

CHARACTERISATION OF VMO1 IN HUMAN TISSUES

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Abstract

The Vitelline membrane outer layer protein 1 (VMO1) was first identified in the Vitelline membrane of the chicken egg. This membrane almost entirely protein that separates the egg white from the yolk in amniotic eggs and acts as the last defensive barrier between pathogens and the egg yolk with the developing embryo. Since its discovery in the vitelline membrane, VMO1 has been isolated from a number of different tissues and animals. The one that would appear to have the largest impact on human health would be its discovery in the Reissner's membrane of mice, whose auditory system has high structural homology to humans. The Reissner's membrane maintains the electrolyte level in the compartments of the inner ear as well as the resting electrochemical potential which are both critical for correct hearing function.

The main aims of the research undertaken in this thesis was to 1) determine the tissues in which VMO1 is or is not expressed in humans and 2) create a genetically modified expression clone of human VMO1 to use in further downstream protein-protein interaction studies. Our hypothesis is that we can amplify *VMO1* from human cell lines based on available bioinformatics data.

The first objective was to determine the gene expression of *VMO1* in multiple human cell lines. RNA was extracted from three commercial cell lines (list) and the integrity of RNA was confirmed by agarose gel electrophoresis by the observation of two rRNA bands. From this cDNA

was made, and PCR analysis performed to identify the expression, or not, of VMO1 in these tissues. Successful amplification of VMO1 from lung cells (A549) lead to the creation of a human *VMO1* expression clone, by transforming competent *Escherichia coli* cells with ligated *VMO-1* insert/pPLUG vector. White colonies were selected for DNA extraction and confirmed as positive using colony PCR and agarose gel electrophoresis. DNA sequencing of the positive clone confirmed the nucleotide sequence as *VMO1*.

The second objective was to validate the commercial antibody for human VMO1 using physiological and immunohistochemical methodology. The immunohistochemistry data suggests that the VMO1 protein is a secreted protein since signal was detected in the P5 mouse inner ear and mouse adult lung. However, multiple bands were observed in the western blot. Further investigation is required to validate the VMO1 antibody to demonstrate that it is indeed specific to recognising its target epitope. The production of recombinant VMO1 protein would be beneficial to address this question.

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List of Abbreviations

bp	Base pairs
DAPI	4',6-diamidino-2-phenylindole
EDTA	Ethylenediaminetetraacetic acid
EST	Expressed sequence tag
FITC	Fluorescein isothiocyanate
IPTG	Isopropyl β -D-1-thiogalactopyranoside
LB	Luria base
LB+	Lura base with ampicillin
MOPS	3-propanesulfonic acid,
MPSS	Massively parallel signature sequencing
NCBI	National Centre for Biotechnology Information
PCR	Polymerase chain reaction
OCT	Optimal cutting temperature compound
RT	Reverse transcriptase enzyme
SDS	Sodium dodecyl sulphate
VMO1	Vitelline membrane outer layer 1
XGAL	5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside

1 Literature Review

1.1 Vitelline Membrane and VMO1

The vitelline membrane is a acellular matrix consisting of almost entirely protein that separates the egg white (albumin) from the yolk in amniotic eggs. This membrane consists of two distinct major layers with the inner layer facing the egg yolk, and the outer facing the albumen, separated by a thin continuous film as shown in Figure 1. The vitelline membrane acts as the last defensive barrier between pathogens and the egg yolk with the developing embryo, and as such, has a direct effect thereupon [2-6]. The vitelline membrane is formed in two separate places during the process of egg development. The inner layer is formed first, taking place inside the ovary no more than 7-10 days before ovulation. The outer layer forms later in a funnel shaped structure called the infundibulum, which is where fertilisation takes place. The differing places of development give the two layers a rather different protein composition and as such distinctly different properties.

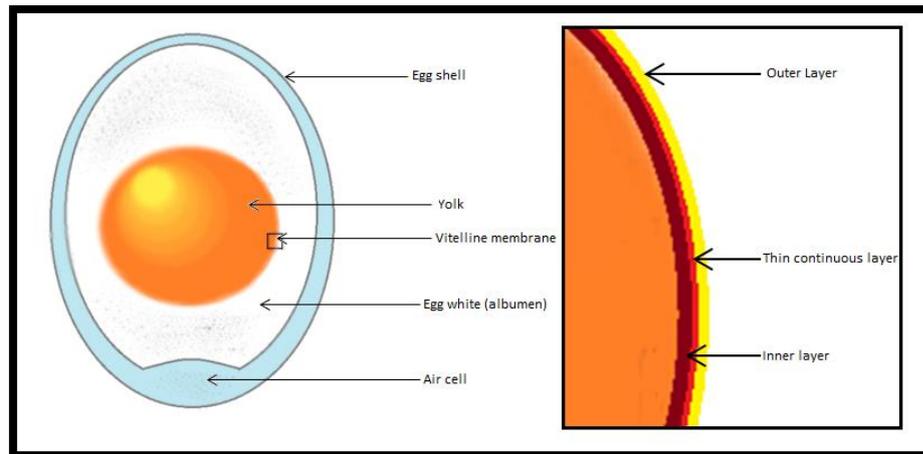


Figure 1: Cross section of the chicken egg to illustrate the structure of the chicken egg and the vitelline membrane with its three layers; the outer vitelline layer, the thin continuous layer and inner perivitelline layer next to the egg yolk (adapted from http://shelka78.files.wordpress.com/2011/12/egg_cross_section.png)

