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Investigation of the potential molecular recognition sites of two human vitamin C transporters.

Site specific mutagenesis to modify certain codons of the coding genes.

A thesis submitted as part of the requirement for the Masters of Research

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Abstract

Vitamin C (L-ascorbic acid), a cofactor for numerous mammalian enzymes and a well-known antioxidant, is renowned for its range of health advantages and protective capabilities against degenerative disorders. The mechanisms regulating the cellular movement of ascorbic acid signify a primary aspect for recognising the roles played by vitamin C in human biology. The ability for this nutrient to be absorbed and occupied in cells is accomplished via the two sodium-coupled proteins, hSVCT1 and hSVCT2. The two transporters have varying roles in relation to ascorbate, nevertheless they are both rely on certain membrane targeting to achieve their essential functions. If the correct localisation for the two proteins is not found they are unable to complete their functions, leading to reduced transport capabilities. Deficiency of vitamin C in humans is fatal and has been linked with an increased chance of cancer and other degenerative diseases, therefore the two protein carriers are crucial for human health. Despite numerous studies evaluating vitamin C's benefits for humans, detailed knowledge on the membrane targeting of the two transporters is still limited. It is unclear how these proteins react to the interaction and presence of new substances entering the human body and how this will affect their functionality. Consequently, the aim of this research is to modify the potential molecular recognition sites on the hSVCT1 and hSVCT2 genes through site directed mutagenesis. This study could therefore indicate regions on the amino acid sequences which could be fundamental for functionality for the two carriers. This knowledge would assist the growth of therapeutic strategies in fighting certain conditions and even in the production of new drugs.

Table of Contents

List of Abbreviations

(h)SVCT	(Human) Sodium-dependent vitamin C transporter
AA	Ascorbic Acid
BLAST	Basic Local Alignment Search Tool
bp	Base Pair
C-terminal	Carboxyl-terminal
cDNA	Complementary DNA
COS	CV-1 (Simian), carrying the SV40 genetic matter
DHA	Dehydro-L-ascorbic acid
DNA	Deoxyribonucleic acid
dNTP	2-deoxyribonucleoside-5-triphosphate
EDTA.Na_2	Ethylenediaminetetraacetic acid
GLUT	Glucose transporter type
GLUT 1-4	Facilitative glucose transporter 1, 2 and 4
GULO	L-guluno-y-lactone oxidase gene
HCl	Hydrochloric acid
HEK	Human Embryonic Kidney
K_a	Acid dissociation constant
kb	Kilobases
K_m	Michaelis constant
LB	Luria-Bertani
Mins	Minutes
mRNA	Messenger RNA
N-terminal	Amino- terminal

NCBI	National Centre for Biotechnology Information
NEB	New England BioLabs
ORF	Open reading frame
PCR	Polymerase Chain Reaction
phSVCT	hSVCT-containing the entry plasmid
PKA	Protein Kinase A
PKC	Protein Kinase C
PMA	+Phorbol 12-myristate 13-acetate
RNA	Ribonucleic acid
rSAP	Shrimp Alkaline Phosphatase
Secs	Seconds
SNP	Single Nucleotide Polymorphism
SOC	Super optimal broth with catabolite repression
SVCT1	Sodium vitamin C Transporter 1
SVCT2	Sodium vitamin C Transporter 2
TAE	Tris-acetate-EDTA.Na_2
Tm	Melting Temperature
Tris	Tris(hydroxymethyl)aminomethane
UTR	Untranslated Region
V_{max}	Maximum reaction rate

List of Figures

List of Tables

1.0 Introduction

Vitamin C (L-ascorbic acid), is a crucial nutrient needed for human survival which is gained through the consumption of certain foods and dietary supplements. The micronutrient is acknowledged across the medical industry due to its involvement in numerous cellular reactions where it acts as a free radical scavenger by reducing the effects of oxidative stress. It is also notoriously known for its involvement in multiple degenerative disease, where it plays a protective role (Van der Reest and Gottlieb., 2012). Due to its water solubility, vitamin C needs specific protein carriers to enable it to move across plasma membranes. As humans do not naturally synthesise the nutrient these proteins are essential to enable absorption into cells, where it can complete its biological functions (Mun et al., 2006). The two human Na+- dependent vitamin C transporters, hSVCT1 and hSVCT2, are recognised to be accountable for the majority of cellular uptake of the nutrient in humans (Gess et al., 2005). It is evident that the human body therefore relies on only two transporters for survival which could seem unstable, particularly when minimal detailed knowledge is understood on their structural and functional influences. Throughout the pharmaceutical industry, new drugs and therapies are continually being developed to manage with the development of new diseases and disorders. It is therefore necessary to understand the potential for adverse drug reactions and how detect them within the human body. Understanding the mechanisms which control and shape a proteins behaviour and interactions with other substances, particularly in relation to vitamin C, is crucial. This knowledge could consequently aid our understanding on how vitamin C can be used as a treatment and preventive for a vast range of degenerative diseases linked with oxidative stress.

Therefore, this research project aims to complete site-specific mutagenesis on the two vitamin C transporters, hSVCT1 and hSVCT2 through molecular cloning techniques. These results can then provide fellow researchers with an insight into the molecular recognition sites of the transporters which are crucial for their functionality and biological activities. This could potentially lead to influencing how the pharmaceutical industry design and develop their medicines.

1.1 Vitamin C, Health and Disease

Vitamin C (L-ascorbic acid, AA) is a fundamental micronutrient needed for normal cellular function and growth. The nutrient is chemically the simplest of the vitamins and consequently was one of the first to be characterised and have its structure determined. It is produced industrially more than any other vitamin and is taken routinely by human beings. The six-carbon lactone is produced from glucose in the liver in the majority of mammalian species, however not by humans, non-human primates and guinea pigs (Williams and Deason, 1967). Those mammals who can synthesise vitamin C do so via the L-gulono-y-lactone pathway (Burk et al., 2006). Humans have an inability to synthesise vitamin C due to mutations in the L-gulono-γ-lactone oxidase (GLO) gene, which codes for the final enzyme utilised in biosynthesis of the nutrient. Clinical expression of vitamin C deficiency, scurvy is a fatal disorder unless suitably treated, therefore humans are required to consume vitamin C to survive (Stone, 1966; McRae, 2008). Symptoms of scurvy include fatigue, painful joints and muscles, fever and bleeding into the skin (Bsoul and Terezhalmy 2004; Montecinos et al. 2007).

Studies into high levels of vitamin C in humans have suggested it has the ability to protect against certain conditions including cardiovascular disease, cataracts, cancer and Parkinson's (Mahdavi et al. 2009; Ravindran et al. 2011; Bennett et al. 2012). The abilities that vitamin C holds in protecting against diseases and conditions routes from its function as a reducing agent, where it can donate an electron and convert to dehydro-L-ascorbic acid (DHA) (Sagun et al., 2005; May 2011). The role of vitamin C in relation to human health has been researched for many years due to its numerous positive abilities and capability to work both inside and outside of cells. The most studied function of ascorbic acid is its primary role to neutralise free radicals due it is water soluble characteristics enabling it to inhibit large quantities of damage (Jelodar, Nazifi and Akbari, 2013). Vitamin C provides a beneficial source of electrons which are donated to the free radicals such as hydroxyl radicals (Klennel, 1949). This action prevents the cells from being damaged by genetic alterations and prevents mutations to the chromosomes (Singh and Gaby, 1991).

Alongside the liver and adrenal glands the greatest levels of vitamin C in the body are found in the brain (140 mg/kg) and neuroendocrine tissue (Anderson et al., 1997). Inside the human body, vitamin C presents complex non-linear pharmacokinetics, in

addition to varying tissue distribution. This is also shown in the brain which is able to retain the vitamin at the deprivation of other tissues and organs, including the liver and kidney, during states of chronic deficiency (Lykkesfeldt et al., 2007). The brain is moderately resistant to vitamin C depletion, suggesting an essential role of the nutrient (Hejazi et al., 2011). AA acts has numerous functions within the brain including; monooxygenase-dependent synthesis of neurotransmitters and neuropeptide hormones, as well as recycling of the enzyme cofactor tetrahydrobiopterin (Lykkesfeldt et al., 2007; Figueroa-Mendez and Rivas-Arancibia, 2015). The nutrient has also been proven to be involved in the physiology of the central nervous system and supporting the structure of particular neurons in addition to modulating the neurotransmission within human bodies (Flagg, Coates and Greenberg, 1995).

Vitamin C levels have been linked to learning and memory with suggestions that combinations of vitamin C and E have advantageous effects reducing memory alterations (Arzi, Hemmati and Razian, 2004; Harrison et al., 2009). Studies conducted by Figueroa-Mendez and Rivas-Arancibia in 2015 have established that vitamin C effects on learning and memory are reliant on the redox balance state. This behaviour and distribution in humans allows the vitamin to act as a factor in a minimal of eight enzymes, within mammals, to defend a range of cells from oxidative stress in addition to the production of collagen (Wang et al. 2002; Valko et al. 2006; Hierro et al. 2013). Evidently these protective qualities of vitamin C make it imperative to fight against a diverse array of diseases.

1.2 Cellular Acquisition and Accumulation of Vitamin C

Several aspects, both endogenous and exogenous, impact vitamin C body levels, primarily vitamin C transporters that control the vitamin's accessibility and plasma and tissue levels. This is in addition to a range of environmental influences and endogenous stresses, for example oxidative stress and disease (Ciocoiu et al., 2007). Vitamin C exists in plasma primarily in its reduced form, ascorbic acid, at concentrations in the normal range of 30-60 mM (Levine et al., 1996; Rivas et al., 2008; Elste et al., 2017). Humans require only a small concentration of the vitamin per day. Research has stated that humans need approximately 100 mg of the nutrient per day to allow for healthy plasma and tissue levels of the vitamin (Levine et al. 1996; Lee et al., 2005). This intake

per day means the plasma levels are approximately 50-100 μM and distributed white blood have concentrations between 1 to 2 mM (Johnston, 2009; Padayatty et al. 2003).

Vitamin C performs the majority of its biological roles intracellularly therefore it is obtained by most cells from the plasma, a procedure that needs the involvement of certain transporters. The oxidised form of the nutrient is transported across cells via facilitated diffusion by the GLUT 1,3 and 4 glucose sensitive transporters, which protects mitochondria from oxidative injury (Huang et al., 2001; KC et al. 2005). DHA has a crucial responsibility in many cells since it can be used to regenerate AA.

DHA is transported from the lumen of the small intestine and reduced to AA, which consequently distributes in the blood (Tu et al., 2017). Physiological research has indicated that blood concentrations of vitamin C only consists of <0.5% of DHA, due to its brief physiological half-life, with the remaining involving AA (Dhariwal et al. 1991; Song et al. 2002). This was verified when oxidised vitamin C was expressed in *Xenopus laevis* oocytes and recognised using an electrochemical detection method (Koshiishi et al. 1998; Rumsey et al. 2000). The reduced form crosses the plasma membrane via the Na+-dependent systems and the SVCT1 and SVCT2 transporters, which transport stereospecifically (Liang et al. 2001). The two SVCT's are surface glycoproteins encoded by two different genes, which have been proven to be very similar in structure, with high sequence homology (Savini et al., 2007). They have distinct functional characteristics and specific tissue distribution which suggests that they hold separate physiological roles within the human body (Savini et al. 2007).

Alongside the apparent competitive inhibition effects of glucose concentration on DHA transport, it is known that the SVCT family are the main vitamin C carriers within mammalian cells. At physiological pH, approximately 99.9% of ascorbate exists as a monovalent anion holding a negative charge, which inhibits it from dispersing through cell membranes (Jin et al., 2005). This emphasises the need for the two transporters, which permit intake of vitamin C into the gastro intestine and consequently into the cells (Omotayo et al. 2015). SVCT1 and SVCT2 both cotransport ascorbic acid and sodium down an electrochemical sodium gradient at a ratio of 1:2, which is sustained by K^+/Na^+ exchange systems (Seno et al., 2004). This specific transport is highly responsive to changes to certain factors such as temperature and pH (Liang et al., 2001; Wohlrab et al. 2017). Studies on Xenopus laevis oocytes and mammalian cells have

proven that SVCT1 and SVCT2 transport activity of ascorbate is only functional when they are activated by sodium (Tsukaguchi et al. 1999; Bürzle et al. 2013; Subramanian et al. 2016).

1.3 The Human Sodium-dependent Vitamin C Transporters

1.3.1 Genetics of the Human Vitamin C Transporters

The general gene structures of the two transporters are very similar, with human SVCT1 possessing 15 exons and human SVCT2 consisting of 17, with most exons being similar sizes. This has been proven through encoding cDNA's which have been replicated for both proteins, taken from human cDNA libraries, allowing the two carrier structures to be mapped (Stratakis et al. 2000; Wang et al. 2000). The two transporters have distinct functional characteristics and specific tissue distribution which suggests that they hold separate physiological roles within the human body (Savini et al. 2007). SVCT1 and SVCT2 are members to a family of nucleobase transporters, involving general purine permease (UapC), uracil transporter (UraA) and membrane-bound uracil permease (PyrP) (Faaland et al., 1998; Meintanis et al., 2000; Wilson, 2005; Savini et al., 2007; Lu et al., 2011). The two carriers have no structural homology with any other mammalian membrane transporter. SVCT1 and SVCT2 are encoded by the SLC23A1 and SLC23A2 genes, respectively (McNulty et al., 2005). The SLC23A1 gene is 16,096 bp long and maps to human chromosome 5q31.2–31.3 (Sotiriou et al., 2002). The SLC23A2 gene maps to chromosome 20p12.2– 12.3 and is ten times larger, at 158,398 bp long(Clark et al., 2002).

A putative structure for SVCT1 and SVCT2 has been calculated by hydropathy analysis. Kyte-Doolittle plots suggest that both transporters are trans-membrane (TM) proteins (Savini et al., 2007). The predicted structure holds 12 membrane-spanning domains, with the N- and the C-termini found on the cytoplasmic side of the membrane (MacDonald, Thumser and Sharp, 2002). The extracellular loop between the 7 and 8 TM domains contains many conserved proline residues, that are required for arrangement, stability and transport efficiency (Liang et al., 2001). Several other conserved proline residues have been located within the TM and could potentially be significant for determining the protein structure (Liang et al., 2001).

Expression of the two SVCT transport proteins is specific depending on the tissue and cell and is controlled by transcriptional regulation of the human solute carrier gene

family 23 (*SLC23*) (Michels et al.2013). In epithelial cells, SVCT1 and SVCT2 are expressed in the apical and basolateral membranes, respectively. Sodium-dependent vitamin C transporter 1 is expressed in the epithelial tissue of the kidney, intestine, liver, lung, and skin (Qiao and May, 2011). Within the kidney, SVCT1 is located in the brush-border membrane of the proximal tubule where it controls uptake of ascorbic acid, consequently acting as a key part in maintaining whole body ascorbate levels (Wang et al. 1999). The SVCT2 protein is greatly expressed in the brain where it is crucial for upholding the high ascorbate levels required for brain function and growth (Meredith et al. 2011).

The transporters can be distinguished due to their kinetic properties which have been proven to be functionally different. SVCT1 has an ascorbic acid Km higher than SVCT2 and a lower affinity for ascorbate meaning it is adapted to high capacity uptake of the vitamin from the diet (Savini et al. 2007). Sodium-dependent vitamin C transporter 2 is positioned in nearly every tissue and cell in the body (Michels et al. 2013). It is categorised as a low capacity, high affinity transporter and can retain lower concentrations of ascorbic acid than SVCT1 (Obrenovich et al., 2006). Through studies using human cell lines and cells with over expressed cloned SVCT1 and SVCT2, it has been established that SVCT1 has an ascorbic acid transport Km of approximately 80-200 uM and approximately 15-25 uM for SVCT2 (Godoy et al., 2007; Rivas et al.,2008). The greater v_{max} value for human SVCT1 can be linked to a higher turnover rapidity.

At acidic pH, the transporters are unable to function at an optimum level, as shown through a reduced binding affinity for ascorbate (Shaghaghi et al. 2016). Both transporters are stereospecific and have an optimum pH of approximately 7.5 , which was measured at 22°C in transfected COS-1 cells, and displayed great specificity for L-ascorbic acid rather than for its stereoisomer (Wang et al. 2000; Liang et al. 2001).

Table 1-1. Comparison of Characteristics of hSVCT1 and hSVCT2

Characteristic	**hSVCT1**	**hSVCT2**
Gene Length	16,096	158,398
Chromosomal Locus	5Q31.2-31.3	20P12.2-12.3
Protein Length	598 amino acids	650 amino acids
SNP'S	4	0
Gene Structure	15 exons	17 exons
Protein Mass	65-80 kDa	65-80 kDa
K_m (affinity)	65-252uM	8-69Um
V_{max} (Capacity)	8-15.8pmol	0.04-1.2pmol
Localisation in Polarised Epithelia	Apical	Basolateral
Optimum pH	7.5 at 22°C	7.5 at 22°C
Distribution in Humans	Kidney, Small Intestine epithelium, Colon and Liver	Widespread

1.3.2 Mutations in *SLC23A1* and *SLC23A2*

As a result of the direct contact of SVCTs with reduced vitamin C and their responsibilities in absorption and tissue accumulation, genetic modifications in *SLC23A1* and *SLC23A2* seem to have the biggest impact on human vitamin C in comparison to other genetic factors (Shaghaghi et al., 2016). Polymorphisms in the genes encoding the transporter proteins are greatly linked with plasma ascorbate levels and potentially effect tissue cellular vitamin C levels (Jimenez-Fernandez et al., 2012). Moreover, changes to genetic sequences of specific proteins that reduce oxidative stress including; haptoglobin, glutathione-S-transferases, affect ascorbate levels in the human body (Michels et al., 2013).

One study has evaluated one splicing variant for SVCT1 which produced a protein holding an insertion of 4 additional amino acids localised in the extracellular loops that connects transmembrane domains 3 and 4 (Rivas et al., 2008). These studies revealed that this splicing variant was unable to make the transporter function correctly due to its inability to carry vitamin C (Wang et al. 1999; Eck et al. 2004). Another splicing variant has been expressed for SVCT2 which is lacking 345 base pairs from the coding

sequence. This concluded in a short protein of 525 amino acids which was not able to transport ascorbic acid and instead acted as an inhibitor. This protein inhibited the transport which is normally facilitated by SVCT2 and it also marginally inhibited SVCT1 functions through the development of protein- protein complexes (Liu et al. 2001).

More than 150 SNPs have been acknowledged in *SLC23A1,* with a minimum of four of the SNPs found in *SLC23A1* located in the coding region (exons 3, 7, and 8), which causes one synonymous and three nonsynonymous alterations to the transporter (Gispert et al., 2000). All nonsynonymous polymorphisms created an operative SVCT1 protein, but each of these transporters displayed declines in ascorbate transport when expressed in *Xenopus laevis* oocytes (Corpe et al., 2010).A vigorous discovery for the influence of *SLC23A1* genetic variation on plasma ascorbate levels was established by Timpson et al. 2010 in a multiple unit assessment in excess of 15,000 individuals within the United Kingdom. One of the SNPs researched was connected with regular, reproducible reduction in circulating vitamin C levels. Generally, for the population in this study, the genetic change was linked with a roughly 6 μM lower plasma or serum ascorbate concentration per allele. The considerable size of *SLC23A2* means that variants in the genetic structure are comparatively recurrent and extensive (Maulén et al., 2002).

Most of the SNPs investigated are either intronic or untranslated regions of *SLC23A2* and therefore do not directly modify the coding of the SVCT2 transporter (Smith, Visioli and Hagen, 2002). Contradictory to variations in *SLC23A1*, genetic alterations in *SLC23A2* are not predicted to have a significant influence on ascorbate homeostasis in the circulation, as SVCT2 controls the tissue build-up the vitamin C from the plasma (Cahill and El-Sohemy 2009).

1.3.3 N-Glycosylation

Analyses indicate that hSVCT1 and hSVCT2 each have a 12-transmembrane structure with cytoplasmic C and N-terminal domains in addition to several consensus sites for glycosylation and phosphorylation (Subramanian et al., 2008). Both SVCT1 and SVCT2 have potential sites where N-glycosylation can occur within the extracellular loops, hSVCT1; Asn^{138} and Asn^{144} with hSVCT2 at; Asn^{188} and Asn^{196}. The sites were located between the predicted transmembrane domains 3 and 4 and an additional site for hSVCT1, Asn^{230}, found between TM 5 and 6. N-glycosylation has been proven to be

needed for transport functionality, targeting, folding, and stability of numerous transporters (Martínez-Maza et al. 2001; Tanaka et al. 2004; Zhou et al. 2005).

Subramanian et al, 2011 studied whether the transport functionality and targeting of the vitamin C transport proteins were affected within human hepatic liver cells when the N-glycosylation sites were mutated. The results showed that removal of these individual sites considerably reduced the expression and therefore the ascorbate uptake by the hSVCT1 and hSVCT2 mutants. The research also proved that mutations of the N-glycosylation sites particularly affected the cell surface targeting of the transporters. Although both transporters had restricted ascorbate uptake, they reacted differently to the mutations, which may have been caused by separate mechanisms. SVCT1 alterations caused a decreased quantity of the transporter being present on the cell membrane compared to SVCT2 whose localisation did not change (Subramanian et al. 2008). The study also indicated that glycosylation may control the allocation of SVCT1 both on cell surface membrane and inside the cell. The results concluded that glycosylation is crucial for the functional expression for both ascorbate transporters.

1.3.4 Phosphorylation

The knowledge of the protein sequences of the two transporters has revealed that they hold five protein kinase C (PKC) phosphorylation sites. These are located on the cytoplasmic surfaces of the proteins. The PKC activating agent PMA, has found to decrease ascorbate uptake by the two transporters when tested in oocytes containing hSVCT1 and hSVCT2 (Takanaga et al. 2004). For SVCT1 the decrease in Vmax is linked with a change in dispersal from the cell surface membrane to inside the cell which contrasts with SVCT2 which remained in the plasma membrane. Reidling et al studied the outcome when all five PKC sites were altered in SVCT1 and SVCT2. Each site was changed from a threonine or serine to an alanine. They found that changing just a singular amino acid did not impact the reduction in ascorbic acid uptake or transport. Indications suggest that other factors such as protein-protein mechanisms may be influencing the level of affects that PMA has on the transporters.

The transporters both demonstrate changes in transport capability upon PKC instigation, particularly SVCT1 (Wu et al. 2007; Figueroa-Méndez and Rivas-Arancibia 2015). These studies have found that it is probable that PKC activation potentially leads to

internalization of hSVCT1 which is thought to be reversible, however it does not affect the hSVCT2 membrane expression (Reidling et al., 2008).

1.3.5 Pathways which Regulate SVCT

Many contributing factors alter the signal transduction pathways that mediate ascorbate uptake, including increased age. Research undertaken by Michels et al (2003) showed that SVCT1 mRNA falls by approximately 45% in rat hepatocytes, aged 24-26 months, compared to juvenile rats, aged 3-5 months. Within humans, the independent functions of the two transporters are essential for transport and survival. Therefore this drop in SVCT1 mRNA will cause the carrier to be defective, which can be fatal. The loss of this protein for humans as they get older can consequently give an indication to the decline in ascorbate levels, caused by altered signalling.

Disturbance to the signalling pathways of SVCT transporters can lead to numerous problems with the uptake of ascorbate. Reduced ascorbate concentrations have been linked to a decrease in vitamin E, glutathione, levels within the body and ultimately causes a disruption in antioxidant protection (Ulrich-Merzenich et al. 2007; Yousef et al. 2012). Reduced defence against antioxidants allows an opportunity for an increase in production of pro-oxidant cells inside the body, which increases with age (Gess et al. 2013; Sorice et al. 2014). This increase in pro-oxidants can potentially increase the activation of PKC, which can lead to a reduction in the transport capacity of SVCT1.

1.4 Vitamin C and Degenerative Diseases

Vitamin C is widely used as a therapeutic agent in many diseases and disorders. Recent pharmacokinetic data has improved the knowledge relating to vitamin C transport and its links with the prevention and treatment of cancer. Since the 1970's research has reviewed the nutrient as a cancer remedy and have investigated the effects of high-dose of AA on the growth and progression of tumours and the mechanisms behind the anti-cancer effect (Chambial et al., 2013).

High levels of vitamin C in the blood is beneficial as the nutrient increases the build-up of hydrogen peroxide (H_2O_2) which is preferentially toxic towards tumour cells (Chen et al., 2007). Individuals with cancer who are given vitamin C intravenously will see plasma levels elevate as the injection bypasses the intestinal absorption system (Harrison, 2012). This is valuable as high doses have been linked to slowing the growth

and spread of prostate, pancreatic, liver and colon cancer. It is also thought to be beneficial in curing individuals with cancer by combining high doses of AA with chemotherapy, making the treatment more effective (Korok et al., 2000; Stephenson et al., 2013). Specific treatments which have increased efficiency when combined with high dose of AA include; arsenic trioxide for ovarian cancer cells and gemcitabine in pancreatic cancer cells (Qazilbash et al., 2008; Monti et al., 2012). Evidence has been accumulating which indicates that intravenous vitamin C may enhance the quality of life of cancer patients, through reduced pain and the need for pain relief medication.

The antioxidant functions of AA are essential for optimal human health in addition to its anti-inflammatory properties and inhibition of tumour metastasis. Absence of the nutrient would increase the levels of reactive oxygen species in the body which stimulates apoptosis, inflammation and initiate interference to genes which facilitate cell adhesion (Jagetia et al., 2007; Sangani et al 2015). Obstructions to the vitamin C transporters can instigate the onset of numerous degenerative diseases such as cardiovascular disease (CVD) which has resilient oxidative damaging factors (Yun et al., 2015). It is evident that sufficient levels of vitamin C and its specific mechanisms of transport are crucial for human health and the prevention of numerous harmful diseases.

1.5 Vitamin C Inhibition

SVCT1 and SVCT2 are low abundance transport proteins and consequently are yet to be quantified by binding or coupling. Vitamin C and flavonoids are both substances widely found within numerous fruits and vegetables that humans ingest (Awad et al., 2001). The reactions of flavonoids with the vitamin C transporters have been investigated as they are predicted to reduce the level of ascorbate uptake within mammals (Berger et al., 2003; Caprile et al., 2009). Song et al, 2002 investigated 12 flavonoids and tested them against SVCT1 transport activity within Xenopus laevis oocytes and Chinese hamster ovary cells. They found that certain flavonoids have novel regulatory abilities that reduce or inhibit ascorbate uptake. They founded that the most potent of all the flavonoids were the flavanols, of which Quercitin is found in most plant-based foods. When tested Quercetin acted as a non-competitive inhibitor which reduced SVCT1 transport efficiency (Biondi et al., 2006). SVCT1 did not transport the flavonoid and the inhibition appeared reversible. Quertcetin appeared the most effective inhibitor due to its structure. The flavonoids ability to reduce ascorbate transport took

place from the intestinal lumen into the cells (Kuo, Morehouse and Lin, 1997; Rietjens et al., 2002). It is evident that the effects of the flavonoids can greatly influence the ascorbate bioavailability, consequently this may alter human daily requirement of vitamin C.

1.6 Drug Transport in relation to SVCT1 and SVCT2

The efficiency of certain therapeutic drugs are frequently limited by negative biological or pharmacokinetic influences including, poor bioavailability or restricted water solubility. Plasma membrane proteins are freely available to drug molecules due to their localisation on the cell surface (Boyer et al., 2005). By joining these drugs with the substrates of their appropriate complementary transporter proteins, drug uptake and oral accessibility can be considerably improved (Luo et al. 2011). It has been established that both vitamin C transporters have the capability to transport other compounds, with the requirement of specific structural conditions (Rumsey et al. 1999; Luo et al. 2008). This suggests that the two ion transporters may have the ability to be areas for site specific drug delivery.

Studies by Dalpiaz et al, 2005 investigated the results of conjugating an anticonvulsant drug with ascorbic acid. The outcome showed that individually the drug does not co-operate with SVCT2, nonetheless in the presence of AA the drug was able to interact and showed anticonvulsant action, enhancing the delivery. These results will provoke further research into the vitamin C transporters and their interactions with specific drugs. If these predictions are correct this knowledge can be utilised for designing and testing of new drugs and medicines.

1.7 Rationale

Establishing how specific types of cells transport vitamin C is essential in recognising how vitamin C homeostasis is controlled across mammalian bodies. Over the past decade substantial developments have been made in our understanding of the systems by which vitamin C distributes into cells. Nevertheless, little knowledge is acknowledged on the mechanisms which control the actions and localisation of these transporter proteins.

Extending our knowledge on the structural operating mechanisms of hSVCT1 and hSVCT2 gives the opportunity to manage or influence the cell surface presence of these

proteins. This could be greatly beneficial to certain people whose health is affected by low ascorbate plasma concentrations as a result of ; genetic, environmental and physical factors. An advanced understanding of these mechanisms would provide the opportunities for tissue specific drug delivery. This would be highly important for the pharmaceutical drug design and medicinal treatment of patients as it could aid the development of new therapeutic medications.

Pinpointing the specific sites on SVCT1 and SVCT2 using approaches of mutagenesis may be important for a better understanding of membrane interaction mechanisms. This may be used for further study into how certain residues can influence the membrane targeting of each transporter. By analysing each protein and nucleotide sequence and consequently altering certain amino acids, it will provide an insight into whether these sites are influential in the mechanisms of the transporters. The created mutants will support future studies on the mechanisms that control targeting of SVCT1 and SVCT2. It will also aid our understanding on how the proteins will react and be affected by drug competition, such as medicines entering the human body.

1.8 Aims and Objectives

The aim of this master's research is to prepare a starter for future evaluation of the molecular recognition sites of the human vitamin C transporters through studying the expected crucial sites on the proteins. To accomplish this aim the following objectives were designed:

Objective 1: To assess hSVCT1 and hSVCT2 coding sequences and protein sequences with the use of bioinformatics tools and literature reviews. This highlights area including reading frames that are likely to play a factor in molecular recognition.

Objective 2: To conduct PCR based site specific mutagenesis on hSVCT1 and hSVCT2's coding regions that are predicted to be important.

Objective 3: To obtain molecular clones through transformation methods and colony growth into phSVCT1 and phSVCT2 plasmids.

Objective 4: To confirm the mutations by sequencing analysis, assisted with bioinformatic approaches.

2.0 Materials and Methodology

2.1 Materials

All reagents used were, where appropriate, molecular biology grade and are listed in Table 2-1. Details of this use of these enzymes are explained in Table 2-2 and the main enzymes used are listed in Table 2-3. Maps of the host plasmids are shown in Figure 2-1. All resources including pipette tips, beakers and flasks were all autoclaved prior to us. COSHH and Record of Risk Assessment forms can be found in Appendix I and II.

Due to the nature of this research a variety of commercial kits were utilised. The QIAPrep- spin MiniPrep Kit to extract hSVCT1, hSVCT2 and JM109, the QIAquick PCR Purification Kit to purify PCR products and the QIAquick DNA Gel Extration Kit used following gel electrophoresis. See Table 2-1 for all commercial kits, their manufacturers and catalogue numbers.

The necessary enzymes included: T_4 DNA Ligase from New England Biolabs, *Taq* Polymerase from Promega Ltd, *Dpn1* from New England Biolabs, restriction endonuclease *XbaI* and restriction endonuclease *HindIII* both from Thermo Fisher. See Table 2-1 for all enzymes, manufacturers and catalogue numbers.

Throughout the research project a number of chemicals were regularly used to undertake the laboratory procedures these include: Ethanol Absolute, Agarose Powder, Tryptone, Yeast Extract, Agar Powder, 1kb Ladder, 5X Flexi Buffer, Magnesium Chloride solution, NEB Buffer 2, BSA, SYBR Safe gel stain and ampicillin 100mg/ml. See Table 2-1 for a list of all chemicals, manufacturers and catalogue numbers and see Table 2-2 for the application of each chemical.

For the protocols to be effective the following equipment and machinery were used: Bio-Rad ChemDoc MP Imaging System for gel electrophoresis, JB Nova water bath, NanoDrop 2000 Spectrophotometer, Shimadzu Spectrophotometer UV-1800, Heraeus centrifuge, centrifuge S3430R, 800W Large Autoclave, Orbital Shaker and Benchmark Heated Magnetic Stirrer.

