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# Expression and Purification of Recombinant VMO1 Protein

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# Abstract

Hearing loss is a common sensory deficit that affects more than 275 million people worldwide. Identifying and understanding the genetic causes which underlie hearing loss can lead to improved diagnostic and treatment strategies. The biological function of Vitelline Membrane Outer Layer 1 homolog (chicken) (VMO1) is of particular interest as the mRNA transcript was previously found to be uniquely expressed and highly abundant in the mouse inner ear.

The aim of this research was to design, clone, express and purify a recombinant VMO1 protein containing a His Tag using the *VMO1* gene amplified from human cell lines. The *VMO1* gene was cloned into the pET28b(+) expression system and bacterial cells were manipulated to express the recombinant VMO1 protein through IPTG induction. The resulting recombinant protein was then purified using a nickel affinity gel. Finally, a commercial VMO1 antibody was tested and validated using the purified recombinant VMO1 protein using western blot analysis.

Bioinformatics analysis was undertaken to develop primers to amplify cDNA of two isoforms of the *VMO1* gene in human cell lines, isoform 1 and isoform 3. Both isoforms of the *VMO1* gene were found to be expressed in two commercial *in vitro* human cancer cell lines; monocytic leukaemia and breast cancer. Following amplification, the isoforms were sub cloned into T-tailed vectors, with isoform 1 being successfully ligated into the pET28b(+) expression vector. Sequencing data confirmed the amplified cloned PCR inserts were *VMO1* transcripts, and the predicted translated sequence was in-frame with the His Tag, resulting in a recombinant protein that was 20 kDa in size.

Expression of the recombinant VMO1 protein was attempted using three bacterial expression strains with successful expression of a 20 kDa protein occurring in the Rosetta™ (DE3) pLysS strain. The recombinant VMO1 protein formed inclusion bodies within bacterial cells following over expression. Western blot analysis of non purified protein samples showed that the 6X His antibody recognised a 20 kDa recombinant protein as expected. However, the commercially available VMO1 antibody detected two bands approximately 20 and 35 kDa in size.

In the future, it is recommended that optimisation of recombinant protein folding methodology, large scale protein induction and purification of the VMO1 protein be completed. In addition, it is recommended to validate the VMO1 antibody by testing *in vitro* human cancer cell lines for the expression of VMO1 protein. Once the specificity of the VMO1 antibody has been demonstrated by western blot analysis, functional assays could be performed to determine the biological function of VMO1. This may provide further insight into the function of VMO1 in the mammalian inner ear and the role it may play in hearing.

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# Abbreviations

| | |
|---|---|
| 3' | Three prime DNA end |
| °C | Degrees Celsius |
| Å | Angstrom(s) |
| β | Beta |
| β-actin | Beta actin |
| μF | Microfarad |
| μg | Microgram(s) |
| μL | Microlitre(s) |
| μM | Micromolar |
| A | Adenine nucleotide |
| AA | Amino acid(s) |
| AHL | Acquired hearing loss |
| APA | Aldosterone producing adenoma |
| APS | Ammonium persulfate |
| BAH | Bilateral adrenal hyperplasia |
| bp | Base pairs |
| BSA | Bovine serum albumen |
| $Ca^{2+}$ | Calcium ion |
| cDNA | Complementary DNA |
| CD | Circular dichroism |
| $Cl^-$ | Calcium ion |
| cm | Centimetre(s) |
| Cm-Cys | Carboxymethyl-cysteine |
| $CO_2$ | Carbon dioxide |
| CSE | Cochlear sensory epithelia |
| Cys | Cysteine |
| Da | Dalton(s) |
| dB | Decibel(s) |
| DEPC | Diethylpyrocarbonate |
| DFNA | Autosomal dominant deafness locus |
| DFNB | Autosomal recessive deafness locus |
| DFNX | X-linked deafness locus |
| $ddH_2O$ | Double distilled water |
| DNA | Deoxyribonucleic acid |
| dNTP | Deoxyribonucleotide |
| DSC | Differential scanning calorimetry |
| *E. coli* | *Escherichia coli* |
| EDTA | Ethylenediaminetetraacetic acid |
| EPA | New Zealand Environmental Protection Authority |
| *et al* | And others |
| FBS | Fetal bovine serum |
| FPKM | Fragments Per Kilobase gene model and Million reads |

| | |
|---|---|
| g | Gram(s) |
| GAPDH | Glyceraldehyde-3-phosphate dehydrogenase |
| GHL | Genetic hearing loss |
| GMO | Genetically modified organism |
| GP | Glycoprotein(s) |
| HEPES | 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid |
| HCl | Hydrochloric acid |
| HL | Hearing loss |
| HPLC-MS/MS | High performance liquid chromatography tandem mass spectrometry |
| Hz | Hertz |
| IHC | Inner hair cells |
| IPTG | Isopropyl β-D-1-thiogalactopyranoside |
| $K^+$ | Potassium ion |
| kDa | KiloDalton(s) |
| kV | Kilovolt(s) |
| L | Litre(s) |
| LB | Luria base |
| MALDI-ToF | Matrix assisted laser desorption/ionisation time of flight |
| MCS | Multiple cloning site |
| MET | Mechanoelectrical transduction |
| min | Minute(s) |
| MIR | Multiple isomorphous replacement |
| mg | Milligram(s) |
| mL | Millilitre(s) |
| mm | Millimetre(s) |
| mM | Millimolar |
| MoCR | Mouse organ of Corti |
| MoSV | Mouse stria vascularis |
| MoVB | Mouse vestibular epithelium |
| MPSS | Massively parallel signature sequencing |
| $mQH_2O$ | Milli-Q water |
| mRNA | Messenger RNA |
| MRT | Mouse Reference Transcriptome |
| MS/MS | Tandem Mass Spectrometry |
| MTT | Mitochondrial mutation deafness |
| $Na^+$ | Sodium ion |
| NCBI | National Center for Biotechnology Information |
| ng | Nanogram(s) |
| nm | Nanometre(s) |
| NMR | Nuclear magnetic resonance |
| OHC | Outer hair cells |
| OoC | Organ of Corti |
| PAGE | Polyacrylamide gel electrophoresis |

| | |
|---|---|
| PCR | Polymerase chain reaction |
| PD | Postnatal day(s) |
| PEG | Polyethylene glycol |
| PVDF | Polyvinylidene fluoride |
| RACC | Rare Codons Calculator |
| RCC | Renal cell carcinoma |
| RNA | Ribonucleic acid |
| RNA-Seq | RNA Sequencing |
| rpm | Revolutions per minute |
| RT | Room temperature |
| RT-PCR | Real time Polymerase chain reaction |
| SAcat | Stretch-activated nonselective cation |
| SDS | Sodium dodecyl sulfate |
| sec | Second(s) |
| SELDI-ToF | Surface-enhanced laser desorption/ionisation time of flight |
| Ser | Serine |
| STR | Short Tandem Repeat |
| T | Thymine |
| TAE | Tris base, acetic acid and EDTA |
| TBS | Tris buffered saline |
| TBST | Tris buffered saline with Tween 20 |
| TE | Tris-EDTA |
| TEMED | Tetramethylethylenediamine |
| ToF-MS | Time of flight - mass spectrometry |
| tRNA | Transfer RNA(s) |
| U | Enzyme units |
| USA | United States of America |
| UV | Ultraviolet |
| VM | Vitelline membrane |
| VMO1 | Vitelline membrane outer homolog 1 (chicken) |
| V | Volt(s) |
| w/v | Weight per volume |
| W | Watts |
| WCL | Whole cell lysate |
| x *g* | Relative centrifugal force |
| X-Gal | 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside |

# CHAPTER ONE
# Introduction and Literature Review

## 1.1 Introduction

Hearing loss (HL), in the form of moderate to profound deafness, is a common sensory deficit thought to affect more than 275 million people worldwide (Hilgert, Smith, & Van Camp, 2009). It can be caused by the partial or complete loss of sound perception. The aim of this thesis is to express and purify a recombinant Vitelline Membrane Outer Layer 1 homolog (chicken) (VMO1) protein that can be used for validation of commercially available antibodies and elucidation of function of the protein through biochemical assays. This protein was first discovered in the chicken egg as part of the vitelline membrane. This membrane plays an integral part in keeping the egg white and developing embryo in the yolk separate during early embryogenesis. Furthermore, the VMO1 protein has been found to be uniquely expressed in the inner ear of the mouse where it is localised to the Reissner's membrane, a two-layered membrane that prevents mixing of the endolymphatic and perilymphatic fluids of the cochlea. The role of VMO1 in the inner ear is unknown. However, it is considered a good candidate for functional investigation studies based on its unique localisation.

This literature review will discuss in depth the definition of hearing loss as it is an issue that affects a large number of people around the world and in New Zealand. In addition to this, the structure of the mammalian ear will be explained in detail for the understanding of how external sound is able to be received by the brain through the ear. Hearing loss (HL) is a complex disorder which can be caused by environmental, genetic, or a combination of both factors. Understanding of the

genetic causes behind hearing loss can provide better insight into treatment strategies. Finally, the biology of VMO1 will be discussed.

## 1.2 Hearing Loss

It is estimated that one in 500 newborns are affected by HL with that rising to 2.7 per 1000 by the age of 5 years and 3.5 per 1000 during adolescence. In New Zealand, data collected by Greville Consulting (2006) from censuses in 1991/92, 1996/97, and 2001/02 indicated that the prevalence of HL is around 400,000 people. Hearing loss also affects the aging population as a condition known as presbycusis. This term refers to all conditions leading to HL in elderly people and is characterised by reduced hearing sensitivity and speech interpretation, and slowed processing of sound. This disorder is of high prevalence causing both social and health problems with about 40% of the over 65 year old population being affected. Untreated presbycusis can lead to people feeling isolated and depressed due to the effects it has on communication with others. However, it can be treated with the use of hearing aids or cochlea implants but cannot be reversed due to the loss of sensory cells in the inner ear during the natural aging process (Gates & Mills, 2005; Huang & Tang, 2010). An unaffected person is able to hear within the range of 20 to 20,000 Hertz (Hz) (Rosen & Howell, 2011). Humans are most sensitive to frequencies that are between 2,000 and 5,000 Hz. Loudness of sound is measured in decibels (dB) with HL defined as mild (30-50 dB), moderate (50-70 dB), severe (70-90 dB), or profound (>90 dB) (Lammens, Verhaert, Devriendt, Debruyne, & Desloovere, 2013).

### 1.2.1 Classification of Hearing Loss

HL can be classified by the abnormalities present in the mammalian ear, age of onset, if it affects one or both ears, or is caused for genetic or environmental

factors. Abnormalities in the outer and middle ears are classified as conductive HL. This dysfunction prevents sound from reaching the inner ear for processing. Sensorineural HL occurs when there is an abnormality in the inner ear or the vestibulocochlear nerve. Also, mixed HL can occur when both conductive and sensorineural HL is present in the individual.

In addition, HL can be further categorised as prelingual or postlingual. If HL is present before speech development in a toddler, it is defined as prelingual. This type of HL can have profound effects on the individual's ability to develop a spoken language and affect educational progress (Bitner-Glindzicz, 2002). In comparison, postlingual HL occurs after speech development with the most common example being age-related HL in the older population (Hilgert et al., 2009). Furthermore, HL can be unilateral, or affecting one ear, or bilateral where it affects both ears. HL can be caused by a number of environmental factors including noise, physical trauma, bacterial or viral infections, ototoxic compounds, such as the antibiotic gentamicin (Turnidge, 2003), and the natural aging process (Gates & Mills, 2005).

HL can be further classified as acquired hearing loss (AHL) or genetic hearing loss (GHL). GHL is present at birth and responsible for more than 50% of prelingual deafness (Smith, Shearer, Hildebrand, & Van Camp, 2014) whereas AHL occurs after birth due to result of an illness or injury. It is estimated that at least two thirds of prelingual HL is thought to have a genetic cause with the remaining cases being caused by environmental factors. The genetic causes of HL can be further classified as syndromic or non-syndromic. Non-syndromic deafness is thought to account for 60-70% of inherited HL, with autosomal recessive inheritance being the most common cause. In addition to this, 10-15% have an

autosomal dominant cause with the remaining being X-linked, mitochondrial, or chromosomal (Bitner-Glindzicz, 2002). When HL is inherited, it is mostly monogenetically caused. Syndromic HL is where the HL is associated with other medical disorders such as Usher syndrome and Pendred syndrome (Hilgert et al., 2009; Martini, Calzolari, & Sensi, 2009; Petit, Levilliers, & Hardelin, 2001).

### 1.2.2 Genetics of Hearing Loss

A number of deafness loci have been identified and been named DFN accordingly. They are then sequentially numbered based on order of discovery. The first locus mapped was DFNA1 in 1992 (Leon, Raventos, Lynch, Morrow, & King, 1992). GHL can be inherited as a Mendelian trait and as such, can be inherited as autosomal dominant (DFNA), autosomal recessive (DFNB), and X-linked (DFNX). GHL can also be inherited through mutations to mitochondria (MTT). Figure 1 shows 80 genes and loci that have been identified in the human genome as playing a role in HL. They are colour-coded based on the inheritance traits they show.

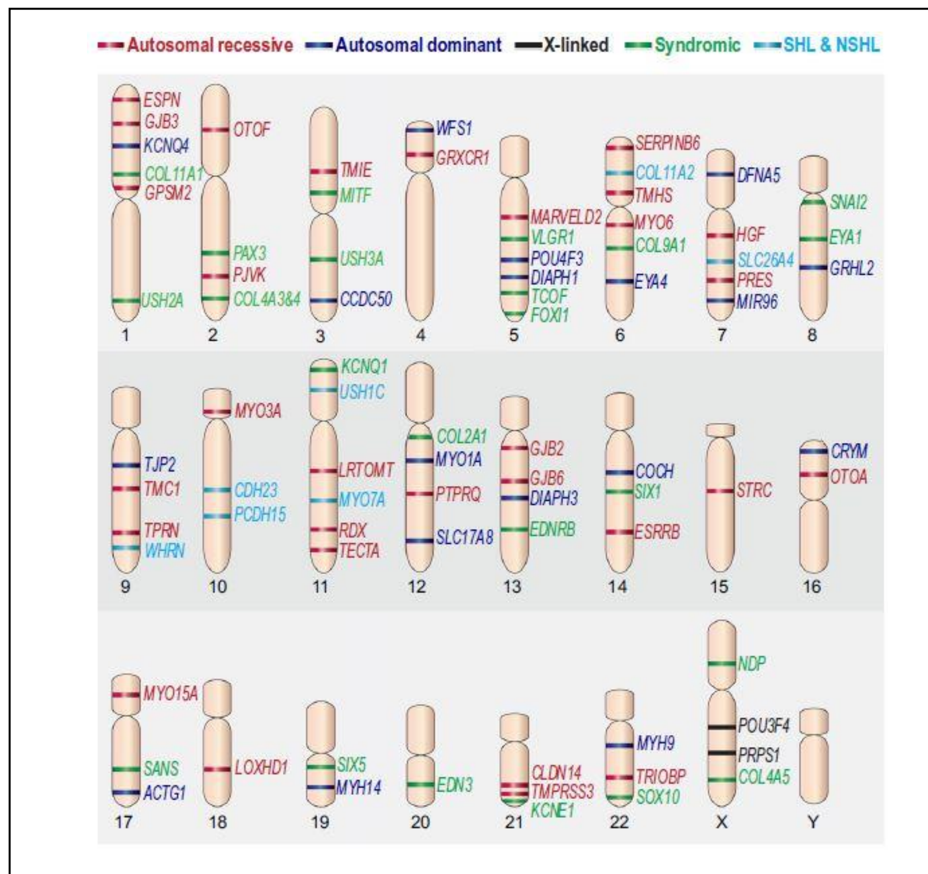


**Figure 1: Genes in the human genome that have been identified as playing a role in hearing loss.** They are grouped by colour according to the inheritance pattern. SHL and NSHL stand for syndromic hearing loss and non-syndromic hearing loss, respectively. When a gene has been implicated as the cause for both of these types of hearing loss, a light blue line is used. All other classifications represent genes that cause non-syndromic hearing loss. Autosomes are numbered 1 through to 22 and the sex chromosomes are identified as X and Y. (Adapted from Dror and Avraham (2010).

Currently, over 130 non-syndromic deafness genes have been identified (Van Camp & Smith, 2005), with more than 1000 mutations relating to deafness-causing genes being reported (Shearer, Hildebrand, Sloan, & Smith, 2011). One such DFNA gene is Pou class 4 homeobox 4 (*POU4F3*), which is a located on the DFNA15 locus positioned at chromosome 5q31-q33. Twelve members of a five-generation Libyan family were diagnosed with progressive HL that segregated as an autosomal dominant trait (Vahava et al., 1998). Onset of HL occurred between 18 – 30 years of age. Following genome-wide screening, the human ortholog of

the mouse deafness gene *Pou3f4* was found located on chromosome Xq21.1. This gene is expressed in postmitotic auditory hair cells and a targeted deletion results in a deaf mouse. The human ortholog, *POU3F4*, codes for a transcription factor that binds DNA at two sites, the POU domain and the $POU_H$ domain. The gene is made up of two exons and upon sequencing of the gene in affected members of this family; it was found that an eight base pair deletion occurred in exon 2. This led to a translational frameshift with four missense amino acids and a premature stop codon in the $POU_H$ domain of the protein thus affecting its function (Bird & Friedman, 2012; Vahava et al., 1998).

Furthermore, DFNX1 through to DFNX5 are five loci that have been mapped to the X chromosome that are linked to non-syndromic HL. Mutations to only three genes have been reported; phosphoribosyl pyrophosphatase synthetase (*PRPS1*), *POU3F4*, and small muscle protein (*SMPX*). Recessive mutations in the DFNX2 locus are the most common of those that are X-linked. However, these alleles are only responsible for a small part of non-syndromic HL. DFNX2 mutations are characterised by rapidly progressive HL and hearing is affected due to the fixation of the stapes footplate in the middle ear (Figure 2). This prevents sound from properly travelling to the inner ear due to lack of movement by the ossicles (Bird & Friedman, 2012; Choi et al., 2013).

The diagnosis of HL caused by genetic mutations has recently become available with the advancement of technology. Before now, technology was limited due to time and cost constraints. Therefore, due to advancements in technology and growing knowledge, new loci and genes responsible for HL will be identified (Shearer & Smith, 2012).

## 1.3 The Mammalian Ear

The mammalian ear, as an organ, plays an important sensory role in the body, aiding in the detection of sound as well as playing a part in balance (Dror & Avraham, 2010). In particular, the human ear is able to hear frequencies between 20 Hertz (Hz) and 20,000 Hz (Hudspeth, 1997). The structure of the ear is composed of three compartments; the outer ear, the middle ear, and the inner ear (Figure 2).

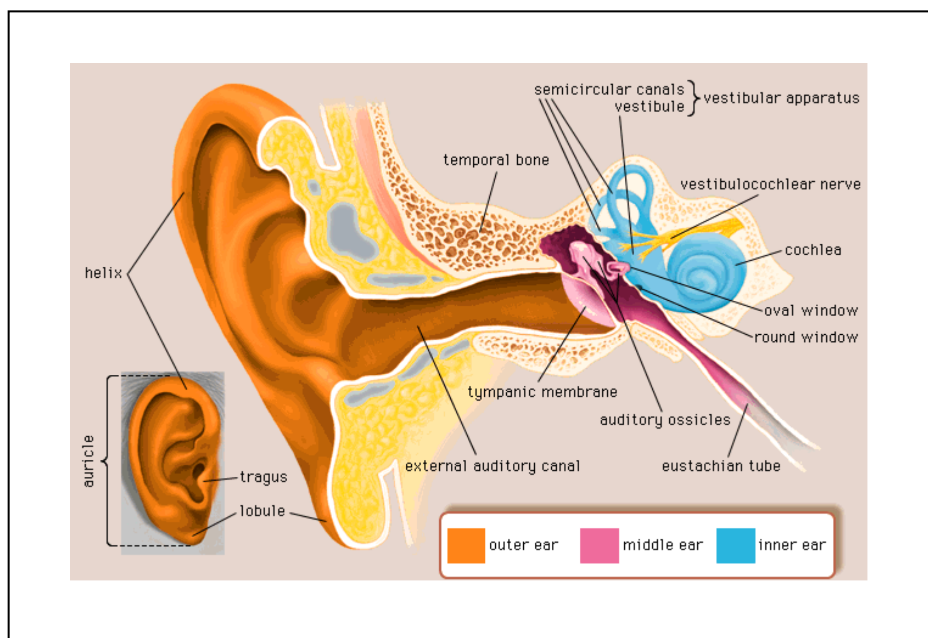


**Figure 2: Anatomy of the human ear.** The three compartments of the ear are shown; outer (orange), middle (pink) and inner (blue) (adapted from Encyclopaedia Britannica).

### 1.3.1 The Outer and Middle Ear

The outer ear consists of the auricle and the external auditory canal. The outer ear transmits sound waves from the environment, through the middle ear and into the inner ear. Sound waves are transferred to the middle ear through the tympanic membrane (ear drum). The middle ear is a hollow space consisting of the Eustachian tube which links the middle ear to the nasopharynx and equalises the air pressure of the middle air to environmental air pressure (Hidir, Ulus, Karahatay, & Satar, 2011). Of importance in the middle ear are the three

ossicles, or bones; malleus, incus, and stapes. They act together to direct sound towards the oval window of the cochlea at the start of the inner ear.

### 1.3.2 The Inner Ear

The inner ear is a fluid-filled organ which turns sound waves into electrical stimuli which can be interpreted by the brain via the cochlear nerve. It is composed of a membranous labyrinth within a cavity of the bony labyrinth (temporal bone) and divided into two systems; the auditory system and the vestibular system (Figure 4A). The auditory system consists of the snail-shaped cochlea which allows for detection of noise whilst the vestibular system contains the three semicircular ducts, the utricle and saccule, all of which aid in balance (Dror & Avraham, 2010). The base, or beginning, of the cochlea is where high-range frequencies (20,000 Hz) are transduced whereas the apex of the cochlea allows for transduction of low frequencies (200 Hz). From the base to the apex of the cochlea, frequencies are tonotopically arranged (Figure 3). As a result, high frequency sounds cause maximum vibrations to the basilar membrane at the base whereas low frequency sounds are detected by the apex of the basilar membrane with mid-range frequencies detected between the base and apex.

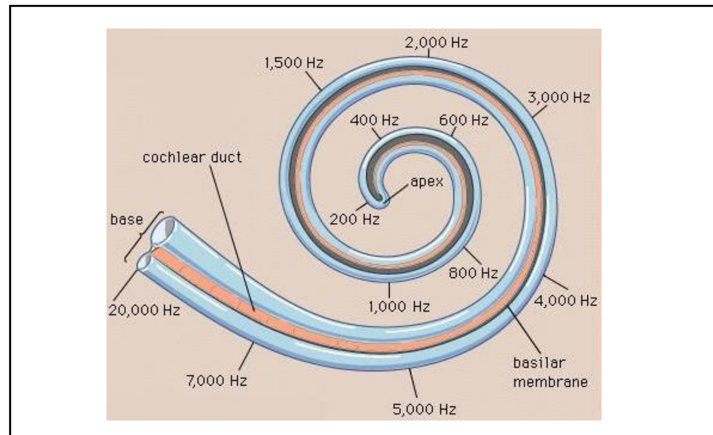


**Figure 3: Arrangement of frequencies detected along the cochlea from the base to the apex.** Frequencies are tonotopically distributed from high frequency to low frequency (adapted from Encyclopaedia Britannica).

A cross-section of the cochlea shows that it is divided into three fluid-filled sections known as scalae (Figure 4A). The scala media, or cochlear duct, contains endolymph, a fluid with a high concentration of potassium ions ($K^+$) (about 150 mM) and a very low concentration of sodium ions ($Na^+$) (1 mM). This ionic composition is similar to intracellular fluid and remains similar across mammals (Raphael & Altschuler, 2003). The scala media is surrounded by the scala vestibuli (vestibular duct) and scala tympani (tympanic duct), both of which contain perilymph. In comparison to endolymph, the perilymph has a high concentration of $Na^+$ (145 mM) and low $K^+$ (3.5 mM) (Petit et al., 2001).

### 1.3.3 Organ of Corti

Within the scala media is the organ of Corti (OoC) which is responsible for the detection of sound. The OoC is located on the basilar membrane, which separates the scala media from the scala tympani, and below the tectorial membrane (Figure 4A). The OoC contains two types of hair cells; a single row of inner hair cells (IHC) and three rows of outer hair cells (OHC). These hair cells play a large role in the translation of mechanical stimuli into an electrical signal that can be

interpreted by the brain. On the apical surface of the hair cells are filaments called stereocilia. They are densely packed into hair bundles with between 20 and 50 stereocilia present per IHC depending on the mammalian species (Raphael & Altschuler, 2003). They connect to adjacent stereocilia via apical tips and interact with neighbouring stereocilia using side-links. The stereocilia of the OHC are connected to the tectorial membrane at the Kimura's membrane, a thickening on the lower surface of it (Richardson, Lukashkin, & Russell, 2008). When sound waves enter the oval window from the middle ear, they are converted to liquid waves within the inner ear (Petit et al., 2001). The liquid waves in the inner ear causes the basilar membrane to vibrate with movements travelling longitudinally across it (Ghaffari, Aranyosi, & Freeman, 2007). This vibration is transferred to the OoC and the hair cells that line it. The movement of the hair bundles causes mechanoelectrical transduction (MET) channels on the tips of the stereocilia to open. This leads to an influx of $K^+$ and calcium ions ($Ca^{2+}$) into the stereocilia which depolarises the hair cells. This causes the release of a neurotransmitter from the hair cells and leads to an electrical signal being sent to the cochlear nerve.

### 1.3.4 Reissner's Membrane

Reissner's membrane is located between the scala media and scala vestibuli, keeping the endolymph and perilymph separate respectively (Figure 4B). The membrane is two cell layers thick with epithelial cells located on the endolymphatic side and mesenchymal cells on the perilymphatic side. These cell layers are separated by a basement membrane (Duvall & Rhodes, 1967; Yeh, Tsai, Lee, Hsu, & Tran Ba Huy, 1997). The membrane is a nonvascular structure in a number of animals including humans, however the rabbit is an exception to this (Johnsson, 1971). Reissner's membrane forms an ionic barrier, controlling the

movement of ions across it. In addition to this, three different types of ion channels have been discovered on Reissner's membrane, located on the epithelial cells (Raphael & Altschuler, 2003; Yeh et al., 1997). Firstly, stretch-activated nonselective cation (SAcat) channels were found to be permeable to $Ca^{2+}$ and are thought to play a role in changing the electrochemical composition in endolymph. Secondly, voltage-sensitive Chloride ($Cl^-$) channels were found to be abundant in the Reissner's membrane. Although it remains unknown what triggers a $Cl^-$ channel response, it has been demonstrated that they work in response to the cell influx of $Ca^{2+}$ that occurs at stretch-activated channels (Kotera & Brown, 1993), one of the other channels found on the membrane. Lastly, $K^+$-selective channels were also found on the epithelial cells of the membrane however they rarely occurred (Yeh et al., 1997). This suggests that the Reissner's membrane plays a role in regulating the ionic composition of the endolymph which is important for the transduction of sound waves into electrical signals.

The next section will discuss the biology of the VMO1 protein. This gene was discovered to be exclusively expressed in Reissner's membrane of the mouse inner ear (Peters et al., 2007).

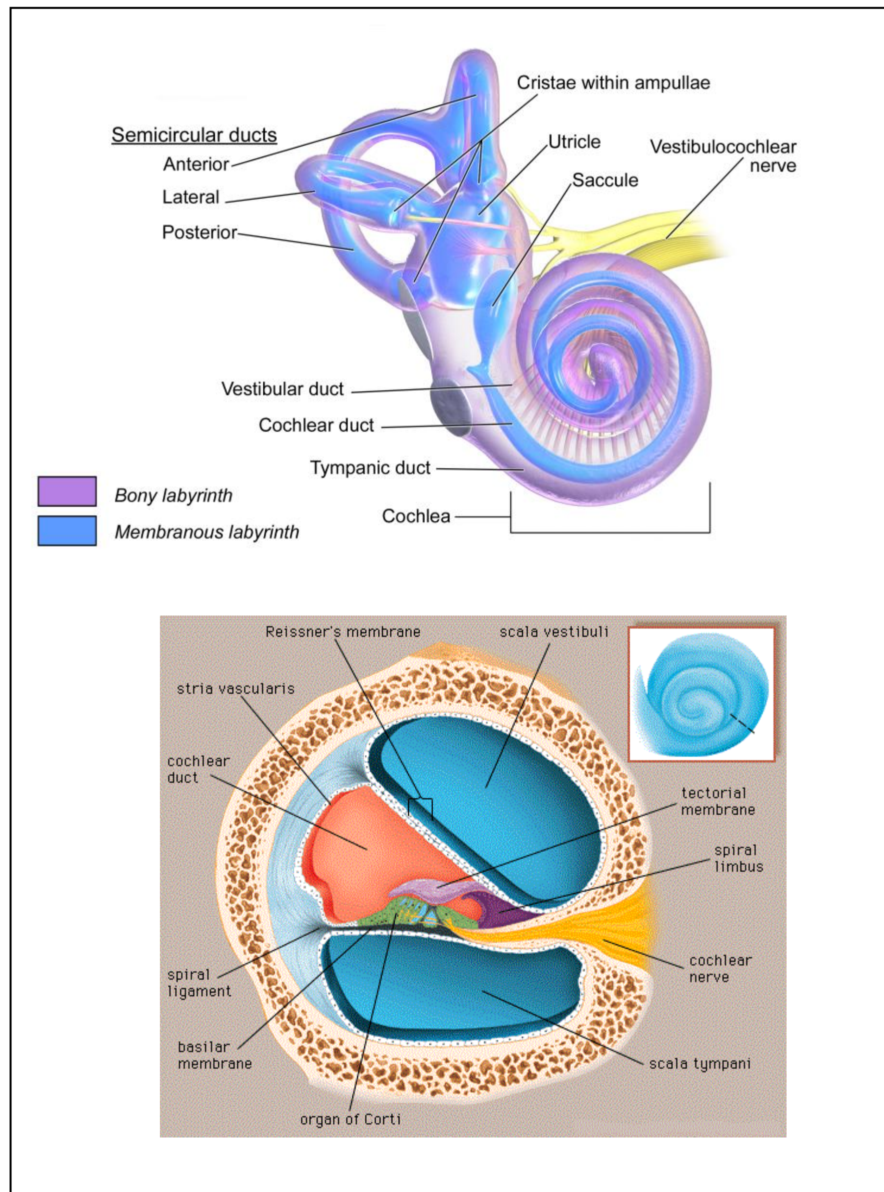


**Figure 4: Anatomy of the mammalian inner ear.** (A) The auditory and vestibular system that make up the inner ear (adapted from Wikipedia). (B) Cross-section of the cochlea in the inner ear showing the three fluid compartments; scala vestibuli, cochlear duct (orange), scala tympani, and location of the hair cells in the organ of Corti (green) (adapted from Encyclopaedia Britannica).

## 1.4 Vitelline Membrane Outer Layer 1 Protein

The human Vitelline Membrane Outer Layer 1 homolog (chicken) (*VMO1*) gene was named after its similarity to chicken VMO1, a protein that was identified in the vitelline membrane (VM) of the chicken egg.

### 1.4.1 VMO1 in the Vitelline Membrane of the Chicken Egg

The VM of a hen's egg is a membrane separating the yolk from the egg white (Figure 5). Two layers are present in the VM, with the outer layer being thicker than the inner layer. Found between these two layers is a thin sheet known as the continuous layer. The VM plays an important role during early embryonic development as it encloses the yolk and provides a base for the expansion of the embryo which eventually ruptures as the embryo expands in size (Bellairs, 1963; Jensen, 1969). The VM acts as a semi-permeable membrane which allows certain materials, such as water and egg albumen, to pass through it (Romanoff & Romanoff, 1949). The inner layer of the VM is laid down around the yolk while it is still in the ovary of the hen and the outer layer is formed around it following ovulation as it passes down the oviduct (Cook, Bellairs, Rutherford, Stafford, & Alderson, 1985). The inner layer is consisted of several kinds of glycoproteins (GP); soluble proteins GP-I, GP-II, GP-III, and the insoluble protein GP-IV. The outer layer is constructed of large macromolecules called ovomucin. To these macromolecules, the soluble proteins, lysozyme, VMO1 and VMO2, are bound (Kido et al., 1995; Kido, Morimoto, & Kim, 1992).

The primary structure of VMO1 was determined by Back, Bain, Vadehra, and Burley (1982) as a basic protein of 163 amino acids in size. The VMO1 protein has a calculated molecular weight of 17,979 Daltons (Da). The mRNA that encodes for *VMO1* has been found to be expressed in the hen oviduct, a region joining the infundibulum to the magnum. However, mRNA expression was not reported in other regions of the hen reproductive system (Kido et al., 1995). Although the function of VMO1 in the VM is unknown, it was found that the protein shares similar activity to lysozyme. Kido et al. (1995) found that VMO1 is

able to synthesise N-acetylchitooligosaccharides from hexasaccharides of N-acetylglucosamines. This is similar to the transferase activity of lysozyme.

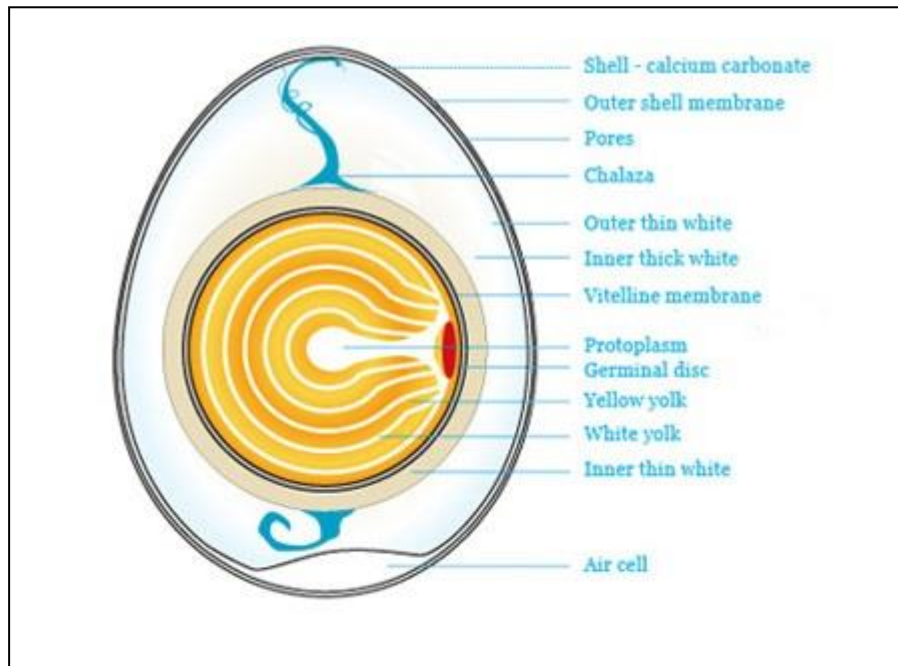


**Figure 5: Structure of the chicken egg showing the location of the vitelline membrane.** The VM separates the egg yolk (yellow and white yolk) from the egg white (inner thin, outer thin and outer thin white) (adapted from http://www.tonis.at/en/das-ei/interesting-facts/the-structure-of-a-chicken-egg.html).

### 1.4.2 Protein Structure of Chicken VMO1

The protein structure of chicken VMO1 was determined by Shimizu et al. (1994) using the multiple isomorphous replacement (MIR) method. Using this method, the crystal structure was determined at 3 angstrom (Å) resolution and allowed for the main chain of VMO1 to be traced in an electron density map. Amino acid analysis using Circular dichroism (CD) spectroscopy, proton nuclear magnetic resonance (NMR) and differential scanning calorimetry (DSC) by Kido et al. (1995) was used to determine the primary structure of VMO1. Their study showed that VMO1 contained high amounts of glycine, serine and other basic amino acid residues. Furthermore, carboxymethylation was used to test for presence of free cysteine resides. If free residues were present, carboxymethyl-cysteine (Cm-Cys)

would be formed. However, no Cm-Cys were found, therefore it was thought all cysteine residues were forming disulfide bridges in the protein structure. In addition, the amino acid analysis found there was no methionine present in the protein, which may be removed post-translationally.

The ribbon representation of VMO1 shows that the secondary structure of the protein is made up of six long beta (β) strands and eight short β-strands (Figure 6A). The presence of β-sheets were determined using both proton NMR and X-ray crystallography (Kido et al., 1995; Shimizu et al., 1994). This structure means the protein is classified as a β-protein. The β-strands were found to sit anti-parallel to one another and grouped into three β-sheets in a so-called Greek key motif formation, named after the Greek art style. As shown in Figure 6B, the first sheet consists of two long and three short β-strands. The second sheet was found to differ slightly from the first, with two long and two short β-strands. It was also discovered that hydrogen bonds were formed between the main chain atoms and side-chain atoms, Serine (Ser) 86, Ser 88 and Ser 130 in the second sheet. The third sheet was comprised of three short β-strands from the N-terminal region of the amino acid sequence and two long β-strands from the C-terminal region. All together, the three β-sheets folded to form a molecule shaped like a flower vase with the dimensions of 30 Å x 30 Å x 45 Å.

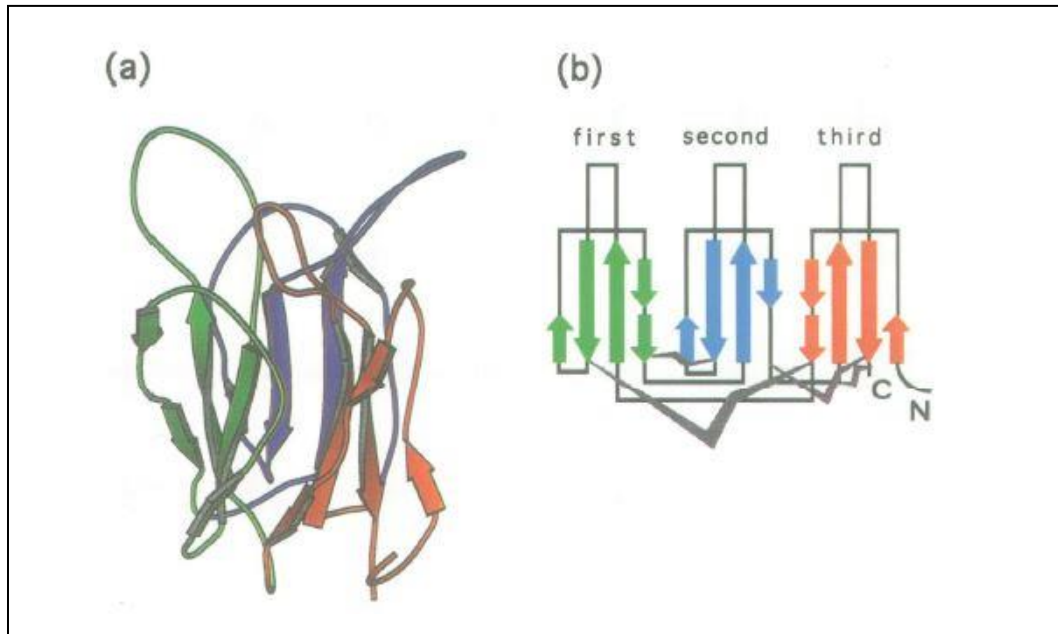


**Figure 6: Schematic diagram of VMO1 protein structure.** (A) Ribbon representation of VMO1 drawn using MOLSCRIPT. The three β-sheets are represented by three different colours; green (1st), blue (2nd) red (third). (B) Diagram of VMO1 showing the formation of the three β-sheets and the direction of the β-strands. Colours used for different strands correspond to the same colours used in Figure 6A (adapted from Shimizu, Vassylyev, Kido, Doi, and Morikawa (1994)).

The internal region of the folded protein structure is filled with side chains of hydrophobic amino acids such as phenylalanine, isoleucine, leucine and valine. In addition to this, hydrogen bonds were found at the top and bottom of this structure. The three β-sheets were held together by three disulfide bridges between cysteine (Cys) residues at the positions Cys26 - Cys57, Cys79 - Cys110, and Cys133 - Cys161 (Shimizu et al., 1994). Proton NMR found that the structure of the protein was stable at 70°C which was normal for the β-prism shape of the VMO1 protein. Although the protein structure has been widely studied and determined, the function of VMO1 remains unknown.

### 1.4.3 VMO1 in Reissner's Membrane of the Mouse Ear

Massively parallel signature sequencing (MPSS) is a technique used for identifying mRNA which is only expressed at low levels in certain cell types. This

method tags each mRNA with a signature near the poly(A) tail, allowing for detection of each product present. This technique was used to generate libraries from inner ear tissues dissected from postnatal day (PD) 7 and 8 aged mice. Three tissue types were used for making the libraries; mouse organ of Corti (MoCR), mouse vestibular epithelium (MoVB), and mouse stria vascularis (MoSV). Interestingly, *Vmo1* was found to be the second most abundant signature present. Additionally, the top ten signatures unique to the inner ear libraries were all present in the MoVB library. A majority of these signatures are from genes that are known to play a role in inner ear function. These included otogelin, in which a mouse mutant model was found to be deaf, and Col9al, in which a mouse knockout model had progressive hearing loss. (Peters et al., 2007).

Using real-time PCR (RT-PCR), *Vmo1* was found to be expressed in the mouse inner ear but not in the mouse liver, kidneys, pancreas, retina, brain, or testes (Peters et al., 2007). In addition to this, in situ hybridisation using an anti-sense probe for *Vmo1* showed that it localised to the RM in the mouse inner ear (Figure 7A). Furthermore, *Vmo1* was not detected in 87 other mouse tissue types that were part of the Mouse Reference Transcriptome (MRT) project. From this data, the authors were able to conclude that *Vmo1* was the first example of a transcript expressed exclusively on the Reissner's membrane.

