Faculty of Science & Technology

Investigating the functionality of the transmembranes in GusB by fusing it with LacY using the Polymerase Chain Reaction.

A dissertation submitted as part of the requirement for Masters by Research

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ABSTRACT

“Investigating the functionality of the transmembranes in GusB by fusing it with LacY using the Polymerase Chain Reaction. “

Glucuronides are produced during the detoxification pathway and removed via the uric pathway. The *Escherichia coli* living within the gut acquire these glucuronides for their survival by utilising the glucuronide transporter (GusB). GusB is encoded by the *gusB* gene which is located within the *gus* operon along with two other structural genes. However, the substrate binding sites of GusB are not currently known and therefore this research project was focused on fusing GusB with a different well-studied secondary transporter known as lactose ‘permease’ (LacY). LacY is a protein which facilitates the movement of lactose molecules across a membrane against the concentration gradient. This protein is been thoroughly studied and its substrate binding sites are known as well as which transmembranes they are located in. Hence, LacY’s structure was used for comparison with GusB as they share structural similarity i.e. 12 transmembranes. For this project, the first 6 membranes of LacY was fused with the last 6 transmembranes of GusB by undergoing fusion polymerase chain reaction (PCR). The PCR method required two steps in which fragments were fused together through overlap extension. Initially, the fusion was successful until a primer design error became evident during restriction digest. This resulted in the primers being redesigned and the PCR was repeated. However, obtaining the fusion since the correction proved difficult and required numerous troubleshoots in which various factors such as $MgCl_2$ concentration, DNA concentration, temperature and extension time was altered. Due to this difficulty, the project did not progress further than PCR but has provided useful information for future troubleshooting and potential determination of the substrate binding sites.

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List of Abbreviations

PCR polymerase chain reaction

ABC ATP-binding cassette

SGLT sodium glucose linked cotransporter

CPA cation- proton activator

LacY lactose ‘permease’

MFS major facilitator superfamily

TM transmembrane

CAP catabolite activator protein

cAMP Cyclic adenosine monophosphate

IPTG Isopropyl-thio-*β*-D-glucuronide

TDG beta-D-galactopyranosyl-1-thio-beta-D-galactopyranoside

LacA thiogalactoside transacetylase

XylE xylose H+ symporter

GusA *β*-D-glucuronidase

GusB glucuronide transporter

X-Gluc 5-bromo-4-chloro-3-indoyl glucuronide

PNPG p-nitrophenyl-*β*-D-glucuronide

PNP p-nitrophenol

UDP-GLcA Uridine 5′-diphosphate-glucuronic acid

UGT UDP-glucuronosyltransferases

G1P glucose-1-phosphate

UTP uridine triphosphate

UGDH UDP-α-D-glucose 6-dehydrogenas

Chapter 1. INTRODUCTION

A wide range of mammal gastrointestinal tracts, including humans, contains a variety of enterobacteria such as *Escherichia coli (E. coli)* (Beaud *et al.* 2005; Martinez-Medina *et al.* 2009). In the gastrointestinal tract, carbon sources such as glucose for metabolism are not readily available; hence, resulting in harsh conditions in which the *E. coli* have to survive (Wallace *et al.* 2010). As a result, *E. coli* have adapted to become scavengers to increase their chances of survival and capabilities to thrive in such difficult conditions (Liang *et al.* 2005). They do this by utilising biological transporters to acquire and metabolise the energy source.

1.1. Biological Transporters

Biological transporters are fundamental components within ion homeostasis as they are responsible for controlling the movement of ions and molecules across cell membranes. Cell membranes are composed of a semi permeable phospholipid bilayer which surrounds the cytoplasm (Cullis and De Kruijff 1979; Kučerka *et al.* 2011). This semi permeable membrane controls the movement of particular ions and molecules in and out of the cell. This movement is achieved by the transporters as they act as 'gate keepers' in areas of the membrane and control the movement of certain substances (Dubyak 2004). The transporters regulate homeostasis in two main ways:

1. Through passive diffusion across the membrane via a concentration gradient

2. Via active transport.

Facilitative transporters (also known as uniporters), are responsible for using passive diffusion as a mechanism for transport. In contrast to passive diffusion, active transport requires energy to control cellular movements as it goes against the concentration gradient. There are two forms of active transport; primary and secondary transport which is utilised by a group of transporters; primary and secondary transporters respectively.

1.1.1. Primary transporters

Primary transporters requires energy to transport molecules against the concentration gradient. ATPases and ATP- binding cassette (ABC) transporters are examples of primary transporters that utilise ATP hydrolysis as their energy source (Hediger *et al.* 2004). ATPases are a family of membrane-bound proteins found in eukaryotes and prokaryotes and are responsible for harnessing the energy generated from the hydrolysis of ATP to control movements of substrates across the membrane (Pedersen and Amzel 1993; Finbow and Harrison 1997; Perzov *et al.* 2001). The ATPase group is comprised of multiple classes of enzymes which vary due to their function. These classes are P-Type, F-Type, V- type and A-type (Hilario and Gogarten 1993; Pedersen 2007). The other well studied primary transporter groups are the ABC transporters.

ABC transporters control the movement of ions across the cytoplasmic membrane against the concentration gradient by hydrolysing ATP to release energy for active transport (Higgins 1992; Linton *et al.* 1998; Law *et al.* 2008; Vasiliou *et al.* 2009). The ABC transporters can transport a variety of substrates and are separated into two categories; exporters and importers. The importers are categorised into a further three types: Type I, Type II and energy coupling factor (ECF) transporters. ABC importers are only present in prokaryotes, whereas ABC exporters are present in both prokaryotes and eukaryotes (Locher 2009; Ter Beek *et al.* 2014; Locher 2016). Despite primary transporters being well studied, their mechanisms and structure is different to the secondary transporter family, which is the focus for this project.

1.1.2. Secondary transporters

Contrarily, cotransporters (also known as secondary transporters), do not require ATP directly to control movements of molecules. Instead, they utilise the electrochemical gradient generated by active transport as the energy source. In addition to this, they couple with ions, for example H^+ ions, to move molecules against their gradients. Additionally, there are two subgroups to divide secondary transporters: antiporters and symporters. Antiporters

transport the molecules in opposite directions and symporters transport molecules or ions alongside an ion in the same direction.

An example of a symporter is the sodium glucose linked cotransporter (SGLT). These transporters are mainly located in the kidneys and intestines (Crane 1965; Wright *et al.* 2011; Harada and Inagaki 2012), and play a fundamental role in the uptake of glucose from renal excretion and glucose homeostasis. In humans, the normal blood glucose concentration is 5.5 mmol/ litre. When these levels start to decline, the SGLT's continuously uptake glucose until levels reach the norm. Likewise, if the levels were higher than the normal, the SGLT's would be inhibited to up taking glucose, thus maintaining the homeostasis (Poudel 2013; Poulsen *et al.* 2015). This cotransporter was first discovered by Crane (1965) when investigating glucose absorption. It was established that the sodium molecules require an electrochemical gradient to move down for the SGLT to uptake the glucose from the kidneys and intestines, and that in fact this electrochemical gradient is maintained by the sodium potassium pump (Lever 1992; Baud *et al.* 2016).

On the other hand, cation-proton antiporter (CPA) family members such as Na^+/H^+ antiporters are found prevalently. They play an important role in the regulation pH and ion homeostasis (Padan 2014; Paulino *et al.* 2014). Initially, their activity was discovered in bacterial cells (West and Mitchell 1974) and has since been identified in other organisms and more widely understood.

One of the most well studied secondary transporters is the lactose 'permease', also known as LacY. This transporter is a symporter responsible for transporting a lactose molecule and proton across the membrane. Numerous studies have shown that this protein structure is composed of 12 transmembranes helices (Abramson *et al.* 2003; Law *et al.* 2008). LacY shares this structural similarity with other transporters and they have been categorised together to form a family known as the major facilitator superfamily (MFS).

1.2. Major Facilitator Superfamily (MFS)

The major facilitator superfamily (MFS) is one of the largest families composed of various secondary membrane transporters which can be found

ubiquitously (Pao *et al.* 1998; Yan 2015). Each transporter is involved in a critical physiological process where it carries nutrients such as lipids, amino acids, substrates or ions across membranes by secondary transporter mechanisms (Saier *et al.* 1999; Yan 2013). Over time, the number of families belonging to the MFS has increased. There are over 70 proteins considered to be part of the MFS; each with a characterised role (Reddy *et al.* 2012; Quistgaard *et al.* 2016). In addition to transportation, various MFS members have vital roles in the signal pathway, metabolism, detoxification and excretion (Pao *et al.* 1998; Dassler *et al.* 2000; Peng *et al.* 2011; Augustin 2010; Chen *et al.* 2016). Furthermore, bioinformatics and biological investigations revealed that MFS proteins tend to have similar structures in terms of being composed of 12 transmembrane helices (TM) that are separated into two bundles comprised of 6 TM's each. TM 1-6 forms the N-domain and TM 7-12 forms the C- domain of the protein (Law *et al.*2008; Reddy *et al.* 2012; Bazzone *et al.* 2016). This knowledge has since been used to distinguish the structure of unknown proteins and classifying them.

Many of the MFS proteins genes, especially in prokaryotes can be found in clusters known as operons. They tend to have a promoter and repressor protein which controls the transcription and is influenced by the availability of the carbon source (Crasnier 1996; Beisel and Storz 2011). However, a phenomenon known as catabolite repression has been shown to positively regulate transcription (Wanner *et al.* 1978; Wong *et al.* 1997). A catabolite activator protein (CAP) binding site tends to be located before the promoter site. CAP is a protein which assists the RNA polymerase to enhance transcription and is regulated by a molecule known as cyclic adenosine monophosphate (cAMP). This molecule's concentration is increased when the carbon source levels are low, thus activating CAP and enabling transcription. When carbon source levels are high, cAMP levels are reduced, hence, CAP is inactivated (Crasnier 1996). This repression is utilised as a form of control by *E. coli* in which it encourages usage of alternative carbon sources and has assisted with their survival in environments where glucose is not readily available (Brückner and Titgemeyer 2002; Kremling *et al.* 2015). This phenomenon been extensively studied in the *lac* operon located in *E. coli.*

1.3. The Lac operon of *E. coli*

The *lac* operon has been extensively studied and is one of the most understood operon systems and it demonstrates the typical operon structure. The *lac* operon is polycistronic as it is controlled by one promoter. In addition to this, there is an operator with 3 operator sites, the *lacI* gene which encodes for a repressor protein and 3 structural genes; *lacZ*; *lacY* and *lacA* (Jacob and Monod 1961; Oehler *et al.* 1990). For this operon to transcribe, the omnipresence of galactosides such as lactose are required within the environment. This is due to the crucial function the lactose fulfils in binding to the repressor protein. This prevents the repressor protein from binding to the operator which would otherwise inhibit gene expression (Garner and Revzin 1981; Oehler *et al.* 1990; Lewis 2013). A potential consequence of lactose scarcity in the environment would be the repression of the operator by the repressor protein *lacI.* This repression results in an operon that would not be expressed, hence, transcription would be inhibited (Gilbert and Müller-Hill 1967). This results in a negatively regulated lac operon as it is not consistently expressed.

1.4. The lactose operon repressor in *E. coli*

The *lacI* repressor was first isolated when experiments involving radioactive isopropyl-thio-galactoside (IPTG) were conducted to test the role of this protein. Gilbert and Müller-Hill's (1966; 1967) findings demonstrated that in lactose depleted environments, transcription was inhibited due to the binding occurring between the protein and the operator. However, when IPTG (an allolactose imitator) was added to the reaction, the DNA was released, and transcription occurred due to the affinity for the operator being reduced and the repressor being induced (Hansen *et al.* 1998; Law *et al.* 2002; Fernández-Castané *et al.* 2012). This was detected and isolated using equilibrium dialysis. This transcription factor has since been extensively researched and has been used as a model for protein-DNA interactions (Stetz *et al.* 2016) and in the analysis of mutagenic specificity (Coulondre *et al.* 1978; Schaaper *et al.*1986; You *et al.* 1999). Following the *lacI* gene is the gene for the first structural gene in this operon, *lacZ.*

1.5. *β*-galactosidase *in E. coli*

The *lacZ* gene encodes for *β*-galactosidase which is responsible for cleaving lactose and allolactose to monosaccharides (Broome *et al.* 2010). Furthermore, this enzyme can catalyse the transgalactosylation of lactose to allolactose which is the product that can repress the *lacZ* gene and in turn regulate the production of *β*-galactosidase (Huber *et al.* 1976; Juers *et al.* 2012). In addition to this, *β*-galactosidase has been commonly used as a reporter gene or as a marker in plasmid recombination. This is due to a scientific phenomenon known as α- complementation (Ullmann *et al.* 1967; Langley *et al.* 1975). *β*-galactosidase is a tetramer composed of four identical monomers which are comprised of two segments; lacZ-alpha and lacZ-omega. After their investigations, it was established that neither lacZ-alpha nor lacZ-omega would function unless both parts were present. This was first demonstrated when an inactive mutant *β*-galactosidase with a deleted sequence was able to function (Ullman 1992). This was due to the α-monomer fragments of the protein having the exact sequence but complete, thus overriding the deleted fragment and restoring the function of the mutant. The following structural gene in the operon is the *lacY* gene which has been extensively studied.

1.6. Lactose 'permease' transporter in *E. coli*

One of the members of the MFS which has been extensively studied is *E. coli* lactose 'permease' (LacY). LacY is encoded by the *lacY* gene and downstream of the *lacZ* gene. It is responsible for the uptake of galactosides such as lactose via active transport and transporting it across the membrane. LacY engages in active transport as a symporter; moving a lactose molecule and proton in the same direction across the membrane into the cell against the concentration gradient by utilising the energy created during downhill H^+ translocation (Foster *et al.* 1983; Venkatesan and Kaback 1998; Kaback 2015). LacY is 417 amino acids long and its structure has been showed to comprise of 12 transmembrane helices with an amino and carboxyl terminal which are both located in the cytoplasmic side of the membrane (see figure 1-1).

In 2003, Abramson *et al.* investigated the crystal structure of this protein by focusing on a mutant of LacY known as C154G. X-ray crystallography at an approximate resolution of 3.6 Å were used to obtain the crystallisation of the mutant's conformation. The X-rays crystallography revealed that in fact C154G has 12 transmembrane helices which are separated into two 6 helix bundles sharing pseudo symmetry. The first 6 transmembrane bundle forms the N domain and the last 6 forms the C domain. The 2 domains are linked by a salt bridge. In between these two bundles is a hydrophilic cavity facing the cytoplasmic side whilst the remained periplasmic side is tightly closed. Bound to this cavity is a lactose homolog known as beta-D-galactopyranosyl-1-thio-beta-D-galactopyranoside (TDG). This led to the understanding of the inward-facing conformation of this mutant as it was restricted to this specific conformation. It also became apparent that for conformational change to occur, a substrate such as galactoside has to bind to the binding site in the cavity to encourage the opening on the periplasmic side. Similar findings were found when the structure of a wild-type lactose permease from *E. coli* underwent an X-ray crystallography with a resolution of 3.6 ångströms (Å) (Guan *et al.* 2007). This mechanism has since become known as the alternating-access model (Smirnova *et al* 2011; Kumar *et al.* 2013). Despite experiments such as site directed alkylation, cysteine residue replacements, and the alternating-access model suggesting an outward-facing conformation, there has been little success with obtaining the crystallisation of this conformation using X-ray. Nonetheless, these revelations have provided other important information regarding the residues required for proton translocation and potential sugar binding sites.

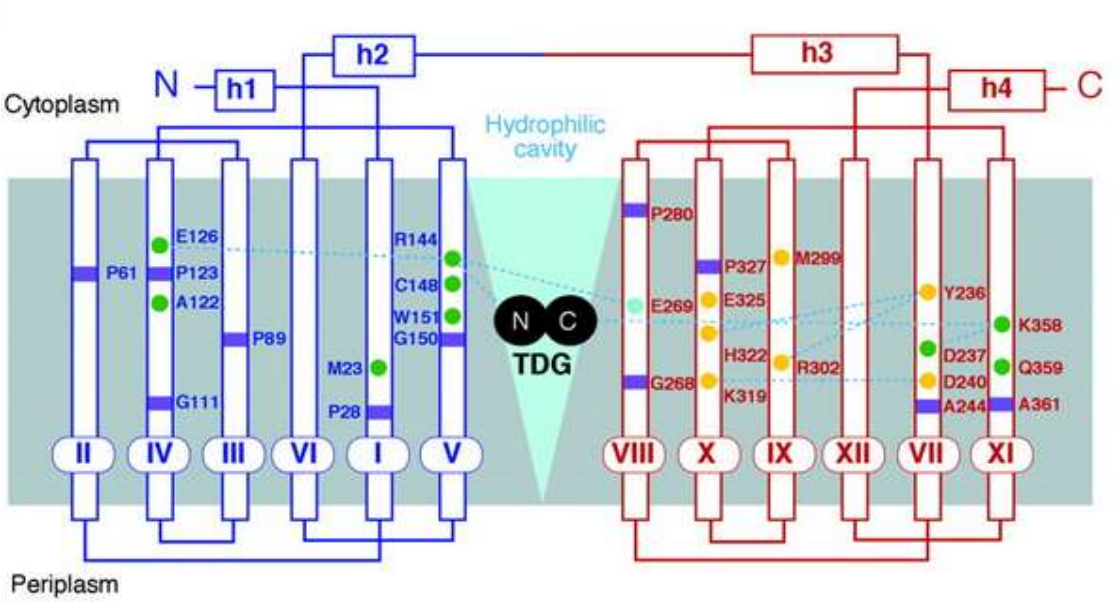

Figure 1-1. Model of Lactose 'permease'. Model illustrating the N- and C- domain which are coloured blue and red respectively. Green and orange coloured residues are the substrate binding and proton translocation sites respectively. The TDG is symbolised by the two black circles (Abramson *et al.* 2003).

1.6.1. Lactose 'permease' proton translocation sites

During the alternating-access mechanism, *β*-galactoside is coupled with a H^+ ion and is symported during the conformation change from inward facing to outward facing. The proton translocation binding sites have been widely studied. One of the experiments used to locate the possible residues was cysteine-scanning mutagenesis (Frillingos *et al.* 1998). During this procedure, all of the amino acids were changed to cysteine, each mutant was then expressed and functionality was tested. Only 18 mutants were unable to catalyse lactose, however based on their properties, only 6 mutants were found to be completely 'irreplaceable'. They are predominantly found in the C-domain, specifically E126, R144, E269, R302, H322 and E325 (see figure 1-2). As this mechanism was being understood, previously suggested proton translocation residues were confirmed to be correct (Abramson *et al.* 2003; Guan and Kaback 2006). Further studies revealed that the deprotonation of E325 may be responsible with the inward to outward facing conformational change. Andersson *et al.* (2012) used molecular dynamics to compare the effect of protonated and deprotonated E325 residues on the confirmation of

LacY in a sugar substrate absent environment. Results demonstrated changes to the salt bridges formed between various residues as well as possible structural change. In addition to this, it became apparent that E269 may be involved with proton translocation and substrate binding.

	Mutant	Active mutants[a] (rate > 20% wt)
A		
Helix I	P31C	P31A, P31G
Helix II	G64C	p(wt)G64A
	D68C	D68S or T/s.s.[d]
Helix IV	G115C	G115A, G115P
	E126C	Not found[e]
Helix V	**R144C**	Not found[e]
	G147C	G147A
Helix VII	D237C	D237C/K358C[f]
	D240C	D240C/K319C[g]
Helix VIII	**E269C**	Not found[h]
Helix IX	**R302C**	Not found[i]
Helix X	K319C	D240C/K319C[g]
	H322C	Not found[j]
	E325C	Not found[k]
	F334C	F334L, F334W
Helix XI	S346C	p(wt)S346C
	T348C	T348S, p(wt)T348C[l]
	K358C	D237C/K358C[f]
B		
Loop I/II	H35C	H35R/H39R
Helix III	L76C	p(wt)L76C
Loop IV/V	K131C	p(wt)K131C
	F140C	p(wt)F140C
Helix V	W151C	W151F
Helix VI	A177C	p(wt)A177C
	L184C	p(wt)L184C
Helix XI	Y350C	Y350F
Helix XII	G380C	Not examined
	L400C	p(wt)L400C

Figure 1-2. Results from cysteine- scanning mutagenesis on Lactose Permease. These results display the lactose transport expression within each mutant, which transmembrane they are located in and their transportation rate. The circled results indicate the possible proton translocation sites due to inactive mutants (amended from Frillingos *et al.* 1998 p. 1284).

1.6.2. Lactose ‘permease’ substrate binding sites

Numerous studies suggest that the majority of substrate binding residues are located within the N-domain. Some of these residues include E126, R144, W151 and E269 (Guan *et al.* 2007). Initially, the protein is in outward- facing conformation and the proton is attracted to the negatively charged amino acid, E269.This triggers the substrate to bind to a residue in the N-domain before a salt bridge is formed between R144 and E269. This salt bridge formation is then believed to cause the conformation change to inward-facing

and releasing the substrate and H^+ (Abramson *et al.* 2003; Mirza *et al.* 2006; Smirnova *et al.* 2009). Therefore, E269 may be responsible for both the binding site and proton translocation.

By understanding the crystallisation of LacY as well as the proton translocation and substrate binding sites, scientists started investigating the binding loop and salt bridges between the N-domain and C-domain 6 TM bundles. Experiments focused on changing residues within the loop/ salt bridge participants to alter the overall charge revealed that in fact, either monomer (6 TM bundle) is still expressed and functions (Sahin-Toth *et al.* 1992; Guan *et al.* 2001; Abramson *et al.* 2003). This knowledge has modified the current understanding on MFS proteins as they theoretically share similar structures. However, this has proved difficult to test with other transporters as they are not as widely understood as LacY. Conversely, the *lacA* gene which is downstream of *lacY* is not fully understood.

1.7. Thiogalactoside transacetylase *in E. coli*

LacA is the final structural gene located within the *lac* operon, after the *lacY* gene. It encodes for an enzyme known as thiogalactoside transacetylase, also known as Galactoside acetyltransferase or LacA. It is approximately 202 amino acids in length and thought to be a trimer (Fowler *et al.* 1985; Lewendon *et al.* 1995). LacA is believed to be involved in the cellular detoxification process but scientists have yet to completely confirm this (Kenneth and Lin 1976; Roderick 2005 p. 568). LacA catalyses the transfer of an acetyl group from acetyl-CoA to the 6-hydroxyl position on a range of substrates such as glucosides and galactosides (Zabin *et al.* 1959; Wang *et al.* 2002). Despite the function of LacA not being completely understood, the *lac* operon is a widely understood system as similar theories have been applied to other transporters in the MFS.