The inner layer is comprised of at least four different glycoproteins (GPI, II, III and IV) and an ordered structure similar to what is seen in connective tissues. The inner membrane is the homologous structure to the mammalian zona pellucida, and as such contains zona pellucida proteins also, notably ZPC/ZX3, ZPI and ZPD. As in the zona pellucida, this layer is involved in the fertilisation process acting primarily as a barrier to sperm. It has also been theorised as an anchor for other cells to adhere to in order to avoid polyspermy, and to facilitate same-species fertilisation only [7, 8].

The outer layer is structurally less ordered, and is composed of several overlapping sub layers. These sub layers are primarily constructed of fine fibrils, interspersed with a high quantity of bound protein. The composition of the outer layer mostly comprises of the macromolecule ovomucin constituting 43% with the soluble proteins lysozyme, vitelline membrane outer layer protein 1 (VMO1) and vitelline membrane outer layer protein 2 (VMO2) (which is now known as β -defensin 11 [9]) bound to it [4, 5].

Lysozyme has the second highest abundance at 32%, with the VMO proteins constituting 20% and 5% respectively to the protein content of the outer layer [10].

The vitelline membrane is present in its complete form at the time of fertilisation in the infundibulum. As with the zona pellucida in mammals it acts as a barrier to sperm, and has been hypothesised as an anchor for other cells to adhere to in order to avoid polyspermy, and to facilitate same-species fertilisation only although this is mostly done by the inner layer [7]. The vitelline membrane does possess qualities that are desirable in a commercial context for its ability to act as a standard for the quality indicator for the shelf life of hens eggs. Whilst the vitelline membrane only persists for four days in a fertilised egg, it has the ability to last a lot longer in the unfertilised egg depending on its storage conditions [3, 4]. Understanding how the membrane degrades might better help to prolong the shelf lives of eggs, and it is due to this commercial interest that the proteins of this outer layer have been looked into. The protein VMO1 is therefore of interest being a major protein constituent in the outer layer, although its function remains elusive.

1.2 VMO1 Structure

Protein structure is a tool that gives us a snapshot of the resulting shape of the protein, and the internal forces that work upon it. Structural homology might also be able to give an indication of potential function of a protein as well. The structure of proteins can be described in many levels, with the primary structure simply being the amino acid sequence. The

secondary structure is how the primary structure interacts with itself through noncovalent bonds and van der Waals forces. These secondary structures determine whether there will be α -helices, β -sheets or turns in the protein, all of which have structural importance to the functioning protein. Tertiary structure is how the secondary structure folds into a three dimensional (3D) shape. This happens usually through folding to form a hydrophobic core through the use of salt bridges, hydrogen and ionic bonds alongside post translational modifications. These all work together to make a distinct shape of a protein, as well as to determine the shape and properties of any active site it may possess, and how it interacts with other proteins [11].

The primary structure of chicken VMO1 is 183 amino acids long, including a 20 amino acid signal peptide leaving a 163 amino acid long mature protein. Signal peptides typically only appear on proteins targeted to a specific area, either to be secreted out of the cell, or to a specific organelle. This alone may signify that VMO1 is a secreted protein, or highly specialised within the cell. In addition there is no methionine (Met) in the protein, which would indicate secretion and post translation modification to the N-terminal of the mature protein. Chemically, VMO1 has a calculated mass of 17,979 Da and an isoelectric point of 10, which is only slightly lower than that of lysozyme which has made it difficult to distinguish in past studies. The amino acid sequence is rich in glycine (Gly) and serine (Ser) as well as the essential amino acids arginine (Arg), lysine (Lys) and histidine (His).

The amino acid sequence of VMO1 contains a thrice repeated amino acid motif of ~53 residues that gives the protein a threefold internal symmetry. This repeat forms a series of β -strands that lie to form a Greek key motif. This motif is characterised by three antiparallel β -sheets connected by short turning strands of two to five amino acid (usually containing Gly or Pro) to make β -hairpins with a fourth β -sheet looping back to lie antiparallel to the first sheet. The resulting structure resembles the decorative key motif found on many pottery pieces and jewellery from the Grecian culture, hence the nomenclature.