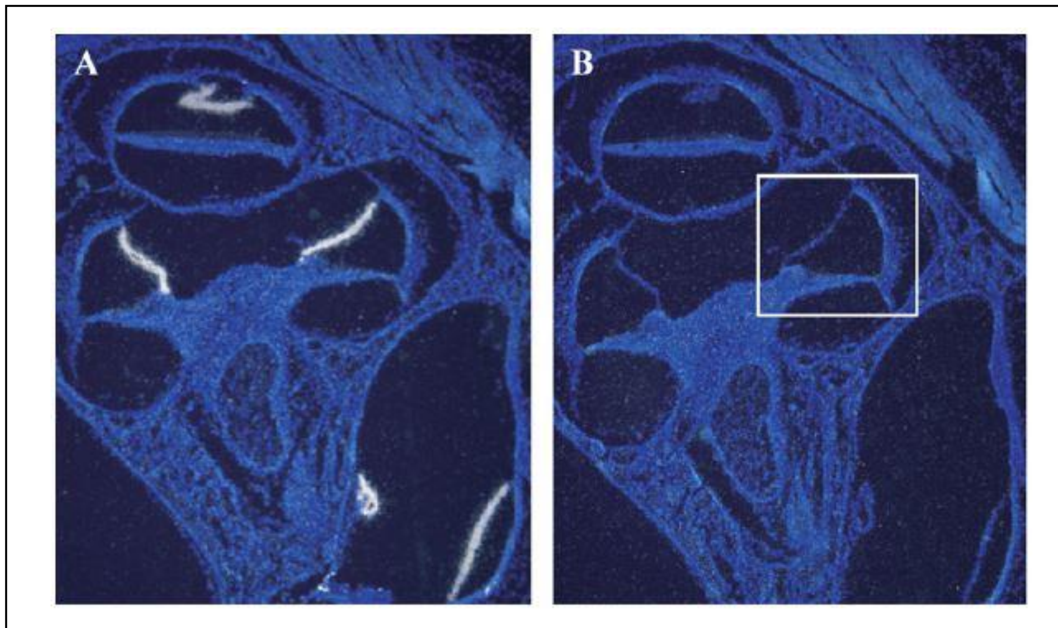


**Figure 7: *In situ* hybridisation of cross sections show localisation of *Vmo1* to the Reissner's membrane in mice.** (A) The antisense probe for *Vmo1* showing positive mRNA expression (white) on RM. (B) Sense probe for *Vmo1* showing no expression in RM (adapted from Peters et al. (2007).

As a result of this data, Vmo1 is of interest as a candidate in human hearing loss and related disorders due to the unique location and level of its expression in the mammalian inner ear. In particular, Meniere's disease, a chronic illness characterised by episodes of vertigo, tinnitus, and hearing loss, this disorder is thought to be caused in part by the rupturing of the extended RM. This is a result of a build up of endolymph in the scala media leading to a feeling of pressure in the inner ear and deafness. This rupturing leads to the symptom of vertigo (Kitajima, Watanabe, & Suzuki, 2011; Nakashima et al., 2007; Sajjadi & Paparella, 2008; Valk, Wit, & Albers, 2006).

### 1.4.4 Identification and Characterisation of VMO1 in Other Organisms

Since the identification and characterisation of VMO1 in the chicken egg, a number of homologues and paralogues have been discovered, including the human homologue.

Bioinformatics analysis of VMO1 by Forrester-Gauntlett (2013) showed that the human (*Homo sapiens*) and mouse (*Mus musculus*) are conserved at the amino acid level with 71.8% identity and 80.2% similarity. In addition to this, the mouse and chicken (*Gallus gallus*) had 47.1% identity and 60.3% amino acid similarity. There was a high level of identity shared with the amino acids responsible for the formation of the chicken VMO1 3-D structure and all eight cysteine residues for disulfide bonds in the chicken VMO1 protein were in identical places in both the mouse and human VMO1 protein.

Within humans, VMO1 has been identified using SELDI-ToF and proteomic studies of urine samples from individuals. One such study by Alves et al. (2013) found VMO1 following tandem mass spectrometry (MS/MS) analysis of urine samples from individuals with renal cell carcinoma (RCC). Although the authors did not purify VMO1, they suggested that it was a secreted protein. In addition to this find, Olivieri et al. (2014) compared urine samples from patients with bilateral aldosterone-producing adenomas to healthy subjects. Two types of these adenomas were used in the study; Aldosterone producing adenoma (APA) and bilateral adrenal hyperplasia (BAH). Protein analysis was performed using nanoHPLC-MS/MS, a type of mass spectrometry, to determine proteins present in the samples. This method was also used to detect the amount of protein present between differing cohorts. VMO1 was found to be down-regulated by 3.57-fold in APA patients compared to the control cohort. It was also down-regulated 2.63-fold in APA patients compared to BAH patients. The reasoning behind this study was to identify biomarkers for diagnosis of differing bilateral aldosterone-producing adenomas in patients.

In addition to chickens, mice and humans, VMO1 has also been identified in several other species. One such study by Chen et al. (2011) investigated the tears of camels during summer and winter to identify seasonal variations between proteins present. Briefly, camel tears from 50 healthy camels were collected and pooled in summer and winter. Proteins were separated using SDS-PAGE and proteins that showed differing expression levels between the two seasons were excised from gels and analysed using MALDI-ToF/ToF-MS for identification purposes. As a result, VMO1 was identified and found to increase in expression in summer compared to winter samples. The presence of VMO1 was further validated using western blot with a rabbit anti-human VMO1 polyclonal antibody. In a follow-up study by Shamsi et al. (2011), tears were collected from healthy camels, sheep, cows, and humans. MALDI-ToF analysis resulted in a 21 kDa protein that was identical to the human VMO1 in camel tears. Following validation using western blot with the aforementioned antibody, VMO1 was also identified in sheep tears but not in cow or human tears. The authors believe that although VMO1 was not detected in cow tears, it may still be present. This is due to there being sufficient changes in the cow VMO1 sequence that would prevent it from binding to the antibody. Finally, it was thought that VMO1 plays a special role in the tear film, keeping the ocular surface healthy.

RNA Sequencing (RNA-Seq) is a new generation sequencing technology used for analysing quantititative RNA expression at a given time. This methodology has been used to investigate the whole transcriptome of normal and noise-traumatised cochlear sensory epithelia (CSE) in rats. Rats were exposed to 120 dB for two hours and tissue samples collected a day later for RNA extraction. RNA-Seq

analysis found VMO1 had a 51.77-fold increase in expression in noise-traumatised rats compared to the control group (Patel et al., 2013).

Two species of catfish, Blue catfish (*Ictalurus furcatus*) and Channel catfish (*Ictalurus punctatus*) are both species used in aquaculture. As a result, studying their health is important for sustaining healthy populations in aquaculture farms. RNA-Seq was used to compare changes in the gills and skin following a seven day fasting period to these fish species. Fasting is common in aquaculture and can increase susceptibility to bacterial infections. Fasted blue catfish were found to have a 3.05-fold down-regulation of VMO1 compared to the fed population. Other genes found to be down-regulated in fasted fish were involved in immune function and metabolism. Fasted channel catfish also showed a 3.55-fold down-regulation of VMO1 when compared to the fed population. However, the function of VMO1 in these fish species remains to be elucidated (Li, Beck, & Peatman, 2014; Liu, Li, Su, Beck, & Peatman, 2013).

Venom gland transcriptomics was applied to a diverse group of advanced snake species by Fry, Scheib, Junqueira de Azevedo, Silva, and Casewell (2012) to identify proteins scaffold types in snake toxins. They found three novel protein scaffold types that had not previously been characterised. One of these was the VMO1 protein in Macleary's water snake (*Enhydris polylepsis*). However, the function of this protein in the venom was not elucidated.

Furthermore, six transcripts of vitelline membrane outer layer 1-like protein were found in the hepatopancreas of Japanese blue crab (*Portunus trituberculatus*) by Wang, Wu, Liu, Zheng, and Cheng (2014). The protein is synthesised there and transported to the developing oocytes via the hemolymph. Therefore, VMO1 plays a similar role in these crabs to the chicken, preventing the yolk and egg

white from mixing in eggs. In addition, VMO1 is localised to the Reissner's membrane in mice (Peters et al., 2007). The Reissner's membrane is involved in keeping endolymph and perilymph fluids in the inner ear from mixing.

Finally, Alföldi et al. (2011) found VMO1 paralogues present in immature eggs from Green anoles (*Anolis carolinensis*). Three paralogues were identified and named VMO1-α, VMO1-β, and VMO1-γ, respectively. Interestingly, VMO1-α was found to have 13 members in its family with positive selection acting for amino acid substitutes found within the negatively-charged cavity of the protein. It is suggested that this region is substrate-binding and modifies the lysozyme-like transferase activity that VMO1 has been demonstrated to possess (Kido et al., 1995). Despite being found in a wide range of tissues in a number of species, the function of VMO1 is yet to be elucidated.

To determine the function of VMO1, a number of molecular strategies can be used. The aims of this research thesis are to design, clone and express a recombinant VMO1 protein using a bacterial expression system and in addition, purify and test the resulting recombinant protein against commercial VMO1 antibodies using western blot analysis. Thus, this will allow further investigation of protein function by the identification of protein partners, and performing biochemical assay to test for transferase activity. The next section will describe the process of producing the recombinant VMO1 protein and the technology used to achieve this.

### 1.5 Recombinant DNA Technology

The term, recombinant DNA technology, refers to a number of techniques that allow for the introduction of a gene from one organism into another organism

(Pasternak, 2005). Of particular interest to this research, are the experimental techniques PCR, DNA cloning, and expression of heterogeneous proteins in bacterial cells, that were used for the development and production of VMO1 protein.

### 1.5.1 Polymerase Chain Reaction

Polymerase Chain Reaction (PCR) is a molecular technique used to amplify a target gene or nucleotide sequence within a DNA sample. The PCR process involves the use of enzymes that allow for the duplication of the specific regions of DNA, making millions of copies of a sequence during the reaction process (Saiki et al., 1988; Verma, Dalal, & Sharma, 2014). Before PCR can be performed, a template DNA sample is required. This is generally obtained through the isolation of messenger RNA (mRNA) from cells and converted to complementary DNA (cDNA) using the enzyme reverse transcriptase. This allows for the exonic DNA to be isolated that is expressed in the cells. This cDNA can then be used as a template for PCR. For the amplification of a specific target sequence, primers which are specific and complementary to the sequence are required. These primers will anneal to the DNA template at their target sequence.

Basically, PCR consists of a number of cycles repeated 25 – 35 times. At the start of the PCR, an initialisation step is required that heats the reaction to 94°C – 96°C for the activation of the DNA polymerase. Following this step, the cycles begin. There are three steps per cycle; denaturation, annealing, and extension. During the denaturation step, the reaction is heated to 90°C – 98°C for around 30 seconds to separate the double stranded DNA molecules. Following this, the annealing step occurs at a temperature that is suitable for the primers. This step allows the primers to anneal to their complementary strand of DNA. The DNA polymerase is

able to bind to the segments where the primers have annealed so that the targeted DNA region can be amplified. Next is the extension step which allows for optimal DNA synthesis for the DNA polymerase. This step synthesises a new DNA strand that is complementary to the DNA template the primer has bound to. Deoxyribonucleotides (dNTPs) in the reaction mix are added to form the double stranded template. After the cycling process has occurred, the reaction is concluded with a final extension stage to end the PCR process (Strachan & Read, 1999).

PCR was used for this research to amplify the *VMO1* gene in cDNA extracted from commercial *in vitro* human cell lines. The PCR product was electrophoresed on agarose gels and purified for isolation of the amplified *VMO1* gene. The next section will briefly describe DNA cloning and how it was applied in this research study.

### 1.5.2 DNA Cloning

DNA cloning is a technique where specific cDNA is inserted into a circular plasmid vector. This plasmid is then transformed into host cells such as the bacterium *Escherichia coli* (*E. coli*). To insert the cDNA into the plasmid, the plasmid is first digested using restriction enzymes to linearise it. This can create either blunt ends, where there are no nucleotide overhangs, or sticky ends where there is an overhang of nucleotides on a single strand of the DNA. The gene of interest can also be generated with restriction enzyme sites on the end of them that are the same as the ones used to digest the plasmid, allowing digestion of the gene to create sticky ends on the 5’ and 3’ ends of the gene. This allows the gene to be ligated into the plasmid using the enzyme T4 ligase to join the two products together (Lodish, 2012).

For the purpose of this thesis, the *VMO1* gene was ligated into two plasmid vectors, pBlueScript II SK (+), a T-tailed cloning vector, and pET28b(+), an expression plasmid vector for producing the recombinant VMO1 protein.

### 1.5.3 The pET Expression System

The pET system is a group of plasmids designed for the cloning and expression of recombinant proteins in *E. coli*. The system was first developed by Studier and Moffatt (1986). The genes which code for the protein are ligated into the pET vector and transformed into the bacterial host cell. The ligated gene is under the control of bacteriophage T7 transcription with expression of the gene being induced by the T7 RNA polymerase in the cells (Rosenberg et al., 1987; Studier, Rosenberg, Dunn, & Dubendorff, 1990). When the plasmid is introduced to a host cell with the T7 RNA polymerase gene under *lacUV5* control, the expression can be induced by introduction of compound Isopropyl β-D-1-thiogalactopyranoside (IPTG) which induces the expression of the *lacUV5* promoter (Sørensen & Mortensen, 2005).

## 1.6 Research Aims and Objectives

The first aim of this research thesis is to design, clone, and express recombinant N-terminal His-tagged VMO1 protein using the pET28b(+) bacterial expression system. The second aim is to purify the resulting protein using a nickel resin column. Finally, the third aim is to validate a commercial VMO1 antibody against the purified protein using western blot methodology to show that it is indeed specific, selective and reproducible. This will support immunohistochemistry data that suggests VMO1 is a secreted protein in the mouse by Forrester-Gauntlett (2013). To accomplish the research aims, the following six objectives are required:

1) Bioinformatics analysis of VMO1 to develop and annotate a graphic molecular view of the designed protein construct using primers with restriction enzyme overhangs;
2) Amplify *VMO1* mRNA from commercial human cell lines and convert to cDNA;
3) Clone the *VMO1* cDNA into the pET28b(+) expression vector;
4) Express recombinant VMO1 protein in *E. coli*;
5) Purify the recombinant VMO1 protein for characterisation and determination of function; and
6) Validate a VMO1 antibody against a range of protein samples including purified and non-purified recombinant VMO1 protein using a western blot.

# CHAPTER TWO
# Methods and Materials

All methods were carried out in C.2.03 Molecular Genetics Laboratory at the University of Waikato (UoW), Hamilton, New Zealand unless otherwise stated. Solutions and buffers were prepared using either autoclaved 15 - 18 Megohm-cm double distilled deionised water ($mQH_2O$) or double distilled water ($ddH_2O$) (Barnstead™ double distilled/deionisation system) as listed in Appendix One. Buffers and solutions required for Ribonucleic acid (RNA) experiments used $mQH_2O$ further treated with 0.1% Diethylpyrocarbonate (DEPC). All glassware was washed in a Miele dishwasher, autoclaved at 120°C for 15 minutes (min) and cooled to room temperature (RT) before use. All centrifugation was carried out on Eppendorf Benchtop Centrifuges at RT unless otherwise stated. Absorbance readings of the optical density measured at a wavelength of 600 nanometres (nm) ($OD_{600}$) were performed on a Bio-Rad SmartSpec™ 3000 spectrophotometer. Laboratory benches were cleaned with 70% ethanol prior to experiments being carried out with aseptic techniques followed. The University of Waikato received HSNO approval (GMD101146) from the New Zealand Environmental Protection Authority (EPA) in 2011 to develop a range of genetically modified non-pathogenic microorganisms, cell lines and zebrafish carrying genes coding for proteins involved in causation of disease, in the evolution of protein stability and cellular functions. The research in this thesis meets these requirements by developing a genetically modified organism (GMO) containing the *VMO1* gene for work towards the elucidation of its protein function. The location and nature of the development and the disposal of the approved genetically modified *E. coli* were in accordance with the APP201152 application submitted to the EPA, and

controls listed in the GMD101146 approval. Details of this application can be found on the EPA website (EPA, 2014).

### 2.1 Isolation and Purification of the *VMO1* Gene

Total RNA was isolated from cultured human *in vitro* cell lines and converted to complementary Deoxyribonucleic acid (cDNA) for use with PCR using primers designed to amplify the *VMO1* genes (Section 2.3). The aim of this first experiment was to identify, isolate and purify *VMO1* genes to make recombinant DNA for cloning purposes.

#### 2.1.1 Mammalian Cell Culture

Cells were grown in a dedicated Cell Culture Laboratory and were kindly donated by Miss Kirsty Mayall and Dr Greg Jacobson (UoW). Three different human cell lines (Table 1) were grown in CELLSTAR® 25 mL tissue culture flasks (Greiner Bio-One, Austria) containing 10 mL of growth media and antibiotics in a 37°C incubator with 5% $CO_2$ atmosphere. Media was changed every two days until cells were 90% confluent before either being split to continue growth of the cell line or processed for RNA extraction. Human ethics approval was not required from the UoW Human ethics committee as these cell lines are commercially available.

**Table 1: Human *in vitro* cell lines tested for *VMO1* gene expression.** For each cell line listed is the tissue of origin, the components of the media they were grown in and if they were adherent or not to the tissue flask. The recipes for the growth media can be found in Appendix 1.

| Cell line name | Tissue of origin | Growth mode | Reference |
|---|---|---|---|
| A549 | Lung carcinoma | Adherent monolayer | Giard et al. (1973) |
| SUM149PT | Breast tissue | Adherent monolayer | Elstrodt et al. (2006) |
| THP1 | Monocyte | Suspension cells | Tsuchiya et al. (1982) |

### 2.1.2 RNA Extraction from Human Cell Lines

All RNA work was performed in a dedicated RNA workspace to prevent degradation of RNA by RNases. The area was cleaned with RNase AWAY™ (Life Technologies, USA) and 70% ethanol prior to use. Sterile RNase- and DNase-free filter pipette tips and dedicated auto pipettes were used and samples were stored in autoclaved 1.7 mL microcentrifuge tubes (Raylab, NZ).

Total RNA was extracted from human cell lines using the TriZol® Reagent method (Invitrogen, USA). Media was removed from the culture flask containing roughly $2x10^6$ cells/mL of either A549 or SUM149PT cells using a sterile 3.5 mL Pasteur pipette (Sarstedt, Germany). Two millilitres of TriZol® Reagent was added directly to the flasks and swirled briefly to lift the adherent cells from the culture flask surface followed by pipetting up and down several times to lyse the cells. Media containing THP1 cells was placed in a 15 mL falcon tube (Biologix, China) and spun down in a Heraeus™ Multifuge 1 S-R centrifuge at 428 x *g* for five min at RT. Media was removed and the pelleted cells were resuspended in 1 mL of TriZol®. A 1 mL aliquot of TriZol® treated cells was transferred to 1.7 mL microcentrifuge tube for RNA extraction. Samples were then incubated at RT for five min to ensure complete dissociation of the nucleoprotein complex. Two millilitres of chloroform was added to each tube to remove protein and shaken vigorously for 15 seconds (sec) followed by a two min incubation at RT. The samples were then centrifuged at 12,000 x *g* for 15 min at 4°C. The upper aqueous phase was removed to a new 1.7 mL microcentrifuge tube and 0.5 µL of Glycogen (20 µg/µL) was added as a co-precipitate of RNA. Next, 500 µL of 100% isopropanol was added to tubes to precipitate the RNA and incubated at RT for 10 min. The tubes were then centrifuged at 12,000 x *g* for 10 min at 4°C to pellet

the RNA. The supernatant was removed and 1 mL of 75% ethanol was added to each tube to precipitate out the RNA in the solution and remove residual salts. Samples were vortexed briefly for 12 sec then centrifuged at 7500 x *g* for five min at 4°C. The supernatant was discarded and the tubes were left to air-dry at RT for 10 min. The RNA pellet was resuspended in 20 µL of RNase-free DEPC $mQH_2O$. RNA samples were then incubated on an Eppendorf Thermomixer® comfort set at 55°C for 15 min to aid resuspension of the pellet.

### 2.1.3 DNase Treatment of Total RNA Extracted from Human Cell Lines

RNA samples were DNase-treated to remove possible genomic DNA contamination from the extraction method. A 5 µL volume containing the reagents (ZymoResearch, USA) listed in Table 2 were added directly to the 20 µL RNA sample from Section 2.1.2 and incubated in a thermomixer for 15 min at 37°C for optimal enzyme activity, 10 min at 65°C to heat inactivate the enzyme, and five min on ice to rapidly cool the sample. The purity and concentration of the RNA samples was determined using a ThermoScientific™ NanoDrop 2000 spectrophotometer and software. Treated RNA samples were either used directly for further downstream applications such as cDNA synthesis or stored at -80°C.

**Table 2: Reagents from the DNase treatment kit (ZymoResearch, USA) used to treat RNA of possible genomic contamination**

| **Reagent** | **Volume (µL)** |
|---|---|
| 10X DNase I buffer | 2.50 |
| DNase I (1 U/µL) | 1.25 |
| 0.1% DEPC $H_2O$ | 1.25 |
| **Total** | **5.00** |

### 2.1.4 cDNA Synthesis

RNA was converted into cDNA using the Tetro cDNA Synthesis Kit (Bioline, USA) so the resulting double stranded cDNA template could be used for downstream PCR reactions. All cDNA synthesis reactions were performed in

sterile RNase- and DNase-free PCR tubes (Axygen, USA). Reagents were thawed on ice and the 5X RT buffer was vortexed briefly to ensure it was completely dissolved. The reaction was prepared as shown in Table 3 to a total volume of 20 µL and mixed gently by pipetting up and down. Oligos $(dT)_{18}$ primers, rather than Random Hexamers, were used to target the poly-A tail of mRNA so only mRNA was converted to cDNA rather than total RNA. PCR tubes were then placed in a Bio-RAD T100 Thermal Cycler for incubation at 45°C for 30 min to anneal the Oligos $(dT)_{18}$ primers to the mRNA and for optimal Reverse Transcriptase activity. Samples were then exposed to 85°C for five min to deactivate Reverse Transcriptase. The samples were cooled on ice for five min. A negative control for each RNA sample was prepared which contained no Reverse Transcriptase (-RT negative control). No cDNA was expected to be synthesised in this reaction and therefore was used as a measure of genomic contamination to the extracted RNA samples.

**Table 3: Reagents used for 20µL cDNA synthesis reaction.**

| **Reagent** | **RNA sample** | **-RT Negative control** |
|---|---|---|
| Total RNA<br>(15.4 – 97.2 ng/µL) | 2 µL | 2 µL |
| Oligos $(dT)_{18}$ primer | 1 µL | 1 µL |
| 10mM dNTPs | 1 µL | 1 µL |
| 5X RT Buffer | 4 µL | 4 µL |
| Ribosafe RNase Inhibitor<br>(10U/µL) | 1 µL | 1 µL |
| Tetro Reverse Transcriptase<br>(200U/µL) | 1 µL | - |
| $mQH_2O$ | 10 µL | 11 µL |
| **Total** | **20 µL** | **20 µL** |

### 2.2 Primer Design

Oligonucleotide primers were designed for PCR, cloning, and DNA sequencing using Geneious version 7.1.7 (Biomatters, http://www.geneious.com) unless otherwise stated. Primers were synthesised by Integrated DNA Technologies Ltd

(IDT) and reconstituted in 1X TE buffer (10mM Tris-HCl, 1mM EDTA, pH 8) to a final concentration of 100 µM and stored at -20°C. Working stocks were diluted to 5 µM using $mQH_2O$ and also stored at -20°C. Primers used for the purpose of this research are listed in Table 4. Three sets of primer pairs (SH1-3) including a Forward (F) and reverse (R) specific for human *VMO1* were designed using the NCBI reference sequences as described below.

### 2.2.1 SH1F and SH1R

SH1F and SH1R primers were designed to amplify the complete *VMO1* isoform 1 gene (NCBI: NM_182566) and allow ligation into a cloning vector for expression of recombinant VMO1. The location of the binding of this primer set is shown on Figure 8. SH1F contained a BamHI restriction enzyme site at the 5' end whilst SH1R contained an EcoRI restriction enzyme site near the 5' end. Extra nucleotides were added so the cleavage site would not be close to the end of the product. This was based on recommendations from New England BioLabs (1999). This allowed for digestion of the PCR product using these enzymes to create complementary sticky ends for ligation into the pET28b(+) and pBlueScript II SK (+) vectors. This primer set predicted a PCR product size of 691 base pairs (bp) due to the addition of an adenine base to each 3' end of the PCR product by the enzyme Taq Polymerase during PCR. This allowed the PCR product to be inserted into pBlueScript II SK (+). Once cut with both restriction enzymes, the product was estimated to be 685 bp in size. This primer set was also used for sequencing reactions for gene confirmation.

### 2.2.2 SH2F and SH2R

SH2F and SH2R primers were designed to amplify the whole *VMO1* isoform 3 gene (NCBI: NM_001144940). Like the primer set, SH1F and SH1R, SH2F

contained a BamHI restriction enzyme site and SH2R an EcoRI restriction enzyme site to create sticky ends for ligation into vectors. For example, the recognition sequence of BamHI is G^GATCC and EcoRl is G^AATTC. The position of the binding of these primers is shown on Figure 8 and a PCR product formed from these primers was predicted to be 389 bp in size. Once cut with BamHI and EcoRl , the resulting product was predicted to be 379 bp.

### 2.2.3 SH3F and SH3R

SH3F and SH3R primers were designed for sequencing of an inner region of the *VMO1* isoform 1 gene (NCBI: NM_182566), forming a product 438 bp in length. The start of SH3F bound to nucleotide 133 and SH3R ended at nucleotide 568, as shown in Table 4.

### 2.2.4 PCR Positive Controls

Primers for use as positive controls in PCR were sourced from the C.2.03 Molecular Genetics Laboratory Primer Database (UoW). There were two primers sets that were used as positive controls. Firstly, the housekeeping gene, Glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) was used as it has been shown to be constitutively expressed across a large number of human tissues (Barber, Harmer, Coleman, & Clark, 2005). HCC37F in conjunction with HCC38R was designed for human *GAPDH* and gives a PCR product of 412 bp. Secondly, the primers HCC30F and HCC31R were used since they have been shown to amplify the *VMO1* gene from the commercial cell line, A549 (Crossan, 2014).

### 2.2.5 LMP10F and LMP11R

LMP10F and LMP11R were obtained from primer stocks in the Molecular Genetics Lab (UoW) and were designed to target the T7 promoter and T7

terminator of pET28b(+) respectively as the *VMO1* gene was ligated between these two regions in the vector. This primer set was also used in colony PCR to check colonies for the insert of the *VMO1* gene into pET28b(+) (Section 2.3.2).

### 2.2.6 Primers for Orientation of Cloned PCR Insert

LMP6F and LMP7R were used in colony PCR (Section 2.3.2) to test the orientation of the *VMO1* insert into the pBlueScript II SK (+) vector. LMP6F was paired with either SH1R or SH2R whilst LMP7R was paired with either SH1F or SH2F. If *VMO1* isoform 1 was successfully ligated into the pBlueScript II SK (+) vector, a band of 789 bp would be observed on an agarose gel following colony PCR with SH1R/LMP6F and SH1F/LMP7R. Alternatively, if *VMO1* isoform 3 was ligated into this vector, a product of 516 bp would be observed using SH2R/LMP6F and SH2F/LMP6F.

**Table 4: Primer sequences used for the research detailed in this thesis.** The nucleotide sequence of primers with restriction enzyme sites are depicted in bold.

| Primer name | Sequence (5'- 3') | Gene target | Melting temperature (°C) | Product length (bp) | Restriction enzymes | Primer Application |
|---|---|---|---|---|---|---|
| SH1F | C**GGATCC**CAGACGCTACAGGATGG | *VMO1* isoform 1 | 63.6 | 691 | BamHI | PCR, Colony PCR |
| SH1R | CCG**GAATTC**AAGAGGTGGGACTAGCCTCC | | 64.8 | | EcoRI | |
| SH2F | CGC**GGATCC**GATTCACAGACGCTACAG | *VMO1* isoform 3 | 65.5 | 387 | BamHI | PCR, Colony PCR |
| SH2R | CCG**GAATTC**AGAAAGCCACTAGGTAGGCG | | 63.6 | | EcoRI | |
| SH3F | GGCGACTGGTTTCACATGTGC | *VMO1* isoform 1 inner region | 62.6 | 438 | | DNA Sequencing |
| SH3R | CCAAAGTCTCCCCAGCTCAG | | 60.0 | | | |
| LMP6F | GTTGTAAAACGACGGCCAGT | M13 | 55.8 | | | DNA Sequencing, Colony PCR |
| LMP7R | AACAGCTATGACCATG | | 45.4 | | | |
| LMP10F | TAATACGACTCACTATAGGG | T7 Promoter | 47.5 | | | DNA Sequencing, Colony PCR |
| LMP11R | GCTAGTTATTGCTCAGCGG | T7 Terminator | 53.4 | | | |
| HCC30F | GGCCTGATTCACAGACGCTA | *VMO1* | 57.0 | 354 | | PCR positive control |
| HCC31R | CTCACTCCATTCGCCCCAGC | | 60.8 | | | |
| HCC37F | CAAGAAGGTGGTGAAGCAGG | HeLa GAPDH | 56.0 | 412 | | PCR positive control |
| HCC38R | GATGGTACATGACAAGGTGC | | 53.6 | | | |

**Figure 8: *VMO1* isoform 1 reference sequence NM_182566 used for designing primer sets SH1F/SH1R and SH3F/SH3R.** The restriction enzyme sites of BamHI and EcoRI on SH1F/SH1R are also shown in dark green. BamHI has a recognition site of G^GATCC and EcoRI has a recognition site of G^AATTC.

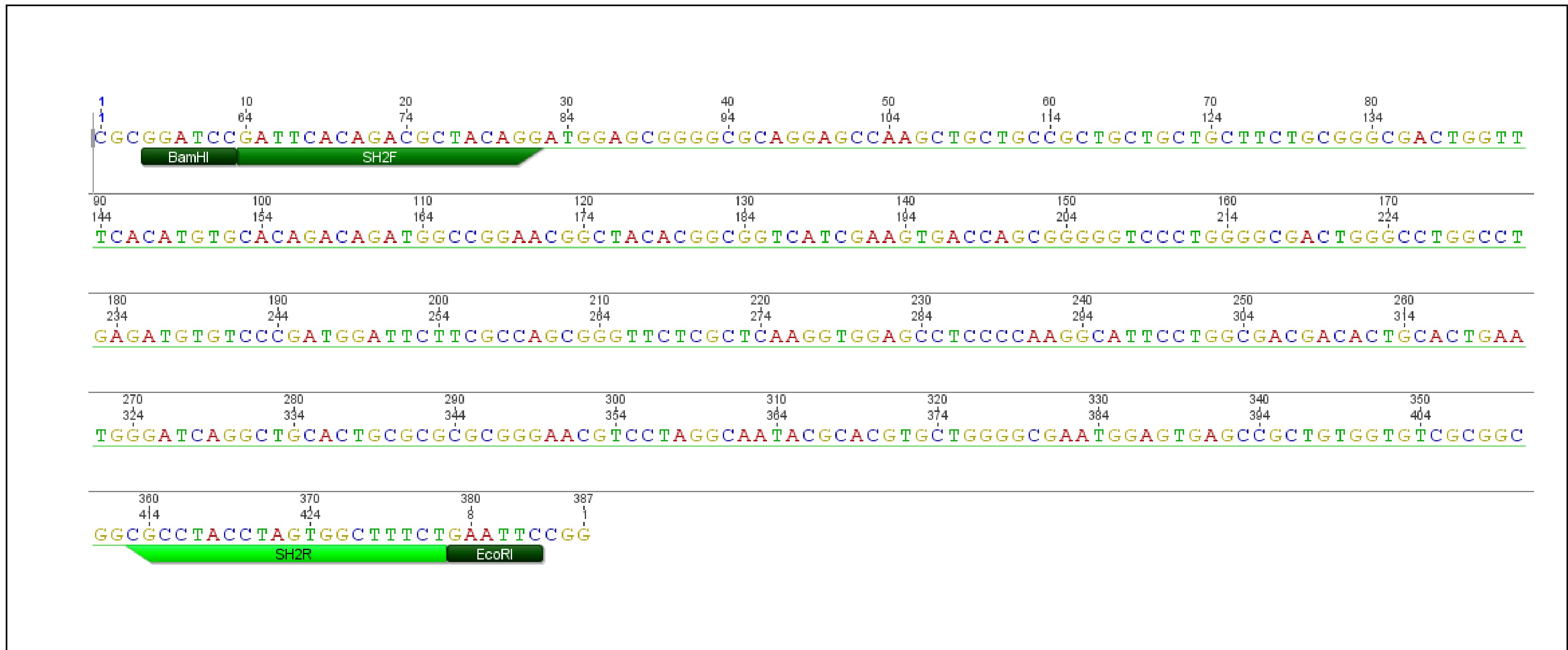


**Figure 9: *VMO1* isoform 3 reference sequence NM_001144940 used for designing primer set SH2F/SH2R.** The restriction enzyme sites on SH2F/SH2R primers are also shown in dark green. BamHI has a recognition site of G^GATCC and EcoRI has a recognition site of G^AATTC.

## 2.3 Polymerase Chain Reaction

PCR was performed using reagents from Solis BioDyne (Estonia) (Table 5). To avoid contamination, master mixes for PCR were prepared in a dedicated PCR cabinet that was UV-treated before each use. Also, dedicated pipettes and sterile DNase- and RNase-free filter tips were used. Next, 18 µL of master mix was aliquoted into sterile 200 µL PCR tubes (Axygen, USA) with F and R primers (Table 4) added to a final concentration of 0.25 µM. Lastly, 1 µL of cDNA was added as template.

**Table 5: Final concentration of reagents used in PCR**

| Reagent | Final concentration | Individual amounts per reaction (µL) |
|---|---|---|
| 10X Buffer B1 (Tris-HCl and $(NH_4)_2SO_4$) | 1X | 2 |
| 25 mM $MgCl_2$ | 1.5 mM | 1.2 |
| 10 mM dNTPs | 200 µM | 0.4 |
| 5 U/µL HOT FIREPol® DNA Polymerase | 0.05 U/µL | 0.2 |
| $mQH_2O$ | – | up to 20 µL |

cDNA extracted from several cell lines were used as the template for amplification and to test for presence of the *VMO1* gene. PCR was carried out in a Bio-Rad T100 Thermal Cycler using the conditions outlined in Table 6. The primer set of HCC37F and HCC38R was used as a positive control for PCR (Section 2.2.4). In addition, each PCR was carried out with negative controls which contained primers and no template cDNA to test for possible contamination. The template cDNA was substituted with $mQH_2O$.

**Table 6: PCR cycle setting for amplification of DNA**

| Step | | Temperature | Time |
|---|---|---|---|
| HOT FIREPol® DNA Polymerase activation | | 95°C | 15 min |
| Cycling x 30 | Denaturing | 95°C | 30 sec |
| | Annealing | 60°C | 30 sec |
| | Extension | 72°C | 1 min |
| Final extension | | 72°C | 5 min |

Following PCR, the amplified products were stored at RT until being loaded onto an agarose gel for analysis.

### 2.3.1 Gradient PCR

Gradient PCR was performed to optimise PCR reactions with primer sets and determine the best annealing temperature for primer sets. This was carried out using the same process as described in Section 2.3 with differing rows of the Bio-Rad T100 Thermal Cycler set to temperatures between 52°C and 62°C.

### 2.3.2 Colony PCR

Colony PCR was performed on bacterial colonies following transformation of ligated vector DNA. This was to test colonies for presence of PCR inserts in cloned vectors or to check the orientation of the PCR insert in the cloned vector. A number of primers were used for colony PCR, as shown in Table 4. Colonies were selected from plates using a sterile toothpick and dipped into a 200 µL PCR tube containing a final concentration of 1X Buffer B1 (Tris-HCl and $(NH_4)_2SO_4$), 1.5 mM $MgCl_2$, 200 µM of dNTPs, 0.05 U/µL HOT FIREPol® DNA Polymerase and 0.25 µM of primers. The sterile toothpick was then streaked out onto Luria broth (LB) agar plates containing selective antibiotics. Colony PCR was performed as per Table 6. Following PCR, the reactions were loaded onto agarose gels for analysis.

### 2.4 Agarose Gel Electrophoresis

Agarose gel electrophoresis was used to separate out fragments of DNA for analysis, purification and quantification, and was carried out in a dedicated gel electrophoresis room as Ethidium bromide, a known carcinogen, was used in the process of making gels. Two agarose gel methods were used for the purpose of this MSc research; low melting point and agarose. In general, gels were cast and run using an Owl™ Separation System. DNA samples were mixed with 2 µL of 6X Loading Dye and loaded into the gel well. Following electrophoresis, gels were photographed using a COHU High Performance CCD camera and Scion Image – Release Beta 4.0.2 software (Scion Corporation). Molecular DNA ladders were used to determine the size of fragments within gels. Weight per volume (w/v) concentrations of gels was based on the size of the fragments being electrophoresed and further applications (Table 7).

**Table 7: Weight per volume (w/v) agarose gels and the purpose each percentage gel was used for.**

| Percentage gel | Amount of agarose added to 35 mL of 1X TAE buffer | Nucleic Acid Analysis |
|---|---|---|
| 0.9% | 0.315 g | Plasmid |
| 1% | 0.35 g | Plasmid |
| 2% | 0.70 g | PCR |

#### 2.4.1 Low Melting Point Agarose Gels for DNA Purification

SeaPlaque® GTG® agarose powder (Lonza, USA) was weighed out in a weigh boat (0.315-0.7 g) and added to a 250 mL flask containing 35 mL of 1X TAE electrophoresis buffer (40 mM Tris-Acetate, 1 mM EDTA, pH 8). This 0.9-2% agarose is suitable for the separation of DNA fragments between 100 bp to 23,000 bp and has a melting temperature of ≤65°C. The flask was then gently swirled and placed in a 650 W microwave where it was heated on medium heat

for one min. The flask was swirled again and microwaved until crystals of agarose were completely dissolved. The solution was cooled by running the flask under cold tap water before adding 2 µL of 10 mg/mL Ethidium bromide and swirling to mix. The solution was poured into a balanced gel caster. A comb containing 6 wells that were 9 mm in width each was inserted into the solution and the gel was left to set at RT before use. The agarose gel was placed in the electrophoresis apparatus and covered with 1X TAE buffer. Agarose gels were run for 30 min at 90 V before being placed on a Safe Imager™ Transluminator (Invitrogen, USA) to visualise the DNA products under blue light and prevent DNA damage. Fragments were purified from the gel using the protocol outlined in Section 2.5.

### 2.4.2 Agarose Gels

Agarose gels were made using HyAgarose™ LE Agarose (Hydragene, USA) powder which was weighed out in a weigh boat and added to 35 mL of 1X TAE buffer in a flask. This agarose was used since it is common reagent in the laboratory that has low electroendosmosis and a standard melting point (≤88°C) resulting in high resolution sharp DNA bands or high clarity and a low background. The amount of agarose added differed depending on the size of the fragments being run on the gel, as shown in Table 7. The flask was microwaved on medium heat to dissolve the agarose powder. Once dissolved, the mixture was cooled by running the flask under cold water and 2 µL of 10 mg/mL Ethidium bromide was added to allow visualisation of DNA fragments. The mixture was poured into an Owl™ Gel casting system with a two combs containing twelve 0.75 cm width wells placed in it and left to set at RT. Gels were run for 30 min at 90 V in 1X TAE buffer and viewed under UV light.

### 2.5 Purification of *VMO1*

Low melting point gels were visualised on a Safe Imager Transluminator (Invitrogen, USA). Using a molecular ladder for reference, the DNA fragment of the correct size was cut from the gel using a scalpel and placed into a 1.7 mL microcentrifuge tube and weighed. Using the ZymoClean™ Gel DNA Recovery Kit (ZymoResearch, USA), three volumes of Agarose Dissolving Buffer was added per volume of gel fragment and incubated at 55°C for 10 min, shaking at 700 rpm in an Eppendorf Thermomixer comfort. The dissolved solutions were loaded into a provided Zymo-Spin Column and placed into a provided Collection tube. The solutions were centrifuged at 16000 x *g* for 30 sec and the flow-through in the Collection tube was discarded. A total volume of 200 µL of DNA Wash Buffer was added to the columns and centrifuged at 16000 x *g* for 30 sec. This step was repeated once more before placing the Zymo-Spin Column into a new 1.7 mL microcentrifuge tube and 20 µL of $mQH_2O$ was added to the column matrix. This was then centrifuged for 30 sec at 16000 x *g* to elute the DNA. DNA quality was analysed using the NanoDrop 2000 Spectrophotometer and accompanying software. Following extraction, DNA was stored at -20°C or used for digestion using restriction enzymes.

#### 2.5.1 Cloning *VMO1* into a T-tailed Vector

Following isolation of the PCR product using the Gel Recovery Kit, the *VMO1* PCR products for isoform 1 and isoform 3 were digested overnight at 37°C, mixing at 700 rpm in an Eppendorf Thermomixer comfort, using the restriction enzymes, BamHI and EcoRI (Roche, USA). A total reaction volume of 40 µL was made as shown in Table 8. Following digestion, the BamHI/EcoRI digested

amplified DNA was purified to remove residual enzymes and salts prior to ligation into vectors.