1.8. Xylose- H+ transporter

LacY is not the only MFS protein to have its inward facing- form crystallised, another secondary transporter also located in *E. coli* has had its structure crystallised. The xylose- H+ symporter, also known as XylE has been widely studied. This transporter is coded by the *xylE* gene which is located in an operon, downstream of *xylA* and *xylB* which encodes for xylose isomerase and xylulokinase respectively (Song and Park 1997). Like other members of the MFS including LacY, this protein is comprised of 12 transmembrane helices with a N- and C- domain based on the cytoplasmic side as shown in figure 1-3. (Davis and Henderson 1987; Henderson and Baldwin 2012). Unlike LacY, this protein has been crystallised in inward- facing conformation as well as outward-facing conformation at 2.6 – 2.9 Å resolution (Yan 2013; Henderson and Baldwin 2013). Furthermore, the proton translocation sites are located within the first 6 transmembranes and the substrate binding sites are within the last 6 transmembranes, yet XylE still utilises the alternating-access mechanism (Sun *et al.* 2012; Henderson and Baldwin 2013). Since this discovery, it has resulted in further investigation into MFS members' transmembrane function as it demonstrates that despite sharing some structural similarities, the functions of each transmembrane can vary.

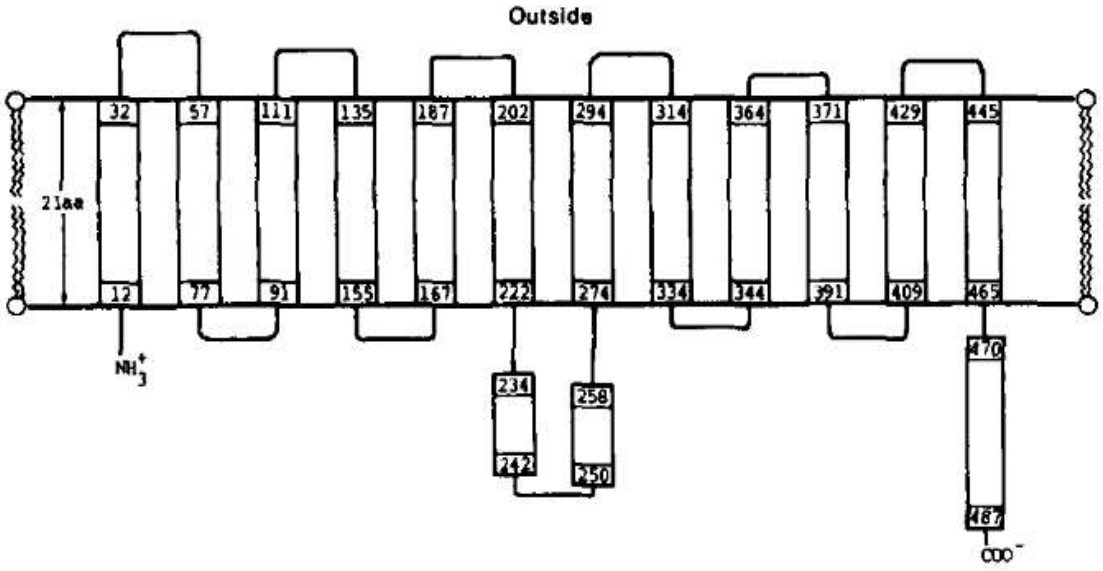

Figure 1-3. A model of the Xylose transporter XylE. A model illustrating the 12 transmembranes of XylE (Davis and Henderson 1987).

1.9. *Gus* Operon in *E. coli*

The *lac* and *xylose* operon are not the only operons found in *E. coli* which requires suitable environmental conditions to operate. The *gus* operon requires the presence of glucuronides in order to function. In similar fashion to the *lac* operon, the *gus* operon is controlled by a repressor, *gusR,* which is located upstream of 3 structural genes; *gusA, gusB* and *gusC* (Wilson *et al.* 1992). The first structural gene, *gusA* encodes for *β*-D-glucuronidase which is responsible for hydrolysing glucuronides into aglycones and glucuronic acid. Downstream of this gene is *gusB* which codes for the glucuronide transporter (GusB) which transports the glucuronides. The final gene in this operon, *gusC*, encodes for an outer membrane associated protein which increases the activity of GusB (Liang et al. 2005). *β*-D-glucuronidase is currently the most extensively researched structural gene from the *gus* operon and has a variety of uses.

1.10. *β*-D-glucuronidase *in E. coli*

β-D-glucuronidase, also known as GusA, is a hydrolase known to be found in prokaryotes and eukaryotes. In *E. coli,* this protein is responsible for the cleaving of glucuronides produced during glucuronidation into aglycones and glucuronic acid. Gus A is composed of 603 amino acids and its transcription is regulated by repressors; *uidR* (*gusR*) and uxuR. These repressors were confirmed when investigations by Novel and Novel (1976) revealed inhibition to the transcription of GusA when bound to the operon (Blanco *et al.* 1985). This also revealed the negative regulation of GusA as the repressors would only bind to the operon when there is a lack of glucuronide, similar to the mechanism of *lacI*.

E. coli, found in the gastrointestinal tract, utilise GusA in order to survive. As soon as an assortment of glucuronides is readily available in the environment, GusA hydrolyses the glucuronides into the aglycones and glucuronic acid components. When compounds are conjugated with glucuronic acid to form glucuronides during glucuronidation, their water solubility is increased and their ability to be absorbed into the bloodstream is reduced (Lee 1995; Prijovich *et al.* 2002; Kaushik *et al.* 2006). However, as GusA hydrolyses the

glucuronides, it separates the glucuronide into the two components. The glucuronic acid is metabolised by the bacteria whereas the aglycone moiety undergoes a phenomenon known as enterohepatic circulation (Wilson *et al.* 1992; Roberts *et al.* 2002; Gloux *et al.* 2011). This process involves the recycling of moieties entering the gastrointestinal tract. As the glucuronides are hydrolysed in the intestines, some of the aglycones moieties formed are absorbed by enterocytes located in the intestinal wall and released into the bloodstream. These moieties are then transported to the liver where they are reabsorbed by hepatocytes to undergo glucuronidation again. On the other hand, the remaining aglycones located in the intestines that were not absorbed continue to pass through the system to be excreted via urine or faeces.

GusA has been widely used as a reporter gene in plants (Jefferson *et al.* 1986). Similar to the theory behind blue/ white staining, GusA is used in transgenic plants which contain substrates known to be cleaved by this enzyme. One such substrate that has been used frequently is chromogenic 5-bromo-4-chloro-3-indoyl glucuronide (X-Gluc) (Jefferson *et al.* 1987; Platteeuw *et al.* 1994; Wilson *et al.* 1995; Yamaguchi *et al.* 2001; Chen *et al.* 2007; Liu *et al.* 2013). Scientists have been able to identify the expressed regions as they would appear blue in colour if this enzyme is present. The application of this knowledge has led to the detection of various gene expressions and is still a commonly used method.

1.11. Glucuronide transporter in *E. coli*

Unlike the LacY transporter which has been extensively studied, the glucuronide transporter (GusB), found in *E. coli,* is not as understood. GusB is a key transporter involved in the survival of *E. coli* in the human intestinal tract. In 2005, Liang et al. determined the biological function and characteristics of this transporter as well as parts of its structure. It is predicted to have 12 transmembranes α-helices as well as an N- and C-terminal in the cytoplasmic side, similar to other MFS members. This protein is thought to be 457 amino acids long and is known to act as a symporter by coupling up with H+ ions to transport glucuronides across the membrane

(Liang 1992; Liang et al 2005). It does this by creating an electrochemical gradient and uses the proton motive force to drive the molecule into the cell.

As a transporter, GusB recognises a wide range of glucuronides formed during detoxification, despite structural differences in the aglycone part of the molecule. However, the function of the transmembrane helices for this protein is unknown and therefore the substrate binding sites locations are unknown. Investigations are being carried out in attempt to locate these sites by using knowledge from other transporters such as LacY and XylE. Moreover, studies are being carried out in attempt to reveal the 3-dimensional structure of GusB.

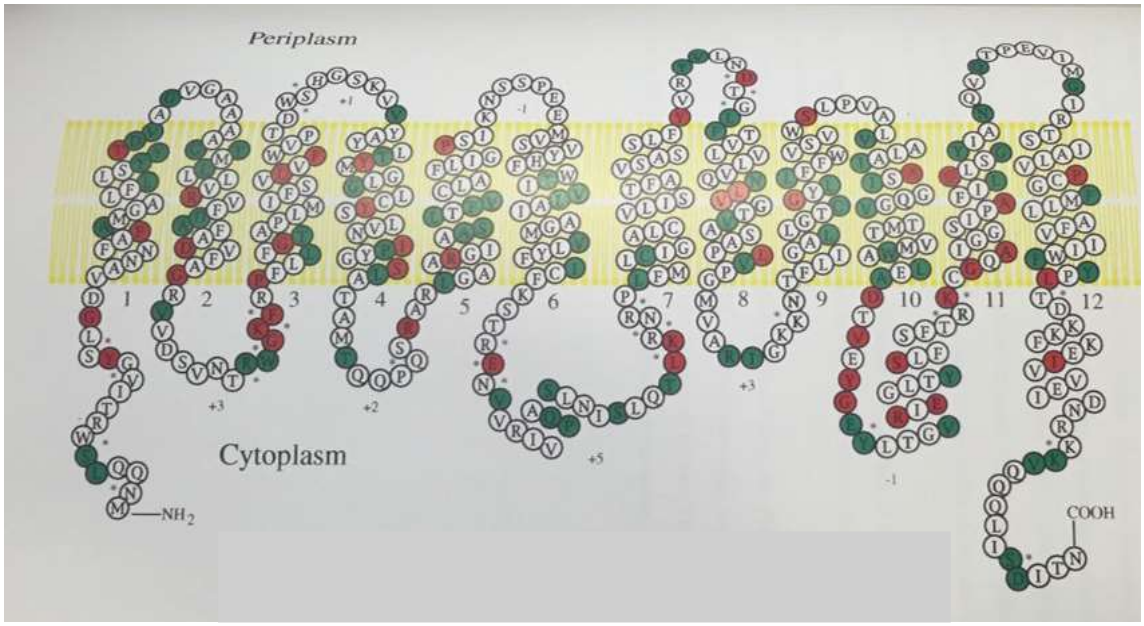

Figure 1-4. A model of the glucuronide transporter GusB. A model illustrating the 12 transmembranes of GusB and its amino acid residues (Liang 1992)

1.12. Synthesis of glucuronides

Glucuronides are carbohydrate compounds formed during a detoxification pathway known as glucuronidation. The glucuronide is composed of two parts; glycone and aglycone. Before glucuronides are formed,uridine 5′-diphosphate-glucuronic acid (UDP-GlcA) needs to be omnipresent as it forms the glycone component. UDP-GlcA is an active sugar substrate primarily found in hepatic tissue and is synthesized in a two-step reaction via the uronic

acid. During its synthesis, glucose-6-phosphate undergoes isomeration to form glucose-1-phosphate (G1P). This reaction is catalysed by phosphoglucomutase which transfers the phosphate group from the 6 to 1 position. G6P then reacts with uridine triphosphate (UTP) to form uridine diphosphate glucose (UDPGlc). This is catalysed by the enzyme uridine diphosphate glucose pyrophosphorylase (Turnquist *et al.* 1974). UDPGlc is then oxidised by UGDH (UDP-α-D-glucose 6-dehydrogenase) to yield UDP-GlcA (Bar-Peled *et al.* 2004). This compound is then used in biosynthesizing of glucuronides in the process known as glucuronidation.

1.13. Glucuronidation

Glucuronidation is phase II of the detoxification pathway that occurs in mammals, predominantly in the liver. During this pathway, enzymes known as UDP- glucuronyltransferase (UGT) catalyse the conjugation of UDP-GlcA with endogenous/xenobiotics such as paracetamol, or endogenous compounds such as bilirubin to form the glucuronide (Burchell and Coughtrie 1989; Margaillan *et al.* 2015). UDP- GlcA does this by acting as a glucuronosyl donor towards a wide range of aglycones with various nucleophilic functional groups (Dutton 1956). Before glucuronidation, the aglycones tend to have higher polarity and are more active as they can penetrate cell membranes. Glucuronidation detoxifies these aglycones by altering their biological structure to form a negatively charged glucuronide. Due to the negative charge, the glucuronide cannot penetrate cell membranes and relies on transporters to transport them across the membrane (Yang *et al.* 2017). In addition to this, the glucuronide tends to have a lower half-life than the parent compounds. Moreover, this results in the reduction of the toxicity of these compounds and increases their water solubility due to the increase in hydrophilic properties, thus allow easier excretion via bile or urine (Jessen *et al.* 2003; Yang *et al.* 2017).

1.14. Rationale

Determining the possible transmembranes responsible for symporting the glucuronides in GusB could have a major effect in the medicine industry. By using other transporters (such as LacY and XylE) which share similar

structures with GusB and applying their findings, this could assist in narrowing down its molecular recognition sites. Once the GusB specific recognition of glucuronides is better understood, this protein transporter could have various uses.

Firstly, this research could impact the current level of knowledge surrounding major facilitator superfamily members. By fusing different parts of transporters (some already known structurally i.e LacY and others not i.e GusB) together which share similar homology, this could provide information regarding their recognition sites, crystal structures and function. Furthermore, the theory and results from this project could be applied to other MFS members to try and understand them more in depth.

Moreover, the research could contribute to the biosensor industry. By having the ability to specify GusB in order to narrow the range of glucuronides recognised, it can be engineering to detect 'drug cheats'. Each drug produces a different type of glucuronide and the glucuronide transporter could be designed to recognise the glucuronides produced by the specific drugs that are being investigated.

By genetically engineering GusB in order to recognise certain glucuronides, this could be used in future disease or disorder detection. Certain glucuronide levels could be monitored based on the amount of transportation carried out by these biosensors. For example, kernicterus is a neurological damage caused to the brain when bilirubin is not detoxified and mostly affects newborns (Shapiro *et al.* 2006; Ahlfors 2010). Therefore by designing the biosensor to recognise bilirubin mono and di-glucuronides which are formed when bilirubin is conjugated with glucuronic acid during detoxification, the levels of this product could be measured and therefore indicate whether levels produced are in the expected range or not.

1.14.1. Aim

The aim of this research is determine the functionality of the transmembranes in the glucuronide transporter, GusB by fusing it with lactose 'permease' (LacY), a well-known protein and locating GusB's possible substrate binding sites.

1.14.2. Objectives

The overall objectives for this research are:

- To design primers for the fusion between *gusB* and *lacY*
- To extract and purify plasmid DNA containing active *gusB* gene and genomic DNA containing the *lacY* gene
- To successfully form a fusion of *gusB* and *lacY* and amplify it through PCR
- To clone the transformants
- To assess the functionality of the transformants using chromogenic glucuronides/ lactose

Chapter 2. MATERIALS AND METHODS

2.1. Chemicals, enzymes and commercial kits used

Due to both genomic and plasmid DNA being used in this project, various kits were used to extract them. The DNeasy Blood and tissue kit (catalogue number 69504) was used to extract genomic DNA from JM109 whereas the QIAprep- Spin Miniprep Kit (catalogue number 27104) was used for plasmid pMJB33 and pTTQ18 extraction. Other kits used once PCR was completed were the QIAquick PCR Purification Kit (catalogue number 28104) in order to 'clean' the PCR products and QIAquick DNA Gel Extraction Kit (catalogue number 28704).

The enzymes required were: *Taq* Polymerase (catalogue number M780A) from Promega Ltd, DpnI (R0176S), restriction endonucleases *Eco*RI (catalogue number R0101S) and *Hind*III (catalogue number R0104S), T_4 DNA ligase (catalogue number M0202S) from New England Biolabs.

Due to the nature of the project, a variety of chemicals were required in order to conduct the research; they were as follows: 1kb ladder, GoTaq® G2 Flexi DNA Polymerase (catalogue number M7801) which contained 5x Flexi Buffer, Green Flexi Colourless Buffer and Magnesium Chloride solution from Promega Ltd; ampicillin 100 mg/ml (catalogue number A5354), 5-bromo-4-chloro-3indoyl glucuronide (catalogue number B5285), p-nitrophenyl-*β*-D-glucuronide (catalogue number N1627) and isopropyl-thio-*β*-D-glucuronide (catalogue number 42897) from Sigma-Aldrich; Ethanol absolute (catalogue number E/0650/17), agarose powder (catalogue number BP1356-500), tryptone (catalogue number BP1421-500), yeast extract (catalogue number BP1422-500) and agar powder (catalogue number BP1423-500) which were from Fisher Scientific; SYBR® safe gel stain (catalogue number S33102) from Invitrogen and NEBuffer 2 (catalogue number B7002S) from New England Biolabs.

2.2. Equipment used

The main equipment needed was as follows: Nanodrop 2000 Spectrophotometer, Peqstar thermocycler for PCR, Bio-Rad power pack for

electrophoresis, Heraeus Biofuge Pico table top microcentrifuge, centrifuge S430R, Bio-Rad ChemiDoc™ MP Imaging System, JB Nova water bath and Shimadzu Spectrophotometer UV-1800.

2.3. Preparation LB media and agar plates

In order to grow the plasmid DNA (pMJB33 and pTTQ18) and genomic DNA (JM109), nutrient rich media's were prepared according to the laboratory manual (Maniatis *et al.* 1982). As not many plates were required, the constituents for making 1 litre of media and agar was halved to make 500ml of each. Table 2-1 summarises the components for Luria-Bertani (LB) media and LB agar media in 500 millilitre.

Table 2-1. The constituents for LB media and LB agar Media for 500 millilitres (Maniatis *et al.* 1982)

LB Media		**LB agar Media**	
Components	**Weight**	**Components**	**Weight**
Bacto-Yeast Extract	2.5g	Bacto- Yeast Extract	2.5g
Bacto- tryptone	5g	Bacto- tryptone	5g
Sodium Chloride	5g	Sodium Chloride	5g
		Agar	7.5g

Both the LB Media and agar media were mixed in glass bottles. Half of the bottles were filled with distilled water and all the ingredients were added and placed on a hot plate for a few minutes using a magnetic stirrer to mix them together and remove the lumps of powder. The bottles were then filled to the 500 millilitre mark with distilled water and autoclaved.

Once the bottles had cooled down, around 25 ml of LB agar media containing no ampicillin was poured in 4 plates and set aside to cool. For the remaining 400ml of media, 400 microlitres (µl) of ampicillin was added to the agar media and mixed by inverting the bottle. As above, 25 ml of agar was then poured into the remaining plates and they were left to set before incubating them at 37 ^{o}C to dry them off for a few hours.

2.4. Isolation of single colonies

The dry agar plates were streaked using a loop. The loop was sterilised and then placed into the 'deep' strain (the 'deep' is from the frozen stock of the *E. coli* strains) containing plasmids MC1061 (pMJB33) and MC1061 (pTTQ18) and the genomic DNA JM109, see Table 2-2. Once placed in the deep, the loop was then streaked on the agar plates and were then left overnight in the incubator at 37 ^{o}C. Once the bacteria colonies had grown, single colonies from each plate were used for inoculation.

Table 2-2. The various *E. coli* strains and their descriptions

***E. coli* Strains**	**Description**
MC1061 (pMJB33)	*E. coli* expression vector which is ampicillin resistant and has an IPTG-inducible *tac* promoter
MC1061 (pTTQ18)	K-12 F^{-} λ^{-} Δ*(ara-leu)7697* [*araD139*]B/r Δ*(codB-lacI)3 galK16 galE15* e14^{-} *mcrA0 relA1 rpsL150*(Str^{R}) *spoT 1 mcrB1 hsdR2*(*r*$^{-}$*m*$^{+}$)
JM109	F´ *traD36 proA*$^{+}$*B*$^{+}$ *lacI*^{q} *Δ(lacZ)M15/ Δ(lac-proAB) glnV44 e14*$^{-}$ *gyrA96 recA1 relA1 endA1 thi hsdR17*

2.4.1. Inoculation and growth of bacteria.

For the inoculation, 10 ml of LB media along with 10 µl of ampicillin was pipetted into a sterilised flask. Using the loop, a single colony from the plates was collected and placed into media, waiting a few seconds to allow the transfer. Once the colony was transferred, the flask was placed into an orbital shaking incubator overnight @ 37^{o}C to allow the bacteria to grow.

2.5. Plasmid DNA Extraction

The bacteria strains from the flasks which contained the pMJB33 and pTTQ18 plasmids respectively underwent mini prep in order to extract the

plasmids using the the QIAprep Spin Miniprep Kit. In addition to this, the genomic DNA from the JM109 strains had to be extracted using the DNeasy Blood and tissue kit.

2.6. PCR Primers

2.6.1. PCR Primer Design

For this project, primers had to be designed in order to fuse the first 6 transmembranes helices of LacY with the last 6 transmembrane helices of GusB. In order to increase the success of this fusion, multiple primers were designed to improve the yield. The primer design followed a set of criteria (Reed *et al.* 2012 p.468) as well as being designed in the 5' -3' direction:

- **Length**- 18-33 nucleotides base-long primers to increase the likelihood of the complementary strands to bind during amplification.
- **Base composition**- the GC content had to be <50% due to GC bonds higher annealing temperate compared to AT. Polypurine and polypyrimidine tracts were avoided when designing the primers.
- **Melting temperature**- the temperature at which both the forward and reverse primers can anneal with the template was calculated to not differ by 3 degrees in order to secure successful PCR reactions.

2.6.2. Primer rehydration and dilution

Upon delivery of the primers, they were lyophilised form and therefore needed rehydrating with $T_{10}E_1$ (10 mM Tris-HCl pH8.0 and 1 mM EDTA.Na_2, pH8.0), see table 2-3 for the respective volumes added to each primer. However, after rehydration of the primers, the final concentrations (100 µM) were too high for PCR and therefore required further diluting to 10 µM. This was done by a 1 in 9 dilution; for every 1 µl of primer, 9 µl water was mixed.

Table 2-3. Table showing the various primers and amount of $T_{10}E_1$ added for dilution

Oligonucleotide Name	Vol added (100pmol/µl)	Final Concentration (µM)
LacYF1	320	100
LacYF2	299	100
LacFY3	284	100
gusBR1	318	100
gusBR2	399	100
lacYgusBF1Y	216	100
LacYgusBF3Y	246	100
lacYgusBR1Y	229	100
lacYgusBR3Y	288	100

2.7. PCR preparation

Before carrying out the PCR reactions, the various components concentration had to be diluted to result in the correct concentration required for the PCR.

2.7.1. Preparation of Deoxynucleotide Triphosphate (dNTP)

One of the components for the PCR reaction is 1 mM of dNTP. This was made by diluting 10 µl of 10 mM dNTP with 90 µl of distilled water.

2.8. PCR procedure

For this project a two-step PCR was carried out; the first step PCR involved amplification of DNA composed of one flanking primer and one fusion primer with an overlapping region to create a fragment. During this process two fragments were produced to use in second step PCR. Second step PCR then fused the two separate fragments from 1st step PCR together in order to create a fusion between the first 6 transmembers of *lacY* and the last 6 transmembranes of *gusB.* Tables 2-4 and 2-5 shows the reagents required for first step and second step PCR respectively with the overall volume being 50 µl.

