

If you decide that the controls in Section 15. are sufficient, skip to section 27. If you decide that the risks are NOT adequately controlled (or you're not sure), then you will need to give special instructions to control the risk.

26. Special Instructions to control the risk:
27. Ensure those affected are informed of the Risks & Controls - Confirm how this will be done e.g. by issuing written instructions:
Copy of this assessment sent to academic in charge of this practical. Verbal instructions are given to students at the start of the practical.

Bournemouth University *COSHH Assessment Form: Qaigen QIAprep Spin kit*

1. Assessor:	**2. Assessment Date:**	**3. Assessment Review:**		
I. Green	18/10/10	Next Review Date: 4/10/11 10/10/12 15/10/13 6/3/15	Reviewed on: Date: 10/10/2011 15/10/12 6/3/14	Reviewed By: I. Green I. Green I. Green
4. Summary of process or method (or make specific reference to written protocol to be used) :				
Use of proprietary kit according to manufacturers instructions.				
5. Key Activity/Task (in relation to exposure potential e.g. mixing, filling, spraying, etc.):		**6. People who could come to harm** (number & roles e.g. students)		
Diluting stock solutions and pipetting solutions. Handling of microorganisms		Staff (4) & Students (69)		
7. Duration of Exposure (minutes, hours and how often):		**8. Location and Conditions of Use** (e.g. lab, room, temp etc.)		
2 hours, several times for staff and PGRs and once for UG students		DG 43C & C221		

	9. Hazardous ingredients: (copy form/add more rows as req'd)	**10. Quantities Used**	**11. Workplace Exposure Limit (WEL)**	**12. Risk/safety phrases**	**13. Actual Potential Route of Exposure** (E.g. by inhalation)	**14.Datasheet Attached? Y/N**
A	Buffer P1	250 ml	N/A	Not hazardous	Skin/eye contact, swallowing, inhalation	Y
B	Buffer PE (concentrate)	250 ml	N/A	Not hazardous	Skin/eye contact, swallowing, inhalation	Y
C	Buffer N3 (Guanidinium chloride & acetic acid)	250 ml	**Acetic acid:** OEL Short-term value: 37 mg/m³, 15 ppm Long-term value: 25 mg/m³, 10 ppm	**Hazard Statements:** H302- Harmful if swallowed H315- Causes skin irritation H319- Causes serious eye irritation **Precautionary Statements:** P301+ P312- IF SWALLOWED: Call a POISON	Skin/eye contact, swallowing, inhalation	Y

				CENTER or doctor/ physician if you feel unwell. P303+P361+P353- IF ON SKIN (or hair): Take off immediately all contaminated clothing. Rinse skin with water/shower P305+P351+P338: IF IN EYES: Rinse cautiously with water for several minutes. Remove contact lenses, if present and easy to do. Continue rinsing.		
D	Buffer P2 (Sodium dodecyl sulphate & sodium hydroxide)	250 ml		**Hazard Statements:** H302- Harmful if swallowed H315- Causes skin irritation H319- Causes serious eye irritation **Precautionary Statements:** P301+ P312- IF SWALLOWED: Call a POISON CENTER or doctor/ physician if you feel unwell. P303+P361+P353- IF ON SKIN (or hair): Take off immediately all contaminated clothing. Rinse skin with water/shower P305+P351+P338: IF IN EYES: Rinse cautiously with water for several minutes. Remove contact lenses, if present and easy to do. Continue rinsing.	Skin/eye contact, swallowing, inhalation	y
E	Buffer PB (guanidinium chloride & Propan-2-ol)	250 ml	**Propan-2-ol** OEL Short-term value: 1250 mg/m³, 500 ppm Long-term value: 999 mg/m³, 400 ppm	**Hazard Statements:** H226- Flammable liquid H302- Harmful if swallowed H315- Causes skin irritation H319- Causes serious eye irritation **Precautionary Statements:** P210- Keep away from heat/sparks/open flames/ hot surfaces – No Smoking P301+ P312- IF SWALLOWED: Call a POISON CENTER or doctor/ physician if you feel unwell. P303+P361+P353- IF ON SKIN (or hair): Take off immediately all contaminated clothing. Rinse skin with water/shower P305+P351+P338: IF IN EYES: Rinse cautiously with water for several minutes. Remove contact lenses, if present and easy to do. Continue rinsing.	Skin/eye contact, swallowing, inhalation	y
F	Lyse Blue	250 ml	N/A	Not hazardous	Skin/eye contact, swallowing, inhalation	y