**Table 8: Reaction mixture used for restriction digestion of *VMO1* PCR products**

| **Reagent** | **Volume** (µL) |
|---|---|
| Purified PCR product | 20 |
| 10X Buffer 3 | 2 |
| 100X BSA (10 mg/mL) | 1 |
| EcoRI (10U/µL) | 2 |
| BamHI (10U/µL) | 2 |
| $mQH_2O$ | 13 |
| **Total Volume** | **40 µL** |

### 2.5.2 Purification of Digested DNA

Following digestion of the *VMO1* products, the ZymoClean™ Genomic DNA Clean and Concentrator Kit™ (ZymoResearch, USA) was used to purify out the amplified DNA from reagents in the restriction digest. In a 1.7 mL microcentrifuge, 200 µL of DNA Binding Buffer was mixed with each of the 40 µL double digest reactions and transferred to a provided Zymo-Spin Column in a collection tube. This was centrifuged at 16000 x *g* for 30 sec and the flow-through was discarded. Next, 200 µL of DNA Wash Buffer was added to the columns and centrifuged at 16000 x *g* for 30 sec. This step was repeated and then the column was transferred to a new 1.7 mL microcentrifuge tube. Finally, 15 µL of prewarmed (55°C) $mQH_2O$ was added to the column matrix, incubated for one min at RT before centrifuging for 30 sec at 16000 x *g* to elute the DNA. The purified, digested *VMO1* PCR products were run on a 2% 1X TAE agarose gel to estimate the concentrations of the DNA. A total volume of 5 µL of each product was mixed with 2 µL of 6X Loading Dye and run on the gel with 5 µL of 100 bp DNA ladder (GenScript, USA) at 90V for 30 min. The brightness of the bands was compared to the DNA ladder whose bands were of known concentrations.

These concentrations were used for the Ligation calculation (Equation 1). This was so the ligation reaction was performed with a molar ratio of 1:3 vector to insert of the known DNA sizes present. The next step was to ligate the purified *VMO1* insert into a cloning vector.

$$ng/\mu L\ PCR\ insert\ required\ = \frac{ng/\mu L\ Vector\ concentration\ x\ PCR\ product\ size}{Vector\ size}\ x\ 3$$

**Equation 1: Ligation calculation used for determining the concentration of PCR product required for a molar ratio of 1:3 vector to insert.** Size is measured in bp

## 2.6 Ligation of Purified *VMO1* into pBlueScript II SK (+) Cloning Vector

Purified *VMO1* was ligated into the pBlueScript II SK (+) vector for means of DNA sequencing and subcloning before being cloned into the pET28b(+) expression vector. pBlueScript II SK (+) is a cloning vector of 2961 bp and the vector map can be found in Appendix Two. The pBlueScript II SK (+), kindly donated by Dr Ray Cursons (UoW), was digested using an EcoRV enzyme to create a linearised blunt vector. This was then tailed using dideoxythymidine triphosphate (ddTTP). This created a vector with a single 3'-overhanging thymine on each of the blunt ends. A 10 µL ligation reaction was prepared in 200 µL PCR tubes as per Table 9. Briefly, the T4 DNA Ligase buffer (Fermentas Thermo Scientific, USA) was mixed by pipetting up and down with the purified *VMO1* insert DNA, digested vector DNA and $mQH_2O$ before being mixed with T4 DNA Ligase (Fermentas Thermo Scientific, USA). The reaction was left overnight at 4°C to increase the number of transformants.

**Table 9: Reagents used for ligation of *VMO1* isoforms into vectors.**

| **Reagent** | **Volume** (µL) |
|---|---|
| 1X T4 DNA Ligase Buffer (40 mM Tris-HCl, 10 mM $MgCl_2$, 10 mM DTT, 0.5 mM ATP) | 1 |
| Purified Vector DNA (5 ng/µL) | 5 |
| Purified *VMO1* PCR product (30 ng/µL) | 1 |
| $mQH_2O$ | 2 |
| T4 DNA Ligase (5 Weiss units/µL) | 1 |
| **Total Volume** | **10 µL** |

### 2.6.1 Preparation of Fresh Chemical Competent *E. coli* DH5α Cells

*Escherichia coli (E. coli)* DH5α were prepared for chemical transformation of pBlueScript II SK (+) and pET28b(+) vectors containing either *VMO1* isoform 1 or isoform 3. *E. coli* DH5α were streaked out on a LB agar plate and grown overnight at 37°C. Two colonies were selected for inoculation of 10 mL of LB broth and grown at 37°C shaking at 800 rpm in a Ratek Orbital Mixer Incubator until the culture appeared turbid. Once turbidity was reached, 1.5 mL amounts of culture were removed and spun down for one min at 16000 x *g* and the supernatant removed. Pellets were then resuspended in 100 µL of $CaCl_2$/PEG solution (0.1 M $CaCl_2$ and 1% PEG 8000) and stored on ice until required for transformation.

### 2.6.2 Chemical Competent Transformation

Following preparation of chemical competent DH5α, the whole ligation reaction (10 µL) was added to the cells and flicked to mix. Cells were left on ice for 30 min with intermittent flicking every five min. Cells were heat shocked in a water bath set to 42°C for one min then placed on ice for one min. Following this, 1 mL of LB broth prewarmed to 37°C was added and cells were left to grow for an hour to allow expression of antibiotic resistance gene(s) at 37°C shaking at 300 rpm in an Eppendorf Thermomixer® Comfort.

### 2.6.3 Selection for Colonies Containing pBlueScript II SK (+) with the *VMO1* Insert

Prior to transformation, LB agar plates containing ampicillin (100 mg/mL) were spread with 4 μL of 2% 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) and 40 μL of 20% Isopropyl β-D-1-thiogalactopyranoside (IPTG). Ampicillin was used to select for growth of colonies containing the vector whereas X-Gal and IPTG were used for blue and white colony screening. The gene of interest was cloned into the multiple cloning site (MCS) of the *lacZ*, a gene in pBlueScript II SK (+) which is responsible for producing the α-peptide of β-galactosidase. The plasmid was transformed into *E. coli* DH5α which contains the gene for production of the ω-peptide for β-galactosidase. If both peptides are produced, the β-galactosidase enzyme is formed and can be detected by X-Gal. This can be cleaved by β-galactosidase to form 5-bromo-4-chloro-indoxyl which oxidises to 5,5'-dibromo-4,4'-dichloro-indigo, a blue coloured pigment. However, the cloning into this site disrupts the production of the a-peptide of the β-galactosidase protein. Following growth for an hour (Section 2.6.2), cells were spun down for one min at 16000 x *g* and resuspended in 100 μL of LB broth before being spread out on the plates containing IPTG/X-Gal. Plates were grown overnight at 37°C. White colonies were selected for colony PCR (Section 2.3.2) to check the orientation of the insert in the vector using primers described in Section 2.2.6. The transformation efficiency could not be calculated due to a low volume of unknown concentration of pBlueScript II SK (+) vector. However, it was noted that the DNA was detected on an agarose gel (Dr. Ray Cursons, personal communication, 12 June, 2014).

### 2.7 pET28b(+) Expression Vector

A frozen glycerol stock of (*E. coli*) DH5α containing pET28b(+) was obtained from communal stocks in the -80°C freezer (UoW). It was streaked out on a LB agar plate containing the antibiotic kanamycin at a final concentration of 30 µg/µL. The plates were left to grow overnight upside down in a 37°C oven. A single colony was selected from the agar plate to inoculate 10 mL of LB broth containing a final concentration of 30 µg/µL kanamycin and left to grow overnight at 37°C in a Ratek Orbital Mixer incubator on speed setting 8. A plasmid map of pET28b(+) can be found in Appendix Two.

#### 2.7.1 Extraction and Purification of pET28b(+)

The pET28b(+) vector was extracted from *E. coli* DH5α using the Zyppy Plasmid Miniprep kit (ZymoResearch, USA) and the manufacturers guidelines. This protocol can extract up to 25 µg of plasmid DNA. For this procedure, all centrifugation was performed at 16000 x *g*. A 1.5 mL volume of overnight bacterial culture was transferred to a 1.7 mL microcentrifuge tube and centrifuged for 30 sec, and the supernatant discarded. The pellet was resuspended in 600 µL of 1X TE buffer pH 8. The 7X Lysis Buffer was preheated to 37°C for 30 min to dissolve the mixture before adding 100 µL to the resuspended cell pellet. It was mixed by inversion six times to ensure complete cell lysis. A 350 µL volume of pre-chilled Neutralisation Buffer was added to the tube and quickly mixed (within two min) by inversion to ensure complete neutralisation of the reaction. As a result, a yellow precipitate formed and was centrifuged for four min. Approximately 900 µL of the supernatant was transferred to the provided Zymo-Spin IIN column, taking care to avoid disturbing the pellet of cellular debris. The column was placed into a provided Collection tube and centrifuged for 15 sec.

The flow-through in the Collection tube was discarded and the column placed back in the original tube. Then, 200 μL of Endo-Wash Buffer was placed in the column and centrifuged for 30 sec before adding 400 μL of Zyppy Wash Buffer and centrifuging for one min. The column was transferred to a new 1.7 mL microcentrifuge tube and 30 μL of prewarmed 55°C $mQH_2O$ was added directly to the column and left to incubate at RT for one min. Finally, the column was centrifuged for 30 sec to elute the plasmid DNA. The concentration and purity of plasmid DNA was determined using the NanoDrop 2000 Spectrophotometer software. The purity of the DNA sample is determined by the 260/280 ratio, and those around ~1.8 are considered pure enough for downstream applications. It was then stored at -20°C or digested by restriction enzymes for later applications.

### 2.7.2 Restriction Digest of pET28b(+)

The pET28b(+) vector was digested using two restriction enzymes, BamHI and EcoRI (Roche, USA). These enzymes were used to create a linearised vector with compatible sticky ends for ligation of the amplified *VMO1* gene. The two enzymes selected were within the MSC of pET28b(+) at position 192 and 198 (Appendix Two). Four reactions were set up according to Table 10. Both enzymes were tested individually as well as a double digest to check that the digestion activity was complete. A negative control was also used as a comparison of non-digested DNA. Following an overnight digest in an 37°C Eppendorf Thermomixer comfort which was shaking at 700 rpm, 5 μL of each reaction was then mixed with 2 μL of 6X Loading Dye and loaded onto a 0.9% 1X TAE gel (Section 2.4). A 5 μL volume of 1 kB Plus DNA Ladder (Invitrogen, USA) was also loaded onto the gel.

**Table 10: Reaction mixes for digestion of pET28b(+) using restriction enzymes.**

| Reagent | Negative control | Double digest | BamHI | EcoRI |
|---|---|---|---|---|
| pET28b(+) () | 5 µL | 12.5 µL | 5 µL | 5 µL |
| Buffer 3 | 1 µL | 2.5 µL | 1 µL | 1 µL |
| 100X BSA (10mg/mL) | 1 µL | 1 µL | 1 µL | 1 µL |
| BamHI (10U/µL) | - | 2.5 µL | 1 µL | - |
| EcoRI (10U/µL) | - | 2.5 µL | - | 1 µL |
| mQ$H_2O$ | 13 µL | 29 µL | 12 µL | 12 µL |
| **Total** | **20 µL** | **50 µL** | **20 µL** | **20 µL** |

The double restriction enzyme digest of pET28b was seen as a single product on the gel of 5362 bp. In addition to this, a linearised product was present on the gel in the single digest reactions. This allowed for positive confirmation that the digest had worked. Following this confirmation, the remaining 35 µL volume of the double digest was mixed with 2 µL of 6X Loading Dye and loaded onto a 1% 1X TAE low melting point gel. Following electrophoresis (Section 2.4.1), a 5362 bp band was gel purified using the ZymoClean™ Gel DNA Recovery Kit (ZymoResearch, USA).

### 2.7.3 Ligation of Purified, Digested pBlueScript II SK (+) -*VMO1* Insert into BamHI/EcoRI Digested pET28b(+) Vector

White colonies containing ligated pBlueScript II SK (+) and *VMO1* gene were selected and analysed using colony PCR to detect for the insertion in the correct orientation (Section 2.4.1). Positive clones were grown in 10 mL of LB and ampicillin (50 µg/µL) broth and the vector DNA purified out as per Section 2.7.1. The cloned *VMO1* gene of approximately 693 bp was cut from pBlueScript II SK(+) vector using the restriction enzymes BamHI and EcoRI to confirm the PCR results prior to DNA sequencing. The restriction digest product was then run on a 2% 1X TAE low melting point agarose gel and purified (Section 2.4.1) before being ligated into BamHI/EcoRI digested pET28b(+) as per the protocol below.

## 2.8 Transformation of Ligated Vectors into Bacterial Competent Cells

Ligated vectors containing the *VMO1* insert were transformed into bacterial cells through either electroporation or chemical transformation. Positive controls were used where possible for calculation of the transformation efficiency. The equation for working out the transformation efficiency is shown in Equation 2. Colonies on the plate were counted and entered into the equation. The amount of DNA added for the transformation was either determined by the concentration provided by the manufacturer as a positive control or by NanoDrop. Transformations are considered of high quality when the cells are worked out as $10^{10}$ transformants/µg for electrocompetent cells and $10^{7}$ transformants/µg for general cloning purposes.

$$\frac{\#\ colonies\ on\ plate}{ng\ of\ DNA\ plated} \times 1000\ ng/\mu g = \text{transformants/µg}$$

**Equation 2: Calculation for determining the transformation efficiency following transformation of vectors into competent cells.**

### 2.8.1 Preparation of Electrocompetent Cells

Electrocompetent cells were produced as they have higher transformation efficiency rates than chemical competent cells (Hanahan & Glover, 1985). *E. coli* DH5α was sourced from glycerol stocks stored at -80°C and streaked out on a LB agar plate and grown overnight at 37°C. The following day, a single colony was used for inoculation of 10 mL of LB broth and grown overnight at 37°C at 200 rpm in a Ratek Orbital Mixer Incubator. The 10 mL overnight culture was used to inoculate 1 L of LB broth and incubated at 37°C until the $OD_{600}$ reached exponential growth phase between 0.5 and 0.7 before being chilled on ice for 15 min. Centrifugation was carried out on a benchtop Heraeus Multifuge 3SR

Plus at 4°C for 15 min at 4500 rpm to pellet the bacterial cells. The culture was centrifuged in cooled, sterile centrifuge buckets and the supernatant was poured off. The pellet was resuspended in 1 L of sterile, chilled 10% glycerol then centrifuged at 4°C for 15 min at 4500 rpm. The glycerol was poured off and the pellet was resuspended in 500 mL of fresh 10% glycerol before being re-centrifuged as before. The glycerol was once again poured off and the pellet resuspended in 20 mL of fresh 10% glycerol. Following a final centrifugation, the pellet was resuspended in 3 mL of 10% glycerol and 50 µL aliquots were made in prechilled 1.7 mL microcentrifuge tubes on ice. The 50 µL aliquots were briefly frozen in liquid nitrogen before being stored at -80°C.

### 2.8.2 Transformation of pET28b(+) and *VMO1* using Electroporation

Electrocompetent *E. coli* DH5α (Section 2.8.1) and *E. coli* BL21 (DE3) strains were used for electroporation. Electrocompetent cells were thawed at RT before being placed on ice. In a prechilled 1.7 mL microcentrifuge tube, 40 µL of electrocompetent cells were mixed with 2 µL of ligated reaction DNA (Section 2.7.3). This volume was transferred to a pre-chilled sterile 0.2 cm Bio-Rad Gene Pulser® Cuvettes. The cuvettes were placed on ice to cool. Right before electroporation, the mixture was transferred to the cuvette and tapped lightly to ensure the mixture was at the bottom of the cuvette. The cuvette was wiped with a paper towel to remove any ice and placed in the safety chamber slide of the Bio-Rad Gene Pulser™ which was set to 25 µF and 2.50 kV, and the pulse controller set to 200Ω. The cuvette was pulsed once at the setting above. Immediately after electroporation, 1 mL of LB broth warmed to RT was added to the cuvette and the cells gently resuspended. This was then transferred to a 1.7 mL microcentrifuge tube and incubated for one hour at 37°C, shaking at 300 rpm to allow for the

expression of antibiotic resistant genes. The cells were then plated onto prewarmed LB agar plates containing kanamycin (30 μg/μL) for selective growth of bacterial cells containing the vector. The transformations were plated in serial dilutions of 1:10 of 10 μL of transformed cells diluted in 90 μL of fresh LB broth, 100 μL of undiluted cells, and the remaining cells spun down and resuspended in 100 μL of fresh LB broth prior to plating. The plates were grown upside down overnight in a 37°C oven. The white *E. coli* DH5α colonies were tested for presence of the *VMO1* insert in the correct orientation using colony PCR. Positive clones were then grown in 5 mL of LB broth containing kanamycin (30 μg/μL) and the vector purified out for DNA sequencing, or transformation into protein expression bacterial strains; *E. coli* BL21 (DE3), *E. coli* BL21 (DE3) pLysS, or Rosetta™ (DE3) pLysS.

### 2.8.3 Transformation of pET28b(+) into *E. cloni*® EXPRESS Chemically Competent Cells

The pET28b(+) vector containing *VMO1* isoform 1 was transformed into *E. cloni*® EXPRESS BL21 (DE3) pLysS cells (Lucigen, USA). Prior to transformation, LB agar plates containing chloramphenicol (34 μg/μL) and kanamycin (30 μg/μL) were prewarmed at 37°C in an oven. LB agar plates containing chloramphenicol (34 μg/μL) and ampicillin (50 μg/μL) were also prewarmed in a 37°C oven for the transformation of the positive plasmid control, pUC19 (10 pg), provided with the kit. pET28b(+) containing *PhoH2* (sourced from Dr Emma Andrews, UoW) was used as positive control for protein expression and also prepared for transformation. The *E. cloni*® cells were removed from the -80°C freezer, thawed on ice and 40 μL of cells were aliquoted into each sterile pre-chilled 14 mL tube (Greiner Bio-One, Austria) on ice. For chemical transformation, the cells were

mixed with 1 μL of vector and stirred briefly for three seconds using a pipette tip. The mixture was incubated on ice for 30 min before being heat shocked in a 42°C water bath for 45 sec. The tubes were incubated on ice for two min before adding 960 μL of RT Expression Recovery Medium (Lucigen, Austria) to the cells. The tubes were placed in a Ratek Orbital Mixer Incubator at 250 rpm for one hour at 37°C to express the antibiotic genes. Following this growth, 200 μL was spread out on the appropriate antibiotic plates and grown overnight at 37°C. Single white colonies were selected for protein expression induction.

### 2.8.4 Transformation of pET28b(+) into Rosetta™ (DE3) pLysS Competent Cells

Transformation of DNA into competent Rosetta™ (DE3) pLysS cells (Novagen, USA) was performed as per manufacturer's instructions. Cells were removed from the -80°C freezer and thawed on ice and 20 μL of cells were aliquoted into sterile, prechilled 1.7 mL microcentrifuge tubes on ice. One microlitre of purified, isolated pET28b (+) containing the *VMO1* isoform 1 insert (Section 2.7.3) was pipetted into the tube and gently mixed by swirling using the pipette tip. In addition to this, a test plasmid control (1 pg) provided by the manufacturer was transformed into the competent cells as they had been displayed to produce more than 2 x $10^6$ colonies/μg plasmid when plated. This reaction was placed on ice for five min before heating tubes in a 42°C waterbath for 30 sec. Tubes were then placed back on ice for two min before adding 80 μL of SOC media provided by the manufacturer. Tubes were placed in an Eppendorf Thermomixer® comfort set at 37°C, shaking at 300 rpm for one hour. Following this, 40 μL of the transformation was pipetted onto a LB agar plate containing chloramphenicol (34 μg/μL) and kanamycin (30 μg/μL) and spread with a sterile glass rod. Also,

40 µL of the control transformation was plated onto a LB agar plate containing ampicillin (50 µg/µg) and chloramphenicol (34 µg/µL) and spread using a glass rod. Plates were placed upside down in a 37°C oven to grow overnight. Following overnight growth, plates were inspected for presence of growth and a single colony containing the pET28b (+)/*VMO1* construct was selected for downstream protein expression applications. In addition, this colony was streaked onto a LB agar plate containing appropriate antibiotics so that downstream applications could be performed using the same colony or produced into glycerol stocks.

### 2.9 DNA Sequencing

DNA Sequencing was carried out by the University of Waikato DNA Sequencing Facility using an Applied Biosystems 3130xl Genetic Analyser. The purpose of sequencing was for confirmation of the *VMO1* nucleotide sequence as well as checking the orientation of the insertion of the gene into the recombinant vectors, pBlueScript II SK (+) and pET28b(+). Sequencing was also used to confirm the *VMO1* gene was in frame in the vector(s) for protein expression and that there were no stop codons present in the open reading frame of the translated nucleotide sequence. Sequencing was carried out using primers provided by the Facility or those listed in Table 4. Primers were provided to the UoW DNA Sequencing Facility at a final concentration of 5 µM in 0.1% DEPC mQ$H_2O$. Vectors containing the *VMO1* gene that were at a concentration of 150 µg/µL, determined by a NanoDrop 2000 Spectrophotometer, were sent for sequencing. The resulting sequences were analysed using Geneious version 7.1.7 (Biomatters, http://www.geneious.com).

## 2.10 Recombinant VMO1 Protein Expression

Recombinant protein expression was optimised using two different bacterial strains and concentrations of IPTG. Firstly, pET28b(+) containing the *VMO1* isoform 1 gene was transformed into electrocompetent *E. coli* BL21 (DE3), and then into commercial *E. cloni*® EXPRESS BL21 (DE3) pLysS cells for protein expression of the recombinant VMO1 protein. This bacterial strain contains the pLysS plasmid that encodes for the T7 lysozyme. This lysozyme lowers the background expression levels of genes that are under the T7 promoter. This does not inhibit the expression of the target gene following induction with IPTG. In addition, a positive pET28b(+) plasmid control for protein expression was included. This construct was developed and obtained as extracted vector DNA from Dr Emma Andrews (UoW) and contains the *PhoH2* from *Mycobacterium smegmatis*. It was used as it had previously been demonstrated to express at the same conditions as the conditions used for this thesis, producing a protein of approximately 50 kiloDalton (kDa) in size and was transformed into the *E. coli* BL21 (DE3) pLysS cells (E Andrews, personal communication, October 2014).

### 2.10.1 Small-scale Time Course Protein Expression Analysis using *E. coli* BL21 (DE3)

A single colony from a transformed agar plate (Section 2.8.2) was selected for inoculation of 2 mL of LB broth with kanamycin (30 µg/µL). The culture was grown at 37°C shaking at 200 rpm in a Ratex Orbital Mixer Incubator until the $OD_{600}$ was between 0.6 and 1. The culture was spun down for 30 sec at 16000 x *g* and the supernatant removed and the pellet resuspended in 2 mL of fresh LB broth and kanamycin (30 µg/µL). Three 14 mL tubes were set up containing 5 mL of LB broth and kanamycin (30 µg/µL). To each tube, 200 µL of the resuspended

bacterial cells was added and incubated at 37°C in a 250 rpm shaking incubator until the $OD_{600}$ measured between 0.4 and 0.6. To two of the culture tubes, 100 mM IPTG was added to the final concentrations of 0.4 mM and 1 mM, respectively. IPTG binds to the lac repressor, allowing genes under the lac operon to be transcribed. One tube was reserved as a control for uninduced cells where no IPTG was added. Following this, the culture tubes were placed in a 37°C shaking incubator at 250 rpm with 500 µL aliquots taken at certain time points (0 hr, 3 hr, 6 hr, 24 hr) for whole cell extraction. These 500 µL aliquots of culture were transferred to 1.7 mL microcentrifuge tubes, spun down for five min at 5000 x *g* at 4°C and then the media was removed. The bacterial cell pellet were resuspended and lysed in 50 µL of Cracking Buffer. Next, 50 µL of 2X SDS Loading Buffer was added and the sample boiled for five min at 99°C. Samples were stored on ice until PAGE gel analysis to observe protein bands in non-induced and induced samples (Section 2.11).

In order to determine if the expressed recombinant protein was soluble or insoluble, 1 mL of whole extract cell sample was centrifuged at 5000 x *g* for five min at 4°C and the media removed. The cell pellet was resuspended in 100 µL of 5X TE buffer (pH 8). Samples were sonicated using a Misonix Sonicator® Ultrasonic Liquid Processor XL2020 with the microtip probe. The sonicator was tuned to manufacturer's instructions before placing the probe in the sample. The sonicator was set to Continuous mode and power setting 1. Samples were sonicated six times for 20 sec pulses with 20 sec between pulses to prevent overheating of the protein samples. Following sonication, the samples were spun at 4°C for five min at 16000 x *g* to pellet out the insoluble fraction. To process the soluble fraction, 50 µL of the supernatant was transferred to a new 1.7 mL

microcentrifuge tube and 50 µL of 2X SDS Loading Buffer was added. The remaining supernatant was discarded from the insoluble fraction and the pellet resuspended in 50 µL each of Cracking Buffer and 2X SDS Loading Buffer. The soluble and insoluble fraction samples were then boiled for five min at 99°C prior to being run on a SDS-PAGE gel and finally stored at -20°C.

### 2.10.2 Protein Expression using *E. cloni*® EXPRESS BL21 (DE3) pLysS

Protein expression of VMO1 isoform 1 was performed in a similar manner to that of the *E. coli* BL21 (DE3) except two changes were made to the LB media. Firstly, filter sterilised 20% glucose was added to the LB broth to a final concentration of 1%. Glucose was used to control non-specific expression. Secondly, chloramphenicol was added to the LB broth to a final concentration of 34 µg/µL to select for the pLysS plasmid. A final concentration of 1mM IPTG was used for protein expression induction and aliquots were taken at varying time points and protein samples were processed for whole cell extracts and soluble and insoluble fractions. Samples were pelleted by centrifugation at 5000 x *g* for five min at 4°C and media was removed and stored at -80°C if samples could not be analysed immediately.

### 2.10.3 Small-scale Time Course Protein Expression Analysis using Rosetta™ (DE3) pLysS

A single colony was selected from a LB agar plate containing chloramphenicol (34 µg/µL) and kanamycin (30 µg/µL) and used to inoculate 5 mL of starter LB broth containing chloramphenicol (34 µg/µL) and kanamycin (30 µg/µL). The starter broth was grown overnight shaking at 200 rpm at 37°C. The following day, 200 µL was used to inoculate two 14 mL tubes of LB broth containing the aforementioned antibiotics. The tubes were grown shaking at 200 rpm at 37°C

until the $OD_{600}$ reached between 0.4 and 0.6, then IPTG (0.4 mM) was added to one of the tubes to induce protein expression whilst the other tube was used as a control. A 0.5 mL sample was taken at time point zero hours from both tubes for analysis of protein expression in the whole cell lysate. The tubes were placed back in a Ratek Orbital Mixer Incubator at 200 rpm at 37°C for 24 hours. At the 24 hour point, 0.5 mL samples were taken from each tube for proteins present in the whole cell lysate and 1 mL samples were taken for fractionated protein samples. All samples were spun at 5000 x *g* for five min at 4°C to pellet cells and supernatant was discarded. Whole cell sample pellets were resuspended in 50 µL of Cracking buffer before adding 50 µL of 2X SDS Loading buffer. The fractionated sample pellets were resuspended in 100 µL of 5X TE buffer and sonicated as per Section 2.10.1. Sonicated samples were centrifuged once again at 5000 x *g* for five min at 4°C to pellet the sample. Following this, 50 µL of supernatant was removed to a new 1.7 mL microcentrifuge tube and mixed with 50 µL of 2X SDS Loading buffer with the rest of the supernatant discarded. The pellet was resuspended in 50 µL of Cracking buffer before adding 50 µL of 2X SDS Loading buffer. Samples were boiled for five min at 99°C on an Eppendorf Thermomixer® comfort set prior to loading 10 µL onto an SDS-PAGE gel for protein separation.

### 2.11 SDS-PAGE Gel

Denaturing sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was used for separation of proteins in whole cell lysates and fractionated samples of bacterial cells. This was to determine the presence of recombinant VMO1 protein expression in bacterial cells. Protein samples were run on three types of gels; premade commercial 10% Mini-PROTEAN® TGX Stain-Free™

Gels (Bio-Rad Laboratories, Inc, USA), premade commercial 12% Express PAGE Gels (GenScript), or 12% hand cast PAGE gels which are recommended for separating out proteins between 20 and 150 kDa (Thermo Fisher Scientific Inc, 2014).

Hand cast gels were made as described in Table 11 with a 4% stacking gel used to focus the protein bands and a 12% separating gel for separation of proteins based on molecular weight. Gels were cast using a Hoefer™ Dual Gel Caster with glass plates, spacers and combs. The glass plates were cleaned with detergent then left to dry. They were cleaned again using 70% ethanol and a small amount of Beckman vacuum grease silicon (USA) was spread evenly along the bottom of the plates to help create a vacuum seal with the caster. The glass plates with the spacers were then placed in the gel caster. The 12% separating gel was prepared in a 50 mL falcon tube (Biologix, China) with the Ammonium persulfate (APS) and Tetramethylethylenediamine (TEMED) added last just before gels were cast to cause the polymerisation of acrylamide. The reagents were mixed gently together before being transferred between the glass plates using a sterile 3.5 mL transfer pipette (Sarstedt, Germany). A layer of 2-butanol was added to the top of the gel once it was poured to exclude air from the top of the gel and help with polymerisation of the gel. Once the gel was set, the 2-butanol was poured off and rinsed using $ddH_2O$. The 4% stacking gel was prepared and added to the top of the separating gel and a comb containing 10 teeth was added to the stacking gel and left to set.

**Table 11: Reagents required for preparing 12% SDS-PAGE hand cast gels with a 4% stacking gel.** Volumes are sufficient to make two gels.

| Reagent | Volume | |
|---|---|---|
| | 12% Separating gel | 4% Stacking gel |
| 22.2% Acrylamide/0.6% Bis (37:1) | 10.81 mL | 2.00 mL |
| 1 M Tris/HCl pH 6.8 | − | 6.54 mL |
| 3 M Tris/HCl pH 8.8 | 2.50 mL | − |
| mQ$H_2O$ | 6.28 mL | 1.25 mL |
| 10% SDS | 200 µL | 100 µL |
| 10% APS | 180 µL | 100 µL |
| TEMED | 26 µL | 10 µL |

### 2.11.1 Gel Electrophoresis

Gel electrophoresis of pre made gels was carried out using the Mini-PROTEAN® Tetra Cell (Bio-Rad Laboratories, Inc, USA) and hand cast gels in a Mini PROTEAN® 3 Cell (Bio-Rad Laboratories, Inc, USA). The glass plates containing the gels were placed in the electrophoresis tanks and covered with 1X Tris-Glycine running buffer (0.025 M Tris-HCl, 0.192 M Glycine, 0.1% SDS, pH 8.5). The premade 12% gels were run using 1X MOPS running buffer (GenScript, USA) Gel combs were removed and the wells rinsed with the running buffer. Protein samples (10 µL) were loaded into the wells and 5 µL of either Precision Plus Protein™ Unstained Standards (Bio-Rad Laboratories, Inc, USA) or PAGE-MASTER Protein Standard Plus (GenScript, USA) protein ladder was added to one of the wells for determination of protein sizes. The SDS-PAGE gel was electrophoresed at 200 V for 45 min until the dye front was approximately a centimetre above the bottom of the gel. The gel was then used for visualisation of protein using Coomassie Blue staining or Bio-Rad Gel Doc™ EZ System. Alternatively, the gel was used for western blotting analysis (Section 2.13).

### 2.11.2 SDS-PAGE Gel Visualisation

Premade 10% gels were visualised using a Bio-Rad Gel Doc™ EZ System using the stain-free plate. The gel was removed from between the glass plates and

placed on the stain-free plate. The plate was then placed in the Gel Doc™ system and the gel imaged using the Image Lab™ Software (Bio-Rad, USA) according to the manufacturer's instructions.

Hand cast gels were visualised using Coomassie Blue R250. The gel was removed from the glass plates and placed into a container and covered with fixing solution (10% acetic acid, 50% methanol in $ddH_2O$) for ten minutes at RT. The fixing solution was removed, and then Coomassie Blue R250 was added to cover and stain the gel overnight, shaking on an Orbital shaker (Bellco Glass Inc., USA) at speed setting four. The following day, the Coomassie Blue R250 was removed and the gel rinsed with $ddH_2O$ before being covered with fixing solution to destain the gel on the shaker for two hours set as above. Once destained, the gel was placed on an Ilford A3 Light Box and photographed for analysis using a COHU High Performance CCD camera and Scion Image – Release Beta 4.0.2 software.

### 2.12 Bradford Assay for Estimation of Protein Concentration

A quick Bradford Assay was performed using Bio-Rad Protein Assay Dye Reagent Concentrate and Bovine serum albumen (BSA) as a standard. Differing concentrations of BSA were made up in 1X PBS from a stock solution of 10 mg/mL Purified BSA (Roche, USA) as per Table 12. Bio-Rad Protein Assay Dye Reagent Concentrate was diluted to 1:4 in $mQH_2O$. This protein assay was used based on the change in colour in response to the concentration of protein added to it as the dye binds to basic and aromatic amino acid residues. Using a 96 well plate, 100 µL of the diluted assay reaction was added to a row of the plate and 1 µL of the differing BSA concentrations were added to each well in ascending concentration order. In the next row, 1 µL of protein samples of

unknown concentrations were mixed with 100 µL of diluted assay reaction. The colour changes were visually compared to the BSA standards for estimation of protein concentration. This was used for quickly estimating the amount of protein to be loaded onto the SDS-PAGE gels for western blots. A more precise measurement can be obtained by measuring the wavelength at 585 nm.

**Table 12: Concentrations of BSA standards made up for the quick Bradford Assay.**

| **Conc. (mg/mL)** | **0** | **0.1** | **0.25** | **0.5** | **1** | **2** | **4** | **6** | **8** | **10** |
|---|---|---|---|---|---|---|---|---|---|---|
| BSA (10 mg/mL) | 0 | 1 | 2.5 | 5 | 10 | 20 | 40 | 60 | 80 | 100 |
| 1X PBS pH 8 | 100 | 99 | 97.5 | 95 | 90 | 80 | 60 | 40 | 20 | 0 |

## 2.13 Western Blot for Detection of Recombinant VMO1 Protein

Following confirmation of presence of recombinant VMO1 protein expression using SDS-PAGE gels to separate out proteins, the samples were run on premade commercial 12% Express PAGE gels and electrophoresed for 30 min at 200 V in a Mini-PROTEAN® Tetra Cell (Bio-Rad Laboratories, Inc, USA) using 1X MOPS running buffer (GenScript, USA). Ten microlitres of protein sample were loaded into wells as well as 4 mg/mL BSA, mouse ear protein extract kindly donated by Miss Blaise Forrester-Gauntlett, and a purified R343A protein, kindly donated by Dr Emma Andrews, which contains a His Tag for positive control with the 6X His Tag antibody (Catalogue number: GTX115045) as it has been previously demonstrated to work (E Andrews, personal communication, January 2015). In addition, 5 µL of each WB-MASTER Protein Standard and PAGE-MASTER Protein Standard Plus (GenScript, USA) protein ladders were both loaded into wells for determination of protein size. The WB-MASTER Protein Standard is specifically designed for western blotting as the proteins present in the

ladder contain IgG banding sites that can bind to primary and secondary antibodies so it can be visualised on the membrane along with the samples.

Followed gel electrophoresis of samples, gels were removed from their encasing and rinsed in dd$H_2O$. Separated proteins on the gels were transferred to a Polyvinylidene Difluoride (PVDF) membrane (Amersham LIFE SCIENCE, UK) using the *e*Blot™ Protein Transfer System (GenScript, USA). The hydrophobic PVDF membrane was dipped into 100% methanol for activation then placed in dd$H_2O$ for one min to rinse. Next, the membrane was placed in *e*Blot™ Equilibrium Buffer for five min. The transfer stack was set up according to manufacturer's instructions. The *e*Blot™ Anode Pad was placed on the anode plate and the pre-wet membrane was placed on top of it. Air bubbles were carefully removed from between the membrane and the anode pad using the supplied shovel. Following this, the gel was placed on top of the membrane and air bubbles carefully removed. The Gel Window was placed on the gel to cover the margin of the membrane. Next, the *e*Blot™ Cathode Pad was placed over the gel and the lid was closed on top of it. The *e*Blot™ Protein Transfer System was run for seven min as recommended for transferring proteins less than 80 kDa in size.

The transferred membranes were placed in containers containing 10% Blocking solution and placed on an orbital shaker at setting four at RT for an hour. The membranes were then rinsed using three washes of 1X Tris Buffered Saline Tween (TBST) for 15 min each. Next, two antibodies, 6X His Tag antibody (GeneTex Inc., USA, Catalogue number: GTX115045) and VMO1 antibody [N1C3] (GeneTex Inc., USA, Catalogue number: GTX106683) were made up to a final ratio of 1:1000 in 10% Blocking solution to a final volume of 2 mL. The mixture was pipetted over the membrane and placed on an orbital shaker at 4°C

overnight to incubate. The following day, antibodies were removed from the membranes and three washes of 15 min with 1X TBST were performed on an orbital shaker at RT. The membranes were incubated for one hour at RT with the secondary antibody, goat anti-rabbit IgG-HRP (Santa Cruz Biotechnology Inc., USA, Catalogue number: sc-2004), made up to a final ratio of 1:5000 in 10% Blocking solution. The membranes were washed three times for 15 min each again with 1X TBST on an orbital shaker at RT before having a final wash in 1X TBST for one hour at RT. Membranes were then rinsed in 1X TBS for 15 min on the orbital shaker at RT.

Proteins were detected on the membranes using the SuperSignal® West Femto Maximum Sensitivity Substrate (ThermoScientific, USA) kit. This kit is an ultrasensitive enhanced chemiluminescent (ECL) substrate for low-femtogram level detection during western blot analysis. It allows for detection of proteins on the membranes that have been probed with primary and secondary antibodies. For the reaction to occur, equal amounts of SuperSignal® West Femto Stable Peroxide Buffer and SuperSignal® West Femto Luminol/Enhancer Solution were combined in a tube and mixed briefly. The reaction was then added to the membranes in equal amounts straight after mixing and placed in a dark cupboard for two min to allow for the reaction to occur.

### 2.13.1 Chemiluminescent Development and Detection of Proteins

Following the reaction of the SuperSignal® West Femto Maximum Sensitivity Substrate with the membrane, the membrane was developed using the FujiFilm Intelligent Dark Box II with the FujiFilm LAS-1000+ camera and accompanying software. The camera was cooled to -25°C before use. The camera was set on chemiluminescence and the light source set to EPI. The membrane was placed on

a black tray in the machine and whilst the door was open, the brightness was adjusted using the FujiFilm LAS-1000+ software. The door was closed and an image taken using the manual setting with the software. The membrane was exposed to chemiluminescence for 45 sec to allow development of the image and the resulting image was saved using the accompanying software.

### 2.13.2 Ponceau S Staining of Western Blot Membrane

After chemiluminescent imaging, membranes were stained using Ponceau S Stain in order to detect proteins present on the membrane. Membranes were covered with the stain for five min and placed on an Orbital shaker (Bellco Glass Inc., USA) on setting 4 at RT. The dye was then poured off and the membrane covered with Ponceau S Destaining solution and placed back on the orbital shaker for 10 min to remove residual stain. The destaining solution was then poured off and membranes were visualised for presence of proteins in each sample.

## 2.14 Small Scale Purification of Recombinant VMO1 Protein

A 5 mL culture flask of Rosetta™ (DE3) pLysS containing the pET28b (+) and *VMO1* construct was induced with IPTG and grown for 24 hours as per Section 2.10.3 for expression of the recombinant VMO1 protein. A final concentration of 1.0 mM IPTG was added for protein induction. Following the 24 hour incubation, the culture was spun down at 5000 x *g* for five min at RT to pellet the cells. The supernatant was removed and the pellet resuspended in 500 µL of NaCl-free Lysis buffer and 500 µL of 2X cOmplete, Mini, EDTA-free Protease Inhibitor solution (Roche, Germany). Samples were then sonicated using a Misonix Sonicator® Ultrasonic Liquid Processor XL2020 with the microtip probe as per Section 2.10.1. Following sonication, the samples were treated with 5 µL of DNase I, RNase-free and 100 µL of 10X Reaction Buffer

(ThermoScientific™, USA). The samples were incubated for 30 min at 37°C in an Eppendorf Thermomixer® comfort. The samples were then centrifuged at 5000 x *g* at 4°C for five min to collect the inclusion bodies. The supernatant was removed and saved for analysis. The inclusion bodies pellets were resuspended in 1X Binding buffer with Urea for the protein purification.

### 2.14.1 Purification of Recombinant VMO1 Protein using HIS-Select® Nickel Affinity Gel

The HIS-Select® Nickel Affinity Gel (Sigma-Aldrich, USA) was used for purification of the recombinant VMO1 protein from the insoluble fraction. The affinity gel solution was mixed gently by inversion before pipetting 50 µL of it into a 1.7 mL microcentrifuge tube. It was spun in a centrifuge at 5000 x *g* for 30 sec at 4°C and the liquid was removed. Next, 250 µL of $mQH_2O$ was added and spun at 5000 x *g* for 30 sec at 4°C and liquid removed. This step was repeated to rinse the solution of the storage buffer than contains ethanol. Next, 200 µL of Equilibration Buffer was added and mixed well with the affinity gel. This mixture was spun at 5000 x *g* for 30 sec at 4°C. The supernatant was removed and discarded before adding 100 µL of the resuspended insoluble fraction. This solution was mixed well and centrifuged at 5000 x *g* for 30 sec at 4°C. The supernatant was saved from this step. Next, the affinity gel was washed with 500 µL of Wash Buffer by mixing briefly then centrifuging at 5000 x *g* for 30 sec at 4°C. The supernatant was collected and saved before repeating the step, again collecting the supernatant. The protein was then eluted from the affinity gel using 50 µL of Elution Buffer at pH 6.0. This was mixed well with the gel and centrifuged at 5000 x *g* for 30 sec at 4°C. The supernatant was collected and the step repeated to collect another supernatant sample. Following this, the affinity

gel was eluted again using 50 μL of Elution Buffer at pH 4.5. This is because recombinant proteins with a histidine tag will not elute at pH 6.0, so it is advised to try a lower pH such as pH 4.5. The solution was mixed well before centrifuging at 5000 x *g* for 30 sec at 4°C. The supernatant was collected and the step repeated to collect another supernatant sample. All collected supernatant samples were processed for analysis with western blot with antibodies.