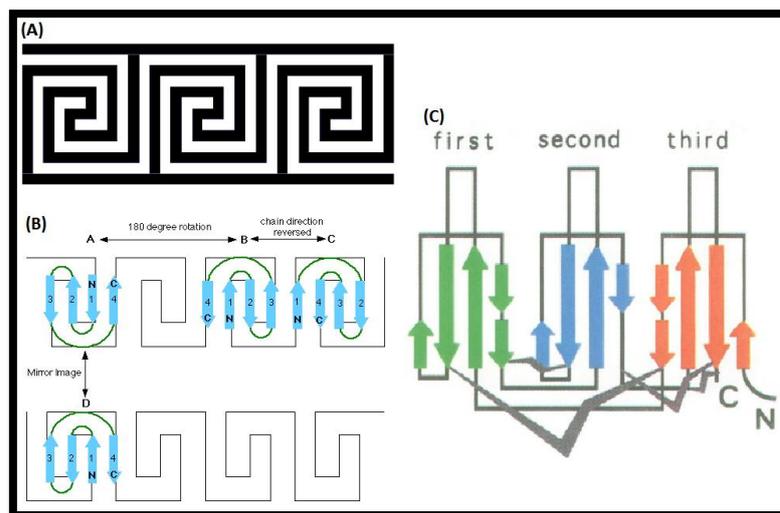


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The three repeats of the β -sheets that make the three Greek key structures are the only secondary structures that contribute to the structure of the VMO1 protein, and by merit of being comprised almost solely of β -

sheets VMO1 is categorised as a β protein. This classification groups it with other proteins such as the β -barrel of human retinol binding protein, the β -propeller protein found in the influenza viral envelope and the immunoglobulin fold of antibodies. The beta sheets fold into a triangular prism shape, to form the tertiary, 3D crystal structure. Each side of the prism is one of the Greek key β -sheets, with the disulphide bonds at the wide bottom end of the prism, and long flexible loops at the 'point.' The base of the prism has an overall positive charge, with the disulphide bonds that are the sole bonds forming the structure as there are no other interactions between the beta sheets. This is in contrast with the flexible loops which are overall negatively charged, and form a cavity that is likewise negatively charged. This cavity is similar in size to that of lysozyme, a well-known hydrolase of polysaccharides and is characteristic of many proteins consisting of Greek key motif structures, which gain functional variability from such loops, and the cavities thus generated are of a size to fit ligands such as oligosaccharides. This would appear to indicate that VMO1 should have an enzymatic hydrolysis or recognition activity on a saccharide substrate which would rely on the acidic residues that give the cavity its negative charge, namely due to containing two Asp residues frequently involved in hydrogen binding between proteins and sugar [2].

1.3 VMO1 in Other Tissues

Since its discovery in the vitelline membrane VMO1 has been isolated from the egg white [12, 13] and egg shell matrix [14]. Its discovery has not been limited to within the chicken either, with VMO1 homologues having

been identified in the genomes and tissues from other animal species. It has been genetically confirmed to exist in the green anole lizard [15], and has been identified through proteomic studies to be in the tissues of other animals. Notably *vmo1* has been found to be expressed in the tear film of camels and sheep [16, 17], the maxillary venom gland of *Philodryas offersii* (South American green racer) [18], and the Reissner's membrane in mice [1].

Firstly the tear film contains a number of lipid, protein and mucous components which act together to form a barrier between the delicate surface of the eye and the outside world. The tear film also acts to nourish and lubricate the surface, and is stabilised by the proteins it contains. These proteins, whilst contributing to the integrity of the tear film also play a large role in modulating the eyes wound healing ability and protection from pathogens. Therefore they are a good marker for studying pathophysiological mechanisms and ocular surface diseases, as well as potentially being used as a marker for ocular health. Several environmental risk factors such as relative humidity, temperature, air velocity, and air particles alter the composition of the tear film, which may cause and or exacerbate the development of ocular discomfort as eye irritation symptoms. Dry eye diseases have also been reported to be linked to other environmental factors such as windy weather, long hours of sunlight and UV radiation, and several types of conjunctivitis can be linked to the seasonal changes of these environmental factors. In the camel tear film VMO1 was shown to increase significantly during the summer months

indicating that its function, whatever that may be, is important for the regulation of the tear film under harsh conditions [17].

Secondly snake venom has the primary role of assisting prey capture, whether by slow death or paralysis of the prey. As such, there is natural selection pressure on both the prey and the snake, with the prey developing tolerance to the venom, and the snakes having evolutionary pressure to vary or optimise the toxins in their venom to circumvent the tolerance. The production of venom starts with the expression of a gene for a normal body protein that undergoes duplication and the copy expressed solely in the venom gland. These proteins are often cysteine rich to confer stability and already bioactive, or important regulatory genes, and the duplicates become mutated through random mutation, unequal crossing over or gene conversion and gain a new function. This process is called neofunctionalisation. Despite knowing that this occurs, few proteomic studies of the venom glands of toxic snakes focus on new venom scaffolds, but instead on new isoforms of existing ones. One study investigated new scaffolds to study toxin evolution over time, and identified the expression of VMO1 within the venom gland of *P. olfersii* [18]. No function was determined for VMO1, nor was it analysed in other tissues to determine if this is a duplicate and thus new venom scaffold. However, since it is expressed in the venom gland then it supports the idea that VMO1 is a secreted protein.