Table 2-1. List of reagents and their manufacturers.

#	Reagent	Manufacturer	Catalogue Number	Contact
1	1kb Ladder	Promega	G5711	www.promega.com
2	5X Green GoTAQ™ buffer	Promega	M7911	www.promega.com
3	10X Buffer (1-4)	NEB	B7000S	www.neb.com
4	Agar	FS	BP1423-500	www.fishersci.com
5	Agarose	FS	V3121	www.fishersci.com
6	Ampicillin	Sigma-Aldrich	A9393	www.sigmaaldrich.com
7	Bacto-tryptone	FS	BPE1421-500	www.fishersci.com
8	Bacto-yeast extract	FS	BP1422-500	www.fishersci.com
9	Bovine Serum Albumin	NEB	B9000S	www.neb.com
10	ddH_20	Acros Organics	345470250	www.acros.com
11	dNTP	Roche	11969064001	www.roche.com
12	Dpn1	NEB	R0176S	www.neb.com
13	EDTA	FS	BP120-500	www.fishersci.com
14	Ethanol	FS	BP2818-500	www.fishersci.com
15	GoTaq Polymerase	Promega	M3001	www.promega.co.uk
16	HCl (Concentrated)	FS	SA49	www.fishersci.com
17	HindIII Enzyme	ThermoFisher	ER0501	www.thermofisher.com
18	Isopropanol	FS	BP2618-212	www.fishersci.com
19	$MgCl_2$	Promega	A3511	www.promega.com
20	NaCl	BDH Lab Supplies	S271-500	www.uk.vwr.com
21	QIAquick Gel Extraction Kit (50)	QIAGEN	28704	www.QIAGEN.com
22	QIAquick PCR Purification Kit (50)	QIAGEN	28104	www.QIAGEN.com

23	QIAprep Spin Miniprep Kit (250)	QIAGEN	27106	www.QIAGEN.com
24	Sepharose	Sigma-Aldrich	CL6B200	www.sigmaaldrich.com
25	Shrimp Alkaline Phosphatase	NEB	M0371S	www.neb.com
26	SYBR Safe DNA gel Stain	Invitrogen	S33102	probes.invitrogen.com
27	T4 DNA Ligase reaction buffer	NEB	B0202S	www.NEB.com
28	Tris Acetate	FS	BPE1331-1	www.fishersci.com
29	Tris Base	FS	BP152-1	www.fishersci.com
30	XbaI Enzyme	ThermoFisher	IVGN0126	www.thermofisher.com
31	Yeast Extract	Sigma-Aldrich	70161-50G	www.sigmaaldrich.com

Table 2-2. Uses of Reagents from Table 2-1.

#	Use
1	Loaded during agarose gel electrophoresis to approximate weight of DNA
2	Loading dye for agarose gel electrophoresis (Enables DNA in samples to sink to the bottom of the wells)
3	Restriction analysis and overhanging ends formation
4	Used to make agar plates for *E.coli* DH5α (phSVCT): Selection/recovery and transformation
5	Used to make a gel for electrophoresis
6	Added to LB agar
7	To make LB medium and agar plates
8	To make LB medium and agar plates
9	Used during restriction digest to stabilise certain enzymes and to prevent adhesion
10	To make buffers, media, agar plates and dilutions
11	Component used during PCR
12	Used to cut methylated DNA
13	To make TE buffers for dilutions
14	Sterilisation of glass transmitter used in transformation
15	Component of PCR
16	To make TRIS-Cl, used as elution buffer in certain gel extractions and to make TE buffers
17	Component which cuts sequences during restriction digest
18	Gel extraction of DNA (Increases the yield of DNA fragments)
19	Component used during PCR
20	Used to make LB agar plates
21	Extraction of PCR products
22	Purification of PCR products
23	Cell lysis and Extraction of phSVCT plasmids

24	Used during purification of PCR products
25	Prevents religation of linearized plasmid DNA
26	Used to dye the agarose gel
27	Ligation of fusion products with the vector
28	To make TE buffers and TAE buffers for Agarose Gel Electrophoresis
29	To make TE buffers and TAE buffers for Agarose Gel Electrophoresis.
30	Component which cuts sequences during restriction digest
31	Component which is used to make LB plates

Table 2-3. Enzymes Used

***E.coli* Strain**	**Chromosomal Genotype**	**Source**	**Use**
DH5α (phSVCT1)	*fhuA2, Δ (argF-lacZ), U169, phoA, glnV44, Ø80 Δ (lacZ),MI5, gyrA96, recA1, relA1, endA1,thi-1, hsdR17. (hSVCT1 cloned in pcDNA3.1, with the V5 epitope and 6-histidine tail.)*	Liang, W-J, (NEB; Liang et al.2017)	Source of plasmid phSVCT1
DH5α (phSVCT2)	*fhuA2, Δ (argF-lacZ), U169, phoA, glnV44, Ø80 Δ(lacZ),MI5, gyrA96, recA1, relA1, endA1,thi-1, hsdR17. (hSVCT2 cloned in pcDNA3.1, with the V5 epitope and 6-histidine tail.)*	Liang, W-J, (NEB; Liang et al.2017)	Source of plasmid phSVCT2
MC1061	*K-12 F^- λ^- Δ(ara-leu)7697 [araD139]B/r Δ(codB-lacI)3 galK16 galE15 e14$^-$ mcrA0 relA1 rpsL150(Str^R) spoT1 mcrB1 hsdR2(r^-m^+)*	Liang, W-J, (NEB; Liang et al.2017)	Sub-cloning of hSVCT cDNA

Table 2-4. *E.coli* Strains Used

***E.coli* Strain**	**Chromosomal Genotype**	**Source**	**Use**
DH5α (phSVCT1)	*fhuA2, Δ (argF-lacZ), U169, phoA, glnV44, Ø80 Δ (lacZ),MI5, gyrA96, recA1, relA1, endA1,thi-1, hsdR17. (hSVCT1 cloned in pcDNA3.1, with the V5 epitope and 6-histidine tail.)*	Liang, W-J, (NEB; Liang et al.2016)	Source of plasmid phSVCT1
DH5α (phSVCT2)	*fhuA2, Δ (argF-lacZ), U169, phoA, glnV44, Ø80 Δ(lacZ),MI5, gyrA96, recA1, relA1, endA1,thi-1, hsdR17. (hSVCT2 cloned in pcDNA3.1, with the V5 epitope and 6-histidine tail.)*	Liang, W-J, (NEB; Liang et al.2016)	Source of plasmid phSVCT2
MC1061	*K-12 F^- λ^- Δ(ara-leu)7697 [araD139]B/r Δ(codB-lacI)3 galK16 galE15 e14$^-$ mcrA0 relA1 rpsL150(Str^R) spoT1 mcrB1 hsdR2(r^-m^+)*	Liang, W-J, (NEB; Liang et al.2016)	Sub-cloning of hSVCT cDNA

2.2 Methodology

2.2.1 Strategy One

To perform the laboratory experiments a relative efficient strategy had to be designed and followed. This strategy is outlined in Figure 2-1.

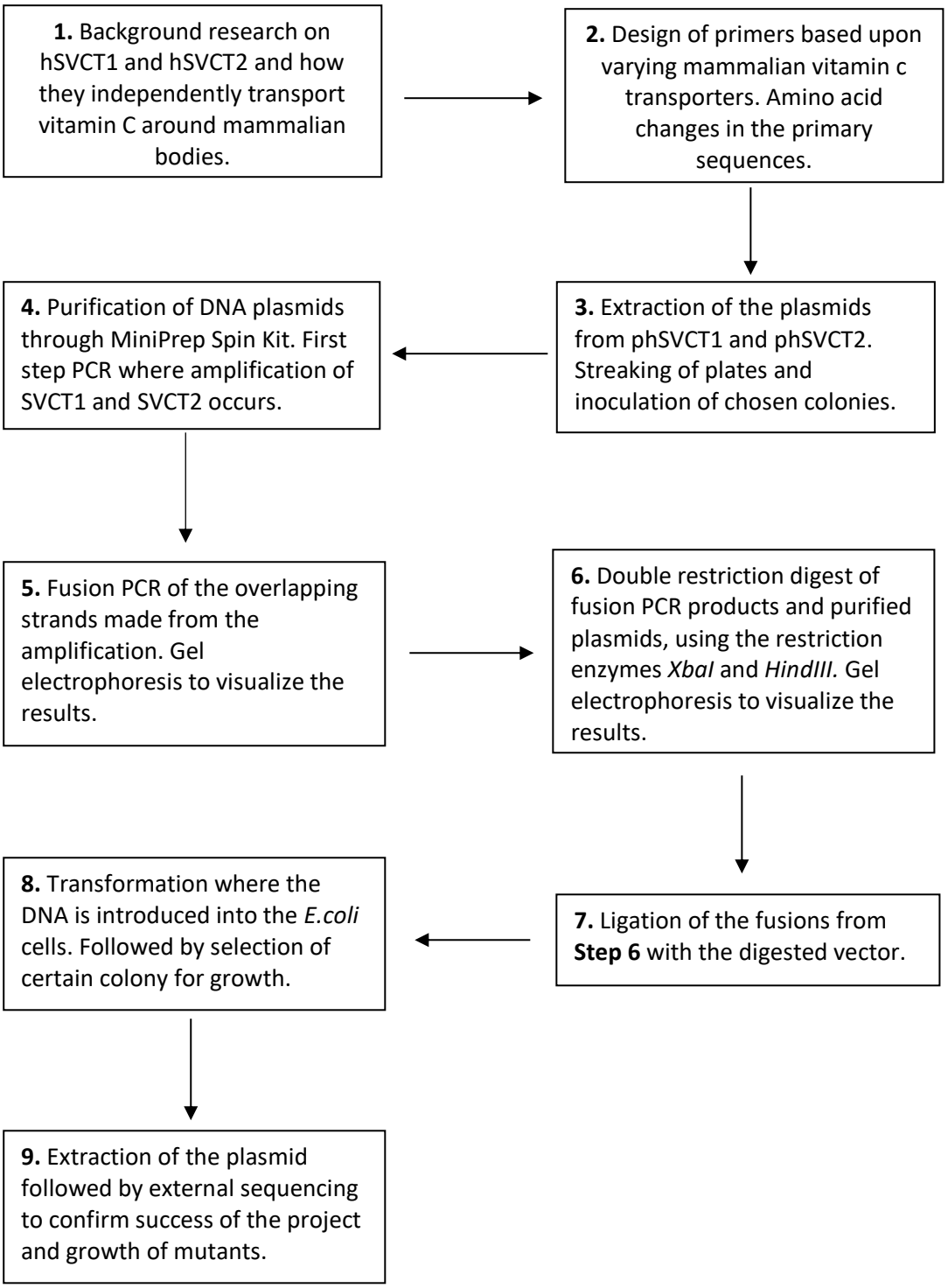


Figure 2-1. Flowchart of Overall Research Strategy

2.2.2 Strategy Two

A new strategy was designed for this project when the initial outline was not successful. The improved strategy was effective and more efficient. Strategy two is outlined in Figure 2-2.

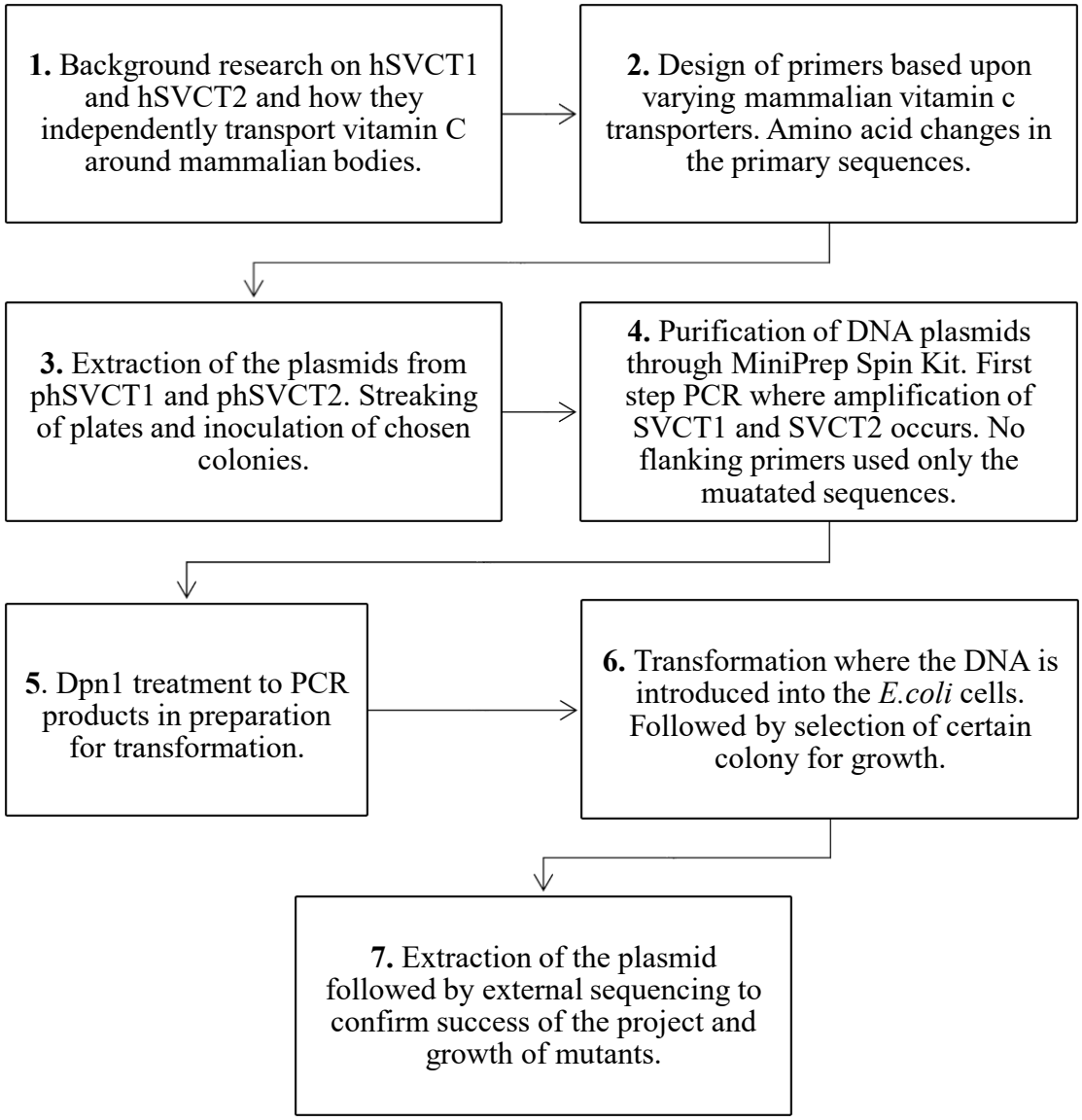


Figure 2-2. Flowchart of strategy two which was utilised to complete the project.

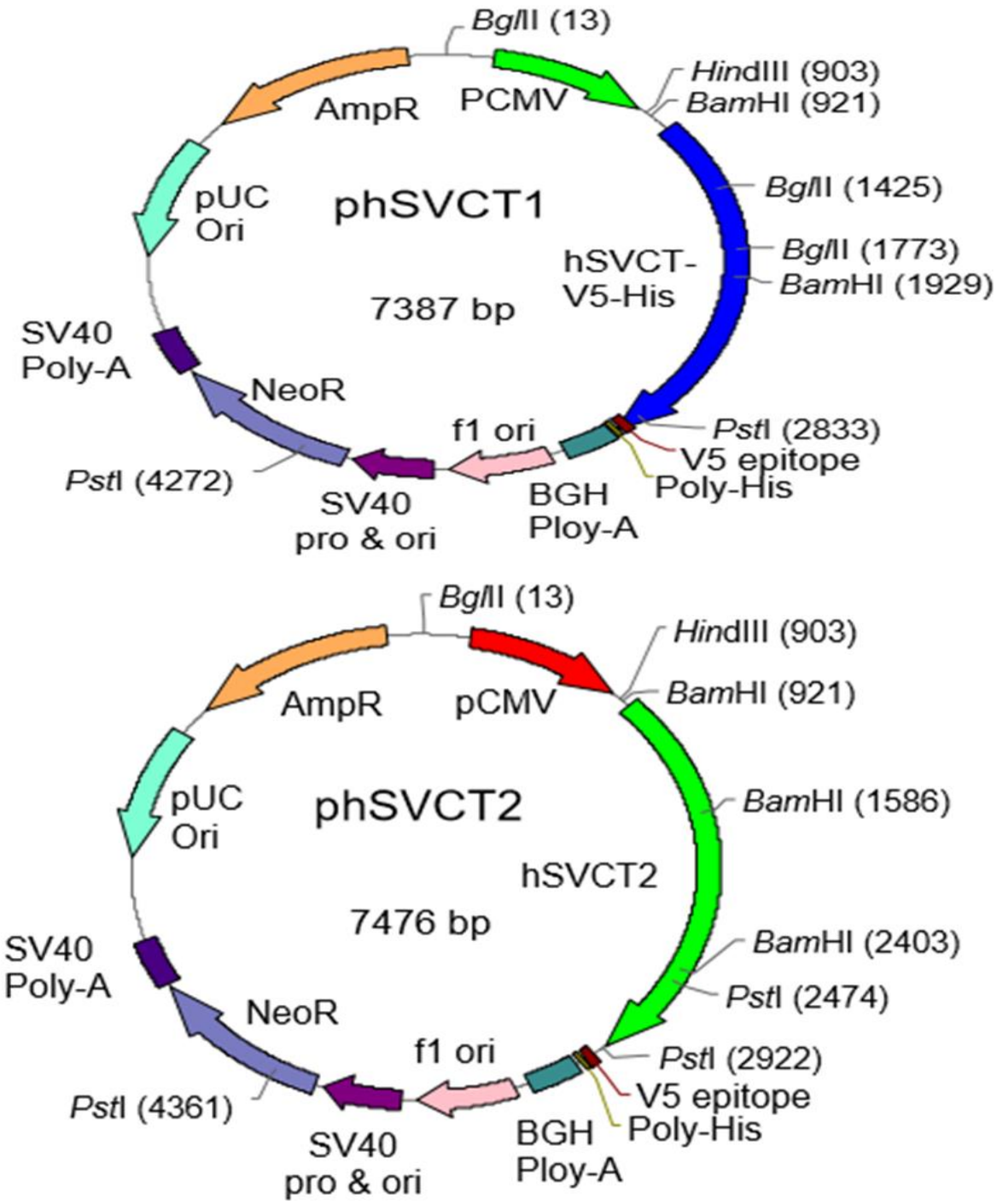


Figure 2-3. Plasmids phSVCT1 and phSVCT2: Mammalian Expression Vectors Used as Parent Clones and Vectors. The plasmid maps were designed using Redasoft Plasmid 1.1 software. hSVCT1 and hSVCT2 were cloned into the plasmids pcDNA3. Primer binding sites have been indicated in green.

2.2.3 Primer Design

Primers were designed based upon the amino acid coding sequences of the two vitamin C transporters. The primers are an important constituent used during the polymerase chain reaction which allow the gene, phSVCT, to be amplified. They also facilitate in the alteration of restriction sites found on the vector and gene. The membrane sequences were compared between numerous organisms, particularly among those who can naturally synthesise vitamin C against those who have to consume or ingest the nutrient to survive. A total of five primers sets were designed for each of the transporters, centred upon altering specific amino acids which appeared to be crucial in their primary structures. The coding sequences of the transporters were found using NCBI databases and the open reading frames were identified using the lalign program. The primers were created for replacing one amino acid with another, based on their charge, properties and location on the protein sequence. To design effective primers, it is crucial that the sequences meet the specific criteria to enable them to anneal to specific regions. The following formula was utilised which allowed the correct annealing temperatures (Ta) and melting temperatures ^{TM} for the primers;

$$Tm= 81.5 + 41 \times CG\% - 675/mers$$

$$Ta= Tm-5°C$$

Adhering to the criteria, primer was created between 18-25 nucleotides long and had calculated annealing temperature between 58-71°C for differing PCR reactions. A forward and reverse primer was designed for each change in amino acid, which were constructed with less than 1°C difference between annealing temperatures. The designed primers were diluted with $T_{10}E_1$ prior to any use during the laboratory process.

2.2.4 Preparation of Essential Solutions, Reagents and Buffers

Tris(hydroxymethyl)aminomethane – hydrochloride (TRIS-HCl) is designed to keep solutions within a pH range of 7.0 to 9.0. To make a 1M solution, 121.14g of Tris was mixed to 800ml of deionised water on a hot plate until fully dissolved, this was then combined with 54ml of 37% HCl, which caused the pH of the solution to reach 8. This specific value was measured using a pH indicator and at pH8 DNAase is prevented from

reacting any further. The final solution was subsequently autoclaved under a media setting, guaranteeing sterilisation.

Ethylenediaminetetraacetic acid (EDTA) was also formed as it is an important component in inactivating enzymes. To make a 0.5 Molar solution of EDTA with a pH near 8.0, 93.06g of EDTA was added to 500ml of deionised water and 11.7g of NaOH pellets were dissolved into the solution to increase to pH 8. The solution was thoroughly mixed using a magnetic stirrer and the pH was confirmed with a pH indicator. At this pH level, the solution was then autoclaved until the solution became transparent.

$T_{10}E_1$ (10mM Tris HCl pH8.0, 1mM EDTA) is needed for diluting both primers and uncut DNA and was made up to 1ml each time. 2µl of EDTA was added to 10µl of Tris-HCl and 988µl of distilled water into a 1.5ml Eppendorf.

To make Luria-Bertanin (LB) ampicillin agar, 100µg/ml of ampicillin stock solution was mixed to the LB agar media constituents, see Table 2-4, this occurred after the components had been autoclaved, mixed on a heat block and cooled (Maniatis, 1989).

Table 2-5. Constituents used to make LB agar.

Constituent	**Mass (grams)**
Tryptone	10
Yeast Extract	5
Sodium Chloride (NaCl)	10
Agar	15

2.2.5 Culture of Bacteria Containing SVCT Plasmids

The host bacteria, DH5α (ph*SVCT1*) and DH5α (ph*SVCT2),* were selected from storage in deep freeze (-80^{o}C) and were streaked onto the prepared ampicillin LB plates under a tungsten loop to separate the pure strain from a single species of the bacteria. The streaking took place using a Bunsen burner, to ensure that all equipment was sterile reducing the chance of contamination. This technique is used to obtain a single colony of bacteria and identifies potential contamination. These streaked plates were then incubated for approximately 14 hours, upside down at 37°C and subsequently at room temperature for 30 minutes to allow for sufficient growth. All plates were stored at 4 ^{o}C

for a maximum of two weeks. After the first stage of incubation, an appropriate single colony was selected from the plate and inoculated into a separate 50ml Falcon tube containing liquid LB medium and ampicillin. These colonies were then grown at 37°C for 18 hours, with continuous shaking (250 rpm).

2.2.6 DNA Quantification with NanoDrop

The DNA Thermo Scientific NanoDrop 2000 Spectrophotometer was utilised which uses UV and visible light to determine the concentrations of the nucleic acids and to quantify the amount of DNA in the samples. To begin, 1µl of water was added acting as 'blank' testing to calibrate the machine. To check the absorbance of the whole DNA sample 1µl of the required plasmid DNA was used. The concentration results indicated whether a dilution to the plasmid was suitable for the PCR process. This was subject to the 260/280 ratio; which should be between 1.80-2.0 and the OD 260 reading that was required between 0.20-0.80. The final nucleic acid concentration must be no more than 50µg for double stranded DNA.

2.2.7 QIAprep@ Spin Miniprep Kit

The QIAprep Spin Miniprep Kit (Qiagen) was utilised for purifying the SVCT1 and SVCT2 plasmid DNA by isolating certain plasmids from bacterial cells. The kit used silica-gel-membrane technology to bind DNA, from which the DNA can be eluted with an elution buffer or water. 50ml of each sample was formed from this protocol and the draw through was pipetted into a new 1.5ml Eppendorf and eluted in $T_{10}E_1$ buffer. 4µl of the DNA sample was then transferred into a new Eppendorf ready to be mixed with 1µl of *GoTaq*5X Green Buffer, in preparation for screening gel electrophoresis.

2.2.8 Polymerase Chain Reaction- PCR

To begin the cloning procedure, 10 fragments were amplified in total; 5 from phSVCT1 and 5 from phSVCT2. The primers needed for first step PCR were initially diluted accordingly into the prepared $T_{10}E_1$, depending on their weight. The preparation for the PCR took place using the constituents shown in the Table 2-5 and a specific programme was designed based upon the annealing temperatures of the created primers. During the running programme the annealing temperature was maintained at 60.0°C, this was

calculated as the average between all the samples, which is important as efficiency and specificity of PCR are strongly affected by the annealing temperature. The course comprised of 95°C for 5 mins and then 28 cycles each at; 94°C for 1 min, an extension of 60°C for 30 seconds, 72°C for 2 mins and finally the last extension of 72°C for 4 mins.

Table 2-6. Constituents used for PCR.

Constituent	**Volume (µl)**	**Final Concentration**
dH_20	33.5	
5X Flexi Buffer	10	1X
1mM dNTP	1	1mM
25mM $MgCl_2$	2	0.2µM
10µM Forward Primer	1	2 µM
10µM Reverse Primer	1	2 µM
DNA Sample	1	
GoTaq DNA Polymerase	0.5	2.5units/ 50µl

In preparation for PCR to successfully generate specific DNA target regions, a solution of 10mM dNTP was formed. This was accomplished using 10µl of dATP, dDTP, dCTP, dGTP joined with 60µl of deionised water. This 100µl solution was then equally divided into falcon tubes and diluted further at a 1:9 ratio so that 1mM of dNTP could be used as a PCR constituent.

2.2.9 QIAquick PCR Purification Kit

QIAquick Kit is used prior to fusion PCR to purify the samples. The purification process eliminates primers, nucleotides, enzymes, salts, agarose, ethidium bromide, and other impurities from DNA samples. Silica-membrane equipment removes the complications and difficulties related with loose resins and slurries. The QIAquick kit uses a simple bind-wash-elute process.

2.2.10 Sepharose Purification

Sepharose CL-6B 200 is used to separate media and resins due its extensive fractionation ranges making it appropriate for characterising or cleaning up samples holding components of diverse molecular weights. The Sepharose beads were used following primer PCR to ensure that a high level of purification took place to remove any remaining impurities, including salts and primers. 15-20ml of Cl-6B 200 was poured into a falcon tube and placed into the refrigerated Denley BR401 centrifuge and span at 3000rpm for 10 minutes. The volume of supernatant was then recorded and removed and an equal volume of $T_{10}E_1$ was added. This process was repeated a further three times until a large volume of supernatant had been removed. To form a filter, glass beads were added to a cut 100ul pipette and approximately 750ul of the prepared CL-6B 200 mixture was added. This was inserted into an Eppendorf and spin column and span in a centrifuge at 9000rpm for 10 minutes until no aqueous solution remained. 45ul of the DNA sample was added to the spin column and centrifuged providing approximately 40ul of pure DNA remaining.

2.2.11 *Dpn1* Treatment

The restriction enzyme *Dpn1* is utilised after PCR due to its ability to digest methylated GATC sites. 1ul of the enzyme Dpn1 was added to the PCR samples alongside 5.5ul of 1XCutSmart®Buffer. The samples were then placed into the incubator for 20 minutes at 80°C to heat shock the enzyme and prevent any further reactions.

2.2.12 Fusion Polymerase Chain Reaction

Fusion PCR involves two parallel PCR amplifications from plasmid templates. PCR fusion of the amplified fragments from the first step PCR occurred through a single overlap extension occurring on PCR fragments from the parallel reactions. The first amplification used 10 parallel reactions, where 5 overlapping regions on each plasmid took place. The fusion PCR took place using a programme which was similar to the initial PCR setting. The lid temperature was also maintained at 110.0°C with an annealing temperature of 60°C. The programme ran using 28 cycles; 95°C for 5’0”, 94°C for 1’0”, an extension of 60°C for 30”, 72°C for 4’30” and a concluding extension of 72°C for 4’30”. Following each PCR procedure, gel electrophoresis took place using

a 1.5 % w/v agarose gel, which was left to proceed for 40 minutes at 70V. Those bands which were required were extracted from the gel using a QIAquick Gel Extraction Kit and were consequently purified.

Table 2-7. Constituents and volumes used for Fusion PCR

Constituent	Volume (µl)	Final Concentration
dH_20	33.5	
5X Flexi Buffer	10	1X
1mM dNTP	1	1mM
25mM $MgCl_2$	2	0.2µM
10µM Forward Primer	1	2 µM
10µM Reverse Primer	1	2 µM
Primary PCR DNA Sample	1	
GoTaq DNA Polymerase	0.5	2.5units/ 50µl

2.2.13 Agarose Gel Electrophoresis

Agarose gel electrophoresis was run following the completion of most stages of the practical project as it separates DNA by size for visualization and purification of the samples (Smith, 1996). The agarose gel was made using 40ml of 1X TAE Buffer (using diluted 50X TAE Buffer), 64g of genetic analysis grade agarose .The components were mixed together in a microwavable flask and then heated until clear and fully dissolved. Once slightly cooled, 4µl of SYBR Safe DNA gel stain is added which binds to the DNA and allows you to visualize the DNA under UV light. The agarose was then poured into a loading tray with a well comb in place and left to set for 20 minutes at room temperature. The agarose gel at 1.5% w/v, was loaded with 5X Green GoTAQ™ buffer as a loading dye. To create a 1kb ladder, required for agarose electrophoresis gels, 100µl of promega 1kb ladder was added to 100µl of 5X Green GoTAQ™ buffer and 300 µl of $T_{10}E_{1.}$ 5µl of a 1kb DNA ladder was loaded alongside the experimental samples in each gel and was run at 70V for 40 minutes. To represent the results from the gel electrophoresis a ChemiDoc™ MP Imaging System is utilised to analyse and easily quantify the samples, the machine uses a camera and UV-and white light illumination.

2.2.14 Restriction Digest

Restriction digest took place with the use of phSVCT1 and phSVCT2 vectors and fusion fragments. This allowed the DNA molecules to be cut into smaller pieces with the use of restriction endonucleases, preparing the DNA for analysis. The chosen restriction enzymes, *XbaI* and *HindIII* cut the DNA into segments at specific restriction sites. The restriction digests were arranged using the components shown in Table 2-7. and the samples were then left in an incubator at 37°C for approximately 3 hours and subsequently stored within the freezer.

Table 2-8. **Constituents and volumes used for Restriction Digest**

Constituent	**Volume (µl)**
dH_20	37
10X NEB Buffer 2	5
BSA (10mg/ml)	1
Plasmid DNA/ PCR DNA	5
Enzyme (20 units/ µl)	2

2.2.15 Making Competent Cells

In preparation for transformation the strain JM109 was grown on LB ampicillin prepared plates by streaking the strain four times in a certain pattern, using a d-loop and a Bunsen burner for sterilisation. The plates were left in the incubator overnight at 37°C for approximately 16 hours at 2500rpm to allow a sufficient number of colonies to grow. Following the growth of JM109 an individual colony was chosen and placed into the media using the d-loop, under sterilised conditions. The colony was inoculated overnight held within a flacon tube and shook in the orbital shaker at 250rpm at 37°C until the solution turned cloudy. Following the inoculation of JM109, 250ul of the competent cells were pipetted into 25ml of LB media and placed into the orbital shaker for a further 2 hours, at the same conditions. The mixture was then measured for absorbance using a Shimadzu UV-1800 Spectrophotometer which gave a log phase reading at A600 of 0.2552Abs. The culture was then chilled on ice and 1ml of cells was harvested by quick centrifuging at 8000rpm for 2 minutes at 4°C. The supernatant was

then discarded and the cells were re-suspended in 500ul of ice cold sterile calcium solution (50mM $CaCl_2$, 10mM Tris HCl, pH 8.0). The suspension was placed into an ice bath for 15 minutes and centrifuged at room temperature for 1 minute at 10,000 rpm. The supernatant was removed and to conclude the cells were re-suspended in 66ul the calcium solution.