### 2.14.2 Western Blot of Protein Purification for Detection

Supernatant samples were collected from the protein purification using the HIS-Select® Nickel Affinity gel. Fifteen microlitres of sample was mixed with 15 μL of 2X SDS Loading Dye. Finally, a western blot was undertaken as described in Section 2.13 to identify purified recombinant VMO1 protein and validate the specificity of the VMO1 antibody against a range of protein samples.

# CHAPTER THREE
# Results

This chapter outlines the results obtained using molecular methods for the amplification of *VMO1* mRNA, the cloning and expression of recombinant VMO1 protein, the validation of antibodies against the recombinant protein using western blotting, and finally, the purification of the recombinant VMO1 protein using HIS-Select nickel affinity gel.

## 3.1 Primers for Amplification of Human *VMO1*

Primers that targeted the full length mRNA of *VMO1* isoforms 1 and 3 were designed using Geneious version 7.1.7 and synthesised by IDT Ltd. The primer set SH1F and SH1R were designed to target the *VMO1* isoform 1 gene and the primer set SH2F and SH2R were designed to target the *VMO1* isoform 3 gene. Primers were tested on cDNA synthesised from RNA extracted from two human cell lines, SUM149PT and THP1. The cDNA was kindly donated by Miss Hannah Crossan (UoW).

### 3.1.1 Validation of Previous Findings

The primer set of HCC30/31 was tested on two cell lines to validate the results found by Crossan (2014). Ten PCR reactions were set up as per Table 5 and Table 6 and the resulting amplicons were run on a 2% 1X TAE agarose gel stained with Ethidium bromide for 30 min at 90 V.

Figure 10 shows the agarose gel electrophoresis results of this PCR. Lanes 1 and 8 were loaded with the 100 bp DNA ladder for the determination of band sizes. Amplification of *VMO1* from SUM149PT cDNA resulted in two bands (Lane 2). A faint band at approximately 350 bp which corresponds with the expected

amplified *VMO1* product of 354 bp in size and a band of nearly 800 bp in size which could be possible genomic DNA contamination or non-specific amplification. However, there is a band present around 800 bp in size in the -RT negative control (i.e., template from cDNA synthesis reaction with no reverse transcriptase added). Therefore, results suggest that the SUMP149PT RNA sample was contaminated with genomic DNA. No bands were present in the negative PCR control (Lane 4). This is an indication that the PCR master mix made contained no DNA contamination from exogenous sources.

The positive PCR control primer set HCC37/38 amplifies *GAPDH*, a consecutively expressed gene in humans. The product size of this gene using these primers is 412 bp which can be seen as a very bright band in lane 5 as expected. Lane 6 was a negative reverse transcriptase reaction using the HCC37/38 primers and no bands were present, indicating no genomic contamination. Lane 7 was a negative control in the PCR reaction and contained no bands of amplified DNA as expected due to no cDNA template being present for amplification.

In comparison, no bands were observed from PCR reactions using THP1 cDNA and HCC30/31 primers (Lane 9). The negative PCR control shows that there was no DNA contamination (Lane 10) whereas the *GAPDH* positive control (Lane 11) resulted in a bright band of expected size and a smaller fainter band present of unknown origin. Lane 12 was a negative PCR control with no THP1 template cDNA present. Therefore, no bands were expected to be present, as shown in this lane, due to no DNA being present for amplification.

In conclusion, *VMO1* gene is expressed in SUM149PT and not THP1 cells. Therefore, our data is not in agreement with Hannah Crossan 2014.

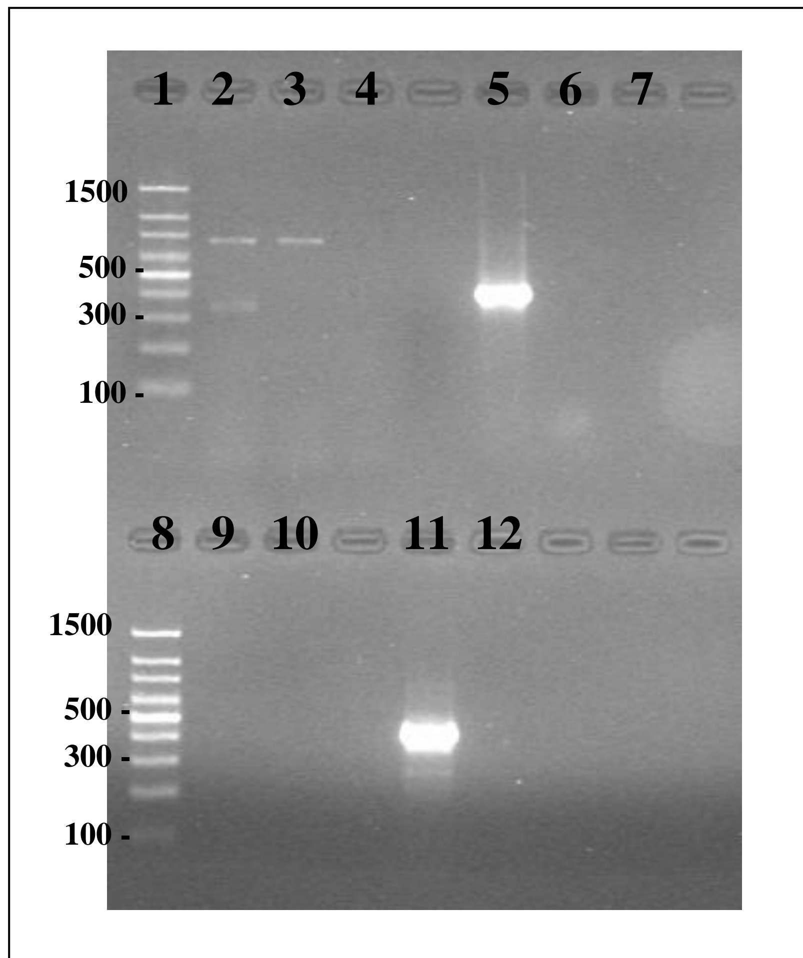


**Figure 10: Amplification of *VMO1* and *GAPDH* from SUM149PT and THP1 cell lines.** PCR products were run on a 2% 1X TAE agarose gel for 30 min at 90 V. Lanes 1 and 8: 100 bp DNA ladder (GenScript, USA); Lane 2: SUM149PT cDNA with HCC30/31; Lane 3: SUM149PT cDNA -RT negative control with HCC30/31; Lane 4: Negative PCR control with HCC30/31; Lane 5: SUM149PT cDNA with HCC37/38; Lane 6: SUM149PT -RT negative control with HCC37/38; Lane 7: Negative PCR control with HCC37/38; Lane 9: THP1 cDNA with HCC30/31; Lane 10: Negative PCR control with HCC30/31; Lane 11: THP1 cDNA with HCC37/38; Lane 12: Negative PCR control with HCC37/38.

### 3.1.2 Gradient PCR

The primer pairs of SH1F/1R and SH2F/SH2R were tested using gradient PCR in order to optimise the PCR protocol for amplification of the expected product size and determine the best annealing temperature. Figure 11 shows the results of the gradient PCR where ranges of annealing temperatures from 55 to 62°C were investigated. Lanes 2 through to 4 contain PCR products run at the annealing temperature of 55.5°C. Lane 2 contained the primer set of SH1F/1R and only contained a faint band below 100 bp in size which is indicative of primer dimers, a common by-product of PCR. This is where the primers in the reaction have attached to one another where complementary bases are present. This leads to amplification of the primer dimer by the Taq polymerase. This indicated that this temperature was too low for this primer set to anneal to the cDNA and allow amplification of the *VMO1* isoform 1 gene. Lane 3 contained the PCR products of primer set SH2F/2R. It shows a faint band of the expected PCR product size of *VMO1* isoform 3 of 387 bp as well as a very faint band below 100 bp of primer dimers. Lane 4 is a negative control for the SH1F/1R primer set and contains no cDNA in the reaction therefore it would be expected to see no bands for amplification of DNA. However, there are bands present below 100 bp from primer dimer formation. Lanes 5 through to 7 contain PCR products run at the annealing temperature of 57.1°C. Lane 5 contains SH1F/1R in the PCR mix and shows no bands other than primer dimers below 100 bp. This indicates that this temperature is not ideal for amplification of the *VMO1* isoform 1 gene. Lane 6 contains SH2F/2R and has no bands other than primer dimers. Therefore, this primer set did not amplify the *VMO1* isoform 3 gene. Lane 7 is a negative control PCR containing the SH2F/2R primer set and no template cDNA to amplify. Therefore, it is expected that there are no bands present however there are primer

dimers present below 100 bp. Both lanes 8 and 9 had annealing temperatures of 60.4°C. Lane 8 contains the primer set SH1F/1R. Multiple bands are present in this lane however the *VMO1* isoform 1 gene, with an expected size of 691 bp, is present in the lane as the brightest band. It is unsure what the other bands present in the lane are without isolating them and sequencing them but they may be caused by non-specific binding of the primer set to the cDNA. Lane 9 contains the primer set of SH2F/2R and shows multiple bands present. However the brightest band in the lane corresponds with the expected size of the *VMO1* isoform 3 gene. Lanes 10 and 11 had an annealing temperature of 62.0°C and used the primer sets of SH1F/1R and SH2F/2R respectively. Lane 10 also shows multiple bands present including primer dimers with the expected product of 691 bp being the brightest band in the lane. Lane 11 shows multiple bands with the brightest band being the expected product of 387 bp in size.

Following the results of the gradient PCR, the annealing temperature of 60°C was selected for the primer sets SH1F/1R and SH2F/2R for future PCR applications.

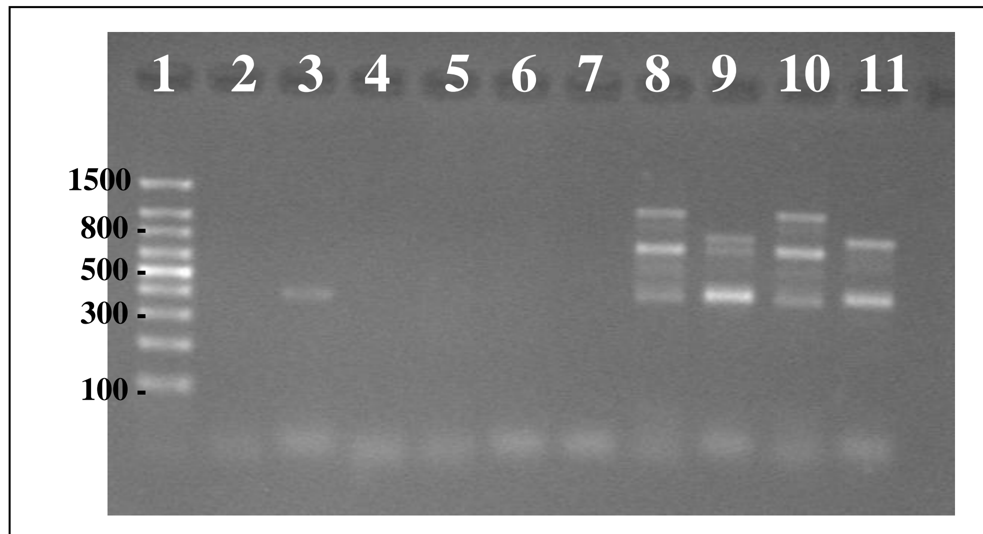


**Figure 11: Gradient PCR of amplified SUM149PT cDNA with SH1F/1R or SH2F/2R primer pairs.** Products were run on a 2% 1X TAE agarose gel for 30 min at 90 V. Lane 1: 100 bp DNA ladder (GenScript, USA). The following primer pair and annealing temperature was used; Lane 2: SH1F/1R at 55.5°C ; Lane 3: SH2F/2R at 55.5°C; Lane 4: Negative PCR control using SH1F/1R at 55.5°C;Lane 5: SH1F/1R at 57.1°C; Lane 6: SH2F/2R at 57.1°C; Lane 7: Negative PCR control using SH2F/2R at 57.1°C; Lane 8: SH1F/1R at 60.4°C; Lane 9: SH2F/2R at 60.4°C ; Lane 10: SH1F/1R at 62.0°C.

### 3.1.3 Unexpected PCR Results.

PCR was performed to amplify the *VMO1* isoform 1 and 3 genes using the primer pairs SH1F/1R and SH2F/2R. The PCR cycle performed was per Table 6. Figure 12 shows the results of this PCR run on an agarose gel. Lane 1 contains the 100 bp DNA ladder (GenScript, USA). Lane 2 contains the SUM149PT cDNA amplified by primer set HCC37/38 which targets the *GAPDH* gene. A band at the expected size, 412 bp, was present in the agarose gel. Therefore, the positive control was successful and the components of the reaction and the thermal cycler conditions were configured properly and working efficiently. In addition, both negative controls worked, showing that no DNA was produced when there was no reverse transcriptase present in the cDNA synthesis reaction (Lanes 3, and 9) or no DNA template added (Lanes 4, 7 and 10). Lanes 5 though to 7 used the primer set SH1F/1R to amplify the *VMO1* isoform 1 gene in SUM149PT cDNA. Lane 5

contained the cDNA synthesised and a PCR product of 691 bp was predicted to be in this lane. However, there were no bands present in the gel despite the primers demonstrating working in Figure 11. Lanes 8 through to 10 were PCR products from the reaction using the primer set SH2F/2R designed to amplify the *VMO1* isoform 3 gene. A product of 387 bp was expected to be amplified by these primers (Lane 8). However, no band was visible suggesting that the primers had not amplified the gene.

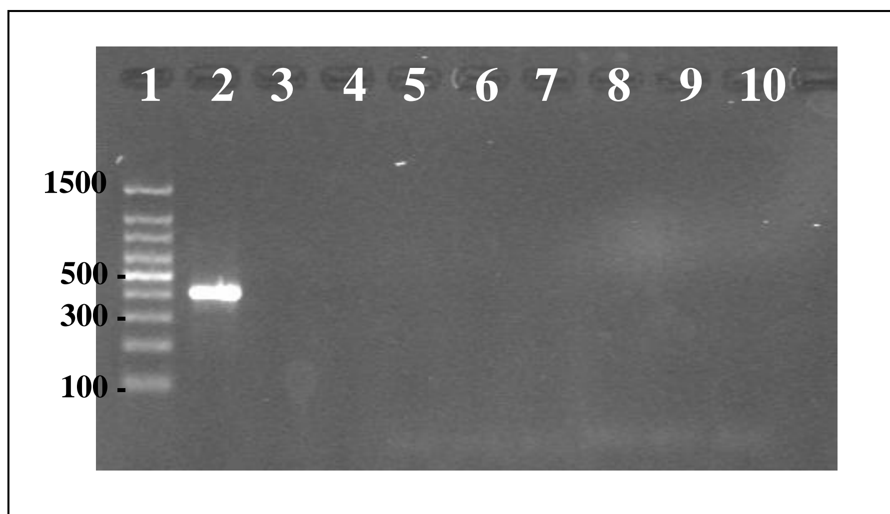


**Figure 12: PCR amplification of SUM149PT cDNA.** PCR products were run on a 2% 1X TAE agarose gel for 30 min at 90 V. Lane 1: 100 bp DNA ladder (GenScript, USA); Lane 2: Positive PCR control with HCC37/38; Lane 3: SUM149PT cDNA negative RT control with HCC37/38; Lane 4: Negative PCR control with HCC37/38; Lane 5: SH1F/1R; Lane 6: SH1F/1R; Lane 7: Negative PCR control with SH1F/1R; Lane 8: SH2F/2R; Lane 9: SUM149PT cDNA negative RT control with SH2F/2R; Lane 10: Negative PCR control with SH2F/2R.

The results of this PCR suggest that the primer sets SH1F/1R and SH2F/2R did not amplify their targeted genes in the reaction. This result suggests that the *VMO1* gene is not expressed in the SUM149PT cell line, compared to mRNA which was provided by Crossan (2014).

## 3.2 Multiple Human Cell Line Testing for Expression of the *VMO1* Gene

Following the results of the PCR amplification of SUM149PT cDNA using the primer sets SH1F/1R and SH2F/2R, it was decided to extract a new batch of RNA from three mammalian cell lines and test the newly synthesised cDNA for the presence of *VMO1* gene expression. The cell lines selected were A549, a lung carcinoma derived cell line, THP1 and SUM149PT.

### 3.2.1 RNA Concentration and Purification using NanoDrop

Following isolation of RNA from the three different cell culture lines, samples were measured using a ThermoScientific™ NanoDrop 2000 Spectrophotometer and accompanying software (Table 13). The RNA was DNase-treated as per Section 2.1.3 prior to testing the quality and quantity using the NanoDrop. RNA was tested to determine concentration and purity to decide if it was suitable for downstream applications such as cDNA synthesis. The 260/280 absorbance ratio of RNA is generally accepted as being pure when it is around 2.0. In addition to this, the 260/230 absorbance ratio was determined by the NanoDrop. This ratio indicates the presence of contamination from organic compounds such as phenol and TriZol used during the extraction process. As a result of these values, a low 260/230 ratio can be caused by glycogen used as a coprecipitate during RNA extraction.

**Table 13: Concentration and purity of RNA extracted from human cell lines.** The 260/230 and 260/280 ratios were used for determining purity and presence of contamination in the samples following extraction.

| Cell line | 260/230 | 260/280 | Concentration (ng/µL) |
|---|---|---|---|
| A549 | 0.79 | 1.80 | 97.2 |
| SUM149PT | 0.69 | 1.68 | 87.0 |
| THP1 | 0.68 | 1.39 | 15.4 |

In conclusion, RNA was extracted from three human cell lines ranging from 15.4 to 97.2 ng/μL and contained impurities. Based on these results, RNA was converted to cDNA using the Tetro cDNA Synthesis Kit (Bioline, USA) as per Section 2.1.4.

### 3.2.2 cDNA Concentration and Purification using NanoDrop

Following cDNA synthesis, the samples were tested for their concentration using a ThermoScientific™ NanoDrop 2000 Spectrophotometer and accompanying software (Table 14). The 260/280 ratio was determined by reading the spectra of the samples at 260 nm and 280 nm respectively. This measure is a good indication of DNA purity as nucleic acids have a maximum absorbance at 260 nm and proteins at 280 nm. However, it is noted that the resulting cDNA samples were not purified and therefore residual salts, RNA, dNTPs and primers may interfere with the reading output. Table 14 shows that THP1 cDNA had a very low concentration in relation to the other two samples. This is due to differences in the concentration of the RNA template (Table 13) in the 2 μL volume.

**Table 14: Concentration and purity of cDNA converted from mRNA extracted from human cell lines.** The 260/230 and 260/280 ratios were used for determining purity and presence of contamination in the samples following extraction.

| Cell line | 260/230 | 260/280 | Concentration (ng/μL) |
|---|---|---|---|
| A549 | 1.99 | 1.60 | 1185.3 |
| SUM149PT | 1.58 | 1.31 | 2022.1 |
| THP1 | 1.07 | 1.24 | 204.0 |

Following determination of cDNA quantity using the NanoDrop, the samples were used for PCR amplification with a number of primer sets.

### 3.2.3 PCR Amplification of cDNA Extracted from Human Cell Lines

PCR was performed on each of these cell lines using the protocol as per Section 2.3 using the primer sets SH1F/1R, SH2F/2R, HCC30/31, and HCC37/38. The results of the PCR amplification of these cell lines are shown in Figure 13. The 100 bp DNA ladder (GenScript, USA) was loaded into the first well of each row on gel for determination of product sizes in the gel. The extracted cDNA from THP1 was loaded into lanes 2 – 6 on the gel. The THP1 cDNA was amplified with SH1F/1R and produced a PCR product of 691 bp as shown in lane 2. There was also an unknown PCR product of approximately 1000 bp produced as shown by a fainter band in lane 2. Amplification of the THP1 cDNA by SH2F/2R produced multiple products as shown in lane 3; however the expected product of 387 bp was present. A band at 691 bp in this lane suggests that this primer set may also amplify the *VMO1* isoform 1 gene. The amplification of the cDNA by HCC30/31 also produced a number of products including the predicted 354 bp product of the *VMO1* gene (Crossan, 2014). Amplification of the *GAPDH* gene by HCC37/38 was performed as a positive control and produced the predicted product as shown in lane 5.

The SUM149PT cDNA was amplified with the same primers as above. These PCR products were loaded in lanes 6 through to 11. The SH1F/1R primers did not produce any PCR products and this was shown in lane 6 as there were no bands present in the gel. SH2F/2R also failed to amplify the cDNA as shown in lane 7. In addition to this, the primer set HCC30/31 was tested against the SUM149PT cDNA as it had previously been demonstrated to work with this cell line (Crossan, 2014). However, this failed to produce the expected product of 354 bp as shown in lane 8. The *GAPDH* gene was amplified by primer set HCC37/38 as a positive

control for this cell line and the product was loaded into lane 9, producing a very bright band of 412 bp in size as expected. Lane 10 was a negative control of the cDNA synthesis reaction using the HCC37/38 primer set with no template DNA present and no products were expected to be amplified and the lane should not have contained bands. However, there is a faint band of a similar size to the *GAPDH* gene product of 412 bp which was loaded into the lane next to it. This suggests that the product has contaminated the next lane, causing the band to be visible. Lane 11 was the negative PCR control using the SH2F/2R primer set with no cDNA present to test for contamination. As expected, there were no PCR products produced as there were no bands present in the gel.

The A549 cDNA was tested with the SH1F/1R primers to determine if the *VMO1* isoform 1 gene would be amplified. The PCR of this was loaded into lane 12 and no bands were detected on the gel suggesting that *VMO1* is not expressed in the A549 cell line. This was the same for the SH2F/2R primer set loaded into lane 14 and the HCC30/31 primer set loaded in lane 15. Neither of these primer sets showed amplification of products. The *GAPDH* gene was amplified in the A549 cDNA and produced a product of the expected size, as shown in lane 16 with a very bright band. The negative A549 cDNA synthesis control was amplified using primer set HCC37/38 and loaded into lane 17 and no bands were present indicating no DNA was present in the reaction as expected. A negative PCR control was performed using HCC30/31 and was loaded into lane 18 and did not produce any products as expected.

Finally, the previously provided SUM149PT cDNA sample was amplified using the same primers as above. The PCR products produced by SH1F/1R was loaded into lane 19 and showed the amplification of a number of products. The expected

product of 691 bp was present in this sample showing that amplification of the expected gene occurred. The amplification by SH2F/2R was loaded into lane 20 and produced a faint band of the expected product size, 387 bp. In addition, an unknown PCR product of around 800 to 900 bp was present on the gel. The amplification of the cDNA by HCC30/31 was loaded into lane 21 and showed the expected product at 354 bp as a faint band as well as a brighter unknown product around 800 bp in size. The *GAPDH* gene was amplified by HCC37/38 as a positive control for the PCR reaction and produced the expected product as seen as a very bright band in lane 22. The negative cDNA synthesis control was tested using primer set HCC37/38 and no bands were expected to be present in the gel. However, there was a product approximately 500 bp in size suggesting that the RNA contained genomic DNA contamination. Therefore, it is recommended that a DNase treatment is applied to future RNA extractions to remove genomic DNA. Finally, a negative PCR control was performed using HCC37/38 and no products from this reaction were detected on the gel as expected.

The results of this PCR indicate that the *VMO1* isoforms 1 and 3 genes are expressed in THP1 cell lines. Also, our results suggest that *VMO1* is not expressed in the lung and breast.

The resulting positive THP1 PCR products were loaded onto a low melting point gel and bands of the expected size were cut from the gel for purification (Section 2.5.2). These purified products were used for ligating into the pET28b(+) vector as per Section 2.3.7.

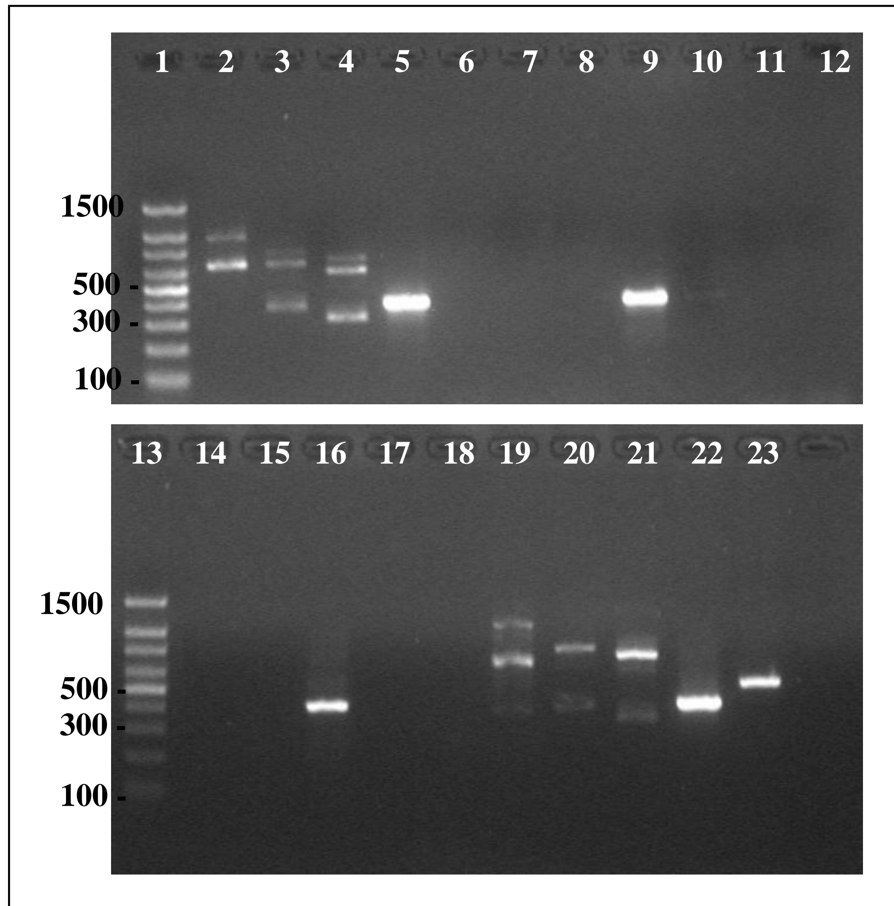


**Figure 13: PCR amplification of cDNA from human cell lines A549, SUM149PT, and THP1 using primers designed to amplify *VMO1* genes.** Annealing temperature was 60°C. PCR products were run on a 2% 1X TAE agarose gel for 30 min at 90 V. Lanes 1 and 13: 100 bp DNA ladder (GenScript, USA); THP1 cDNA: Lane 2: SH1F/1R; Lane 3: SH2F/2R; Lane 4: HCC30/31; Lane 5: HCC37/38. SUM149PT cDNA: Lane 6: SH1F/1R; Lane 7: SH2F/2R; Lane 8: HCC30/31; Lane 9: HCC37/38; Lane 10: negative RT control with SH2F/2R; Lane 11: Negative PCR control with SH2F/2R. A549 cDNA: Lane 12: SH1F/1R; Lane 14: SH2F/2R; Lane 15: HCC30/31; Lane 16: HCC37/38; Lane 17: Negative RT control with HCC37/38; Lane 18: Negative PCR control with HCC30/31. Provided SUM149PT cDNA (Crossan, 2014): Lane 19: SH1F/1R; Lane 20: SH2F/2R; Lane 21: HCC30/31; Lane 22: HCC37/38; Lane 23: Negative RT control with HCC37/38; Lane 24: Negative PCR control with HCC37/38.

### 3.3 Digestion of pET28b Vector using Restriction Enzymes

The pET28b(+) vector was digested overnight at 37°C using the BamHI and EcoRI enzymes. Four reactions were set up; a negative control with no restriction enzymes, a double digest, and two tubes each containing one each of the enzymes. Figure 14 shows the agarose gel results of the digest. Multiple bands are observed for the negative control (Lane 2) due to the differing conformations of plasmid DNA from top to bottom on the gel; nicked/relaxed circle, linear, supercoiled, and

circular, single stranded DNA. Despite the pET28b(+) vector being 5368 bp in size, these four conformations will migrate through the gel at differing speeds and show bands at different places relative to size in comparison to the DNA ladder. Lane 3 shows the pET28b(+) vector which has been digested with both BamHI and EcoRI. This digest cuts out a small section of six base pairs from the vector, making a linear product of 5362 bp in size with sticky overhangs for cloning in of the *VMO1* DNA. Both lanes 4 and 5 show pET28b(+) digested with either BamHI or EcoRI respectively. This was performed to show that the enzymes worked individually. Lane 5 shows multiple bands which is due to incomplete digestion of the entire vector in the reaction by the EcoRI enzyme. Therefore, some of the conformations of the plasmid DNA can be seen.

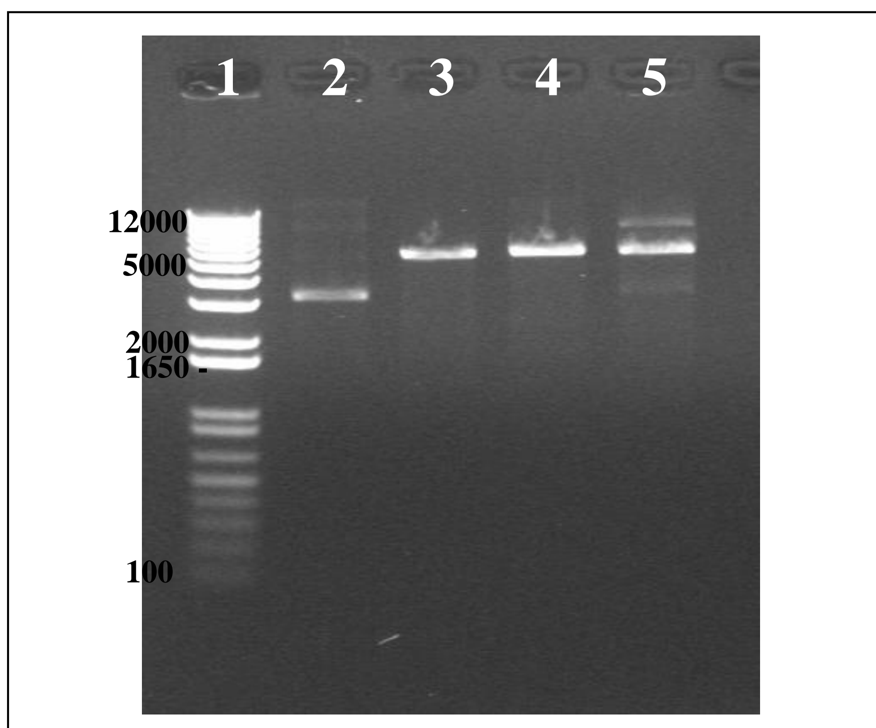


**Figure 14: Restriction enzyme digest of pET28b(+) using EcoRI and BamHI.** Products were run on a 0.9% 1X TAE agarose gel for 30 min at 90 V. Lane 1: 1KB+ DNA Ladder (Invitrogen, USA); Lane 2: Control digest containing no enzymes; Lane 3: Double digest containing both enzymes; Lane 4: Digest using BamHI; Lane 5: Digest using EcoRI.

The next step was to load the remaining 45 µL double digested pET28b(+) sample on a 1% TAE LMP agarose gel stained with 2 µL of Ethidium bromide. The gel was run for 30 min at 90 V before being viewed on a Safe Imager™ Transluminator (Invitrogen, USA) to prevent damage to the DNA present in the gel. The band was cut from the gel and recovered using the ZymoClean™ Gel DNA Recovery Kit and purified using the ZymoClean™ Genomic DNA Clean and Concentrator Kit™. The resulting product was run on a gel to estimate the concentration of the product for ligation of the vector with purified *VMO1* DNA (Figure 15).

### 3.4 Estimation of concentration of products

Amplified *VMO1* isoforms 1 and 3 were recovered from a LMP gel using the ZymoClean™ Gel DNA Recovery Kit and digested overnight at 37°C using the restriction enzymes, BamHI and EcoRI. This was performed to create sticky overhangs on the DNA fragments for cloning into the pET28b(+) vector. Following this, the digested reactions were purified using the ZymoClean™ Genomic DNA Clean and Concentrator Kit™ leaving 15 µL of each purified product. Wells were loaded with 5 µL of product mixed with 2 µL of 6X Loading dye. The 100 bp DNA ladder (GenScript, USA) was used for estimation of concentration of products and determination of size. GenScript states that their 500 bp indicator in the ladder has a concentration of 20 ng/µL and all other bands having a concentration of 10 ng/µL. Products were run on a 2% 1X TAE agarose gel for 30 min at 90 V (Section 2.5.2).

Figure 15 shows the gel that was used for the estimation of the concentrations of each of the products run on it. These estimations were used to calculate the Ligation calculation (Equations 3 and 4). Lane 1 was loaded with the 100 bp DNA

ladder (GenScript, USA). Lane 2 was loaded with the purified digested *VMO1* isoform 1 PCR product. This was estimated as having a concentration of 25 ng or 5 ng/µL based on the volume loaded onto the gel. Lane 3 was loaded with purified digested *VMO1* isoform 3 PCR product. This was also estimated as having a concentration of 25 ng or 5 ng/µL. Lane 4 was loaded with the purified, digested pET28b(+) vector. This was estimated to have a concentration of 150 ng or 30 ng/µL based on the brightness of the band in comparison to the 500 bp indicator in the DNA ladder. These estimations were used for calculations for the ligation reaction.

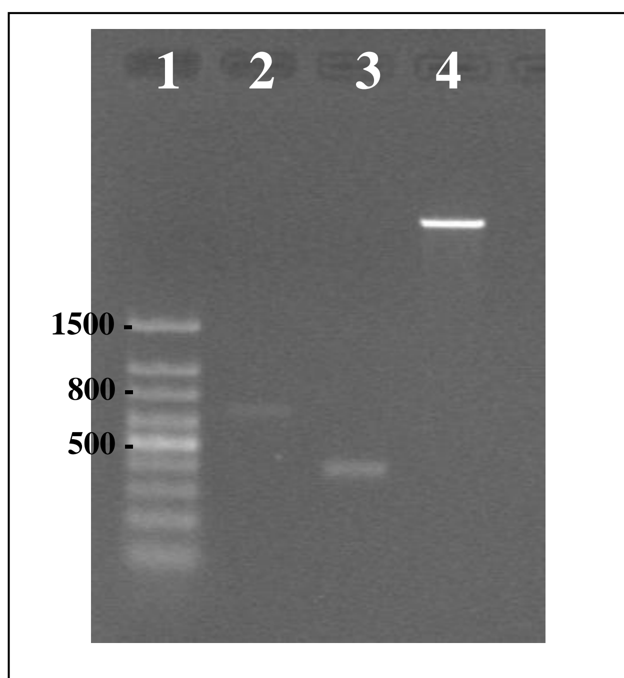


**Figure 15: Estimation of concentration of purified products for ligation calculations.** The purified products were loaded on a 2% 1X TAE agarose gel and run for 30 min at 90 V. Lane 1: 100 bp DNA ladder (GenScript, USA); Lane 2: Purified, BamHI/EcoRI digested *VMO1* isoform 1 gene; Lane 3: Purified, BamHI/EcoRI digested *VMO1* isoform 3 gene; Lane 4: Purified, BamHI/EcoRI digested pET28b(+) vector.

### 3.4.1 Ligation

Estimations of DNA concentrations of purified products were used for the calculation of amount of product (Equations 3 and 4) needed for the ligation

reaction so that a molar ratio of 1:3 of vector to insert was optimal for cloning purposes. The *VMO1* isoform 1 gene insert was calculated as requiring 11.6 ng/µL for the ligation reaction. As the concentration of this product was estimated at 5 ng/µL (Figure 15), 3 µL was added to the ligation reaction along with 1 µL of the purified, digested pET28b(+). The *VMO1* isoform 3 gene was calculated as needing 6.5 ng/µL in the ligation reaction and as a result, 5 µL of the product was added to the ligation reaction with 1 µL of purified, digested pET28b(+). The ligated products were used for transformation into *E. coli* DH5α using electroporation (Section 2.8.2).

$$ng/\mu L\ PCR\ insert\ required\ = \frac{30\ ng/\mu L\ x\ 691\ bp}{5362\ bp}\ x\ 3 = 11.6\ ng/\mu L$$

**Equation 3: Ligation calculation of the *VMO1* isoform 1 gene concentration needed to ligate into the pET28b(+) vector.**

$$ng/\mu L\ PCR\ insert\ required\ = \frac{30\ ng/\mu L\ x\ 387\ bp}{5362\ bp}\ x\ 3 = 6.5\ ng/\mu L$$

**Equation 4: Ligation calculation of the VMO1 isoform 3 gene concentration needed to ligate into the pET28b(+) vector.**

## 3.5 Transformation of pET28b(+)/*VMO1* Construct into Electrocompetent *E. coli* DH5α

Following overnight ligation of the *VMO1* gene into the pET28b(+) (Section 2.7.3), the ligated product was transformed into electrocompetent *E. coli* DH5α and plated onto LB agar plates containing kanamycin. A positive control of pET28b(+) was also transformed for calculation of the transformation efficiency as per Equation 3. To calculate this efficiency, the numbers of colonies present on the LB agar plate were counted. A value higher than $10^9$ is considered as being

high efficiency grade; however the efficiency was calculated as being lower than $10^6$ which is considered low for electrocompetent cells. Despite having a low transformation efficiency of 2.7 x $10^5$, colonies transformed with the pET28b(+)/*VMO1* isoform 1 construct were present on the LB agar and kanamycin plate. A number of these colonies were tested using colony PCR (Section 2.3.2) for confirmation that the ligation of the *VMO1* gene was successful into the pET28b(+) vector.

**3.5 1 Gradient PCR of T7 Primers**

Gradient PCR was performed on purified pET28b(+) vector DNA using the primer set LMP10F/11R in order to determine the best annealing temperature. The LMP10F primer was designed to anneal to the T7 promoter of the pET28b(+) vector and LMP11R to the T7 terminator of pET28b(+), resulting in an amplicon of 373 bp in size. These regions were selected to amplify from as the MCS of pET28b(+) falls between these two regions and this is where the *VMO1* gene has been cloned into. The PCR was set up as per Section 2.3.1.

Figure 16 shows the results of the gradient PCR. Lane 1 was loaded with 5 µL of 100 bp DNA ladder (GenScript, USA). Lane 2 contained the PCR negative control with no template DNA and no bands were expected, as seen, to be present as a test for contamination. Lane 3 contained the PCR product with an annealing temperature of 52.0°C. As there were no bands present, this indicated that the primers did not anneal to the pET28b(+) vector at this temperature. The PCR product with an annealing temperature of 54.3°C was loaded into lane 4. This lane contained a band at 373 bp, as expected to be seen. However it contained a faint band around 250 bp in size of unknown identity. When the PCR had an annealing temperature of 55.7°C, there was a band at 379 bp but the band of around 250 bp

was also brighter compared to at 54.3°C, as seen in lane 5. The final annealing temperature tested was 58.0°C and produced two products of 379 bp and 250 bp, however the band at 250 bp was fainter than that at 55.7°C. The annealing temperature of 60°C was not tested in this gradient PCR however it was used for downstream applications with this primer set to attempt to obtain a single band of the correct size when visualised on an agarose gel.

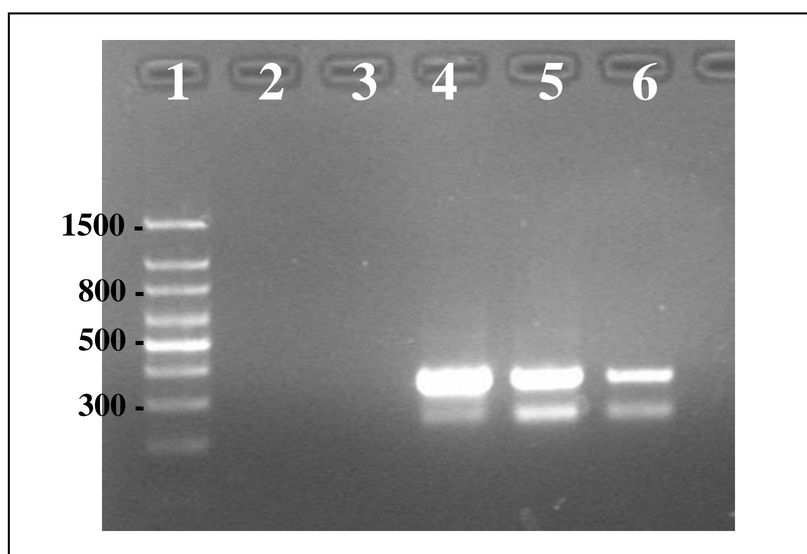


**Figure 16: Gradient PCR using the primer set LMP10F and LMP11R.** LMP10F binds to the T7 promoter in the pET28b(+) vector. LMP11R binds to the T7 terminator in the pET28b(+) vector. Products were loaded on a 2% 1X TAE agarose gel and run for 30 min at 90 V. Lane 1: 100 bp DNA ladder (GenScript, USA); Lane 2: Negative PCR control with no template DNA at 52.0°C annealing temperature; Lane 3: Purified pET28b(+) vector with LMP10F/11R at 52.0°C annealing temperature; Lane 4: Purified pET28b(+) vector with LMP10F/11R at 54.3°C annealing temperature; Lane 5: Purified pET28b(+) vector with LMP10F/11R at 55.7°C annealing temperature; Lane 6: Purified pET28b(+) vector with LMP10F/11R at 58.0°C annealing temperature.