G	RNase A solution	250 ml		**Hazard Statements:** H334- May cause allergy or asthma symptoms or breathing difficulties if inhaled. H315- Causes skin irritation H319- Causes serious eye irritation **Precautionary Statements:** P303+P361+P353- IF ON SKIN (or hair): Take off immediately all contaminated clothing. Rinse skin with water/shower P305+P351+P338: IF IN EYES: Rinse cautiously with water for several minutes. Remove contact lenses, if present and easy to do. Continue rinsing.	Skin/eye contact, swallowing, inhalation	y
H	Buffer EB	250ml	N/A	Not Hazardous	Skin/eye contact, swallowing, inhalation	y

15. Control Measures

Engineering controls:
Ensure good general ventilation

Personal protective equipment:
Standard lab PPE required – lab coat, nitrile gloves (or equivalent) and safety spectacles.

General protective and hygienic measures:
Keep away from foodstuffs, beverages and
Immediately remove all soiled and contaminated
Wash hands before breaks and at the end
Avoid contact with the eyes and skin.

Storage:
Keep receptacle tightly sealed, otherwise, no special requirements.

Buffer PB – keep away from ignition sources.

.

C, D, E & G react dangerously with: Acids, alkalis and oxidising agents
In addition to the above E also reacts dangerously with: Alcohols and amines

Now mark in the letters from the list of 'Hazardous Ingredients' above to indicate potential danger:

16. Indication of Danger				17. Route of Exposure		18. Chemical State		19. Flammability		20. Volatility		21. Dust rating	
Very Toxic		Irritant	D	Inhalation	ALL	Solid		Flammable	E	Low	ALL	Low	ALL
Toxic		Sensitiser		Skin Contact	ALL	liquid	ALL	Highly flammable		Medium		Medium	
Corrosive		Carcinogen		Eye Contact	ALL	Gas/vapour		Extremely flammable		High		High	
Harmful	C E G	Mutagenic		Swallowing	ALL			Oxidising					
Biological Agent		Toxic to reproduction		Injection				Explosive					

22. First Aid Procedures (as advised from Material Safety Data Sheet)

If inhaled	If skin contact	If eye contact	If swallowed	If injected
Supply fresh air; consult doctor immediately	Immediately wash with water and	Rinse opened eye for several minutes (15 min) under running water	Call for a doctor immediately.	Call for a doctor immediately.

23. Spillage Procedures: ⟶	Ensure adequate ventilation and follow control measures in section 15 above.
24. Disposal Arrangements	Absorb with liquid-binding material (sand, diatomite, acid binders, universal binders, sawdust).

Collection	Swill down sink	Evaporation	In normal waste	Other
✓				

25. Are the risks adequately controlled? (Write 'Yes' or 'No'):	yES

If you decide that the controls in Section 15. are sufficient, skip to section 27. If you decide that the risks are NOT adequately controlled (or you're not sure), then you will need to give special instructions to control the risk.

26. Special Instructions to control the risk:

27. Ensure those affected are informed of the Risks & Controls - Confirm how this will be done e.g. by issuing written instructions:

Bournemouth University *COSHH Assessment Form: Plasmid digest*

1. Assessor:	**2. Assessment Date:**	**3. Assessment Review:**		
I. Green	18/10/10	Next Review Date: 4/10/11 9/10/14 27/05/16	Reviewed on: Date: 9/10/13 27/05/15	Reviewed By: I. Green E. Franklin
4. Summary of process or method (or make specific reference to written protocol to be used) :				
Buffers and enzymes are mixed with extracted bacterial DNA plasmids				
5. Key Activity/Task (in relation to exposure potential e.g. mixing, filling, spraying, etc.):		**6. People who could come to harm** (number & roles e.g. students)		
Pipetting solutions		Staff (4) & Students (69)		
7. Duration of Exposure (minutes, hours and how often):		**8. Location and Conditions of Use** (e.g. lab, room, temp etc.)		
Up to 8 hours, several times per annum		Laboratories DG43C & C221		