2.2.16 Transformation

7µl of the plasmid DNA mixture was added to 200 µl of the competent cells on ice and was then left to incubate at 37°C for 20 minutes. The DNA mixture was then heat shocked at 42°C, which occurred using a water bath for two minutes, and the samples were instantly transferred straight back onto ice for a further 2 minutes. Each sample was added to 330 µl of SOC media (2% tryptone, 0.5% yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2$, 10 mM $MgSO_4$, and 20 mM glucose.) and incubated further at 37°C for 60 minutes.

200 µl of those incubated cells were transferred onto "pre-dried" ampicillin plates which contained LB agar (100 µg/ml), where glass beads were utilised to ensure that the solution was spread across the entire plate. These loaded plates were left to incubate over night for approximately 17 hours at 37°C upside down, to enable sufficient growth.

Following incubation, the transformation samples were purified using the QIAprep® Spin Miniprep Kit. They were later digested for an hour at a temperature of 37°C using the same procedure as previously stated in 2.2.14 using the restriction enzymes, *XbaI* and *HidIII.*

To show the successful samples, a final electrophoresis gel was run for 40 minutes at 70 w/v which compared the uncut DNA against the digested DNA. Prior to the making the gel, the uncut DNA had been diluted using 9µl of prepared $T_{10}E_1$ to 1µl of the sample, this ensured that the bands did not appear too bright on the gel and reduced the chance of smearing.

3.0 Results

3.1 Strategy One

3.1.1 Primer Design

To complete the PCR- based site directed mutagenesis, primers were specifically constructed for successful laboratory research. Throughout the creating process numerous factors were involved including, the final annealing temperature and melting temperature, the G/C content, the primer sequence length and the number of di-nucleotide repeats. Strategy steps 1 and 2 were effectively completed. The physical properties and template-binding sites of the primers are displayed in Table 3-1.

Table 3-1. Initial Primer Design

Primer	Nucleotide Sequence (5'- 3')	Annealing Temperature (ºC)	Amino Acid Change	Source	Design
pcDNA3F	CCACTGCTTACTGGCTTATCG	65.3		Eurofins	C.D*/ W-J.L**
pcDNA3R	GCCCTCTAGACTCGAGCG	66.3		Eurofins	C.D*/ W-JL**
VTC1P190SF	GTCACCCCCACTGTCTCC	66.3	Proline to Serine	Eurofins	CD*/ W-J.L**
VTC1P190SR	GGAGACAGTGGGGGTGAC	66.3		Eurofins	CD*/ W-J.L**
VTC1V62GF	ACCATCGCCGTGCCCTTC	66.3	Valine to Glycine	Eurofins	CD*/ W-J.L**
VTC1V62GR	GAAGGGCACGGCGATGGT	66.3		Eurofins	CD*/ W-J.L**
VTC1I96LF	ACCACTCTCATCCAGACCAC	65.3	Isoleucine to Leucine	Eurofins	CD*/ W-J.L**
VTC1I96LR	ACCACTCTCATCCAGACCAC	65.3		Eurofins	CD*/ W-J.L**
VTC1L277FF	ACAGACGTGCTGCCCACAG	66.3	Leucine to Valine	Eurofins	CD*/ W-J.L**

VTC1L277FR	CTGTGGGCAG CACGTCTGT	66.3		Eurofins	CD*/ W-J.L**
VTC1N385SF	GTCCAGTCCC AACATTGGC	64.7	Asparagine to Serine	Eurofins	CD*/ W-J.L**
VTC1N385SR	GCCAATGTTG GGACTGGAC	64.7		Eurofins	CD*/ W-J.L**
VTC2A174VF	CATTTTTGGCC CCTGCTCGA	65.3	Alanine to Valine	Eurofins	CD*/ W-J.L**
VTC2A174VR	TCGAGCAGGG GCCAAAAATG	65.3		Eurofins	CD*/ W-J.L**
VTC2K184RF	TCTTTAGATAA ATGGAAATGT AACAC	66.3	Lysine to Arginine	Eurofins	CD*/ W-J.L**
VTC2K184RF	GTGTTACATTT CCATTTATCTA AAGACAGG	66.3		Eurofins	CD*/ W-J.L**
VTC2Q353GF	GATGCCAGGC AAGGCGTG	66.3	Glutamine to Arginine	Eurofins	CD*/ W-J.L**
VTC2Q353GR	CACGCCTTGCC TGGCATC	66.3		Eurofins	CD*/ W-J.L**
VTC2D133GF	GTGGGGTACG ACCAGTGG	66.3	Aspartic Acid to Glycine	Eurofins	CD*/ W-J.L**
VTC2D133GR	CCACTGGTCGT ACCCCAC	66.3		Eurofins	CD*/ W-J.L**
VTC2I381LF	CCGGTGTCATC GGCATGC	66.3	Isoleucine to Leucine	Eurofins	CD*/ W-J.L**
VTC2I381LR	GCATGCCGAT GACACCGG	66.3		Eurofins	CD*/ W-J.L**

Table 3-2. Dilutions, concentrations and purity of hSVCT1 and hSVCT2

Plasmid	Dilution Ratio Water (µl): DNA (µl)	Concentration (ug/ ml)	OD260 Reading (Concentration of nucleic acid)	$A_{260/280}$ (Purity ratio)
hSVCT1 (1)	3:1	17.3	0.345	1.81
hSVCT1 (2)	2:1	29.3	0.587	1.86
hSVCT2 (1)	5:1	20.7	0.414	1.86
hSVCT2 (2)	3:1	21.4	0.429	1.89

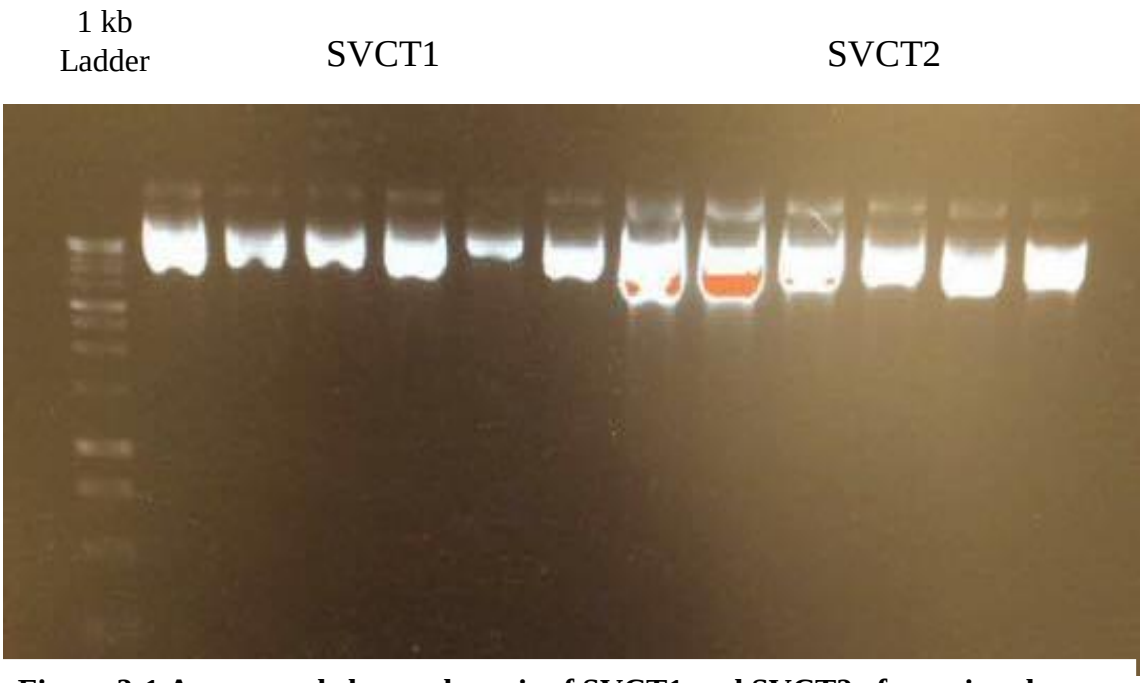


Figure 3-1 Agarose gel electrophoresis of SVCT1 and SVCT2 after using the QIAprep@ Spin Miniprep Kit. The agarose gel shows successful miniprep of the samples, hSVCT1 and hSVCT2, which illustrate supercoiled DNA. The bright staining of the bands suggest too much DNA is in the samples however this is expected following using the miniprep kit. The image was taken on a Bio-Rad ChemiDoc^{TM} MP imaging system with the use of UV light. Samples were used for both strategy one and strategy two. The method and reagents utilised are stated in 2.2.7 and the volumes of reagents, primers and their sources are described in Materials 2.0.

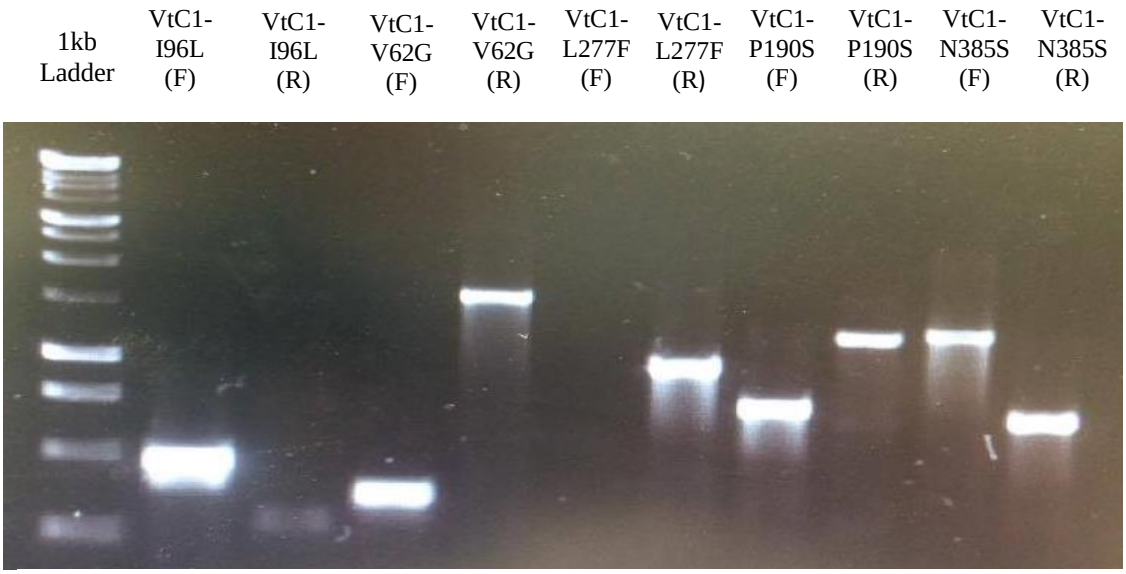
3.1.2 Extraction of Plasmids phSVCT1 and phSVCT2

Prior to completing PCR, the concentrations of the plasmids SVCT1 and SVCT2 were measured using the DNA Nanodrop. It was crucial to ensure that the current concentrations were close to a value of 50ng/ul as this would alter the volumes of plasmid added during PCR. Furthermore, the optical density at 250nm was required to be between 0.2-0.8 and the OD260/0D280 ratio was between 1.8-2.0. Certain dilutions were made to the samples using distilled water until the necessary values were within the range. See Table 3-2 for the results of the plasmid concentrations.

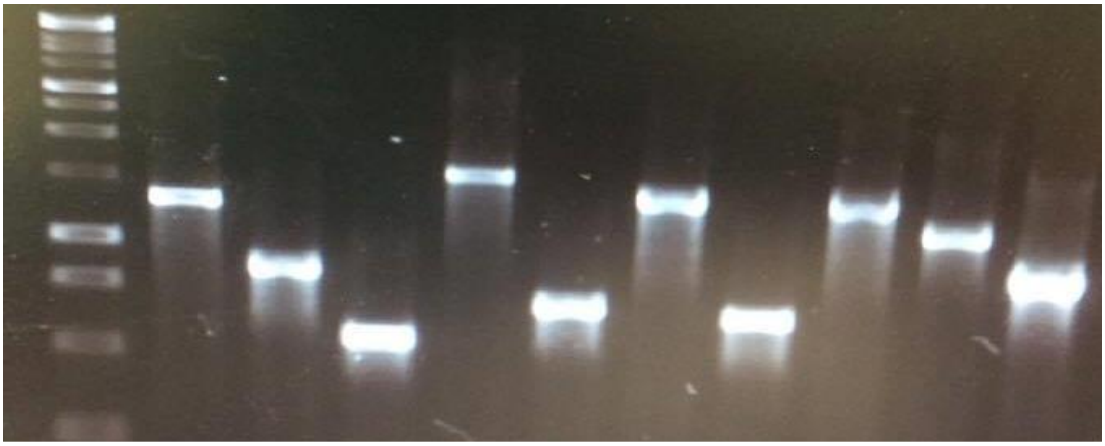
Stage 3 was positively completed. The plasmids phSVCT1 and phSVCT2 were successfully extracted and purified from the *E.coli* DH5α cells, in preparation to be utilised as both parent clones and vectors. Figure 3-1 shows the TAE agarose electrophoresis gel results for the following the inoculation of SVCT1 and SVCT2 after miniprep. The use of the QIAprep® Spin Miniprep Kit was efficient allowing approximately 8µg of plasmid DNA taken from each innoculon.

3.1.3 First Step Polymerase Chain Reaction of human SVCT1 and human SVCT2 samples.

Step 4 was successful on the first attempt as displayed in Figure 3-2 and Figure 3-3. The constructed primers and chosen PCR settings were beneficial for the amplification of the overlapping hSVCT fragments, this allowed ~2µg of desired product to be taken from each plasmid template. Purification was effective and consequently there was approximately 5ng/µl of required PCR product within each 50µl sample. Bands 2 and 5 in Figure 3-2 are both faint in the image however they were visible by the human eye following completion of the gel electrophoresis. The two bands matched the predicted sizes in comparison to the 1kb ladder and consequently these samples were used during the next stage of the process along with the other successful samples.



3-2 Agarose gel electrophoresis of the first attempt at primary PCR for hSVCT1 samples. The image shows that the gel electrophoresis was successful for all samples, excluding one. The bands display bright staining on the gel which would indicate too much DNA in the samples. All successful samples are located at the correct weights based on their sequence lengths. The image was taken on a Bio-Rad ChemiDoc™ MP imaging system with the use of UV light. The method and reagents utilised are stated in 2.2.8 and the volumes of reagents, primers and their sources are described in Materials 2.0.



3-3 Agarose gel electrophoresis of the hSVCT2 samples from the first attempt at primary PCR. The image shows that primary PCR was successful for all samples as the bands are located in the correct position in relation to the 1kb ladder, for their expected weight. All band staining is too bright which suggest too much DNA in the samples. The image was taken on a Bio-Rad ChemiDoc™ MP imaging system with the use of UV light. The method and reagents utilised are stated in 2.2.8 and the volumes of reagents, primers and their sources are described in Materials 2.0.

3.1.4 Fusion Polymerase Chain Reaction

Strategy step 5 was completed successfully using gradient PCR and is illustrated in Figure 3-4. All of the first step PCR products were fused with the corresponding remaining overlapping fragments to result in a total of 10 fused products. Five fusion products were formed for hSVCT1 and another five for hSVCT2. The results in Figure 3-4 are following multiple attempts to reach successful fusions at the correct sizes. The bands were then extracted from the gel using the QIA gel extraction kit to remove any unwanted DNA or impurities from the samples which may obstruct the next stage.

3.1.5 Plasmid and Fusion Product Restriction Digest using *Xba*I and *Hind*III.

Step 6 was the double digestion using *Hind*III and *Xba*I simultaneously, in addition to the plasmids phSVCT1 and phSVCT2 DNA. The plasmid restriction digest was successful on the first attempt as shown in Figure 3-5. This would have allowed the plasmid DNA to be linearized, forming overhanging ends that were complementary to

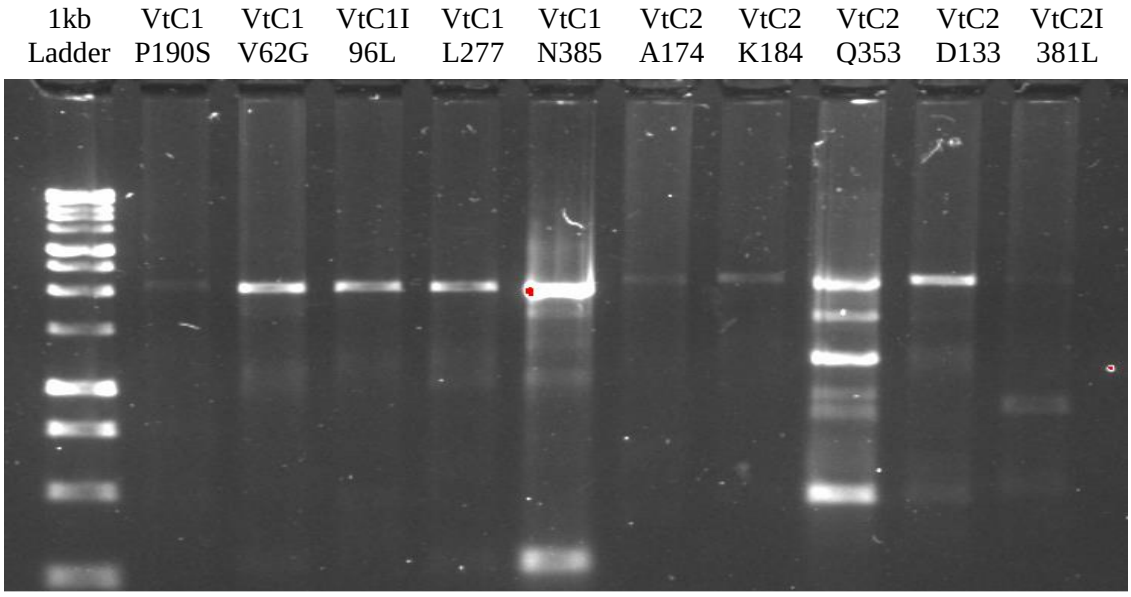


Figure 3-4 Agarose gel electrophoresis of fusion PCR products. The gel electrophoresis was run using Bio-Rad 1-D gel electrophoresis equipment at 70V for 50 minutes, 4ul of the DNA sample was loaded into the 1.5% gel. The bands for each sample are at approximately 2kb which is accurate corresponding with their predicted sizes. The method and reagents utilised are stated in 2.2.12 and the volumes of reagents, primers and their sources are described in Materials 2.0.

those which were formed during the previous stage. However, the restriction digest of the DNA samples was unsuccessful following multiple attempts, see Figure 3-6.

Multiple troubleshoots were attempted for restriction digest of the DNA samples including altering the DNA concentration, volume loaded into the wells, gel electrophoresis running times, restriction enzyme volumes and BSA volume. Nevertheless, all electrophoresis gels appeared blank with no bands visible to the human eye or via the Bio-Rad ChemiDoc^{TM} MP imaging system. Therefore, it was evident that the concentrations and purity of the samples needed to be checked to ensure that a viable volume of DNA was in the sample. The Nanodrop results are shown in the Table 3-3 which indicate the concentration of required DNA is minimal.

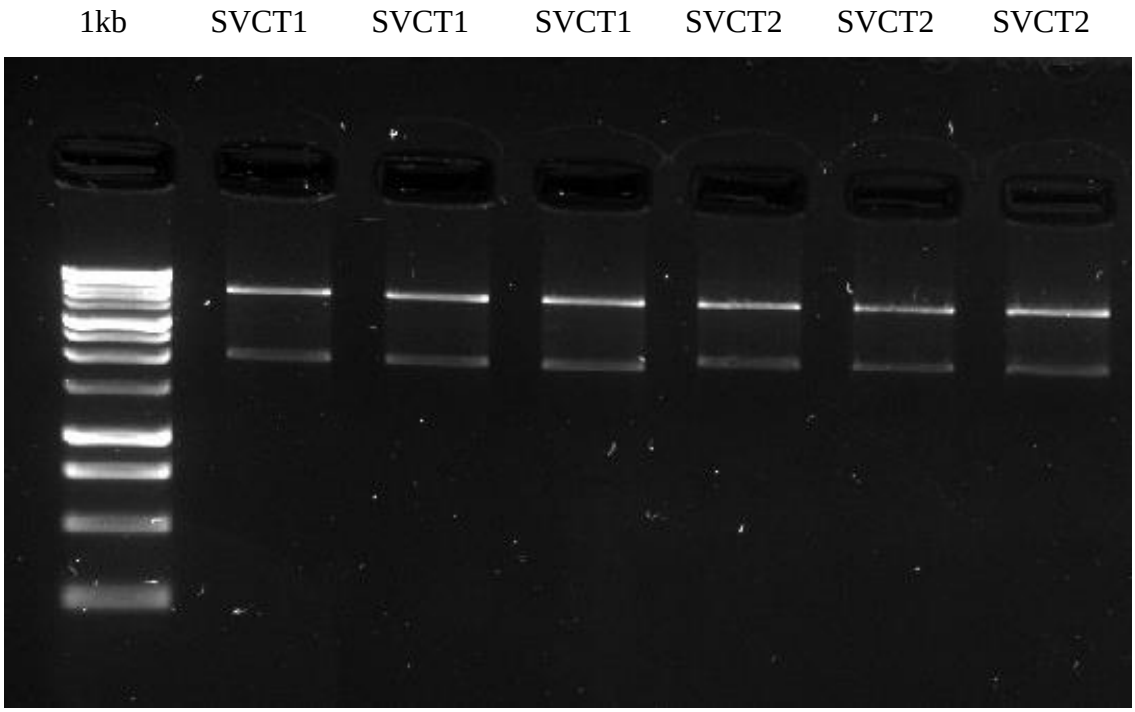


Figure 3-5 Restriction digest of the plasmids SVCT1 and SVCT2. Restriction digest of the two SVCT plasmids using the restriction enzymes *XbaI* and *HindIII***.** The gel electrophoresis was run using Bio-Rad 1-D gel electrophoresis equipment at 70V for one hour, 8ul of the DNA sample was loaded into the 1.5% gel. Apparent restriction fragment lengths shown by the bands are as expected for phSVCT1 and phSVCT2.

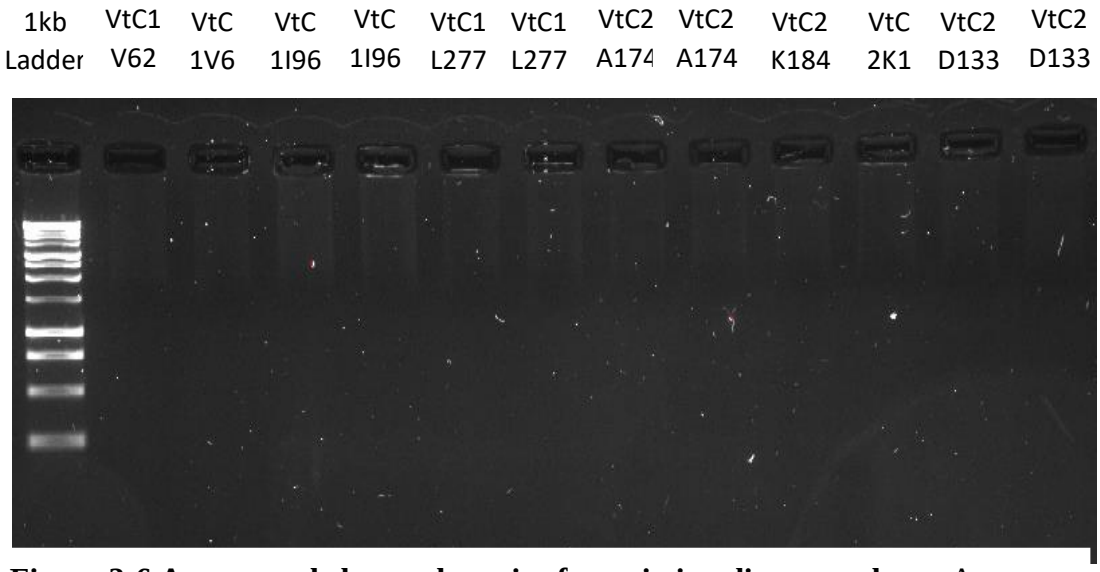


Figure 3-6 Agarose gel electrophoresis of restriction digest products. Agarose gel electrophoresis of the digested samples using the restriction enzymes *HindIII* and *XBaI* which are incubated for 4 hours. It is evident that the restriction digest was not successful for any of the samples, no bands are visible to the human eye or via the image. The image was taken on a Bio-Rad ChemiDoc™ MP imaging system with the use of UV light. The method and reagents utilised are stated in 2.2.14 and the volumes of reagents, primers and their sources are described in Materials 2.0.

Table 3-3. Concentrations and purity of samples following restriction digest.

Sample	Concentration (ng/ul)	OD260 Reading (Concentration of nucleic acid)	$A_{260/280}$ (Purity Ratio)
VtC1V62G	21.6	0.433	0.63
VtC1I96L	26.3	0.525	0.68
VtC1IL277F	10.0	0.200	0.65
VtC2A174V	6.5	0.130	0.53
VtC2K184R	27.2	0.543	0.62
VtC2D133G	18.8	0.542	0.11

The Nanodrop results show that although the overall concentration of the samples is good, the required DNA purity is very low suggesting that the samples may be contaminated, or the DNA has been lost during previous stages. Consequently, it was apparent that using these samples would provide unsuccessful results and it would be beneficial to begin the laboratory process from the beginning.

3.2 Strategy Two

3.2.1 Primer Design

To make the improved strategy successful, slight alterations were made to the primer sequences. These improvements included alterations to the sequence lengths, G/C content and amino acid changes. These improvements were made to increase the effectivity of the project and to increase the possibility of changes to the transport functions of SVCT.

1 and SVCT2. Strategy steps 1 and 2 were effectively completed and primer designs are displayed in the Table 3-4.

Table 3-4. Revisited Primer Design

Primer	**Nucleotide Sequence (5'-3')**	**GC-Content %**	**Annealing Temperature (°C)**	**Amino Acid Change**	**Source**	**Design**
pcDNA 3F	CCACTGCTTACTGGCTTATCG		65.3		Eurofins	C.D*/ W-J.L**
pcDNA 3R	GCCCTCTAGACTCGAGCG		66.3		Eurofins	C.D*/ W-JL**
mVC1 P190SF	GTCACCTCCACTGTCTCC	61.1	58.2	Alanine to Valine	Eurofins	C.D*/ W-J.L**
mVC1 P190SR	GGAGACAGTGGAGGTGAC	61.1	58.2		Eurofins	C.D*/ W-J.L**
mVC1 L277EF	ACAGACGTGCAGCCCAC	63.2	61.0	Leucine to Valine	Eurofins	C.D*/ W-J.L**
mVC1 L277ER	CTGTGGGCTGCACGTCTGT	63.2	61.0		Eurofins	C.D*/ W-J.L**

mVC1 N385S F	GTCCAGTCCCAGCATTGGC	63.2	61.0	Asparagine to Serine	Eurofins	C.D*/ W-J.L**
mVC1 N385S R	GCCAATGCTGGGACTGGAC	63.2	61.0		Eurofins	C.D*/ W-J.L**
mVC2 K184E F	CCTGTCTTTAGATGAATGGAAATGTAACAC	36.7	62.7	Lysine to Arginine	Eurofins	C.D*/ W-J.L**
mVC2 K184E R	GTGTTACATTTCCATTCATCTAAAGACAGG	36.7	62.7		Eurofins	C.D*/ W-J.L**
mVC2 Q353G F	GATGCCAGGCGAGGCGTG	72.2	62.8	Glutamine to Arginine	Eurofins	C.D*/ W-J.L**
mVC2 Q353G R	CACGCCTCGCCTGGCATC	72.2	62.8		Eurofins	C.D*/ W-J.L**
mVC2 D133G F	GTGGGGTACGGCCAGTGG	72.2	62.8	Aspartic Acid to Glycine	Eurofins	C.D*/ W-J.L**
mVC2 D133G R	CCAACTGGCCGTACCCCAC	72.2	62.8		Eurofins	C.D*/ W-J.L**

3.2.2 Extraction of Plasmids phSVCT1 and phSVCT2

Stage 3 was successful. Extraction of plasmids phSVCT1 and phSVCT2 were taken from Strategy 1. These had previously been inoculated and were extracted using the QIAprep® Spin Miniprep Kit. The TAE gel electrophoresis results are shown in Figure 3-1.

3.2.3 Primary PCR of hSVCT1 and hSVCT2 samples

Stages four and five, primary PCR and *Dpn1* treatment were successful. The annealing temperature and programme setting were designed based on the style of PCR being utilised and the primers. This form of PCR does not include the use of flanking primers and only the mutated primers and plasmids. Consequently, the programme settings are lengthened to allow enough time for the PCR to work. *Dpn1* treatment was successfully completed to ensure the majority of methylated DNA was removed.

3.2.4 Transformation

Stage six, transformation appeared successful. The transformation procedure was first optimised using a positive control (undigested hSVCT1 and hSVCT2 vectors) which gave rise to many colonies as predicted. This indicated that the transformation procedure was relatively reliable and that any colony growth on the plates would be successful transformation of the mutants. Transformation of the mutated samples into competent *JM109 E.coli* appeared successful for three of samples. Transformation of samples mVC1P190S, mVC1L277E and mVC2D133G were unsuccessful with no colonies formed (Figure 3-7). Transformation for samples mVC1N385S, mVC2K184E and mVC2Q353G formed colonies following overnight incubation at 37 ºC. Sample mVC1N385 formed 5 visible colonies, mVC2K184E shows 3 visible colonies and mVC2Q353G formed 7 colonies (Figure 3-8). Although these appeared successful the transformation efficiency is lower than expected for the amount of DNA which was added. Following this transformation procedure, it was expected that each sample would arise to 10-15 colonies forming on each plate, therefore these results were lower than predicted.

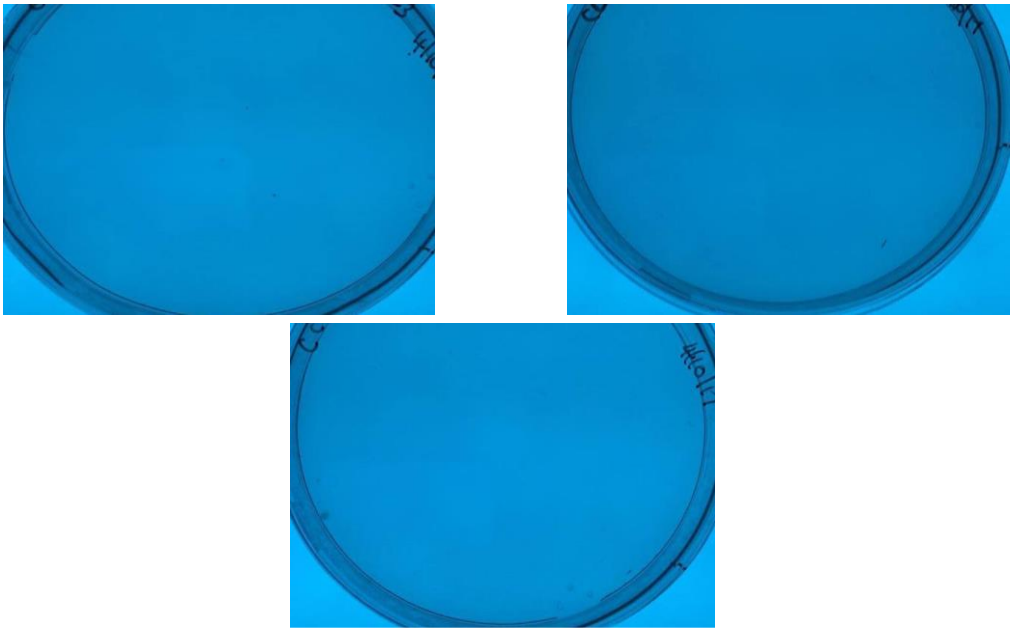


Figure 3-7. Attempted Transformation of Competent *E.coli* with mutated samples. The transformation procedure is stated in Methods. The photographs were taken using a light box with a f/5.6 for 3 seconds. No colonies formed for mVC1P190S, mVC1L277E and mVC2D133G following overnight incubation at 37 ^{o}C.