### 3.5.2 Colony PCR of Transformed *E. coli* DH5α for Confirmation of Construct Presence

White colonies were selected from plates following electroporation to test for positive *VMO1* isoform 1 inserts using Colony PCR. Briefly, colonies were selected using a sterile toothpick and dipped into a tube containing PCR reagents

and primers designed to target the T7 promoter (LMP10F) and T7 terminator (LMP11R) as per Section 2.3.2. In total, 12 colonies were tested for confirmation that *VMO1* had been ligated into the pET28b(+) vector. The PCR results are shown in Figure 17 and shows that the positive control of undigested pET28b(+) without an insert amplified with the LMP10F/11R primers, resulting in expected PCR product size of 379 bp in size (Lane 2). A single band was obtained by using the annealing temperature of 60°C instead of 58°C as this temperature produced two products (Figure 16, lane 6). A negative PCR was also performed to test for contamination in the PCR reaction. No bands were expected to be present due to no template DNA being present for amplification. This reaction was loaded into lane 3 and no bands were visualised in the gel, indicating that no contamination occurred. All 12 colonies tested via colony PCR resulted in a band at 373 bp which indicated that the ligation had been unsuccessful. If the *VMO1* isoform 1 gene had been inserted into the MCS, a product of 1064 bp was expected to be amplified in the PCR. The PCR conditions were set to meet this as it is recommended that an extension time of 30 sec to one min is used per 1 kB sized product (Cheng, Chang, Gravitt, & Respess, 1994). These lanes also contained faint bands below 100 bp which could be from DNA present in the bacterial colonies or PCR primer dimers.

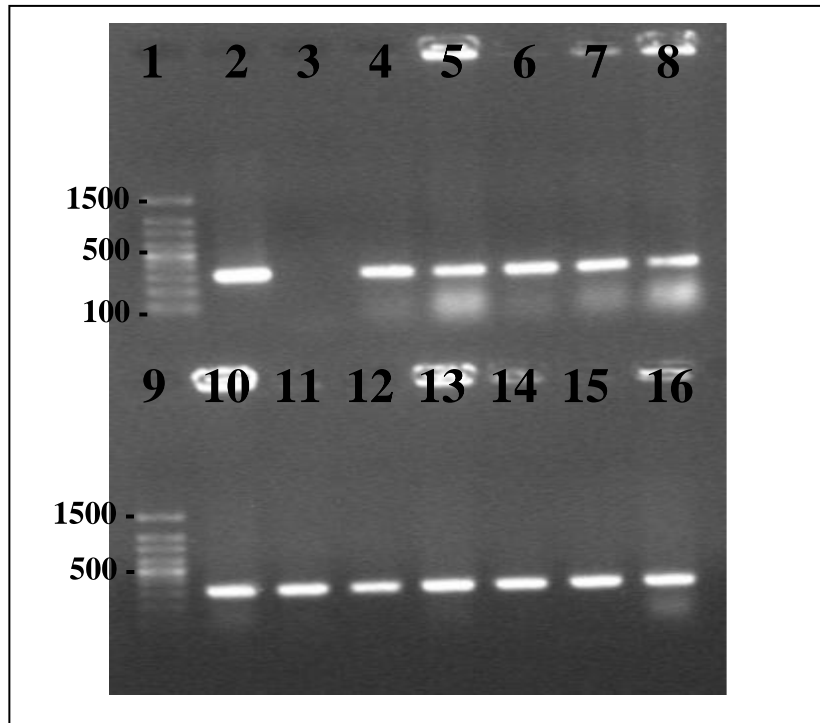


**Figure 17: Colony PCR of *E. coli* DH5α inserted with the pET28b(+) vector to check for presence of the *VMO1* isoform 1 gene insert.** The PCR products were loaded on a 2% 1X TAE agarose gel and run for 30 min at 90 V. Primers used were LMP10F and LMP11R. Lanes 1 and 9: 100 bp DNA ladder; Lane 2: Positive PCR control; Lane 3; Negative PCR control; Lanes 4 – 8 and 10-16: Colonies selected from the transformed plate.

### 3.6 Ligation and Transformation of the pBlueScript II SK (+)/*VMO1* Constructs into Chemical Competent *E. coli*

Following the unsuccessful transformation of the *VMO1* isoform 1 gene into the pET28b(+) vector, ligation of the gene into a T-tailed pBlueScript II SK (+) vector was attempted. In addition to this, the *VMO1* isoform 3 gene was also ligated into the pBlueScript II SK (+) vector. The ligated products were then transformed into chemically competent *E. coli* DH5α and plated onto an LB agar plate containing ampicillin. The plates were also spread with IPTG and X-Gal for blue and white colony screening. Plates were grown overnight at 37°C and the next day investigated for the presence of white colonies (Figure 18). These colonies were selected for Colony PCR using the LMP6F primer which binds to the M13

Forward primer and the LMP7R primer which binds to the M13 Reverse primer in the pBlueScript II SK (+) vector.

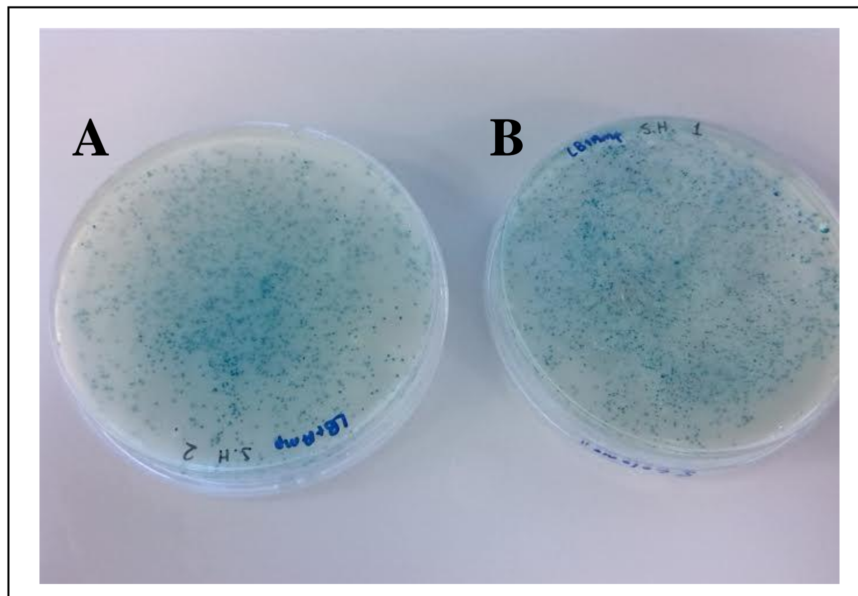


**Figure 18: Chemical competent *E. coli* DH5α cells contained the pBlueScript II SK (+) vector.** Colonies were plated onto LB agar plates containing ampicillin for selected growth of colonies containing the vector. The plates were also spread with IPTG and X-Gal for blue and white colony screening. Following transformation, plates were grown upside down at 37°C overnight. Plate A contains colonies transformed with the pBlueScript II SK (+) vector containing the *VMO1* isoform 3 gene. White colonies were selected for Colony PCR using primers LMP6F and LMP7R. Plate B contains colonies transformed with the pBlueScript II SK (+) vector containing the *VMO1* isoform 1 gene. White colonies were selected for Colony PCR using primers LMP6F and LMP7R.

### 3.6.1 Colony PCR of pBlueScript II SK (+)/*VMO1* genes

White colonies on the LB plates were selected for Colony PCR to test for presence of the *VMO1* inserts into the pBlueScript II SK (+). The primers selected were LMP6F for the M13 Forward primer and LMP7R for the M13 Reverse primers. Both these primers anneal to a specific region on the pBlueScript II SK (+) vector, on either side of the ligation site (Appendix Two). Ten microlitres of PCR product were mixed with 2 µL of 6X Loading dye and loaded into a 2% 1X TAE agarose gel stained with Ethidium bromide. The gel was run for 30 min

at 90 V to separate out the products then viewed under a UV light and imaged (Figure 19).

The PCR products from white colonies selected from the plate with the pBlueScript II SK (+)/*VMO1* isoform 1 construct were loaded into lanes 2 through to 6. An expected product of 918 bp was seen on the gel as a bright band in all of these wells showing that each of the colonies contained the *VMO1* isoform 1 gene inserted into the vector. These lanes also contained very faint bands above 1500 bp in size which could be non-specific amplification during PCR. Also, primer dimers below 100 bp in size were observed in each PCR reaction.

The PCR products from white colonies selected from the plate with the pBlueScript II SK (+)/*VMO1* isoform 3 construct were loaded into lanes 7 through to 11. An expected PCR product of 614 bp was seen in all of these lanes, confirming that the ligation had been successful. In addition to this, primer dimers less than 100 bp in size were present in each of the lanes.

Following these results, the five positive colonies containing the pBlueScript II SK (+)/*VMO1* isoform 1 construct and five positive colonies containing pBlueScript II SK (+)/*VMO1* isoform 3 construct were tested again using Colony PCR to determine the direction of the inserted genes. The 3´ A-overhang PCR product can ligate into the 3´ T-overhangs vector in either orientation. Therefore, it is important that the orientation is known so that the cloned *VMO1* insert can be digested and ligated into pET28b(+) and the coding of the VMO1 recombinant protein is in-frame.

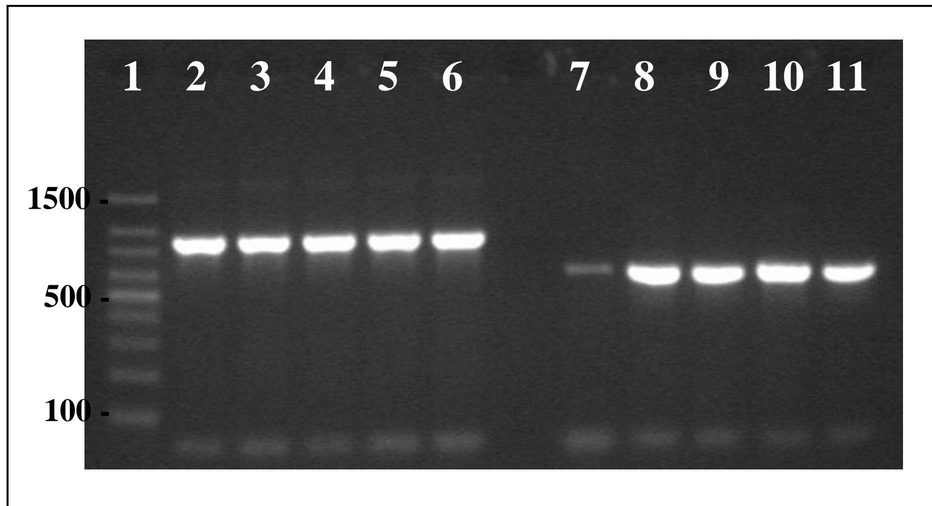


**Figure 19: Colony PCR results testing *E. coli* DH5α transformed with the pBlueScript II SK (+) vector ligated with either *VMO1* isoform 1 or 3.** PCR products were run on a 2% 1X TAE agarose gel stained with Ethidium bromide for 30 min at 90 V. Lane 1: 100 bp DNA ladder (GenScript, USA); Lanes 2 – 6: White colonies tested for presence of *VMO1* isoform 1 gene in the pBlueScript II SK (+) vector using the primer set LMP6F/LMP7R; Lanes 7 – 11: White colonies tested for presence of *VMO1* isoform 3 gene in the pBlueScript II SK (+) vector using the primer set LMP6F/7R.

### 3.6.2 Directional Colony PCR using LMP7 and LMP6

Firstly, the SH1R primer was used in combination with LMP6F as the SH1R primer would anneal to the 3' end of the *VMO1* isoform 1 gene and the LMP6F to the M13 Forward primer sequence in the pBlueScript II SK (+)/*VMO1* isoform 1 construct. With this primer pair, a band of 789 bp in size is expected. In addition to this, the same colonies were tested with SH1F and LMP7R, amplifying from the 5' start of the *VMO1* isoform 1 gene and ending at the M13 Reverse primer sequence in the pBlueScript II SK (+)/*VMO1* isoform 1 construct. This would result in the amplification of a of 820 bp band. If the gene was inserted in the correct orientation, the start of the *VMO1* gene would be closest to the M13 Forward primer region and the end of it closest to the M13 Reverse primer.

The same process was repeated to colonies containing the pBlueScript II SK (+)/*VMO1* isoform 3 construct using SH2F with LMP7R and SH2R with LMP6F.

Five positive clones for pBlueScript II SK (+)/*VMO1* isoform 1 construct were tested via Colony PCR with the SH1R and LMP6F primers. Only three colonies resulted in a positive result bright band of 789 bp in size. Thus, indicating that the gene was inserted in the correct direction (Figure 20, Lanes 3, 5, and 6). Smearing was also present which could be caused by extracellular debris from the colonies. The same five colonies were again tested but with the primers SH1F and LMP7R. Only three colonies resulted in a positive result bright band of 820 bp in size. Thus, indicating that the gene was inserted in the correct direction (Figure 20, Lanes 8, 10, and 11).

The pBlueScript II SK (+)/*VMO1* isoform 3 construct was also tested with Colony PCR to determine the direction. Lanes 13 through to 17 were loaded with the PCR products of amplification of the pBlueScript II SK (+)/*VMO1* isoform 3 construct with the SH2F and LMP7R primers. The expected product from this amplification was 516 bp and can be seen in lanes 13, 14, and 16, indicating that the gene was inserted in the right direction in these colonies. Also present in these lanes were unknown products of approximately 350 bp. Lanes 15 and 17 contained products about 700 bp in size that were also unknown. The same colonies were tested with SH2R and LMP6F and these products were loaded into lanes 18 through to 22. Lanes 18, 19, and 21 contained products of the expected size of 485 bp. Lane 20 did not show any amplification. Lane 22 contained faint bands of both approximately 700 bp and 150 bp in size that were unknown PCR products.

In addition to this, the four primer pairs used in this PCR were used as a negative control with no template DNA used as a test for the presence of contamination (Lanes 24 - 27). All lanes did not show that these PCR contained products, showing that the PCR master mix or primers did not introduce contamination.

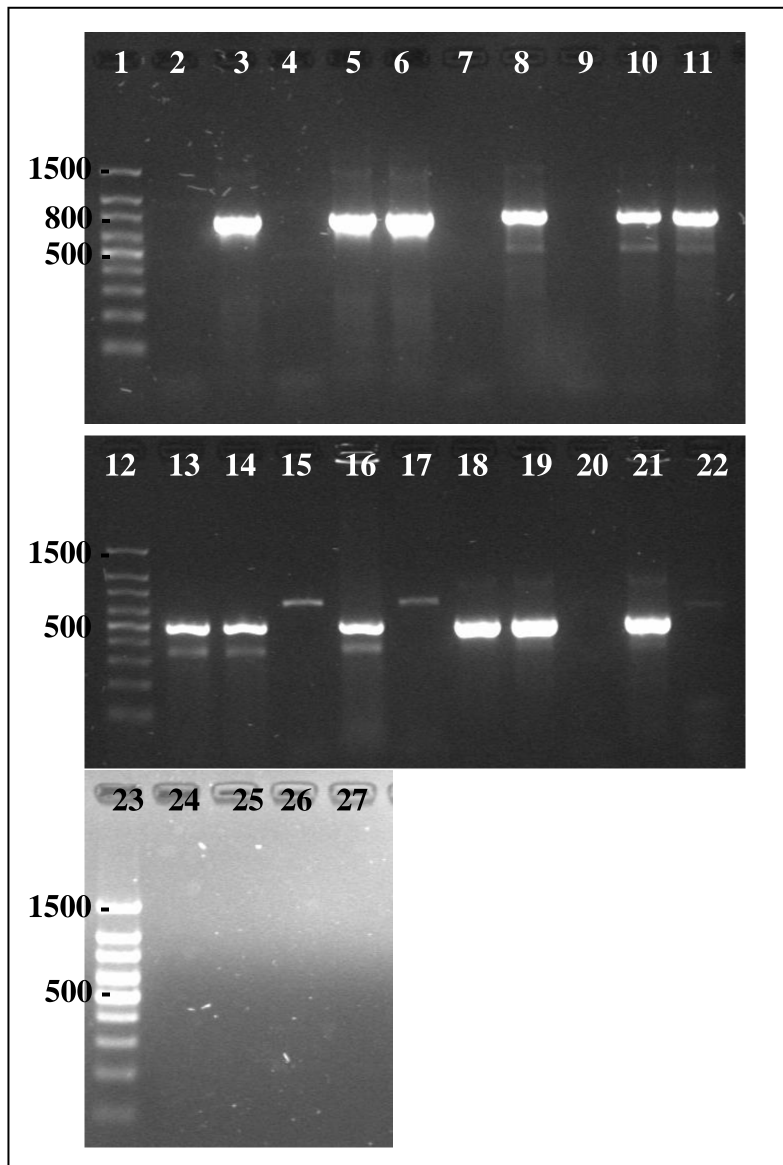


**Figure 20: Directional Colony PCR on *E. coli* DH5α colonies containing the recombinant pBlueScript II SK (+) with either *VMO*1 isoform 1 or 3.** PCR products were loaded onto a 2% 1X TAE agarose gel stained with Ethidium bromide and run for 30 min at 90 V. Lanes 1, 12 and 23: 100 bp DNA ladder (GenScript, USA); Lane 2 - 6: SH1R and LMP6F; Lanes 7 - 11: SH1F and LMP7R; Lanes 13 - 17: SH2F and LMP7R Lanes 18 - 22: SH2R and LMP6F; Negative PCR control: Lane 24: SH1R and LMP6F; Lane 25: SH1F and LMP7R; Lane 26: SH2F and LMP7R; Lane 27: SH2R and LMP6F.

The *E. coli* DH5α colonies with the pBlueScript II SK (+)/*VMO1* isoforms 1 and 3 that tested positive for the inserts in the correct direction were used to inoculate LB broth with ampicillin. The DNA constructs were then extracted from the bacterial cells using the Zyppy Plasmid Miniprep kit (ZymoResearch, USA) as per Section 2.7.1. The concentration and purity of the purified plasmids was then

measured using the NanoDrop (Table 15). The total yield of DNA was between 5.9 and 14.8 µg, respectively.

The 260/280 ratio of 1.85 was considered as pure for DNA for pBlueScript II SK (+)/*VMO1* isoform 1 and 1.79 for pBlueScript II SK (+)/*VMO1* isoform 3. Therefore, the plasmid samples were sent for sequencing by the Waikato DNA Sequencing Facility due to meeting the concentration requirements and having negligible contamination.

**Table 15: NanoDrop results for determination of concentration and purity of the extracted plasmid sample.**

| Sample | 260/230 | 260/280 | Total Yield (ng) | Concentration (ng/µL) |
|---|---|---|---|---|
| pBlueScript II SK (+)/*VMO1* isoform 1 | 1.87 | 1.85 | 14,751 | 491.7 |
| pBlueScript II SK (+)/*VMO1* isoform 3 | 1.56 | 1.79 | 5865 | 195.5 |

### 3.7 DNA Sequencing of the pBlueScript II SK (+) Vectors containing *VMO1* genes

The Waikato DNA Sequencing Facility uses an Applied Biosystems 3130xl Genetic Analyser for the purposes of sequencing the DNA. Sequencing results were provided as ab1 files that were uploaded to Geneious version 7.1.7. These files were analysed for peak quality in the chromatograms as well as for errors in nucleotide calls during the sequencing process. Noise at the start and end of the sequences were removed as well. Sequencing was performed using the T7 and T3 primers provided by the facility on both of the constructs as the genes had been inserted between these two regions. Both the sequences for each primer were aligned to form consensus sequences for each of the constructs. A sequence length of 852 bp was required to cover the whole length of the isoform 1 gene, whereas a whole sequence length of 544 bp was required to cover the entire isoform 3 gene.

These consensus sequences were then aligned to reference sequences obtained from NCBI.

The sequencing results for pBlueScript II SK (+)/*VMO1* isoform 1 are shown in Figure 21. Image A shows the results using the T7 primer. The sequence was of general good quality; however the sequence was rather short. Image B shows the sequencing results using the T3 primer. There was a lot of noise present at the start of the sequence which had to be removed before alignment of the T7 and T3 sequence. This sequence was also shorter than the expected sequence length that can be provided by the Applied Biosystems 3130xl Genetic Analyser. Once aligned, the consensus sequence was aligned with the reference sequence for VMO isoform 1 mRNA (NCBI: NM_182566) using the ClustalW2 alignment tool. This alignment is shown in Figure 22. The consensus sequence was not long enough to align to the complete reference sequence. Therefore, primers to cover the internal region of the gene were later designed to overcome this problem. The matches to the reference sequence are indicated with an asterisk symbol (*) under the alignment. The pairwise sequence identity score was given as 91.21% which indicates that it is the *VMO1* isoform 1 gene.

The sequencing results for pBlueScript II SK (+)/*VMO1* isoform 3 are shown in Figure 23. Image A shows the results using the T7 primer. The sequence was of general good quality with noise at the start of the sequence that was removed prior to alignment. Image B shows the sequencing results using the T3 primer. There was a lot of noise present at the start of the sequence which had to be removed before alignment of the T7 and T3 sequence. Both sequences were of long length, providing more detail of the sequence for comparison to the reference sequence. Once aligned, the consensus sequence that was formed was aligned with the

reference sequence for *VMO1* isoform 3 mRNA (NCBI: NM_001144940) using the ClustalW2 alignment tool. This alignment is shown in Figure 24. The reference sequence was able to align to the whole consensus sequence. The pairwise sequence identity score for this alignment was given as 99.47% showing that the PCR product inserted into the vector was a very good match to the reference and was the *VMO1* isoform 3 gene.

The confirmation that the two inserts into the vector were what they were thought to be allowed for downstream applications. The *VMO1* isoform 1 gene was digested out of the pBlueScript II SK (+) vector using EcoRI and BamHI restriction digest enzymes (Section 2.7.3), ligated into the pET28b(+) vector and sequenced to ensure that the *VMO1* region was covered, no mutations were introduced and the protein was in-frame with the His Tag. Alternatively, the pBlueScript II SK (+) vector/*VMO1* could be resequenced with internal primers and alternative sequencing primers such as M13, SK and KS (Appendix Two)

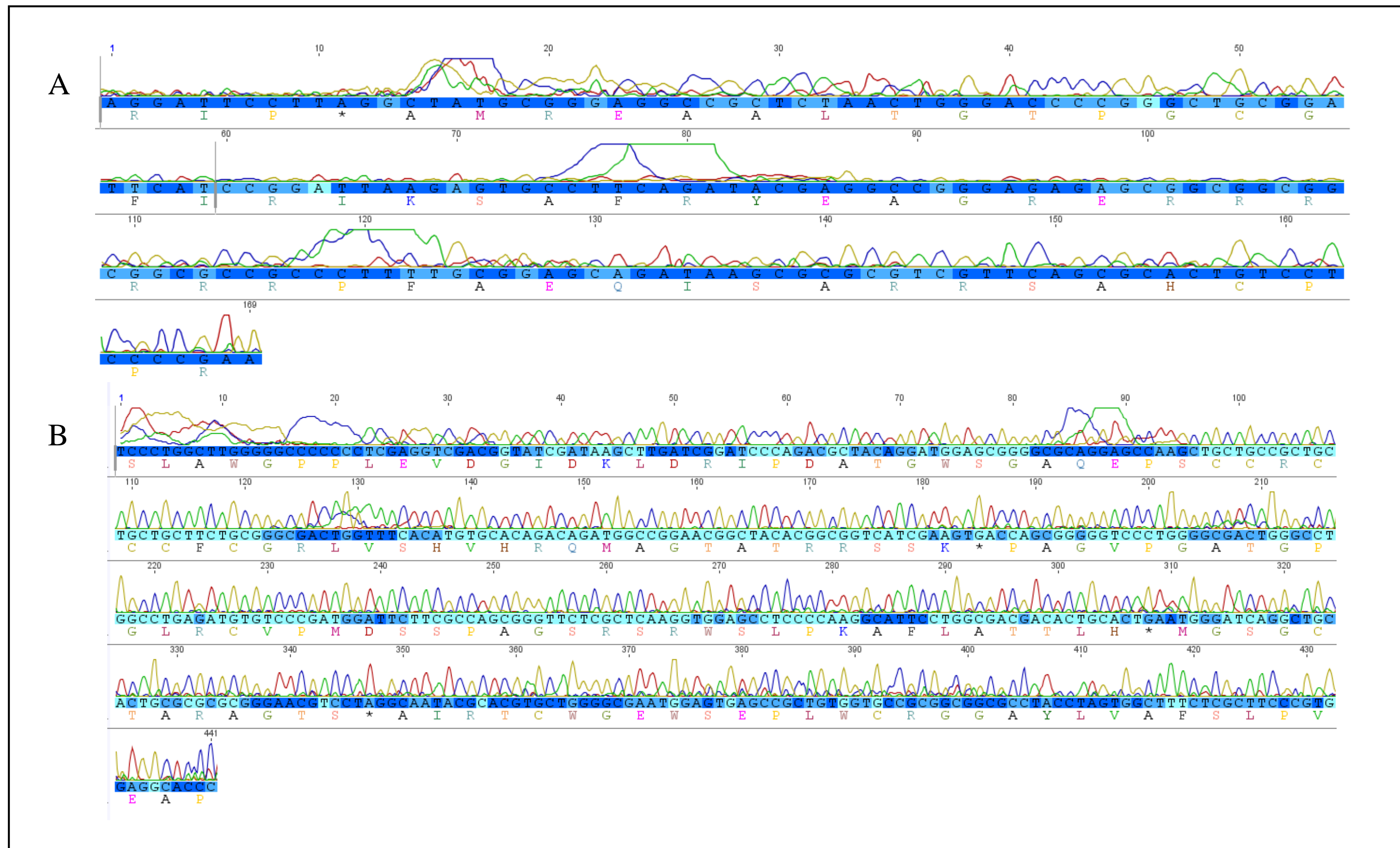


**Figure 21: DNA Sequence Chromatograms generated by Geneious version 7.1.7.** Image A is the raw sequence data of pBlueScript II SK (+)/*VMO1* isoform 1 sequenced with the T7 promoter primer provided by the Waikato DNA Sequencing Facility. Image B is the raw sequence data of pBlueScript II SK (+)/*VMO1* isoform 1 sequenced with the T3 primer provided by the Waikato DNA Sequencing Facility. Both sequences were aligned to generate a consensus sequence that was aligned to the reference sequence for *VMO1* isoform 1 (NCBI: NM_182566).

```
Iso1      TCGAGGTCGACGGTATCGATAAGCTTGATCGGATCCCAGACGCTACAGGATGGAGCGGGG 60
RefSeq    --------------------------GATC---CCACAGACGCTACAGGATGGAGCGGGG 31
                                    ****    * ************************

Iso1      CGNAGGAGCCAAGCTGCTGCCGCTGCTGCTGCTTCTGCGGGCGACTGGTTTCACATGTGC 120
RefSeq    CGCAGGAGCCAAGCTGCTGCCGCTGCTGCTGCTTCTGCGGGCGACTGGTTTCACATGTGC 91
          ** *********************************************************

Iso1      ACAGACAGATGGCCGGAACGGCTACACGGCGGTCATCGAAGTGACCAGCGGGGGTCCCTG 180
RefSeq    ACAGACAGATGGCCGGAACGGCTACACGGCGGTCATCGAAGTGACCAGCGGGGGTCCCTG 151
          ************************************************************

Iso1      GGGCGACTGGGCCTGGCCTGAGATGTGTCCCGATGGATTCTTCGCCAGCGGGTTCTCGCT 240
RefSeq    GGGCGACTGGGCCTGGCCTGAGATGTGTCCCGATGGATTCTTCGCCAGCGGGTTCTCGCT 211
          ************************************************************

Iso1      CAAGGTGGAGCCTCCCCAAGGCATTCCTGGCGACGACACTGCACTGAATGGGATCAGGCT 300
RefSeq    CAAGGTGGAGCCTCCCCAAGGCATTCCTGGCGACGACACTGCACTGAATGGGATCAGGCT 271
          ************************************************************

Iso1      GCACTGCGCGCGCGGGAACGTCCTAGGCAATACGCACGTG-------------------- 340
RefSeq    GCACTGCGCGCGCGGGAACGTCCTAGGCAATACGCACGTGGTAGAGTCCCAGTCTGGAAG 331
          ****************************************

Iso1      CTGGGGCGAATGGAGTGAGCCGCTGTGGTGCCGCGGCNTGCGCCTACCTAGTGGCTTTCT 400
RefSeq    CTGGGGCGAATGGAGTGAGCCGCTGTGGTGTCGCGGCG-GCGCCTACCTAGTGGCTTTCT 390
          ****************************** ******  *********************

Iso1      CGCTTCCCGTGGAGGCACCCC--------------------------------------- 421
RefSeq    CGCTTCGCGTGGAGGCACCCACGACCCTCGGTGACAACACAGCAGCGAACAACGTGCGCT 450
          ****** **************

Iso1      ------------------------------------------------------------
RefSeq    TCCGCTGTTCAGACGGCGAGGAACTGCAGGGGCCTGGGCTGAGCTGGGGAGACTTTGGAG 510


Iso1      ------------------------------------------------------------
RefSeq    ACTGGAGTGACCATTGCCCCAAGGGCGCGTGCGGCCTGCAGACCAAGATCCAGGGACCTA 570


Iso1      ------------------------------------------------------------
RefSeq    GAGGCCTCGGCGATGACACTGCGCTGAACGACGCGCGCTTATTCTGCTGCCGCAGTTGAA 630


Iso1      -------------------------------------------------------
RefSeq    CGGCGCCGCCGCCGCCGCTCTCTCCCGGGCCAGGAGGCTAGTCCCACCTCTTGAATT 687
```

**Figure 22: Alignment of sequencing data to the reference sequence for confirmation of *VMO1* isoform 1 gene.** Iso1 refers to the generated consensus sequence of sequencing data from sequences shown in Figure 21. RefSeq refers to the reference sequence for *VMO1* isoform 1 (NCBI: NM_182566). Sequences were aligned using ClustalW2 (http://www.ebi.ac.uk/Tools/msa/clustalw2/). * represents identical nucleotides between the consensus sequence and the reference sequence.

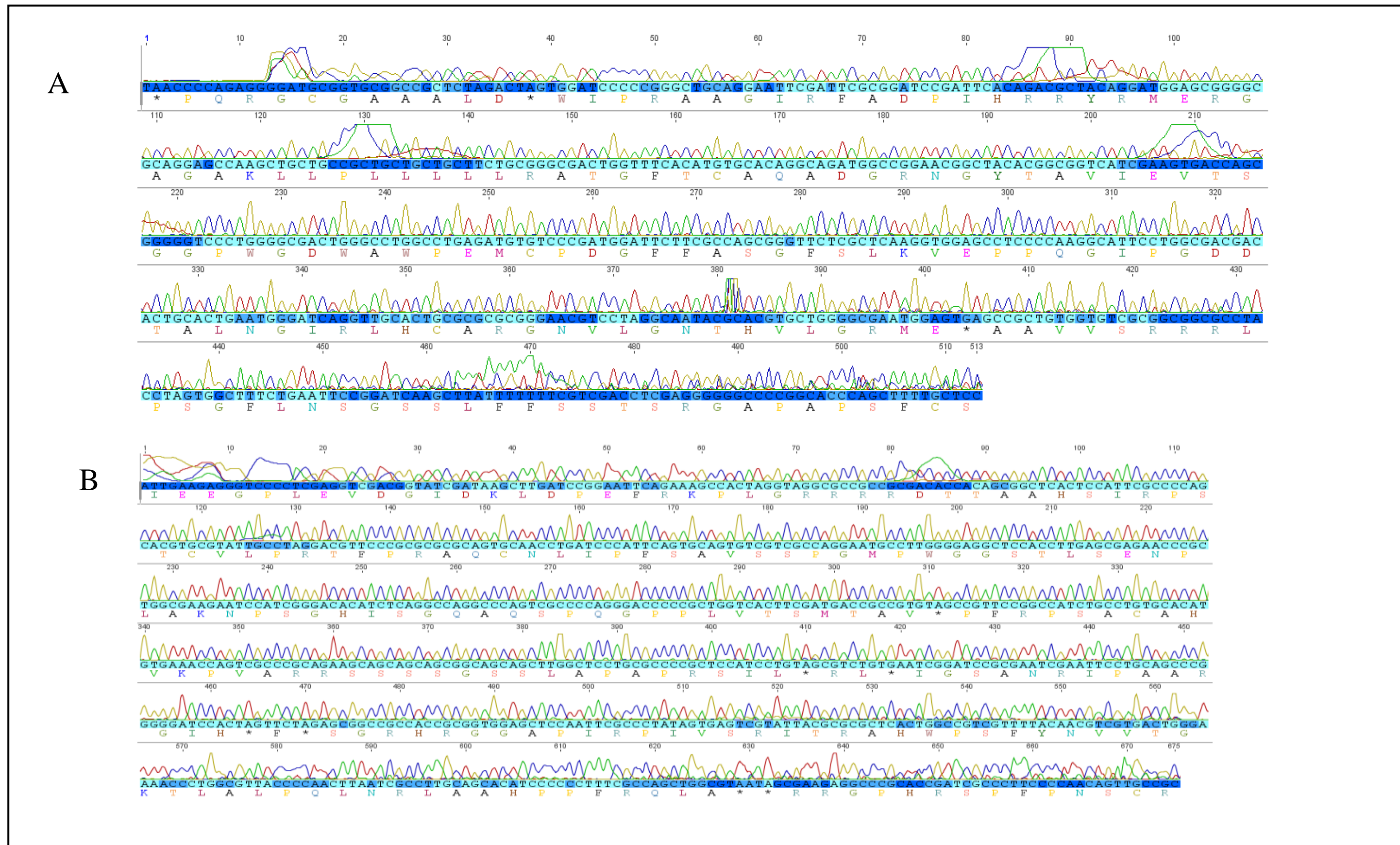


**Figure 23: DNA Sequence Chromatograms generated by Geneious version 7.1.7.** Image A is the raw sequence data of pBlueScript II SK (+)/*VMO1* isoform 3 sequenced with the T7 promoter primer provided by the Waikato DNA Sequencing Facility. Image B is the raw sequence data of pBlueScript II SK (+)/*VMO1* isoform 3 sequenced with the T3 primer provided by the Waikato DNA Sequencing Facility. Both sequences were aligned to generate a consensus sequence that was aligned to the reference sequence for *VMO1* isoform 3 (NCBI: NM_001144940).

```
Iso3      GCGGCAACTGTTTGGGGAAGGGCGATCGGTTGCGGGCCTCTTCGCTATTACGCCAGCTGG 60
RefSeq    ------------------------------------------------------------


Iso3      CGAAAGGGGGGATGTGCTGCAAGGCGATTAAGTTGGGTAACGCCAGGGTTTTCCCAGTC 120
RefSeq    ------------------------------------------------------------


Iso3      ACGACGTTGTAAAACGACGGCCAGTGAGCGCGCGTAATACGACTCACTATAGGGCGAATT 180
RefSeq    ------------------------------------------------------------


Iso3      GGAGCTCCACCGCGGTGGCGGCCGCTCTAGAACTAGTGGATCCCCCGGGCTGCAGGAATT 240
RefSeq    ------------------------------------------------------------


Iso3      CGATTCGCGGATCCGATTCACAGACGCTACAGGATGGAGCGGGGCGCAGGAGCCAAGCTG 300
RefSeq    ---------GATCCGATTCACAGACGCTACAGGATGGAGCGGGGCGCAGGAGCCAAGCTG 51
                   ***************************************************

Iso3      CTGCC-GCTGCTGCTGCTTCTGCGGGCGACTGGTTTCACATGTGCACAGGCAGATGGCCG 359
RefSeq    CTGCC-GCTGCTGCTGCTTCTGCGGGCGACTGGTTTCACATGTGCACAGACAGATGGCCG 110
          ***** ******************************************* **********

Iso3      GAACGGCTACACGGCGGTCATCGAAGTGACCAGCGGGGGTCCCTGGGGCGACTGGGCCTG 419
RefSeq    GAACGGCTACACGGCGGTCATCGAAGTGACCAGCGGGGGTCCCTGGGGCGACTGGGCCTG 170
          ************************************************************

Iso3      GCCTGAGATGTGTCCCGATGGATTCTTCGCCAGCGGGTTCTCGCTCAAGGTGGAGCCTCC 479
RefSeq    GCCTGAGATGTGTCCCGATGGATTCTTCGCCAGCGGGTTCTCGCTCAAGGTGGAGCCTCC 230
          ************************************************************

Iso3      CCAAGGCATTCCTGGCGACGACACTGCACTGAATGGGATCAGGTTGCACTGCGCGCGCGG 539
RefSeq    CCAAGGCATTCCTGGCGACGACACTGCACTGAATGGGATCAGGCTGCACTGCGCGCGCGG 290
          ******************************************* ****************

Iso3      GAACGTCCTAGGCAATACGCACGTGCTGGGGCGAATGGAGTGAGCCGCTGTGGTGTCGCG 599
RefSeq    GAACGTCCTAGGCAATACGCACGTGCTGGGGCGAATGGAGTGAGCCGCTGTGGTGTCGCG 350
          ************************************************************

Iso3      GCGGCGCCTACCTAGTGGCTTTCTGAATTCCGGATCAAGCTTATCGATAYCGTCGACCTC 659
RefSeq    GCGGCGCCTACCTAGTGGCTTTCTGAATT------------------------------- 379
          *****************************

Iso3      GAGGGGGGCCCCGGCACCCAGCTTTTGCTCC 690
RefSeq    -------------------------------
```

**Figure 24: Alignment of sequencing data to the reference sequence for confirmation of *VMO1* isoform 3 gene.** Iso3 refers to the generated consensus sequence of sequencing data from sequences shown in Figure 23. RefSeq refers to the reference sequence for *VMO1* isoform 3 (NCBI: NM_001144940). Sequences were aligned using ClustalW2 (http://www.ebi.ac.uk/Tools/msa/clustalw2/). * represents identical nucleotides between the consensus sequence and the reference sequence.

### 3.8 Transformation of the pET28b(+)/*VMO1* Isoform 1 Construct into Electrocompetant *E. coli* DH5α

Following partial confirmation that the PCR inserts into the pBlueScript II SK (+) vectors were the *VMO1* genes using DNA sequencing, the inserts were digested out using restriction enzymes and gel purified. The inserts were ligated into the pET28b(+) vectors then transformed into electrocompetent *E. coli* DH5α. Transformed cells were plated onto LB agar plates with kanamycin to select for cells containing the pET28b(+) vector. The transformation efficiency was uncalculated due to there being a very high number of colonies on the undiluted plate which was greater than 200. Therefore the 1:10 diluted plate was used for selecting colonies due to the colonies being easier to select. Sixteen of the colonies transformed with the pET28b(+)/*VMO1* isoform 1 construct that grew on the plate were tested using colony PCR with the primer set LMP10F/LMP11R (Figure 25). Sixteen white colonies were tested and three were nine were positive (Lanes 2, 4, 7, 12 – 16, and 19) as the expected size of the PCR product of 1051 bp was observed on the agarose gel. The colonies tested that were amplified and loaded into lanes 3, 5, 8, 9, and 18 of the agarose gel showed products of around 360 bp in size, indicating that the *VMO1* isoform 1 gene had not been ligated into the vector. Lanes 6 and 17 did not show bands present indicating that the PCR did not work for these colonies. Finally, a negative PCR control loaded into lane 20 which showed no presence of amplification indicating that there was no contamination in the PCR master mix.

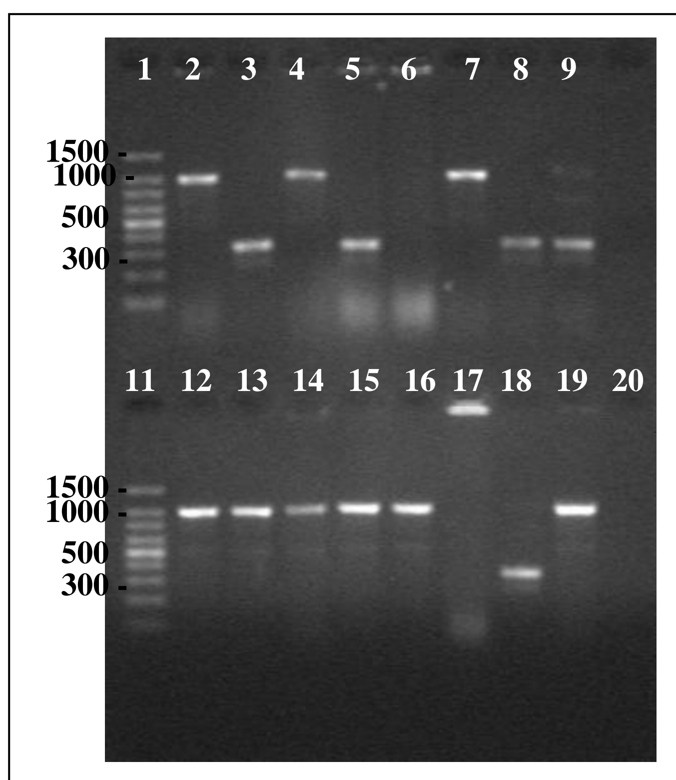


**Figure 25: Colony PCR of electrocompetent *E. coli* DH5α cells transformed with the pET28b(+)/*VMO1* isoform 1 construct.** PCR products were loaded on a 2% 1X TAE agarose gel stained with Ethidium bromide. The gel was run for 30 min at 90 V. Lanes 1 and 10: 100bp DNA ladder (GenScript, USA); Lanes 2 – 9, 11 – 19: White colonies tested with LMP10F and LMP11R; Lane 20: Negative PCR control with LMP10F and LMP11R.

After confirmation that the ligation had worked, the positive colony in lane 15 was selected for growth and extraction of the vector for DNA sequencing. The vector was extracted using the Zyppy Plasmid Miniprep (ZymoResearch, USA) and the purity and concentration of the extracted plasmid DNA was measured (Table 16). The total yield of DNA obtained was 5271 ng. The extracted vector met the requirements of concentration for DNA sequencing as listed in Section 2.9. The 260/230 ratio indicated that there was negligible contamination from organic solvents and the 260/280 ratio indicated the DNA was of high purity.