Table 2-4. Components of first Step PCR equalling to 50 µl

Component	Volume (µl)	Final Concentration
Distilled H20	33.5	-
5x Flexi Buffer	10	-
1mM dNTP	1	20µM
25mM MgCl2	2	1000 µM
*10µM Forward Flanking Primer	1	0.2 µM
*10 µM Reverse Mutagenic Primer	1	0.2µM
DNA Template	1	42.7ng
Taq polymerase	0.5	5u/µl
Total	50	-

*PCR reactions were also set up using the Forward fusion primers and Reverse flanking primers

Before undergoing second step PCR, the PCR products from the first step PCR were loaded on a 1.5% agarose gel and underwent electrophoresis at 70 V for 40 minutes. The gel was then assessed using the ChemiDoc MP Imaging system in order to show the separate DNA bands based on their molecular weight. All of the successful fragments were then purified using the Qaigen PCR Purification kit. After purification, every sample was treated *Dpn1* by doing the following reaction; 5.5 µl cutsmart buffer and 1 µl of *Dpn1* was added to the PCR product tube before being incubated at 80 ^{o}C for 20 minutes.

Once all the successful samples were treated with *Dpn1*, the successful fragments were paired up with its respective pair and undergone second step PCR in order to obtain a fusion. The products were then analysed via an electrophoresis gel and molecular sizes were checked by comparing them to the 1 Kb ladder.

Table 2-5. Components of second Step PCR

Component	Volume (µl)	Final Concentration
Distilled H_20	32.5	-
5x Flexi Buffer	10	-
1 mM dNTP	1	20µM
25 mM $MgCl_2$	2	1000 µM
10 µM Forward Flanking Primer	1	0.2 µM
10 µM Reverse Flanking Primer	1	0.2µM
*DNA Template	2	30-50ng
Taq polymerase	0.5	5u/µl
Total	50	-

*1 µL of DNA template from each corresponding fragment in the relevant pair

After second stage PCR, the products underwent agarose gel electrophoresis in order to check the success of the fusion. The gel was run at 70 V for 40 minutes.

During PCR, the repeating cycles include denaturation, annealing and polymerization of the DNA. Table 2-6 shows the settings chosen.

Table 2-6. The PCR settings used for this project- a gradient temperature was used

Steps	Temperature (ºC)	Time (minutes)	Purpose
1	95	5	Denaturation
*2	94	1	Denaturation
*3	63.5 ± 1.5	0.5	Annealing
*4	72	2	Polymerization
5	72	4	Completion of the extension
6	8	60	Storage

*Stages 2-4 is the cycle which was repeated 30 times.

2.9. Gel extraction

After running fusion PCR, the samples were loaded onto a gel and ran at 70 V for 1 hour in order to separate the bands as much as possible. The fusion bands were then excised from the agarose gel using sharp scalpels. The gel slices were then weighed in microcentrifuge tubes (refer to the appendix- lab book for results) and the QIAquick Gel Extraction Kit protocol was followed to isolate the DNA fragments, remove the agarose and purify the DNA. This resulted in 50 µl of samples

2.10. Restriction Digest

In order to successfully ligate the PCR products, the DNA has to be cleaved with restriction enzymes *Eco*RI and *Hind*III to create sticky ends. In addition to this, the pTTQ18 vector had to be cleaved using the same restriction enzymes to prepare for insertion of the PCR product during ligation. The overall digestion time lasted 4 hours with both *Eco*RI and *Hind*III restriction enzymes being added at the start of the reaction and the reaction was incubated at 37 ^{o}C throughout.

Table 2-7. Components for restriction double digest of the second step PCR product and Plasmid

Components	**H183D (µl)**	**pTTQ18 (µl)**
Distilled H20	37	37
10 X Buffer 2	5	5
BSA 10mg/ml	1	1
Plasmid pTTQ18	-	5
DNA fragments	5	-
*Eco*RI µ/ml	1	1
*Hind*III µ/ml	1	1

2.11. Ligation

Due to the digested pTTQ18 vector having phosphorylated areas, it had to be treated with Shrimp Alkaline Phosphatase (SAP) in order to remove them. This was done before ligation. The whole vector plasmid sample was treated

with 1 µl SAP and 5 µl cut smart buffer and was incubated 37 degrees for 30 minutes.

For ligation, the total volume was no more than 20 µl for each reaction (see Table 2-8 for the components). The reaction mixtures were kept overnight at 16^{o}C before being used for transformation.

Table 2-8. Components required for ligation of DNA fragment (H183D) and plasmid vector pTTQ18

	Volume (µl)				
Components	**L1**	**L2**	**L3**	**C1**	**C2**
Vector (30-50ng/µl)	1	1	1	1	1
Insert (30-50ng/µl)	1	2	3	-	-
10x Ligase Buffer	2	2	2	2	2
T4 Ligase	1	1	1	-	1
H_2O	15	14	13	17	16

2.12. Making competent cells

To make competent cells, Bacterial strain MC1061 was grown in no ampicillin LB media overnight at 37 ^{o}C in an orbital shaker (250rpm). 250 µl of the overnight MC1061 culture was then inoculated into fresh no ampicillin LB media and grown for 2 hours at 37 ^{o}C with vigorous shaking (250rpm) to assist the bacteria to reach log phase (Light absorbance at A600 = 0.2-0.3). Immediately after reaching log phase, 1 ml of cells were harvested by centrifugation at 8000rpm and 4 ^{o}C for 2 minutes. The supernatant was discarded and the cells were resuspended in 0.5 ml of ice-cold sterile calcium solution (50 mM $CaCl_2$, 10 mM Tris-HCl, pH 8.0). The suspension was placed in an ice bath for 15 minutes before being centrifugated again at 10,000 rpm for 1 minute at room temperature. The supernatant was discarded and the cells were resuspended in 66 µl ice-cold calcium solution.

2.12.1. Transformation

During the transformation, 10 µl of the ligated samples (L1, L2, L3, C1 and C2) were pipetted into 200 µl of the previously made competent cells and

were left to incubate for 30 minutes on ice. These samples were then heat shocked at 42 ^{o}C for 2 minutes in a water bath before being immediately placed in a slushy ice bath to chill for 2 minutes. 330 µl of LB media containing no ampicillin was pipetted into each sample before incubating the cells at 37 ^{o}C with vigorous shaking of 250 rpm for 60 minutes. After incubation, 200 µl of the incubated cells were plated onto ‘pre dried’ Lb agar plates containing ampicillin and spread with glass beads to ensure the cells dispersion. The plates containing the cells were then incubated overnight at 37 ^{o}C before being examined the following day and photographed.

2.13. Transformant confirmation

Potential transformant colonies from the agar plates were inoculated overnight in LB media containing ampicillin. 1ml of cells was then harvested and extracted using the QIAprep Spin Miniprep Kit. Some of the extracted plasmid DNA underwent restriction digest using *Eco*RI or *Hind*III (refer to Section 2.9) in order to form a comparison between cut and uncut DNA on an agarose gel. If the plasmid was successfully cut, a linearized band of approximately 6 kb would be visible on the gel.

Chapter 3. RESULTS

3.1. Open Reading Frame (ORF) analysis

In order to design the primers, ORF's for *lacY* and *gusB* had to be obtained. This was done by using online softwares and tools such as ExPASy translate tool (http://web.expasy.org/translate/) or NCBI (https://www.ncbi.nlm.nih.gov/). Another important feature of these tools is the start and stop codons are highlighted which assisted with identifying protein genomes within the sequences.

3.2. Primer Design

Primers were designed with some assistance from my supervisor. For this experiment, 11 primers were initially designed in order to increase the chances of getting a successful fusion; 3 forward flanking primers, 2 reverse flanking primers and 6 fusion primers. All of the primers were designed following the criteria mentioned in the methods chapter (Section 2.5.1). In addition to this, a third restriction enzyme recognition site (*Bam*HI) was incorporated within the fusion primer linkers. Once all the primers were ordered, primers with the closest annealing temperatures were paired together to increase the potential of having successful fusions.

Table 3-1. Forward primer designs for the LacY part of the fusion

Name	Primer	Annealing temp	Designer	Synthesizer
LacYF1	5'TAATGGAATTCGTATTTCGCGTAAGGAAATCCA-3'	62.6°C	CC*/WJL*	Eurofin Genomics
LacYF2	5'-TAATGGAATTCCCCGTATTTCGCGTAAGG-3'	61.8°C	CC*/WJL*	Eurofin Genomics
LacYF3	5'TAATGGAATTCCCGTATTTCGCGTAAGGAAATC-3'	64.5°C	CC*/WJL*	Eurofin Genomics

*CC= Christine Ciocan *WJL= Wei-Jun Liang

Table 3-2. Reverse primer designs for the GusB part of the fusion

Name	Primer	Reverse Complement	Annealing Temp	Designer	Synthesizer
gusBR1	GCAATTAATCAGCGATATCACTAATTAA	5'-TAATGAAGCTTTTAATTAGTGATATCGCTGATTAATTGC -3'	64.1°C	CC*/WJL*	Eurofin Genomics
gusBR2	CAATTAATCAGCGATATCACTAATTAA	5'-TAATGAAGCTTTTAATTAGTGATATCGCTGATTAATTG -3'	62.1°C	CC*/WJL*	Eurofin Genomics

*CC= Christine Ciocan *WJL= Wei-Jun Liang

Table 3-3. Original forward and reverse (including the linker) primer designs

Name	Primer	Annealing Temperature
lacYgusB F1	5'-TAATGGAATTCTAGGTGCCAACGGTGGATCCTGGTCCGTCATTG-3'	65.3 °C
lacYgusB F2	5'-TAATGGAATTCTAGGTGCCAACGGTGGTGGATCCGGTAGTCCGTCATTGAAT-3'	66.8 °C
lacYgusB F3	5'-TAATGGAATTCGTGCCAACAGTGGTGGATCCGGTCCGTCATTGAATA-3'	63.9 °C
lacYgusB R1	5'-TAATGAAGCTTCAATGACGGACCAGGATCCACCGTTGGCACCTA-3'	65.3 °C
lacYgusB R2	5'-TAATGAAGCTTATTCAATGACGGACTACCGGATCCACCACCGTTGGCACCTA-3'	66.8 °C

lacYgusB R3	5'- TAATGAAGCTTTATTCAATGACGGACCG GATCCACCACTGTTGGCAC-3'	63.9 °C

Key

*Hin*dIII Restriction enzyme

*Eco*RI Restriction enzyme

gusB section

lacY section

Primer

Linker with *Bam*HI restriction site included

Extra nucleotides

3.3. Plasmid Extraction

Before proceeding with PCR, the concentrations of plasmid DNA pMJB33 and pTTQ18 as well as the concentration of genomic DNA JM109 were measured with a DNA Nanodrop. It was important to keep the concentrations as close to 50 ng/µl as possible. In addition to this, the OD260 (Optical Density at 260 nm) reading was kept between 0.2-0.8 and the OD260/OD280 ratio was kept in between 1.8-2 as that is the acceptable 'purity' range. If any of these readings were too high, the DNA samples were diluted until the figures were in the necessary range. See Table 3-4 for the results from the Nanodrop.

Table 3-4. Concentration, purity and dilution factors of genomic DNA JM109 and plasmid pMJB33 **and pTTQ18**

Plasmid	**Water (µl): DNA (µl) dilution ratio**	**Concentration (ng/ µl)**	**OD260 Reading (Concentration of nucleic acid in sample)**	**OD260/OD 280 Ratio (Purity ratio)**
JM109	-	19.7	0.395	1.86
pMJB33	-	157.3	3.074*	1.87
pMJB33	8:1	39.9	0.797	1.81
pTTQ18	-	17.6	0.352	1.84

***Reading was too high and therefore diluted

3.4. First round PCR- Amplification of DNA fragments Attempt 1

For the first round PCR, 5 fragments were amplified with 4 of the genes being successful amplified (samples 2, 3, 4 and 5). Bands 2 and band 5 were very faint but were visible to the human eye (refer to Appendix- lab book p.8 for pairings). A 1 kb ladder was loaded onto every gel in order to estimate weights for each band and compare the actual sizes with predicted sizes to confirm whether they are the desired fragments.

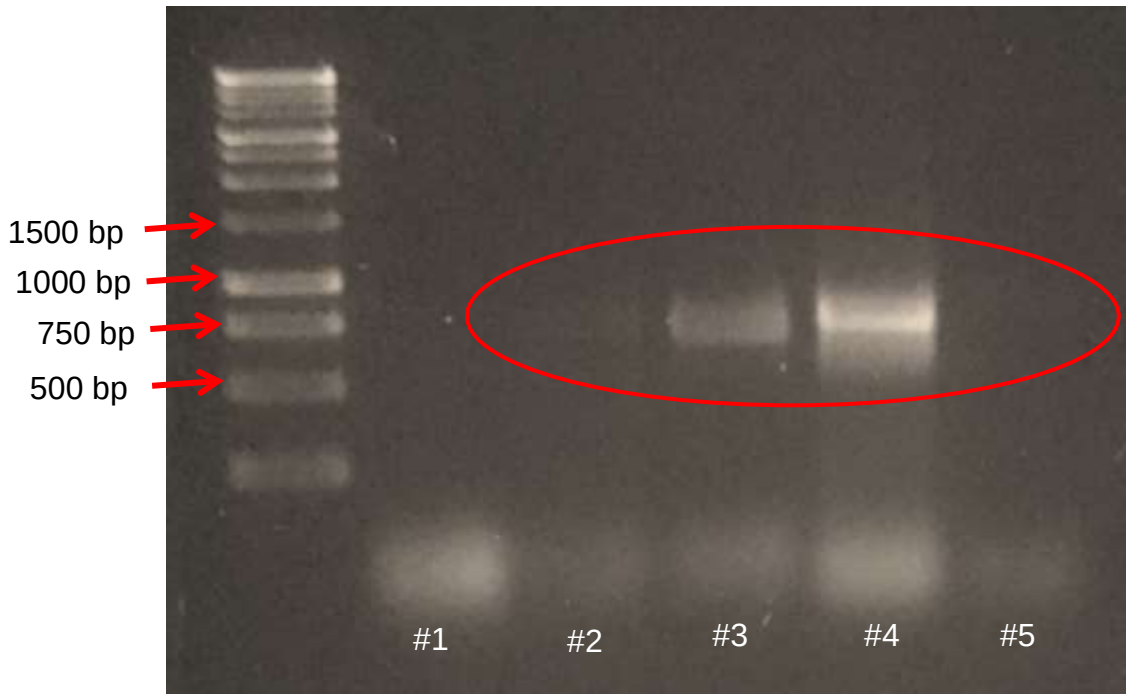

Figure 3-1. Evaluation of first round PCR Products (#1, #2, #3, #4, #5) by agarose gel electrophoresis. The visualised bands on the gel are the correct predicted size of each fragment, thus suggesting successful amplification. Lane 1: 1 kb ladder. Lane 2: No band visible. Lane 3: LacY fragment 2 (667 bp). Lane 4: LacY fragment 3 (661 bp). Lane 5: GusB fragment 1 (756 bp). Lane 6: GusB fragment 2 (755 bp).

1.5% agarose gel was used and ran for 40 minutes at 70 V

3.5. DNA purification of successful samples from first round PCR

The successful fragments (#2, #3, #4 and #5) from first stage PCR were purified in order to remove impurities from the previous reaction (refer to Appendix p.8 for pairings). This was carried out in attempt to improve the chances of getting a successful fusion. As illustrated, there is a bit of smearing still evident after purification, however it was decided to progress onto the next step due to time constraints.

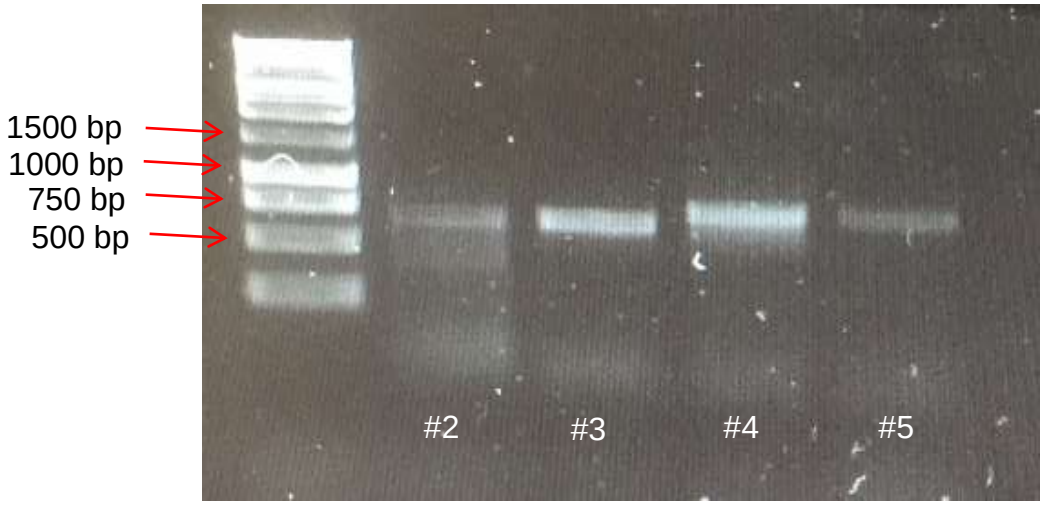

Figure 3-2. Evaluation of DNA purification of the first round PCR Products (#2, #3, #4, #5) by agarose gel electrophoresis. The visualised bands on the gel are the purified samples from the first stage PCR. Lane 1: 1 kb ladder, Lane 2: LacY fragment 2 (667 bp). Lane 3: LacY fragment 3 (661 bp). Lane 4: GusB fragment 1 (756 bp). Lane 5: GusB fragment 2 (755 bp).

1.5% agarose gel was used and ran for 40 minutes at 70 V

3.6. Evidence of successful fusions formed using two step PCR

As the project progressed, aims were being met such as forming a fusion between the two proteins by using two step PCR. As shown below, there were 4 successful fusions following four troubleshoots to improve the band quality and reduce smearing (see Appendix- lab book pages 13-17 for the gels from the other troubleshoots). Following the procedure, these bands were excised and extracted using the gel extraction kit and then underwent restriction digest.

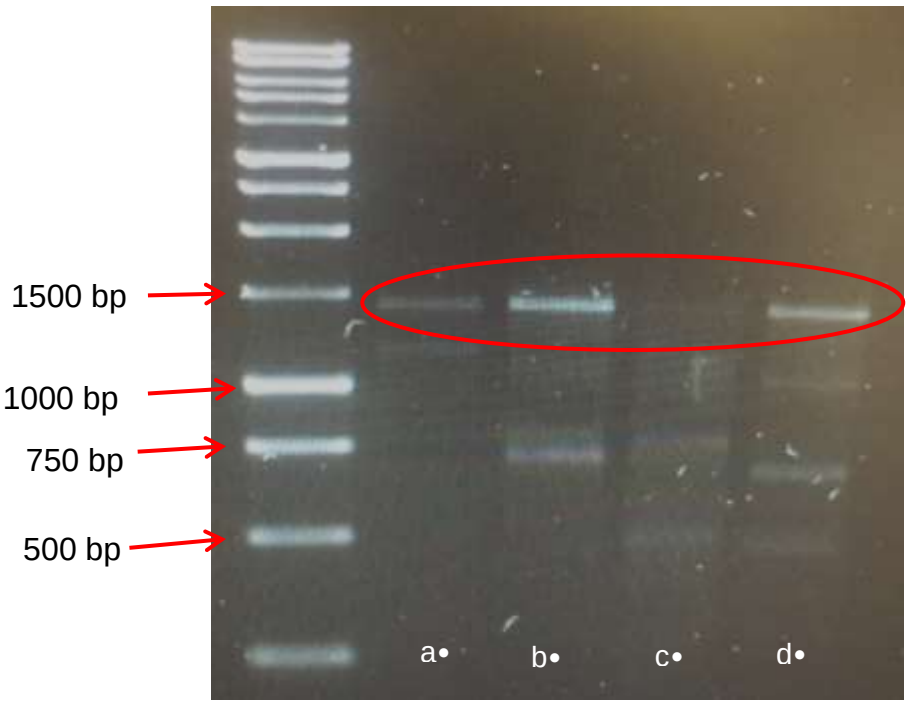

Figure 3-3. Evaluation of the second stage PCR Products by agarose gel electrophoresis. After troubleshoot 4 consisting of reduced cycle numbers and reduced magnesium concentration, the smearing and unwanted band fragments have been reduced compared to the first troubleshoot (refer to Appendix- lab book 16). All four samples (circled above) were excised, extracted and purified. Lane 1: 1kb ladder. Lane 2: fusion formed between LacY fragment 2 and GusB 2. Lane 3: fusion formed between LacY fragment 3 and GusB 1. Lane 4: fusion formed between LacY fragment 2 and GusB 1. Lane 5: fusion formed between LacY fragment 3 and GusB 2.

1.5% agarose gel was used and ran for 40 minutes at 70 V

3.7. Restriction Digest 1 of successful Fusion

Samples a•, b• and d• from fusion (see Figure 3-3) underwent restriction double digest. Similar to the digestion of the plasmid, the samples were cleaved with *Eco*RI and *Hind*III following the protocol stated in methods (Section 2.9). It was decided to not digest sample c• as after gel extraction, the band was extremely faint (refer to Appendix- lab book p.19). As bands were faint after gel extraction (refer to Appendix- lab book p.19), each sample had two sets which underwent restriction digest which were later combined into one sample to increase DNA concentration. As evident on the gel, there is still 1.4 kb estimated band sizes visible after restriction digest which indicates that the digest appeared successful. However, due to the multiple bands being visible, the restriction digest was repeated with new DNA sample from the PCR fusions.

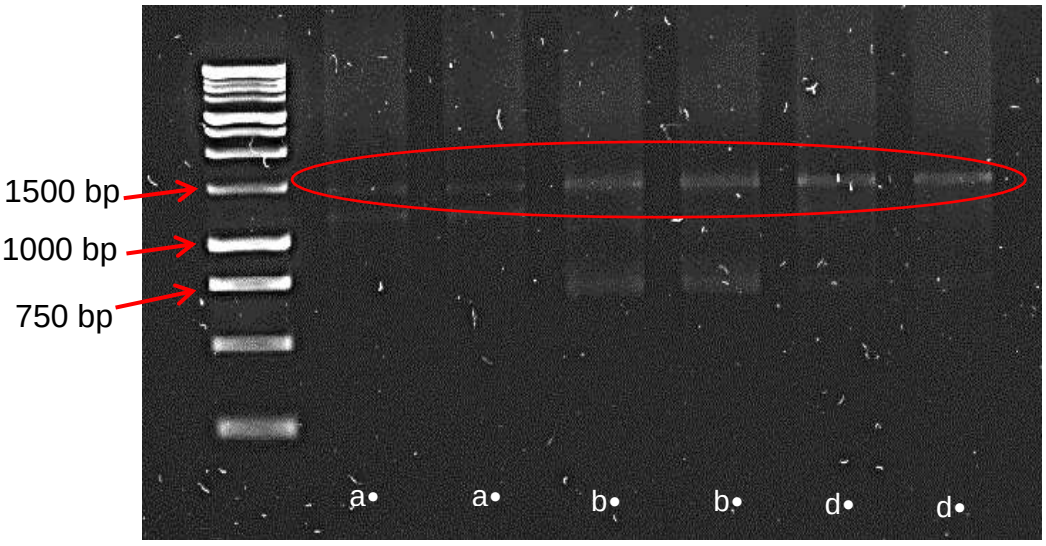

Figure 3-4. Evaluation of the restriction digest of PCR samples. As evident on the agarose gel, the fusion bands for samples a•, b• and d• are still present after undergoing double digest. There is also other bands present which could indicate contamination. This prompted a repeat of digest with new samples. Lane 1: 1 kb ladder. Lane 2 and 3: Fusion 1 double digested with *Eco*RI and *Hind*III. Lane 4 and 5: Fusion 2 double digested with *Eco*RI and *Hind*III. Lane 5 and 6: Fusion 3 double digested with *Eco*RI and *Hind*III.