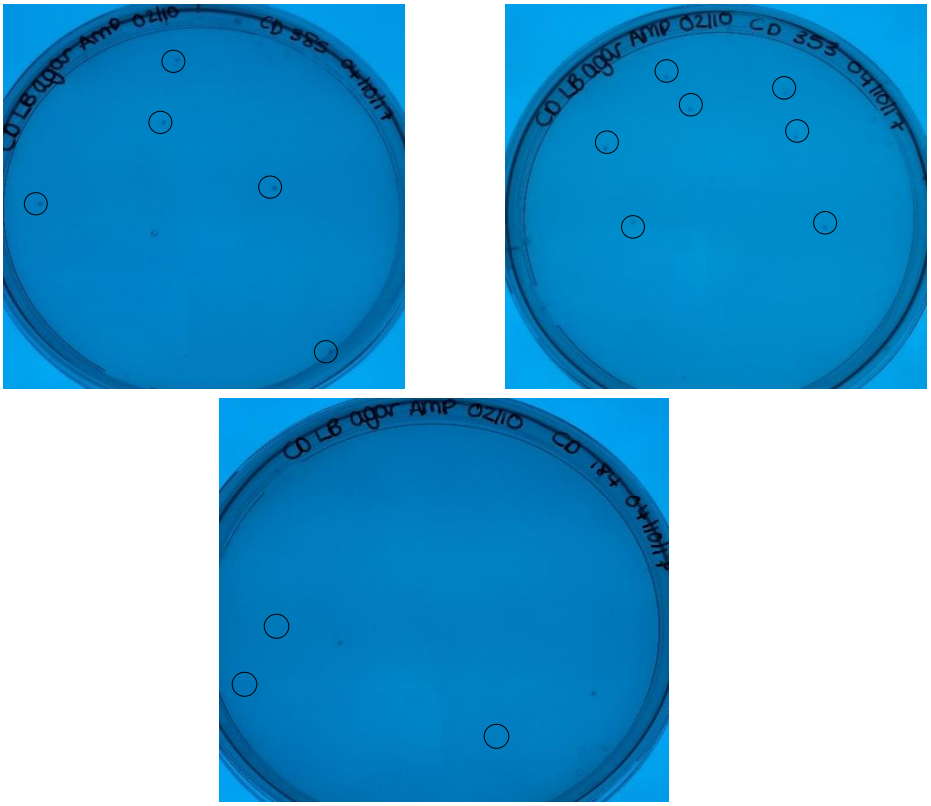


Figure 3-8. Attempted Transformation of Competent *E.coli* with mutated samples. The transformation procedure is stated in Methods. The samples mVC1N385S, mVC2K184E and mVC2Q353G formed colonies following overnight incubation at 37 ^{o}C. mVC1N385 formed 5 visible colonies, mVC2K184E 3 visible colonies and mVC2Q353G formed 7 colonies. The photographs were taken using a light box (to visualise the colonies clearly) with a f/5.6 for 3 seconds.

3.2.5 MiniPrep and Restriction Digest using *Xba*I and *Hind*III.

Step seven was effective with the use of the QIAprep® Spin Miniprep Kit following the protocol stated in the Methods. A total of five colonies were chosen from the successful samples; two for mVC1N385S, two for mVC2Q353G and one chosen from mVC2K184E. For each colony two sets of miniprep was completed in case of any errors occurring during the procedure, this would ensure that the samples can be successfully sequenced. Following successful miniprep and prior to the samples being sequenced a restriction digest was completed using the restriction enzymes *Xba*I and *Hind*III. The restriction digest protocol is stated in the Methods and both samples were left for 4 hours to be incubated at 37 ^{o}C. The results in Figure 3-9 shows the cut and the uncut versions of each sample to compare whether the DNA is supercoiled, nicked or linear.

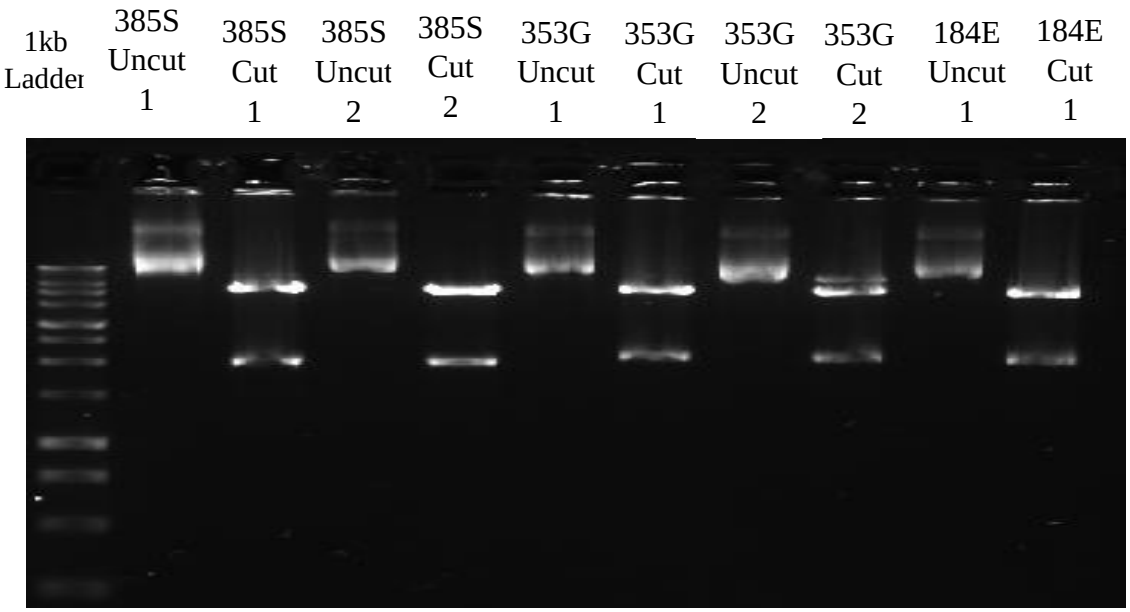


Figure 3-9. Gel Electrophoresis results after restriction digest using *Xba*I and *Hind*III. The restriction digest procedure and protocol are stated in the Methods 2.1.14. The gel electrophoresis was run using Bio-Rad 1-D gel electrophoresis equipment at 70V for one hour, 4ul of the DNA sample was loaded into the 1.5% gel. Apparent restriction fragment lengths shown by the bands are as expected between the cut and the uncut DNA.

3.2.6 Sequencing

The samples were analysed at an external company to confirm that there is a successful mutation within the sequences. The results showed that the sequencing reactions were all effective therefore suggesting that the DNA was clean. Nevertheless, the intended mutagenesis was not successful at the targeted sites and the samples being analysed

were very similar to the original hSVCT1 and hSVCT2 sequences. However, certain mutations are present in the samples which had not been predicted or planned that are likely to have occurred during the PCR procedure. Figures 3-10, Figure 3-11, Figure 3-12 and Figure 3-13 indicate where these mutations are located using pairwise sequence alignment, EMBOSS Needle of the nucleotide sequences. These mutations are stated in comparison to the original hSVCT1 and hSVCT2 published sequences. Unintended mutations have been located in the following sequences; mVC1N385S (1), mVC1N385S (2), mVC2K184E (1) and mVC2K184E (2).