**Table 16: NanoDrop results of pET28b(+)/*VMO1* isoform 1 construct for determination of purification and concentration.**

| **Sample** | **260/230** | **260/280** | **Concentration (ng/µL)** |
|---|---|---|---|
| pET28b(+)/*VMO1* isoform 1 | 1.68 | 1.81 | 175.7 |

### 3.8.1 Sequencing of pET28b(+)/*VMO1* Isoform 1 Construct

The extracted pET28b(+)/*VMO1* isoform 1 construct was sequenced using the Waikato DNA Sequencing Facility and primers were provided to the facility. The construct was sequenced using LMP10F, SH1F and LMP11R to cover the entire insert. Sequences were analysed using Geneious version 7.1.7. Raw sequences using these primers can be found in Appendix Three. The alignment of these three sequence files showed that the inner region of the gene had not been sequenced. As a result, the primer set SH3F/SH3R was designed to target this region (Section 2.2.3). Following sequencing of the construct with these two primers, the four sequences from LMP10F, SH1F, SH3F, and SH3R were aligned using the ClustalW2 alignment tool to form the consensus sequence as shown in Figure 26. Furthermore, Geneious version 7.1.7 was used for annotation of the consensus sequence (Figure 27) showing the sections of the sequence that are part of the pET28b(+) vector including the His Tag.

With confirmation of the recombinant *VMO1* gene being in-frame with the His Tag in the pET28b(+) vector, the DNA construct was transformed into electrocompetent *E. coli* BL21 (DE3) cells for protein induction using IPTG.

```
SH3F       ------------------------------------------------------------
SH3R       ------------------------------------------------------------
LMP10      ATAATTTTGTTTAACTTTAAGAAGGAGATATACCATGGGCAGCAGCCATCATCATCATCA
SH1F       ------------------------------------------------------------


SH3F       ------------------------------------------------------------
SH3R       ------------------------------------------------------------
LMP10      TCACAGCAGCGGCCTGGTGCCGCGCGGCAGCCATATGGCTAGCATGACTGGTGGACAGCA
SH1F       ------------------------------------------------------------


SH3F       ------------------------------------------------------------
SH3R       ------------------------------------------------------------
LMP10      AATGGGTCGGGATCCCAGACGCTACAGGATGGAGCGGGGCGCAGGAGCCAAGCTGCTGCC
SH1F       ------------------------------------------------------------


SH3F       ------------------------------------------------------------
SH3R       ------------------------------------------------------------
LMP10      GCTGCTGCTGCTTCTGCGGGCGACTGGTTTCACATGTGCACAGACAGATGGCCGGAACGG
SH1F       --TGCTGCTGCTTCTGCGGGCGACTGGTTTCACATGTGCACAGACAGATGGCCGGAACGG


SH3F       ------GGCGGTCATCGAAGTGACCAGCGGGGGTCCCTGGGGCGACTGGGCCTGGCCTGA
SH3R       ------------------------------------------------------------
LMP10      CTACACGGCGGTCATCGAAGTGACCAGCGGGGGTCCCTGGGGCGACTGGGCCTGGCCTGA
SH1F       CTACACGGCGGTCATCGAAGTGACCAGCGGGGGTCCCTGGGGCGACTGGGCCTGGCCTGA


SH3F       GATGTGTCCCGATGGATTCTTCGCCAGCGGGTTCTCGCTCAAGGTGGAGCCTCCCCAAGG
SH3R       ------------------------------------------------------------
LMP10      GATGTGTCCCGATGGATTCTTCGCCAGCG-------------------------------
SH1F       GATGTGTCCCGATGGATTCTTCGCCAGCGGGTTCTCGCTCAAGGTGGA------------


SH3F       CATTCCTGGCGACGACACTGCACTGAATGGGATCAGGCTGCACTGCGCGCGCGGGAACGT
SH3R       --------GCGACGACACTGCACTGAATGGGATCAGGCTGCACTGCGCGCGCGGGAACGT
LMP10      ------------------------------------------------------------
SH1F       ------------------------------------------------------------


SH3F       CCTAGGCAATACGCACGTGCTGGGGCGAATGGAGTGAGCCGCTGTGGTGTCGCGGCGGCG
SH3R       CCTAGGCAATACGCACGTGCTGGGGCGAATGGAGTGAGCCGCTGTGGTGTCGCGGCGGCG
LMP10      ------------------------------------------------------------
SH1F       ------------------------------------------------------------


SH3F       CCTACCTAGTGGCTTTCTCGCTTC
SH3R       CCTACCTAGTGGCTTTCTCGCTTC
LMP10      ------------------------
SH1F       ------------------------
```

**Figure 26: Alignment of DNA sequences generated using LMP10F, SH3F, SH3R, and SH1F primers.** This alignment was used to generate the consensus sequence for identification of the recombinant *VMO1* gene within the pET28b(+) vector. Sequences were aligned using ClustalW2 (http://www.ebi.ac.uk/Tools/msa/clustalw2/).

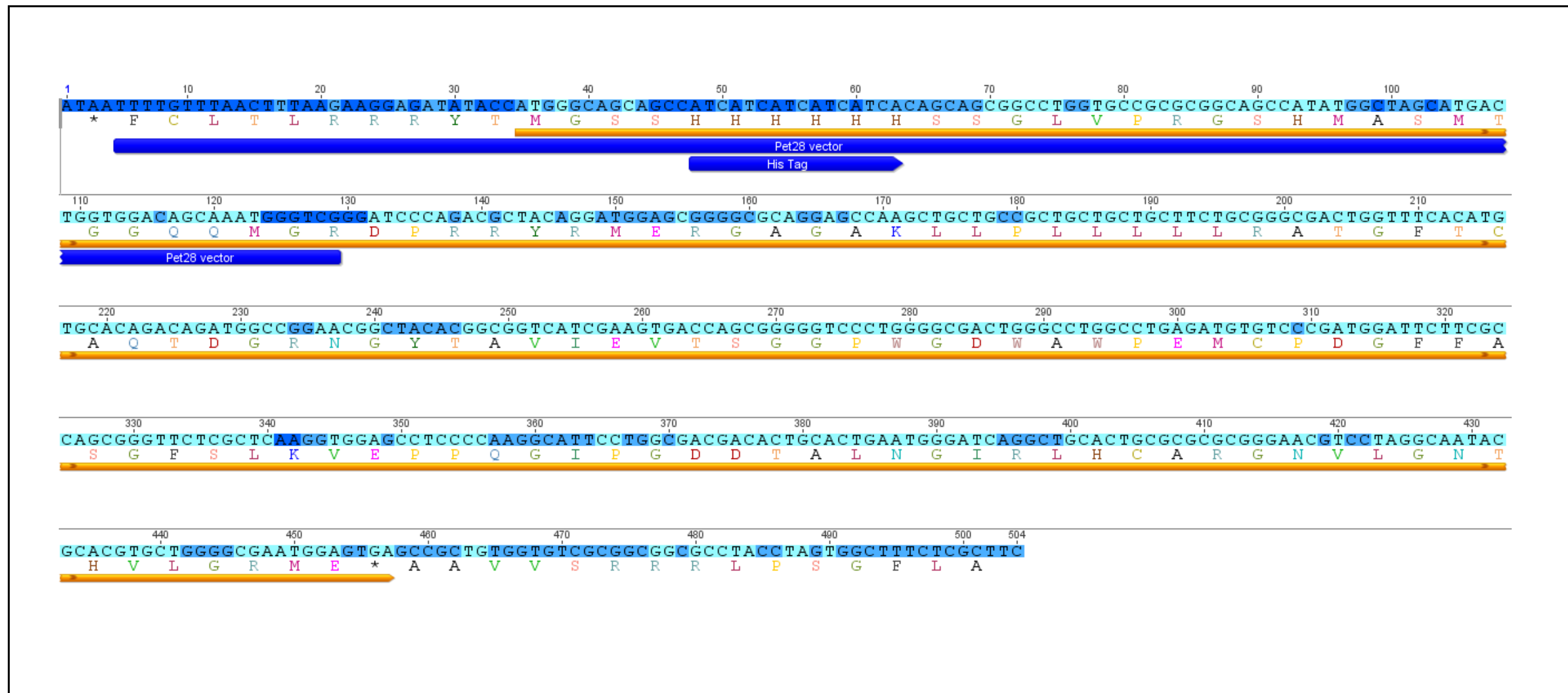


**Figure 27: Consensus sequence formed from alignment of sequences generated by SH1F, SH3F, SH3R, and LMP10F primers.** The consensus nucleotide sequence (1-504) was annotated using Geneious version 7.1.7. The start codon is at position 36 and the stop codon at position 456 with the yellow line indicating the recombinant protein (AA) that will be expressed within bacterial cells following induction using IPTG. The labelled blue line represents the pET28b(+) vector sequence (1-129) and the in-frame His Tag (48-63) that resides within the recombinant protein.

### 3.9 Protein Expression in *E. coli* BL21 (DE3)

The pET28b(+)/*VMO1* isoform 1 construct was transformed into electrocompetent *E. coli* BL21 (DE3) bacterial cells. The transformed cells were then plated on LB agar plates with kanamycin for selection of the vector. After overnight growth, the plate was inspected for growth and 16 colonies were present. A single colony was selected for inoculation of LB broth for protein expression. Two final concentrations of IPTG were used for protein induction; 0.4 mM and 1.0 mM, respectively. In addition to this, a control broth was grown that was not induced. Samples were taken at 0 hour, 3 hours, 6 hours and 24 hours post-induction from all broths. Whole cell lysate samples were prepared for each sample taken and loaded onto a hand cast 12% separating SDS-PAGE gel with a 4% stacking gel. The Plus Protein™ Unstained Standards (Bio-Rad Laboratories, Inc, USA) ladder was used for determination of protein sizes in the samples. The SDS-PAGE gels were run for 45 min at 200 V then fixed with fixing solution and stained with Coomassie Blue R250 for visualisation of the bands. Gels were visualised on a white light and imaged. The induced recombinant VMO1 protein was expected to be 20 kDa in size and represented as a bright band on the SDS-PAGE following induction with IPTG. However, no bright bands were seen on the gel at this size in the induced samples. Therefore, induction was suspected to have not worked. There was a bright band detected at in the 6 hours and 24 hours non-induced samples less than 20 kDa in size but it is unknown what protein caused these bright bands (Figure 28B, lanes 2 and 5).

It was decided to transform the pET28b(+)/*VMO1* isoform 1 construct into *E. cloni* BL21 (DE3) pLysS cells as these cells contain the pLysS plasmid. This

plasmid acts to prevent expression of genes under the control of the T7 promoter prior to induction using IPTG.

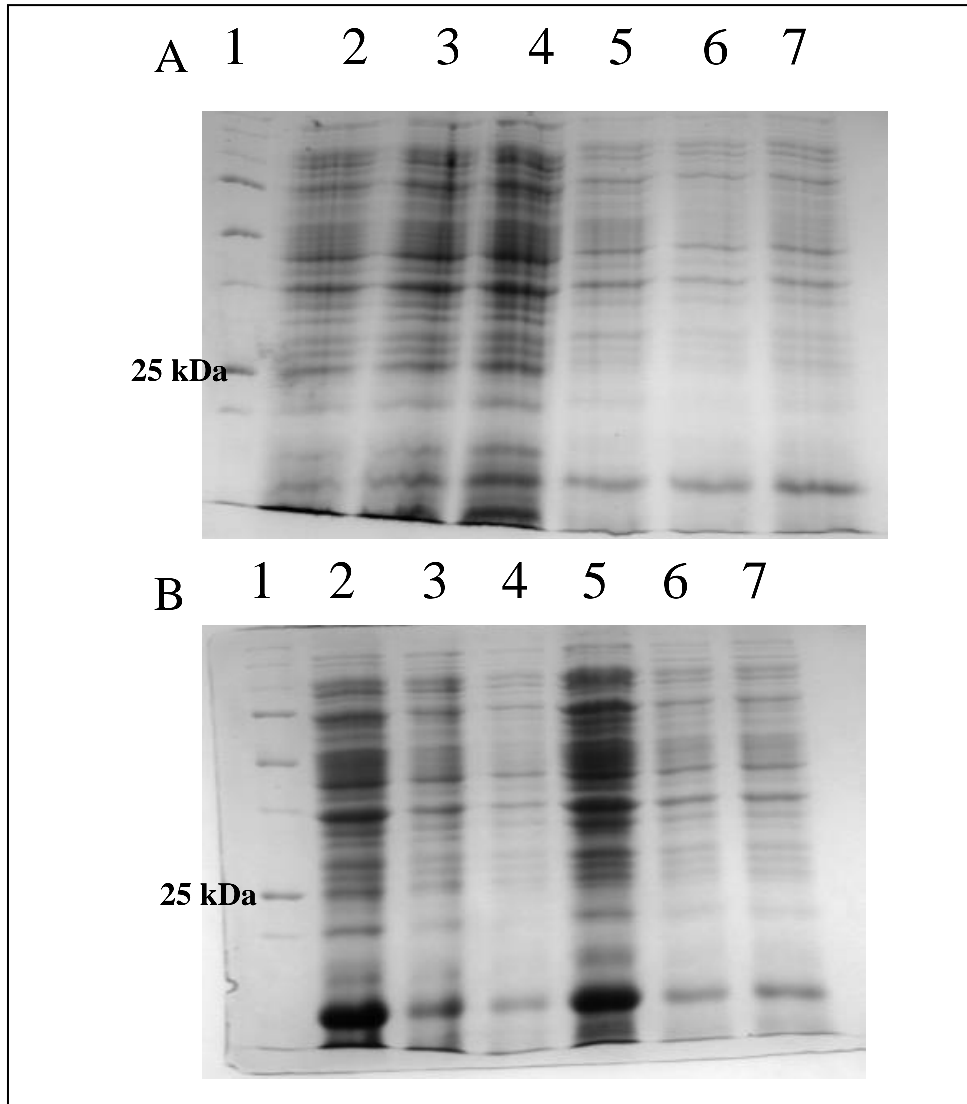


**Figure 28: Protein induction of *E. coli* BL21 (DE3) cells using IPTG.** Two final concentrations of IPTG were used, 0.4 mM and 1.0 mM as well as a non-induced control. Whole cell lysate samples were taken at 0 hours, 3 hours, 6 hours, and 24 hours post-induction and loaded onto hand cast 12% separating SDS-PAGE gels. The gels were run for 45 min at 200 V and stained with Coomassie Blue R250 for visualisation of proteins on a white light and imaged. **Gel A)** Lane 1: Precision Plus Protein™ Unstained Standards ladder (Bio-Rad Laboratories, Inc, USA); Lane 2: Non-induced 0 hours; Lane 3: 0.4 mM 0 hours: Lane 4: 1.0 mM 0 hours; Lane 5: Non-induced 3 hours; Lane 6: 0.4 mM 3 hours: Lane 7: 1.0 mM 3 hours. **Gel B)** Lane 1: Precision Plus Protein™ Unstained Standards ladder (Bio-Rad Laboratories, Inc, USA); Lane 2: Non-induced 6 hours; Lane 3: 0.4 mM 6 hours; Lane 4: 1.0 mM 6 hours: Lane 5: Non-induced 24 hours; Lane 6: 0.4 mM 24 hours; lane 7: 1.0 mM 24 hours.

### 3.10 Protein Expression in *E. cloni* BL21 (DE3) pLysS

The pET28b(+)/*VMO1* isoform 1 construct was transformed into chemically competent *E. cloni* BL21 (DE3) pLysS cells as per manufacturer's instructions. In addition to this, a control vector was provided by Dr Emma Andrews that contained the *PhoH2* gene from *M. smegmatis* in the pET28b(+) vector. This was also transformed into the bacterial cells. The transformations were plated onto LB plates containing appropriate antibiotics and grown overnight. The pET28b(+)/*VMO1* isoform 1 plates were counted for number of colonies and found to have 152 colonies present on the plate. Protein expression was induced using IPTG at a final concentration of 1.0 mM. A non-induced broth was also grown for both protein expression experiments. Samples for whole cell lysate were taken at 0 hours, 3 hours, 6 hours, and 24 hours and processed for PAGE electrophoresis. Figure 29 shows that non-specific protein expression across all four sample timeframes occurred. In addition to this, the positive control, PhoH2 protein did not show induced expression as the expected protein size was 50 kDa was not observed on the PAGE gel (E Andrews, personal communication, October, 2014). The recombinant VMO1 protein was also not expressed at high levels, as expected, following induction with IPTG.

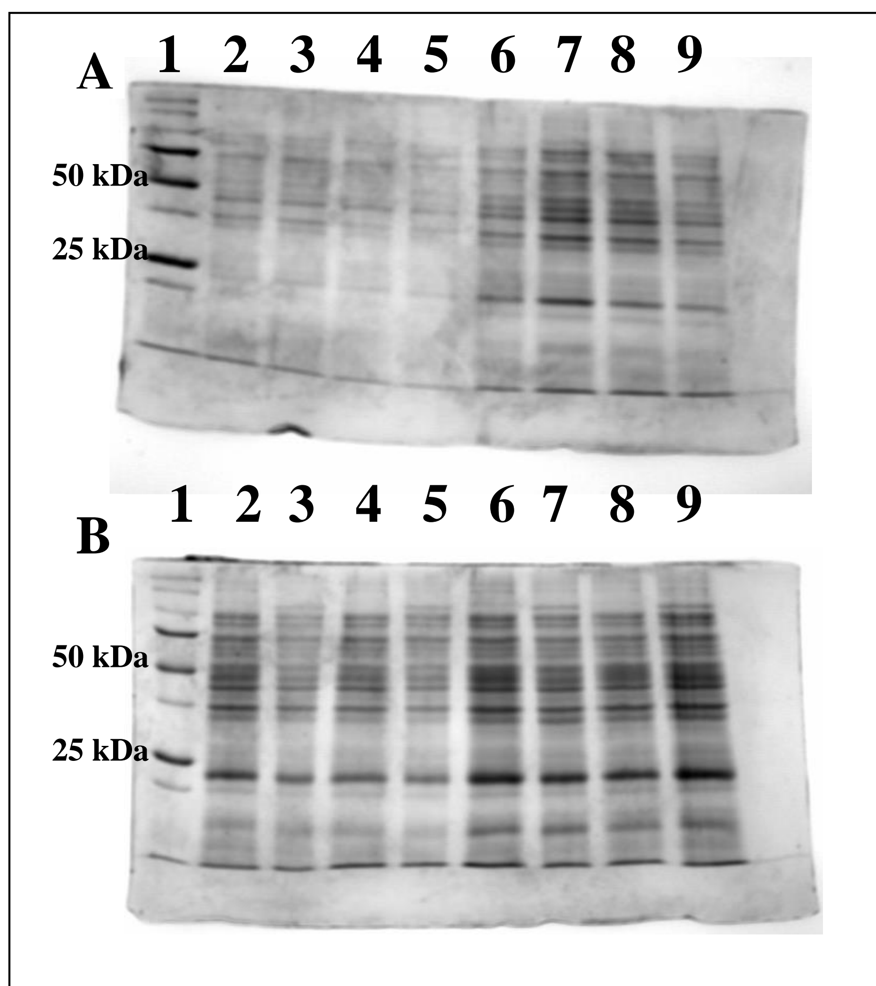


**Figure 29: Protein induction of *E. cloni* BL21 (DE3) pLysS cells using IPTG.** The final concentration of IPTG used was 1.0 mM as well as a non-induced control. In addition, a positive control protein, PhoH2 was used. Whole cell lysate samples were taken at 0 hours, 3 hours, 6 hours, and 24 hours post-induction and loaded onto hand cast 12% separating SDS-PAGE gels. The gels were run for 45 min at 200 V and stained with Coomassie Blue R250 for visualisation of proteins on a white light and imaged.
**Gel A)** Lane 1: Precision Plus Protein™ Unstained Standards ladder (Bio-Rad Laboratories, Inc, USA); Lane 2: Non-induced PhoH2 0 hours; Lane 3: 1.0 mM IPTG PhoH2 0 hours; Lane 4: Non-induced VMO1 0 hours; Lane 5: 1.0 mM IPTG 0 hours; Lane 6: Non-induced PhoH2 3 hours; Lane 7: 1.0 mM IPTG PhoH2 3 hours; Lane 8: Non-induced VMO1 3 hours; Lane 9: 1.0 mM IPTG 3 hours
**Gel B)** Lane 1: Precision Plus Protein™ Unstained Standards ladder (Bio-Rad Laboratories, Inc, USA); Lane 2: Non-induced PhoH2 6 hours; Lane 3: 1.0 mM IPTG PhoH2 6 hours; Lane 4: Non-induced VMO1 6 hours; Lane 5: 1.0 mM IPTG 6 hours; Lane 6: Non-induced PhoH2 24 hours; Lane 7: 1.0 mM IPTG PhoH2 24 hours; Lane 8: Non-induced VMO1 24 hours; Lane 9: 1.0 mM IPTG 24 hours.

### 3.10.1 Glucose Inhibition for Protein Expression

Following non-expression of the recombinant VMO1 protein in these bacterial cells using standard conditions, glucose at a final concentration of 1% was introduced to the LB broth containing selective antibiotics during growth of the bacterial cells before induction with 1 mM of IPTG. Introduction of a glucose source to bacterial cells leads to low levels of cAMP in the cells. As a result, the *lac* promoter transcription is low as well. Therefore non-specific transcription and expression is suppressed until IPTG is introduced to the cells for T7 RNA polymerase expression (Novy & Morris, 2001). Samples for whole cell lysate were taken at 0 hours and 24 hours and processed for PAGE electrophoresis. Samples were loaded onto a 12% hand cast separating gel SDS-PAGE gel with a 4% stacking gel. Samples were electrophoresed for 45 min at 200 V for separation of proteins. The gel was stained with Coomassie Blue R250 Stain and visualised under white light. Very little protein was present on the gel, seen as very faint bands, in the 0 hour whole cell lysate samples (Figure 30, lanes 2 - 5). This indicates that the non-specific protein expression was hindered by the presence of the glucose. The 24 hour whole lysate sample failed to show expression of the recombinant VMO1 protein when induced with glucose (lane 9) with proteins in this sample appearing lower in concentration on the gel. In addition to this, no rather bright bands were present at 50 kDa in the positive control of PhoH2 from *M. smegmatis* in the 24 hour post-induction sample (lane 7).

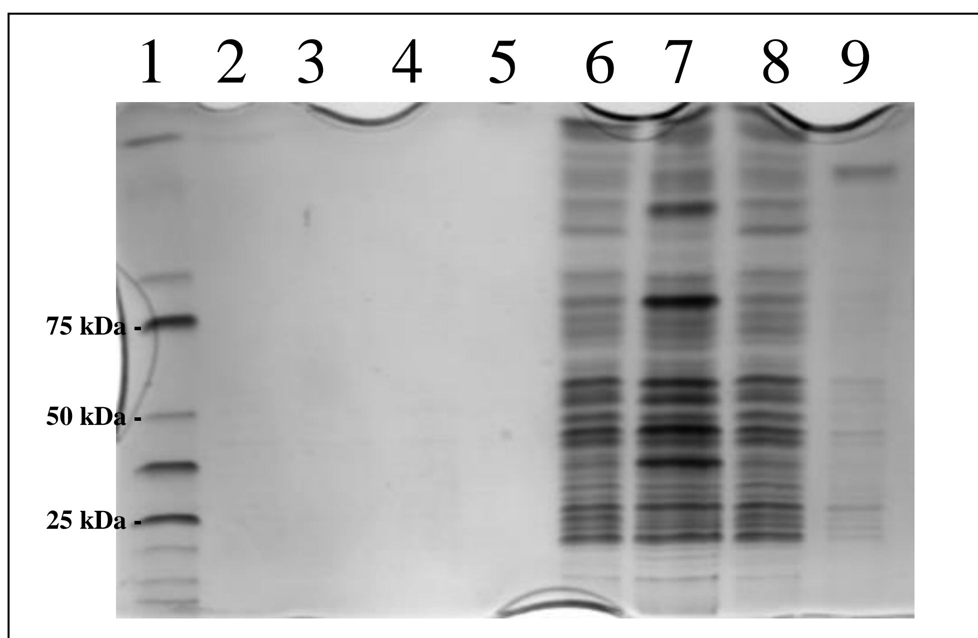


**Figure 30: Protein induction of *E. cloni* BL21 (DE3) pLysS cells using IPTG and 1% Glucose.** The final concentration of IPTG used was 1.0 mM as well as a non-induced control. In addition, a positive control protein, PhoH2 was used. Whole cell lysate samples were taken at 0 hours and 24 hours post-induction and loaded onto hand cast 12% separating SDS-PAGE gels. The gels were run for 45 min at 200 V and stained with Coomassie Blue R250 for visualisation of proteins on a white light and imaged. Lane 1: Precision Plus Protein™ Unstained Standards ladder (Bio-Rad Laboratories, Inc, USA); Lane 2: Non-induced PhoH2 0 hours; Lane 3: 1.0 mM IPTG PhoH2 0 hours; Lane 4: Non-induced VMO1 0 hours; Lane 5: 1.0 mM IPTG 0 hours; Lane 6: Non-induced PhoH2 24 hours; Lane 7: 1.0 mM IPTG PhoH2 24 hours; Lane 8: Non-induced VMO1 24 hours; Lane 9: 1.0 mM IPTG 24 hours.

Due to non-expression of the recombinant VMO1 protein in these bacterial cells using two methods for protein induction, the DNA sequence generated from sequencing data (Figure 26) was investigated for the presence of rare codons using the RACC (available at http://nihserver.mbi.ucla.edu/RACC/). The *VMO1* nucleotide sequence was found to contain the rare codons AGG, AGA, and CGA, which code for arginine as well as the rare isoleucine codon, ATA, and the rare leucine codon, CTA. To overcome this codon bias, the bacterial cell line, Rosetta™ (DE3) pLysS cells (Novagen, USA) was purchased and the pET28b(+)/*VMO1* isoform 1 construct was transformed into these bacterial cells for protein induction.

### 3.11 Protein Expression in Rosetta™ (DE3) pLysS

The pET28b(+)/*VMO1* Isoform 1 construct was transformed into chemical competent Rosetta™ (DE3) pLysS cells as per manufacturer's instructions. The cells were plated onto LB agar plates with chloramphenicol and kanamycin for the selection of cells containing the construct and pLysS plasmid. Following overnight growth of the plates, a single colony was selected for growth in LB broth and protein induction using IPTG (final concentration 1.0 mM). Samples were taken at 0 hours and 24 hours post-induction for analysis of protein expression in the whole cell lysates. In addition to this, samples were taken at 24 hours post-induction for analysis of proteins in the soluble and insoluble fractions. Samples were processed and loaded onto a 4% stacking gel with 12% separating gel. The PAGE-MASTER Protein Standard Plus ladder (GenScript, USA) was used for determination of protein sizes present in the SDS-PAGE gel. The products were electrophoresed for 30 min at 200 V, fixed with fixing solution and stained with Coomassie Blue R250 for detection of proteins present on the gel. The gel was visualised on a white light and imaged (Figure 31). Bright protein bands just under 20 kDa were detected in the 24 hour induced whole cell lysate (Figure 30, lane 5) and the 24 hour induced insoluble fraction (Figure 31, lane 9). These bands indicated that the recombinant VMO1 protein had been expressed in these cells following induction with 1.0 mM IPTG. It also indicated that the protein was insoluble.

Following these results, the protein was tested against antibodies using western blotting methodology.

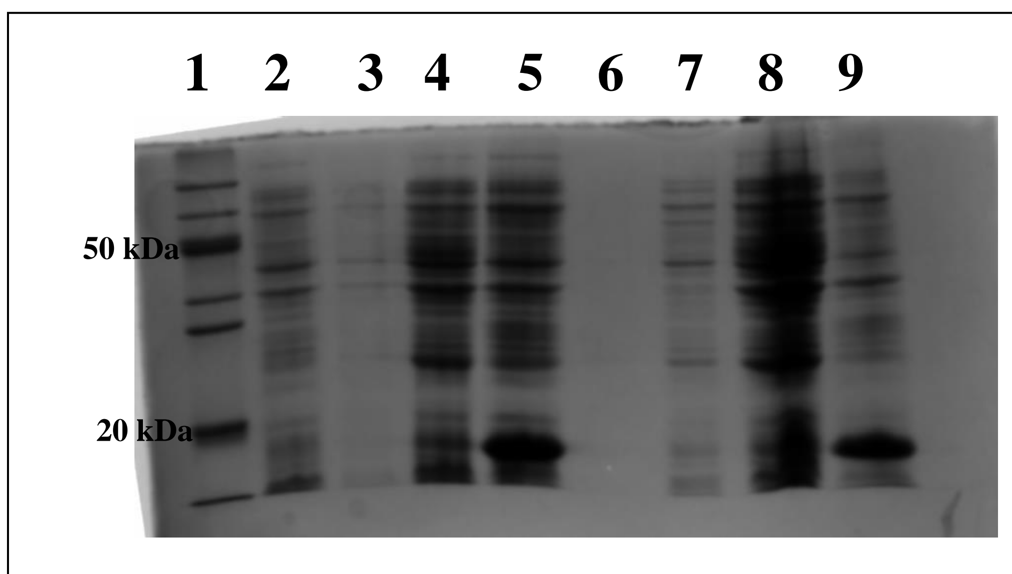


**Figure 31: Protein induction of Rosetta™ (DE3) pLysS cells using 1 mM IPTG.** Whole cell lysate (WCL) samples were taken at 0 hours and 24 hours post-induction. Samples for soluble and insoluble fractions were taken at 24 hours post-induction. Samples were loaded onto hand cast 12% separating SDS-PAGE gels. The gels were run for 45 min at 200 V and stained with Coomassie Blue R250 for visualisation of proteins on a white light and imaged. Lane 1: PAGE-MASTER Protein Standard Plus ladder (GenScript, USA); Lane 2: Non-induced 0 hours WCL, Lane 3: 1.0 mM IPTG 0 hours WCL; Lane 4: Non-induced 24 hours WCL; Lane 5: 1.0 mM IPTG 24 hours WCL; Lane 6: Non-induced 24 hours soluble fraction; Lane 7: 1.0 mM IPTG 24 hours soluble fraction; Lane 8: Non-induced 24 hours insoluble fraction; Lane 9: 1.0 mM IPTG 24 hours insoluble fraction.

### 3.11.1 Western Blotting of Protein Expression in Rosetta™ (DE3) pLysS cells

The 24 hour post-induced whole cell lysate, and the 24 hour post-induced insoluble and soluble fractions were loaded onto commercial 12% Express PAGE gels. Two gels were run, one to be tested against each of the primary antibodies, 6X His Tag antibody and VMO1 antibody. In addition to these samples, 5 µL each of WB-MASTER Protein Standard and PAGE-MASTER Protein Standard Plus (GenScript, USA) protein ladders were both loaded into wells for determination of protein size. The WB-MASTER Protein Standard consists of seven recombinant proteins with each of the proteins containing an IgG banding site that is able to bind to primary or secondary antibodies. Samples were loaded onto the gels as per Section 2.13 and run for 30 min at 200 V. Prior to loading the

proteins into the gel, the concentration of the mouse ear protein extract was determined using a quick Bradford Assay against BSA standards (Section 2.12). The concentration was determined as approximately 1 mg/mL and 10 µL was used for preparing the sample for SDS-PAGE gel analysis.

#### 3.11.1.1 6X His Tag Antibody

The results of the western blot indicated that the 6X His Tag antibody had bound to proteins present in the 24 hour IPTG induced whole cell lysate and 24 hour IPTG induced insoluble fraction at 20 kDa, and the purified R343A protein at 50 kDa and 35 kDa (Figure 32). No binding of the antibody occurred to the BSA protein or the 24 hour IPTG induced soluble fraction samples. The membrane was then stained using Ponceau S stain to show that the transfer of proteins onto the membrane had been successful (Figure 33). Low concentrations of protein were present in lane 4, the 24 hour post-induction soluble fraction. The WB-MASTER Protein Standard (GenScript, USA) in lane 7 did not stain from the Ponceau S Stain which may be due to the proteins present being too low in concentration for the stain. The Ponceau S Stain has been demonstrated to detect proteins with a concentration between 250 and 500 ng on nitrocellulose membranes (Salinovich & Montelaro, 1986). Following these results, a purification protocol of the recombinant VMO1 protein was undertaken using the HIS-Select Nickel Affinity Gel (Section 2.14.1).

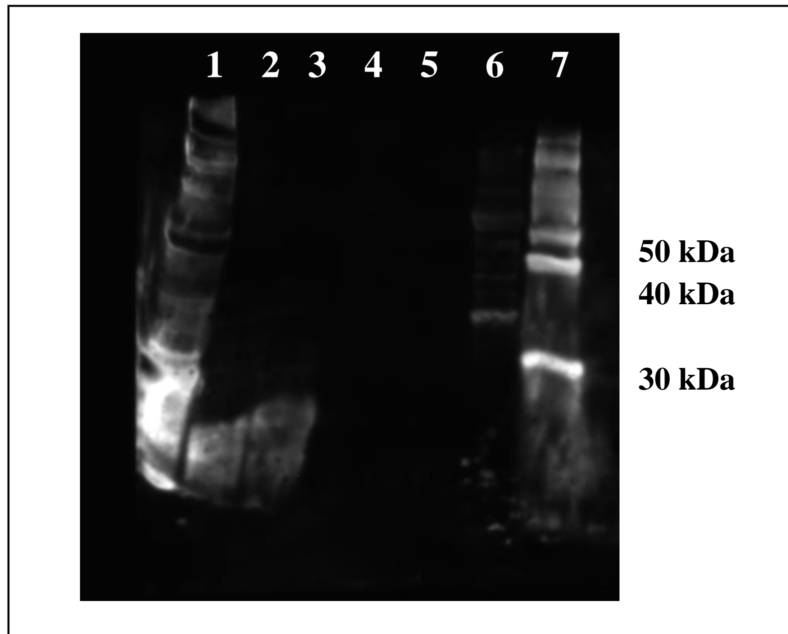


**Figure 32: Western blot using 6X His Tag antibody.** The goat anti-rabbit IgG-HRP was used as the secondary antibody. Chemiluminescence was allowed to develop for two min using ECL developing solution. The membrane was imaged using the FujiFilm Intelligent Dark Box II with the FujiFilm LAS-1000+ camera. Lane 1: PAGE-MASTER Protein Standard Plus (GenScript, USA); Lane 2: 24 hour IPTG WCL; Lane 3: 24 hour IPTG insoluble fraction; Lane 4: 24 hour IPTG soluble fraction; Lane 5: 4 mg/mL BSA; Lane 6: Purified R343A protein; Lane 7: WB-MASTER Protein Standard (GenScript, USA).

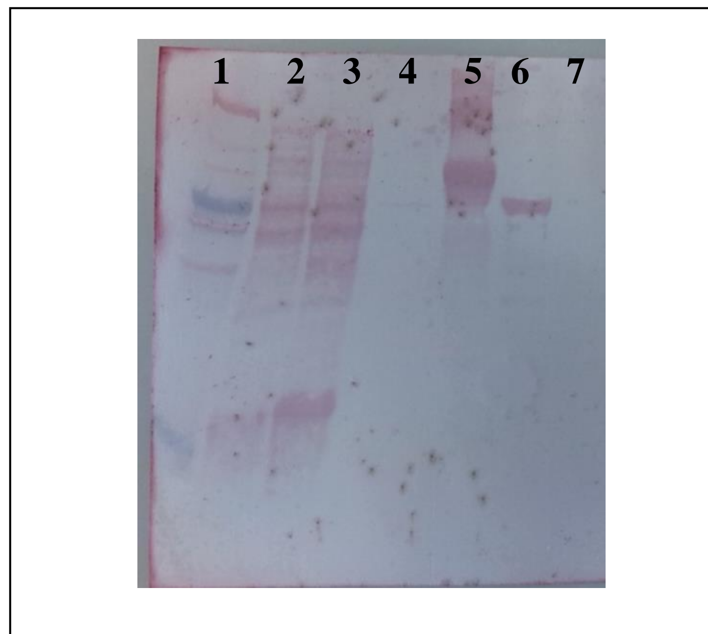


**Figure 33: Staining of PVDF membrane using Ponceau S stain**
Lane 1: PAGE-MASTER Protein Standard Plus (GenScript, USA); Lane 2: 24 hour IPTG WCL; Lane 3: 24 hour IPTG insoluble fraction; Lane 4: 24 hour IPTG soluble fraction; Lane 5: 4 mg/mL BSA; Lane 6: Purified R343A protein; Lane 7: WB-MASTER Protein Standard (GenScript, USA).

#### 3.11.1.2 VMO1 Antibody

The results of the western blot indicated that the VMO1 antibody had bound to proteins present in the 24 hour IPTG induced whole cell lysate and 24 hour IPTG induced insoluble fraction at approximately 20 kDa (Figure 34). Additionally, there appears to be a smear present at 35 kDa which is of unknown identity in lane 2. However, the chemiluminescence was very faint and smeared on the membrane due to the transfer of the proteins from the SDS-PAGE gel onto the PVDF membrane being affected. In addition to this, the western blot indicated that no binding of the antibody occurred to the negative control (BSA protein) as expected or the 24 hour IPTG induced soluble fraction samples in lanes 4 and 6 respectively. Surprisingly, the 1 mg/mL of mouse ear protein extract, provided by Miss Blaise Forrester-Gauntlett (UoW), did not show presence of a protein binding to the VMO1 antibody as previously demonstrated (Forrester-Gauntlett, 2013). This may have been due to degradation of the proteins present in the sample over a period of time.

Following western blotting with the antibody, the PVDF membrane was then stained using Ponceau S stain to show that the transfer of proteins onto the membrane had occurred (Figure 35). Low concentrations of protein were present in lanes 2 and 3, which were the 24 hour post-induction whole cell lysate and 24 hour post-induction insoluble fraction. In particular, the 24 hour post-induction soluble fraction in lane 4 was rather light compared to other samples. Lane 5 which contained the mouse ear sample showed a smear indicated that protein degradation had occurred. The negative control protein, BSA, was shown to be a bright band present, indicating it had been transferred to the membrane but when the membranes were probed with the antibodies, interactions had not occurred as

expected. The expected size of BSA is approximately 66.5 kDa; however on the PVDF membrane, this was seen as a smear with a bright band of around this size indicating that the BSA sample had denatured.

Following the probing of the PVDF membrane with the VMO1 antibody showing that the recombinant VMO1 protein was present in the insoluble fraction, a small scale protein purification experiment was undertaken to purify the protein.

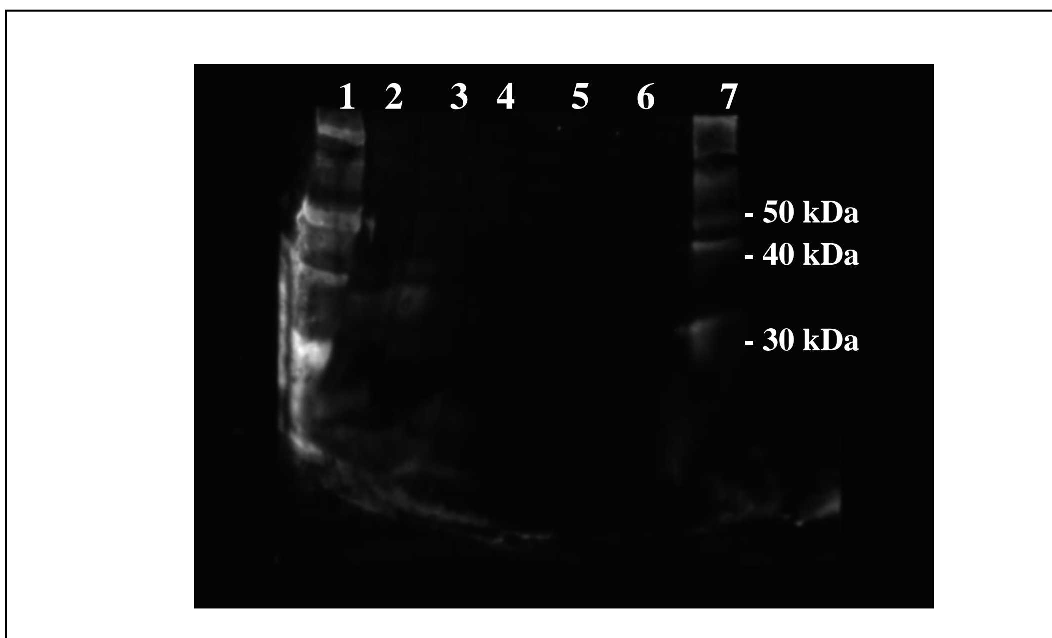


**Figure 34: Western blot using VMO1 antibody.** The goat anti-rabbit IgG-HRP was used as the secondary antibody. Chemiluminescence was allowed to develop for two min using ECL developing solution. The membrane was imaged using the FujiFilm Intelligent Dark Box II with the FujiFilm LAS-1000+ camera. Lane 1: PAGE-MASTER Protein Standard Plus (GenScript, USA); Lane 2: 24 hour IPTG WCL; Lane 3: 24 hour IPTG insoluble fraction; Lane 4: 24 hour IPTG soluble fraction; Lane 5: Mouse Ear protein extract; Lane 6: 4 mg/mL BSA; Lane 7: WB-MASTER Protein Standard (GenScript, USA).