1.5% agarose gel was used and ran for 40 minutes at 70 V

3.8. Restriction Digest of plasmid

The plasmid pTTQ18 underwent restriction double digest according to the protocol mentioned in Methods (Section 2.9) to linearize the plasmid vector and prepare it for ligation by cutting it twice. The enzymes used to cleave the plasmid were *Eco*RI and *Hind*III and as expected, a linearized 4.5 kb estimated size band was present on the gel after restriction digest, indicating successful cleaving of the vector.

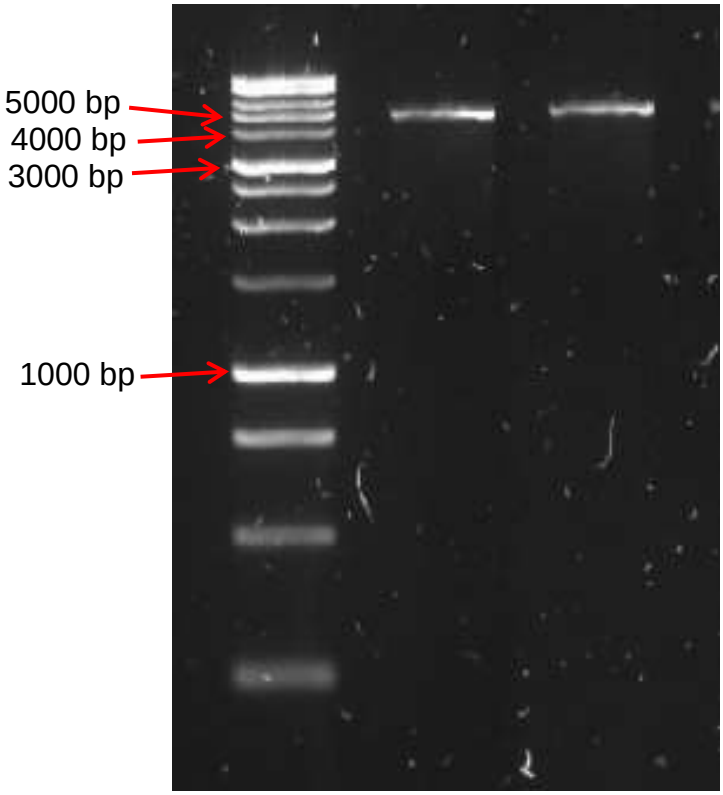

Figure 3-5. Evaluation of the restriction digest of plasmid pTTQ18. A linearized band location in between 4000 bp and 5000 bp indicates a successful digest of the plasmid. As it was a double digest, the smaller fragment which was cut from the fragment was not visible on the gel due to it being smaller than 250 bp in size. Lane 1: 1 kb ladder. Lane 2 and 3: pTTQ18 plasmid double digested with *Eco*RI and *Hind*III (4.5 kb).

1.5% agarose gel was used and ran for 40 minutes at 70 V

3.9. Restriction Digest repeat of PCR fusions

Despite the previous restriction digest of the PCR fusions being successful, the double digest was repeated with new samples and fresh enzymes to reduce the amount of unwanted bands and contamination. The enzymes used were *Eco*RI and *Hind*III. As evident below, the sample bands were no longer visible on the gel. However nanodrop confirmed that DNA was still present (see Appendix- lab book p.21). This prompted investigation as the fusion was no longer visible after digest when it was expected to have 1.4 kb bands present.

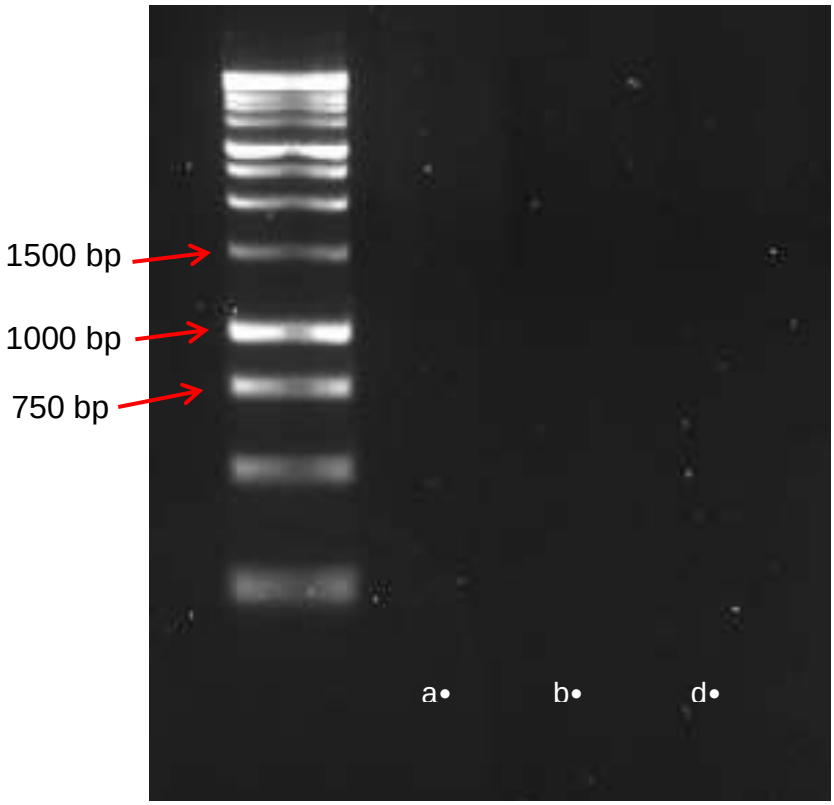

Figure 3-6. Evaluation of the restriction digest repeat of PCR samples. The restriction digest was repeated in attempt to reduce the amount of bands visible on the gel. However, no bands are visible on the gel despite the expectation of 1.4 kb bands being present. This prompted investigation to identify the cause. Lane 1: 1 kb ladder. Lane 2, 3 and 4: no band evident after double digest with *Eco*RI and *Hind*III.

1.5% agarose gel was used and ran for 40 minutes at 70 V

3.10. Restriction Digest repeat of PCR fusions

After further investigation into the cause of the digest bands no longer being present on the gel, it became evident that there was an error in the initial primer designs (see 3.2.). By error, there were more than 2 restriction sites on the fragment which resulted in the fragment being cleaved into more than two fragments. These primers were then corrected and redesigned along with the assistance of Dr WJ Liang- creating 4 new fusion primers.

Table 3-5. Corrected forward and reverse (including the linker) primer designs

Name	Primer	Annealing Temperature	Designer	Synthesizer
lacYgusB F1Y	5'-TAGGTGCCAACGGTGGATCCGTTCCGTCATTG-3'	65.3 °C	CC*/WJL*	Eurofin Genomics
lacYgusB F3Y	5'-TAGGTGCCAACAGTGGTGGATCCGTTCCGTCATTGAATA-3'	63.9 °C	CC*/WJL*	Eurofin Genomics
lacYgusB R1Y	5'-CAATGACGGAACGGATCCACCGTTGGCACCTA-3'	65.3 °C	CC*/WJL*	Eurofin Genomics
lacYgusB R3Y	5'-TATTCAATGACGGAACGGATCCACCACTGTTGGCACCTA-3'	63.9 °C	CC*/WJL*	Eurofin Genomics

*CC= Christine Ciocan *WJL= Wei-Jun Liang

Key

gusB section

lacY section

Linker with *Bam*HI restriction site included

3.11. First round PCR- Amplification of DNA fragments (Attempt 2)

Since redesigning the primers, the first step PCR was repeated to correct the design error. The previous fusion formed (see 3.6) was used as the template (refer to Appendix- lab book p.31 for pairings). During the first step PCR, 4 fragments were amplified and they were all successful as illustrated on the gel. This particular troubleshoot consisted of reducing the magnesium concentration.

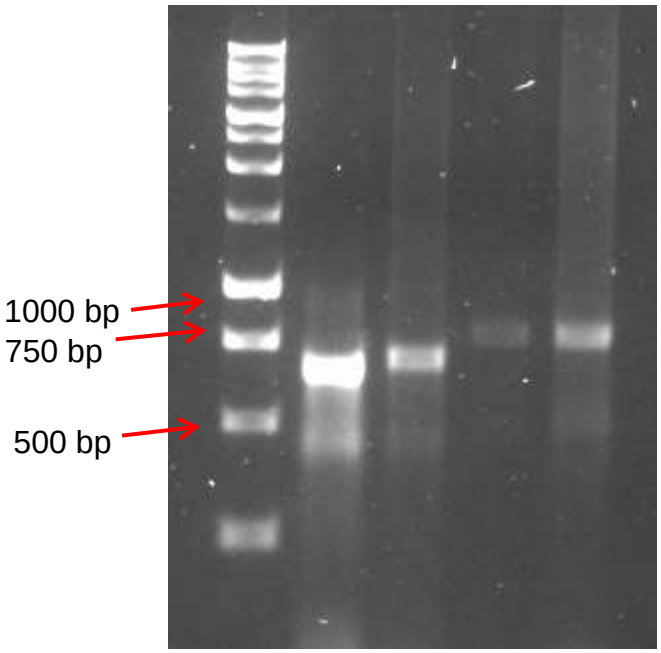

Figure 3-7. Evaluation of first round PCR repeat Products by agarose gel electrophoresis. The visualised bands on the gel are the correct predicted size of each fragment, thus suggesting successful amplification. Lane 1: 1 kb ladder, Lane 2: LacY fragment 1 (659 bp). Lane 3: LacY fragment 2 (666 bp). Lane 4: GusB fragment 1 (755 bp). Lane 5: GusB fragment 2 (758 bp).

1.5% agarose gel was used and ran for 40 minutes at 70 V

3.12. DNA purification of successful 1st step PCR samples (Attempt 2)

The chosen successful fragments from first stage PCR were purified to ensure the samples are cleaner and impurities were removed. As seen below, there was some slight smearing and unwanted bands still visible. However due to time constraint it was decided to continue with the project. Samples 5 and d• are from two other troubleshoots that were carried out (refer to Appendix- lab book pages 28, 29 and 32).

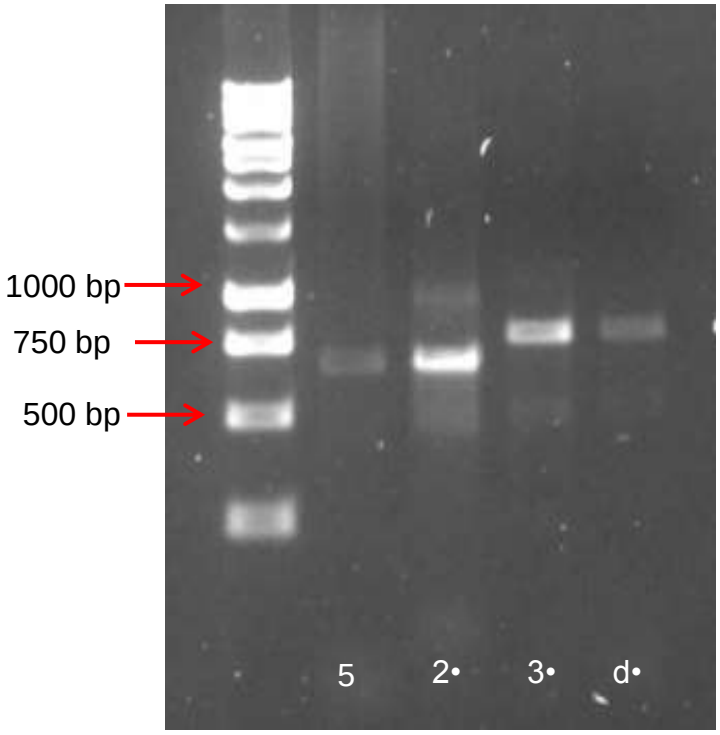

Figure 3-8. Evaluation of DNA purification of the first round PCR Products by agarose gel electrophoresis. The visualised bands on the gel are the purified samples from the first stage PCR. Lane 1: 1 kb ladder, Lane 2: LacY fragment 1 purified (659 bp). Lane 3: LacY fragment 2 purified (666 bp). Lane 4: GusB fragment 1 purified (755 bp). Lane 5: GusB fragment 2 purified (758 bp).

1.5% agarose gel was used and ran for 40 minutes at 70 V

3.13. Evidence of successful fusions formed using two step PCR (Attempt 2)

The second stage comprised of first step PCR products being paired up together (refer to Appendix- lab book p.35 for pairings) and being amplified to produce the fusion. The expected fusion band should be around 1.4 kb in size. There were multiple troubleshoots done to ensure a successful fusion. The image below is the result of the third troubleshoot (see appendix- lab book p.33 and p.34 for the other troubleshoots) where a gradient temperature PCR was run. As evident on the gel, there are faint fusion bands of the correct estimated size as well as a lot of smearing and unwanted bands present.

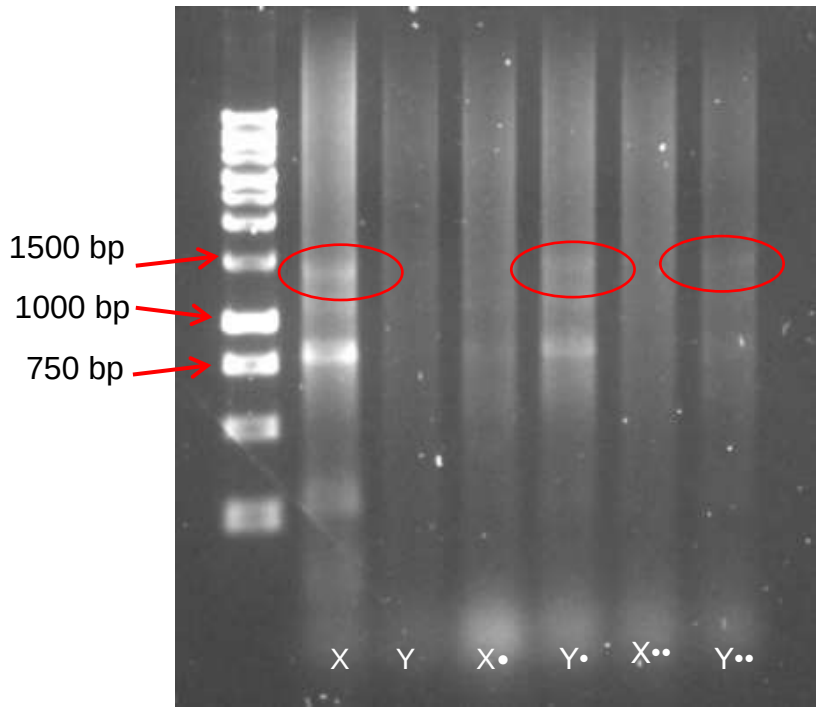

Figure 3-9. Evaluation of the second stage PCR repeat products. After 3 troubleshoots, there is still a lot of smearing and unwanted bands visible. Lane 1: 1kb ladder. Lane 2 potential fusion formed between LacY fragment 1 and GusB 1 at 58 degrees. Lane 3. No potential fusion band. Lane 4: No potential fusion band. Lane 5: potential fusion formed between LacY fragment 2 and GusB 2 at 60 degrees. Lane 6: No potential fusion band. Lane 7: potential fusion formed between LacY fragment 2 and GusB 2 at 62 degrees

1.5% agarose gel was used and ran for 40 minutes at 70 V

3.14. First round PCR- Amplification of DNA fragments (Attempt 3)

It was decided to attempt first step PCR again for a final time and without using the previous fusion as the DNA template (refer to Appendix- lab book p.36 for pairings). In addition to this, this repeat was focused on reducing the smearing as much as possibly to ensure a successful fusion. This was done by altering the magnesium concentration between the samples (see Appendix- lab book p.36 for constituents). As seen below (Figure 3-10), only 2 out of 8 samples had the correct band sizes. The two bands visible are the *lacY* part required for the fusion, thus troubleshooting was required to ensure bands for the *gusB* half (Figure 3-11). The troubleshooting to obtain the *gusB* fragments consisted of altering the temperature. After all the troubleshooting attempts, the chosen fragments were then extracted and purified before continuing with the second step PCR.

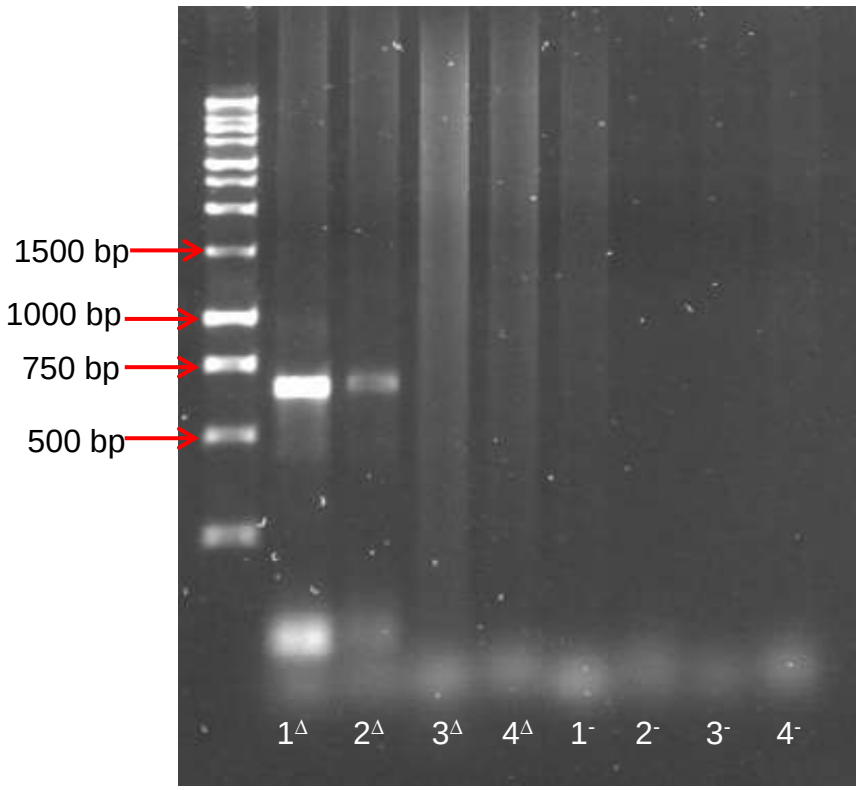

Figure 3-10. Evaluation of first round PCR repeat 3 products by agarose gel electrophoresis. Only two out of 8 samples had bands. Despite smearing still being evident, it had reduced as the magnesium concentration was decreased. Lane 1: 1 kb ladder. Lane 2: LacY fragment 1 (659 bp). Lane 3: LacY fragment 2 (666 bp). Rest of the lanes: No bands evident.
1.5% agarose gel was used and ran for 40 minutes at 70 V

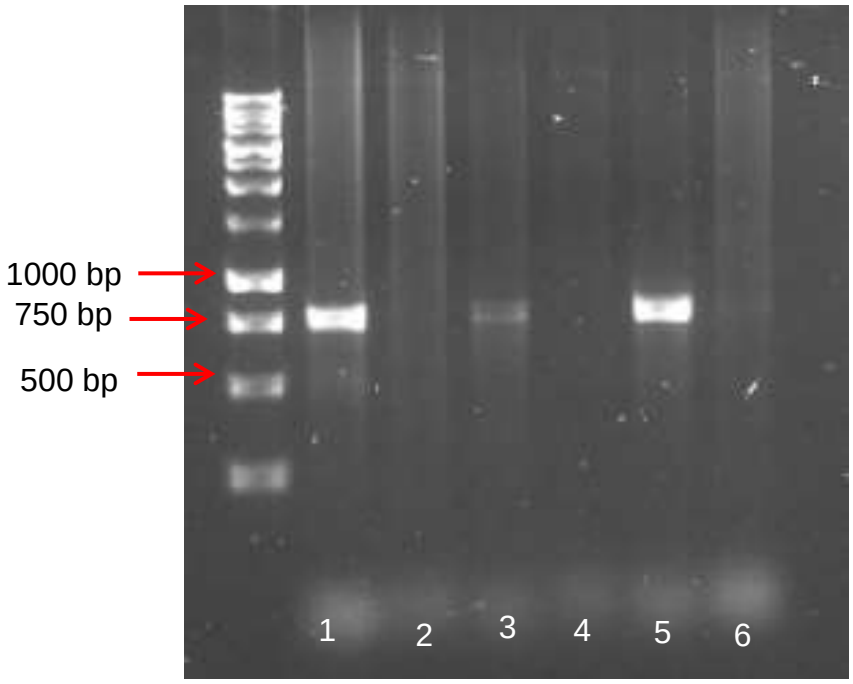

Figure 3-11. Evaluation of first round PCR repeat 3 *gusB* fragments by agarose gel electrophoresis. Out of 6 samples, 3 bands were successfully produced with the correct fragment size of 755 bp. There is some smearing evident but the fragments were excised, extracted and purified. Lane 1: 1 kb ladder. Lane 2, 4 and 6: GusB fragment (755 bp).

1.5% agarose gel was used and ran for 40 minutes at 70 V

3.15. Final second step PCR attempt (Attempt 3)

Various variables were altered in attempt to form a fusion during attempt 3. Nonetheless, after three second step PCR troubleshoots (refer to Appendix- lab book p.44 for pairings), no fusion band appeared and therefore attempts were unsuccessful (refer to Appendix- lab book p. 41-45). Figure 3-12 is the result from the third troubleshooting where a gradient temperature was run, along with an increase in GoTaq polymerase concentration. Despite obtaining an unsuccessful fusion, unwanted bands have been reduced as the troubleshooting progressed. Due to time constraint, ligation, transformation and the functionality testing could not be completed.