Thirdly the inner ear is comprised of the cochlea and vestibular which both play an important role in the translation of sound into nerve impulse. The organ of Corti, located within the cochlea is the end point for sound, and is

structurally comprised of three rows of sensitive but vulnerable outer hair cells, and one row of less sensitive and less vulnerable inner hair cells. It is attached to the basilar membrane, and partially covered by the tympanic membrane[19]. The cochlea itself contains three chambers of differing ionic strengths, the scala tympani, and scala vestibuli which both contain perilymph, and the scala media containing endolymph. Perilymph is similar to blood plasma with a high concentration of Na^+ at 140mM and a low concentration of K^+ at 4mM, whilst endolymph has the opposite with a high concentration of K^+ at 150mM and low Na^+ at 1mM making it more similar to intracellular fluid [20]. These two fluids maintain a resting electrochemical potential of between +80 - +100mV relative to the interior of the hair cells, important for their continued correct function [21]. The endolymph filled scala media and perilymph filled scala vestibuli are separated by the Reissner's membrane, a double layered membrane which acts as a diffusion barrier between the two differing electrochemical fluids. The Reissner's membrane acts as a facilitator of electrolytes between the two fluids maintaining their composition, and through that the resting electrochemical potential that is critical for correct hearing function [22]. Therefore it is of interest to investigate VMO1 in the inner ear, and specifically these structures, in order to better understand the molecular mechanisms of hearing. However, the inner ear is difficult to study and it is only through the use of animal models that it can be looked at in any great depth. Using a mouse model, RNA transcripts for *Vmo1* were obtained from the inner ear, and the *Vmo1* gene was shown to be localised to the Reissner's membrane using in situ hybridisation. Such specific localisation

would appear to indicate a significantly important function for *Vmo1* in the mouse inner ear, especially *Vmo1* in the mouse inner ear, especially as *Vmo1* gene expression was not found in 87 tissues included in the Mouse Reference Transcriptome or reverse transcribed in adult liver, kidney, pancreas, retina, brain, and testes [1].

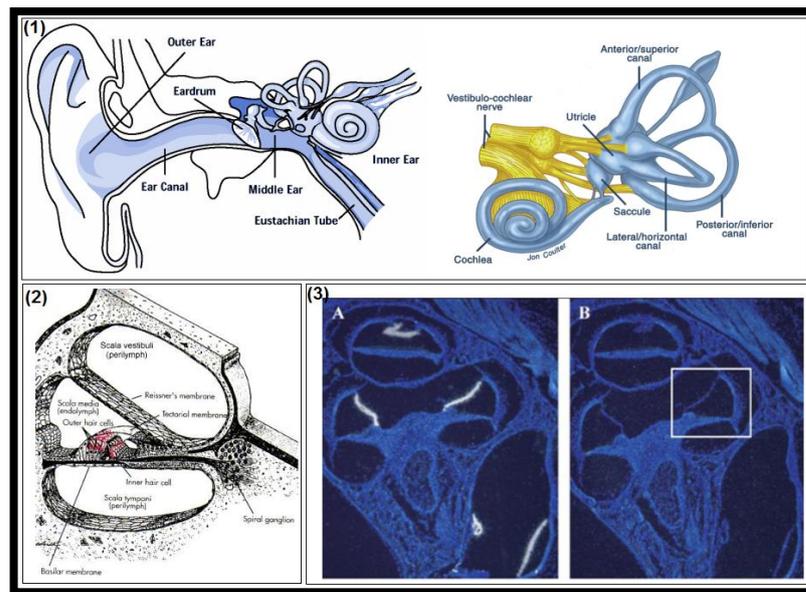


Figure 3: Diagram showing the different parts of the ear. (1) Shows the full human ear, which shares a high homology to the mouse ear, with the outer, middle, and inner ear shown as well as a close up of the inner ear (adapted from <https://www.health.ny.gov/publications/4815/> and <http://www.earsite.com/what-is-acoustic-neuroma>). (2) A cross section of the cochlea showing the important structures therein (adapted from http://download.videohelp.com/vitalis/med/diagram_of_cochlea.htm). (3) In situ hybridisation of the cross section of a P5 mouse cochlea using a radioactively labelled *Vmo1* antisense probe which binds to

Despite having an unknown function VMO1 seems to play an important role in all of the tissues that it appears in, being of either high abundance as in the vitelline membrane and tear film, or being highly localised, as with the Reissner's membrane in mice. Being able to elucidate a function for this protein may help with the understanding of key mechanisms, and may be able to confer information about the pathophysiology of some

diseases. In the next section, VMO1 and its potential in humans will be reviewed.

2 VMO1 in humans

With the implication that VMO1 has a significant role in the tissues it is identified in, then VMO1 could potentially be a candidate for a pathophysiological indicator of disease, particularly in humans. Whilst it was not found in the tear film of humans [16], the high level of structural homology between the mouse and human ear could mean that the high specificity of *Vmo1* in the mouse Reissner's membrane suggests a significant role for hearing. As such, VMO1 would be a suitable candidate protein for hearing loss and balance disorders caused within the inner ear. In addition, it could provide screening opportunities for some disorders that are difficult to diagnose.

However, before being able to attribute any disease causation by VMO1, a function must be elucidated. Even so, there is little known about VMO1 in regards to human expression beyond not being found in tear film [16], and that it has been found in very low abundance within human urine samples which would signify again that VMO1 is a secreted protein [23].