	9. Hazardous ingredients: (copy form/add more rows as req'd)	**10. Quantities Used**	**11. Workplace Exposure Limit (WEL)**	**12. Risk/safety phrases**	**13. Actual Potential Route of Exposure** (E.g. by inhalation)	**14.Datasheet Attached? Y/N**
A	Tris - HCl	1 L	N/A	Not a hazardous substance or mixture according to Regulation (EC) No. 1272/2008. This substance is not classified as dangerous according to Directive 67/548/EEC.	Inhalation, skin/eye contact, ingestion	Y
B	100 mM $MgCl_2$	>10 ml	N/A	Not a hazardous substance or mixture according to Regulation (EC) No. 1272/2008. Not a hazardous substance or mixture according to EC-directives 67/548/EEC	Inhalation, skin/eye contact, ingestion	Y
C	Dithiothreitol (R*,R*)-1,4-Dimercaptobutane-2,3-diol	1 L	N/A	**Hazard Statements:** H315- Causes skin irritation H319- Causes serious eye irritation **Precautionary Statements:** P305+P351+338- IF IN EYES: Rinse cautiously with water for several minutes. Remove	Inhalation, skin/eye contact, ingestion	Y

				contact lenses, if present and easy to do. Continue rinsing.		
D	EDTA	250g	N/A	**Hazard Statements:** 319- Causes serious eye irritation **Precautionary Statements:** P305+P351+338- IF IN EYES: Rinse cautiously with water for several minutes. Remove contact lenses, if present and easy to do. Continue rinsing.	Inhalation, skin/eye contact, ingestion	Y
E	Bovine serum albumen	100 g	N/A	None listed	Inhalation, skin/eye contact, ingestion	Y
F	NaCl	500 g	N/A	None - not hazardous	Inhalation, skin/eye contact, ingestion	Y
G	Glycerol	2.5 L	N/A	None - not hazardous	Inhalation, skin/eye contact, ingestion	Y

15. Control Measures

Engineering controls:
1) Only use concentrated Dithiothreitol in a fume cupboard
2) Ensure good general ventilation

Personal; protective equipment
1) Prevent contact with eyes, skin and clothing – lab coat, nitrile gloves (or suitable alternative) and safety spectacles must be worn at all times.

Other controls:
1) Wash thoroughly after handling. Wash thoroughly after handling.
2) minimise dust generation

Storage:
Store in a tightly closed container.
Store in a cool, dry, Well-ventilated area away from incompatible substances - oxidisers
No special precautions indicated.

Dithiothreitol – Store below 4°C

Now mark in the letters from the list of 'Hazardous Ingredients' above to indicate potential danger:

16. Indication of Danger				17. Route of Exposure		18. Chemical State		19. Flammability		20. Volatility		21. Dust rating	
Very Toxic		Irritant	C, D	Inhalation	All	Solid	A B	Flammable		Low	A B D E F G	Low	C G
Toxic		Sensitiser		Skin Contact	All	liquid	All	Highly flammable		Medium	c	Medium	A B D F E
Corrosive		Carcinogen		Eye Contact	All	Gas/vapour		Extremely flammable		High		High	
Harmful	c	Mutagenic		Swallowing	All			Oxidising					
Biological Agent		Toxic to reproduction		Injection				Explosive					

22. First Aid Procedures (as advised from Material Safety Data Sheet)

If inhaled	If skin contact	If eye contact	If swallowed	If injected
Remove from exposure and move to fresh air immediately. If not breathing, give artificial respiration. If breathing is difficult, give oxygen. Get medical aid if cough or other symptoms appear.	Flush skin with plenty of water for at least 15 minutes while removing contaminated clothing and shoes. Get medical aid if irritation develops or persists. Wash clothing before reuse.	Flush eyes with plenty of water for at least 15 minutes, occasionally lifting the upper and lower eyelids. If irritation develops, get medical aid.	Never give anything by mouth to an unconscious person. Do NOT induce vomiting. If conscious and alert, rinse mouth and drink 2-4 cupfuls of milk or water. Get medical aid if irritation or symptoms occur.	Get medical aid immediately

23. Spillage Procedures: →	Absorb spill with inert material (e.g. vermiculite, sand or earth, then place in suitable container. Avoid runoff into storm sewers and ditches which lead to waterways. Clean up spills immediately, observing precautions in the Protective Equipment section. Remove all sources of ignition. Provide ventilation.
24. Disposal Arrangements	

Collection	Swill down sink	Evaporation	In normal waste	Other
Dithiothreitol	Other substances			

25. Are the risks adequately controlled? (Write 'Yes' or 'No'):	Yes

If you decide that the controls in Section 15. are sufficient, skip to section 27. If you decide that the risks are NOT adequately controlled (or you're not sure), then you will need to give special instructions to control the risk.

26. Special Instructions to control the risk:
27. Ensure those affected are informed of the Risks & Controls - Confirm how this will be done e.g. by issuing written instructions:
1. Practical leader will be given a copy of this assessment. 2. A briefing will be given to the students before the practical.