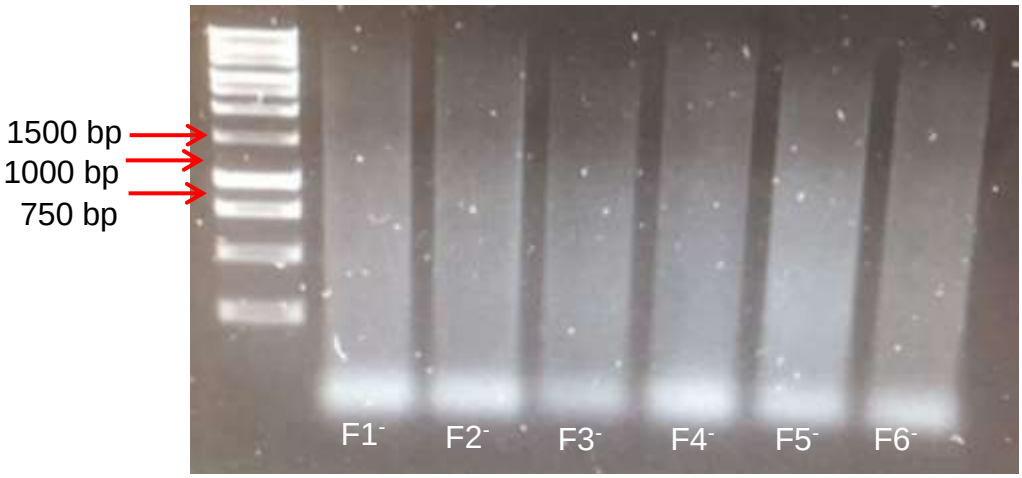

Figure 3-12. Evaluation of the second stage PCR Products by agarose gel electrophoresis. After 3 troubleshoots, there has been no fusion band of 1.4 kb in size present. Unwanted bands are no longer visible, however, the smearing is still evident throughout the troubleshoot attempts. Lane 1: 1 kb ladder. Lanes 2-7: No fusion bands present.

1.5% agarose gel was used and ran for 40 minutes at 70 V

Chapter 4. DISCUSSION

The glucuronide transporter, a MFS family member, is a crucial transporter in ensuring *E. coli* survival in the mammalian intestines. GusB recognises a wide range of readily available glucuronides produced during the detoxification process of glucuronidation (Liang 1992; Liang et al 2005). This transporter then transports these across the bacterial membrane and into the cell where *β*-D-glucuronidase converts it into a carbon source (Wilson *et al.* 1992; Roberts *et al.* 2002). Understanding the protein interactions between GusB and the other proteins in the *gus* operon, has led to a more thorough understanding of the mechanism and importance of their interaction.

Moreover, understanding the mechanism and structure of one of the most well studied MFS proteins, LacY, has provided a fundamental part of this project. There has been in-depth studies on this transporter's structure, specifically the N-domain being responsible for substrate binding and the C-domain containing the proton translocation sites (Frillingos *et al.* 1998; Abramson *et al.* 2003; Guan and Kaback 2006; Guan *et al.* 2007). As GusB and LacY are members of the MFS, known information can be applied to and compared between the two as they have similar structures i.e 12 transmembranes and cytoplasmic loop (Reddy *et al.* 2012). By using this knowledge and applying it to GusB, it may assist future deciphering of its molecular recognition sites.

4.1. Results

There has been some success in terms of achieving the objectives started in chapter 1. At the start of the project, progress was being made in terms of forming a fusion between the transmembranes of *lacY* and *gusB* using the PCR fusion method. As seen on Figures 3-1, 3-2 and 3-3, this was easily achieved with good results throughout as shown by DNA electrophoresis. However, the DNA bands were not as clean as hoped for and this was evident by the smearing. This could be an indication of possible contamination caused by equipment not being completely sterile or due to solutions and kits being shared by multiple students. However, after spending

a few weeks on running various troubleshoots in attempt to clean the bands, it was decided to persevere with the project.

The successful fusion formed between the *lacY* and *gusB* genes underwent double restriction digest to prepare the fragment for ligation. Initially, the double digest appeared to be successful as bands at approximately 1.4 kb in size was still visible on the agarose gel. However, smearing and unwanted bands were evident (see Figure 3-4), thus the digest was repeated to try and result in cleaner bands on the electrophoresis gel as it could impact the success of the ligation and transformation later in the project.

The double digest was repeated as shown on Figure 3-6. However, the fragment was no longer visible on the agarose gel after digest, especially as a 1.4 kb band was expected to be visible. Initially it was thought the restriction enzymes may have been the issue, however, this was quickly eliminated during the digest of the plasmid as the correct band size of approximately 4.5 kb was visible on the agarose gel.

To attempt to isolate the cause, additional restriction digests troubleshoots were carried out. The next restriction digest troubleshoot involved increasing the amount of DNA sample in the digest sample as it was thought the dilution factor from the gel extraction may have been an issue. The one band visible after gel extraction was quite faint and around 5 ng/µl. Initially 5 µl of DNA sample was added to a total volume of 50 µl. This resulted in a dilution factor of 10, thus reducing the DNA concentration and therefore the band may not be visible on the gel. In order to overcome this issue, more DNA volume was added to the reaction, however there was still no band evident on the gel which prompted further investigation.

This led to further investigation into the possible cause of the band no longer being present on the gel. After consulting with Dr Wei-Jun Liang, it became evident that there was an error with the primer design. The first set of fusion primers (refer to results) had more restriction sites designed within the sequence than it should have; overall there should have been 1 *Eco*RI restriction site and 1 *Hind*III site, however, there was in fact 3 restriction sites for each enzyme as restriction sites were added onto the end of the fusion

primers by human error. This issue resulted in the fusion fragment being cut into multiple small fragments, hence the fusion band no longer being visible on the gel. As this was a vital error, the fusion primers had to be redesigned to fix this mistake (refer to Results) by deleting the restriction sites on the end of each fusion primer and the project was repeated from first step PCR with the new primers.

The whole PCR procedure was repeated from the beginning. First step PCR was repeated with the corrected primers. For the DNA template in this PCR, the previous fusion formed (Figure 3-3) was used to improve the chances of a successful fusion. Numerous troubleshoots were required to achieve the correct estimated sized bands (see Appendix- lab book p.29-32). The correct bands were then paired up (refer to Appendix for the pairings) and underwent fusion PCR. Unfortunately, this proved to require various troubleshoots as the fusion bands were difficult to achieve. Based on previous troubleshoots findings, the $MgCl_2$ was the first factor to be altered. $MgCl_2$ is a cofactor for the *Taq* polymerase (GoTaq polymerase) and can affect its specificity and fidelity (Eckert and Kunkel 1991). *Taq* polymerase is a thermostable enzyme isolated from *Thermus aquaticus* and is responsible for DNA replication (Chien *et al.* 1976; Tindall and Kunkel 1988). However, for Go *Taq* polymerase to function, it requires a cofactor, in this case magnesium to assist in binding the dNTP to the DNA sequence (Lorenz 2012). Determining the optimal volume of $MgCl_2$ in the reaction is crucial as it can decrease the specificity of the enzyme and therefore reduce its efficiency, possibly resulting in incorrect fragments being replicated. Troubleshoots containing 1 mM and 0.5 mM $MgCl_2$ were carried out, however there appeared to be no significant differences between the bands (refer to Appendix- lab book p.33 and p.41-44).

Following this troubleshoot, multiple PCR gradients runs were done to find the optimal temperature to produce the fusion. Firstly, the PCR were carried out at the calculated average annealing temperature. However, this proved unsuccessful as very faint or no fusion bands were present, only smearing was evident. A gradient PCR was then performed on numerous occasions, ranging from 64-58 degrees. In addition to this, it was thought that the

extension time may have been too generic at first as each enzyme has its own extension rate. *Taq* polymerase replication rate is approximately 1 kb per minute (personal communication with Dr WJ Liang, September 2017) therefore the extension time was changed according to the size of the fragment, this case approximately 1.4 kb in size and therefore the PCR extension time was set to 1.5 minutes. This change made no significant difference to obtaining a fusion. As this continued to prove unsuccessful and it was decided to restart the whole PCR procedure again.

The PCR procedure was repeated again, starting with first step PCR. The DNA samples used were the original JM109 and pMJB33 samples instead of the previous fusion (see Figure 3-3 in Results). Similar conditions were used for this procedure based on the previous results and this was unsuccessful. In fact, the first step PCR had 4 troubleshoots and the most successful bands were chosen for the fusion PCR. However, to increase the chance of successful fusions, the chosen samples were purified followed by being gel extracted to clean the samples as much as possible. Once the corrected bands were achieved (see Results 3.14 and Appendix-lab book p. 36-39), fusion PCR was attempted once again.

The fusion PCR proved difficult again with further troubleshoots being carried out. Fusion bands were not visible on the agarose gels, despite Nano drop confirming DNA being present (see Appendix- lab book p.45). Altered factors included; temperatures ranging from 64-54 degrees and different magnesium chloride concentrations as before. Another factor which underwent troubleshooting was the amount of DNA sample pipetted into the reaction. As seen on Figures 3-10 and 3-11 in the results chapter, the *gusB* fragments were brighter than the *lacY* fragments, this suggesting more DNA was present. Due to this, it was decided to do a 2:1 ratio of DNA sample into the PCR mix in attempt to gain a fusion. In addition to this, to encourage the GoTaq enzyme to replicate and fuse the fragments, its concentration was increased by adding 1 µl instead of the usual 0.5 µl. As seen on Figure 3-12 in the results chapter, this still seemed to make no difference to obtaining a fusion. However, the amount of unwanted bands has reduced throughout the PCR troubleshooting. Unfortunately, due to time constraint the

troubleshooting had to be stopped. Nonetheless, understanding the theory of PCR and troubleshooting procedures have improved throughout the project and have been vital in understanding how to improve for future research.

4.2. Challenges faced

4.2.1. Primer design error

For designing the primers used in this project, a protocol was followed (refer to Method) to result in successful bands being present on the agarose gel. Initially this was a difficult task due to the high content of cytosine and guanine residues in the *lacY* and *gusB* genome. This in turn added a bit of complication as the primers were designed to be within 1 degree of each other in attempt to increase the chances of successful annealing during PCR. However, due to the different amount of guanine and cytosine bases in each primer, it led to annealing temperatures being within 2 degrees of each other.

One of the major challenges throughout this project was the struggle to obtain the fusion formed of *lacY* and *gusB* after correcting the primer design error (see Section 3.2. in Results). As there were numerous factors which could have influenced the success of the PCR, there were many troubleshoots involving altering these factors. However, it became evident that altering one factor made no significant difference to the lack of fusion and it may have been due to a combination of factors. It proved difficult as various factor combinations were tested, however it could be refined in future work.

4.2.2. Unwanted bands and smearing

Another challenge was having multiple unwanted extra bands within samples. Samples were treated with *Dpn*I which targets the methylated areas on the DNA (Lacks and Greenberg 1975). They were also purified with the Qiagen PCR Purification kits. However, very faint extra bands were still visible on the gel. Consequently, this could have led to non-specific binding during PCR and result in an increase of extra bands. Moreover, smearing has been evident throughout the PCR results. Despite steps were taken to reduce this contamination, it remained unclear what was the exact cause of this. Therefore, samples underwent gel extraction after PCR purification in attempt

to reduce these two issues and get rid of unwanted DNA/ possible contaminants.

4.2.3. *Taq* polymerase

By running gradient PCR, the aim was to find the optimum temperature for GoTaq polymerase to fuse the two 1st step PCR fragments and replicate the fragment. As the gradient ranged from 64- 54 degrees, this could have impacted *Taq* polymerase's fidelity as lowering the temperature can encourage non-specific binding. Furthermore, GoTaq polymerase does not have proof reading ability, thus if an error was made in the sequence, it would still be amplified (Huang *et al.* 1992; Kunkel 1992; Cline *et al.* 1996). This may have resulted in the extra bands evident on the agarose gels.

4.2.4. Nanodrop

The nanodrop spectrophotometers was helpful for giving an indication on DNA concentration and purity. However, it may have not always been accurate with its readings. The A260/280 ratio gives an indication of the purity of the sample with 'pure' samples being around 1.8-2.0 (Teare *et al.* 1997; Desjardins and Conklin 2010). However, all proteins absorb a certain wavelength, thus when taking the PCR fusion attempt samples, it could have given an inaccurate reading due to additional proteins other than the fusion being present in the sample or due to contamination. Therefore the nanodrop readings was taken as estimations and used along with the estimation of DNA concentrations from the agarose gel bands.

4.3. Future research

Despite the aim of this project not being fulfilled, this project has provided useful information which would assist it progressing further and completing the aim in the future. The PCR troubleshoots carried out during this project have provided a good starting guideline for future work. In addition to this, the future work can focus on clearing up the DNA, reducing the extra bands and smearing and ensuring a fusion is formed with the first 6 transmembranes of *lacY* and the 6 transmembranes of *gusB.*

Furthermore, the functionality of potential transformants should be assessed using chromogenic substrates and spectrophotometric monitoring. The chromogenic substrates which would be used for this testing are 5-bromo-4-chloro-3-indoyl glucuronide (X-Gluc) and *p*-nitrophenyl-*β*-D-glucuronide (pNPG). If transportation is present, *β*-D-glucuronidase would cleave these glucuronides, resulting in a particular colour change; cleaved X-Gluc and pNPG resulting in a blue coloured aglycone sediment and yellow coloured soluble *p*-nitrophenol (pNP) respectively. The pNP colouring isn't as evident to the naked eye as the X-Gluc, thus the absorbance intensity should be measured using a spectrophotometer at 405nm wavelength. These readings directly correlated to the concentration of pNP.

Another aspect on improving future work may be using a thermostable enzyme with polymerase function which has 3'-5' proof reading abilities and higher fidelity than *Taq* polymerase. One potential enzyme may be the *Pfu polymerase (*Cline *et al.* 1996; McInerney *et al.* 2014*).* This could assist in reducing non-specific binding and mutational fragments and therefore increasing the chances of the correct fragment being replicated.

Future PCR runs should also be focussed on forming a fusion with the transmembranes swapped round i.e first 6 transmembranes of *gusB* and last 6 transmembranes of *lacY.* Furthermore, these fusions should be repeated but with XylE instead of LacY. LacY and XylE have substrate binding sites in different transmembranes (refer to Chapter 1), yet it is unknown in GusB. Therefore by fusing each half with GusB and testing its functionality using chromogenic glucuronides, this could narrow down the possible substrate binding sites by indicating which transmembranes they are located in. Moreover, as structures of XylE and LacY have been crystallised, future experiments could indicate which structure GusB is more similar to as its 3D structure is currently unknown and could assist with crystallising GusB's structure.

Additionally, future experiments should investigate the expression of N-domain (first 6 transmembranes) and C-domain (last 6 transmembranes) separately as this could give an indication to the possible 3D structure of GusB. In addition to this, if the domains can be independently expressed, it

could indicate that the cytoplasmic loop is not functionally significant and investigations could be focused on the specific transmembranes, their function and topology.

The biotechnology industry is a growing industry, especially in drug design and biosensors. Once the specific binding sites in the glucuronide transporter have been located, this could prove an important finding when designing biosensors. Genetically engineered GusB with specific binding sites could act as a biosensor to detect drug use/ drug cheating. Glucuronides are products formed during the detoxification pathway and one way of disposing them is via urine. Biosensors could be designed to detect glucuronides produced by the detoxification of certain drugs in the urine, even in low concentrations. Furthermore, this method may become more favourable as it is less invasive than blood testing or taking saliva samples.

Chapter 5. CONCLUSION

This research project was designed in attempt to narrow down the possible transmembranes containing the substrate binding sites of the glucuronide transporter. Unfortunately, producing a fusion between the first 6 transmembranes of LacY and the last 6 transmembranes of GusB proved unsuccessful and therefore the aim could not be answered. Nonetheless, there was some success in terms of completing the objectives (see Chapter 1). Objectives 1 and 2, designing fusion primers and extracting DNA containing the *gusB* and *lacY* genes respectively were successfully completed. Objective 3, successfully forming a fusion between the *gusB* and *lacY* genes using the PCR method was partially successful. The fusion was formed before the primer error was corrected (see Results chapter). Unfortunately this proved difficult to achieve again after the correction. Further troubleshooting is required to obtain the fusion which could then be functionally tested and provide possible insight into the substrate binding site locations within GusB. As mentioned in the discussion, by understanding the importance of each 6 transmembrane bundle of GusB, it could bring scientists closer to determining the exact substrate binding sites locations and establishing the 3D structure of this protein which would impact the science industry.

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Chapter 7. APPENDIX

Gus Operon

CTGGTCAGAAATATGGCGTTTGACCTGGGTGAAAAAAATATTCGGGTAA
ATGGCATTGCGCCGGGGGCAATATTAACCGATGCCCTGAAATCCGTTAT
TACACCAGAAATTGAACAAAAAATGTTACAGCACACGCCGATCAGACGT
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CTGCTGCGAGCTGGGTAAGCGGACAAATTCTCACCGTCTCCGGTGGTG
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GTGTTGTTTACCGGGATAAACTCATCAACGTCTCTGCTAAATAACTGGCA
GCCAAATCACGGCTATTGGTTAACCAATTTCAGAGTGAAAAGTATACGAA
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GCCAGAGAGATTTTTTCAGAAAATGGATTTCACAGTGCCTCGATGAAAGC
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TCGCCGATGCAGATATTCGTAATTATGCGGGCAACGTCTGGTATCAGCG

CGAAGTCTTTATACCGAAAGGTTGGGCAGGCCAGCGTATCGTGCTGCGT

TTCGATGCGGTCACTCATTACGGCAAAGTGTGGGTCAATAATCAGGAAG

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CGTATGTTATTGCCGGGAAAAGTGTACGTATCACCGTTTGTGTGAACAAC

GAACTGAACTGGCAGACTATCCCGCCGGGAATGGTGATTACCGACGAAA

ACGGCAAGAAAAAGCAGTCTTACTTCCATGATTTCTTTAACTATGCCGGG

ATCCATCGCAGCGTAATGCTCTACACCACGCCGAACACCTGGGTGGAC

GATATCACCGTGGTGACGCATGTCGCGCAAGACTGTAACCACGCGTCTG

TTGACTGGCAGGTGGTGGCCAATGGTGATGTCAGCGTTGAACTGCGTG

ATGCGGATCAACAGGTGGTTGCAACTGGACAAGGCACTAGCGGGACTTT

GCAAGTGGTGAATCCGCACCTCTGGCAACCGGGTGAAGGTTATCTCTAT

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GCAACGCGTAAACTCGACCCGACGCGTCCGATCACCTGCGTCAATGTAA

TGTTCTGCGACGCTCACACCGATACCATCAGCGATCTCTTTGATGTGCT

GTGCCTGAACCGTTATTACGGATGGTATGTCCAAAGCGGCGATTTGGAA

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TGGTACTGGTGCAAAACCTGGTTGGTACTGTGGCATCGGCACCGCTGGT
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TGGTCACTGCCGGTGGCGTTGGTTGCGTTGGCCATCGCTTCAATTGGTC
AGGGCGTTACCATGACCGTGATGTGGGCGCTGGAAGCTGATACCGTAG
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ACTATTCTCATTTACCCGTAAATGCGGTCAGGCAATCGGAGGTTCAATTC
CTGCCTTTATTTTGGGGTTAAGCGGATATATCGCCAATCAGGTGCAAAC
GCCGGAAGTTATTATGGGCATCCGCACATCAATTGCCTTAGTACCTTGC
GGATTTATGCTACTGGCATTCGTTATTATCTGGTTTTATCCGCTCACGGA
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CAGGAGCTGTCAGTTTGAGTTGCGCGTTCGCCTGCGCGACCATGTCTTC
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ACTACGCCTAGCGCAACAATGACGCCTACCGCTACCAGCGATTTATTCA
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TTATAAGCCTTTAATAAGCTT

Lac Operon

GACACCATCGAATGGCGCAAAACCTTTCGCGGTATGGCATGATAGCGCC
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TCGGTGATAACGTACTGATTGCACCCAACGTTACTCTTTCCGTTACGGGA
CACCCTGTACACCATGAATTGAGAAAAAACGGCGAGATGTACTCTTTTCC
GATAACGATTGGCAATAACGTCTGGATCGGAAGTCATGTGGTTATTAATC
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CACAAAAGACATTCCACCAAACGTCGTGGCGGCTGGCGTTCCTTGTCGG
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CGGAAAACGTACTGGTGCAAAACGCAGGGTTATGATCATCAGCCCAACG
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GCGCAACAACGGCAGGCAGCGCCACCAGGCGAGTGTTTGATACCAGGT
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CCGCCCATCAGAGCCGCGGACCACAGCCCCATCACCAGTGGCGTGCGC
TGCTGAAACCGCCGTTTAATCACCGAAGCATCACCGCCTGAATGATGCC
GATCCCCACCCCACCAAGCAGTGCGCTGCTAAGCAGCAGCGCACTTTG
CGGGTAAAGCTCACGCATCAATGCACCGACGGCAATCAGCAACAGACT
GATGGCGACACTGCGACGTTCGCTGACATGCTGATGAAGCCAGCTTCC
GGCCAGCGCCAGCCCGCCCATGGTAACCACCGGCAGAGCGGTCGAC

Primer Design

>gusB

MGALFLLSYYTDVAGVGAAAAGTMLLLVRVFDAFADVFAGRVVDSVNTRW
GKFRPFLLFGTAPLMIFSVLVFWVPTDWSHGSKVVYAYLTYMGLGLCYSLV
NIPYGSLATAMTQQPQSRARLGAARGIAASLTFVCLAFLIGPSIKNSSPEEMV
SVYHFWTIVLAIAGMVLYFICFKSTRENVVRIVAQPSLNISLQTLKRNRPLFML
CIGALCVLISTFAVSASSLFYVRYVLNDTGLFTVLVLVQNLVGTVASAPLVPG
MVARIGKKNTFLIGALLGTCGYLLFFWVSVWSLPVALVALAIASIGQGVTMTV
MWALEADTVEYGEYLTGVRIEGLTYSLFSFTRKCGQAIGGSIPAFILGLSGYI
ANQVQTPEVIMGIRTSIALVPCGFMLLAFVIIWFYPLTDKKFKEIVVEIDNRKK
VQQQLISDITN

>lacY

MYYLKNTNFWMFGLFFFFYFFIMGAYFPFFPIWLHDINHISKSDTGIIFAAISLF
SLLFQPLFGLLSDKLGLRKYLLWIITGMLVMFAPFFIFIFGPLLQYNILVGSIVG
GIYLGFCFNAGAPAVEAFIEKVSRRSNFEFGRARMFGCVGWALCASIVGIMF
TINNQFVFWLGSGCALILAVLLFFAKTDAPSSATVANAVGANHSAFSLKLALE
LFRQPKLWFLSLYVIGVSCTYDVFDQQFANFFTSFFATGEQGTRVFGYVTT
MGELLNASIMFFAPLIINRIGGKNALLLAGTIMSVRIIGSSFATSALEVVILKTLH