A bioinformatics approach is able to answer a few questions about human VMO1. Firstly *VMO1* is located on human chromosome 17 p13.2, and contains four mRNA transcript variants, ranging from 669bp to 821bp long. Three out of the four transcripts contain three exons, with no differences between any transcripts at the first exon, and the majority of the variation in the second. Indeed, transcript 2 has the longest exon 2, whilst transcript variant four lacks exon two entirely. Exon three is identical throughout the

transcripts except for variants 3 and 4, which lack the first base, with all sequences being obtained from the nucleotide database of the National Centre for Biotechnology Information (NCBI) website. Appendix three shows the alignments.

Expressed sequence tag (EST, retrieved from the Unigene database <http://www.ncbi.nlm.nih.gov/UniGene/clust.cgi?ORG=Hs&CID=122561&MAXEST=37>) data having been generated over a number of tissues through both RNA sequencing and microarray technology. ESTs are short subsections of complementary DNA (cDNA) that can be used to identify a gene. RNA sequencing uses Massively Parallel Signature Sequencing (MPSS) to generate libraries of mRNA transcripts in order to quantify and identify them. It works by tagging PCR products amplified for the cDNA made from mRNA transcripts, and amplifying them before being attached to microbeads and sequenced on a flow cell and the sequences analysed [1]. Microarray on the other hand uses short sections of nucleotides to hybridise the cDNA to a biochip which then undergoes sequencing to measure the expression levels of a gene. Through the use of both these technologies, the EST library shows that VMO1 is potentially expressed in a number of tissues (Table 1).

Human Tissues	Methodology
Cerebellum, frontal lobe, heart, kidney, liver, testis	RNA Sequencing
Adipose, blood, breast, bronchus, colon, conjunctiva, frontal lobe, fertilised oocyte, liver, lung, myometrium, nasopharynx, nose, pituitary, prostate, putamen, vagina, trachea, temporal lobe, spleen, skin, skeletal muscle, sinus, retina	Microarray

Table 1: EST data for VMO1

2.1 Aim, Hypothesis and Objectives

2.1.1 Aim

The main aims of the research undertaken in this thesis were to 1) characterise tissues in which *VMO1* is or is not expressed in humans. 2) To create a genetically modified expression clone of human *VMO1* to use in further downstream protein-protein interaction studies. 3) To validate a *VMO1* antibody for use in immunohistochemistry.

2.1.2 Hypothesis

EST data shows that there are a number of tissue types in which *VMO1* is expressed. Our hypothesis is that we can amplify *VMO1* from human cell lines of these tissues.

2.1.3 Objectives

The first objective was to determine the gene expression of *VMO1* in multiple human cell lines using the following molecular methods to:

- a) Culture human cell lines under physical containment level two (PC2) conditions;
- b) Extract mRNA and convert to cDNA; and
- c) Use polymerase chain reaction (PCR) to amplify *VMO1* transcripts.

The second objective was to create a human *VMO1* expression clone using the following molecular methods to:

- a) Prepare *Escherichia coli* (*E. coli*) competent cells;
- b) Ligate *VMO1* PCR product from above into an expression construct;

- c) Transform construct into competent *E. coli* cells;
- d) Extract construct DNA and amplify *VMO1*; and
- e) Sequence the PCR product to verify it is *VMO1*.

The third objective was to validate the antibody, using the following physiological and immunohistochemical methods to:

- a) Dissect organs from deceased mice and prepare protein lysates of tissue for western blot analysis or tissue for histology;
- b) Embed organs in both optimal cutting temperature media (OCT) and paraffin for sectioning on the cryotome/microtome respectively;
- c) Test *VMO1* antibody against housekeeping antibodies using immunohistochemistry; and
- d) Analyse *VMO1* binding and background using fluorescent microscopy.

This data will allow us to begin to address the question of where *VMO1* is expressed in humans, as well as to validate the commercial *VMO1* antibody. In addition the immunohistochemistry may tell us whether *VMO1* protein is secreted. Additionally this research will also allow for the future study of the human *VMO1* protein, and the properties it has through the development of a recombinant human *VMO1* expression clone.

3 Materials and Methods

Unless otherwise stated all processes were carried out within the C.2.03 Molecular Genetics Lab at the University of Waikato, Hamilton, New Zealand. All sequencing was performed at the Waikato DNA Sequencing Facility at the University of Waikato. All solutions were made up using autoclaved 15-18 megohm-cm double distilled deionised water (mQH₂O) (Barnstead double distilled/deionisation system) except for RNA work where mQH₂O was further treated with 0.1% Diethylpyrocarbonate (DEPC). All chemicals and solvents (salts, buffers, organic solvents,) were obtained from Sigma-Aldrich® Co and all molecular biology reagents (enzymes and buffers) were obtained from Invitrogen unless otherwise stated. All experiments were carried out aseptically on bench tops cleaned with 70% ethanol, and in the case of RNA work, also wiped with RNA Away™ decontamination reagent supplied by Ambion®. All glassware was washed in the dishwasher and then autoclaved before use. All solution and buffer recipes can be found in Appendix one.