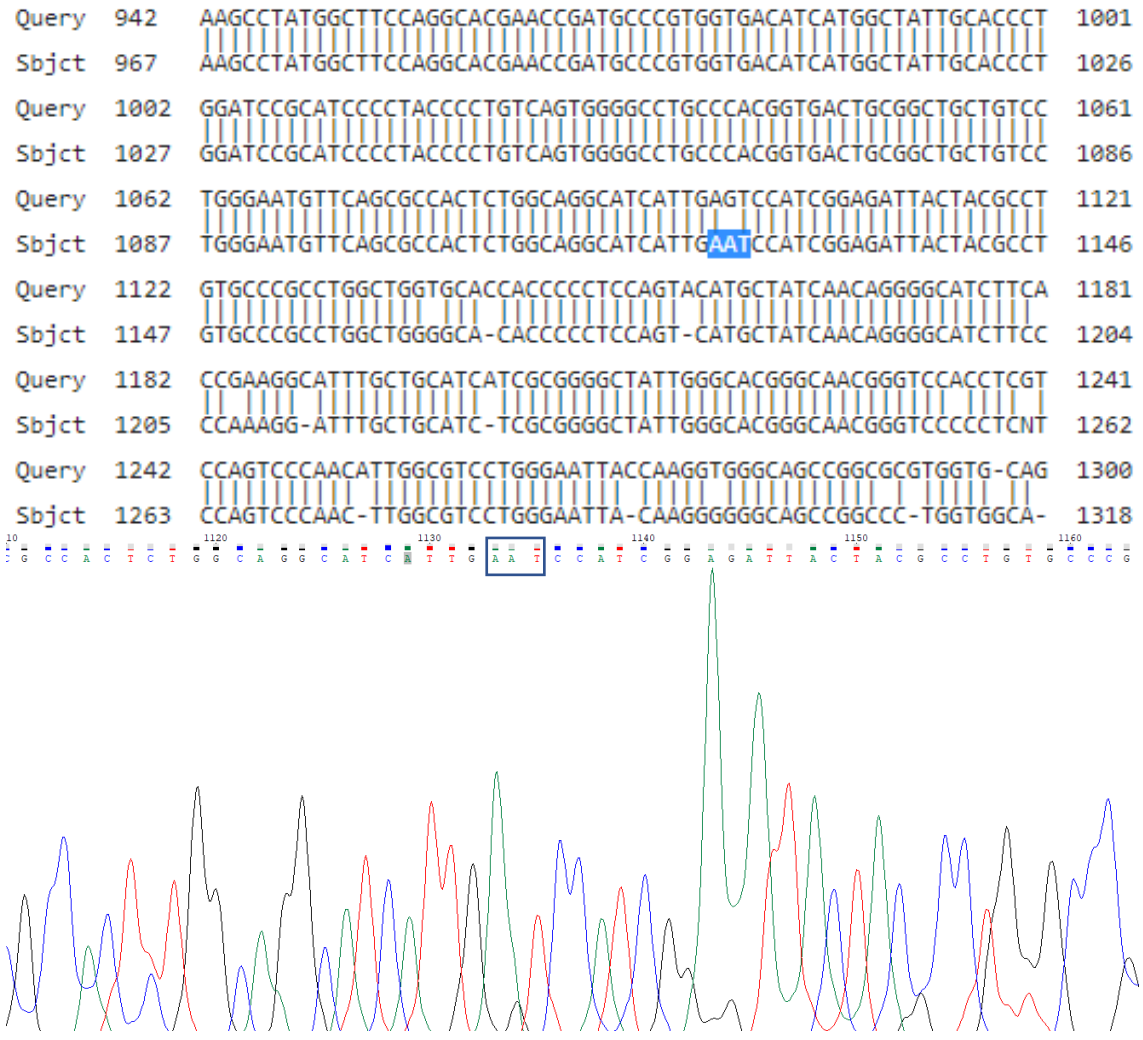


Figure 3-10. Results of the pairwise alignment of the resultant mVC1N385S (1) and (2) sequences with the published hSVCT1 sequence and Chromas results. The pairwise alignment was calculated using the EMBOSS Needle tool which calculated the optimal global alignment of the two sequences. The pairwise alignment settings were fixed to gap open penalty-10, gap extension penalty- 0.5, the Matrix as DNAFull and the output format as 'pair'. The results show that there is high similarity between the sequences and an unintended mutation from AGT (Serine) to AAT (Asparagine). Chromas 2.6.4 software was utilised to analyse the sequence and confirm that this mutation was present.

```
Query  1062  TGGGAATGTTCAGCGCCACTCTGGCAGGCATCATTGAGTCCATCGGAGATTACTACGCCT  1121
Sbjct  1082  TGGGAATGTTCAGCGCCACTCTGGCAGGCATCATTGAATCCATCGGAAATTACTACCCCT  1141

Query  1122  GTGCCCGCCTGGCTGGTGCACCACCCCCTCCAGTACATGCTATCAACAGGGGCATCTTCA  1181
Sbjct  1142  GTGCCCGCCTGGCTGGGGCACCACCCCCTCCAGGACATGCTATCAACAGGGGCATCTTTC  1201

Query  1182  CCGAAGGCATTTGCTGCATCATCGCGGGGCTATTGGGCACGGGCAAC-GGGTCCACCTCG  1240
Sbjct  1202  CCGAAGGCATTTGCTGCTTCTTCCCGGGGCTATTGGGGCCGGGGCACGGGGTCCACCTCG  1261

Query  1241  TCCAGTCCCAACATTGGCGTCCT-GGGAATTACCAAGGTGGGGCAGCCGG-CGCGTGGTGC  1298
Sbjct  1262  TCCAGTCCCAACATTGGCGTCCTGGGGAATTACCAAGGGGGGCACCCGGGCGCG-GGTGG  1320

Query  1299  AG-TATGGTGCGGCTAT-CATGCTGGTCCTGGGCACCATCGGCAAGTTCACGG-CCCTCT  1355
Sbjct  1321  AAATAAGTTGCGG-TATACATGCGGGTCCTGGGAACCATCGGGAAATTACCGGNCCCT-T  1378

Query  1356  TCGCCTCGCTCCCTGACCCCATCCTGGGGGGCATGTTCTGCACTCTCTTTGGCATGATTA  1415
Sbjct  1379  TCNCCTCGTTCCCTGAACCCCTCNGGGGGGGGAATTNTC-GAACTC-CTTTNGGG-GATAA  1435

Query  1416  CAGCTGTGGGGCTGTCCAACCTGCAATTTGTGGACATGAACTCCTCTCGCAACCTCTT-C  1474
Sbjct  1436  CA-C-GTGGGGGGGTTCCACCCGAAATTTG-GGAAAGAAACTC-TCTCG-ACCCTCTTTC  1490
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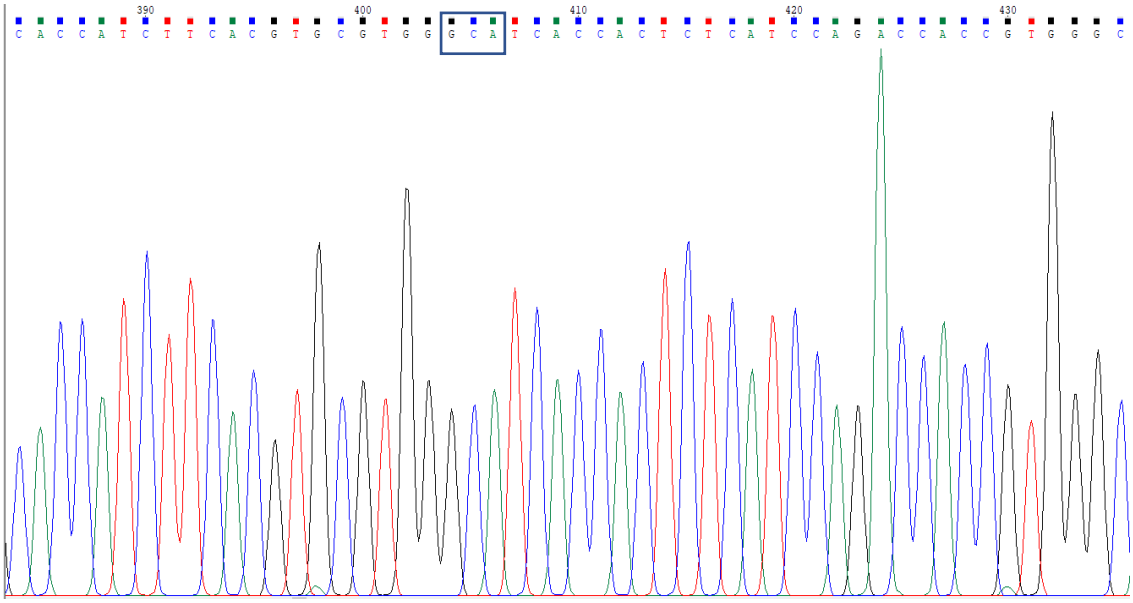

Figure 3-11. Results of the pairwise alignment of the resultant mVC1N385S (2) sequence with the published hSVCT1 sequence and Chromas results. The pairwise alignment was calculated using the EMBOSS Needle tool which calculated the optimal global alignment of the two sequences. The pairwise alignment settings were fixed to gap open penalty-10, gap extension penalty- 0.5, the Matrix as DNAFull and the output format as 'pair'. The results show that there is high similarity between the sequences and an unintended mutation from GGC (Alanine) to GCA (Glycine). Chromas 2.6.4 software was utilised to analyse the sequence and confirm that this mutation was present.

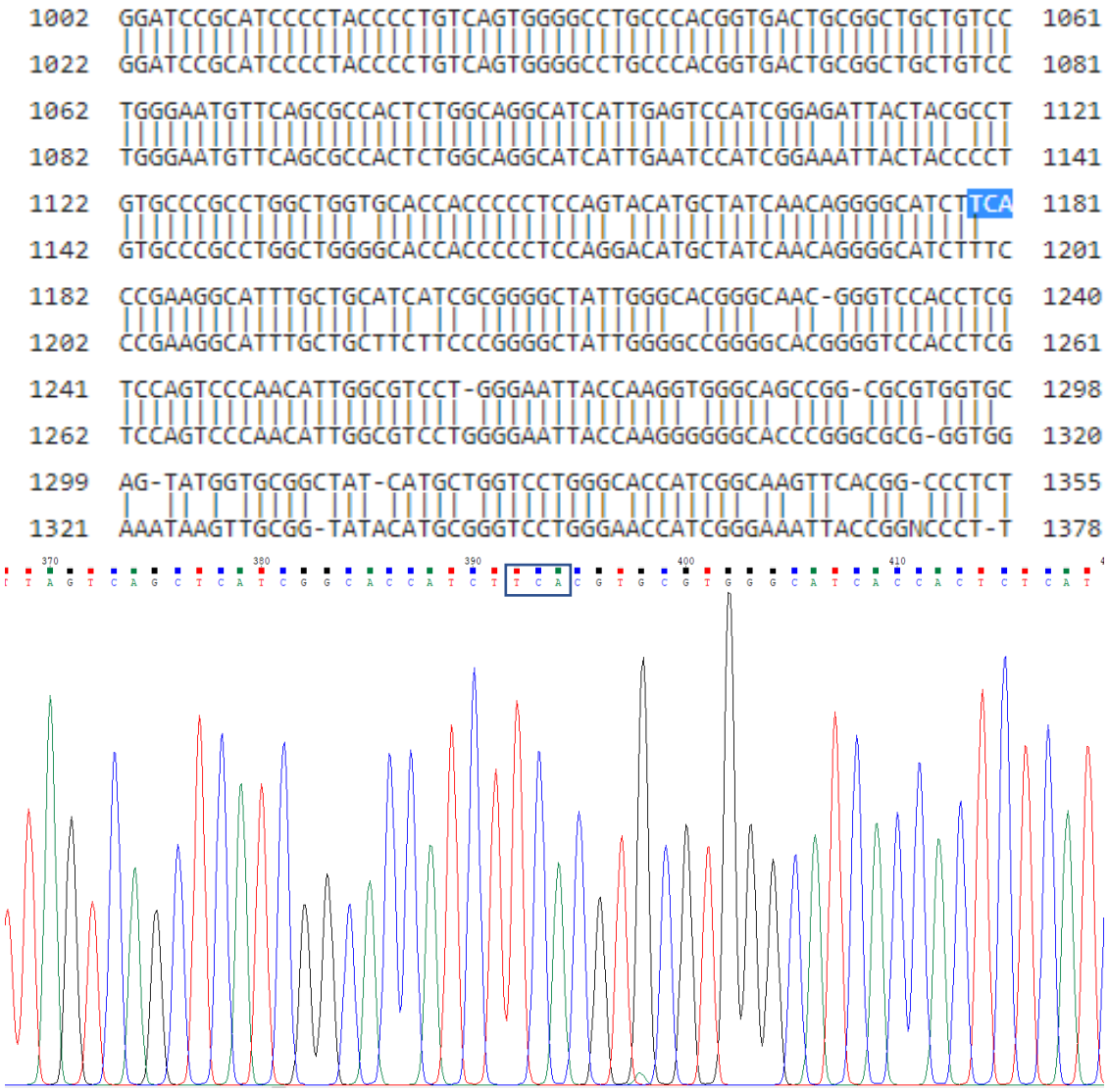

Figure 3-12. Results of the pairwise alignment of the resultant mVC1N385S (2) sequence with the published hSVCT1 sequence and Chromas results. The pairwise alignment was calculated using the EMBOSS Needle tool which calculated the optimal global alignment of the two sequences. The pairwise alignment settings were fixed to gap open penalty-10, gap extension penalty- 0.5, the Matrix as DNAFull and the output format as 'pair'. The results show that there is high similarity between the sequences and an unintended mutation from TCA (Serine) to TTC (Phenylalanine). Chromas 2.6.4 software was utilised to analyse the sequence and confirm that this mutation was present.

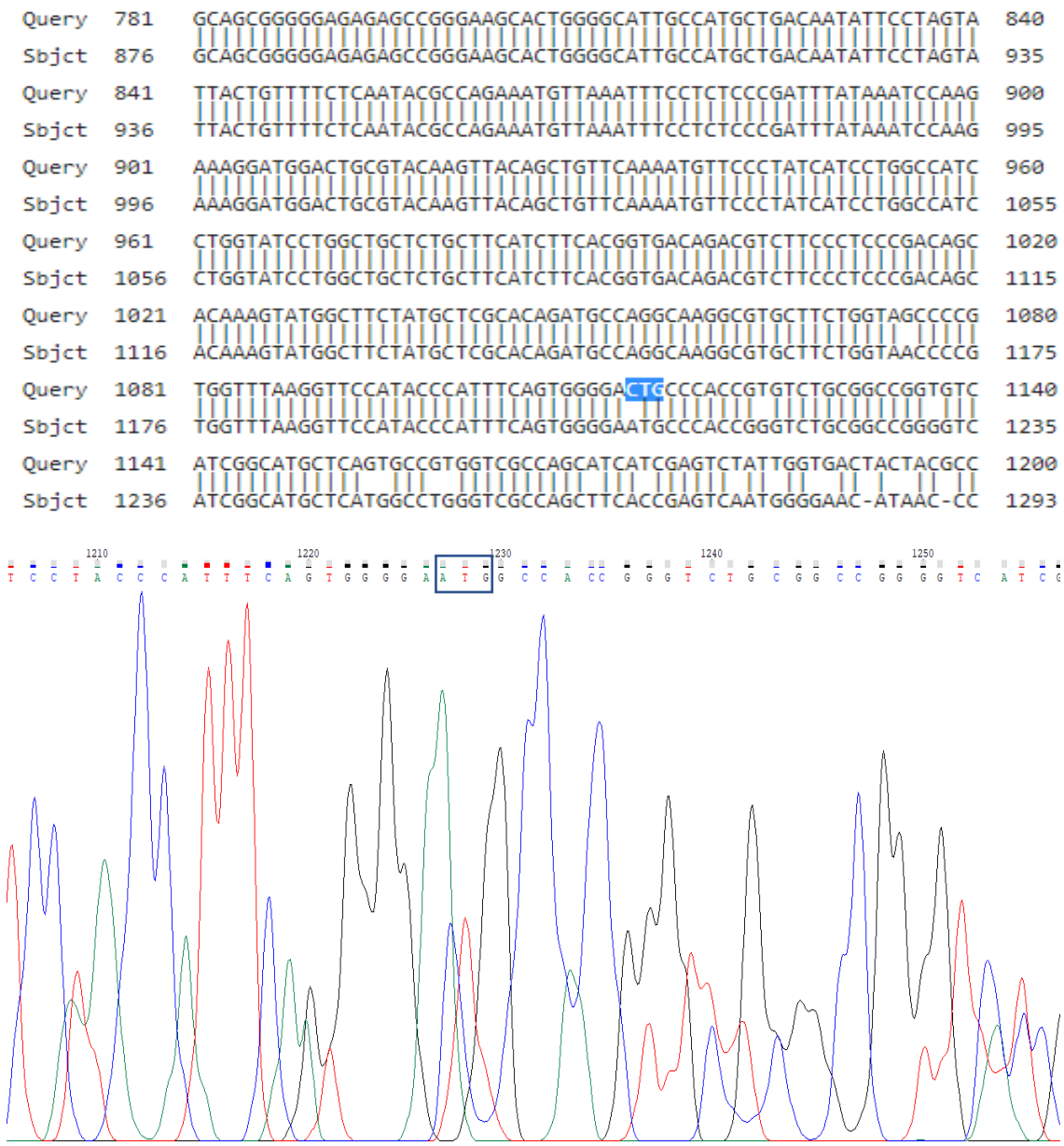

Figure 3-13. Results of the pairwise alignment of the resultant mVC2K184E (1) and (2) sequences with the published hSVCT1 sequence and Chromas results. The pairwise alignment was calculated using the EMBOSS Needle tool which calculated the optimal global alignment of the two sequences. The pairwise alignment settings were fixed to gap open penalty-10, gap extension penalty- 0.5, the Matrix as DNAFull and the output format as 'pair'. The results show that there is high similarity between the sequences and an unintended mutation from CTG (Leucine) to ATG (Methionine). Chromas 2.6.4 software was utilised to analyse the sequence and confirm that this mutation was present.

4.0 Discussion

4.1 Discussion of Results

The purpose of this research masters was to complete site-directed mutagenesis by specifically altering sites on the protein sequences of the two human vitamin C transporters. This would purposely create mutations in the sequences which consequently modifies the coding genes. The sites were chosen due to two factors; their location on the sequence and the structure of the amino acid acting as the mutation. The site of the point mutation could potentially be crucial for the transporter functionality and potential mechanisms that regulate its activity (Inoue, 2017). Studies on the SVCT1 and SVCT2 structure and function have taken place for the last decade, however minimal knowledge is known on how these proteins will react to new substances entering the human body. Across the medical and pharmaceutical industry new medicines are regularly and rapidly being designed and although they are thoroughly tested, little understanding is known on how they will influence the two vitamin C transporters (Jacobs et al., 2015). This knowledge could be vital for individual's health, particularly those who are at risk of low ascorbate levels and could be beneficial during the design of new medicines (Schlueter and Johnston, 2011).

The strategy and planning of this research was scheduled prior to any laboratory work commenced, this included a Gantt chart which reviewed the stages which should be accomplished per month. The majority of the objectives for this project were all met within the time frame across the 11-month period. The successful objectives were achieved with numerous troubleshoots to try and attain the ultimate results and successful clones. This included external sequencing and bioinformatic sequencing to test whether the mutated samples were grown into the original plasmid (Brown et al., 2014). The strategy was altered mid-way through the research with an improved and shorter method to make it more efficient due to time factors. Due to this change certain, stages were removed that had previously been completed but were unsuccessful including; fusion PCR, restriction digest and ligation.

4.2 Major Findings

1) Primers were successfully designed based upon the amino acid location on the primary structure of the transporters with the use of bioinformatics tools to check the protein and

nucleotide sequences. The mutations of the designed primers were also based on the varying amino acid structures.

2) The primary PCR was successful for all mutants using both the original and improved method.

4.3 Implications of the Results

The initial growth of the SVCT strains (provided by WJ Liang, Liang et al 2001; 2002) were effective and provided constituents needed for PCR that were correctly diluted. The E. coli strains *DHα (phSVCT1)* and *DHα (phSVCT2)* both hold a B-lactamase gene on their plasmids, which enables their selection and recovery in LB ampicillin media (Larsson et al., 2015). The two plasmids were extracted successfully, and the gel electrophoresis showed the DNA to be located at ~8-10kb.

Understanding the formations of circular DNA is essential; 3 conformations exist, supercoiled, open-circular and linear. Within the laboratory, on completion of precise plasmid preparation, most DNA is formatted as supercoiled however a number will tolerate single strand nicks, where they are in a relaxed conformation (Smith 1996). It is known that supercoiled DNA runs faster during gel electrophoresis through the gel matrix in comparison to linear DNA (Bendel and James 1983). This knowledge was consequently crucial when analysing the electrophoresis gels after they had been run, to understand the format of the DNA.

For plasmid extraction the DNA showed to have two topological forms as two bands appeared on the gel as shown in Figure 3-1. These were nicked and relaxed which is expected for plasmids which have been extracted from the bacterial cell. Primary PCR was successful for each strategy with bands located at the correct weight. The programme for PCR was designed based upon the annealing temperatures of the primers which on average was 61.0^{o}C. This temperature appeared to provide optimal conditions for successful PCR and reduced the chances of non-specific binding. GoTaq® Flexi DNA Polymerase and $MgCl_2$ were used during all PCR reactions which improved and amplified the templates considerably as they both have a large influence on primer hybridization and enzyme fidelity. To confirm the results of PCR, gel

electrophoresis was completed and the band weights were between ~0.5 and ~1kb which is expected for these amplicons.

Fusion PCR was successful during the first strategy of this research following several attempts, all bands were located at the correct weight in comparison to the 1kb ladder. The programme initially utilised the identical primary PCR programme. However, after an unsuccessful first attempt the programme was altered to reduce the annealing temperature to 60.0 ^{o}C. This gave another negative result where no bands appeared on the electrophoresis gel. The second troubleshoot doubled the volume of template DNA from 2ul to 4ul which resulted in visible bands however at this stage they were too bright and certain bands emerged at the incorrect size. From these results it appeared beneficial to analyse the samples using the nanodrop to check the concentrations. The samples were measured and diluted accordingly so that each sample had approximately 5ng of the required DNA. Following the corrected dilutions to these samples the fusion was successful for all 10 mutants. In addition to the QIA Purification Kit a technique involving the use of sepharose and Cl-6B to filter through the sample. The results represented the efficiency of this method which is improved and more effective than the QIA Purification Kit.

For restriction digest the enzymes *Xba*I and *Hind*III were chosen as they appeared to be the most suitable for cleaving the restriction sites and producing highly reactive 'sticky ends'. Both enzymes have similar reaction kinetics allowing them both to be involved in the digest reaction at the same time with similar incubation conditions. The restriction digest was successful on the first attempt for the diluted plasmids SVCT1 and SVCT2, which were consequently extracted using the QIA gel extraction kit. Attempts at restriction digest on the purified fusion products were unsuccessful following three troubleshoots. The first troubleshoot involved doubling the volume of DNA sample from 5ul to 10ul and incubating the samples at 37°C for 4 hours. To complete the agarose gel 4ul of the sample was loaded into the wells however the results showed no bands appearing on the gel. For the second troubleshoot the same constituents and volumes were used however new batches of the enzymes and NEB Buffer 2 were ordered to ensure that no contamination was occurring. This presented the same results as previously where no bands appeared on the gel following 4ul of DNA being loaded into the wells. The final troubleshoots used 10ul of DNA sample to be digested however for the gel electrophoresis 8ul of the sample was loaded, to increase the chances of a

visible band with the correct weight. This final attempt was also not successful suggesting that an insufficient amount of DNA was in the samples.

Another limitation became apparent after the incompletion of restriction digest, throughout the process all electrophoresis gels were prepared at 1.5%. Due to the sizes of the predicted bands being between 1-7kb long it would have been beneficial to prepare the electrophoresis gels with a concentration of agarose at 0.8%. This would have increased the resolution and resulted in a greater separation between bands of a similar size. This factor could have consequently affected whether bands appeared on the gel following restriction digest. Due to this unsuccessful stage, the DNA sample concentrations were checked using the Nanodrop which confirmed that low concentrations of the required DNA were present in these samples. It is unclear if these were lost during the extraction process or if other factors such as the quality of the restriction enzymes affected the result.

The Table 3-3 shows the Nanodrop results following the final attempt at restriction digest. The results show that the OD A260 readings, which is a quantity measure for nucleic acids, are within the expected values as they fall between 0.2 and 0.8. The 260/280 ratio provided the explanation why the restriction digest was not successful. The ratio should be between 1.8-2.0 therefore these results are considerably low to what was expected. As the 260/280 ratio measures the purity of the samples, it suggests that little pure DNA was in the samples and that high levels of contaminants are present.

Due to these results a new strategy was designed to allow the research to be completed during the time frame and to achieve the growth of mutated hSVCT1 and hSVCT2 sequences. A new form of PCR was researched and used during this strategy of the project. 'Round-the-horn' PCR for site-specific mutagenesis was used where both primers contain the mutations and they are phosphorylated so that the PCR products can be ligated into a circle. This technique only requires small primers and if a band appears during gel electrophoresis it indicates that the reaction was successful and they must contain the mutations. The procedure is effective as it rapidly amplifies the plasmid which causes increases in yield and transformation efficiency.

To successfully complete the revisited primary PCR no flanking primers were involved, only the plasmid and the mutant forward and reverse primers. The volumes and constituents of primary PCR were altered and therefore the programme was also

adapted. The new programme ran for approximately six hours and the following settings were utilised; heat the lid to 100°C , 95°C for 5 mins, then 30x cycles each at 94°C for 1 min ,an extension of 61°C for 30 seconds, 72°C for 10 mins and a final extension at 72°C for 4 mins. The annealing temperature was adjusted to 61.0ºC as this was the average temperature of all six samples, this provided optimum conditions thereby increasing the efficiency of the PCR reaction. Following primary PCR and *Dpn1* treatment, the new strategy was utilised with transformation and growth of the mutations into the plasmid as the next step.

Transformation for human SVCT containing plasmids into competent *E.coli* appeared successful for three out of the six samples. To provide good experimental data, the positive controls, SVCT1 and SVCT2 plasmids were plated. The results were as predicted with an abundance of colonies growing, confirming the competency of the transformation procedure. Overall only 1-10 tranformants grew per plate which is fewer than predicted, this could be linked to several factors. One limitation suggests that an insufficient number of cells were plated and therefore the volume of cells would need to be adjusted to obtain a desirable number of colonies. It would have also been beneficial to pre-warm the medium and plates, to increase the probability of colony growth. The final factor that may have altered the transformation efficiency was the amount of DNA used, for 1-10ng of DNA approximately 50-100ul of competent cells should be utilised. A suitable selection of colonies grew onto the LB plates for samples mVC1N385S and mVC2Q353G and two individual growths were selected, depending on their size and distribution on the plate. Sample mVC2K184E had only three visible colonies on the plate, therefore, only one colony was chosen to be sequenced.

Following the inoculation of each colony into LB media, the samples successfully underwent miniprep using the QIAMiniPrep kit and were stored in the freezer. To confirm the success of the results a restriction digest took place to compare the cut and uncut samples. The digest confirmed the apparent successful growth of the clones and confirmed the transformation efficiency as the DNA appeared supercoiled. The samples were sent to an external sequencing company to analyse the samples and test them within mammalian cells.

Changes in DNA associated with mutation can lead to errors in the protein sequences and creating non-functional protein. To function optimally, each amino acid and its

location is crucial for a protein to work (Choi and Chan, 2015). When a mutation cooccurs it can alter the functioning of the transporter proteins, thereby affecting the whole human body. The concluding results for each sample showed that all sequencing reactions were functioning suggesting that the mutagenesis methodology was completed effectively and efficiently and therefore the DNA was clean. This result also confirms that the designed and revisited strategy was successful and that it can be applied to other scientific research in the future. However, the intended mutagenesis was not successful at the targeted sites and therefore the sequences are highly similar to hSVCT1 and hSVCT2. The cause for the mutagenesis to be unsuccessful will have likely occurred during the PCR process where the forward and reverse primers did not meet. Furthermore, it is unclear how effective the *Dpn1* treatment was during this procedure and consequently not all methylated DNA will have been digested.

The sequencing results indicated that the samples had additional mutations which were not intended. These mutations were predominantly found in samples mVC1N385S (1) and (2) and one mutation found in mVC2K184E. Although these substitutions were not intentional they may still affect the transport functionality and ability of the two vitamin C transporters (Kitzman et al., 2017). The first located point mutation is shown in Figure 3-10 which substitutes Serine to Asparagine. Both amino acids are fairly small in size, polar and are non-essential to humans as they can be naturally synthesised. Due to their similar biochemical properties, particularly as they are both polar, it is likely this is a conservative mutation and therefore there would only be a small effect to the function of the hSVCT1 transporter.

Figure 3-11 shows the mutation from Alanine to Glycine which occurs in the SVCT1 transporter. These amino acids have similarities as they are classed as aliphatic and hydrophobic however their functions differ. Alanine is non-polar and has a side chain which is very non-reactive consequently it is not involved in protein function (Klatte and Wendish, 2015). Glycine holds a hydrogen on its side chain making it more flexible and likely to reside on protein structures playing a distinctive role in their functions. Therefore, there is a possibility that this mutation in the hSVCT1 transporter may improve or reduce the transport activity of the protein. Figure 3-12 shows the mutation in SVCT1 from Serine to Phenylalanine. Serine is small in size, polar and classed as hydroxyl where Phenylalanine is classed as aromatic and hydrophobic (Wu, 2009). Similarly, both amino acids are fairly non-reactive and have side chains which do not

affect protein functioning, although Phenylalanine can be involved with substrate recognition.

The final mutation was found in sample mVC2K184E and is shown in Figure 3-13 and indicates that Leucine has changed to Methionine. Both amino acids are stated as hydrophobic, where Leucine is classed as aliphatic. The structure, size and biochemical properties are all very similar between these amino acids and therefore it is unlikely that the SVCT2 transporter function would be altered or reduced (Hansen et al., 2017).

Mutagenesis of the desired targets were unsuccessful, however after evaluating the sequences it is evident that certain mutations, between Alanine and Glycine, may influence the protein transport activity. Certain artificial molecules were also produced during the PCR reaction, which may have been caused by too many cycles in the PCR programme (Wu, 2009). The cause of the mutations during this research is unclear however one factor includes undesired reactions between different template molecules (Miyazawa, 2013). The insert of mutations to the final samples may also be linked to the DNA polymerase used during PCR which needs to be high fidelity. Finally, the quality of the dNTP's can occasionally lead to mutations in sequences, where they have been stored incorrectly or used at inappropriate concentrations.

4.4 Significance of the Research in the Field

The functioning sequencing reactions of the samples suggest that the technique chosen of 'Round-the-Horn' PCR and transformation methods were effective and highlights the procedures flexibility and efficiency. Although the targeted mutations did not occur the unintended changes provide an opportunity for further research into whether these amino acid substitutions will affect the efficiency and functionality of the transporters, when studied on mammalian cells.

Modifications in expression or functionality of SVCT1 and SVCT2 have not yet been associated with human disease, and no substances have been linked to influence either of the two transporters in the clinical environment (Wohlrab, Phillips and Dachs, 2017). However, the importance of the transporters for sustaining cellular ascorbate concentrations and the importance of vitamin C for human health is evident (Gaziano et al., 2009; Stephenson et al., 2013). This research will also aid the design of new pharmaceutical drugs and medicines to ensure that they will not affect the two vitamin

C transporters. This will benefit those individuals whose overall health is influenced by low ascorbate levels or to those whose are using high levels of vitamin C to aid a specific condition (Yousef et al. 2012; Figueroa-Méndez and Rivas-Arancibia 2015). Further studies into whether these samples have influenced the transport activity of the proteins will provide a better understanding on how important vitamin C is for humans and other mammals who cannot naturally synthesise the micronutrient. In humans, who are unable to naturally synthesise ascorbic acid, a dysfunctional SVCT1 or SVCT2 can instigate a significant reduction of ascorbate and clinical consequences occur, particularly during pregnancy (Carr et al.,2014). Understanding the mechanisms and factors that regulate the uptake of vitamin C within humans can aid the design of therapeutic strategies for degenerative diseases, including cancer.

4.5 Limitations of this Project

The main limitations associated with this research project are linked to time constraints and availability of the required constituents. As there were only nine months available for the practical elements of this research each stage had to be completed by a certain date. This reduced the available time to complete as many troubleshoots needed to improve the quality of the results. This includes reducing smearing on electrophoresis gels, improved nanodrop results and repeating stages where necessary if no results were found for a particular sample. The initial background study, use of bioinformatic tools and design of strategy 1 was completed efficiently. However, unforeseen troubleshooting took up a substantial amount of time and supplies during this research leading to the first stages taking longer than expected. Following the unsuccessful restriction digest, the decision was made to design a new strategy that would be effective within the available time. This required further knowledge in relation to laboratory skills and understanding of the two transporter proteins. With the availability of more time strategy 2 could have been repeated numerous times until a successful clone was formed, thereby achieving all objectives of the project. Although time constraints were a factor throughout this research, the availability of laboratory equipment and most importantly the constituents, also moulded how productive the nine months were.

During this study, all procedures were completed within the university laboratory which is shared between undergraduates, masters students and PhD students. Therefore, during

busy semesters certain equipment was not always readily available or faulty and practical work had to be delayed. Furthermore, resources including; GoTaq Polymerase, *Dpn1* enzyme, dNTP, pipette tips and falcon tubes were used continually by many students and therefore rapidly ran out. Many components were not replaced and had to be ordered which used up valuable laboratory time. Without these products most of stages could not be completed and the research was further delayed. Building an effective strategy to complete site directed mutagenesis, whilst withstanding reliability and functionality of the two transporters, is a challenging and extensive procedure. During the early stages of the practical work, troubleshoots were positively conquered and these techniques were utilised at later phases during the study, nevertheless due to the number of obstacles occurring it was apparent that not enough time was available to complete the research. These troubleshoots in addition to complications with resources potentially hindered the project's completion.

5.0 Conclusion and Further Work

5.1 Conclusion

The aim of this study was to complete site directed mutagenesis on the two human vitamin C transporter coding sequences, to highlight at a molecular level what governs their behaviour and functionality. Objectives 1 and 2 were achieved, with analysis of the coding sequences and designing of primers required for the laboratory procedures. Primary and secondary PCR were achieved, providing fusion products which contained the altered amino acid on each sequence. Objective three was not reached due to numerous troubleshoots being unsuccessful and the final transformation clones did not contain the intended mutations. Sequencing provided confirmation that the mutagenesis was not completed at these sites, however mutation at other regions on the sequences did take place. After analysing these changes and where they are located, it is evident that these substitutions could be beneficial in providing insights into the transport activities of hSVCT1 and hSVCT2 (Kocot and Luchowska-Kocot, 2017).

These clones are consequently available to be identified and used for additional research, to test if there is any influence on the two transporter proteins. hSVCT1 and hSVCT2 are the primary carriers of vitamin C, which has been linked with supporting the prevention of numerous disorders and conditions. Due to numerous predictions from sequence analysis, inadequate amounts of experimental data and lack of knowledge concerning the molecular behaviour of human SVCT1 and human SVCT2, expression and additional valuation of these final samples could be significantly advantageous.

5.2 Future Work

Full completion of the objectives of this research project are highly feasible with the opportunity of more time for troubleshooting at each stage and optimisation of the conditions to make the laboratory procedures effective. It would be beneficial to increase the number of codons being modified on the sequences to highlight more regions which are important for the transporter functionality. Furthermore, it would be advantageous to complete site directed mutagenesis using both strategies to compare their efficiency and indicate which is more effective in relation to this research. Transfecting and expressing the final samples into mammalian cells would provide an insight into whether these unintended mutations affect the transport activity of the

proteins. It could also highlight the potential molecular recognition sites, areas for substrate binding and regions involved with PKC and PKA regulatory pathways (Pantavos et al., 2014).

Similar studies could include domain swapping of the hSVCT proteins and sub cloning of those hybrids into expression vectors to evaluate the molecular mechanisms of the two transporters. This could provide a better understanding of the transport systems and aid the design, development and modifications to pharmaceuticals. It could also provide detailed knowledge on how both transporters individually react to interactions with current and new substances entering the human body. Ultimately this research on the vitamin C transporters could benefit pharmacogenomics, pharmacology and human health.

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Appendix I -COSHH Form

Bournemouth University *COSHH Assessment Form*

1. Assessor:	2. Assessment Date:	3. Assessment Review:		
Camilla Domeneghetti		Next Review Date:	Reviewed on: Date:	Reviewed By:
4. Summary of process or method (or make specific reference to written protocol to be used) :				
Site-specific mutagenesis, PCR and Electrophoresis.				
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 reagents, loading agarose gels, staining agarose gels and making TAE buffer.		Myself, my supervisor (Wei-Jun Liang) and other students who will be sharing the laboratory with me.		
7. Duration of Exposure (minutes, hours and how often):		**8. Location and Conditions of Use** (e.g. lab, room, temp etc.)		
3-7 hours; 4-5 days a week.		Laboratory- room temperature and availability of equipment. Fume Cupboard- temperature for making TAE.		

	9. Hazardous ingredients: (copy form/add more rows as req'd)	10. Quantities Used	11. Workplace Exposure Limit (WEL)	12. Risk/safety phrases	13. Actual Potential Route of Exposure (E.g. by inhalation)	14.Datasheet Attached? Y/N
A	Tris Base	240g	None	Irritant	Eyes, skin and ingestion.	N
B	EDTA	100ml; 0.5M	None	Irritant	Eyes, skin and ingestion.	N
C	SYBR Safe Dye	100ml	None	None	Eyes, skin, ingestion and inhalation.	N
D	Acetic Acid (TAE Buffer)	60ml	250mg/m^{-3} 10ppm	Corrosive/ Flammable	Eyes, skin, ingestion and inhalation.	N
E	Boric Acid	80mM	None	R60 and R61	Eyes, skin, ingestion and inhalation.	N

15. Control Measures

To follow all laboratory procedures; no eating or drinking within the laboratory.
Wear appropriate laboratory clothing, always have long hair tied up and ensure that gloves and safety goggles are always worn.
Always add acid to water and use fume cupboard when needed.
Always clear any spillages within the laboratory and wash any equipment following use.

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	A B	Inhalation	D E	Solid	A	Flammable	D	Low		Low	
Toxic		Sensitiser		Skin Contact	A-E	liquid	BCDE	Highly flammable		Medium	DE	Medium	
Corrosive	D E	Carcinogen		Eye Contact	A-E	Gas/vapour	D E	Extremely flammable		High		High	
Harmful		Mutagenic	C	Swallowing	A-E			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
Fresh Air	Wash immediately with water and see doctor if the symptoms continue.	Rinse thoroughly with water and visit hospital if pain or loss of vision continues.	Seek medical attention immediately.	Na/

23. Spillage Procedures: ⟶

24. Disposal Arrangements

Collection	Swill down sink	Evaporation	In normal waste	Other

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

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:

By presenting completed COSHH form before any laboratory work begins.

Appendix II-Initial Research Ethics

Bournemouth University

Initial Research Ethics

Note: ***All researchers*** must complete this brief checklist to identify any ethical issues associated with their research. Before completing, please refer to the BU *Research Ethics Code of Practice* which can be found www.bournemouth.ac.uk/researchethics. School Research Ethics Representatives (or Supervisors in the case of students) can advise on appropriate professional judgement in this review. A list of Representatives can be found at the aforementioned webpage. **Sections 1-5 must be completed by the researcher and Section 6 by School Ethics Representative/ Supervisor prior to the commencement of any research.**

1 RESEARCHER DETAILS						
Name	Camilla Domeneghetti					
Email	i7685183@bournemouth.ac.uk					
Status	☐ Undergraduate	☒ Postgraduate	☐ Staff			
School	☐ BS	☐ AS	☐ DEC	☐ HSC	☐ MS	☒ ST
Degree Framework & Programme	Masters of Research, Molecular Cloning					

2 PROJECT DETAILS	
Project Title	
Project Summary *Sufficient detail is needed; include methodology, sample, outcomes etc*	
Proposed Start & End Dates	25/01/17-10/01/2018
Project Supervisor	Wei-Jun Liang
Framework Project Co-ordinator	Wei-Jun Liang

3 ETHICS REVIEW CHECKLIST - PART A				
I	Is approval from an external Research Ethics Committee (e.g. Local Research Ethics Committee (REC), NHS REC) required/sought?		☒ Yes	☐ No
II	Is the research solely literature-based?		☐ Yes	☒ No
III	Does the research involve the use of any dangerous substances, including radioactive materials?		☒ Yes	☐ No
IV	Does the research involve the use of any potentially dangerous equipment?		☒ Yes	☐ No
V	Could conflicts of interest arise between the source of funding and the potential outcomes of the research? (see section 8 of BU Research Ethics Code of Practice).		☐ Yes	☒ No
VI	Is it likely that the research will put any of the following at risk:	Living creatures?	☐ Yes	☒ No
		Stakeholders?	☐ Yes	☒ No
		Researchers?	☐ Yes	☒ No
		Participants?	☐ Yes	☒ No
		The environment?	☐ Yes	☒ No
		The economy?	☐ Yes	☒ No
VII	Does the research involve experimentation on any of the following:	Animals?	☐ Yes	☒ No
		Animal tissues?	☐ Yes	☒ No
		Human tissues (including blood, fluid, skin, cell lines)?	☐ Yes	☒ No
		Genetically modified organisms?	☐ Yes	☒ No
VIII	Will the research involve prolonged or repetitive testing, or the collection of audio, photographic or video materials?		☒ Yes	☐ No
IX	Could the research induce psychological stress or anxiety, cause harm or have negative consequences for the participants or researcher (beyond the risks encountered in normal life)?		☐ Yes	☒ No
X	Will the study involve discussion of sensitive topics (e.g. sexual activity, drug use, criminal activity)?		☐ Yes	☒ No
XI	Will financial inducements be offered (other than reasonable expenses/ compensation for time)?		☐ Yes	☒ No
XII	Will it be necessary for the participants to take part in the study without their knowledge / consent at the time?		☐ Yes	☒ No
XII	Are there problems with the participant's right to remain anonymous?		☐ Yes	☒ No
XIV	Does the research *specifically* involve participants who may be vulnerable?		☐ Yes	☒ No
XV	Might the research involve participants who may lack the capacity to decide or to give informed consent to their involvement?		☐ Yes	☒ No

4 ETHICS REVIEW CHECKLIST - PART B	
Please give a summary of the ethical issues and any action that will be taken to address these.	
Ethical Issue:	**Action:**

5 RESEARCHER STATEMENT			
I believe the information I have given is correct. I have read and understood the BU Research Ethics Code of Practice, discussed relevant insurance issues, performed a health & safety evaluation/ risk assessment and discussed any issues/ concerns with a School Ethics Representative/ Supervisor. I understand that if any substantial changes are made to the research (including methodology, sample etc), then I must notify my School Research Ethics Representative/ Supervisor and may need to submit a revised Initial Research Ethics Checklist. By submitting this form electronically I am confirming the information is accurate to my best knowledge.			
Signed	C. Domeneghetti	**Date**	25/01/2017

6 AFFIRMATION BY SCHOOL RESEARCH ETHICS REPRESENTATIVE/ SUPERVISOR		
Satisfied with the accuracy of the research project ethical statement, I believe that the appropriate action is:		
The research project proceeds in its present form	☒ Yes	☐ No
The research project proposal needs further assessment under the School Ethics procedure*	☐ Yes	☒ No
The research project needs to be returned to the applicant for modification prior to further action*	☐ Yes	☒ No
** The School is reminded that it is their responsibility to ensure that no project proceeds without appropriate assessment of ethical issues. In*		

Appendix III- Record of Risk Assessment

REF SciTech- /

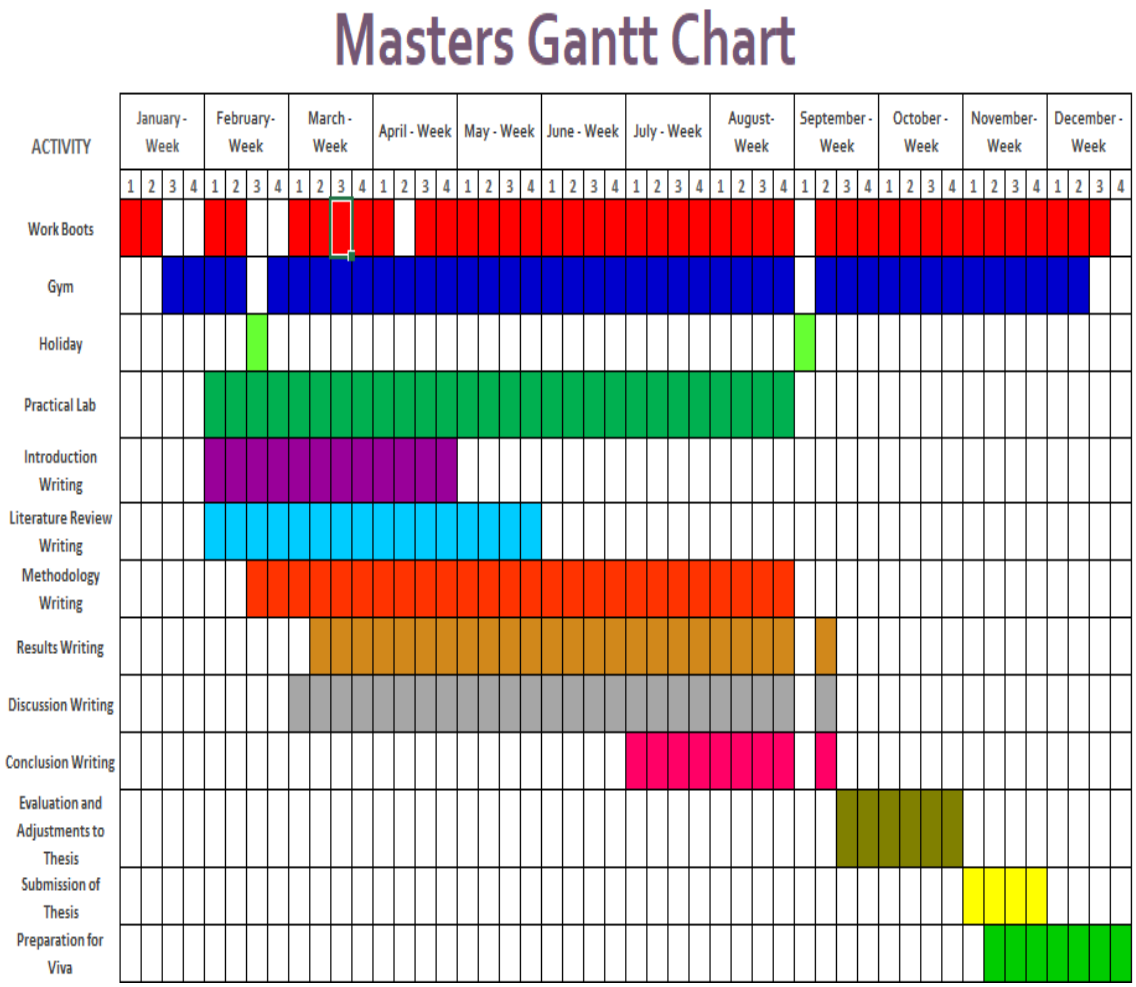
Faculty of Science & Technology

RECORD OF RISK ASSESSMENT

NAME:	Camilla Domeneghetti	PROGRAMME(S) OR PROJECT:	MRes Molecular Cloning
MOBILE TEL NOS:	07469218048	PROJECT AUTHORISATION:	*F&R/IPO Signature:*
	Please note that mobile must be kept on at all times		
TITLE OF ACTIVITY:	Molecular cloning of hSVCT1 and hSVCT2 human vitamin C transporters	DATE(S) OF ACTIVITY:	January 2017- January
LOCATION:	PhD Labratory	DATE OF ASSESSMENT:	
EQUIPMENT:	PCR Machine, Electophoresis equipment, fume cupboard	VEHICLE HIRE:	

HAZARD/RISK	PERSONS AT RISK	RISK			ACTION		
		A PROBABILITY (5)	B SEVERITY (5)	(A * B)	WHAT	WHO	WHEN
Tris Base (Irritant)	Myself and fellow students	2	3	6	Wear appropriate gloves, lab coat and safety glasses	Myself	At all times when handling this reagent
EDTA (Irritant)	Myself and fellow students	2	3	6	Wear appropriate gloves, lab coat and safety glasses	Myself	At all times when handling this reagent
Acetic Acid (TAE) (Corrosive/ Flammable)	Myself and fellow students	2	4	8	Wear appropriate gloves, lab coat and safety glasses	Myself	At all times when handling this reagent
Boric Acid (R60 and R61)	Myself and fellow students	2	4	8	Wear appropriate gloves, lab coat and safety glasses	Myself	At all times when handling this reagent
SYBR Safe Dye	Myself and fellow students	2	2	4	Wear appropriate gloves, lab coat and safety glasses	Myself	At all times when handling this reagent
Fume Cupboard Use	Myself and fellow students	3	4	12	Wear appropriate gloves, lab coat and safety glasses and always add acid to water.	Myself	At all times when handling this reagent

Appendix IV- Masters of Research Gantt Chart



Appendix V - hSVCT1 and hSVCT2 Deduced Amino Acid Sequences

hSVCT1
Nucleotide sequence (1897 nucleotides):

CGCCCGGGCAGGTCCTTTGTCAAGTCATCCCCTCTTCTCCTCAGGAACTGCTCAAACCTGTGCCCCAAAG
ATGAGGGCCCAGGAGGACCTCGAGGGCCGGACACAGCATGAAACCACCAGGGACCCCTCGACCCCGCTAC
CCACAGAGCCTAAGTTTGACATGTTGTACAAGATCGAGGACGTGCCACCTTGGTACCTGTGCATCCTGCT
GGGCTTCCAGCACTACCTGACATGCTTCAGTGGTACCATCGCCGTGCCCTTCCTGCTGGCTGAGGCGCTG
TGTGTGGGCCACGACCAGCACATGGTTAGTCAGCTCATCGGCACCATCTTCACGTGCGTGGGCATCACCA
CTCTCATCCAGACCACCGTGGGCATCCGGCTGCCGCTGTTCCAGGCCAGTGCCTTTGCATTTCTGGTTCC
AGCCAAAGCCATACTGGCTCTGGAGAGATGGAAATGCCCCCCGGAAGAGGAGATCTACGGTAACTGGAGT
CTGCCCCTGAACACCTCTCATATTTGGCACCCACGGATACGGGAGGTCCAGGGTGCAATCATGGTGTCCA
GCGTGGTGGAGGTGGTGATTGGCCTGCTGGGGCTGCCTGGGGCCCTGCTCAACTACATTGGGCCTCTCAC
AGTCACCCCCACTGTCTCCCTCATTGGCCTTTCTGTCTTCCAAGCTGCTGGCGACCGAGCTGGCTCCCAC
TGGGGCATCTCAGCTTGCTCCATTCTCCTGATCATCCTCTTCTCCCAGTACCTGCGCAACCTCACCTTCC
TGCTGCCTGTCTACCGCTGGGGCAAGGGCCTCACTCTCCTCCGCATCCAGATCTTCAAAATGTTTCCTAT
CATGCTGGCCATCATGACCGTGTGGCTGCTCTGCTATGTCCTGACCTTGACAGACGTGCTGCCCACAGAC
CCAAAAGCCTATGGCTTCCAGGCACGAACCGATGCCCGTGGTGACATCATGGCTATTGCACCCTGGATCC
GCATCCCCTACCCCTGTCAGTGGGGCCTGCCCACGGTGACTGCGGCTGCTGTCCTGGGAATGTTCAGCGC
CACTCTGGCAGGCATCATTGAGTCCATCGGAGATTACTACGCCTGTGCCCGCCTGGCTGGTGCACCACCC
CCTCCAGTACATGCTATCAACAGGGGCATCTTCACCGAAGGCATTTGCTGCATCATCGCGGGGCTATTGG
GCACGGGCAACGGGTCCACCTCGTCCAGTCCCAACATTGGCGTCCTGGGAATTACCAAGGTGGGCAGCCG
GCGCGTGGTGCAGTATGGTGCGGCTATCATGCTGGTCCTGGGCACCATCGGCAAGTTCACGGCCCTCTTC
GCCTCGCTCCCTGACCCCATCCTGGGGGGCATGTTCTGCACTCTCTTTGGCATGATTACAGCTGTGGGGC
TGTCCAACCTGCAATTTGTGGACATGAACTCCTCTCGCAACCTCTTCGTGCTGGGATTTTCCATGTTCTT
CGGGCTCACGCTGCCCAATTACCTGGAGTCCAACCCTGGCGCCATCAATACAGGCATTCTTGAAGTGGAT
CAGATTCTGATTGTGCTGCTGACCACGGAGATGTTTGTGGGCGGGTGCCTTGCTTTCATACTTGACAACA
CAGTGCCAGGGAGCCCAGAGGAGCGTGGTCTGATACAGTGGAAAGCTGGGGCTCATGCCAACAGTGACAT
GTCTTCCAGCCTCAAGAGCTACGATTTCCCCATTGGGATGGGCATAGTAAAAAGAATTACCTTTCTGAAA
TACATTCCTATCTGCCCAGTCTTCAAAGGATTTTCTTCAAGTTCAAAAGATCAGATTGCAATTCCAGAAG
ACACTCCAGAAAATACAGAAACTGCATCTGTGTGCACCAAGGTCTAA

Translation:

MRAQEDLEGRTQHETTRDPSTPLPTEPKFDMLYKIEDVPPWYLCILLGFQHYLTCFSGT
IAVPFLLAEALCVGHDQHMVSQLIGTIFTCVGITTLIQTTVGIRLPLFQASAFAFLVPA
KAILALERWKCPPEEEIYGNWSLPLNTSHIWHPRIREVQGAIMVSSVVEVVIGLLGLPG
ALLNYIGPLTVTPTVSLIGLSVFQAAGDRAGSHWGISACSILLIILFSQYLRNLTFLLP
VYRWGKGLTLLRIQIFKMFPIMLAIMTVWLLCYVLTLTDVLPTDPKAYGFQARTDARGD
IMAIAPWIRIPYPCQWGLPTVTAAAVLGMFSATLAGIIESIGDYYACARLAGAPPPPVH
AINRGIFTEGICCIIAGLLGTGNGSTSSSPNIGVLGITKVGSRRVVQYGAAIMLVLGTI
GKFTALFASLPDPILGGMFCTLFGMITAVGLSNLQFVDMNSSRNLFVLGFSMFFGLTLP
NYLESNPGAINTGILEVDQILIVLLTTEMFVGGCLAFILDNTVPGSPEERGLIQWKAGA
HANSDMSSSLKSYDFPIGMGIVKRITFLKYIPICPVFKGFSSSSKDQIAIPEDTPENTE
TASVCTKV

hSVCT2

Nucleotide sequence (1953 nucleotides):

ATGATGGGTATTGGTAAGAATACCACATCCAAATCAATGGAGGCTGGAAGTTCAACAGAAGGCAAATACG
AAGACGAGGCAAAGCACCCAGCTTTCTTCACTCTTCCGGTGGTGATAAATGGAGGCGCCACCTCCAGCGG
TGAGCAGGACAATGAGGACACTGAGCTCATGGCGATCTACACTACGGAAAACGGCATTGCAGAAAAGAGC
TCTCTCGCTGAGACCCTGGATAGCACTGGCAGTCTGGACCCCCAGCGATCAGACATGATTTATACCATAG
AAGATGTTCCTCCCTGGTACCTGTGTATATTTCTGGGGCTACAGCACTACCTGACATGCTTCAGCGGCAC
GATCGCAGTGCCCTTCCTGTTGGCCGATGCCATGTGTGTGGGGTACGACCAGTGGGCCACCAGCCAGCTC
ATTGGGACCATTTTCTTCTGTGTGGGAATCACTACTTTGCTACAGACAACGTTTGGATGCAGGTTACCCC
TGTTTCAGGCCAGTGCTTTTGCATTTTTGGCCCCTGCTCGAGCCATCCTGTCTTTAGATAAATGGAAATG
TAACACCACAGATGTTTCAGTTGCCAATGGAACAGCAGAGCTGTTGCACACAGAACACATCTGGTATCCC
CGGATCCGAGAGATCCAGGGGGCCATCATCATGTCCTCACTGATAGAAGTAGTCATCGGCCTCCTCGGCC
TGCCTGGGGCTCTACTGAAGTACATCGGTCCCTTGACCATTACACCCACGGTGGCCCTAATTGGCCTCTC
TGGTTTCCAGGCAGCGGGGGAGAGAGCCGGGAAGCACTGGGGCATTGCCATGCTGACAATATTCCTAGTA
TTACTGTTTTCTCAATACGCCAGAAATGTTAAATTTCCTCTCCCGATTTATAAATCCAAGAAAGGATGGA
CTGCGTACAAGTTACAGCTGTTCAAAATGTTCCCTATCATCCTGGCCATCCTGGTATCCTGGCTGCTCTG
CTTCATCTTCACGGTGACAGACGTCTTCCCTCCCGACAGCACAAAGTATGGCTTCTATGCTCGCACAGAT
GCCAGGCAAGGCGTGCTTCTGGTAGCCCCGTGGTTTAAGGTTCCATACCCATTTCAGTGGGGACTGCCCA
CCGTGTCTGCGGCCGGTGTCATCGGCATGCTCAGTGCCGTGGTCGCCAGCATCATCGAGTCTATTGGTGA
CTACTACGCCTGTGCACGGCTGTCCTGTGCCCCACCCCCCCCCATCCACGCAATAAACAGGGGAATTTTC
GTGGAAGGCCTCTCCTGTGTTCTTGATGGCATATTTGGTACTGGGAATGGCTCTACTTCATCCAGTCCCA
ACATTGGAGTTTTGGGAATTACAAAGGTCGGCAGCCGCCGCGTGATACAGTGCGGAGCAGCCCTCATGCT
CGCTCTGGGCATGATCGGGAAGTTCAGCGCCCTCTTTGCGTCCCTTCCGGATCCTGTGCTGGGAGCCCTG
TTCTGCACGCTCTTTGGAATGATCACAGCTGTTGGCCTCTCTAACCTGCAGTTCATTGATTTAAATTCTT
CCCGGAACCTCTTTGTGCTTGGATTTTCGATCTTCTTTGGGCTCGTCCTTCCAAGTTACCTCAGACAGAA
CCCTCTGGTCACAGGGATAACAGGAATCGATCAAGTGTTGAACGTCCTTCTCACAACTGCTATGTTTGTA
GGGGGCTGTGTGGCTTTTATCCTGGATAACACCATCCCAGGCACTCCAGAGGAAAGAGGAATCCGGAAAT
GGAAGAAGGGTGTGGGCAAAGGGAACAAATCACTCGACGGCATGGAGTCGTACAATTTGCCATTTGGCAT
GAACATTATAAAAAAATACAGATGCTTCAGCTACTTACCCATCAGCCCAACCTTTGTGGGCTACACATGG
AAAGGCCTCAGGAAGAGCGACAACAGCCGGAGTTCAGATGAAGACTCCCAGGCCACGGGATAG

Translation:

MMGIGKNTTSKSMEAGSSTEGKYEDEAKHPAFFTLPVVINGGATSSGEQDNEDTELMAI
YTTENGIAEKSSLAETLDSTGSLDPQRSDMIYTIEDVPPWYLCIFLGLQHYLTCFSGTI
AVPFLLADAMCVGYDQWATSQLIGTIFFCVGITTLLQTTFGCRLPLFQASAFAFLAPAR
AILSLDKWKCNTTDVSVANGTAELLHTEHIWYPRIREIQGAIIMSSLIEVVIGLLGLPG
ALLKYIGPLTITPTVALIGLSGFQAAGERAGKHWGIAMLTIFLVLLFSQYARNVKFPLP
IYKSKKGWTAYKLQLFKMFPIILAILVSWLLCFIFTVTDVFPPDSTKYGFYARTDARQG
VLLVAPWFKVPYPFQWGLPTVSAAGVIGMLSAVVASIIESIGDYYACARLSCAPPPPIH
AINRGIFVEGLSCVLDGIFGTGNGSTSSSPNIGVLGITKVGSRRVIQCGAALMLALGMI
GKFSALFASLPDPVLGALFCTLFGMITAVGLSNLQFIDLNSSRNLFVLGFSIFFGLVLP
SYLRQNPLVTGITGIDQVLNVLLTTAMFVGGCVAFILDNTIPGTPEERGIRKWKKGVGK
GNKSLDGMESYNLPFGMNIIKKYRCFSYLPISPTFVGYTWKGLRKSDNSRSSDEDSQAT
G

Appendix VI- Initial Primer Design for hSVCT1 and hSVCT2

hsvct1