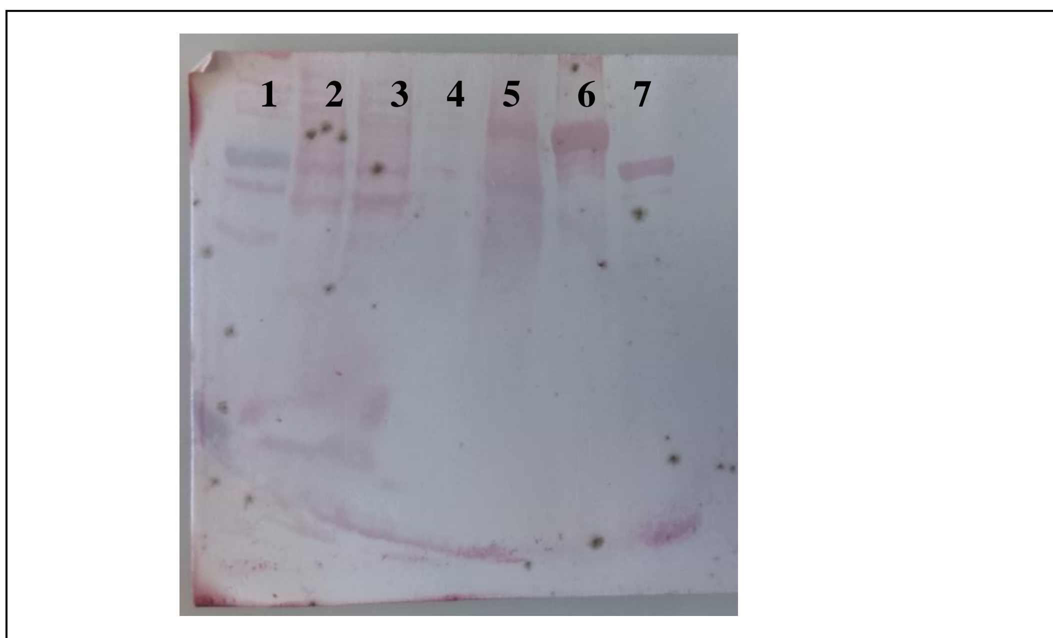


**Figure 35: Staining of PVDF membrane using Ponceau S stain**
Lane 1: PAGE-MASTER Protein Standard Plus (GenScript, USA); Lane 2: 24 hour IPTG WCL; Lane 3: 24 hour IPTG insoluble fraction; Lane 4: 24 hour IPTG soluble fraction; Lane 5: Mouse Ear protein extract; Lane 6: 4 mg/mL BSA; Lane 7: WB-MASTER Protein Standard (GenScript, USA).

### 3.12 Protein Purification of Recombinant VMO1 Protein using HIS-Select Nickel Affinity Gel

A small scale protein purification protocol was undertaken (Section 2.14) to purify the recombinant VMO1 protein from the insoluble fraction sample. Briefly, the 100 µL of the sample was mixed with the nickel affinity gel and centrifuged. The gel was washed with Wash buffer before using the Elution buffer to unbind the protein from the gel. Samples were collected following loading of the whole insoluble fraction (1), washing of the gel with Wash buffer (2A and 2B), washing with the Elution buffer at pH 6.0 (3A and 3B), and washing with Elution buffer at pH 4.5 (4A and 4B). Samples were then prepared for SDS-PAGE gel electrophoresis and transferred to a PVDF membrane for probing with antibodies using western blotting methodology.

### 3.12.1 Western Blot of Protein Purification Samples using 6X His Tag Antibody

Samples were taken during the wash and elution steps to examine if the protein had become unbound during the protein purification process. The western blot results show that the recombinant VMO1 protein was not present in any of the samples taken during the purification process as no His Tag antibody bound to any of the protein present (Figure 36). In addition, the antibody did bind to the positive control (purified R343A protein in lane 9) as expected and not to the negative control protein (BSA in lane 10). However there were two bright bands present in lane 9 and it is unknown what the smaller sized product was. It did not stain on the membrane with Ponceau S Stain which suggests it was of very low concentration.

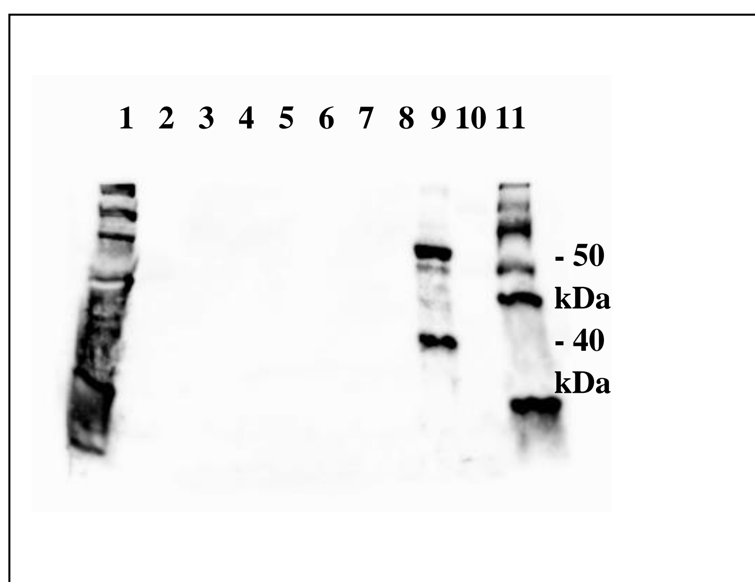


**Figure 36: Western blot probing using 6X His Tag antibody.** Chemiluminescence was allowed to develop for two min using ECL developing solution. The membrane was imaged using the FujiFilm Intelligent Dark Box II with the FujiFilm LAS-1000+ camera. Lane 1: PAGE-MASTER Protein Standard Plus (GenScript, USA); Lane 2: Sample 1; Lane 3: Sample 2A; Lane 4: Sample 2B; Lane 5: Sample 3A; Lane 6: Sample 3B; Lane 7: Sample 4A; Lane 8: Sample 4B; Lane 9: Purified R343A; Lane 10: BSA; Lane 11: WB-MASTER Protein Standard (GenScript, USA).

The membrane was then stained using Ponceau S stain to show proteins present on the membrane that were not detected using chemiluminescence (Figure 37). No proteins were detected in the lanes containing samples from the protein purification. Therefore, it was deduced that the recombinant VMO1 protein had not been purified by this protocol. The purified R343A protein and BSA that were used as controls were detected on the membrane, which were both seen at the correct size.

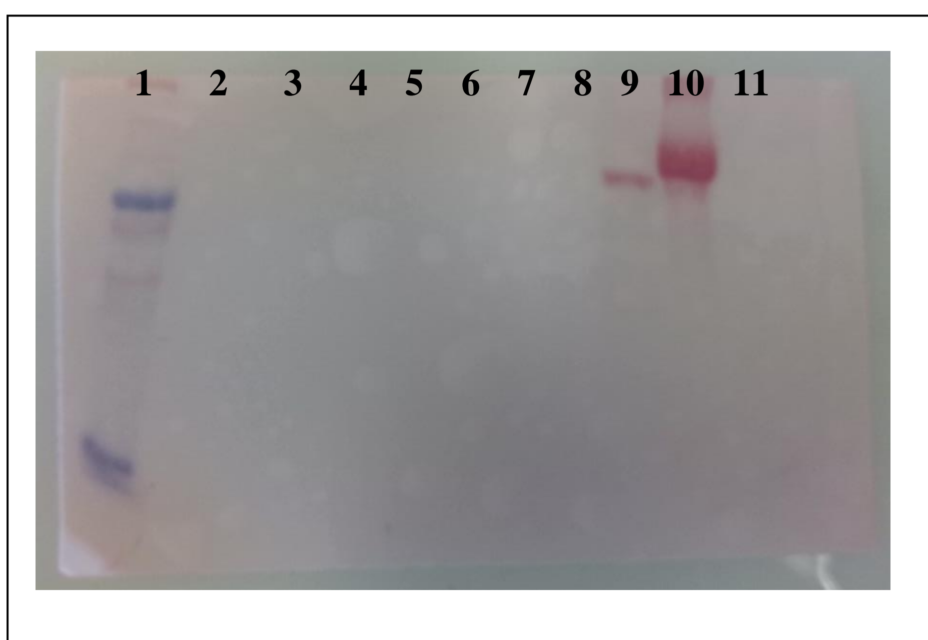


**Figure 37: Staining of PVDF membrane using Ponceau S stain**
Lane 1: PAGE-MASTER Protein Standard Plus (GenScript, USA); Lane 2: Sample 1; Lane 3: Sample 2A; Lane 4: Sample 2B; Lane 5: Sample 3A; Lane 6: Sample 3B; Lane 7: Sample 4A; Lane 8: Sample 4B; Lane 9: Purified R343A; Lane 10: BSA; Lane 11: WB-MASTER Protein Standard (GenScript, USA).

### 3.12.2 Western Blot of Protein Purification Samples using VMO1 Antibody

Probing of the samples from protein purification was repeated but using the VMO1 antibody as the primary antibody. However, a 24 hour control insoluble fraction was loaded on the gel as a control instead of the R343A protein. In addition, BSA was loaded onto the gel as a negative control. The western blot (Figure 38) shows that the VMO1 primary antibody did not detect presence of the

recombinant VMO1 protein in any of the samples taken during the protein purification process suggesting that the protein was not present or purified as a result (Lanes 2 - 8). A protein was detected in lane 9 by the VMO1 antibody that was around 50 kDa in size, however it is not known that this protein was as the recombinant VMO1 protein was predicted as around 20 kDa in size. The VMO1 antibody did not detect the BSA in lane 10 as expected.

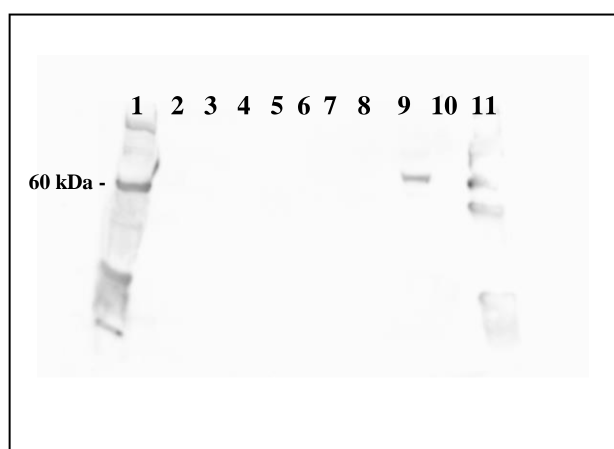


**Figure 38: Western blot probing using VMO1 antibody [N1C3] (Catalogue number: GTX106683) as the primary antibody and goat anti-rabbit IgG-HRP (Santa Cruz Biotechnology Inc., USA) as the secondary antibody.** Chemiluminescence was allowed to develop for two min using ECL developing solution. The membrane was imaged using the FujiFilm Intelligent Dark Box II with the FujiFilm LAS-1000+ camera. Lane 1: PAGE-MASTER Protein Standard Plus (GenScript, USA); Lane 2: Sample 1; Lane 3: Sample 2A; Lane 4: Sample 2B; Lane 5: Sample 3A; Lane 6: Sample 3B; Lane 7: Sample 4A; Lane 8: Sample 4B; Lane 9: 24 hour control insoluble fraction sample; Lane 10: 4 mg/mL BSA; Lane 11: WB-MASTER Protein Standard (GenScript, USA).

Following western blotting, the membrane was stained with Ponceau S stain to show presence of proteins in the samples loaded in the gel. The stain detected protein in the 24 hour control insoluble fraction and BSA sample as well as the PAGE-MASTER Protein Standard Plus ladder (Figure 39). However no recombinant VMO1 protein was detected in the samples taken during the protein purification process. This indicates that there was no protein present in the samples taken throughout the experiment and that the protein was unable to be purified using this method.

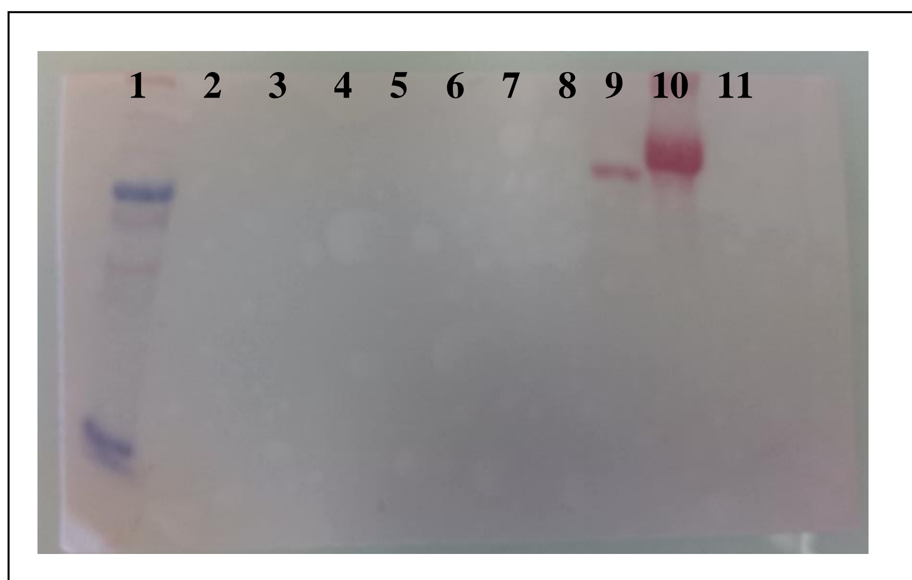


**Figure 39: Staining of PVDF membrane using Ponceau S stain**
Lane 1: PAGE-MASTER Protein Standard Plus (GenScript, USA); Lane 2: Sample 1; Lane 3: Sample 2A; Lane 4: Sample 2B; Lane 5: Sample 3A; Lane 6: Sample 3B; Lane 7: Sample 4A; Lane 8: Sample 4B; Lane 9: 24 hour control insoluble fraction; Lane 10: BSA; Lane 11: WB-MASTER Protein Standard (GenScript, USA).

# CHAPTER FOUR
# Discussion

The aims of this research were to design and express a recombinant VMO1 protein containing a His Tag that would allow for purification using a nickel resin column. These aims were addressed using a bacterial pET expression system with the *VMO1* mRNA being isolated from commercially available human cell lines. In addition to this, antibodies were tested against the recombinant VMO1 protein for validation using western blotting methodology. This chapter will review the results achieved from six research objectives that were outlined in Chapter 1 (Section 1.6) and includes a discussion on the scientific difficulties encountered, strategies used to overcome these difficulties, and future research recommendations to determine the function of VMO1.

## 4.1 Objective 1 – Bioinformatics

For this research, two isoforms of the *VMO1* gene were selected for the development of recombinant protein; isoform 1 and isoform 3. The *VMO1* isoform 1 mRNA product is predicted to be a translated protein that is 202 amino acids in size with a calculated molecular mass of 21.5 kDa. The isoform 1 transcript variant contains three exons. The *VMO1* isoform 3 mRNA is predicted to form a protein with 102 amino acids and a calculated molecular mass of 10.8 kDa. This transcript variant also has three exons but contains an alternative splice site at the 3' end of the second exon, resulting in a frameshift and a smaller protein. Isoform 1 was selected as the commercial antibody used in this research was designed to bind specifically to this protein product. Therefore, it is very important that the VMO1 antibody is tested against isoform 1 protein to

demonstrate antigen specificity, especially since this antibody will be used for future studies to determine protein function. In addition, the isoform 3 product was selected to test if the antibody was able to recognise and bind to other isoforms of the VMO1 protein.

The pET28b(+) vector is part of the pET expression system which can be used for expression of recombinant proteins in bacterial cells. Genes of interest can be cloned into the MCS of the vector and be under the control of a T7 promoter for expression (Appendix Two). The T7 RNA polymerase which is produced by the expression hosts (λDE3 lysogen) binds to the T7 promoter.

This vector was selected due to the presence of two hexahistidine peptide tags which allows for the detection of the expressed recombinant protein using a His antibody, and purification using a nickel resin column. The gene of interest, *VMO1*, was inserted into the MCS of the vector which had been digested with the two restriction enzymes, EcoRI and BamHI. These enzymes were selected for digestion of the vector due to the recognition sites for these enzymes only being present once each in the vector so it would only be digested at these sites. The selection for inserting the gene into this region also allowed the gene to be downstream from the T7 promoter and His Tag and the correct open reading frame for both protein expression and purification purposes.

The primers for targeting the two isoforms were designed with restriction enzyme sites near the start and end of the products (Figures 8 and 9). This was to allow for digestion of the PCR products with these enzymes to create sticky overhangs on the products. This allowed the sticky overhangs to be ligated into their complementary overhangs on the pET28b(+) vector. This helped to ensure that the

gene was ligated into the vector in the correct direction for translation. Finally, the recombinant proteins were designed to have an N-terminal His Tag.

## 4.2 Objective 2 – Amplification of *VMO1* mRNA from Commercial Human Cell Lines

RNA was provided from two cell lines, SUM149PT (breast cancer) and THP1 (monocytic leukaemia) and was converted to cDNA using the Tetro cDNA synthesis kit. The oligo $(dT)_{18}$ primers were selected for the conversion as they selectively target the poly(A) tail on the 3' end of mRNA in a total RNA sample. This was to ensure that the mRNA coding for protein was selected for.

Following cDNA synthesis, PCR was performed using the primer set HCC30/31 for the amplification of *VMO1*. In addition to this, a positive PCR control, *GAPDH*, was used to test that the cDNA synthesis was successful as the expected band of 412 bp was observed on the agarose gel. The results of this PCR suggested that the *VMO1* gene was expressed in SUM149PT cells as a band of 354 bp was observed. However, this is not in agreement with previous data (Crossan, 2014). The *VMO1* gene was not amplified in the THP1 cDNA. The PCR results also indicated that the SUM149PT RNA was contaminated with genomic DNA due to the presence of a band at 800 bp in size (Figure 10) in the – RT control sample. Following on from this, gradient PCR was used to determine the best annealing temperature with the two primer sets that were designed with restriction enzyme sites with SUM149PT cDNA (Figure 11).

### 4.2.1 PCR Optimisation

Gradient PCR was performed using the primer sets SH1F/1R and SH2F/2R to determine the best annealing temperature for the primers. Results showed that the

annealing temperature of 60°C was the best for each of the primer sets due to the brightness of bands of the expected size on the agarose gel despite multiple bands being present in the lanes from non-specific amplification by the primers and differing *VMO1* isoforms (Figure 11). The band of the expected size could be easily gel purified and DNA sequenced for cloning purposes but the band was rather faint indicating a low concentration of DNA present in the sample. Loss of DNA concentration could occur over the downstream applications such as gel purification. However, due to the presence of genomic DNA contamination, a new RNA sample was harvested from cultured SUM149PT cells. Therefore, the RNA could be DNase-treated to remove this contamination prior to downstream applications such as real-time PCR. The new RNA was DNase-treated and converted to cDNA as per Section 2.1.4. Prior to cDNA synthesis, the RNA was checked for quality and quantity using a NanoDrop Spectrophotometer. The RNA was read at wavelengths of 230 nm, 260 nm, and 280 nm. Nucleic acids and proteins have absorbance maxima of 260 nm and 280 nm, respectively. The ratio of these two wavelengths, the 260/280 ratio, is used to determine the purity of the sample. A ratio of around 2.0 is determined as being a pure RNA sample. The 260/280 ratio was determined as 1.68. This 260/280 ratio indicated that the SUM149PT RNA sample was contaminated with proteins absorbing near the 280 nm wavelength (Table 13).

Additionally, the 260/230 ratio is often used as an indication of contamination to samples with a ratio of 2.0 – 2.2 indicating that RNA is free from organic compounds. The 260/280 ratio for the SUM149PT RNA sample was 0.69, which is lower than expected. However, it is suggested that the glycogen used as a coprecipitate during the TriZol extraction process can be a result of these low

ratios. In addition, the TriZol method uses a number of organic compounds and chaotropic reagents for the extraction process such as phenol, guanidinium thiocyanate, and chloroform (Chomczynski & Sacchi, 1987) which can contaminate the sample during the extraction process. These three compounds can also affect the 260/230 ratio when present in nucleic acid samples, leading to lower readings at these spectra. Contamination could be overcome by using commercially available spin-column kits for RNA extraction from cell lines. In addition, these kits overcome the use of dangerous chemicals such as chloroform and are faster than the traditional method for the RNA extraction of around three hours.

### 4.2.2 Unexpected PCR Results

The newly synthesised SUM149PT cDNA as used as a template for PCR using the primer sets of SH1F/SH1R and SH2F/2R. Additionally, a PCR positive control of *GAPDH* was used. Furthermore, the primer set HCC30/31 was used as a positive control for *VMO1* expression since it had been demonstrated to amplify SUM149PT cDNA previously (Figure 10). Following amplification, *GAPDH* control showed a presence of a single bright band as expected on the agarose gel which indicates that the PCR had not been hindered by any contamination present (Figure 12). However, the results were surprising due to a lack of amplification of any products by primers designed for *VMO1* (SH1F/1R, SH2F/2R, and HCC30/31) despite having previously amplifying gene products in other SUM149PT cDNA. Alternatively, these discrepancies could be due to RNA degradation, use of oligo $(dT)_{18}$ primers targeting poly(A) mRNA or issues arising from working with human cancer cell lines such as misidentification, genetic instability and phenotypic drift.

RNA may have been degraded by RNases present or due to it being less stable than DNA over time. Furthermore, the RNA was not electrophoresed on a denaturing agarose gel due to the low yield of RNA obtained. However, if RNA was run on a gel, observations of clear bands representing the 28S and 18S ribosomal RNA would indicate that the RNA was intact. If the RNA had been degraded then there would be a smear present. Alternatively, the RNA could be tested using a Bioanalyzer with an RNA kit that tests the integrity of the RNA samples and gives it an RNA Integrity Number (RIN) based on the software's algorithms. The scores range from 1 to 10, with 1 representing totally degraded RNA and 10 intact RNA (Schroeder et al., 2006). Therefore, it is recommended that the RNA be tested for degradation.

cDNA synthesis was carried out using oligo $(dT)_{18}$ primers with the Tetro cDNA Synthesis Kit. The synthesis of cDNA can also be performed using random hexamer primers. These primers bind to a series of random hexameric sequences that are found in eukaryotic RNA. However, it was not made clear whether previous research involved making cDNA using either oligo $(dT)_{18}$ primers or random hexamer primers. This could be a reason for the discrepancy in the results as the *VMO1* mRNA may have had a shortened poly(A) tail or lacked one which would not have been bound to by the oligo $(dT)_{18}$ primers during cDNA synthesis. Although a majority of the known mRNA in mammalian cells are polyadenylated at the 3' end, a number of other types of RNA are known to lack these tails, such as ribosomal RNA (Yang, Duff, Graveley, Carmichael, & Chen, 2011). Therefore, the true representation of RNA abundance could have been missed by using primers that select for mRNA rather than the total RNA population. As a result, it

is recommended to repeat cDNA synthesis using random hexamer primers on the SUM149PT RNA extract.

Misidentification of human cell lines with cross-contamination occurring between cell lines can cause misleading results (Masters et al., 2001). This could be overcome by authentication of the cell line using an approved DNA method such as short tandem repeat (STR) profiling (Geraghty et al., 2014; Masters et al., 2001). STR profiling amplifies a number of polymorphic STR loci using primers that are commercially available. Along with fluorescent detection, the PCR products are given a numerical code based on the product length. Reference standards have been made for 253 human cell lines including 33 human cancer cell lines. Therefore, this allows cell lines to be matched to the reference standards for confirmation of the cell line. Therefore, it is recommended to correctly identify a cell line using a recommending DNA based method prior to undertaking research with it to confirm that it is the intended cell line.

Alternatively, the differences seen between the two SUM149PT cDNA samples could be due to genetic instability and phenotypic drift. This can occur the longer a cell line is kept culturing.

In addition, cancer cell lines can also have defects with *p53*, a gene responsible for repairing DNA damage (Nigro et al., 1989). Therefore, mutations can occur more frequently in these cell lines. This results in the genotype of the cell lines possibly changing over time with continuous cell line cultures. Finally, phenotypic instability can lead to a lack of expression of genes in the cells. This can be caused by incorrect culturing conditions leading to losses and gains in expression profiles (Geraghty et al., 2014).

It is noted that the passage number between the two flasks of sourced SUMP149PT cells was less than five passages and the growth conditions were the same. ATCC recommends that cell culture should be limited to five passages for use in experiments (ATCC, 2013). Therefore, it is most likely that RNA degradation, absence or presence of a shorter poly(A) tail in the *VMO1* mRNA transcript, or cell line misidentification are plausible reasons to explain our data.

### 4.2.3 Expression of *VMO1* in Human Cell Lines

To address the discrepancy in gene expression results, it was decided to extract RNA from two cell lines that were readily available. The cell lines used were a new source of THP1, and A549, a lung carcinoma cell line. The RNA was extracted using the TriZol method and DNase-treated. RNA concentration and purity was determined using a NanoDrop Spectrophotometer. The 260/230 ratio for the A549 RNA was 0.79 and 0.68 THP1 RNA, respectively. These results indicate the presence of organic compounds in the extracted RNA samples. Low 260/230 ratios can also be caused by glycogen which was used for co precipitation of the RNA during the extraction protocol.

Also, the 260/280 ratios were measured. THP1 RNA had a 260/280 ratio of 1.39 and A549 had a 260/280 ratio of 1.80. These ratios indicated that both samples had protein contamination causing a lower than expected ratio for pure RNA samples. This could be due to contamination from proteins during phase separation with the TriZol and chloroform.

Finally, the concentrations of the RNA samples were determined with THP1 having a concentration of 15.4 ng/µL and A549 a concentration of 97.2 ng/µL, respectively. From these two RNA samples, cDNA was produced as per Section 2.1.4 and used as a template for PCR. The PCR positive control, *GAPDH*,

indicated that the cDNA had been amplified in each of the samples meaning that the cDNA quality was suitable for PCR. This was due to the presence of a bright band of expected size on an agarose gel following electrophoresis of PCR products (Figure 13).

The cDNA from three cell lines, A549, SUM149PT, and THP1, were amplified using a number of primer sets designed to amplify the *VMO1* gene (Table 4). PCR products were loaded onto an agarose gel for separation of products (Figure 13). The results of this PCR were unexpected due to the presence of amplification in the THP1 cDNA of PCR products of the correct size. No amplification of *VMO1* was found in the cDNA from A549 and SUM149PT. The products amplified in the THP1 cDNA were cut from the gels and purified so they could be ligated into vectors. Sequencing was also performed to confirm that the PCR products were in fact the *VMO1* genes. Following this, the next step was to ligate into the expression vector, pET28b(+), to produce a recombinant VMO1 protein.

### 4.3 Objective 3 – Cloning of *VMO1* cDNA into a Bacterial pET Expression System

Initially, it was attempted to ligate the purified *VMO1* isoforms into the pET28b(+) vector using T4 DNA Ligase at a concentration of 1 U/µL. Following the ligation reaction, the products were transformed into electrocompetent *E. coli* DH5α. Cells were plated onto LB agar plates containing selective antibiotics for growth of cells only containing the pET28b(+) vector. A number of colonies on the plate were tested for the presence of the insert into the pET28b(+) vector using colony PCR. The results indicated that although the vector had been transformed into the cells, the insert had not been ligated into it in a number of colonies tested (Figure 17).

It was decided to attempt two new approaches for cloning the *VMO1* genes. Firstly, a new T4 DNA Ligase was sourced from Dr Ray Cursons (UoW). This T4 DNA Ligase was at a concentration of 5 U/µL. Secondly, it was decided to subclone the *VMO1* genes into the pBlueScript II SK (+) vector, a T-tailed cloning vector. Ligation of the *VMO1* isoforms was performed as per Section 2.6 before being transformed into chemical competent *E. coli* DH5α. Colonies grown on an LB agar plate with selective antibiotics were tested for the presence of the inserts as well as directional colony PCR to confirm the inserts were ligated into the pBlueScript II SK (+) vector in the correct direction. Additionally, sequencing confirmation that the inserts were *VMO1* genes was performed by the Waikato DNA Sequencing Facility. Following sequencing confirmation, the *VMO1* isoform 1 gene was digested out of the pBlueScript II SK (+) vector using EcoRI and BamHI and ligated into the digested pET28b(+) vector using the T4 DNA Ligase at 5 U/µL. Transformation into electrocompetent *E. coli* DH5α and colony PCR confirmed the successful ligation of the gene into the vector (Figure 25). Sequencing results also showed it was inserted in-frame of the His Tag of the pET28b(+) vector for purification (Figure 27).

It would be recommended to test the T4 DNA Ligase activity at 1 U/µL by digesting Lambda DNA with the HindIII restriction digest enzyme. It is expected that the digested products would be ligated back together with the T4 DNA Ligase and the results could be visualised on an agarose gel. This would confirm whether the T4 DNA Ligase was active or not.

Additionally, a phosphatase, such as calf-intestinal alkaline phosphatase, can be used on the digested pET28b(+) vector prior to ligation. This works to prevent

self-ligation of the vector by dephosphorylation of the 5'-phosphorylated termini of the vector DNA so the linearised ends of the vector cannot bind back together.

Finally it would be recommended to ligate the *VMO1* isoform 3, as well as other isoforms into the pET28b(+) vector to produce other recombinant VMO1 proteins for testing against antibodies and functional assays.

### 4.3.1 Sequencing Confirmation of *VMO1*

DNA sequencing of the recombinant *VMO1* isoform 1 DNA clone was performed by an Applied Biosystems 3130xl Genetic Analyser which has the capability of sequencing 950 bp when a long-read run is performed according to manufacturer's standards. The sequencing data generated from the T7, SH1F, and SH1R primers did not cover the entire length of the 1051 bp product. Therefore internal primers, SH3F and SH3R, for this isoform were designed to cover the inner region of the isoform 1 gene between position 133 and position 568. A 504 bp consensus sequence was generated from the resulting four overlapping contigs, LMP10F, SH1F, SH3F, and SH3R. The VMO1 sequence was from position 149 to position 457 which aligned 100% with the *VMO1* reference sequence (GenBank Accession number: NM_182566.2). The recombinant protein sequence was between position 35 and position 457 resulting in a 423 nucleotide fragment. In addition, no stop codons were introduced into the open reading frame under the influence of the T7 promoter. Therefore, our data shows that *VMO1* gene is expressed in the THP1 cell.

Following the successful ligation of the *VMO1* isoform 1 gene into the pET28b(+) vector, the next step was to transform the construct into a *E. coli* host strain for protein expression.

### 4.4 Objective 4 – Expression of Recombinant VMO1 Protein in *E. coli*

The expression of recombinant VMO1 protein was accomplished through manipulation of *E. coli* cells using the pET expression system along with induction of expression using IPTG. IPTG is a compound that mimics allolactose, a lactose metabolite. It is able to bind to the lac repressor in bacterial cells, releasing it from the lac operator. This allows the lac operon to begin transcription of genes under control of this operon (Marbach & Bettenbrock, 2012).

Initially, the pET28b(+)/*VMO1* isoform 1 construct was transformed into electrocompetent *E. coli* BL21 (DE3) cells. Protein induction using two final concentrations of IPTG (0.4 mM and 1.0 mM) were tested as per Section 2.10.1. These two concentrations were used as per recommendations by Novagen (2000). In addition, a control was used that was not induced with IPTG to compare expression of proteins between non-induced and induced cells. Following induction with IPTG, samples were taken at a number of time points (0 - 24 hrs) from each of the non-induced control, 0.4 mM IPTG induction, and 1.0 mM IPTG induction. Samples were prepared for SDS-PAGE analysis and protein expression was assessed following staining of the gel. An expected VMO1 protein of around 20 kDa in size was expected on the gel as a rather bright band in induced cells following expression induction using IPTG. However, this was not seen. To address this, an alternative *E. coli* host strain was investigated.

The pET28b(+)/*VMO1* isoform 1 construct was transformed into *E. cloni* BL21 (DE3) pLysS cells. These cells contain the pLysS plasmid which prevents the basal expression from the T7 promoter using T7 lysozyme. The T7 lysozyme inhibits the transcription of the T7 RNA polymerase which plays a role in promoting expression of genes downstream from the T7 promoter. Following

induction with IPTG, the DE3 lysogen expresses the T7 RNA polymerase which can bind to the T7 promoter for expression (Zhang & Studier, 1997). Protein expression was induced as per Section 2.10.2 with IPTG at a final concentration of 1.0 mM along with a non-induced control sample. The samples were run on an SDS-PAGE gel for protein separation. Analysis of the protein samples indicated that the recombinant protein was not expressed in these cells. Therefore, glucose was introduced to the LB broth and the expression induction repeated. The use of glucose in the broth was aimed to control non-specific expression and up-regulate the expression of the recombinant VMO1 protein (Novy & Morris, 2001; Pan & Malcolm, 2000). However, upon analysis of the protein extracts run on an SDS-PAGE gel (Figure 30), it found that the glucose greatly inhibited protein expression over the time course with very low concentrations of protein visualised on the gel after 24 hours of expression induction. Additionally, the positive control that was transformed into the bacterial cells did not show expression of the expected 50 kDa sized *PhoH2* protein. This protein had previously been expressed in these bacterial cells using the same conditions for the expression of the recombinant VMO1 protein. Therefore due to non-expression, the temperature of the incubator was checked using a thermometer and new IPTG was made. It has been found that the incubator has fluctuations in the temperature and does not stay at the set temperature (Sari Karppinen, personal communication, March 2015). Due to time constraints, it was not possible to induce and express the 50 KDa positive control protein construct. Finally, the recombinant VMO1 protein did not appear to be over-expressed in any of the samples based on the protein expression shown on the PAGE gel.

Finally, a third *E. coli* strain, Rosetta™ (DE3) pLysS cells, was used for the expression of the recombinant VMO1 protein. This strain was selected to overcome the issues of potential codon bias. Codon bias occurs in *E. coli* cells with major codons being used for highly expressed genes in the cells and rare codons in genes that are expressed at low levels. If a heterologous gene is introduced to the *E. coli* cells and expression is induced using a reagent such as IPTG, the cells can encounter issues with not having the appropriate transfer RNAs (tRNA) present to keep up with translation (Kane, 1995). The *VMO1* nucleotide sequence contained rare codons for the amino acids arginine, isoleucine, and leucine. Following transformation of the pET28b(+)/*VMO1* isoform 1 construct into these bacterial cells, induction of protein expression was carried out and the proteins analysed using SDS-PAGE gels. The gels indicated over-expression of a protein approximately 20 kDa in size in both the 24 hour post-induction whole cell lysate and the 24 hour post-induction insoluble fraction. This indicated that the VMO1 protein was insoluble and found in inclusion bodies formed inside the bacterial cells.

The next step was to test the protein samples against both the VMO1 antibody and His antibody for confirmation that the recombinant VMO1 protein had been produced in these bacterial cells. This was performed using western blotting methodology.

### 4.5 Objective 5 – Purification of Recombinant VMO1 Protein

Purification of the recombinant VMO1 protein was attempted using a HIS-Select® Nickel Affinity Gel (Sigma-Aldrich, USA). A small scale purification was attempted first to determine the right conditions for purification. Protease inhibitors were used during sample preparation to prevent the degradation of

proteins present in the sample. Additionally, the sample was treated with DNase I to remove genomic DNA contamination from the protein sample. DNA present in the sample can cause it to have high viscosity which can prevent protein analysis.

Two differing pHs of Elution buffer were used for the protein purification. Elution of the protein samples from the gel was first attempted with an Elution buffer at pH 6.0, followed by an Elution buffer at pH 4.0. This is due to some recombinant proteins with a His Tag not eluting at pH 6.0 so a pH as low as 4.5 can be attempted.

Samples were collected throughout the purification process and prepared for SDS-PAGE separation of proteins and western blotting. Probing of the membrane with the VMO1 antibody indicated that the recombinant protein was not present in any of the samples collected during the protein purification (Figure 38). Additionally, probing with the His antibody did not bind to protein in the samples collected from the protein purification experiment (Figure 36). Furthermore, Ponceau S staining of the membrane failed to detect protein on the membranes in the purification samples despite detecting the presence of the controls for the western blotting (Figures 37 and 39). The recombinant protein not binding to the gel could be due to a number of issues. Firstly, recombinant protein was not correctly folded into its native confirmation and the epitope was hidden. Secondly, the conditions of the purification were optimal for binding by the protein and therefore it would be recommended to repeat the small scale purification and check the protein sample pH was between 7.0 and 8.0. Additionally, the protein sample from which an aliquot was loaded onto the affinity gel showed the presence of a white precipitate a day later following storage of the sample at room temperature.

Therefore, it is likely that the protein precipitated out of solution during the purification process.

Over expression of the heterologous protein in the *E. coli* cells can lead to their aggregation in the cytoplasm, forming an insoluble inclusion body (Tsumoto, Ejima, Kumagai, & Arakawa, 2003). These inclusion bodies must be solubilised and the protein refolded correctly. Solubilisation of the inclusion body can be achieved through the use of detergents such as urea and guadinium hydrochloride. Following solubilisation proteins in the inclusion body, the proteins need to be correctly folded using dialysis and buffers for refolding of the protein (Tsumoto et al., 2003). Therefore, it would be recommended to attempt such strategies to solubilise the inclusion bodies for successful protein purification.

Protein refolding and solubility of the protein can be improved through the use of molecular chaperones which are co-expressed in the *E. coli* cells with the recombinant protein (Sørensen & Mortensen, 2005). However these methods can still prevent the correct folding of the recombinant protein due to properties of the protein. The *E. coli* cytoplasm is in a reducing state where disulfide bonds are prevented from forming. This is through two pathways, the thioredoxin system and the glutathione system. These pathways contribute to preventing the formation of disulfide bonds in proteins (Hannig & Makrides, 1998). As shown by Kido et al. (1995), there are disulfide bridges holding together β-sheets together in the chicken VMO1 protein. Furthermore, Forrester-Gauntlett (2013) found through bioinformatics analysis that the presence of the eight cysteine residues for forming the disulfide bonds in the chicken VMO1 protein are in identical places in both the mouse and human VMO1. Therefore, the presence of these cysteines that play a role in the correct folding of the VMO1 protein may have been

prevented from forming disulfide bridges. This prevention of disulfide bonds can be overcome by disrupting the *trxB* and *gor* genes that encode for the two pathways and there are *E. coli* strains available that lack these genes. Therefore, it would be recommended to trial these strains to determine if the recombinant protein can be folded correctly in these cells (Sørensen & Mortensen, 2005).

## 4.6 Objective 6 – Validation of the VMO1 Antibody against the Recombinant VMO1 Protein

The VMO1 antibody was validated against both non-purified and purified protein samples for detection of the presence of the recombinant VMO1 protein. Firstly, western blotting performed on both the 24 hour IPTG induced insoluble fraction and whole cell lysate indicated the presence of a protein that bound to the VMO1 antibody at approximately 20 kDa (Figure 34). There was a protein of 35 kDa size detected in the whole cell lysate sample, however, it is unknown what this protein could be. The identity of this protein could be identified using mass spectrometry to determine its amino acid sequence.

As demonstrated in the western blot image (Figure 34), the VMO1 antibody did not bind to the mouse ear extract provided by Miss Blaise Forrester-Gauntlett (UoW). Previously, this sample was demonstrated to be bound to by the VMO1 antibody. This sample was made from inner ears extracted from four mice specimens aged 28 days or older. The inner ears were pooled together to increase the protein yield during the extraction process (Forrester-Gauntlett, 2013). Due to the absence of binding of the antibody to the protein, it is possible that there has been degradation of the sample over time. Therefore, it would be recommended to extract a new mouse ear protein lysate as per the protocol outlined by Forrester-Gauntlett (2013) and probe this new sample with the VMO1 antibody.

Western blotting performed on the purified protein samples using the VMO1 antibody was not able to detect the presence of the recombinant protein (Figure 38). This indicated that the protein had not been purified during this process. Therefore, it is recommended that protein purification be repeated to obtain a pure recombinant VMO1 protein to validate against the VMO1 antibody. Furthermore, a protein of around 50 kDa in size was detected in lane 9 on the membrane by the VMO1 antibody in the 24 hour control insoluble fraction. It is unknown what this protein could be or why it was not detected on the western blot of the non-purified protein samples (Figure 34). Mass spectrometry could be used to determine what this protein was based on the amino acid sequence.

## 4.7 Future Recommendations

There are three goals that need to be addressed to continue the investigation of VMO1 function. Firstly, the discrepancies between the results reported in this thesis and published data regarding the expression of the *VMO1* gene in human cell lines. Secondly, the large scale purification of recombinant VMO1 isoform 1 protein. Thirdly, the function of VMO1 protein needs to be determined through a number of assays. These three goals are discussed in more detail below.

### 4.7.1 Characterisation of VMO1 in Human Cell Lines

The Human Protein Atlas is a publicly available on-line database with access available to millions of high-resolution images of proteins distributed in 44 different human tissues, 20 different cancer cell tissues from patients, and 46 human cell lines (Pontén, Jirström, & Uhlen, 2008; Uhlén et al., 2015). Data provided by the Human Protein Atlas shows that RNA-Seq has identified the presence of *VMO1* mRNA in eight of the 46 human cell lines that data is available for. RNA expression is measured as number of Fragments Per Kilobase gene

model and Million reads (FPKM). This value is calculated and an average FPKM value is assigned based on replicate values to give an abundance score. These FPKM thresholds are categorised into low, medium, or high RNA abundance. Within cell lines, non-detection is indicated with a score of 0 – 1, low 1 – 20, medium 20 – 50, and high >50.

Interestingly, the Human Protein Atlas indicates that the *VMO1* mRNA is present in the A549 cell lines based on RNA-Seq data. The FPKM score for this data was given as 2 based on two samples tested. This indicates that the *VMO1* mRNA is at low abundance in these alveolar basal epithelial cells. In comparison, data from the protein atlas suggests that *VMO1* mRNA is not present in HeLa cells, yet a commercial antibody available from ProteinTech was validated using protein extracted from HeLa cells (cervical cancer) and HEK-293 cells (Human Embryonic Kidney), resulting in the observation of a 22 kDa protein (ProteinTech, 2015).