MFEVPFLLVGCFKYITSQFEVRFSATIYLVCFCFFKQLAMIFMSVLAGNMYES
IGFQGAYLVLGLVALGFTLISVFTLSGPGPLSLLRRQVNEVA

Fusion of first 6 transmembranes of lacY with last 6 transmembranes of gusB

MYYLKNTNFWMFGLFFFFYFFIMGAYFPFFPIWLHDINHISKSDTGIIFAAISLF
SLLFQPLFGLLSDKLGLRKYLLWIITGMLVMFAPFFIFIFGPLLQYNILVGSIVG
GIYLGFCFNAGAPAVEAFIEKVSRRSNFEFGRARMFGCVGWALCASIVGIMF
TINNQFVFWLGSGCALILAVLLFFAKTDAPSSATVANAVGANPSLNISLQTLK
RNRPLFMLCIGALCVLISTFAVSASSLFYVRYVLNDTGLFTVLVLVQNLVGTV
ASAPLVPGMVARIGKKNTFLIGALLGTCGYLLFFWVSVWSLPVALVALAIASI
GQGVTMTVMWALEADTVEYGEYLTGVRIEGLTYSLFSFTRKCGQAIGGSIP
AFILGLSGYIANQVQTPEVIMGIRTSIALVPCGFMLLAFVIIWFYPLTDKKFKEI
VVEIDNRKKVQQQLISDITN

GusB and LacY LALIGN

```
Waterman-Eggert score: 85;  23.7 bits; E(1) <  0.013
19.7% identity (51.3% similar) in 304 aa overlap (1-278:23-
298)

                10        20        30        40
50
gusB   MGALFLLS--YYTDVAGVGAAAAGTMLLLVRVFDAFADVFAGRVVDSVNTR----
WG---
       ::: : .   .  :.  .. . .: ..  . .:. . . . : . :... :
:
lacY
MGAYFPFFPIWLHDINHISKSDTGIIFAAISLFSLLFQPLFGLLSDKLGLRKYLLWIITG
            30        40        50        60        70
80

                60        70        80        90       100
gusB   ---
KFRPFLLFGTAPLMIFSVLVFWVPTDWSHGSKVVYAYLTYMGLGLCYSLVNIPYGSL
          : ::..:  .::. ...::         :: :   ::     :.:..
..
lacY   MLVMFAPFFIFIFGPLLQYNILV---------GSIVGGIYL-----
GFCFNAGAPAVEAF
            90       100               110           120

      110          120       130       140       150       160
gusB   ATAMTQQPQ---
SRARLGAARGIAASLTFVCLAFLIGPSIKNSSPEEMVSVYHFWT---I
          .... .   .:::. .  : :   ..: . :    .:.:.        . ::
lacY   IEKVSRRSNFEFGRARMFGCVGWALCASIVGIMF----TINNQ--------
FVFWLGSGC
      130       140       150       160                   170

            170       180       190           200         210
gusB   VLAIAGMVLYFICFKSTRENVVRIVAQP----SLNISLQTLKRNRPLFM--
LCIGALCVL
       .: .: ....      .  .:.  :.      ::...:. ... .  :.
::. :.
lacY
ALILAVLLFFAKTDAPSSATVANAVGANHSAFSLKLALELFRQPKLWFLSLYVIGVSCTY
        180       190       200       210       220       230

        220       230         240       250       260
270
gusB   ISTFAVSASSLFYVRYVLNDTG--
LFTVLVLVQNLVGTVASAPLVPGMVARIGKKNTFLI
        ..:  . ...:   .. .. :  .:  .. . .:... .   ..: .. :::
::..:.
lacY   -DVFDQQFANFFTSFFATGEQGTRVFGYVTTMGELLNA-
SIMFFAPLIINRIGGKNALLL
         240       250       260       270        280
290
```

```
gusB   GALL
       .. .
lacY   AGTI


>--
 Waterman-Eggert score: 61;  18.6 bits; E(1) <  0.36
22.1% identity (57.4% similar) in 68 aa overlap (227-285:86-
153)

        230       240       250              260       270
gusB   LFYVRYVLNDTGLFTVLVLVQNLVGTV-------
ASAPLVPGMVARIGKKNTFLIGA--L
       .:   ...    :.   .:: ..:: .       :.:: : ... ........:
.:   .
lacY
MFAPFFIFIFGPLLQYNILVGSIVGGIYLGFCFNAGAPAVEAFIEKVSRRSNFEFGRARM
          90       100       110       120       130       140

       280
gusB   LGTCGYLL
       .:  :. :
lacY   FGCVGWAL
         150

>--
 Waterman-Eggert score: 51;  16.5 bits; E(1) <  0.86
26.7% identity (60.0% similar) in 75 aa overlap (194-264:316-
389)

           200       210       220       230         240
250
gusB   ISLQTLKRNRPLFMLCIGALCVLISTFAVSASSLFYVRYV--
LNDTGLFTVLVLVQNLVG
       . :.::.  .  :.: .: .  . : : :  :. .:.     ... ... . ::.
:.
lacY   VILKTLHMFEVPFLL-
VGCFKYITSQFEVRFSATIYLVCFCFFKQLAMIFMSVLAGNMYE
         320       330       340       350       360
370

              260
gusB   TVA--SAPLVPGMVA
       ...  .: :: :.::
lacY   SIGFQGAYLVLGLVA
          380
```

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

Plasmid Digest

Bournemouth University *COSHH ASSESSMENT FORM*

1. Assessor:	2. Assessment Date:	3. Assessment Review:		
WJ Liang	16/02/2017	**Next Review Date:**	**Reviewed on: Date:**	**Reviewed By:**
4. Summary of process or method (or make specific reference to written protocol to be used) :				
Culturing bacteria , DNA extraction from bacterial cultures, PCR amplification and fusion, electrophoresis, making transformants, restriction digest, functionality test				
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)		
Mixing chemicals using pipettes, handling bacteria, weighing powders		Staff and students in lab		
7. Duration of Exposure (minutes, hours and how often):		**8. Location and Conditions of Use** (e.g. lab, room, temp etc.)		
3- 8 hours – 3 days a week		Dorset House DNA Lab		

	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	Multiple *E. coli* strains	100ml	N/A	Non-pathogenic	Eye/Skin contact, swallowing, inhalation	N/A
B	LB agar	2L	N/A	Not hazardous	Eye/Skin contact, swallowing, inhalation	N/A
C	$CaCl_2$	500g	N/A	Irritating to eyes - Wear goggles and gloves to avoid contact with skin	Eye/Skin contact, swallowing, inhalation	N/A
D	Ampicillin	10g	N/A	Irritating to eyes, respiratory system and skin.	Eye/Skin contact, swallowing, inhalation	N/A

Risk Assessment

				- Wear goggles and gloves, if any contact with eyes rinse immediately with water and seek medical advice		
E	DNTP (10mM)	>10ml	N/A	Not hazardous	Eye/Skin contact, swallowing, inhalation	N/A
F	Restriction enzymes such as EcoRI, HindIII and bamHI	10ml>	N/A	Not hazardous	Eye/Skin contact, swallowing, inhalation	N/A
G	Agarose	100g	N/A	Not hazardous	Eye/Skin contact, swallowing, inhalation	N/A
H	TAE	2-4L	N/A	Irritating to eyes, respiratory system and skin. Wear goggles and gloves, if any contact with eyes rinse immediately with water and seek medical advice	Eye/Skin contact, swallowing, inhalation	N/A

15. Control Measures

Following Lab procedures – no eating or drinking in lab, tie your hair back, wipes spillages and clean surfaces, wear protective equipment, wash hands at the end of the lab session

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, H	Inhalation	All	Solid	A, B, G	Flammable		Low	All	Low	
Toxic		Sensitiser		Skin Contact	All	liquid	A-H	Highly flammable		Medium		Medium	
Corrosive		Carcinogen		Eye Contact	All	Gas/vapour		Extremely flammable		High		High	
Harmful		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 and seek medical advice	Wash immediately with water and rinse thoroughly	Rinse open eye for a few minutes under running water. If symptoms persist seek medical advice	Wash mouth with water and seek medical advice	Seek urgent medical attention
23. Spillage Procedures: →	**Ensure sufficient ventilation and clear up as stated in lab procedure if possible**			
24. Disposal Arrangements				
Collection	**Swill down sink**	**Evaporation**	**In normal waste**	**Other**
X	X		X	
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:
- Briefing before practical - Supervisor has acknowledged this assessment

Initial Review

Notes: *The 1st Review is an important milestone in the programme of study, which must be completed by 6 months (FT) or 12 months (PT).*

The purpose is to enhance the original proposal, to demonstrate your understanding of the research project, to identify any H&S / ethical issues; and to establish the timescale for the research and agreed research plan and future activities. Progress is discussed with and reviewed by the Supervisory Team and assessed by an Independent Academic.

Continuation is dependent on successful completion of this stage.

Text fields will expand as required. Use the TAB key to move to the next field.

1. POSTGRADUATE RESEARCHER DETAILS		
Student number	4538459	
Full name	Christine Ciocan	
Mode of study	Full Time √	Part Time
Date of registration	23/01/2017	

2. INITIAL REVIEW: RESEARCH TO DATE

Abstract (*c. 500 words)*

The major facilitator superfamily (MFS) is composed of various membrane transporters which can be located ubiquitously. Each transporter is involved in a crucial physiological process where it carries molecules across membranes by differing transport mechanisms. The transporters act as symporter, antiporter or uniporter in order to transport the molecules. In this research project, the focus will be on two secondary membrane transporters which share similar homologies but are involved in different processes. They are: Lactose permease LacY and the glucuronide transporter GusB. LacY is a well-known

secondary transport protein which facilitates the movement of lactose molecules across a membrane by utilising the movement of H^+ ions down the concentration gradient. GusB on the other hand plays a role in acquiring glucuronides for enterobacteria *Escherichia coli* for their survival. Contrarily, the structure of GusB is not as fully understood. In this project, the first 6 membranes of LacY will be fused with the last 6 transmembranes of GusB by undergoing fusion PCR. The functionality of this fusion would then be tested. If the results are successful and the fusion still transports molecules, it could suggest that; as previously predicted, the structures of GusB and LacY are similar. Therefore, this discovery (if successful), could bring scientists a step closer to understanding the glucuronide transporter and other MFS members.

Title of thesis

Investigating the functionality of a fusion between two major facilitator superfamily members; LacY and GusB using a fusion Polymerase Chain Reaction.

Background to the topic *(This is the context to your research and should include reference to the academic literature c. 500 words)*

The major facilitator superfamily (MFS) is one of the largest secondary carrier superfamilies in the natural world. It is compiled of various membrane transporter families that can be found in both prokaryotic and eukaryotic cells organisms. Examples of transporters which are members of the MFS include; glucose transporters (GLUT), lactose permease (LacY), xylose transporter (XylE), glucuronide transporter (GusB) and melibiose transporter (MelB).The members of the MFS can act as symporters, antiporters or uniporters in order to transport ions, substrates and other molecules across membranes (Reddy *et al.* 2012; Yan 2015; Quistgaard *et al.* 2016). Each transporter is involved in a different physiological process, yet structurally they can be quite similar. For this project two members of the MFS will be fused together and the functionality will be tested. The two MFS members

focussed on in this project are lactose permease and the glucuronide transporter.

Lactose permease is a well-studied transporter located in *Escherichia coli (E.coli).* It is a symporter that carries lactose across the phospholipid layer by utilising the movement of H^+ ions down the concentration gradient. This transporter is composed of twelve transmembrane helices with N and C terminal domains (Abramson *et al.* 2003). Due to the fact that this protein has been well studied, scientists have been able to formulate a crystal structure (Stroud 2007) and reveal its conformation and binding sites when transporting lactose.

On the other hand, GusB is not as well-known as LacY. *E.coli* which survive in the human gut have used this transporter in order to survive. GusB transports glucuronides, which are formed during glucuronidation, into the *E.coli* to use as a carbon source. Similarly to LacY, GusB has twelve transmembrane α-helices (Liang 1992; Ishii 2013 p.199) with N and C domains. However the substrate recognition sites are not yet known for this protein. Therefore, by applying the existing knowledge from LacY, the aim of this study is to narrow down the possible recognition site locations.

Abramson, J., Smirnova, I., Kasho, V., Verner, G., Kaback, H.R. and Iwata, S., 2003. Structure and mechanism of the lactose permease of Escherichia coli. *Science* [online], 301 (5633), 610-615.

Ishii, N., 2013. Two-Dimensional Crystalline Array Formation of Glucuronide Transporter from Escherichia coli by the Use of Polystyrene Beads for Detergent Removal. *The Journal of Membrane Biology* [online], 246 (1), 199-207.

Liang, W.J., 1992. The Glucuronide Transport System of Escherichia coli. Thesis (PhD). University of Cambridge.

Quistgaard, E. M., Low, C., Guettou, F. and Nordlund, P., 2016. Understanding transport by the major facilitator superfamily (MFS):

structures pave the way. *Nature Reviews: Molecular Cell Biology* [online], 17 (2), 123-132.

Reddy, V. S., Shlykov, M. A., Castillo, R., Sun, E. I. and Saier Jr, M. H., 2012. The major facilitator superfamily (MFS) revisited. *The FEBS journal* [online], 279 (11), 2022-2035.

Stroud, R. M., 2007. Transmembrane transporters: An open and closed case. *Proceedings National Academy of Sciences of the United States of America* [online], 104 (5), 1445-1446.

Yan, N., 2015. Structural Biology of the Major Facilitator Superfamily Transporters. *Annual Review of Biophysics* [online], 44, 257-283.

Research questions – the aims of the research *(This should take the form of a short, achievable statement(s) informing the reader of the purpose of the study c. 500 words)*

The aim of this research is to assess the functionality of a fusion formed between two members of the major facilitator superfamily (MFS) members; in this case the fusion composed of LacY and GusB.

Objectives of the research *(These must be measurable and achievable c. 500 words)*

The overall objectives for this research are:

- To design primers for the fusion between *gusB* and *lacY*
- To extract and purify plasmid DNA containing active *gusB* and *lacY* genes
- To successfully form a fusion of *gusB* and *lacY* and amplify it through PCR
- To clone the transformants
- To assess the functionality of the transformants using chromo-genic glucuronides/ lactose

Outline of proposed methodology *(Including data collection, analysis and sampling, c. 500 words)*

This research will occur in a laboratory. It will consist of fusing two separate transporters genes (*lacY* and *gusB*) that belong to the same family. This will be carried out using two step PCR to fuse the first half (6 transmembranes) of LacY with the last 6 membranes of GusB. The first stage of PCR involves fusing the forward primer of *lacY* with the reverse primer of *lacY/ gusB* linker and the *lacY/gusB* linker forward with the *gusB* reverse respectively by using appropriate annealing temperatures. Once these fragments have been formed, the second stage of PCR will be carried out in an attempt to fuse the two separate fragments formed previously. Electrophoresis would be performed after every PCR in order to confirm base pair sizes of the fragments and to illustrate the DNA quality. This fused gene will then be ligated into a vector which had previously undergone restriction digest. Once the gene is successfully ligated, cloning will be the next part of the process in which functionality would be tested and the clone will be sent for sequencing.

Please describe any ethical, health & safety or risk issues related to your proposed study *(Outline what they are and how they will be addressed c. 250 words each)*

Ethical issues	N/A
Health & safety issues	Using *E.coli* strains, using chemical reagents, using biological reagents
Other risk Issues	

Proposed timescale for the work *(Outline the plan for completing the work within the period of registr ation. Identify all major milestones of the work and indicate how long each will take. c. 250 words)*

The milestones achieved so far are:

- Decided on topic and project aim
- Primers for the fusion have been designed
- Lab work has started- Miniprep has been completed, DNA extraction has been completed, the current stage of this project is using PCR in order to obtain a fusion.

For this project, the aim is to hand in the final piece of work in November. As it currently stands the project is achievable due to existing experience with the methodology, lab procedures and troubleshooting from previous undergraduate IRP research. The idea is to complete the practical work by August/ September time to allocate enough time for the write up.

Agreed research plan and activities for the forthcoming months
(Outline what you are planning to achieve in the forthcoming months, identify all major milestones of the work and indicate how long each will take c. 250 words)

In the next few months, the main focus will be to complete the practical work as well as focusing on completing the introduction for the thesis. The major milestones are:

- Undergo stage 1 and stage 2 of PCR in order to fuse the two transporters (2-4 months)
- Once the transporters are fused, performing a restriction digest and carry out ligation (1 month)
- Start writing up the thesis- working on the introduction and method (2-4 months)
- Continue reading papers

How will your research contribute to new knowledge in this field?
(*c. 250 words)*

This research could impact the current level of knowledge surrounding major facilitator superfamily members. By fusing different

parts of transporters (some already known structurally and others not) together which share similar homology, information could be applied to the less understood proteins such as GusB and other transporters in order to understand their functions and crystal structures. Furthermore, the theory and results from this project could be applied to other MFS members to decipher their structure.

In addition to this, the research could contribute to the biosensor industry. Biological sensors could be engineered to detect 'drug cheats' or disorders by monitoring levels of molecules that are transported by certain sensors. For example, a biosensor which is designed with LacY or GLUT 2 could be used to detect glucose/galactose malabsorption as the functionality rate of the biosensor could indicate the molecule levels.

3. COLLABORATIVE RESEARCH

Do you intend conducting fieldwork or research using private archives or working in collaboration with others?

Yes No X If relevant, a letter signifying appropriate permission will need to be appended.

Collaborating Establishment *A letter of support from the collaborating establishment confirming agreed arrangements must be attached. If no collaboration has been arranged, a brief explanation should be given in all cases.*	
Address of Collaborating Establishment	
Relationship between work to be undertaken in the collaborating establishment and at the University	

Lab Book

Continued from page number

Page number 1

5/4/17

1982 - T Maniatis → molecular cloning

LB media - per litre

Bactro-tryptone 10g
Bacto- yeast extract 5g
NaCl 10g

1. Poured 500 ml of distilled water into autoclaved glass bottle and placed on hot plate (with stirer inside)
2. Add the powders from above to the bottle and stir - after mixing, fill to 1 litre with distilled water
3. Autoclave media

LB agar media - per litre

Bacto-tryptone 10g
Bacto-yeast extract 5g
NaCl 10g
Agar 15g

Steps are the same as for LB media!

After autoclaving media, cool down media and then add ampicillin (if needed) before pouring into petri dishes

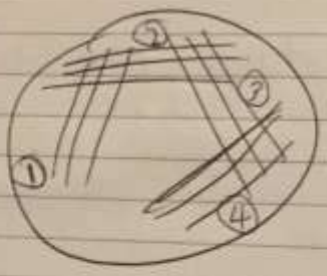
per litre of media = 1000 µl ampicillin

* only use ampicillin plasmid DNA - ~~pGEM~~ (pMJB33)
not genomic DNA (JM109)
- Petri dishes were left to cool on the side

Performed by	Date	Countersigned by	Date	Continued on page number
[signature]	5/4/17	[signature]	5/4/17	

Transfer + extraction → heat loop first to sterilise

1. The loop is placed in the "deep" and then streaked on agar place (3 streaks)
2. The loop is then placed into flame to sterilise (blue flame) and then dip in centre of petridish before streaking again → place lid after each streak
3. Repeat 2 more times



4. Inaubate overnight (37°C) ⇒ lid down, jelly up

★ Gas [redacted] wasn't working in our lab so we used ethanol burners

Innoculation 11/4/17 - 12/4/17

- Pour 10 ml of LB media into a sterilised centrifuge tube/flask
  ↳ add ampicillin for PMJB33 but not JM109!)
- Place foil on top (not too tight) and label strain
- When choosing colony from plates choose the colony of similar colour to the rest
- Sterilise top first (in flame) → then [redacted] pick a colony and collect with loop. Then place

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[redacted]	5/12/4/17	[redacted]	12/4/17	

loop into flask, leaving it for a few seconds before transferring colony by rotating loop into media quickly

- Once completed, place flasks in orbital shaker incubator for [redacted] overnight @ 37°C.

Blood and tissue kit – extracting genomic DNA (JM109)

12/4/17

1. Place 1ml of overnight media into autoclaved eppendorfs – do 4/5 eppendorfs
2. Centrifuge for 5 mins at 300 x g (190 rpm). Resuspend in 200 µl PBS. Add 20 µl proteinase K. [redacted]
3. Add 200 µl Buffer AL. Mix thoroughly by vortexing
4. Add 200 µl ethanol. Mix by thoroughly vortexing
5. Pipette the mixture into a DNeasy Mini spin column placed into a 2ml collection tube. Centrifuge at ≥ 6000 x g (8000 rpm) for 1 minute. Discard the flow-through collection tube. [redacted]
6. Place the spin column in a new 2ml collection tube. Add 500 µl Buffer AW1. Centrifuge for 1 min at ≥ 6000 x g. [redacted] Discard the flow-through and collection tube.

Performed by	Date	Countersigned by	Date	Continued on page number
[redacted]	12/4/17	[redacted]	12/4/17	

7. Place the spin column in a 2ml collection tube, add 500 µl buffer AW2, centrifuge for 3 min at 20 000 xg (14, 000 rpm). [redacted] Discard the flow-through and collection tube

8. Transfer the spin column to a new 1.5 ml or 2 ml microcentrifuge tube

9. Elute the DNA by adding 200 µl Buffer AE to the center of the spin column membrane Incubate for 1 min at room temperature (15-25°C) Centrifuge for 1 min at ≥6000 xg

Miniprep 13/4/17

1. Pillett 1-5 ml bacterial overnight culture by centrifugation at >8000 rpm for 3 mins @ room temp

2. Resuspend pelletted bacterial cells in 250 µl buffer P1 and transfer to a microcentrifuge tube
↳ vortex

3. Add 250 µl buffer P2 and mix thoroughly by inverting the tube 4-6 times until the solution becomes clear - do not proceed more than 5 mins
↳ if using Lyse blue reagent the solution became blue

4. Add 350 µl buffer N3 and mix immediately and thoroughly by [redacted] vortexing (hold first & last upside down) and then place in ice bath for 30 - 60 mins
↳ if using lyse blue reagent the solution will turn colourless

Performed by	Date	Countersigned by	Date	Continued on page number
[redacted]	13/4/17	[redacted]	13/4/17	

5. Centrifuge for 10 mins @ 13,000 rpm &
6. Apply 800µl supernatant from step 5 to the spin column by pipetting. Centrifuge for 30-60's and discard flow-through
7. Wash the spin column by adding ~~0.75ml / 750µl~~ 500µl Buffer PB. Centrifuge for 30-60's and discard flow through
8. Wash the spin column by adding 0.75ml / 750µl ~~buffer~~ buffer PE. Centrifuge for 30-60's and discard flow through.
9. Centrifuge for 1 min to remove residual wash buffer (PE)
10. Place the ~~column~~ column in a clean 1.5 eppendorf. Incubate for 5 mins and then to elute DNA add 50µl buffer EB to the center of the spin column, Let stand for 1 min and then centrifuge for 1 min.