```
ctcaggaactgctcaaacctgtgccccaaagatgagggcccaagaggacctcgagggccgg
  S  G  T  A  Q  T  C  A  P  K  M  R  A  Q  E  D  L  E  G  R
gcacagcatgaaaccaccagggacccctcgaccccgctacccacagagcctaagtttgac
 A  Q  H  E  T  T  R  D  P  S  T  P  L  P  T  E  P  K  F  D
atgttgtacaagatcgaggacgtgccaccttggtacctgtgcatcctgctgggcttccag
 M  L  Y  K  I  E  D  V  P  P  W  Y  L  C  I  L  L  G  F  Q
cactacctgacatgcttcagtggtaccatcgccgtgcccttcctgctggctgaggcgctg
 H  Y  L  T  C  F  S  G  T  I  A  V  P  F  L  L  A  E  A  L
tgtgtgggccacgaccagcacatggttagtcagctcatcggcaccatcttcacgtgcgtg
 C  V  G  H  D  Q  H  M  V  S  Q  L  I  G  T  I  F  T  C  V
ggcatcaccactctcatccagaccaccgtgggcatccggctgccgctgttccaggccagt
 G  I  T  T  L  I  Q  T  T  V  G  I  R  L  P  L  F  Q  A  S
gcctttgcatttctggttccagccaaagccatactggctctggagagatggaaatgcccc
 A  F  A  F  L  V  P  A  K  A  I  L  A  L  E  R  W  K  C  P
ccggaagaggagatctacggtaactggagtctgcccctgaacacctctcatatttggcac
 P  E  E  E  I  Y  G  N  W  S  L  P  L  N  T  S  H  I  W  H
ccacggatacgggaggtccagggtgcaatcatggtgtccagcgtggtggaggtggtgatt
 P  R  I  R  E  V  Q  G  A  I  M  V  S  S  V  V  E  V  V  I
ggcctgctggggctgcctggggccctgctcaactacattgggcctctcacagtcaccccc
 G  L  L  G  L  P  G  A  L  L  N  Y  I  G  P  L  T  V  T  P
actgtctccctcattggcctttctgtcttccaagctgctggcgaccgagctggctcccac
 T  V  S  L  I  G  L  S  V  F  Q  A  A  G  D  R  A  G  S  H
tggggcatctcagcttgctccattctcctgatcatcctcttctcccagtacctgcgcaac
 W  G  I  S  A  C  S  I  L  L  I  I  L  F  S  Q  Y  L  R  N
ctcaccttcctgctgcctgtctaccgctggggcaagggcctcactctcctccgcatccag
 L  T  F  L  L  P  V  Y  R  W  G  K  G  L  T  L  L  R  I  Q
atcttcaaaatgtttcctatcatgctggccatcatgaccgtgtggctgctctgctatgtc
 I  F  K  M  F  P  I  M  L  A  I  M  T  V  W  L  L  C  Y  V
ctgaccttgacagacgtgctgcccacagacccaaaagcctatggcttccaggcacgaacc
 L  T  L  T  D  V  L  P  T  D  P  K  A  Y  G  F  Q  A  R  T
gatgcccgtggtgacatcatggctattgcaccctggatccgcatcccctaccctgtcag
 D  A  R  G  D  I  M  A  I  A  P  W  I  R  I  P  Y  P  C  Q
tggggcctgcccacggtgactgcggctgctgtcctgggaatgttcagcgccactctggca
 W  G  L  P  T  V  T  A  A  A  V  L  G  M  F  S  A  T  L  A
ggcatcattgagtccatcggagattactacgcctgtgcccgcctggctggtgcaccaccc
 G  I  I  E  S  I  G  D  Y  Y  A  C  A  R  L  A  G  A  P  P
cctccagtacatgctatcaacaggggcatcttcaccgaaggcatttgctgcatcatcgcg
 P  P  V  H  A  I  N  R  G  I  F  T  E  G  I  C  C  I  I  A
gggctattgggcacgggcaacgggtccacctcgtccagtcccaacattggcgtcctggga
 G  L  L  G  T  G  N  G  S  T  S  S  S  P  N  I  G  V  L  G
attaccaaggtgggcagccggcgcgtggtgcagtatggtgcggctatcatgctggtcctg
 I  T  K  V  G  S  R  R  V  V  Q  Y  G  A  A  I  M  L  V  L  S
ggcaccatcggcaagttcacggccctcttcgcctcgctccctgaccccatcctgggggggc
 G  T  I  G  K  F  T  A  L  F  A  S  L  P  D  P  I  L  G  G
atgttctgcactctctttggcatgattacagctgtggggctgtccaacctgcaatttgtg
 M  F  C  T  L  F  G  M  I  T  A  V  G  L  S  N  L  Q  F  V
gacatgaactcctctcgcaacctcttcgtgctgggattttccatgttcttcgggctcacg
 D  M  N  S  S  R  N  L  F  V  L  G  F  S  M  F  F  G  L  T
ctgcccaattacctggagtccaaccctggcgccatcaatacaggcattcttgaagtggat
 L  P  N  Y  L  E  S  N  P  G  A  I  N  T  G  I  L  E  V  D
cagattctgattgtgctgctgaccacggagatgtttgtgggcgggtgccttgctttcata
 Q  I  L  I  V  L  L  T  T  E  M  F  V  G  G  C  L  A  F  I
cttgacaacacagtgccagggagcccagaggagcgtggtctgatacagtggaaagctggg
 L  D  N  T  V  P  G  S  P  E  E  R  G  L  I  Q  W  K  A  G
gctcatgccaacagtgacatgtcttccagcctcaagagctacgatttccccattgggatg
 A  H  A  N  S  D  M  S  S  S  L  K  S  Y  D  F  P  I  G  M
ggcatagtaaaaagaattacctttctgaaatacattcctatctgcccagtcttcaaagga
 G  I  V  K  R  I  T  F  L  K  Y  I  P  I  C  P  V  F  K  G
```

```
ttttcttcaagttcaaaagatcagattgcaattccagaagacactccagaaaatacagaa
 F  S  S  S  S  K  D  Q  I  A  I  P  E  D  T  P  E  N  T  E
actgcatctgtgtgcaccaaggtctgaaaaatgacttccaggaaaggaagcatggtatat
 T  A  S  V  C  T  K  V  -  K  M  T  S  R  K  G  S  M  V  Y
aacaggaaaagaaaactacatggggaaccagaagacctaagcctgaaatcccagccctgc
 N  R  K  R  K  L  H  G  E  P  E  D  L  S  L  K  S  Q  P  C
ccctaactaacttctgtgtaaactcagataagtcacctttctctgggattcaaatttttg
 P  -  L  T  S  V  -  T  Q  I  S  H  L  S  L  G  F  K  F  L
catcagttaaaaaaaaaggggtgggggggaatgggccaaagtctgagtcttagagacttg
 H  Q  L  K  K  K  G  W  G  G  M  G  Q  S  L  S  L  R  D  L
taccaatgttatgctatgtctctaaatctttactctcctaagtagacttgtcagcatcta
 Y  Q  C  Y  A  M  S  L  N  L  Y  S  P  K  -  T  C  Q  H  L
ggaagaacagctagaaattttcctctgtgatattttagactgcaagttgaaaaaaaaaaa
 G  R  T  A  R  N  F  P  L  -  Y  F  R  L  Q  V  E  K  K  K
aaaaaaaaa
 K  K  K
```

1) Annealing Temperature = 71.3- 5= 66.3°C
Forward Primer= VtC1P190SF
5'-GTCACCCCCACTGTCTCC-3'
Reverse Primer= VtC1P190SR
5'- GGAGACAGTGGGGGTGAC -3'
Change Proline (CCC) to Serine (TCC)
2) Annealing Temperature = 71.3-5=66.3°C
Forward Primer= VtC1V62GF
5'-ACCATCGCCGTGCCCTTC-3'
Reverse Primer= VtC1V62GR
5'-GAAGGGCACGGCGATGGT-3'
Change Valine (GTG) to Glycine (GGG)
3) Annealing Temperature = 70.3-5=65.3°C
Forward Primer = VtC1I96LF
5'-ACCACTCTCATCCAGACCAC-3'
Reverse Primer= VtC1I96LR
5'-GTGGTCTGGATGAGAGTGGT-3'
Change Isoleucine (ATC) to Leucine (CTC)
4)Annealing Temperature= 71.3-5=66.3°C
Forward Primer = VtC1L277FF
5'-ACAGACGTGCTGCCCACAG-3'
Reverse Primer= VtC1L277FR
5'- CTGTGGGCAGCACGTCTGT -3'
5) Annealing Temperature= 69.7-5=64.7°C
Forward Primer= VtC1N385SF
5'-GTCCAGTCCCAACATTGGC-3'
Reverse Primer= VtC1N385SR
5'-GCCAATGTTGGGACTGGAC-3'
Change Asparagine (AAC) to Serine (AGC)

>hSVCT2

```
atgatgggtattggtaagaataccacatccaaatcaatggaggctggaagttcaacagaa
 M  M  G  I  G  K  N  T  T  S  K  S  M  E  A  G  S  S  T  E
ggcaaatacgaagacgaggcaaagcacccagctttcttcactcttccggtggtgataaat
 G  K  Y  E  D  E  A  K  H  P  A  F  F  T  L  P  V  V  I  N
ggaggcgccacctccagcggtgagcaggacaatgaggacactgagctcatggcgatctac
 G  G  A  T  S  S  G  E  Q  D  N  E  D  T  E  L  M  A  I  Y
actacggaaaacggcattgcagaaaagagctctctcgctgagaccctggatagcactggc
 T  T  E  N  G  I  A  E  K  S  S  L  A  E  T  L  D  S  T  G
agtctggacccccagcgatcagacatgatttataccatagaagatgttcctccctggtac
 S  L  D  P  Q  R  S  D  M  I  Y  T  I  E  D  V  P  P  W  Y
ctgtgtatatttctggggctacagcactacctgacatgcttcagcggcacgatcgcagtg
 L  C  I  F  L  G  L  Q  H  Y  L  T  C  F  S  G  T  I  A  V
cccttcctgttggccgatgccatgtgtggggtacgaccagtggccaccagccagctc
 P  F  L  L  A  D  A  M  C  V  G  Y  D  Q  W  A  T  S  Q  L
attgggaccatttcttctgtgtgggaatcactactttgctacagacaacgtttggatgc
 I  G  T  I  F  F  C  V  G  I  T  T  L  L  Q  T  T  F  G  C
aggttacccctgtttcaggccagtgcttttgcatttttggcccctgctcgagccatcctg
 R  L  P  L  F  Q  A  S  A  F  A  F  L  A  P  A  R  A  I  L
tctttagataaatggaaatgtaacaccacagatgtttcagttgccaatggaacagcagag
 S  L  D  K  W  K  C  N  T  T  D  V  S  V  A  N  G  T  A  E
ctgttgcacacagaacacatctggtatccccggatccgagagatccaggggccatcatc
 L  L  H  T  E  H  I  W  Y  P  R  I  R  E  I  Q  G  A  I  I
atgtcctcactgatagaagtagtcatcggcctcctcggcctgcctggggctctactgaag
 M  S  S  L  I  E  V  V  I  G  L  L  G  L  P  G  A  L  L  K
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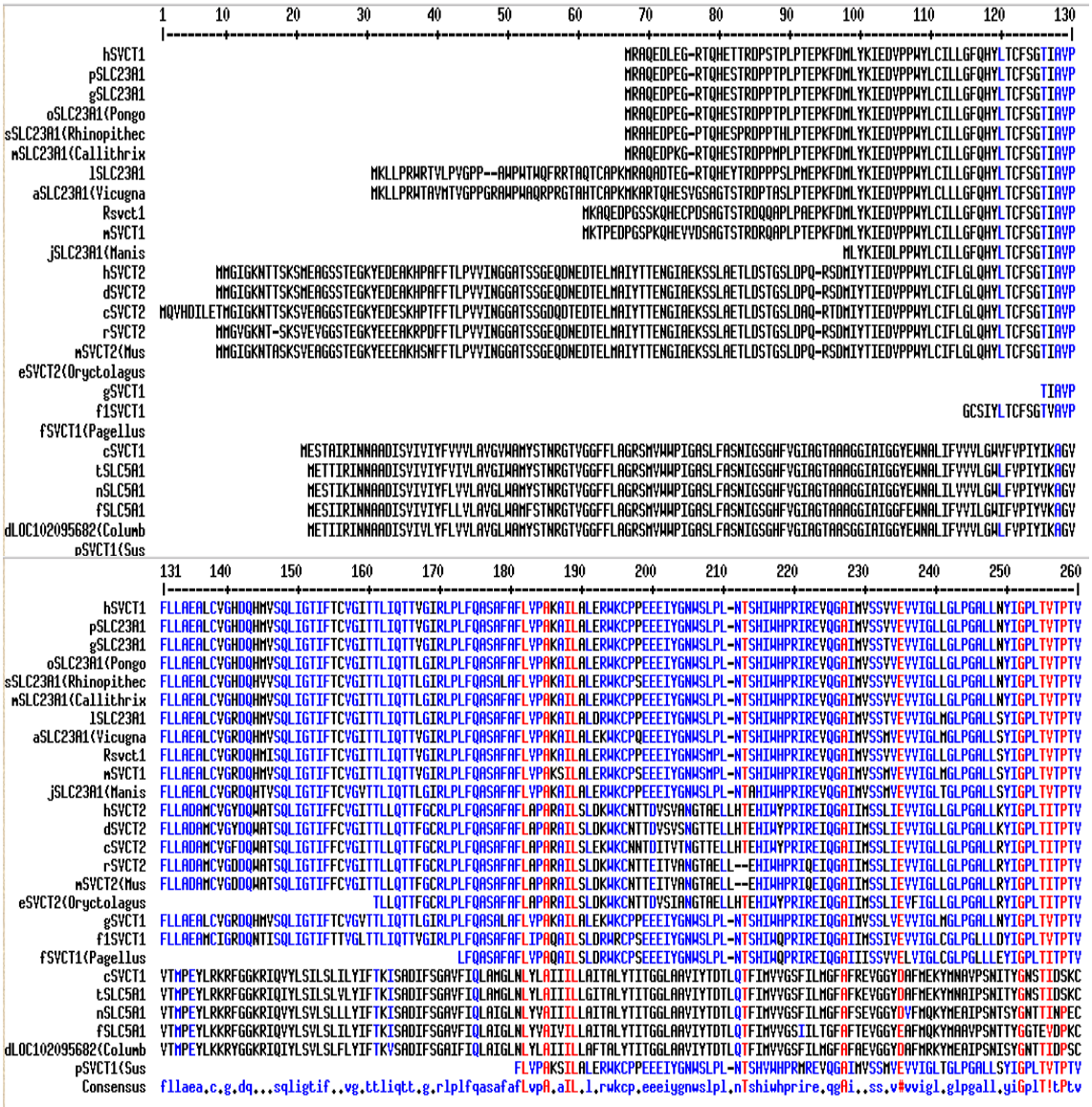
 Y  I  G  P  L  T  I  T  P  T  V  A  L  I  G  L  S  G  F  Q
gcagcgggggagagagccgggaagcactggggcattgccatgctgacaatattcctagta
 A  A  G  E  R  A  G  K  H  W  G  I  A  M  L  T  I  F  L  V
ttactgttttctcaatacgccagaaatgttaaatttcctctcccgatttataaatccaag
 L  L  F  S  Q  Y  A  R  N  V  K  F  P  L  P  I  Y  K  S  K
aaaggatggactgcgtacaagttacagctgttcaaaatgttccctatcatcctggccatc
 K  G  W  T  A  Y  K  L  Q  L  F  K  M  F  P  I  I  L  A  I
ctggtatcctggctgctctgcttcatcttcacggtgacagacgtcttccctcccgacagc
 L  V  S  W  L  L  C  F  I  F  T  V  T  D  V  F  P  P  D  S
acaaagtatggcttctatgctcgcacagatgccaggcaaggcgtgcttctggtagccccg
 T  K  Y  G  F  Y  A  R  T  D  A  R  Q  G  V  L  L  V  A  P
tggtttaaggttccatacccatttcagtggggactgcccaccgtgtctgcggccggtgtc
 W  F  K  V  P  Y  P  F  Q  W  G  L  P  T  V  S  A  A  G  V
atcggcatgctcagtgccgtggtcgccagcatcatcgagtctattggtgactactacgcc
 I  G  M  L  S  A  V  V  A  S  I  I  E  S  I  G  D  Y  Y  A
tgtgcacggctgtcctgtgccccacccccccccatccacgcaataaacaggggaattttc
 C  A  R  L  S  C  A  P  P  P  P  I  H  A  I  N  R  G  I  F
gtggaaggcctctcctgtgttcttgatggcatatttggtactgggaatggctctacttca
 V  E  G  L  S  C  V  L  D  G  I  F  G  T  G  N  G  S  T  S
tccagtcccaacattggagttttgggaattacaaaggtcggcagccgccgcgtgatacag
 S  S  P  N  I  G  V  L  G  I  T  K  V  G  S  R  R  V  I  Q
tgcggagcagccctcatgctcgctctgggcatgatcgggaagttcagcgccctctttgcg
 C  G  A  A  L  M  L  A  L  G  M  I  G  K  F  S  A  L  F  A
tcccttccggatcctgtgctgggagccctgttctgcacgctctttggaatgatcacagct
 S  L  P  D  P  V  L  G  A  L  F  C  T  L  F  G  M  I  T  A
gttggcctctctaacctgcagttcattgatttaaattcttcccggaacctctttgtgctt
 V  G  L  S  N  L  Q  F  I  D  L  N  S  S  R  N  L  F  V  L
ggattttcgatcttctttgggctcgtccttccaagttacctcagacagaaccctctggtc
 G  F  S  I  F  F  G  L  V  L  P  S  Y  L  R  Q  N  P  L  V
acagggataacaggaatcgatcaagtgttgaacgtccttctcacaactgctatgtttgta
 T  G  I  T  G  I  D  Q  V  L  N  V  L  L  T  T  A  M  F  V
gggggctgtgtggcttttatcctggataacaccatcccaggcactccagaggaaagagga
 G  G  C  V  A  F  I  L  D  N  T  I  P  G  T  P  E  E  R  G
atccggaaatggaagaagggtgtgggcaaagggaacaaatcactcgacggcatggagtcg
```

```
 I  R  K  W  K  K  G  V  G  K  G  N  K  S  L  D  G  M  E  S
tacaatttgccatttggcatgaacattataaaaaaatacagatgcttcagctacttaccc
 Y  N  L  P  F  G  M  N  I  I  K  K  Y  R  C  F  S  Y  L  P
atcagcccaacctttgtgggctacacatggaaaggcctcaggaagagcgacaacagccgg
 I  S  P  T  F  V  G  Y  T  W  K  G  L  R  K  S  D  N  S  R
agttcagatgaagactcccaggccacgggatag
 S  S  D  E  D  S  Q  A  T  G  -
```

1)Annealing Temperature =70.3-5=65.3°C
Forward Primer= VtC2A174VF
5'-CATTTTTGGCCCCTGCTCGA-3'
Reverse Primer= VtC2A174VR
5'-TCGAGCAGGGGCCAAAAATG-3'
Change Alanine (GCC) to Valine (GTC)
2) Annealing Temperature = 71.3-5=66.3°C
Forward Primer= VtC2K184RF
5'-CCTGTCTTTAGATAAATGGAAATGTAACAC-3'
Reverse Primer= VtC2K184RR
5'-GTGTTACATTTCCATTTATCTAAAGACAGG-3'
Change Lysine (AAA) to Arginine (AGA)
3) Annealing Temperature = 71.3-5=66.3°C
Forward Primer= VtC2Q353GF
5'-GATGCCAGGCAAGGCGTG-3'
Reverse Primer= VtC2Q353GR
5'-CACGCCTTGCCTGGCATC-3'
Change Glutamine (CAA) to Arginine (CGA)
4)Annealing Temperature = 71.3-5=66.3°C
Forward Primer= VtC2D133GF
5'-GTGGGGTACGACCAGTGG-3'
Reverse Primer= VtC2D133GR
5'- CCACTGGTCGTACCCCAC-3'
Change Aspartic Acid (GAC) to Glycine (GGC)
5) Annealing Temperature =71.3-5=66.3°c
Forward Primer= VtC2I381LF
5'-CCGGTGTCATCGGCATGC-3'
Reverse Primer= VtC2I381LR
5'- GCATGCCGATGACACCGG-3'
Change Isoleucine (ATC) to Leucine (CTC)(CTC)

Appendix VII- Multi Align Images for Varied Species



Appendix VIII- Global Alignment of hSVCT1 and hSVCT2

```
# Program: needle
# Rundate: Tue 07 2017 11:32:03
# Commandline: needle
#    -asequence emboss_needle-I20160512-133536-0770-85028315-pg.asequence
#    -bsequence emboss_needle-I20160512-133536-0770-85028315-pg.bsequence
#    -datafile EDNAFULL
#    -gapopen 10.0
#    -gapextend 0.5
#    -endopen 10.0
#    -endextend 0.5
#    -aformat3 pair
#    -snucleotide1
#    -snucleotide2
# Align_format: pair
# Report_file: stdout
 Aligned_sequences: 2
# 1: hsvct1
# 2: hsvct2
# Matrix: EDNAFULL
# Gap_penalty: 10.0
# Extend_penalty: 0.5
# Length: 2039
# Identity:    1236/2039 (60.6%)
# Similarity:  1236/2039 (60.6%)
# Gaps:         398/2039 (19.5%)
# Score: 3695.5

hsvct1             1 --------------------------------------------------
0

hsvct2             1 AAGACGAGGCAAAGCACCCAGCTTTCTTCACTCTTCCGGTGGTGATAAAT
50

hsvct1             1 -------------------ATGAG--GGCCCAGGAGGAC------CTCGA
23
                                       .||||  ||.|.|.||||||      ||| |
hsvct2            51 GGAGGCGCCACCTCCAGCGGTGAGCAGGACAATGAGGACACTGAGCTC-A
99

hsvct1            24 GGGC-----------CGG-ACACAGCAT----GAAACCACCAGGGACCCC
57
                        .|||           ||| |.||.||||    ||||     ||.|..|.|
hsvct2           100 TGGCGATCTACACTACGGAAAACGGCATTGCAGAAA-----AGAGCTCTC
144

hsvct1            58 TC-----GACCC-------CGCT----------ACCCACAGAGCCTAAGT
85
                        ||     |||||       |.||          ||||.|||.|     .|
hsvct2           145 TCGCTGAGACCCTGGATAGCACTGGCAGTCTGGACCCCCAGCG-----AT
189

hsvct1            86 TTGACATGTTGTACAAGATCGAGGACGTGCCACCTTGGTACCTGTGCATC
135
                        ..||||||.|.||.|..||.||.||.||.||.||.||||||||||.||.
hsvct2           190 CAGACATGATTTATACCATAGAAGATGTTCCTCCCTGGTACCTGTGTATA
239

hsvct1           136 CTGCTGGGCTTCCAGCACTACCTGACATGCTTCAGTGGTACCATCGCCGT
185
                        .|.||||||..|.|||||||||||||||||||.||.||.|||||.||
hsvct2           240 TTTCTGGGGCTACAGCACTACCTGACATGCTTCAGCGGCACGATCGCAGT
289

hsvct1           186 GCCCTTCCTGCTGGCTGAGGCGCTGTGTGTGGGCCACGACCAG-----CA
230
                        ||||||||||.||||.||.||..||||||||||..||||||||     ||
```

```
hsvct2          290 GCCCTTCCTGTTGGCCGATGCCATGTGTGTGGGGTACGACCAGTGGGCCA
339

hsvct1          231 CATGGTTAGTCAGCTCATCGGCACCATCTTCACGTGCGTGGGCATCACCA
280
                    |     .||.||||||||.||.|||||.|||...||.|||||.||||||.|
hsvct2          340 C-----CAGCCAGCTCATTGGGACCATTTTCTTCTGTGTGGGAATCACTA
384

hsvct1          281 CTCTCATCCAGACCACCGTGGGCAT-CCGGCTGCCGCTGTTCCAGGCCAG
329
                    ||.|..|.|||| ||.|||..|.|| |.||.|.||.|||||.||||||||
hsvct2          385 CTTTGCTACAGA-CAACGTTTGGATGCAGGTTACCCCTGTTTCAGGCCAG
433

hsvct1          330 TGCCTTTGCATTTCTGGTTCCAGCCAAAGCCATACTGGCTCTGGAGAGAT
379
                    |||.||||||||||.|||..||.||...||||||.|||.||.|.||.|.||
hsvct2          434 TGCTTTTGCATTTTTGGCCCCTGCTCGAGCCATCCTGTCTTTAGATAAAT
483

hsvct1          380 GGAAATG---CCCCCC-------------------GGAAGAGGAGATCTA
407
                    |||||||   |.||.|                   ||||.||.|||.||
hsvct2          484 GGAAATGTAACACCACAGATGTTTCAGTTGCCAATGGAACAGCAGAGCT-
532

hsvct1          408 CGGTAACTGGAGTCTGC-CCCTGAACACCTCTCATATTTGGCACCCACGG
456
                      ||          ||| |.|.||||||       ||.|||.|.||.|||
hsvct2          533 --GT----------TGCACACAGAACAC-------ATCTGGTATCCCCGG
563

hsvct1          457 ATACGGGAGGTCCAGGGTGCAATCATGGTGTCCAGCGTGGTGGAGGTGGT
506
                    ||.||.|||.|||||||.||.||||||..||||||....||.|.||.||.||
hsvct2          564 ATCCGAGAGATCCAGGGGGCCATCATCATGTCCTCACTGATAGAAGTAGT
613

hsvct1          507 GATTGGCCTGCTGGGGCTGCCTGGGGCCCTGCTCAACTACATTGGGCCTC
556
                    .||.|||||.||.||.||||||||||.||.||.||.|||||.||.||..
hsvct2          614 CATCGGCCTCCTCGGCCTGCCTGGGGCTCTACTGAAGTACATCGGTCCCT
663

hsvct1          557 TCACAGTCACCCCCACTGTCTCCCTCATTGGCCTTTCTGTCTTCCAAGCT
606
                    |.||..|.||.|||||.||..||||.||||||||.||||..|||||.||.
hsvct2          664 TGACCATTACACCCACGGTGGCCCTAATTGGCCTCTCTGGTTTCCAGGCA
713

hsvct1          607 GCTGGCGACCGAGCTGGCTCCCACTGGGGCATCTCAGCTTGCT--CCATT
654
                    ||.||.||..||||.||....||||||||||..|  |.||||  |.||.
hsvct2          714 GCGGGGGAGAGAGCCGGGAAGCACTGGGGCATTGC--CATGCTGACAATA
761

hsvct1          655 CTCCTGATCATCCTCTTCTCCCAGTACCTGC--GCAA-CCTCACCTTCCT
701
                    .||||..|..|.||.||.||.||.|||  ||  |.|| ..|.|..|||||
hsvct2          762 TTCCTAGTATTACTGTTTTCTCAATAC--GCCAGAAATGTTAAATTTCCT
809

hsvct1          702 GCTGCCTGTCTA-----CC-------GCTGGGGCAAGGGCCTCACTCTCC
739
                     ||.||..|.||     ||       |.|||                .|
hsvct2          810 -CTCCCGATTTATAAATCCAAGAAAGGATGG-----------------AC
841
```