9. GusB and XylE pair alignment using the Lalign software on NCBI.

```
n-w opt: -25  Z-score: 83.6  bits: 24.8 E(1): 0.00039
global/global (N-W) score: -25; 19.3% identity (48.4% similar) in 517 aa
overlap (1-491:1-457)

               10        20        30        40        50        60
XylE   MNTQYNSSYIFSITLVATLGGLLFGYDTAVISGTVESLNTVFVAPQNLSESAANSLLGFC
       :: : .   : . .:  . ... :.. .         : . ..   ... .::...: .
GusB   MNQQLSWRTIVGYSLGDVANNFAFAMGALF-------LLSYYTDVAGVGAAAAGTMLLLV
               10        20        30               40        50

               70        80         90       100       110
XylE   VASALIGCIIGGALGGYCSNRFGR-RDSLKIAAVLFFISGVGSAWPELGFTSINPDNTVP
        .   .. ...: .    ..:.:. :  : .... ..: .:   :    ..  . ...:
GusB   RVFDAFADVFAGRVVDSVNTRWGKFRPFLLFGTAPLMIFSVLVFWVPTDWS--HGSKVVY
            60        70        80        90       100         110

     120       130       140         150       160       170
XylE   VYLAGYVPEFVIYRIIGGIGVGLASMLS--PMYIAELAPAHIRGKLVSFNQFAIIFGQLL
       .::. :.   . : ...    .::. ..  :.  :.:. :  ::          : ..:
GusB   AYLT-YMGLGLCYSLVNIPYGSLATAMTQQPQSRARLGAA--RG----------IAASLT
              120       130       140       150

       180       190       200       210       220       230
XylE   VYCVNYFIARSGDASWLNTDGWRYMFASECIPALLFLMLLYTVPESPRWLMSRGKQEQAE
         :. ..:. :   :  .     : : .  . :.  ..: .   .: :           :
GusB   FVCLAFLIGPSIKNSSPEEMVSVYHFWT-IVLAIAGMVLYFICFKSTR-----------E
      160       170       180       190       200

       240       250       260       270       280       290
XylE   GILRKIMGNTLATQAVQEIKHSLDHGRKTGGRLLMFGVGVIVIGVMLSIFQQFVGINVVL
       ...: .   .:   ..: .:..          :.:. .:.. .     ... :.     :
GusB   NVVRIVAQPSLNI-SLQTLKRNRP--------LFMLCIGALCV-----LISTFAVSASSL
        210        220               230       240            250

       300       310       320       330       340       350
XylE   YYAPEVFKTLGASTDIALLQTIIVGVINLTFTVLAIMTVDKFGRKPLQIIGALGMAIGMF
       .:.  :..  :  : ..:.:.. ::..  .  : ..  : ..:.:   .::::  . :..
GusB   FYVRYVLNDTGLFTVLVLVQNL-VGTVASAPLVPGM--VARIGKKNTFLIGALLGTCGYL
            260       270       280       290       300

       360       370       380       390       400       410
XylE   SLGTAFYTQAPGIVALLSMLFYVAAFAMSWGPVCWVLLSEIFPNAIRGKALAIAVAAQWL
        .   .   . :  .. :.:     . .:.   : :.: ..       :. :. .:  . :
GusB   LFFWVSVWSLPVALVALAIASIGQGVTMT---VMWALEADTVE---YGEYLT-GVRIEGL
     310       320       330          340          350        360

       420       430       440              450
XylE   ANYFVSWTFPMMDKNSWLVAHFHNGFSYWIYG-------CMGV-------------LAAL
       .  . :.:    .  .  .  :  :.: .: .        ::.              :: .
GusB   TYSLFSFTRKCGQAIGGSIPAFILGLSGYIANQVQTPEVIMGIRTSIALVPCGFMLLAFV
            370       380       390       400       410       420

       460       470       480          490
XylE   FMWKFVPETKGKTLEELEALWEPETKKTQQTA---TL
       ..: : : :  : ..:. .  . . :  ::     :
GusB   IIW-FYPLTD-KKFKEIVVEIDNRKKVQQQLISDITN
             430        440       450
```

10. **GusB and SV2A pair alignment using the Global Lalign software on NCBI (GusB is the top sequence stating Query and SV2A is the bottom sequence stating Sbjct).**

AAH45111.1 Synaptic vesicle glycoprotein 2A [Homo sapiens]