- P1 buffer = Tris, EDTA, glucose, RNase, Lyse blue
- P2 buffer = SDS, alkaline
- N3 buffer = sodium/potassium acetate = neutralised immediately → place on ~~stay cold~~ ice after vortexing
  - ↳ DNA is stabled
  - ↳ Plasmid DNA to mix into solution = "swimming out"
- PB = binding buffer
- PE = wash buffer

Performed by	Date	Countersigned by	Date	Continued on page number
	13/4/17	[illegible]	13/4/17	

Continued from page number

Page number 6

Diluting primers

13/4/17

Need to make TE, to dilute primers
→ make in eppendorfs
2 µl EDTA (0.5 molar)*, 10 µl Tris (1 Molar)
988 µl water

- Add the various volumes according to the primer sheet
- Volume added is TE
- After adding solution, leave for at least 20 mins before starting PCR

Oligo name	volume added (100 pmol/µl)
LacY F1	320
LacY F2	299
LacY F3	284
gusB R1	318
gusB R2	399
lacYgusB F1	299
lacYgusB F2	358
lacYgusB F3	290
lacYgusB R1	321
lacYgusB R2	311
lacYgusB R3	266

* Dilute 100µM primers to 10µM: 1µl primer + 9µl H_2O

Nanodrop

19/4/17

- measuring DNA with DNA nanodrop → measure concentration of DNA. Programme = nucleic acids
- place 1µl distilled water on the 2 stands and take a blank reading.

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- Wipe first stand and place 1µl of DNA and click measure

Plasmid name	Conc (ng/µl)	260 reading (0.2 - 0.8 ↑)	260/280 ratio (1.8 - 2 ↑)
JM109 1	19.5	0.390	1.87
JM109 2	23.6	0.472	1.92
JM109 3	16.9	0.339	1.92
JM109 4	17.9	0.359	1.94
JM109 5 ★	19.7	0.395	1.86
JM109 6	13.9	0.271	1.94
★PTQ18 1	17.6	0.352	1.84
PTQ18 2	11.5	0.230	1.68
MJB33 1 -	157.3	3.074	1.87
MJB33 2	156.7	3.134	1.87
MJB33 3	174.7	3.495	1.87
MJB33 4	153.3	3.066	1.87
MJB33 5	150.8	3.017	1.88
MJB33 6 -	146.5	2.930	1.87
MJB33 1 water:DNA			
↳ MJB33 2:1	88.3	1.767	1.85
5:1	55.9	1.118	1.87
7:1	43.6	0.872	1.85
★ 8:1	39.9	0.797	1.81
MJB33 6			
↳ MJB33 3:1	34.4	0.688	1.84

Making dNTP 10mM → 1µM

Dilute 10µM to make 1µM = 9µl = H_2O, 10µl dNTP

Performed by [signature] Date 19/4/17

Countersigned by [signature] Date 19/4/17

Continued on page number

Continued from page number

Page number 4

PCR (first step PCR) 19/4/17

Setting up PCR:

1. H_2O = 33.5 µl
2. 5 x Flexi buffer = 10 µl
3. 1 µM dNTP = 1 µl
4. 25 mM $MgCl_2$ = 2 µl
5. 10 µM forward Primer = 1 µl
6. 10 µM Reversed Primer = 1 µl
7. DNA sample = 1 µl
8. Go Taq Polymerase = 0.5 µl

62.6 63.9 = 63.2

1. Lac4F1 + Lac4gusBR3
2. Lac4F2 61.8 + Lac4gusBR3 63.9 = 62.85
3. Lac4F3 64.5 + Lac4gusBR1 65.3 = 64.9
4. GusBR1 64.1 + Lac4gusBF1 65.3 = 64.7
5. GusBR2 62.1 + Lac4gusBF3 63.9 = 63

Ran PCR at 2 temperatures

1, 2, 5 were run at 63.4 °C
3, 4 were run at 64.3 °C

Gel electrophoresis

↳ TAE 1X solution
↳ 40 ml TAE, 0.64 g agarose, 4 µl SYBR safe
- run gel for 40 mins at 70V

Performed by [signature] Date 19/4/17 | Countersigned by [signature] Date 19/4/17 | Continued on page number

Continued from page number

Protein purification (Qiagen) 25/4/17

1. Add 5 volumes buffer PB to 1 volume of the PCR reaction and mix. If it goes orange/violet add protocol
2. To bind DNA, apply the sample to the column and centrifuge for 30-60's. Discard flow-through and place the column back in the same tube
3. To wash, add 750 µl buffer PE to the column and centrifuge for 30-60's. Discard flow-through and place the column back in the same tube
4. Centrifuge the column once more for 1 min to remove residual wash buffer
5. Place each column in a 1.5 eppendorf
6. To elute DNA, add 50 µl buffer EB (10mM: Cl, pH 8.5) or water (pH 7-8.5) to the center of the column and centrifuge the column for 1 min. For increased DNA concentration, add 30 µl elution buffer to the center of the membrane, let stand for 1 minute, and then centrifuge.

Purified sample #2 #3 #4 #5 as they had 'bands. → treated with DpnI: 5.5 µl extracted buffer, 1 µl DpnI and incubate at 80°C for 20 mins

Fusion PCR 26/4/17

32.5 µl = H_2O
10 µl = 5x flexi buffer
1 µl = 1mM dNTP
2 µl = 25mM $MgCl_2$
2 µl = PCR product forward
2 µl = PCR product reverse
0.5 µl = GoTaq polymerase

a = sample 2 + sample 5
b = sample 3 + sample 4
c = sample 2 + sample 4
d = sample 3 + sample 5

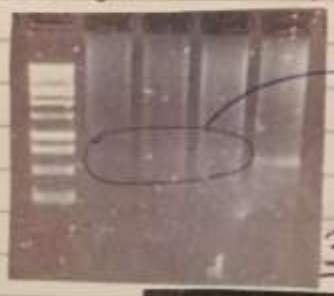
PCR nn @ 63.4 = a, c, d
64.3 = b

WRONG = DIDNT ADD TEMPLATE PRIMERS

Performed by [signature] Date 25/4/17 | Countersigned by [signature] Date 25/4/17 | Continued on page number

2/5/17

- Ran gel from fusion PCR from last week
- only sample d had a band but it might be the wrong size → band seems to be ~750bp?



→ faint bands

fusion troubleshoot #1

3/5/17

32.5 µl H_2O
10 µl flexi buffer
1 µl dNTP (1mM)
2 µl $MgCl_2$ (25mM)
2 µl PCR product forward
2 µl PCR product reverse
0.5 µl Go Taq

Temperatures were changed for this troubleshoot:
60.7°C and 65°C were the two chosen temperatures

a' = sample 2 + sample 5
b' = sample 3 + sample 4
c' = sample 2 + sample 4
d' = sample 3 + sample 5
} ran at 60.7°C

WRONG → DIDN'T ADD PLANT DNA PRIMERS

a^2 = sample 2 + sample 5
b^2 = sample 3 + sample 4
c^2 = sample 2 + sample 4
d^2 = sample 3 + sample 5
} ran at 65°C

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[redacted]	3/5/17	[redacted]	3/5/17	

fusion troubleshoot #2

9/05/17

31.5 µl or 33.5 µl H_2O
10 µl Flexi buffer
1 µl DNTP
3 µl or 1 µl $MgCl_2$
2 µl forward
2 µl reverse
0.5 µl Taq

Temp = 64°C

Sample #1 = sample 2 + 5
#2 = 3 + 4
#3 = 2 + 4
#4 = 3 + 5

} 3 µl $MgCl_2$, 31.5 µl H_2O

#5 = 2 + 5
#6 = 3 + 4
#7 = 2 + 4
#8 = 3 + 5

} 1 µl $MgCl_2$, 33.5 µl H_2O

not sure if 3 µl or 1 µl of $MgCl_2$ was added

WRONG → DIDN'T ADD FLANKING PRIMERS

↳ could explain the streaks
↳ could explain the bands in the incorrect locations

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[signature]	9/5/17	[signature]	9/5/17	

Trouble shoot #3 - fusion PCR 11/05/2017

Start over

H_2O = 30.5 µl
flexi buffer = 10 µl
dNTP = 1 µl
$MgCl_2$ = 2 µl
PCR forward flanking = 1 µl
PCR reverse flanking = 1 µl
DNA template = 4 µl (2 of each strand from 1st step)
Go taq = 0.5 µl

[redacted] fusion PCR started from scratch as realised that [redacted] outer primers weren't being added in fusion when it needed to be

a^3 = sample 2 + sample 5 + LacF3 + gusR1
b^3 = sample 3 + sample 4 + LacF3 + gusR1
c^3 = sample 2 + sample 4 + LacF3 + gusR1
d^3 = [redacted] sample 3 + sample 5 + LacF3 + gusR1

} 52.3°C

a^4 = sample 2 + Sample 5 + LacF3 + gusR1
b^4 = Sample 3 + sample 4 + LacF3 + gusR1
c^4 = sample 2 + sample 4 + LacF3 + gusR1
d^4 = sample 3 + sample 5 + LacF3 + gusR1

} 54.3°C

[redacted]

1, 5, 9, 13 = Sample 2, sample 5 + Lac4F3 + gusBR1
2, 6, 10, 14 = sample 3, sample 4 + Lac4F3 + gusBR1
3, 7, 11, 15 = sample 2, sample 4 + Lac4F3 + gusBR1
4, 8, 12, 16 = sample 3, sample 5 + Lac4F3 + gusBR1

★ see page 15 for gel images

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Troubleshoot #4

16/5/17

30.5 µl H_2O
10 µl flexi buffer
1 µl DNTP
2 µl $MgCl_2$
1 µl PCR forward flanking
1 µl PCR reverse flanking
4 µl DNA template
0.5 µl Go Taq

32.5 µl H_2O
10 µl flexi buffer
1 µl DNTP
2 µl $MgCl_2$
1 µl PCR forward flanking
1 µl PCR reverse flanking
2 µl DNA template
0.5 µl Go Taq

Not running gel due to realisation that PCR programming has been wrong

Fusion PCR from scratch

8/6/17

30.5 µl H_2O
10 µl flexi buffer
1 µl DNTP
2 µl $MgCl_2$
1 µl PCR forward
1 µl PCR Reverse
4 µl DNA
0.5 µl Go Taq

32.5 H_2O
10 µl flexi
1 µl DNTP
2 µl $MgCl_2$
1 µl forward
1 µl Reverse
2 µl DNA
0.5 µl Go Taq

1–3 ran @ 62.6°C
4 ran @ 63.1°C
9–11 ran @ 64.3°C
12 ran @ 64.9°C

5–7 ran @ 626°C
8 ran @ 63.1°C
13–15 ran @ 643°C
16 ran @ 64.9°C

Performed by [redacted] Date 8/6/17

Countersigned by [redacted] Date 8/6/17

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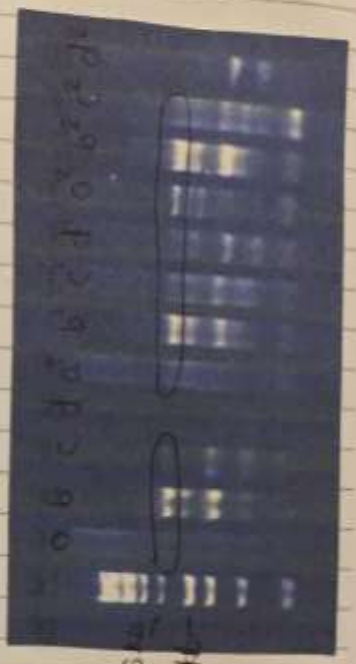
PCR Troubleshoot #2. 14/6/17

samples a-d ran @ [redacted] 64°C

[redacted] 31.5 µl H_2O
10 µl flexi buffer
1 µl DNTP
1 µl $MgCl_2$
1 µl forward primer
1 µl reverse primer
4 µl DNA
0.5 µl Go Taq



samples a'-d' ran @ 58°C

30.5 µl H_2O
10 µl flexi buffer
1 µl DNTP
2 µl $MgCl_2$
1 µl forward primer
1 µl reverse primer
4 µl DNA
0.5 µl Go Taq

samples a^2 - d^2 ran @ 58°C

~~30~~ 31.5 µl H_2O
10 µl flexi buffer
1 µl DNTP
1[redacted] µl $MgCl_2$
1 µl forward primer
1 µl reverse primer

- there are fusion bands with the correct banding size present. However there are multiple other bands and therefore the PCR troubleshoots will focus on reducing these [redacted] other bands.

Performed by [redacted] Date 14/6/17

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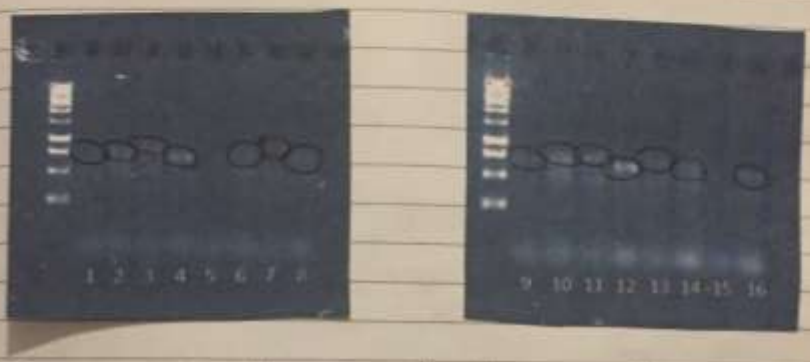
4ul DNA
0.5ul Go Taq

Gel images from fusion PCR from scratch (8/6/17)



- There are bands present, however they are incorrect sizes which indicates that there was no fusion for these PCR troubleshoots. Number 5 and 15 had no fusion or even bands

fusion PCR - changing DNA amount 27/6/17

- a', b, c, d' worked best from last PCR

for a' + b' + c' : 33.5 ul H_2O
10 ul flexi
1 ul dNTP
1 ul $MgCl_2$
1 ul forward
1 ul revers
2 ul DNA

a' = 58°C
b' + c' = 64°C

0.5 ul GoTaq

Performed by [redacted] Date 27/6/17

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d" : 32.5 µl H_2O
10 µl flexi
1 µl DNTP
d" = 58°C 2 µl $MgCl_2$
1 µl forward
1 µl reverse
2 µl DNA
0.5 µl GoTaq

If ya can see faint band on gel then its about 5ng

Options following PCR (seen above):

- Increase cycle number in order to improve faint bands → lower cycle numbers to v. smears
- Play around with annealing temps
- Repeat with unchanged Mg^{2+} + DNA
- Maybe reduce template concentration to try and reduce smears?

PCR troubleshoot - reduced cycle numbers (to 25) 6/7/17

for a", b", c" = 58°C

33.5 µl H_2O
10 µl flexi
1 µl DNTP
1 µl $MgCl_2$
1 µl forward
1 µl reverse
2 µl DNA
0.5 µl GoTaq

Performed by [signature] Date 6/7/17 | Countersigned by [signature] Date 6/7/17 | Continued on page number

For d° = 64°C

32.5µl H_2O
10µl Flexi
1µl DNTP
2µl $MgCl_2$ → not sure if put 1µl or 2µl by error
1µl forward
1µl reverse
2µl DNA
0.5µl Go Taq

PCR troubleshoot using PCR mix (2X) 5/7/17

- for this PCR reaction a prepared PCR master mix (2X) #K0171 from thermo fisher was used.

- Mix contains: 0.05 U/µl Taq DNA polymerase, reaction buffer, 4mM $MgCl_2$, 0.4mM of each DNTP
- water (nuclease free) was used

30 cycles used

Samples 1°-4° = 25µl PCR mix
1µl forward primer
1µl Reverse primer
2µl DNA template
21µl H_2O

1°-3° = 58°C
4° = 64°C

samples 1-4 = 25µl PCR mix
1µl forward primer
1µl reverse primer
4µl DNA template
19µl H_2O

1-3 = 58°C
4 = 64°C

Performed by [signature] Date 5/7/17

Countersigned by [signature] Date 6/7/17

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Gel extraction of fusions 2/8/17.

- Ran gel for 60 mins @ 70V
- only put 4 µl of DNA as the previous bands were good bands → bands were smeary 2/8/17, will run another gel.
- Gel to then be cut with sharp scalpel and Gel extraction kit (Cat No 28704) protocol followed

Protocol:

1. Excise the DNA fragment from the agarose gel with a clean, sharp
2. Weigh the gel slice in a colorless tube/eppendorf. Add 3 volumes Buffer QG to 1 volume gel
3. Incubate at 50°C for 10 min (or until the gel slice has completely dissolved). Vortex the tube every 2-3 min to help dissolve gel. After the gel slice has dissolved completely, check that the color of the mixture is yellow (similar to Buffer QG without dissolved agarose)
4. Add 1 gel volume isopropanol to the sample and mix
5. Apply sample to the QIAquick column and centrifuge for 1 min. Discard flow-through and place the column back in the tube
6. If the DNA will subsequently be used for sequencing, in vitro transcription or microinjection, add 500 µl Buffer QG to the spin column and centrifuge for 1 min. Discard flow through.
7. To wash, add 750 µl Buffer PE to the column and centrifuge for 1 min.

↳ if the DNA will be used for salt-sensitive applications (e.g. Sequencing, blunt-ended ligation), let the column stand 2-5 min after addition of Buffer PE then

Performed by	Date	Countersigned by	Date	Continued on page number
[redacted]	2/8/17	[redacted]	2/8/17	

[redacted] centrifuge for 1 min. Discard flow through.

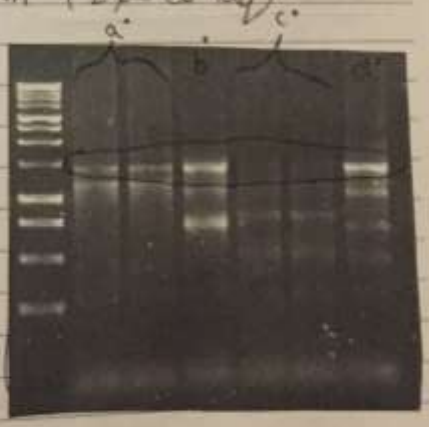
8. Place column in a clean eppendorf
9. To elute DNA, add 50 µl Buffer EB to the center of the column and centrifuge for 1 min. for increased DNA concentration, add 30 µl Buffer EB to the centre of the column, let the column stand for 1 min, then centrifuge for 1 min. After the addition of Buffer EB, to the membrane, increasing the incubation time to up to 4 min can increase the yield of purified DNA

Gel extraction of fusions

30/8/17

- Tried extraction on 24/8 but gel fell apart.
- Ran gel for 1 hr today and loaded the following:

- 2 bands of sample a° (8µl DNA + 2µl Go Taq)
- 1 band of sample b° (8 µl DNA + 2 µl Go Taq)
- 2 bands of sample c° (8 µl DNA + 2 µl Go Taq)
- 1 band of sample d° (8 µl DNA + 2 µl Go Taq)



It was then decided to cut out [redacted] sample a°, b° and d°

↳ Sample c° had very faint bands and it was decided to leave it
↳ scalpel was used to cut the bands on light box
↳ eppendorfs were weighed without gel inside and [redacted] then with gel inside and the difference was calculated

PTO →

Performed by	Date	Countersigned by	Date	Continued on page number
[redacted]	30/8/17	[redacted]	30/8/17	

	eppendorf Without gel inside	eppendorf with gel inside	Difference	QG amount
①	0.988	1.262	0.274	0.822
②	0.994	1.108	0.114	0.342
④	0.993	1.103	0.110	0.330

Samples in blue box in freezer → next step is R digest.

Restriction digest of PCR product + Plasmid (PTG18) 18/9/17

37 µl H_2O
5 µl 10 x Buffer 2
1 µl BSA
5 µl plasmid PTG18 / PCR product
1 µl EcoRI
1 µl HindIII

Digest was incubated at 37°C for 4 hours and a gel was ran
↳ PTG18 had 1 band on gel = digest went well

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↳ PCR products had no bands visible = too little DNA in digest or DNA is lost
↳ Decision is to run gel extracted samples on a gel to confirm DNA is still present and then digest will be done with more PCR product sample

Redo of Digest for PCR products. 21/9/17

- ran gel extracted samples from 30/8/17 on gel at 70V for 40 mins to see if DNA is present.
↳ there were no bands visible on the gel so a nanodrop was done to see if any DNA present.

sample	conc ng/µl	OD 260	260/280
a (PCR)	44.2	0.883	1.71
b (PCR)	38.4	0.767	1.75
d (PCR)	44.2	0.883	1.73
a (extracted)	5.3	0.106	4.52
b (extracted)	6.2	0.123	4.64
d (extracted)	5.9	0.119	2.52
a (R digest)	24.5	0.490	0.72
b (R. digest)	17.9	0.358	0.72
d (R. digest)	17.6	0.351	0.67
PTG18 (R digest)	19.2	0.384	0.66

Possible contamination?