```
hsvct1          740 TCCGCA-----TCCAGATCTTCAAAATGTTTCCTATCATGCTGGCCATCA
784
                    |.||.|     |.|||.|.||||||||||.||||||||.|||||||||.
hsvct2          842 TGCGTACAAGTTACAGCTGTTCAAAATGTTCCCTATCATCCTGGCCATCC
891

hsvct1          785 TGACCGT---GTGGCTGCTCTGCTATGTCCTGACCTTGACAGACGTGCTG
831
                    ||   ||   .|||||||||||||...||.|.||..|||||||||| ||.
hsvct2          892 TG---GTATCCTGGCTGCTCTGCTTCATCTTCACGGTGACAGACGT-CTT
937

hsvct1          832 CCC-----ACAGACCCA-AAAGCCTATGGCTTCCAGGCACGAACCGATGC
875
                    |||     ||||   || ||||  |||||||||.|.||.||.||.|||||
hsvct2          938 CCCTCCCGACAG---CACAAAG--TATGGCTTCTATGCTCGCACAGATGC
982

hsvct1          876 C--------CGTGGTGACATCATGGCTATTGCACCCTGGATCCGCATCCC
917
                    |        ||||    |.|| |||   |.||.||.|||.|.....|.||
hsvct2          983 CAGGCAAGGCGTG----CTTC-TGG---TAGCCCCGTGGTTTAAGGTTCC
1024

hsvct1          918 CTACCCCTGTCAGTGGGGCCTGCCCACGGTGACTGCGGCTGCTGTCCTGG
967
                    .|||||.|.|||||||||.||||||||.|||.|||||||.|.||||.|.|
hsvct2         1025 ATACCCATTTCAGTGGGGACTGCCCACCGTGTCTGCGGCCGGTGTCATCG
1074

hsvct1          968 GAATGTTCAGCGCCACTCTGG----CAGGCATCATTGAGTCCATCGGAGA
1013
                    |.|||.||||.|||   .|||    || |||||||.|||||.||.||.||
hsvct2         1075 GCATGCTCAGTGCC---GTGGTCGCCA-GCATCATCGAGTCTATTGGTGA
1120

hsvct1         1014 TTACTACGCCTGTGCCCGCCTGGCTGGTGCACCACCCCCTCCAGTACATG
1063
                    .|||||||||||||.||.|||.|..||||.||||||||.||..|.||.|
hsvct2         1121 CTACTACGCCTGTGCACGGCTGTCCTGTGCCCCACCCCCCCCCCATCCACG
1170

hsvct1         1064 CTATCAACAGGGGCATCTTCACCGAAGGCATTTGCTG----CATCATCGC
1109
                    |.||.||||||||.||.|||...||||||.|.|.|||    |.|.||
hsvct2         1171 CAATAAACAGGGGAATTTTCGTGGAAGGCCTCTCCTGTGTTCTTGAT---
1217

hsvct1         1110 GGGGC-TATTGGGCACGGGCAACGGGTCCACCTCGTCCAGTCCCAACATT
1158
                      ||| ||||.||.||.||.||.||.||.||.||.|||||||||||||||
hsvct2         1218 --GGCATATTTGGTACTGGGAATGGCTCTACTTCATCCAGTCCCAACATT
1265

hsvct1         1159 GGCGTCCTGGGAATTACCAAGGTGGGCAGCCGGCGCGTGGTGCAGTATGG
1208
                    ||.||..|||||||||.|||||.||||||||.||||||.|.||||..||
hsvct2         1266 GGAGTTTTGGGAATTACAAAGGTCGGCAGCCGCCGCGTGATACAGTGCGG
1315

hsvct1         1209 TGCGGCTATCATGCTGGTCCTGGGCACCATCGGCAAGTTCACGGCCCTCT
1258
                    .||.||..|||||||.|..|||||||..|||||.||||||..|||||||
hsvct2         1316 AGCAGCCCTCATGCTCGCTCTGGGCATGATCGGGAAGTTCAGCGCCCTCT
1365
```

```
hsvct1          1259 TCGCCTCGCTCCCTGACCCCATCCTGGGGGGCATGTTCTGCACTCTCTTT
1308
                     |.||.||.||.||.||.||..|.|||||.|.|.||||||||||.||||||
hsvct2          1366 TTGCGTCCCTTCCGGATCCTGTGCTGGGAGCCCTGTTCTGCACGCTCTTT
1415

hsvct1          1309 GGCATGATTACAGCTGTGGGGCTGTCCAACCTGCAATTTGTGGACATGAA
1358
                     ||.|||||.||||||||.||.||.||.||||||||.||..|.||..|.||
hsvct2          1416 GGAATGATCACAGCTGTTGGCCTCTCTAACCTGCAGTTCATTGATTTAAA
1465

hsvct1          1359 CTCCTCTCGCAACCTCTTCGTGCTGGGATTTTCCATGTTCTTCGGGCTCA
1408
                     .||.||.||.||||||||.|||||.||||||||.||.|||||.||||||.
hsvct2          1466 TTCTTCCCGGAACCTCTTTGTGCTTGGATTTTCGATCTTCTTTGGGCTCG
1515

hsvct1          1409 CGCTGCCCAATTACCTGGAGTC-CAACC--CTGGCGCCATCAATACAGGC
1455
                     ..||.||.|.|||||| .||.| .||||  ||||     ||   |||||.
hsvct2          1516 TCCTTCCAAGTTACCT-CAGACAGAACCCTCTGG-----TC---ACAGGG
1556

hsvct1          1456 AT-TCTTGAAGTGGATCAGATTCTGATTGTGCTGCTGACCACGGAGATGT
1504
                     || .|..||| |.||||||..|..|||..||.||.||.||.||.|..||||
hsvct2          1557 ATAACAGGAA-TCGATCAAGTGTTGAACGTCCTTCTCACAACTGCTATGT
1605

hsvct1          1505 TTGTGGGCGGGTGCCTTGCTTTCATACTTGACAACACAGTGCCAGGGAGC
1554
                     ||||.||.||.||..|.|||||.||.||.||.|||||..|.|||||.|..
hsvct2          1606 TTGTAGGGGGCTGTGTGGCTTTTATCCTGGATAACACCATCCCAGGCACT
1655

hsvct1          1555 CCAGAGGAGCGTGGTCTGATACAGTGGAAAGCTGG-----GGCTCATGCC
1599
                     ||||||||..|.||   .||.|   |||||  |||     ||.|...|.|
hsvct2          1656 CCAGAGGAAAGAGG---AATCC---GGAAA--TGGAAGAAGGGTGTGGGC
1697

hsvct1          1600 AACAGTG-ACATGTC-TTC--CAGCCTCAAGAGCTACGATTTCCCCATTG
1645
                     || ||.| |||..|| .||  |.||.|..||...|||.||||.||..|||
hsvct2          1698 AA-AGGGAACAAATCACTCGACGGCATGGAGTCGTACAATTTGCCATTTG
1746

hsvct1          1646 GGATGGGCATAGTAAAAAGAATTAC-----CTTTCTGAAATACATTCCTA
1690
                     |.|||..|||  ||.|||.||.|||     ||   |.|..|||.|.||.|
hsvct2          1747 GCATGAACAT--TATAAAAAAATACAGATGCT---TCAGCTACTTACCCA
1791

hsvct1          1691 TCTGCCCAGTCTT---------------CAAAGGATTTTCTTCAAGTTCA
1725
                     ||.||||||..|||               .|||||     |.|||.|
hsvct2          1792 TCAGCCCAACCTTTGTGGGCTACACATGGAAAGG-----CCTCAGG----
1832

hsvct1          1726 AAAGA-----------------TCAGATTGCAATTCCAGAAGACACTCCA
1758
                      ||||                 ||||||          |||||||| |||
hsvct2          1833 -AAGAGCGACAACAGCCGGAGTTCAGAT----------GAAGACTC-CCA
1870

hsvct1          1759 GAAAATACAGAAACTGCATCTGTGTGCACCAAGGTCTAA   1797
                     |        |..||.|.||            ||
```

Appendix IX- Local Alignment of hSVCT1 and hSVCT2

```
Query: ./wwwtmp/lalign26697.1.seq

  1>>>hsvct1 598 bp - 598 aa
Library: ./wwwtmp/lalign266551.2.seq
      650 residues in     1 sequences

Statistics: (shuffled [500]) MLE statistics: Lambda= 0.1594;  K=0.01575
 statistics sampled from 1 (1) to 500 sequences
Threshold: E() < 10 score: 40
Algorithm: Smith-Waterman (SSE2, Michael Farrar 2006) (7.2 Nov 2010)
Parameters: BL50 matrix (15:-5), open/ext: -12/-2
 Scan time:  0.040

>>hsvct2 650 bp                                                (650 aa)
 Waterman-Eggert score: 2636;  612.2 bits; E(1) <  2e-179
68.3% identity (88.5% similar) in 546 aa overlap (28-572:86-629)

        30        40        50        60        70        80
hsvct1 KFDMLYKIEDVPPWYLCILLGFQHYLTCFSGTIAVPFLLAEALCVGHDQHMVSQLIGTIF
       . ::.: ::::::::::.::.:::::::::::::::::::.:.:::.::  .::::::::
hsvct2 RSDMIYTIEDVPPWYLCIFLGLQHYLTCFSGTIAVPFLLADAMCVGYDQWATSQLIGTIF
          90       100       110       120       130       140

        90       100       110       120       130       140
hsvct1 TCVGITTLIQTTVGIRLPLFQASAFAFLVPAKAILALERWKCPPEEEIYGNWSLPL-NTS
        ::::::::::: : :::::::::::::.::.:::.:..:::   .   .: .  : .:
hsvct2 FCVGITTLLQTTFGCRLPLFQASAFAFLAPARAILSLDKWKCNTTDVSVANGTAELLHTE
         150       160       170       180       190       200

        150       160       170       180       190       200
hsvct1 HIWHPRIREVQGAIMVSSVVEVVIGLLGLPGALLNYIGPLTVTPTVSLIGLSVFQAAGDR
       :::.::::::.:::::..::..:::::::::::::::.::::::.::::.::::: :::::.:
hsvct2 HIWYPRIREIQGAIIMSSLIEVVIGLLGLPGALLKYIGPLTITPTVALIGLSGFQAAGER
         210       220       230       240       250       260

        210       220       230       240       250       260
hsvct1 AGSHWGISACSILLIILFSQYLRNLTFLLPVYRWGKGLTLLRIQIFKMFPIMLAIMTVWL
       ::.::::.  .:.:..::::: ::. : ::.:.  :: :  ..:.::::::.:::.. ::
hsvct2 AGKHWGIAMLTIFLVLLFSQYARNVKFPLPIYKSKKGWTAYKLQLFKMFPIILAILVSWL
         270       280       290       300       310       320

        270       280       290       300       310       320
hsvct1 LCYVLTLTDVLPTDPKAYGFQARTDARGDIMAIAPWIRIPYPCQWGLPTVTAAAVLGMFS
       ::...:.:::.: :   ::: ::::::  .. .:::...::: ::::::::.::.:.::.:
hsvct2 LCFIFTVTDVFPPDSTKYGFYARTDARQGVLLVAPWFKVPYPFQWGLPTVSAAGVIGMLS
         330       340       350       360       370       380

        330       340       350       360       370       380
hsvct1 ATLAGIIESIGDYYACARLAGAPPPPVHAINRGIFTEGICCIIAGLLGTGNGSTSSSPNI
       :..:.:::::::::::::. :::::.:::::::.::. :.. :..:::::::::::::
hsvct2 AVVASIIESIGDYYACARLSCAPPPPIHAINRGIFVEGLSCVLDGIFGTGNGSTSSSPNI
         390       400       410       420       430       440

        390       400       410       420       430       440
hsvct1 GVLGITKVGSRRVVQYGAAIMLVLGTIGKFTALFASLPDPILGGMFCTLFGMITAVGLSN
       ::::::::::::.: :::.::.:: ::::.::::::::.::..:::::::::::::::
hsvct2 GVLGITKVGSRRVIQCGAALMLALGMIGKFSALFASLPDPVLGALFCTLFGMITAVGLSN
         450       460       470       480       490       500

        450       460       470       480       490       500
hsvct1 LQFVDMNSSRNLFVLGFSMFFGLTLPNYLESNPGAINTGILEVDQILIVLLTTEMFVGGC
       :::.:.:::::::::::::.::::.::.::..::  . :::  .::.: ::::: ::::::
hsvct2 LQFIDLNSSRNLFVLGFSIFFGLVLPSYLRQNP--LVTGITGIDQVLNVLLTTAMFVGGC
         510       520       530         540       550       560

        510       520       530       540       550       560
```

```
hsvct1 LAFILDNTVPGSPEERGLIQWKAGAHANSDMSSSLKSYDFPIGMGIVKRITFLKYIPICP
       .::::::::.::.:::::. .:: :.  ..   ....::..:.::.:.:.   ..:.:: :
hsvct2 VAFILDNTIPGTPEERGIRKWKKGVGKGNKSLDGMESYNLPFGMNIIKKYRCFSYLPISP
           570       580       590       600       610       620

        570
hsvct1 VFKGFS
       .: :..
hsvct2 TFVGYT

>--
 Waterman-Eggert score: 66;  21.2 bits; E(1) <  0.15
17.8% identity (55.0% similar) in 129 aa overlap (159-287:458-582)

      160       170       180       190       200       210
hsvct1 AIMVSSVVEVVIGLLGLPGALLNYIGPLTVTPTVSLIGLSVFQAAGDRAGSHWGISACSI
       .:. .... ...:..:  .::.  . :  :  ..    .... :.:    .   ...
hsvct2 VIQCGAALMLALGMIGKFSALFASL-PDPVLGALFCTLFGMITAVGLSNLQFIDLNSSRN
       460       470       480       490       500       510

      220       230       240       250       260       270
hsvct1 LLIILFSQYLRNLTFLLPVYRWGKGLTLLRIQIFKMFPIMLAIMTVWLLCYVLTLTDVLP
       :... :: .   . ..:: :   . :.     : ... ..:.       : .. : ...:
hsvct2 LFVLGFSIF---FGLVLPSYLRQNPLVTGITGIDQVLNVLLTTAMFVGGCVAFILDNTIP
        520          530       540       550       560       570

      280
hsvct1 TDPKAYGFQ
         :.  :..
hsvct2 GTPEERGIR
           580
>--
 Waterman-Eggert score: 64;  20.7 bits; E(1) <  0.2
20.3% identity (58.0% similar) in 69 aa overlap (351-410:210-278)

              360       370             380       390          400
hsvct1 PPVHAINRGIFTEGICCIIAGLLGTGN------GSTSSSPNIGVLGIT---KVGSRRVVQ
       : .. :. .:.  ..  .. ::::  .      :  . .:.....:..    .: :   .
hsvct2 PRIREIQGAIIMSSLIEVVIGLLGLPGALLKYIGPLTITPTVALIGLSGFQAAGERAGKH
     210       220       230       240       250       260

             410
hsvct1 YGAAIMLVL
       .: :.. ..
hsvct2 WGIAMLTIF
     270
598 residues in 1 query   sequences
650 residues in 1 library sequences
 Scomplib [36.3.5e Nov, 2012(preload8)]
 start: Tue Feb 07 15:45:31 2017 done: Tue Feb 07 15:45:32 2017
 Total Scan time:  0.040 Total Display time:  0.040