3.1 Preparation of VMO1 Recombinant DNA

Below is detailed all methods used during the process of creating recombinant DNA for the study of VMO1. Recombinant DNA allows us to clone the VMO1 DNA into a bacterial plasmid vector, once the mRNA has been extracted from tissue samples. Our sources for the mRNA in this study are human cell lines that have been grown in DMEM + glucose and penicillin-streptomycin antibiotic. We choose to use the mRNA from the

cells, as opposed to DNA, for the additional information it can tell us about whether VMO1 is expressed in these tissue types. The mRNA is then converted to cDNA, and undergoes PCR to check the quality and size, before being ligated into T t-tailed vector and transformed into competent bacterial cells through either electroporation or heat shock. Once incorporated, the competent cells can be prepared in either a mini- or maxi-prep and the plasmid isolated for direct use, or for incorporation into an expression vector for protein studies.

3.1.1 General Materials

Unless stated otherwise these statements hold true for methods outlined below. All RNA centrifugation was carried out using an Eppendorf bench top centrifuge model 5424R, whilst all other centrifugation was carried out on an Eppendorf bench top centrifuge model 5415R both with a maximum speed of 16.1xg. Sterile aerosol tips were Sorenson™ low binding MultiGuard aerosol barrier tips supplied by Sigma-Aldrich® Co, else MULTIMAX Low Retention Pipet Tips from BioExpress and used with Eppendorf auto pipettes. All 1.5mL and 2mL micro centrifuge tubes were supplied by Raylab New Zealand Ltd, whilst all 0.6mL tubes were supplied by Axygen®. All falcon tubes were Cellstar® branded supplied by Greiner Bio-one. Nanodrop qualification and quantification was carried out using a Thermo Scientific™ Nanodrop 2000 spectrophotometer and accompanying software. Agarose gel electrophoresis was carried out using Hydragene HyAgarose™ agarose and TAE buffer, run at 90V for 30 minutes. All thermo cycler incubations were carried out in an Eppendorf thermo mixer comfort machine, while PCR was carried out in a Bio-Rad

Laboratories Inc. T100 thermal cycler. All electroporation was carried out using a Bio-Rad Laboratories Inc. Gene Pulser® and Pulse Controller set at 25 capacitance, 200 resistance, and 250 voltage. 100bp DNA ladder was sourced from Solis Biodyne. All plates were made using LB+ agar media unless stated otherwise.

3.1.2 In Vitro Cultivation of Human Cells

Frozen cells were obtained from storage in the -80°C Cell Culture Inventory, Department of Biological Sciences, University of Waikato. Cells were revived in DMEM + glucose and penicillin-streptomycin antibiotic and grown in a 37°C incubator at 5% CO₂ by Kirsty Mayall. Cell lines used were the lung carcinoma A549 cells and white blood leukocyte THP-1 cells donated by Gregory Jacobson, and cervical cancer HeLa cells donated by Ryan Martinus. A cell concentration of 10⁶ was required for efficient RNA extraction.

3.1.3 RNA Extraction from Human Cell Lines

All RNA work was performed on a dedicated RNase free bench, with its own dedicated filter tips for auto pipettes to be used for RNA work only. All reagents used are stored there for RNA use only to decrease RNase contamination. Bench and instruments were wiped down with RNA Away™ decontamination reagent before and after use to also minimise RNase contamination.

RNA extraction was performed on all cell lines. All media was first removed from T25 tissue culture flasks by aspiration and cells were washed with 1-2mL 1x PBS solution. The PBS was then aspirated off.

500 μ L GITC was then added over the surface of the cells in order to lyse them, and the liquid pipetted up and down until gluggy, then transferred to a 1.5mL micro centrifuge tube, and 1/10volume of 2M sodium acetate was added in order to precipitate RNA. The tube was mixed thoroughly by inversion for 15 seconds. 500 μ L phenol (pH4) was added in fume hood, and shaken vigorously for 1 minute, before being placed on the rotating wheel for 5 minutes. Phenol is important for removing protein, and pH4 specifically keeps the RNA in solution as opposed to pH8 for DNA. Further protein removal was facilitated by adding 100 μ L bromochloropropane (chloroform could be substituted here, but is more toxic) and emulsified (shake vigorously for 15 seconds) before being placed on a rotating wheel for 5 minutes. The sample was then centrifuged at maximum speed for 10minutes at 4°C. The aqueous layer was then transferred to a new 1.5mL tube. The organic layer can either be discarded, or used for protein extraction.

One tenth volume of 2M sodium acetate was added to the transferred aqueous layer and mixed thoroughly by inversion before adding 500 μ L phenol (pH4) in fume hood. Also in fume hood, 200 μ L of chloroform was added to aid in further protein removal, for the purest RNA. The sample was then emulsified for 1 minute, before being placing on the rotating wheel for 5 minutes then centrifuged at maximum speed for 10minutes at 4°C. The aqueous layer was again removed to new tube.

An equal volume of ice cold isopropanol was added to the sample, and incubated at -20°C for 10minutes. Centrifuged at maximum speed or 10minutes at 4°C, then removed and discarded the supernatant. The

pellet was washed with 500µL of 60% ethanol to remove excess salts and dehydrate pellet. The centrifugation was repeated, and all ethanol removed. The pellet was air dried for 5 minutes before being resuspended in 30µL DEPC treated water. The RNA concentration and quality were measured on a 2% agarose gel. The sample was then either used directly in downstream applications or stored at -80°C.