Our PCR data suggests that *VMO1* is expressed in SUM149PT and THP1 cell lines. The Human Protein Atlas has data on two breast adenocarcinoma cancer cell lines; MCF7 and SK-BR-3 – IHC. Interestingly, MCF is positive for VMO1 RNA, whereas SK-BR-3 is not. Additionally, the THP1 cell line reported an FPKM of 0.0 and 0.2 respectively for two samples analysed using RNA-Seq. This indicates that there was no detection of *VMO1* RNA in the samples from this cell line. Furthermore, there has been no antibody probing data made available for these cell lines.

In addition to this, the VMO1 protein was stained for using a VMO1 antibody (Sigma-Aldrich, USA, Product name: HPA023038) on 48 normal human tissue samples as well as human cancer samples of the 20 most common cancer types.

Results showed that the VMO1 protein was present in five of the 12 breast cancer tissue samples at high levels and seven of the 12 breast cancer tissue samples at medium levels. However, it is noted that the Protein Atlas VMO1 antibody differs to the one used for the purposes of this study.

In conclusion, it would be recommended in the future to purchase this new antibody and test both antibodies on protein lysate extracted from the three human cell lines used in this study; SUM149PT, A549, and THP1 but also include HeLa and HEK-293 cells. In addition, harvest RNA from these cultured cells, run extracted RNA on a denaturing agarose gel, synthesise cDNA using random hexamer primers and design new *VMO1* primers to target the four isoforms. The outcome of this may resolve the true expression of VMO1 in human cell lines and the discrepancies in the results presented in this thesis and current data available on-line. Alternatively, with human ethics approval RNA could be extracted from a snap-frozen or a formalin-fixed, paraffin-embedded biopsy tissue specimen.

### 4.7.2 Recommendations for Purifying Recombinant VMO1 Protein

A number of recommendations were outlined in Section 4.5 to solubilise the inclusion bodies for successful protein purification. Following optimisation of small scale protein expression and purification of the VMO1 protein from inclusion bodies, the next step would complete a large scale protein induction, extraction and purification experiment. It would then be advisable to make the protein more specific through cleavage at the thrombin site of the purified recombinant protein. This cleavage site is located between the hexahistidine tag and the start of the VMO1 protein and would remove 13 amino acids from the beginning of the recombinant protein. This step would form a protein with no additional tags and therefore closely resembles the native protein (Hefti, Dixon, &

Vervoort, 2001). The resulting protein could then be concentrated and purified using a size exclusion column which would remove the cleaved His Tag peptide and protease from the sample. Finally, a Bradford assay would be performed to determine the concentration of the purified protein against BSA standards, and a western blot run to confirm that the VMO1 antibody recognises the further purified recombinant protein.

### 4.7.3 Future Determination of VMO1 Protein Function

Once the recombinant VMO1 protein has been concentrated and purified, a number of studies can be investigated to determine the function of the VMO1 protein and if it plays a significant role in the mechanism of hearing. Firstly, the stability of the protein needs to be determined. This will address the ideal conditions that the VMO1 protein can be stored at to prevent degradation or precipitation of the protein out of solution. For example, this would involve the implementation of Circular dichroism spectroscopy (Greenfield, 2006).

Secondly, it is recommended that the transferase activity of VMO1 be assessed. It has been found that the chicken VMO1 protein possesses the ability to synthesise N-acetylchitooligosaccharides from hexasaccharides of N-acetylglucosamines which is similar to the function of the lysozyme (Kido et al., 1995). Therefore it would be interesting to determine whether the recombinant human VMO1 has the same or similar properties.

Thirdly, to determine protein partners of VMO1, a pull-down assay is recommended. The pull-down assay is a technique which can be used to determine the physical interactions between proteins and for determining protein-protein interactions. This could be used to determine which proteins interact with VMO1. The technique is a form of affinity purification where the "bait" protein,

VMO1, is captured to an affinity ligand that is immobilised. The VMO1 protein would then be incubated with “prey” proteins in a cell lysate. Unbound protein is washed away that has not interacted with the captured VMO1 protein. The captured VMO1 protein along with its protein partners are eluted using a number of methods such as competitive analytes, low pH, or reducing buffers and the products are analysed using SDS-PAGE gels (Golemis & Adams, 2002).

Finally, it would be recommended to test inner ear protein extracts from mice exposed to loud noise compared to control mice. Previous studies using RNA-Seq of rat cochleae has shown that *Vmo1* had a 51.77 fold increase in expression following exposure to loud noise in comparison to the control group (Patel et al., 2013). The aim of this study was to determine the changes in gene expression following acoustic trauma. Therefore, it would be recommended to repeat this experiment in another animal model, such as the mouse whose Vmo1 protein is 92% similar to the rat Vmo1 amino acid sequence, to see if similar results are obtained in regards to changes of gene expression for the *Vmo1* gene in the inner ear. Finally, protein samples could be obtained from the separate groups for analysis by SDS-PAGE and western blotting methodology to test for presence of the VMO1 protein in the samples.

# CHAPTER FIVE
# Conclusion

The first aim of this research was to design, clone, and express a recombinant VMO1 protein. This was achieved through the use of the pET28b(+) expression system and *VMO1* mRNA amplified from the THP1 human cell line. Sequencing data confirmed that the *VMO1* gene was expressed in this cell line despite data from the Human Protein Atlas suggesting otherwise.

The second aim was to purify the recombinant VMO1 protein using a nickel resin column. Although this aim was not achieved due to complications with the purification procedure, a number of techniques to overcome these issues have been discussed for future purification attempts.

Lastly, the third aim was to validate a commercially available VMO1 protein against the recombinant protein. It was demonstrated that the antibody was able to bind to the recombinant protein using western blotting methodology, indicating that the antibody was able to identify the recombinant protein and could be reproduced successfully.

In conclusion, the findings presented in this thesis suggest that *VMO1* mRNA of both 387 bp and 691 bp is expressed in THP1 human cell line and insoluble VMO1 recombinant protein of approximately 20 kDa in size can expressed and be recognised by two antibodies; VMO1 and His Tag. With optimisation and purification, a large scale induction of recombinant protein would be beneficial for future functional assays such as a pull-down assay to identify interacting protein partners. This may provide further insight into the function of VMO1 in the inner ear and the role it may play in hearing.

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# APPENDIX ONE
# Buffers and Solutions

**A549 growth medium**

To make 100 mL:

| | |
|---|---|
| 89 mL | RPMI-1640 Media |
| 10 mL | Fetal Bovine Serum |
| 1 mL | 100X Penicillin/Streptomycin |

**37:1 Acrylamide: BIS (22%)**

| | |
|---|---|
| 22.2 g | Acrylamide |
| 0.6 g | Bisacrylamide |

Make up to 100 mL with $ddH_2O$, store at 4°C in a light protected bottle

**50 mg/mL Ampicillin**

| | |
|---|---|
| 50 mg | Ampicillin |
| 1 mL | $mQH_2O$ |

Filter sterilise, store at -20°C.

**100 mg/mL Ampicillin**

| | |
|---|---|
| 100 mg | Ampicillin |
| 1 mL | $mQH_2O$ |

Filter sterilise, store at -20°C.

**10% Ammonium persulfate (APS)**

| | |
|---|---|
| 0.1 g | Ammonium persulfate |
| 1 mL | $ddH_2O$ |

Store at -20°C.

**10% Blocking solution**

| | |
|---|---|
| 2.5 g | Nonfat Dry Milk powder |

Make up to 25 mL in 1X TBST. Store at 4°C for up to three days.

**75% Ethanol**

| | |
|---|---|
| 750 mL | 100% reagent grade ethanol |
| 250 mL | 0.1% DEPC $mQH_2O$ |

**$CaCl_2$/PEG solution**

To make 10 mL:

| | |
|---|---|
| 9.9 mL | 0.1 M $CaCl_2$ |
| 10 µL | 1% PEG8000 |

**Chloramphenicol**
34 mg Chloramphenicol
1 mL Ethanol
Filter sterilise, store at -20°C

**2X cOmplete, Mini, EDTA-free Protease Inhibitor solution**
1 tablet 2X cOmplete, Mini, EDTA-free Protease Inhibitor
5 mL $mQH_2O$
Store at 4°C for one week or freeze aliquots at -20°C for long term storage

**Coomassie blue stain**
0.25 g Coomassie blue brilliant stain R-250
90 mL Methanol
10 mL Glacial acetic acid
Make up to 200 mL with $ddH_2O$

**Cracking Buffer**
3.6 g Urea
Make up to 10 mL with 2X SDS Loading Buffer

**0.5M EDTA**
186.1 g disodium EDTA.$2H_2O$
Add to 800 mL of $mQH_2O$ and stir with magnetic mixer. Adjust pH to 8.0 with NaOH and adjust volume to 1 L with $mQH_2O$. Autoclave.

**Elution Buffer pH 4.5**
0.1M Sodium phosphate
8 M Urea
Dissolve in $ddH_2O$. Adjust pH to 4.5 with appropriate acids or bases.

**Elution Buffer pH 6.0**
0.1M Sodium phosphate
8 M Urea
Dissolve in $ddH_2O$. Adjust pH to 4.5 with appropriate acids or bases.

**Equilibration Buffer**
0.1 M Sodium phosphate
8 M Urea
Dissolve in $ddH_2O$. Adjust pH to 8.0 with appropriate acids or bases.

**20% Glucose**
To make 10 mL
2 g *D*-Glucose
10 mL $mQH_2O$
Filter sterilise. Aliquot into 2 mL tubes, store at -20°C

**10% Glycerol**

| | |
|---|---|
| 100 mL | Glycerol |
| 900 mL | mQ$H_2O$ |

Autoclave

**80% Glycerol**

| | |
|---|---|
| 160 mL | Glycerol |
| 40 mL | mQ$H_2O$ |

Autoclave

**100mM IPTG**

| | |
|---|---|
| 2.38 g | IPTG |
| 100 mL | dd$H_2O$ |

Filter sterilise and aliquot into 1 mL tubes. Store at -20°C.

**Kanamycin**

| | |
|---|---|
| 30 mg | Kanamycin |
| 1 mL | mQ$H_2O$ |

Filter sterilise, store at -20°C.

**LB broth**

| | |
|---|---|
| 25.0 g | LB |

Add to 1 L of distilled $H_2O$ and autoclave.

**LB plates**

| | |
|---|---|
| 25 g | LB |
| 15 g | Agar |

Make up to 1 L with mQ$H_2O$. Autoclave. Cool to ~50°C. Add antibiotics if necessary and pour. Once set, refrigerate upside down.

**6X Loading Dye**

| | |
|---|---|
| 3 mL | Glycerol |
| 25 mg | Bromophenol Blue |
| 20 µL | Xylene Cyanole |

Make up to 10 mL with sterile mQ$H_2O$.

**Lysis Buffer**

| | |
|---|---|
| 1 mL | 1 M Tris-HCl pH 7.5 |
| 6.25 mL | 4 M NaCl |
| 5 mL | Glycerol |
| 50 µL | TritonX-100 |

Make up to 50 mL with dd$H_2O$

**Lysis Buffer (NaCl - free)**

| | |
|---|---|
| 0.2 mL | 1 M Tris-HCl pH 7.5 |
| 1 mL | Glycerol |
| 10 μL | TritonX-100 |

Make up to 5 mL with dd$H_2O$

**1X Phosphate buffered saline (PBS)**

| | |
|---|---|
| 8 g | NaCl |
| 0.25 g | KCl |
| 0.2 g | $KH_2PO_4$ |
| 1.15 g | $Na_2HPO_4$ |

Make to 1 L with mQ$H_2O$

**Ponceau S stain**

| | |
|---|---|
| 0.1 g | Ponceau S |
| 0.1 g | Acetic acid |

Make to 1 L with mQ$H_2O$

**Ponceau S Destaining solution**

| | |
|---|---|
| 2.5 mL | Acetic acid |

Make up to 250 mL with sterile mQ$H_2O$

**0.4 M Sodium phosphate**

| | |
|---|---|
| 2.839 g | Disodium phosphate (anhydrous) |
| 50 mL | Sterile dd$H_2O$ |

Use at final concentration of 0.1 M.

**10% SDS**

| | |
|---|---|
| 1 g | SDS |

Dissolve in 8 mL of mQ$H_2O$ then make to 10 mL.

**2X SDS Loading Buffer**

To make 10 mL:

| | |
|---|---|
| 1 mL | 1 M Tris.HCl pH 6.8 |
| 3 mL | 100% Glycerol |
| 4 mL | 10% SDS |
| 10 mg | Bromophenol Blue |

Make up to 10 mL with sterile mQ$H_2O$

Prior to use, add 20 μL of β-Mercaptoethanol to 1 mL of 2X SDS Loading Buffer

**SUM149PT growth medium**

To make 50 mL:

| | |
|---|---|
| 46.2 mL | Ham's F12 Nutrient Mixture |
| 2.5 mL | Fetal Bovine Serum |
| 500 µL | 1 M HEPES |
| 250 µL | 1 mg/mL Insulin |
| 50 µL | 1 mg/mL Hydrocortisone |
| 500 µL | 100 X Penicillin/Streptomycin |

**THP1 growth medium**

To make 100 mL:

| | |
|---|---|
| 89 mL | RPMI-1640 Media |
| 10 mL | Fetal Bovine Serum |
| 1 mL | 100X Penicillin/Streptomycin |

**1X TAE Buffer**

| | |
|---|---|
| 20 mL | 50X TAE Buffer |
| 980 mL | dd$H_2O$ |

**50X TAE Buffer**

| | |
|---|---|
| 242 g | Tris |
| 57.1 mL | Glacial Acetic acid |
| 100 mL | 0.5 M EDTA, pH 8 |

Make to final volume of 1 L with dd$H_2O$

**1X Tris Buffered Saline (TBS)**

| | |
|---|---|
| 100 mL | 10X TBS |
| 900 mL | Sterile mQ$H_2O$ |

**10X Tris Buffered Saline (TBS)**

| | |
|---|---|
| 24.2 g | Tris base |
| 80 g | NaCl |

Adjust pH to 7.6 with HCl. Make up to 1 L with sterile mQ$H_2O$.

**1X TBST**

| | |
|---|---|
| 100 mL | 10X TBS |
| 1 mL | Tween20 |

Make up to 1 L with sterile mQ$H_2O$.

**1X Tris-EDTA buffer pH 8.0**

To make 200 mL:

| | |
|---|---|
| 2 mL | 1M Tris |
| 0.4 mL | 0.5M EDTA |

Make up to 200 mL with mQ$H_2O$. Check pH and adjust with acid/base if necessary.

**5X Tris-EDTA Buffer pH 8.0**

| | |
|---|---|
| 12.5 mL | 1 M Tris-HCl pH 8.0 |
| 2.5 mL | 0.5 M EDTA pH 8.0 |

Adjust volume to 250 mL with $mQH_2O$, autoclave

**1X Tris-Glycine running buffer**

| | |
|---|---|
| 100 mL | 10X Tris-Glycine Buffer |
| 900 mL | $ddH_2O$ |

**10X Tris-Glycine Buffer**

| | |
|---|---|
| 30 g | Tris |
| 144 g | Glycine |
| 10 g | SDS |

Make up to 1 L using $ddH_2O$.

**1M Tris-HCl pH 8.0**

| | |
|---|---|
| 121.1 g | Tris base |

Make up to 800 mL of $mQH_2O$ and adjust to correct pH. Autoclave.

**8M Urea**

| | |
|---|---|
| 4.8048 g | Urea |
| 10 mL | Sterile $ddH_2O$ |

**Wash Buffer**

| | |
|---|---|
| 0.1M | Sodium phosphate |
| 8 M | Urea |

Dissolve in $ddH_2O$. Adjust pH to 6.3 with appropriate acids or bases.

**X-Gal**

| | |
|---|---|
| 20 mg | X-Gal |
| 1 mL | Dimethyl formamide |

Store at -20°C.

# APPENDIX TWO
# Vector Maps

## pET28b(+) Vector

This vector is an expression system that is designed to produce protein within the bacterial host cell it was transformed into through induction with IPTG. The *VMO1* gene was cloned into the vector between the EcoRI and BamHI restriction enzyme sites prior to transformation.

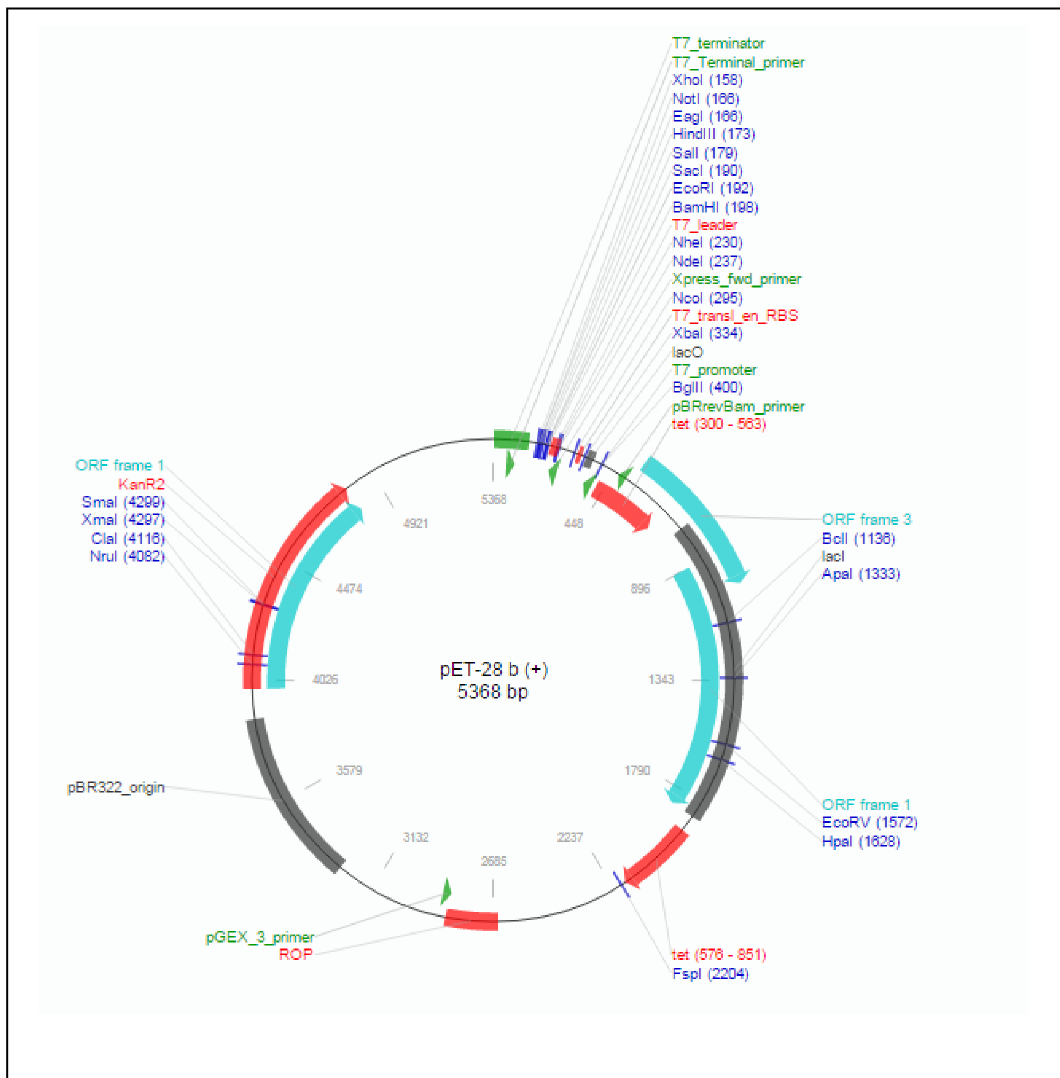


**Figure 40: pET28b(+) vector map.** Depicted is the multiple cloning site, restriction enzyme sites, sequencing primer sites (green), and kanamycin antibiotic resistance gene (red) (AddGene).

**pBlueScript II SK (+) Vector**

The pBlueScript II SK (+) plasmid is a cloning vector that can be used for blue and white colony screening for presence of the gene insert using IPTG and X-Gal. It also contains an ampicillin resistance gene used for growth selection of cells containing the plasmid on LB agar plates with ampicillin. This vector was used due to difficulties cloning the *VMO1* gene directly into the pET28b(+) vector.

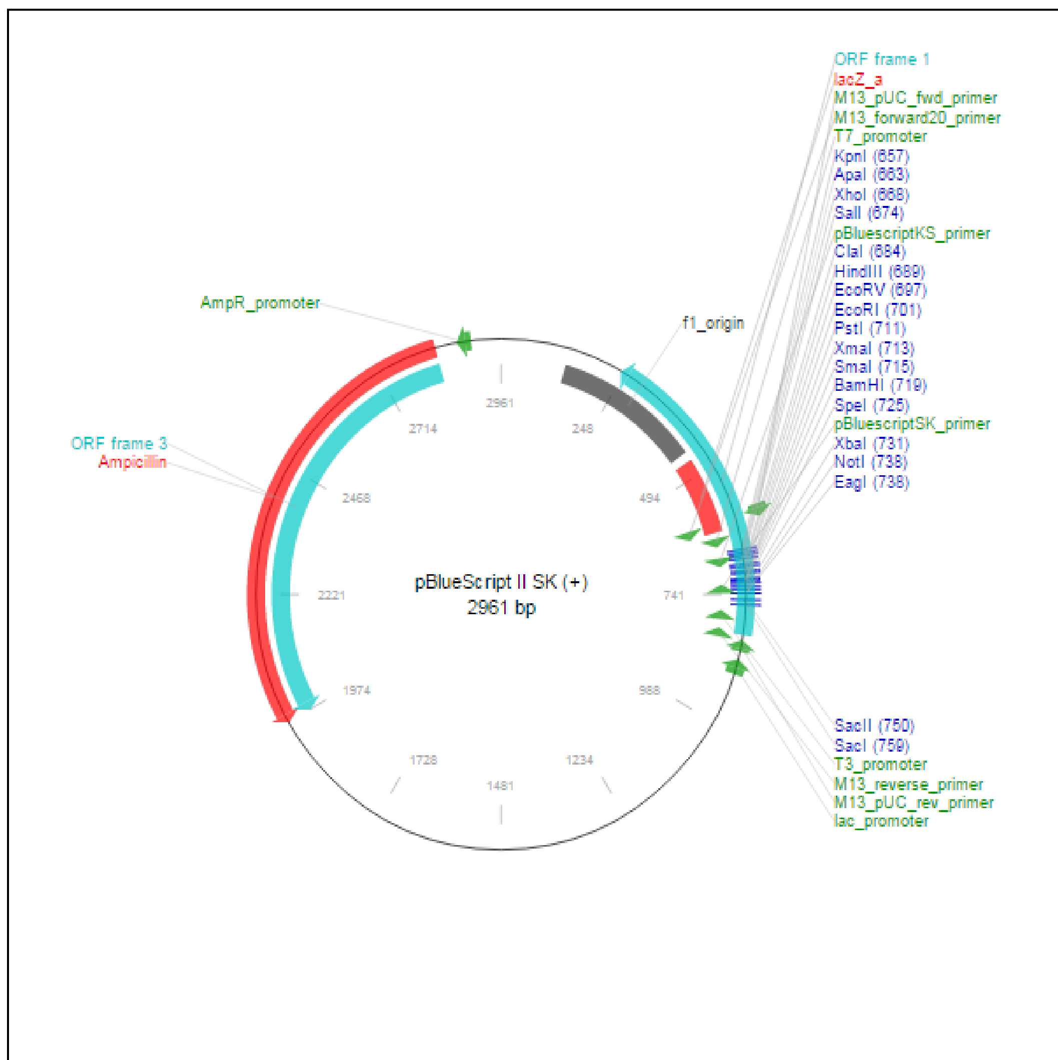


**Figure 41: pBlueScript II SK (+) vector map.** Shown is the multiple cloning site positions of restriction enzyme sites, sequencing primer sites (green), ampicillin antibiotic resistance gene (red) (AddGene).

# APPENDIX THREE
# Sequencing Data

Sequencing was provided by the Waikato DNA Sequencing Facility of UoW. Sequencing was carried out on an Applied Biosystems 3130xl Genetic Analyser. Sequence outputs were analysed using Geneious version 7.1.7 (http://www.geneious.com, Kearse et al., 2012).

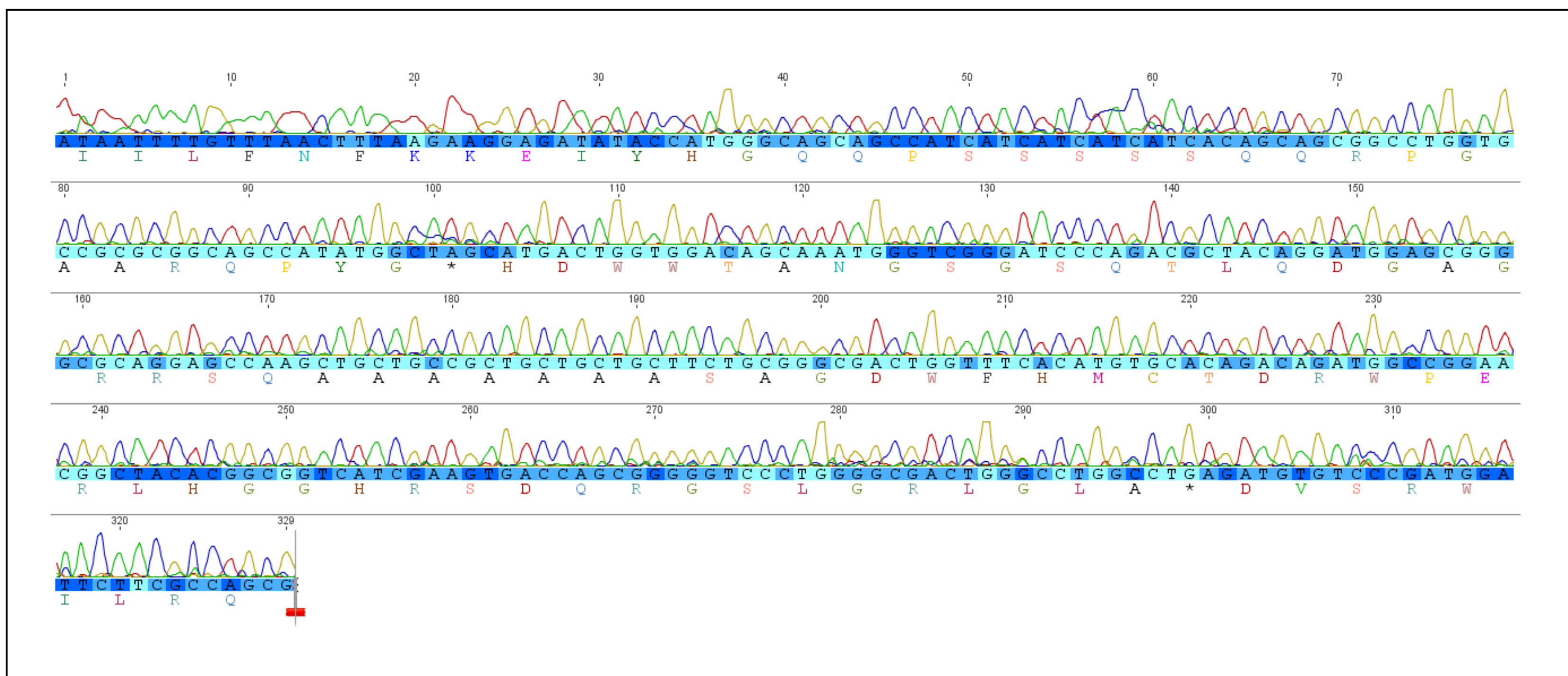


**Figure 42: Sequencing results of pET28b(+)/*VMO1* isoform 1 construct using LMP10F.** The sequence has been edited for quality and used to form a consensus sequence for confirmation of the ligation into the vector and to check it is in-frame with the His Tag for purification. Sequencing was provided by Waikato DNA Sequencing Facility and edited using Geneious version 7.1.7.

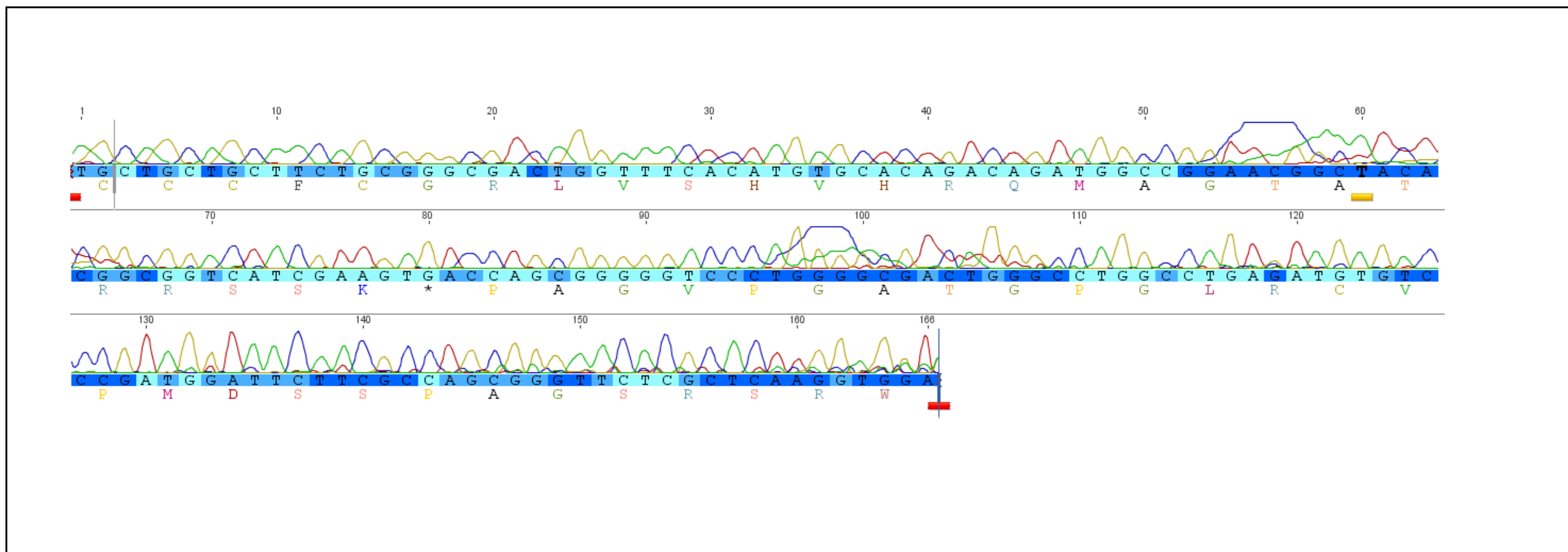

**Figure 43: Sequencing results of pET28b(+)/*VMO1* isoform 1 construct using SH1F.** The sequence has been edited for quality and used to form a consensus sequence for confirmation of the ligation into the vector and to check it is in frame with the His Tag for purification. Sequencing was provided by Waikato DNA Sequencing Facility and edited using Geneious version 7.1.7.

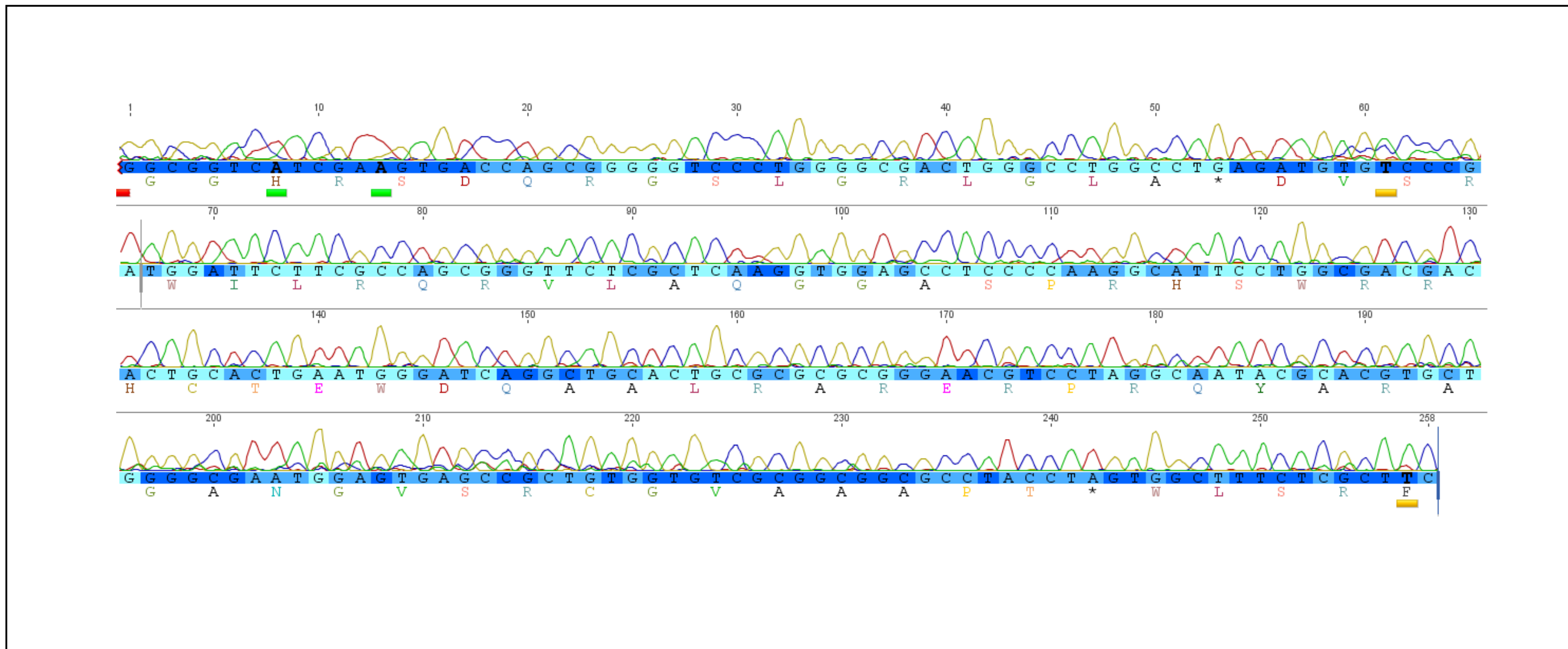

**Figure 44: Sequencing results of pET28b(+)/*VMO1* isoform 1 construct using SH3F.** The sequence has been edited for quality and used to form a consensus sequence for confirmation of the ligation into the vector and to check it is in-frame with the His Tag for purification. Sequencing was provided by Waikato DNA Sequencing Facility and edited using Geneious version 7.1.7.

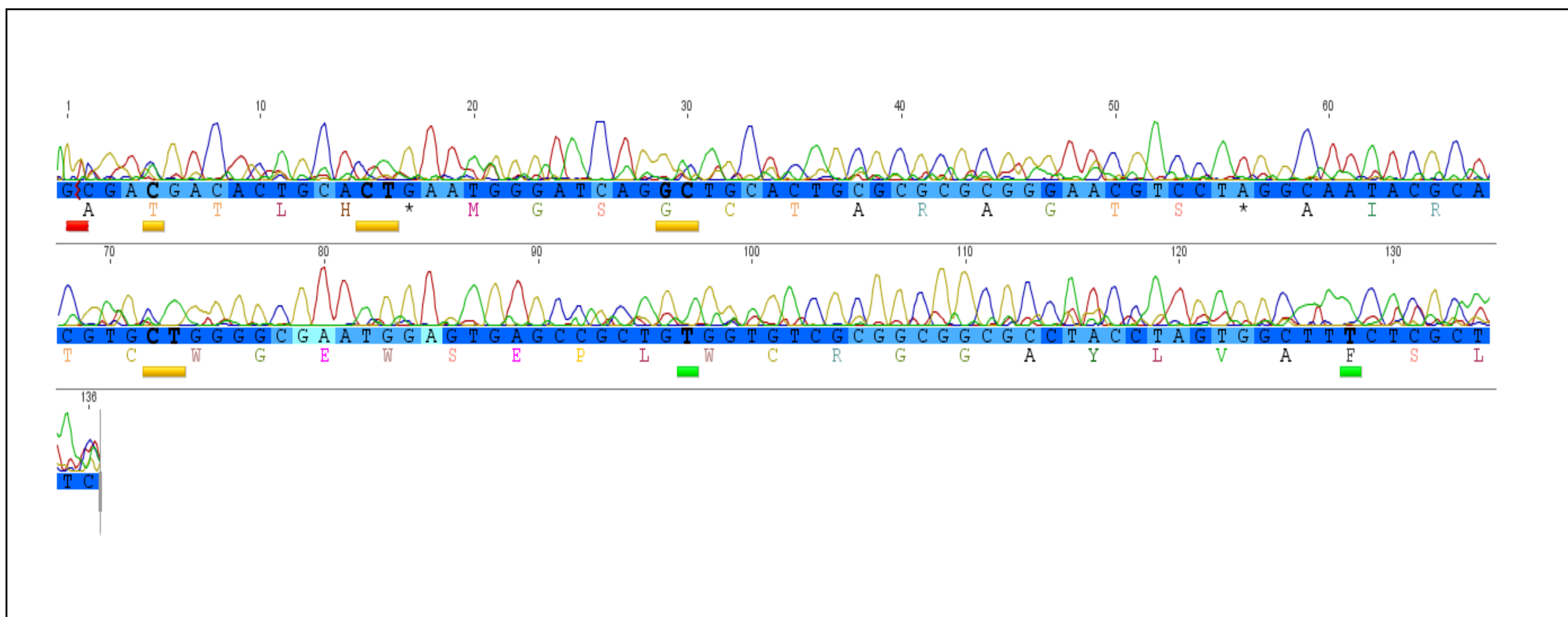


**Figure 45: Sequencing results of pET28b(+)/*VMO1* isoform 1 construct using SH3R.** The sequence has been edited for quality and used to form a consensus sequence for confirmation of the ligation into the vector and to check it is in-frame with the His Tag for purification. Sequencing was provided by Waikato DNA Sequencing Facility and edited using Geneious version 7.1.7.

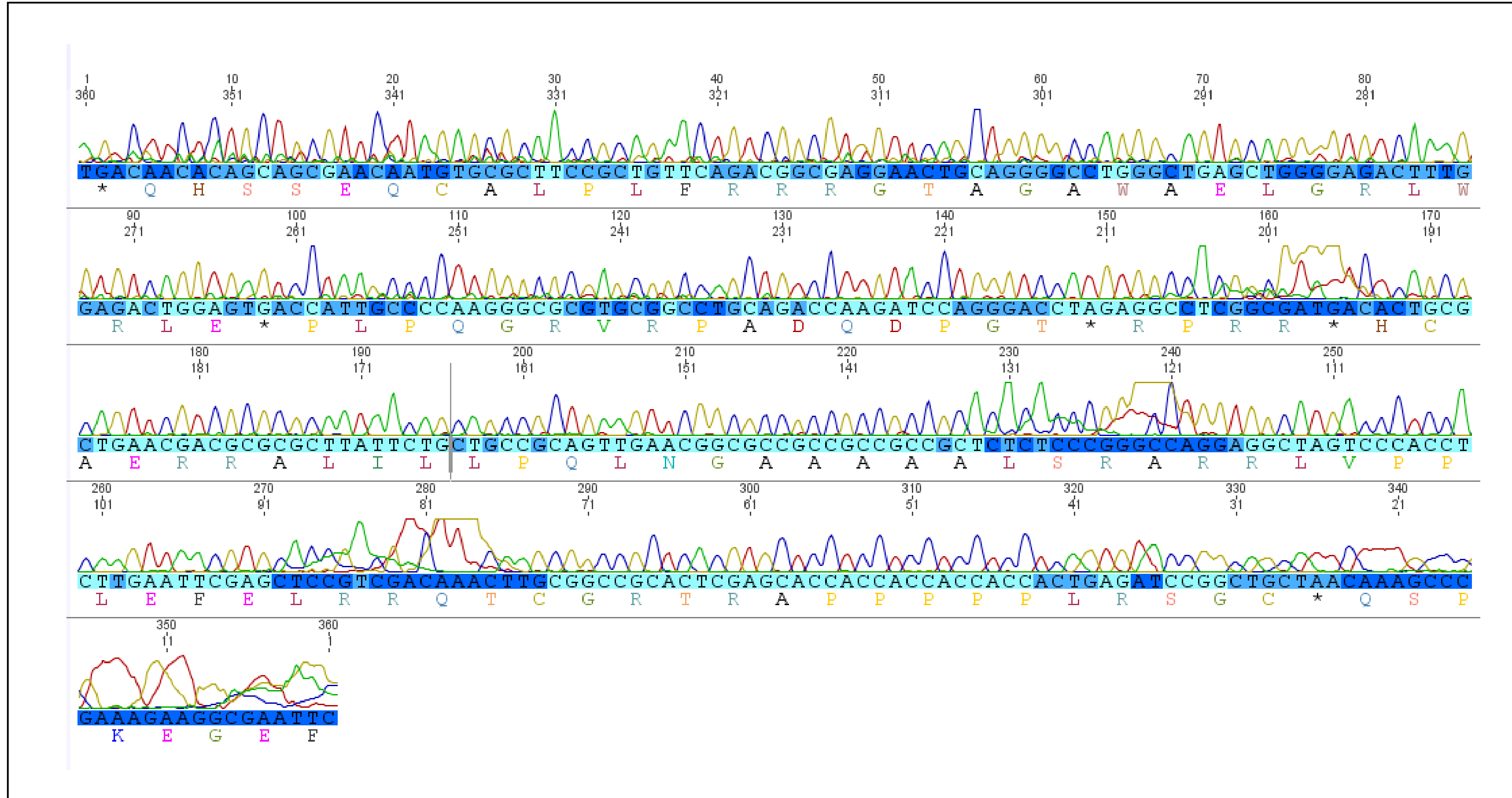

**Figure 46: Sequencing results of pET28b(+)/*VMO1* isoform 1 construct using LMP11R.** The sequence was used for confirmation of the ligation into the vector and to check it is in-frame with the His Tag for purification. Sequencing was provided by Waikato DNA Sequencing Facility and data interpreted using Geneious version 7.1.7.