Function used was LALIGN [36.3.5e Nov, 2012(preload8)]
```

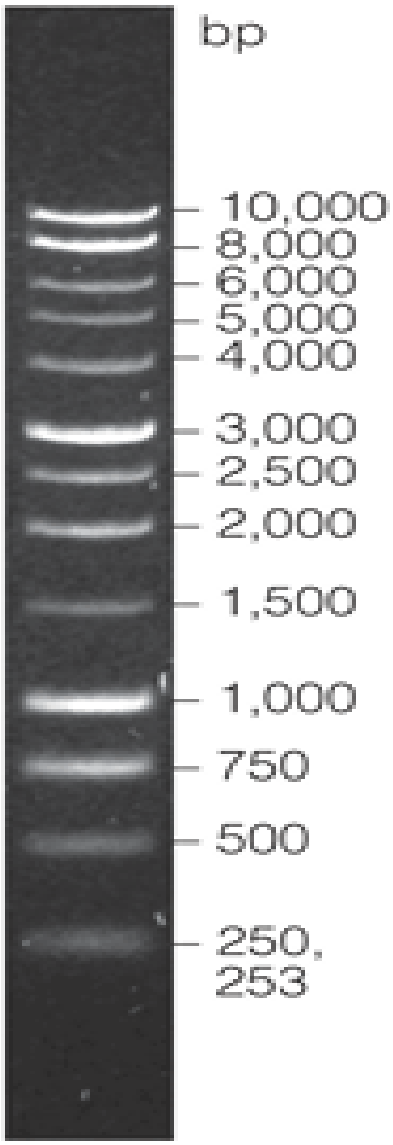
Appendix X- GIBCO-BRL Oligonucleotide Tm Table

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	48.3	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

Appendix XI- 1kb Ladder (Promega; Catalogue No. G5711)



Appendix XII- Research Notebook Copies

Continued from page number	Page number 1

05/04/2017 Making LB agar / Streaked.

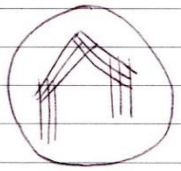
Autoclaved using "instrument setting" (approx 1hr). - beakers, eppendorfs and yellow pipette tips.

Prepared LB agar using:

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

This was mixed using a hot plate and mixing magnets and distilled water was added up to 1 Litre.
This solution was then autoclaved under "media" setting.
- It was left to cool and then 1000µl ampicillin was added.
Plates were then poured using all amp media (approx 40 in total).
They were left to set overnight on the bench.

Pre-pared LB agar (already cooled) was streaked.
This used a flame and a d-loop and pc SVCT1 / SVCT2 was streaked:



These plates were left to incubate overnight at 37°C to allow the colony to grow.

Performed by	Date	Countersigned by	Date	Continued on page number
C Domeneghetti			5/4/17	

06/04/2017. Making LB Media / Innoculation.

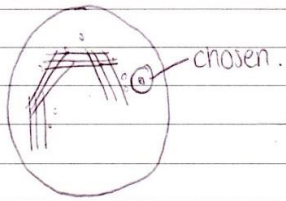
Bacto ~~kg~~ tryptone - 10g
Bacto yeast extract - 5g
Bacto NaCl - 10g.
} 1 Litre. - does not solidify as no agar added.

Mixed using a hot plate and mixing magnets and filled to 1 litre ~~of~~ using distilled water.

It was then autoclaved under "media" setting.
Amp was then added at 1µl / 1ml ratio to the LB media.

(Approx ~~25~~ 50 ml of LB media was added to a conical flask. and ~~25~~ 50 µl of ampicellin was added.)

At 11am the plates which were incubated overnight were taken out and a colony was picked up with the d-loop. The d-loop was then placed into the media (in the conical flask) and swirled around for a few minutes. At around 4pm the media (and colony) were added into the orbital shaking incubator at 250 rpm at 37°C overnight.



(Add glycerol stock before miniprep if you want to be able to freeze the overnight culture and not have to re-innoculate. Can freeze up to 80°C.).

Performed by	Date	Countersigned by	Date	Continued on page number
C. Domaracka		[illegible]	6/4/17	

11/04/2017 Spin Mini Prep Kit (QIAGEN).

1) Pelleted 1ml of the overnight culture by centrifuge at 13,000rpm (table-top microcentrifuge) for 1minute at room temp (15-25°C).

2) Removed the suspension (leaving the pellet at the bottom of the eppendorf). Then added 250µl of P1 buffer (which had LyseBlue added) and vortexed.

3). Added 250µl of P2 buffer and mixed by inverting 4-6 times.

4) Added 350µl of N3 buffer and vortexed. Then immediatly placed it into an ice bath for approx 5 minutes. (to stop the lysis reaction proceeding for longer than 5 mins.

5) Centrifuge for 10mins at 13,000rpm in a table-top microcentrifuge.

6) Remove the supernatant (800µl) and pipette into the middle of the spin column + centrifuge for 1min. (discard flow-through).

7) Wash the QIA prep spin column by adding 0.5ml Buffer PB. centrifuge for 1min and discard flow-through.

8) Wash the QIA prep spin column by adding 0.75ml Buffer PE. Centrifuge for 1min and discard flow-through.

9) Centrifuge again for 1min to remove residual wash buffer.

10) Place the QIA prep column (top part only) into a 1.5ml microcentrifuge tube (eppendorf). Incubate for 5 mins and elute the DNA by adding 50µl Buffer EB to the centre of the spin column.

remove ethanol

11) Stand for 1minute and centrifuge for 1minute.

Performed by	Date	Countersigned by	Date	Continued on page number
C. Dem			11/4/17	

11/04/17. Gel electrophoresis.

To make the electrophoresis gel:

40ml TAE (x1) - (if 50x of TAE only available you need to dilute by 1/50)
0.6g agarose

Heat in a microwave (putting blue roll on the top to cover the conical flask) for approx 1 minute at 30 second intervals until fully dissolved and clear.
- Then let to cool until able to hold and add cybersafe (4ul) and mix. (takes 20min) to set).

Loading the gel:

5ul ladder (made of 300ul TiOE1
100ul 1kb DNA ladder
100ul 5x Green GoTaq Buffer).

4ul DNA and 1ul 5x GreenGoTaq Buffer.

Load the wells and run at 70V for 40minutes.
Black end (-) at the top.
Red end (+) at the bottom.

Performed by	Date	Countersigned by	Date	Continued on page number
C. Domeneghetti			11/4/17	

19/04/17 NanoDrop 2000 - Spectrophotometer - Thermo Scientific.

Nanodrop: - to test how much DNA we have.

OD260 /OD280 = Should be around ~~0.8~~ 1.8 (if less than 1.8 dna is less pure + may have contaminents).
between.

Talbe of results for Nanodrop:

Name	conc (ug/ml)	(needs to be 0.2-0.8) OD260	(1.8-2.0) 260/280.
1) SVCT1 (1) ✓	103.6	2.072	1.88 = 60µl H_2O
3:1	17.3	0.345	1.81 = 20µl dna sample.
SVCT1 (2) x	102.4	2.049	1.90
SVCT1 (3)	80.8	1.616	1.87
2:1 ✓	29.3	0.587	1.86 = 40µl H_2O 20µl DNA sample.
3:1 x	15.7	0.315	1.77
SVCT1 (4)	47.7	0.955	1.90
2:1 x	14.6	0.292	1.89
SVCT1 (5) x	33.1	0.663	1.90
SVCT1 (6) x	54.1	1.083	1.88
2:1	15.7	0.314	1.86
SVCT2 (1) x	52.6	1.052	1.88
2:1	14.6	0.293	1.87
SVCT2 (2)	206.0	4.121	1.91
5:1 ✓	20.7	0.414	1.86 = 50µl H_2O 10µl dna sample.

Performed by	Date	Countersigned by	Date	Continued on page number
C. Domeneva			19/4/17	

Name	conc (µl/ml)	OD260	OD 260/280.
SVCT2 (3)	160.5	3.211	1.91
4:1 ×	9	0.179	1.88
SVCT2(4)	221.4	4.428	1.91
4:1 ×	16.7	0.335	1.81
3:1 ✓	21.4	0.429	1.89 = 60µl H2O 20µl dna sample
SVCT2(5)	145.0	2.899	1.92
2:1 ×	23.0	0.461	1.90
SVCT2(6)	89.4	1.787	1.91
2:1 ×	20.1	0.402	1.93

To use the Nanodrop 2000 :

- use autoclaved water (1µl) as the blank. Place this on the front and back raised section on the machine. (The run blank on the computer).
- Once completed wipe the front section with a dry tissue.
- Then add 1µl of dna sample and find out results.

If the readings are too high you can dilute the samples and test again.

- Can store products in the freezer.

To make dNTP for PCR you add 1µl of 10mM dntp and 9µl of distilled water.

Performed by	Date	Countersigned by	Date	Continued on page number
C ~~Domenegan~~			19/4/17	

19/04/17. making TioE1 and PCR.

Make in eppendorfs: for 1ml = 2µl EDTA
10µl TRIS
988µl water.

Primers: TioE1 dilutes the primers.

Main Forward = PCDNA3F
Main Reverse = PCDNA3R.

VtC1P190SF you add 371µl TioE1
VtC1P190SR you add 179µl TioE1

VtC1V62GF you add 411µl TioE1
VtC1V62GR you add 230µl TioE1

VtC1I96LF you add 341µl TioE1
VtC1I96LR you add 241µl TioE1

VtC1L277FF you add 218µl TioE1
VtC1L277FR you add 311µl TioE1

VtC1N385SF you add 364µl TioE1
VtC1N385SR you add 248µl TioE1.

SVCT1

VtC2A174VF you add 278µl TioE1
VtC2A174VR you add 205µl TioE1

VtC2K184RF you add 284 TioE1
VtC2K184RR you add 267 TioE1

VtC2Q353GF you add 238 TioE1
VtC2Q353GR you add 301 TioE1

SVCT2.

Performed by	Date	Countersigned by	Date	Continued on page number
C. Domenech			19/4/17	

VtC2D133GF you add 240µl TioE1
VtC2D133GR you add 288µl TioE1
VtC21381LF you add 274µl TioE1
VtC21381LR you add 248µl TioE1
} SVCT2.

You add the appropriate amount of TioE1 to each of the primers.

PCR

1) Dilute the primers (again) using 1µl of the primers and 9µl of distilled water. (in eppendorfs)

To set up pcr you need the small pcr tubes:

H2O = 33.5µl
5x flexi buffer (grey) = 10µl
1mM dNTP = 1µl
25mM $MgCl_2$ = 2µl
10µM forward primer = 1µl
10µM reverse primer = 1µl
DNA sample = 1µl = SVCT1 /SVCT2 diluted
GoTAQ polymerase = 0.5µl (needs to go straight into PCR after).

} all reagents should be kept on ice.

When adding the primers you add:

The main forward primer (pCDNA3F) and the mini reverse or main reverse and mini forward
eg. 1µl pCDNA3F and VtCIP190SR(1µl)
1µl pCDNA3R and 1µl VtCIP190SF.

PCR cycle: (1hr 58 minutes long)
1) Heat Lid to 110.0°C
2) Temp 95.0°C for 5 minutes
3) 28x cycles: temp 94°C for 1min
60°C for 30 seconds
72°C for 2 minutes.
4) → Then 72°C for 4mins
5) → Finished.

Performed by	Date	Countersigned by	Date	Continued on page number
C Domeneg			19/4/17	

Electrophoresis gel for first-time PCR samples 20/04/2017

1st gel = SVCT1

Ladder
V+C1I96L(F) = pCDNA3F + V+C1I96LR
V+C1I96L(R) = pCDNA3R + V+C1I96LF
V+C1V62G(F) = pCDNA3F + V+C1V62GR
V+C1V62G(R) = pCDNA3R + V+C1V62GF
V+C1L277F(F) = pCDNA3F + V+C1L277FR ✗ ? Needs PCR to be done.
V+C1L277F(R) = pCDNA3R + V+C1L277FF ✗ ?
V+C1P190S(F) = pCDNA3F + V+C1P190SR
V+C1P190S(R) = pCDNA3R + V+C1P190SF
V+C1N385S(F) = pCDNA3F + V+C1N385SR
V+C1N385S(R) = pCDNA3R + V+C1N385SF

2nd gel = SVCT2

Ladder
V+C2I381L(F) = pCDNA3F + V+C2I381LR
V+C2I381L(R) = pCDNA3R + V+C2I381LF
V+C2D133G(F) = pCDNA3F + V+C2D133GR
V+C2D133G(R) = pCDNA3R + V+C2D133GF
V+C2K184R(F) = pCDNA3F + V+C2K184RR
V+C2K184R(R) = pCDNA3R + V+C2K184RF
V+C2A174V(F) = pCDNA3F + V+C2A174VR
V+C2A174V(R) = pCDNA3R + V+C2A174VF
V+C2Q353G(F) = pCDNA3F + V+C2Q353GR
V+C2Q353G(R) = pCDNA3R + V+C2Q353GF

The gels were ~~ran~~ run for 40minutes at 70V.

Performed by	Date	Countersigned by	Date	Continued on page number
C. Domen			20/4/17	

~~Fusion PCR~~. PCR Purification.

Before completing Fusion PCR you have to purify the samples using the QIAquick® PCR purification kit.

1) Add 20µl PCR sample into an eppendorf and add 100µl Buffer PB. (make sure the mixture isn't orange / violet - if so read protocol instructions)

2) To bind the DNA apply the sample to a QIAquick column and centrifuge at 13rpm for 1minute. Once completed discard the flow through. (000)

3) To wash, add 750µl Buffer PE to the QIAquick column and centrifuge at 13,000 rpm for 1 minute. Once completed discard the flow through.

4) Centrifuge the QIAquick column once more in the 2ml collection tube for 1min to remove the residual wash buffer.

5) Place the QIAquick column in a clean 1.5ml eppendorf.

6) To elute the DNA, add 50µl Buffer EB to the center of the QIAquick membrane and centrifuge for 1minute.

~~7)~~

Following this we added 5.5µl of the Cut Buffer (1x CutSmart® Buffer) and 1ul dpn1 to each pcr sample before fusion pcr.
Then add the samples into the heated mixing block at no speed but at 80°c for 20minutes.

DPN1 is used to get rid of template dna. DPN1 only cuts methylated DNA so the PCR products are untouched.

Electrophoresis gel of each sample needs to be completed at 70V for 40 minutes.

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

26/04/2017

Electrophoresis gels for purified pcr products.

1st gel = SVCT1

Ladder.
V+C1I96L(F)
V+C1I96L(R)
V+C1V62G(F)
V+C1V62G(R)
V+C1P190S(F)
V+C1P190S(R)
V+C1N385S(F)
V+C1N385S(R).

2nd gel = SVCT2.

Ladder
V+C2I381L(F)
V+C2I381L(R)
V+C2D133G(F)
V+C2D133G(R)
V+C2K184R(F)
V+C2K184R(R)
V+C2A174V(F)
V+C2A174V(R)
V+C2Q353G(F)
V+C2Q353G(R)

Performed by	Date	Countersigned by	Date	Continued on page number
C. [redacted]		[redacted]	26/4/17	

26/04/2017

Fusion PCR.

H_2O = 32.5µl
5x Flexibuffer (grey) = 10µl
1mM dntp = 1µl
25mM $MgCl_2$ = 2µl
1st PCR sample (F) = 2µl
1st PCR sample (R) = 2µl
GOTAQ polymerase = 0.5µl

PCR fusion was then run using the peqlab machine on a cycle which was 3hrs 8minutes long.
Lid temperature = 110°C
The cycle was repeated 28 times:
94.0°C for 1min
60.0°C for 30mins
72.0°C for 4.30mins
The close cycle was 72°C for 4.0minutes.

An electrophoresis gel was then run (1.5%) for 40minutes at 70V.

Electrophoresis gel for 1st time fusion:

SVCT1:
- VtC1I96L
- VtC1V62G
- VtC1P190S
- VtC1N385S

SVCT2:
- VtC2I381L
- VtC2D133G
- VtC2K184R
- VtC2A174V
- VtC2Q353G

Performed by	Date	Countersigned by	Date	Continued on page number
[signature]		[signature]	26/4/17	

11/05/2017 Fusion Repeated.

H_2O = 30.5 µl
Flexi buffer = 10µl
dNTP = 1µl
$MgCl_2$ = 2 µl
PCR forward flanking = 1µl (PCDNA3F)
PCR Reverse flanking = 1µl (PCDNA3R).
DNA template = 1+µl (2µl from 1st PCR (F) + 2µl from 1st PCR (R)
GO TAQ = 0.5µl.

Fusion pcr under "cc amplification" setting.

V+C1I96L = V+C1I96L(F) + V+C1I96L(R) + PCDNA3F + PCDNA3R.
V+C1V62G = V+C1V62G(F) + V+C1I[redacted]62G(R) + PCDNA3F + PCDNA3R.
V+C1P190S = V+C1P190S(F) + V+C1P190S(R) + PCDNA3F + PCDNA3R
V+C1N385S = V+C1N385S(F) + V+C1N385S(R) + PCDNA3F + PCDNA3R
V+C2I381L = V+C2I381L(F) + V+C2I381L(R) + PCDNA3F + PCDNA3R
V+C2D133G = V+C2D133G(F) + V+C2D133G(R) + PCDNA3F + PCDNA3R
V+C2K184R = V+C2K184R(F) + V+C2K184R(R) + PCDNA3F + PCDNA3R
V+C2A174V = V+C2A174V(F) + V+C2A174V(R) + PCDNA3F + PCDNA3R
V+C2Q353G = V+C2Q353G(F) + V+C2Q353G(R) + PCDNA3F + PCDNA3R.

What the fusion products have inside ↑ plus the other normal constituents eg. H_2O, flexi buffer, dNTP, $MgCl_2$ and GOTAQ.

Performed by	Date	Countersigned by	Date	Continued on page number
[redacted]		[redacted]	11/5/17	

04/07/17 Fusion Electrophoresis

Ladder
V+C1I96L - results x2 bands
V+C1V62G - results x2 bands
V+C1P190S - results x2 bands
V+C1N385S - results x2 bands.
V+C2I381L
V+C2D133G
V+C2K184R
V+C2A174V
V+C2Q353G

06/07/17 Fusion Repeated (3rd time)
PCR using PCR Master mix (2X) -thermo scientific -K0171.

PCR master mix (2X) = 25ul
pcr forward flanking primer (pCDNA3F) = 1ul
pcr reverse flanking primer (pCDNA3R) = 1ul
DNA template = 2ul (1ul from 1st pcr (F)) and (1ul from 1st pcr (R)).
H2O = 21ul

PCR master mix (2X) = Taq DNA polymerase
reaction buffer
$MgCl_2$
dNTP.

Performed by	Date	Countersigned by	Date	Continued on page number
			4/7/17	

07/07/2017

"04/07/17" fusion bands had to many nanograms of DNA.
So primary pcr bands are still appearing on gels. This is because dpN1 may have not cut all primary pcr products.
Nanodrop needs to be approx 5ng.

Nanodrop SVCT1	ng/lul (close to 5ng)	OD260	260/280
original →	58.7	1.174	1.89
9(H2O) : 1(dna)	4.2	0.084	1.77 ✓

Primary pcr SVCT1 for next week.

H2O = 34.5ul
5 x flexibuffergrey = 10ul
1mM dNTP = 1ul
25mM MgCl2 = 1ul
10mM forward primer = 1ul
10mM reverse primer = 1ul
DNA sample = 1ul
GoTaq polymerase = 0.5ul.

Nanodrop SVCT2	ng/lul	OD260	260/280.
original	28.2	0.565	1.85
5:1	6.0	0.120	1.98.
6:1	5.6	0.112	1.67
7:1	3.8	0.075	2.12 ✓ (used).

Performed by	Date	Countersigned by	Date	Continued on page number
C.			7/7/17	

10/07/17 Gel electrophoresis for primary PCR after new nanodrop readings.

Ladder
V+C1P190SF 7
V+C1P190SR 7
V+C1V62GF 7
V+C1V62GR 7
V+C1I96LF SVCT1 17
V+C1I96LR 14
V+C1L277FF 15
V+C1L277FR 15
V+C1N385SF 12
V+C1N385SR 13

Ladder
V+C2A174VF 17
V+C2A174VR
V+C2K184RF 10
V+C2K184RR 12
V+C2Q353GF SVCT2 13
V+C2Q353GR 16
V+C2D133GF
V+C2D133GR 16
V+C2I381LF 13
V+C2I381LR 18

Performed by	Date	Countersigned by	Date	Continued on page number
			10/7/17	

10/07/17

To make TioE1 for 1000µl (1ml) = 2µl EDTA
10µl TRIS
988µl distilled (autoclaved) water.

Need to rehydrate the beads with TioE1.

CL6B needs to be swirlled until the residue at the bottom is fully mixed.
CL-6B 200 - Sigma - Product code: 10019748 17.

Add 15-20ml of CL-6B into a new falcon tube and use the refrigerated centrifuge (DENLEY BR401) and spin at 3000rpm for 10mins (timed). Make sure the centrifuge is balanced. (KB)

- You should be able to see the supernant after it has been spinned. Remove the supernat and add an equal amount of TioE1.
Then spin again. - repeat whole process 3 times.

Supernatant = clear liquid at the top. Make sure you leave half a ml above the bottom.

use glass beads to block the whole 100µl pipette. (cut tip first).
make uncle in eppendorf using the syringe.
Pipette approx 750µl of the CL-6B mixture which has been spun. make sure to pipette from the same depth each time. Fill eppendorf to the top but do not overflow. - Do not disturb the sand at the bottom.

Spin the eppendorfs in the normal centrifuge at 9000 rpm for 10 mins.
Spin until no aqueous solution at the top and discard.
Add 45µl of DNA sample to the top and replace bottom eppendorf with a clean spin column.
Should have approx 40µl of pure DNA at the bottom (no salts and primers etc).
- if not then spin again.

Performed by C. Donovan	Date	Countersigned by	Date 10/7/17	Continued on page number

12/07/17

DPN1 treatment for primary pcr products.

Dilution of 10x cutsmart buffer to 1x cutsmart buffer by adding 10ul of cutsmart to 90ul distilled water.

Add 5.5ul of the cutbuffer and 1ul of dpn1 to each sample.
Add samples to a heated mixing block for 20mins at no speed. (80°C)

Spin column purification of samples using sepharose beads as shown on page 17.

Gel electrophoresis:

Ladder
V+C1P190SF
V+C1P190SR
V+C1V62GF
V+C1V62GR
V+C1I96LF
V+C1I96LR
V+C1L277F
V+C1L277R
V+C1N385SF
V+C1N385SR

SVCT1

Ladder
V+C2A174VF
V+C2A174VR
V+C2K184RF
V+C2K184RR
V+C2Q353GF
V+C2Q353GR
V+C2D133GF
V+C2D133GR
V+C2I381LF
V+C2I381LR

SVCT2

Performed by	Date	Countersigned by	Date	Continued on page number
[signature]		[signature]	12/7/17	

18/07/2017

Second time a of purification on the same samples because small trace in gel from 12/7 when dna was added repeated centrifuge for 10mins at 9,000rpm (x3). Protocol for purification is detailed on page 17.

Nanodrop + dilutions.

Name	conc (ng/ul)	OD A260 (0.2-0.8)	260/280 (1.8-2)
SVCT1			
• 190F	5.3	0.106	1.71
190R	5.1	0.102	1.53
62F	10.7	0.214	~~one~~ 1.72
62R	6.2	0.124	1.69
• 96F	6.1	0.121	1.82
96R	8.8	0.175	1.61
277F	4.7	0.093	1.65
277R	6.3	0.125	1.94
385F	8.7	0.175	~~on~~ 1.52
385R	5.3	0.106	1.73
• 174F	4.4	0.088	1.76
174R	4.7	0.095	1.72
• 184F	4.5	0.091	1.89
• 184R	5.6	0.112	1.72
SVCT2			
• 353F	4.0	~~0.078~~ 0.081	1.66
353R	5.0	0.099	1.93
• 133F x	5.5	0.109	1.74
• 133R			
• 381F	7.4	0.147	1.58
• 381R	4.5	0.090	2.38

• had no band on gels.

Performed by	Date	Countersigned by	Date	Continued on page number
[signature]		[signature]	18/7/17	

03/08/17. Fusion of 1st and 2nd glass bead purified primary perprodu
Fusion: (+dpN1).

H2O. 33.5 µl
Flexi buffer = 10.0 µl
dntp (1mM) = 1 µl
MgCl2 = 1µl
pcr forward flanking = 1µl (pCDNA3F)
pcr reverse flanking = 1µl (pCDNA3R)
DNA template = 2µl (1µl F) + (1µl R).
Gotaq = 0.5 µl

190 with dash
62 with dash
96 with dash
277 with dash
385 with dash
174 with dash
184 with dash
353 with dash
381 with dash
} Purified with glass beads x1 (not diluted as normal).

190 without dash but '2'
62 without dash but '2'
96 without dash but '2'
277 without dash but '2'
385 without dash but '2'
174 without dash but '2'
184 without dash but '2'
353 without dash but '2'
381 without dash but '2'.
} purified with glass beads x2. (not diluted as normal).

Performed by	Date	Countersigned by	Date	Continued on page number
C. Dan			3/8/17	

Continued from page number	Page number 21

03108/17.

cts To make new gel electrophoresis:

Agarose: 1.12g
TAE : 70ml
6µl sybersafe.

Load 10µl ladder
8µl dna
2µl dye.

loading ladder.
190
62
96
277
space
385
174
space
184
space
353
381

} 1st purified with beads (x1).

Performed by	Date	Countersigned by	Date	Continued on page number
C. Dan			3/8/17	

23/08/17. Repeat Fusion with beaded primary pcr products.

H_2O = 29.5µl
Flexi Buffer = 10µl
dntp = 2µl
$MgCl_2$ = 2µl
pcr forward flanking = 1µl
pcr reverse flanking = 1µl
dna template = 2µl (f) + 2µl (r)
Gotaq = 0.5µl.

More DNA added and more dntp / $MgCl_2$.

New fusion "cc amplification setting":

1) Heat lid to 110°C
2) Temp 95°C for 5mins
3) Cycle (x28):
   94.0°C for 1 min
   65.4°C for 30~~mins~~
   72.0°C for 2.30mins.
4) Close cycle
5) 72°C for 4mins
6) Store at 8.0°C.

(72.0°C for 4mins changed to 2.30mins)
(60.0°C for 30secs changed to 65.4°C).

Performed by	Date	Countersigned by	Date	Continued on page number
~~C. Dorman~~			23/8/17	

Ladder
VtC1P190S
VtC1V62G *
VtC1I96L *
VtC1L277 *
VtC1N385S
VtC2A174V *
VtC2K184R *
VtC2Q353G
VtC2D133G *
VtC2I381L

30/08/17 Gel Extraction.

Gel Loaded:

Ladder.
VtC1V62G
VtC1V62G.
VtC1I96L
VtC1I96L
VtC1L277
VtC1L277
VtC2A174V
VtC2A174V
VtC2K184R
VtC2K184R
VtC2D133G.
VtC2D133G.

Made larger gel so used 1.28 grams agarose and 80ml TAE (1X)
Gel set for 1 hr.

Performed by	Date	Countersigned by	Date	Continued on page number
C Domean			30/8/17	

30/08/17 Gel extraction continued

When loading the gel used 10ul ladder, 8ul dna and 2ul Gotaq greendye
Gel ran at 70V for 1hour to allow a good seperation.
Fusion bands on all however 184R (x2) were very faint.
Gel extraction using the scalpal and the light box to cut each individual band out. Prior to this the eppendorfs were weighed.

1) -62	0.993	1.232	0.239	0.717
2) -96	0.988	1.158	0.170	0.510
3) -277	0.985	1.132	0.147	0.441
4) -174	0.984	1.255	0.271	0.813
5) -184	0.995	1.174	0.179	0.537
6) -133	0.995	1.167	0.172	0.516.
	(eppendorf only)	(gel + eppendorf)	(gel only)	(QG added)

1) QG is added at 3 volumes to 1 to the gel. (ul in table above).
2) Incubate at 50°C for 10minutes (vortex inbetween every 3mins). This is to dissolve the gel and make sure the colour is yellow.
3) Add 1 gel volume (as shown in table above) of isopropanol to the sample and mix.
4) Place in a QIA quick spin column (the sample) and centrifuge for 1min at 13,000rpm and discard the flow through.
5) 500ul buffer QG to the column and centrifuge for 1min at 13,000rpm. Discard flow through.
6) To wash, add 750ul buffer PE and centrifuge for 1min. Leave the column to stand for 5mins and centrifuge for 5mins.
7) To elute dna add 50ul Buffer EB and centrifuge for 1min.
8) Store in freezer.

18/09/17 Restriction Digest

37ul H_2O
5ul 10x NEB Buffer 2
1ul 10x BSA
5ul DNA Sample (fusion products which have been gel extracted)
1ul Hind III
1ul XBaI

For plasmid:

37ul H_2O
5ul 10x NEB Buffer 2
1ul 10x BSA
5ul plasmid (phSVCT1 and phSVCT2)
1ul Hind III
1ul XBaI

Incubate at 37°C for 4hours.

Gel to test if plasmid digest worked:
Large tray so: 128grams agarose
80ul TAE (1x)
Left to set for 40mins.
When loading the gel 10ul ladder, 8ul dna and 2ul gotaq.
Gel ran at 70v for 40mins. (12:55pm)

Ladder
SVCT1
SVCT1 NO Bands.
SVCT1 DID NOT WORK!
GOP
SVCT2
~~SVCT2~~
SVCT2
SVCT2.

Performed by	Date	Countersigned by	Date	Continued on page number
C. Dom		18/9/17		

	Eppendorf Only	Gel + Eppendorf	Gel Only	QG Added
SVCT1	0.995.			
SVCT2(1)	0.984			
SVCT2(2)	0.994			

↑ did not work when running gel

Taken from the start from the ~~deep~~ last years!! X (not correct).

Prepared plates for transformation (hopefully on thursday).
Constituents used are shown on page(1).

2 x 1 litre ~~non amp~~ agar
1 x 500ml LB agar.

5 x No amp agar plates made.
27 x AMP agar plates made.

Performed by	Date	Countersigned by	Date	Continued on page number
[signature]		[signature]	18/9/17	

19/09/17 Restriction Digest:

① Plasmid digest using SVCT1 and SVCT2 diluted samples from 19/04/17 (page 5). SVCT1 number (1) used at 3:1 ratio. SVCT2 number (2) used at a ratio of 5:1.

H_2O	37ul
NEB Buffer 2 (10X)	5ul
10 x BSA	1 ul
Plasmid (SVCT1 or SVCT2)	5ul
HindIII	1ul
XBaI	1ul.

Placed into incubator at 9.20am for 4hrs at 37°C.

② Electrophoresis gel to check dna digestions from 18/9/17. Gel made with the large tray using 1.28g agarose at 80ul TAE. Left to set for 40mins.

New 1X TAE was made using 50X TAE (20ml) and 980ml H_2O. Total = 1 litre 1X TAE.

Ladder

SVCT1:
- V+C1V62G
- V+C1V62G
- V+C1I96L
- V+C1I96L
- V+C1L277F
- V+C1L277F

→ NO Bands on gel! DID NOT WORK!

SVCT2:
- V+C2A174V
- V+C2A174V
- V+C2K184R
- V+C2K184R
- V+C2D133G
- V+C2D133G

Performed by	Date	Countersigned by	Date	Continued on page number
C. Doma			19/9/17	

Digest repeated for dna samples:

32ul	H_2O
5ul	10x NEB Buffer 2
1ul	10x ~~Buffer~~ BSA
10ul	DNA Sample.
1ul	Hind III
1ul	XBaI.

Incubate at 12.10pm until 4.10 at 37°C.

① Plasmid digest worked for SVCT1 and SVCT2.

Ladder
SVCT1
SVCT1
SVCT1
SVCT2
SVCT2
SVCT2.

When running the gel the larger ~~ladder~~ tray was used.
Gel electrophoresis showed two bands for each SVCT1 + SVCT2

When completing gel extraction only the TOP band was extracted not the bottom + then gel extraction kit was used.

	Eppendorf only(g)	Eppendorf + gel (g)	Gel Only(g)	Buffer QG Added (ul)
SVCT1 (1)	0.996	1.144	0.148	0.444
SVCT1 (2)	0.987	1.075	0.088	0.264
SVCT2(1)	0.995	1.204	0.209	0.627
SVCT2(2)	1.001	1.073	0.072	0.216

Performed by [signature] Date

Countersigned by [signature] Date 19/9/17

Continued on page number

20/09/19

Gel electrophoresis of dna sample restriction digests from 19/09/17.

Ladder.

SVCT1
- V+C1V62G
- V+C1V62G
- V+C1I96L
- V+C1I96L
- V+C1L277F
- V+C1L277F

NO Bands ON GEL!

DID NOT WORK.

SVCT2.
- V+C2A174V
- V+C2A174V
- V+C2K184R
- V+C2K184R
- V+C2D133G
- V+C2D133G.

Nanodrop to test dna rdigest samples which did not work

Name	conc (ng/ul)	OD A260 (0.2-0.8)	260/280 (1.8-2)
V+C1V62G	21.6	0.433	0.63
V+C1I96L	26.3	0.525	0.68
V+C1IL277F	10.0	0.200	0.65
V+C2A174V	6.5	0.130	0.53
V+C2K184R	27.2	0.543	0.62
V+C2D133G	18.8	0.542	0.11

Performed by [signature] Date | Countersigned by [signature] 20/9/17 Date | Continued on page number

Nanodrop 260/280 Ratio of samples which did not work are far too low. This means there is not enough DNA in the samples!. Reasons could be:

- enzymes (xBaI and HindIII) are protein, and therefore purity is too low as they reduce it.
- DNA dilutions were not correct.
- not enough DNA loaded in the gel so when extracting a lot is lost.

Restriction digest did not work therefore alternative route has been chosen.

① Primary PCR but without flanking primes!
Change 72°C to 10mins.
dpn1 treatment.

② Straight to ligation.
If a clone is successful then it is close to guaranteeing it is the correct one.

Performed by	Date	Countersigned by	Date	Continued on page number
[signature]		[signature]	20/9/17	

27/09/17. Primary PCR changed.

Primary PCR using diluted primers. No flanking primers used (pCDNA3R + pCDNA3F). Into one pcr tube you put both the forward and revese mutated primes together.

H_2O = 33.5ul.
5x flexi buffer (grey) = 10ul
1mM dntp = 1ul
25mM $MgCl_2$ = 2ul
Forward mutated primer = 1ul
Revese mutated primer. = 1ul
DNA sample (SVCT1 or SVCT2) = 1ul
GOTAQ polymerase = 0.5ul

} all reagents remained on ice.

eg. VtC1I96L(F) and VtC1I96R(R) into the same pcr tube.
In total there are 10 pcr tubes from the 20 primes.

PCR cycle:
1) Heat Lid to 110°C
2) Temp 95.0°C for 5mins
3) 28x cycles: temp 94°C for 1min
60°C for 30 seconds
72°C for 10 mins ← changed from 2mins 30secs.

Total running time = 5hrs 40mins.

No gel following PCR only dpn1 treatment to cut the methyjated DNA.
NO FUSION
NO digest
NO Ligation.
Straight to transformation.

Performed by	Date	Countersigned by	Date	Continued on page number
[signature]		[signature]	27/9/17	

Dpn1 treatment :
10x cutsmart buffer diluted to 1x cutsmart buffer by adding 90ul H_2O and 10ul buffer.

5.5ul dpn1 cutsmart buffer to each per sample.
1ul dpn1.

Straight away place in a heating block at 80°C for 20mins to heat shock.

28/09/17. Transformation prep.

MC1061 which was grown on plates (colony chosen) and innoculated into LB no amp media.
- 15ml of media measured and one colony chosen using the d-loop and left in the orbital shaker overnight (250rpm) at 37°C.
Put in at 7:15pm and taken out at 10:10am. (sautica should be cloudy)

29/09/17 Transformation.

1) using the innoculated MC1061 measure 250ul and pipette into 25ml of LB media. Place in the orbital shaker for 2hrs at 37°C and 250rpm.

2) Check the MC1061 mixture is at log phase (A600 = 0.2 → 0.3) by adding 1ml into a cuvette and measuring in a spectrophotometer. (measures the absorbance).

3) Chill culture on ice and harvest 1ml of cells by quick centrifuging at 8000rpm for 2mins at 4°C.

4) Discard the supernatant then resuspend cells in 500ul of ice-cold calcium solution (sterile) - (50mM $CaCl_2$, 10mM Tris HCl, pH 8.0).

5) Place the suspension in a ice bath for 15mins then centrifuge at 10,000 rpm for 1 minute at room temperature.

6) Discard the supernatant and resuspend the cells in 1/15 (66ul) of ice-cold (sterile) calcium solution.

7) Transformation

7) 10ul of dna sample add to 200ul competent cells on ice (MC1061)
8) Incubate on ice for 20-30minutes.
9) Heat shock the cells at 42°C for 2mins in a waterbath and immediatley return the tubes into an ice bath to chill for 1-2 mins.
10) Add 330ul LB media and incubate the cells at 37°C with vigorous shaking for 60mins.

11) Plate 200ul of incubated cells onto "pre-dried" amp LB agar plates using the glass beads.

12) Grow the cells on the plate at 37°C overnight.

Performed by	Date	Countersigned by	Date	Continued on page number
			29/09/17	

Transformation results from 29/08/17 were not completed.
First step of innoculation meant that the A600 was 0.5837 and 0.6936 (too high).
Potentially grew too much overnight although was left for 14hrs (approx).

02/10/17 Making LB agar + plates.

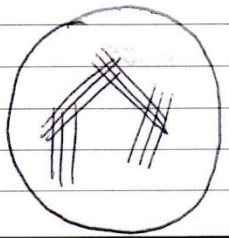
No trytone in lab so LB agar miller was used which contain):

Tryptone 10g
Yeast Extract 5g
Sodium Chloride 10g
Agar 15g.

40 grams 'LB agar miller' was added to 1 litre flask and filled with water. The soution was mixed on the mixing block (magnetic) and then autoclaved under "media setting".

Once the media had cooled 1000ul of ampicillin was added to the media and lightly shaken to mix. Approx 20 plates were made and left to cool and then stored in the fridge.

Alongside this a 500ml flask of media (LB agar) was made and autoclaved + cooled. No amp was added and 2x plates were made.
The deep MC1061 (MJB33) strain was taken from the deepfreezer and streaked using a d-loop. The d-loop was sterilised with a flame.



Performed by	Date	Countersigned by	Date	Continued on page numbe
[redacted]		[redacted]	2/10/17	

The first 3 streaks were ~~dipped into the deep~~ made by dipping the d-loop into the deep and streaking. Then the d-loop was sterilised and the next 3 streaks were made (without putting it back into the deep strain). Repeated until all (x4) streaks were made.

The two plates were then placed into the incubator at 37°C overnight (approx 16 hrs) to allow enough colonies to grow.

03/10/17

A certain colony was chosen and put into 15ml of LB media to be inoculated overnight. The colony was picked up using a pipette and put directly into the media. The inoculation was left for 16hrs at 37°C at 250rpm.

04/10/17

Transformation + preparation of competent cells.
Protocol was followed as shown on page 33.

* A total of 4 eppendorfs were filled to 1 litre with combined competent cells (approx 55 made for 15 plates) ~~for~~.

A600 (log phase readings)
1) 0.2638 Abs
0.2683 Abs
0.2513 Abs
0.2552 Abs.

} wavelength = 600nm.
LB used as blanc.
absorbance set to 0 at start.

Colonies were left to grow in the incubator at 37°C for 16hours.

Performed by	Date	Countersigned by	Date	Continued on page number
[signature]		[signature]	3/10/17	

05/10/17.

Colonies grew overnight however a maximum of 10 per plate.
"174" had no growth at all.
Images of plates were taken using the UV light box.

At 4pm a maximum of 2 colonies per plate were picked up using the sterile α-loop and placed into 15ml LB media.

The media was shaken at 250rpm at 37°C for 16hrs (overnight) until the media went cloudy.

The plates can be stored in the fridge for a maximum of 2 weeks.

06/10/17.

media (inoculated) was taken out of the orbital shaker after 16hrs.
The media had turned cloudy.
Samples undergo miniprep as stated on page (3).

Samples

96 - 1 (1)(2)(3)	96-2 (1)(2)(3).	
62 - 2 (1)(2)(3)		→ only one colony picked
277 - 1 (1)(2)(3)	277 - 2(1)(2)(3)	
190 - 1 (1)(3)	190 - 2(1)(2)(3)	
385 - 1 (1)(2)	385 - 2 (1)(2)(3).	
381 - 1 (1)(2)	381 - 2 (1)(2)(3)	
133 - 1 (1)(2)	133 - 2 (1)(2)	
184 - 1 (1)(2)(3)		→ only one colony picked.
353 - 1 (1)(2)(3)	353 - 2 (1)(2)(3)	

Samples were stored in eppendorfs in the freezer.

Performed by	Date	Countersigned by	Date	Continued on page number
[signature]		[signature]	6/10/17	

07/10/17 Restriction digest after miniprep.

H_2O	– 32ul
10x NEB buffer 2	– 5ul
10x BSA	– 1ul
DNA sample.	– 10ul
Hind III	– 1ul
XBaI	– 1ul.

Left for 4hrs at 37°C (no shaking). XbaI and HindIII added at the same time.

Samples used

96-1 (1) and 96-2(1).
62-2 (1) only
277-1 (1) and 277-2 (1)
190-1 (1) and 190-2 (1)
385-1 (1) and 385-2 (1)
381-1 (1) and 381-2 (1)
133-1 (1) and 133-2(1)
184-1 (1) only
353-1(1) and 353-2(1).

Ladder 1 (larger gel so 1.28gram used and 80ml TAE).
- 8ul DNA to 2ul gotaq.

Ladder 2 (smaller gel) 0.64g agarose and 40ml TAE.
- 4ul DNA to 1ul gotaq.

Both gels run for approx 1hr at 70V.

Performed by	Date	Countersigned by	Date	Continued on page number
[redacted]		[redacted]	7/10/17	

Continued from page number | Page number 38

1) Ladder

96-1 (1) uncut
96-1 (1) cut
96-2 (1) uncut
96-2 (1) cut ← *
277-1 (1) uncut
277-1 (1) cut
277-2 (1) uncut
277-2 (1) cut
190-1 (1) uncut
190-1 (1) cut
190-2 (1) uncut
190-2 (1) cut.
385-1 (1) uncut
385-1 (1) cut
385-2 (1) uncut
385-2 (1) cut
* 62-2 (1) uncut
62-2 (1) cut

} SVCT1

2) Ladder

381-1 (1) uncut
381-1 (1) cut
381-2 (1) uncut
381-2 (1) cut
133-1 (1) uncut
133-1 (1) cut
133-2 (1) uncut
133-2 (1) cut
184-1 (1) uncut
184-1 (1) cut
353-1 (1) uncut
353-1 (1) cut.
353-2 (1) uncut
353-2 (1) cut.

} SVCT2.

Performed by	Date	Countersigned by	Date	Continued on page numbe
[signature]		[signature]	7/10/17	

23/10/17 New primers (dilution and pcr).

mVC1P190SF	391ul TioE1 added	(58.2)
mVC1P190SR	120ul TioE1 added	(58.2)
mVC1L277EF	278ul TioE1 added	(61.0)
mVC1L277ER	379ul TioE1 added	(61.0)
mVC1N385SF	371ul TioE1 added	(61.0)
mVC1N385SR	254ul TioE1 added	(61.0)
mVC1K184EF	222ul TioE1 added	(62.7)
mVC2K184ER	263ul TioE1 added	(62.7)
mVC2Q353GF	155 ul TioE1 added	(62.8)
mVC2Q353GR	336 ul TioE1 added	(62.8)
mVC2D133GF	192 ul TioE1 added	(62.8)
mVC2D133GR	429 ul TioE1 added.	(62.8)

Average (61.4)°C.

Each primer diluted further by adding 9ul distilled H_2O to 1ul primer sample (into new eppendorf).
eg. 36ul water and 4ul primer.

Primary PCR with no flanking primers (pcdna3F + pcDNA3R).

H_2O = 33.5ul
Gotaq flexi buffer = 10ul
dntp = 1ul
25mm $MgCl_2$ = 2ul
Forward mutated primer = 1ul
Reverse mutated primer = 1ul
DNA sample (SVCT1 or SVCT2) = 1ul
Gotaq polymerase = 0.5ul

} all remained on ice.

eg. mVC1P190SF and mVC1P190SR added into the same eppendorf.
6 pcr tubes from the 12 primers.

Performed by	Date	Countersigned by	Date	Continued on page number
[signature]		[signature]	23/10/17	

Primary PCR setting:

1) Heat lid to 110.0°C
2) Temp 95.0°C for 5mins
3) 30x cycles : temp 94°C for 1 min
61°C for 30 seconds
72°C for 10minutes

Total running time = 6hrs and 5mins.

To make LB media (no amp) to grow MC1061 (MJB33).

Bacto tryptone = 2.5g
Bacto yeast extract = 1.25g
NaCl = 2.5g
Agar = 3.75g.

24/10/17.

Repeated primary pcr, however change the programme setting to 28 cycles rather than 30.

dpn1 treatment on the pcr products.
10x cut buffer was diluted to 1x (9 H_2O : 1 cutbuffer).
Heat shock for 20 mins at 80°C.

Performed by	Date	Countersigned by	Date	Continued on page number
[signature]		[signature]	24/10/17	

Continued from page number	Page number 41

30/10/17.

Growing MC1061 (JM109B33) overnight again as previous was not a successful growth. (not enough individual colonies).

New plates made using 'LB agar miller' - molecular genetics powder - BP 1425-500.

for 250ml 10grams of powder was used.
The beaker was filled to 250ml with distilled water and mixed using the mixing plate. (+ magnets).

Plates were incubated overnight at 37°C for 16 hours.

31/10/17.

Growth of MC1061 successful however after talking to uj and background research it will be better to use JM109 or DH5α as the competent cells.

JM109 grown or prepared no amp plates. (Streaked the same as on page 1).
16 hours at 37° C.

- Growth of JM109 was not successful (v. little colonies on both plates). unsure whether the plates had not dried enough - too much water?

Re-grow JM109 tomorrow.

Performed by	Date	Countersigned by	Date	Continued on page number
C. D			30/10/17	

01/11/17.

Prep prepared no amp plates were dried in the incubator for approx 30 minutes before streaking. (ensure no excess water).

JM109 streaked and grown overnight at 37°C for 16 hours.

- Again, little growth of JM109 - will try with small colony to innoculate and check whether they reach the log phase.

06/11/17.

1)- Streaking DH5α (x2 plates)

2)- Innoculating JM109 overnight in the orbital shaker. ×

1) 16 hrs at 37°C (incubater)

2) ~~Or~~ 250rpm at 37°C placed into conical flasks for 16hrs. ×

Streaked MC1061
Streaked DH5α (SVCT1)
Streaked DH5α (SVCT2).

DH5α + MC1061 did not grow enough on the plates.

Performed by	Date	Countersigned by	Date	Continued on page number
			6/11/17	

Continued from page number | Page number 43

03/11/17 Transformation.

Innoculation of JM109 overnight until the solution went cloudy (approx 16hrs).
3 colonies were chosen from 2x streaked plates of JM109.
After 16hrs 250ul of innoculated cells were pipetted into 25ml lb media and incubated at 37°C for a further 2 hours.

Taken out after 1hr 45mins absorbance results:

0.5342
0.5187
0.6044
0.4845
0.5281

} all too high (0.2-0.3 expected).

Using the same streaked plates, try again but incubate for less time.
14-15hrs instead.

09/11/17

Innocuation of JM109 overnight was completed for 15 hours.
A total of 6 conical flasks each had a colony placed into it.

When re-innoculating only left for 1hour and 30mins to check if the log phase is reached, results: (should be between 0.2-0.3).
Absorbance too low:

0.1486
0.1841
0.1518
0.1623
0.1697
0.31354

} all too low.

LB = blanc. (no amp)

Performed by	Date	Countersigned by	Date	Continued on page number
C. Dan			8/11/17	

09/11/17

The innoculation was left for a further 20 minutes to reach the log phase.
Results:

1) 0.4060
2) 0.3419
3) 0.3610
} too high

4) 0.2602
5) 0.2911
6) 0.2830
} log phase (0.2-0.3)

1, 2, 3 were too high so were discarded.

4) 5) 6) were at a good asorbance so were used.

For a total of 40 eppendorfs only (4) and (6) were needed.
(5) was discarded.

The protocol followed is stated on page(33) - "Transformation prep" and "Transformation":

(Calcium sterile solution had been pre-made. : 50mM $CaCl_2$)
10mM Tris-HCl
pH 8.0.

After re-suspending the cells on 66ul of calcium solution on ice- The samples were combined into eppendorfs to 1-1.5ml. A total of 3 eppendorfs were filled. (enough for 15 plates).
The competent cells then remained on ice for 2hours (as storage) prior to transformation.

Performed by	Date	Countersigned by	Date	Continued on page number
[signature]		[signature]	9/11/17	

To make LB media (as previous one was + 2 weeks old):

Bacto tryptone - 10g
Bacto yeast extract - 5g
Bacto NaCl - 10g
} 1 litre

- half for 500ml flask.

10/11/17 Transformation results

Control SVCT1 = successful growth (many colonies)
SVCT2 = successful growth (many colonies)

m190 = no growth
m277 = no growth
m385 = 2-4 colonies (fairly large)
m184 = 2-3 colonies (normal size)
m353 = 1 colony only
m133 = no growth.

13/11/17.

2x colonies picked from 385
2x colonies picked from 184
1x colony picked from 353.

Each colony was placed into a conical flask (autoclaved) containing 25ml of LB media.

They were incubated at 37°C at 250rpm overnight for 16hrs. (until cloudy).

14/11/2017

Miniprep of overnight incubated colonies in LB media.
Protocol is stated on page ③.

m385 (1) 1
m385 (1) 2
m ~~18~~385 (2) 1
m 385 (2) 2
m184 (1) 1
m184 (1) 2
m 184 (2) 1
m184 (2) 2
m353 (1) 1
m353 (1) 2.

2 x miniprep per colony (2ml taken from 25ml LB media per colony incase any problems during miniprep stages).

Once miniprep completed restriction digest:

H_2O - 37ul
10X NEB Buffer 2 - 5ul
10x BSA - 1ul
DNA sample (from miniprep) - 5ul
HindIII - 1ul
XBaI - 1ul.

Both restriction enzymes added at the same time. Samples left to incubate for 4 hours at 37°C.

Performed by	Date	Countersigned by	Date	Continued on page number
C ~~Dawson~~		[signature]	14/11/17	