3.1.4 RNA Agarose Gel Electrophoresis

Quality and concentration of RNA was determined through comparison to a 100bp ladder on a 1% agarose gel.

Whilst the nanodrop also quantifies the RNA, and can show the contamination from carry over solvents or protein, the agarose gel is a better indication of whether the quantification is true of the RNA or is also measuring DNA, as well as what condition the RNA is in. Ideally when run on a 1% agarose gel, pure RNA would show two distinct bands for the 16S and 18S ribosomal bands and little else. Any background smear would indicate that there is degradation of the RNA and a bright band near the top by the wells indicates genomic DNA contamination.

3.1.5 cDNA Synthesis

The Tetro cDNA Synthesis Kit was supplied by Bioline Australia and used according to the manufacturer's directions. In brief, a master mix was prepared in a PCR tube according to Table 2, and mixed by pipetting, and spun briefly to ensure all contents get to the bottom of the tube.

Total RNA	<i>n</i> µl
Primer: Oligo (dt) ₁₈ , Random hexamers, or gene specific	1µl
10mM dNTP mix	1µl
5x RT buffer	4µl
Ribosafe RNase Inhibitor	1µl
Tetro RT (200u/µl)	1µl
DEPC treated water	Up to 20µl

Table 2: Primer mix for cDNA synthesis

Samples were incubated at 45°C for 30 minutes in a thermo mixer to allow reverse transcription of the RNA template. If random hexamers were used they were incubated for an additional 10 minutes 25°C prior to the 45°C incubation to allow the primers to anneal to their target sequence. The reaction was then terminated by heat inactivation with an incubation of 85°C for 5 min, followed by chilling on ice for 2 minutes. The cDNA sample was then either used immediately for PCR and downstream applications or stored at -20°C.

3.1.6 Primer Design

Primers for PCR were designed using the mRNA sequence for transcript variant 1 accessed from the NCBI website. The primer design tool, also from their website was used to generate the primers, and only those that

hit *VMO1* only, and were exon-exon spanning were selected. Exon-exon spanning is required to be able to distinguish between products that come from the cDNA made from *VMO1* mRNA and genomic DNA.

3.1.7 PCR

PCR was carried out on all cDNA samples to ensure that the fragments were of the right size for *VMO1* before any further downstream applications were carried out. Positive controls used were primers for the housekeeping genes *β-actin* and *Glyceraldehyde 3-phosphate dehydrogenase (GAPDH)*.

A master mix was prepared as in Table 3 using sterile aerosol tips to avoid contamination of reaction. 15µL of the master mix was added to 0.6µL PCR tubes. To this was added 2µL of both forward and reverse primers (Table 4), from 2.5mM working stocks, and 1µL template DNA before being placed in the PCR machine and run according to Table 5.

10X Buffer	2µL
25mM MgCl ₂	1.2µL
10mM dNTPs	0.4µL
5U/µL HOT FIREpol® taq polymerase (Solis Biodyne, Estonia)	0.1µL
MilliQ H ₂ O	11.3µL

Table 3: PCR master mix

GAPDH forward	CAAGAAGGTGGTGAAGCAGG
GAPDH reverse	GATGGTACATGACAAGGTGC
VMO1 forward 1	GGCCTGATTCACAGACGCTA
VMO1 forward 2	GCCTGAGATGTGTCCCGATG
VMO1 reverse	CTCACTCCATTCGCCCCAGC

Table 4: Nucleotide sequence of primers. GAPDH product length is 412bp, VMO1 forward 1 with the reverse is 354bp and VMO1 forward 2 with the reverse is 184bp based on transcript variation 1.

95°C initial activation	15 minutes	
95°C activation	20 seconds	} x30 cycles
60°C annealing	30 seconds	
72°C extension	45 seconds	
72°C final extension	5 minutes	

Table 5: PCR cycling conditons

3.1.8 Ligation

Ligation was carried out using the pLUG-Prime® TA-cloning kit from iNtRON Biotechnology, Korea, and used according to manufacturer's instructions. The insert to vector ratio was figured out using Equation 1.

$$\text{ng PCR product} = \frac{50\text{ng} \times \text{PCR product size (bp)}}{\text{X molar ratio}}$$

Equation 1: Insert to vector ration equation

Briefly, a master mix was set up as seen in Table 6 in a 0.6µL PCR tube. The reaction was mixed by pipetting, and briefly spun to ensure all contents at the bottom of tube. Reactions were left at 4°C overnight to ensure maximum ligation efficiency. Controls were set up using the

positive supplied by the kit, and a negative containing no PCR products. Reactions were transformed as outlined in the next section the next day.