Sequence ID: **Query_22605** Length: **742** Number of Matches: **1**

Range 1: 1 to 742 Graphics ▼ Next Match ▲

NW Score	Identities	Positives	Gaps
-264	116/759(15%)	200/759(26%)	319/759(42%)

```
Query  1    MNQQLSWRAIVGYSLGDVANNFAFAMGALFLLS----------------------------  33
            M +    RA       D+A           +
Sbjct  1    MEEGFRDRAAFIRGAKDIAKEVKKHAAKKVVKGLDRVQDEYSRRSYSRFEEEDDDDDFPA  60

Query  34   ----YYTDVAGVGAAAAGTMLLLVRVFDAFADVFAG---------------RVVDSV---  71
                YY           G          D   +++ G                R+ D
Sbjct  61   PSDGYYRGEGTQDEEEGGASSDATEGHDEDDEIYEGEYQGIPRAESGGKGERMADGAPLA  120

Query  72   -------------------------------------NTRWGKFRPFLLF--GTAPLMIF  92
                                                   G+F+  L F  G A LM
Sbjct  121  GVRGGLSDGEGPPGGRGEAQRRKEREELAQQYEAILRECGHGRFQWTLYFVLGLA-LMAD  179

Query  93   SVLVFWV----PTD-----WSHSSKVVYAYLTYMGLGLCYSLVNIPYGGSLATAMTQQPQS  143
             V VF V    P+       S S+K +   + Y+G+ +   L    +G LA
Sbjct  180  GVEVFVVGFVLPSAEKDMCLSDSNKGMLGLIVYLGMMVGAFL----WGGLAD--------  227

Query  144  RARLGAARGIAASL-----------------TFVCLAFLIGPSIKNSSP-----------  175
               RLG  + +  SL                 TF+    L G  I  S P
Sbjct  228  --RLGRRQCLLISLSVNSVFAFFSSFVQGYGTFLFCRLLSGVGIGGSIPIVFSYFSEFLA  285

Query  176  -----EEMVSVYHFWPIVLAIAGMVLYFIC--------------FKSTRENVVRIVAQPS  216
                 E +  +  FW I    A  + + I                F S R  V+ + A PS
Sbjct  286  QEKRGEHLSWLCMFWMIGGVYAAAMAWAIIPHYGWSFQMGSAYQFHSWRVFVL-VCAFPS  344

Query  217  L-KISLQTLKRNRPLFMLCIG----AVCVLISTFAVSASSLFYVRYVLNDTGLFTV----  267
            +  I   T +   P F L  G    A  VL      +  +  +   V + T + T+
Sbjct  345  VFAIGALTTQPESPRFFLENGKHDEAWMVLKQVHDTNMRAKGHPERVFSVTHIKTIHQED  404

Query  268  -LVLVQNLVGTV-----ASAPLVPGMV-----ARIGKKNTFLIGALLGTCGYLLF--FWV  314
             L+ +Q+  GT         A  + G V     +  G +   +   ++G    + F  + +
Sbjct  405  ELIEIQSDTGTWYQRWGVRALSLGGQVWGNFLSCFGPEYRRITLMMMGVWFTMSFSYYGL  464

Query  315  SVWSLPVA--LVALAIASI-----GQGVT-MTVMWALEADTVEYGEYLT----GVRIEGL  362
            +VW    +   L A+  AS       G+ V  +T  + LE       G+Y       G+R++ +
Sbjct  465  TVWFPDMIRHLQAVDYASRTKVFPGERVEHVTFNFTLENQIHRGGQYFNDKFIGLRLKSV  524

Query  363  TY--SLF------------SFTRKCG-----------------------------------  374
            ++  SLF            +F  R C
Sbjct  525  SFEDSLFEECYFEDVTSSNTFFRNCTFINTVFYNTDLFEYKFVNSRLINSTFLHNKEGCP  584

Query  375  -QAIGGSIPAFILGLSGYIANQAQTPEVIMGI-------------RTSIALVPCGFM--  417
                G    A+++    ++   A  P  I+                  + ++ V C F+
Sbjct  585  LDVTGTGEGAYMVYFVSFLGTLAVLPGNIVSALLMDKIGRLRMLAGSSVMSCVSCFFLSF  644

Query  418  ------LLAFVIIW-----------------FYPLTDKK---------------------  433
                 ++A + ++                   YP +DK+
Sbjct  645  GNSESAMIALLCLFGGVSIASWNALDVLTVELYP-SDKRTTAFGFLNALCKLAAVLGISI  703

Query  434  --------------FKEIVVEIDNRKKVQ-QQLINDITS  457
                        F    + + +   ++  +    +
Sbjct  704  FTSFVGITKAAPILFASAALALGSSLALKLPETRGQVLQ  742
```