Possible attempt to clean DNA:
- re precipitation followed by ethanol wash, extended air drying and resuspension in fresh volume of TE or pure water

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[redacted]	21/9/17	[redacted]	21/9/17	

It was [redacted] decided to attempt restriction digest on PCR products before extraction

↳ 37°C for 4 hours
↳ gel ran for 50 minutes @ 70V
↳ loaded 8µl DNA + 2µl Green Taq
↳ bands were visible for all samples - PCR product bands were quite faint

Gel extraction

	eppendorf	with gel (g)	With gel	Difference	QG
2 bands 1 band	1	0.983	1.177	0.194	0.582
2 bands 1 band	2	0.985	1.117	0.132	0.396
2 bands 1 band	3	0.994	1.112	0.118	0.354
2 bands 1 band	4	0.984	1.025	0.041	0.123

Extracted gel as instructed on protocol - ran gel for 40 mins @ 70V - no bands present

27/9/17

Plan: reamplify DNA via PCR
run gel + extract DNA (loading lots of wells)
R digest for 4 hours
Run gel + extract
Ligation

PCR

[redacted] a", b", c" = 33.5µl H_2O
10µl flexi buffer
1µl DNTP
1µl $MgCl_2$
58°C

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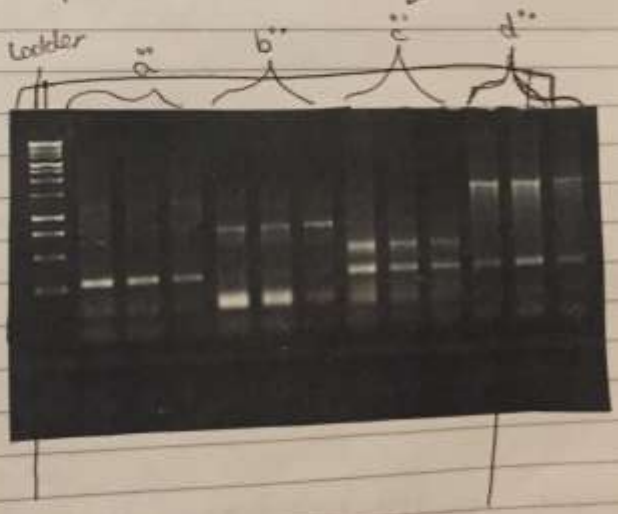
1 µl forward (lac4F3)
1 µl Reverse (GusBR1)
2 µl DNA (Fusion PCR products)
0.5 µl GoTaq

d'' = 32.5 µl H_2O
64°C 10 µl flexi buffer
1 µl DNTP
2 µl $MgCl_2$
1 µl forward
1 µl Reverse
2 µl DNA
0.5 µl GoTaq

For gel, each sample is going to be loaded into 3 wells; 2 X 16 µl sample + 4 µl Green Taq
1X 8 µl sample + 2 µl Green Taq

Gel image example :



▬ = 16 µl DNA
4 µl Dye

— = 8 µl DNA
2 µl Dye

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for gel extraction:

without gel(g)	with gel(g)	difference	QG	
1) 0.995	1.181	0.186	0.558	3 bands
2) 0.994	1.223	0.229	0.687	3 bands
3) 0.996	1.130	0.134	0.402	3 bands

Gel extraction had one definite band (sample d")

R. digest of PCR 28/9/17

ä dig 1, b" & dig 1, d" dig 1 = 37 µl H_2O
5 µl Buffer 2
1 µl BSA
5 µl DNA
1 µl ECOR1
1 µl HindIII

ä" & dig 2, b" dig 2, d" dig 2 = 32 µl H_2O
5 µl Buffer 2
1 µl BSA
10 µl DNA
1 µl ECOR1
1 µl HindIII

Samples are incubated for 4 hrs @ 37°C

See PG 26 for gel extract table

-ran gel : 2X 16µl DNA + 4 µl Dye
1 X 8µl DNA + 2 µl Dye

- sample d had a very faint bands but decided to extract all samples

ä + b" more left

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sample	conc	OD 260	260/280
a" extracted	7.9	0.157	4.81
b" extracted	12.4	0.248	2.97
d" extracted	10.1	0.202	2.57

Ran gel after extraction - samples had no visible bands except for PTTQ18 R.dig! → decided to still go ahead with ligation

Ligation

- Before ligation, vector PTTQ18 was digested with rSAP (get rid of phosphorylation)
  - 1μl rSAP - shrimp alkaline
  - 5μl cut smart buffer

	1:1 (L1)	1:2 (L2)	1:3 (L3)	C1	C2
vector	1	1	1	1	1
Insert	1	2	3	-	-
10x Ligase buffer	2	2	2	2	2
T4 Ligase	1	1	1	-	1
H_2O	15	14	13	17	16

Ligation incubated at 16°C overnight for 16 hrs before moving onto Transformation

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	Without gel (g)	with gel (g)	Difference	[redacted] Q6
1)	0.995	1.181	0.186	0.558
2)	0.994	1.223	0.229	0.687
3)	0.996	1.130	0.134	0.402

Tube 1 = Sample d" R.dig ~~restricted~~

Tube 2 = PTTQ18 1 R.dig

Tube 3 = pTTQ18 2 R.dig.

~~Transf~~ Transformation prep 3/10/17

Inoculation of MC1061 (no amp)
↳ LB media no amp used
↳ 15ml of media measured and put into a falcon tube, then one colony from MC1061 plate was put into media using pipette tip and left in orbital shaker overnight (37°C, 250rpm)

Making competent cells 4/10/17

1. 250 µl from overnight MC1061 culture pipetted into 25ml of LB no amp media
   ↳ Placed in orbital shaker for 2 hrs (37°C, 250rpm) to get to log phase (A600 = 0.2–0.3)
2. After 2 hrs, 1ml of E. coli harvested and put into a curette to measure log phase in a spectrophotometer (measure absorbance)
3. Chill culture on ice and harvest 1ml of cells by quick centrifuging at 8000rpm for 2 mins at 4°C

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4. Discard the supernatant then resuspend cells in 500 µl of ice-cold calcium (sterile) - (50 mM $CaCl_2$, 10 mM Tris HCl pH 8.0)
5. Place the suspension in an ice bath for 15 mins then centrifuge at 10,000 rpm for 1 minute at room temperature
6. Discard the supernatant and resuspend the cells in 1/5 (66µl) of ice-cold (sterile) calcium solution.

Transformation

7. 10 µl of DNA sample (ligated sample) added to 200 µl competent cells on ice [redacted] (MC1061)
8. Incubate on ice for 20-30 mins
9. Heat shock the cells at 42°C for 2 mins in a water bath and immediately return the tubes into an ice bath to chill for 1-2 mins
10. Add 330 µl SOC or LB (no amp) media and incubate the cells at 37°C with vigorous shaking for 60 mins
11. Plate 200 µl of incubated cells [redacted] onto "pre-dried" amp LB agar plates using glass beads
12. Grow the cells on the plate overnight at 37°C

→ Took 54 eppendorfs to make ±4 mL of competent cells.

Checked plates and there was no growth on any plates
→ Decided to redo [redacted] restriction digest as used all of sample from last time to increase success rate

22 µl H_2O
5 µl Buffer 2
1 µl BSA
20 µl DNA → decided to double DNA amount due to having very faint bands previously (used sample of "extracted" 27/9/17)
1 µl ECORI
1 µl HINDIII

No Growth on plates

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[redacted]	4/10/17	[redacted]	4/10/17	

Nano drop

Sample	conc. (ng/µl)	OD 260	260/280
d" extracted (27/9/17)	20.9	0.419	1.74
d" R dig 2 (5/10/17)	7.2	0.145	0.58

The reason to why the fusion was being lost after digest is due to the primer design - there were more restriction sites than there should have been and therefore the fragment was cut more than it should be → New primers were designed.

Redo from scratch 18/10/17

1st step PCR

sample 1= Lac4F3 + LacGusBR1 = 62.2, 64.2
sample 2= Lac4F3 + Lac4GusBR3 = 62.9, 64.9
sample 3= GUSBR1 + Lac4GusBF14 = 62.7, 64.7
sample 4= GUSBR1 + Lac4GusBF34 = 62.9, 64.9

PCR set up:

H_2O = 33.5µl
Flexi Buffer = 10µl
1µM DNTP = 1µl
25mM $MgCl_2$ = 2µl
10µM F Primer = 1µl
10µM R Primer = 1µl
DNA sample = 1µl
GoTaq Polymerase = 0.5µl

(sample extracted) Fusion template ← ↓ ran out for 6,7,8

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Before doing PCR, the new primers had to be diluted with $T_{10}E_1$

Oligo name	volume for 100pmol/µl
lacYgusBF14	216
lacYgus BR14	229
lacYgusBF34	246
lacYgus BR34	288

Primers were then diluted to make 10mM = 1µl Primer + 9µl H_2O

Samples 1 – 4+ 5' contain the fusion template (Sample d.. extracted) as DNA template

Samples 5+6 contains JM109 5 (see pg 7) as template

samples 7+8 contains MDB3318:1 (see pg 7) as template

Gradient PCR

Temp	
62	sample 1+4
52.4	
62.9	sample 2+3
63.3	
63.7	
64.1	sample 5, 5'+8
64.6	sample 7
65.0	sample 6

- Ran Gel at 70 V for [redacted] 40 mins
- Order in gel 1, 2, 3, 4, 5', 5, 6, 7, 8

= Very bright bands too much DNA + some smearing → to reduce $MgCl_2$ as could be introducing mutation or increase # of cycles

Performed by [redacted] Date 18/10/17

Countersigned by [redacted] Date 18/10/17

Continued on page number

-Run gel for sample d'' reamplified
-gel extract band (if too bright then needs diluting)
-dilute 1 in 50?

Use gel extraction as template for 1st step PCR
↳ also change $MgCl_2$ to 1 µl

Without gel.	With	Diff	QG
0.993	1.045	0.052	0.104.

Reamplified previous fusion (d' 6/7/17 + d'' 21/9/17)

sample d' = 32.5 µl H_2O
10 µl flexi buffer
1 µl DNTP
2 µl $MgCl_2$
1 µl F Primer
1 µl R Primer
2 µl DNA sample
0.5 µl Go Taq

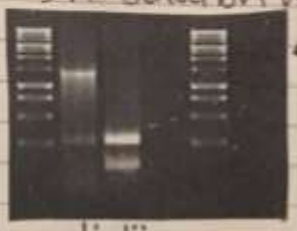
Sample d'' = 33.5 µl H_2O
10 µl flexi buffer
1 µl DNTP
2 µl $MgCl_2$
1 µl F Primer
1 µl R Primer
1 µl DNA sample
0.5 µl Go Taq

Both samples were run at 64°C

- Sample d'' fusion has been successfully amplified.

- Sample d'' gel extraction has also been successful with a faint band being present → no dilution was required

Sample	Conc	OD 260	260/280
→ d'	5.2	0.103	1.64
1:1 d	3.8	0.075	1.79



d' d''

first-step PCR re-run 16/10/17

Sample 1° = Lac4F3 + Lac4GusBR14 = 62°C
sample 2° = Lac4F3 + Lac4GusBR34 = 62.9°C
Sample 3° = GusBR1 + Lac4GusBF14 = 62.9°C
sample 4° = GusBR1 + Lac4GusBF34 = 62°C

PCR set up= [redacted]

34.5 µl H_2O
10 µl Buffer
1 µl DNTP
[redacted]
1 µl $MgCl_2$
1 µl F Primer
1 µl R Primer
1 µl DNA Sample (d'' extracted)
0.5 µl GoTaq

1° 2° 3° 4°

- ↓ cycles (from 30 to 25)
- [redacted]
- ↑ temp

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PCR Troubleshoot #3 - change $MgCl_2$: DNA + temp

35.5 H_2O

10 µl Buffer

1 DNTP

0.5µl $MgCl_2$

1µl F Primer

1µl R Primer

0.5µl DNA template [0.5µl [redacted] etc]

0.5µl GoTaq

samples were run at gradient temp

Temp	
62	sample a + a
62.4	
62.9	sample b+c
63.3	
63.7	
64.1	sample a' + d'
64.6	sample c'
65.0	sample b'

a b c d a' b' c' d'

20/10/17

Samples chosen to purify from the 3 troubleshoots:

sample 5 (18/10/17) = Lac4F3 + Lac4GusBR14 ✓

sample 2' (19/10/17) = Lac4F3 + Lac4GusBR34

sample 3' (19/10/17) = GusBR1 + Lac4GusBF14

sample d' (19/10/17) = GusBR1 + Lac4GusBF34

samples were purified but sample fragment + smearing inside = went ahead anyway.

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Purified samples then treated with DPNI = 1µl DPNI and 5.5µl Cutsmart buffer @ 20°C for 20 mins

PCR fusion #1

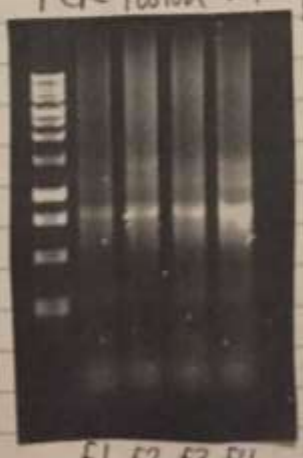
32.5µl H_2O	33.5µl H_2O
10µl Buffer	10µl Buffer
2µl $MgCl_2$	1µl $MgCl_2$
1µl DNTP	1µl DNTP
1µl F Primer	1µl F Primer
1µl R Primer	1µl R Primer
2µl DNA	2µl DNA
0.5µl GoTaq	0.5µl GoTaq

sample 5 + sample 3° = F1 F3 =63.5
sample 2° + sample 4° = F2 F4 =63.5

1st step purification

5 2° 3° 4°

PCR fusion #1 Both samples ran at 63.3



F1 F2 F3 F4

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Troubleshoot 2 – Fusion PCR 23/10/17

F1° + F2°
65.5°C

32.5 µl H_2O
10 µl Buffer
2 µl $MgCl_2$
1 µl DNTP
1 µl F Primer
1 µl R Primer
2 µl DNA
0.5 µl GoTaq

F3° + F4° = 63.5
F5° + F6° = 65.5

33.5 µl H_2O
10 µl Buffer
2 µl $MgCl_2$
1 µl DNTP
1 µl F Primer
1 µl R Primer
1 µl DNA
0.5 µl GoTaq

Ran gel – all samples have smearing and no fusion bands visible

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Troubleshoot 3 – fusion PCR 23/10/17

decided to run PCR @ 3 temps 58°C, 60°C, 62°C

32.5 µl H_2O
10 µl Buffer
2 µl $MgCl_2$
1 µl DNTP
1 µl Primer (F)
1 µl Primer (R)
2 µl DNA
0.5 µl Go Taq

samples X +4 = 58°C
samples X' +4' = 60°C
samples X'' +4'' = 62°C

- sample: X, X', X'' = sample 5' + sample 3'
- sample 4, 4', 4'' = sample 2' + sample 1'

X 4 X' 4' X'' 4''

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25/10/17

Nanodrop of samples

sample	conc (ng/ul)	OD260	260/280	H2O:D ratio
5	8.8	0.175	1.08	
	3.8	0.076	0.97	1:1
2°	7.0	0.140	1.12	
	4.1	0.081	1.05	1:1
3°	5.9	0.118	0.97	
	2.5	0.050	0.82	1:1
d°	6.2	0.124	0.97	
	2.2	0.045	0.88	1:1

1st step PCR again

31/10/17

33.5ul H_2O	34.5ul H_2O
10ul Buffer	10ul buffer
1ul DNTP	1ul DNTP
2ul $MgCl_2$	1ul $MgCl_2$
1ul F Primer	1ul F Primer
1ul R Primer	1ul R Primer
1ul DNA	1ul DNA
0.5ul GoTaq	0.5ul GoTaq
1° → 4°	1- → 4-

1= Lac4F3 + Lac4GusBR14 + JM109 → ran at 65°

2= Lac4F3 + Lac4GusBR34 + JM109 → ran at 64.2

3= GusBR1 + Lac4GusBF14 + MJB33 → ran at 64.2

4= GusBR1 + Lac4GusBF34 + MJB33 → ran at 64.2

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1/11/17

- ran gel from yesterdays PCR - 2 bands evident and smearing evident across samples with 2μl $MgCl_2$

↳ Too much Mg in compared to DNA (1μl).
↳ ~~Possibly~~ Possibly too long extension time?
↳ not enough DNA sample?

Plan → repeat PCR with 1μl $MgCl_2$ and 2μl DNA ~~of the~~ with the same programme.

1st step PCR troubleshoot #2°

33.5μl H_2O
10μl Buffer
1μl DNTP
1μl $MgCl_2$
1μl F Primer
1μl R Primer
2μl DNA
0.5μl GoTaq

1° = Lac4F3 + Lac4GusBR1 + JM109 → 65.1
2° = Lac4F3 + Lac4GusBR3 + JM109 → 64.3
3° = GusBR1 + Lac4GusF1 + MJB33 → 64.3
4° = GusBR1 + Lac4GusF3 + MJB33 → 64.3

ran gel → no bands evident therefore going to lower temp on next PCR → do a range as may not be annealing

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1st step PCR troubleshoot #3 2/11/17

- Plan is to do PCR at 3 different temps (temp gradient) to see if annealing improves (62, 60, 58)

33.5µl H_2O
10µl buffer
1µl DNTP
1µl $MgCl_2$
1µl F Primer
1µl R Primer
2µl DNA → ~~Vac~~ JM109 5
0.5µl GoTaq MOB331 8:1

W, X, Y, Z = 62
W', X', Y', Z' = 60
W^2, X^2, Y^2, Z^2 = 58

Samples W, X, W', X'?, W^2, Y^2 and Z^2? had band!

W, W', W^2 = LacYF3 + LacYGusBR14, JM109
X, X', X^2 = LacYF3 + LacYGusBR34, JM109
Y, Y', Y^2 = GusBR1 + LacYGusBF14, MOB33
Z, Z', Z^2 = GusBR1 + LacYGusBF34, MOB33

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1st step PCR troubleshoot – Gus fragments 10/11/17

- Plan is to run 2 separate PCRs today;

1) More Gus DNA concentration sample at 62°, 60° and 58° = 6 samples (PCR)

2) run perious gus sample + new concentration sample at 62°, 60° and 58° but with 1 min polymerization instead of 2 = (12 samples)

→ Decided to hold fire with this and attempt fusion instead

33.5µl H_2O
10µl buffer
1µl DNTP
1µl $MgCl_2$
1µl F Primer
1µl R Primer
2µl DNA → used MDB331 T:1
0.5µl Go Taq

1, 2 = 62
3, 4 = 60
5, 6 = 58

1, 3, 5 = GusBR1, LacYgusBF14, MDB33
2, 4, 6 = GusBR1, LacYGusBF34, MDB33

Plan for the rest of the day =
- Purify successful lacY fragments → do any gel extraction needed to clear bands
- Run fusion PCR

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Decided to purify samples:

W, X, W', X'

very faint bands visible on gel after purification

- Decided to do gel extraction on successful gus samples from previous ie take (pg 32) = sample 3' + sample d'

Loaded: 1 X 16μl DNA + 4μl [redacted] Dye
1 X 8μl DNA + 2μl Dye

for each sample

Haven't got time to do fusion PCR or run gel after gel extraction → will do this next week

Gel extraction

sample	weight before	after	Diff	QG
3'	0.998	1.098	0.100	0.300
d'	~~0.998~~ 0.988	1.1195	0.117	0.351

will run gel extraction gel next week

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15/11/17.

Plan = - DPNI treatment to purified samples from 10/11/17
- NN gel of gel extracted samples from 10/11/17
- NN PCR fusion (temp gradient, diff $MgCl_2$ amounts, Diff DNA amount)

DPNI treatment of samples W, X, W'
→ 1 µl DPNI, 5.5 µl Cutsmart buffer @ 80°C for 20 mins.

Cut smart buffer needs to be 1X not 5X = dilute by doing 1µl sample and 4µl water

[redacted] meant to be diluted (0 times, not 5!

how to calculate concentration with dilution factor =
initial conc × $\frac{1}{\text{dilution factor}}$ = final conc

PCR fusion Troubleshoot 1 - Temp Gradient + different $MgCl_2$

32.5µl H_2O	33.5µl H_2O
[redacted] 10µl buffer	10 µl buffer
1µl DNTP	1µl DNTP
2µl $MgCl_2$	1µl $MgCl_2$
1µl F Primer	1µl F Primer
1µl R Primer	1µl R Primer
2µl DNA	2µl DNA
0.5µl GoTaq	0.5µl GoTaq

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samples F_1 = sample 3° + sample W } 64
F2 = sample d° + sample X
F3 = sample 3° + sample W } 62
F4 = sample d° + sample X
F5 = sample 3° + sample W } 60
f6 = sample d° + sample X
F7 = sample 3° + sample W } 58
f_8 = sample d° + sample X

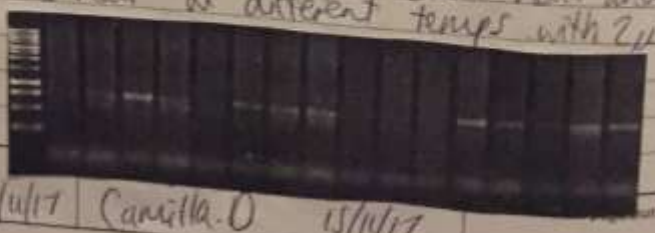
2µl $MgCl_2$

f_9 = sample 3° + sample W } 64
F_{10} = sample d° + sample X
F_{11} = sample 3° + sample W } 62
F12 = sample d° + sample X
f13 = sample 3° + sample W } 60
F14 = sample d° + sample X
F15 = Sample 3° + sample W } 58
F16 = sample d° + sample X

1µl $MgCl_2$

Ran gel of PCR fusion attempts + gel extractions from last week

↳ gel extractions have good faint bands (correct size) with no smearing
↳ No fusion bands formed from PCR, theres a lot of smearing throughout (across the whole column) and bands visible are gusB fragment size? Not sure why - temp + $MgCl_2$ differences doesn't appear to have made much difference
↳ plan = do PCR with 2µl LacY half and 1µl GusB half at different temps with 2µl $MgCl_2$



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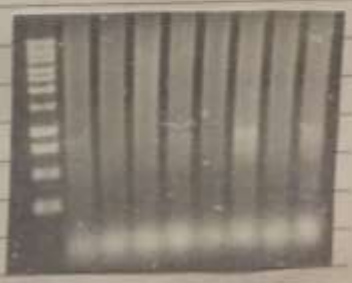
PCR fusion Troubleshoot 2

23/11/17

Plan – NN pcr with 2 µl lacI and 1 µl GusB at 4 different temps (64, 62, 60, 58)

31.5 µl H_2O
10 µl Buffer
1 µl DNTP
2 µl $MgCl_2$
1 µl F Primer
1 µl R Primer
3 µl DNA
0.5 µl Go Taq

(64) F1° = sample 3 + sample W
F2° = sample d + sample X
(62) F3° = sample 3 + sample W
F4° = sample d + sample X
(60) F5° = sample 3 + sample W
F6° = sample d + sample X
(58) F7° = sample 3 + sample W
F8° = sample d + sample X



Plan – · 2 mins extension time
· 1 µl $MgCl_2$ and 0.5 µl $MgCl_2$ (after fusion)
· 1 µl Go Taq – agarose
lower temperatures
· 28 cycles
· 60 – 54 °C temp gradient (55 ℃)

NCBI – map drawing plasmid 32 } draw

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PCR fusion Troubleshoot 3 29/11/17

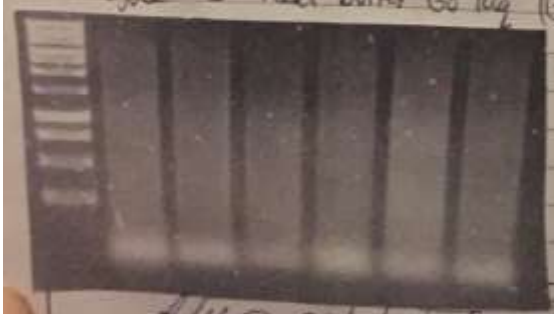
32µl H_2O
10µl Buffer
1µl [redacted] DNTP
1µl $MgCl_2$
1µl F Primer
1µl R Primer
3µl DNA → 1µl Go part, 2µl Lac part
1µl Go Taq

F1 = sample 3° + sample W } 56°C
F2 = sample d° + sample X
F3 = sample 3° + sample W } 55°C
F4 = sample d° + sample X
F5 = Sample 3° + sample W } 54°C
F6 = sample d° + sample X

Change cycles from 30 to 28
Extention time is back to 2 mins

[redacted] To make 1kb ladder:

300µl $dT_{10}E_1$
100µl DNA ladder → G571A 3153704 1Kb DNA ladder Promega
100µl 5X flexi buffer Go Taq (Green) → vortex → M891A Promega



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Nanodrop of samples

sample	conc ng/µl	OD 260	260/280
3° (10/11/17)	8.0	0.160	1.85
d° (10/11/17)	9.4	0.188	1.65
W (10/11/17)	3.8	0.077	1.40
X (10/11/17)	4.4	0.089	1.43

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Countersigned by Camilla D. Date 29/11/17

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