11. **GusB and MelB pair alignment using the Global Lalign software on NCBI (GusB is the top sequence stating Query and MelB is the bottom sequence stating Sbjct).**

QOH83994.1 melibiose:sodium transporter MelB [Escherichia coli]

Sequence ID: **Query_49957** Length: **473** Number of Matches: **1**

Range 1: 1 to 473 Graphics ▼ Next Match

NW Score	Identities	Positives	Gaps
393	128/482(27%)	226/482(46%)	35/482(7%)

```
Query  1    MNQQLSWRAIVGYSLGDVANNFAFAMGALFLLSYYTDVAGVGAAAAGTMLLLVRVFDAFA  60
            M+  +S    + Y  G    +FA  +  ++L+ YYTDV G+     GT+ L+ R++DA
Sbjct  1    MS--ISMTTKLSYGFGAFGKDFAIGIVYMYLMYYYTDVVGLSVGLVGTLFLVARIWDAIN  58

Query  61   DVFAGRVVDSVNTRWGKFRPFLLFGTAPLMIFSVLVFWVPTDWSHSSKVVYAYLTYMGLG  120
            D   G +V++  +RWGKF+P++L GT    +   L+F     +  ++++V+  +TY+  G
Sbjct  59   DPIMGWIVNATRSRWGKFKPWILIGTLANSVILFLLFSAHL-FEGTTQIVFVCVTYILWG  117

Query  121  LCYSLVNIPYGSLATAMTQQPQSRARLGAARGIAASLTFVCLAFLIGPSIKNSSPEEMVS  180
            + Y++++IP+ SL   +T   + R +L       ASL     A +  P +      +
Sbjct  118  MTYTIMDIPFWSLVPTITLDKREREQLVPYPRFFASLAGFVTAGVTLPFVNYVGGGDRGF  177

Query  181  VYHFWPIVLAIAGMVLYFICFKSTRENVVRIVAQPSLKISLQTLK-------RNRPLFML  233
             +  + +VL    +V   I  ++  E V     QPS + S  TLK       +N  L  L
Sbjct  178  GFQMFTLVLIAFFIVSTIITLRNVHE-VFSSDNQPSAEGSHLTLKAIVALIYKNDQLSCL  236

Query  234  CIGAVCVLISTFAVSASSLFYVRYVLNDTGLFTVLVL---VQNLVGTVASAPLVPGMVAR  290
               A+   +++  ++  +++Y  YV+ D  LF   +      NLV  V    LV  +  R
Sbjct  237  LGMALAYNVASNIITGFAIYYFSYVIGDADLFPYYLSYAGAANLVTLVFFPRLVKSLSRR  296

Query  291  IGKKNTFLIGALLGTCGYLLFFWVSVWSLPVALVALAIASIGQGVTMTVMWALE----AD  346
            I      ++  L  +CG LL   +  +   V +V   IA I   V   + W L+    AD
Sbjct  297  ILWAGASILPVL--SCGVLLLMALMSYHNVVLIV---IAGILLNVGTALFWVLQVIMVAD  351

Query  347  TVEYGEYLTGVRIEGLTYSLFSFTRKCGQAIGGSIPAFILGLSGYIANQAQTPEVIMGIR  406
            TV+YGEY   VR E + YS+ +   K G A      A +LG+ GY+ N  Q+ + + ++G++
Sbjct  352  TVDYGEYKLHVRCESIAYSVQTMVVKGGSAFAAFFIAVVLGMIGYVPNVEQSTQALLGMQ  411

Query  407  TSIALVPCGFMLLAFVIIW-FYPLTDKKFKEIVVEI-DNRKKVQQQ---------LINDI  455
              +  +P  F ++  ++ + FY L     + I + + D  +KV  +          ++D+
Sbjct  412  FIMIALPTLFFMVTLILYFRFYRLNGDTLRRIQIHLLDKYRKVPPEPVHADIPVGAVSDV  471

Query  456  -T  456

Sbjct  472  KA  473
```

12. **A 3D representation of the predicted structure of the GusB protein:** This includes the alpha helices and beta strand which interact to the tertiary stricture as seen below. Below is a key showing which amino acids can be found in each helices the colour on the key relates to the colour of the helices seen. The site of mutations made in this project has been highlighted on the sequence using red boxes.