	Experimental	Positive control	Negative control
10x Ligation buffer A	1 μ L	1 μ L	1 μ L
10x Ligation buffer B	1 μ L	1 μ L	1 μ L
TA-cloning vector	2 μ L	2 μ L	2 μ L
PCR product	x μ L (see equation)	-	-
T4 DNA ligase	1 μ L	1 μ L	1 μ L
Control DNA	-	3 μ L	-
Deionised water	X μ L (to make 10 μ L reaction)	X μ L (to make 10 μ L reaction)	X μ L (to make 10 μ L reaction)

Table 6: Ligation master mix

3.1.9 Transformation

3.1.9.1 Electrocompetent cells

All electroporated ligation was into the *E. coli* DH5 α cell line sourced from the Department of Biology (University of Waikato, New Zealand).

All *E. coli* DH5 α cells are stored in 50 μ L aliquots at -80°C, and are thawed completely on ice before use. A 50 μ L aliquot of cells is then transferred into an electroporation cuvette. To this, 1 μ L of the ligation mix was added,

and the cuvette placed in the electroporator. Once electroporated, 1mL of pre-warmed LB+ broth was added into the cuvette, pipetted up and down then all liquid removed to a 1.7mL microcentrifuge tube. Tube was placed in a 35°C shaking incubator for 1 hour, before 100µL of the mix was spread on an XGAL/IPTG plate, which allows for the selection of blue and white colonies to screen for transformations containing the insert, and incubated at 37°C overnight.

3.1.9.2 Chemically competent cells

Chemical transformation was carried out using *E. cloni*® 10G chemically competent cells, sourced from Lucigen according to the manufacturer's instructions. They were stored at -80°C until use, and thawed completely on ice, and 40µL aliquoted into a PCR tube.

Firstly, the ligation reaction was heat inactivated by incubation the reaction at 70°C for 15 minutes prior to transformation. 1-4µL of heat inactivated ligation reaction was then added to the PCR tube containing 40µL of cells and stirred briefly with pipette tip. The mix was then incubated on ice or 30 minutes, followed by heat shock in a water bath at 42°C for 45 seconds before being returned to ice for 2 minutes. 1mL recovery medium was then added to the cells, and incubated at 37°C for 1 hour, shaking at 250rpm, before being spread on XGAL/IPTG plates and incubated overnight at 37°C.

3.1.9.3 Mix and Go competent cells

The Mix & Go *E. coli* Transformation Kit and Buffer Set were supplied by Zymo Research and used as per manufacturer's instructions. Briefly. The

competent cells were prepared by growing *E. coli* cells in Zymobroth™ shaking at 250rpm until they reached an optical density between 0.4 and 0.6 at 600nm. Cells were placed on ice for 10 minutes, then centrifuged at 4°C at 1600xg for 10 minutes. Supernatant was removed and pellet resuspended in 5mL ice cold 1x wash buffer (supplied). Centrifugation was repeated and supernatant discard. 5mL ice cold 1x competent buffer (supplied), aliquoted and stored at -80°C for future use, or used straight away for transformation.

Trnasformation with these competent cells was done by adding 5µL of plasmid DNA to an aliquot of thawed 100µL of Mix & Go cells. These were then left on ice for one minute before being plated onto XGAL/IPTG plates and incubated overnight at 37°C.

3.1.10 Colony PCR

Colony PCR was performed on randomly selected white colonies to screen for the presence of VMO1. PCR master mix was set up as per Table 2, and 2µL of both forward and reverse primers added. Colonies were then picked with a sterile wooden toothpick and dipped into the PCR mix before being streaked on a plate which was left to incubate overnight at 37°C.

PCR was then performed as per section 3.1.7. The results were run on a 2% agarose gel against a 1kb ladder.

3.1.11 Plasmid Isolation

Plasmids were sourced from sourced from the Department of Biology (University of Waikato, New Zealand) and the alkaline lysis isolation protocol used was sourced from the Plant Biotechnology Resource & Outreach Centre at Michigan State University.

Firstly, 2ml of Luria broth (LB) was inoculated with a single bacterial colony obtained from the transformed XGAL/IPTG plates, and incubated overnight at 37°C at a vigorous shaking speed (approximately 360rpm or higher). Samples were then centrifuged at 10,000xg for 5 minutes at room temperature to pellet culture, the supernatant was discarded. The pellet was resuspended pellet using 200µL of ice cold Solution I, to lyse the bacterial cell wall, and 20µg/ml RNase A, to remove RNA, followed by vortexing for 1 minute to mix. 200µL of Solution II was added and mixed thoroughly by gentle inversion to disrupt the protein-protein and lipid-protein complexes prior to incubation at room temperature for 5 minutes. Add solution III to precipitate the chromosomal DNA, protein/membrane complexes before centrifuging the sample at full speed for 5 minutes at room temperature and the supernatant transferred to a new 1.7mL microcentrifuge tube. The DNA was precipitated by adding 0.7 volumes of ice cold 100% isopropanol to the transferred supernatant and mixed thoroughly by gentle inversion before being incubated at room temperature for 5 minutes. The sample was then centrifuged at full speed for 10 minutes at room temperature and the supernatant discarded. The pellet was then resuspended in 200µl TE buffer.

200µl of 1:1 phenol:chloroform pH8, (to optimise for DNA) was added to the sample in the fume hood and mixed well by inversion for 5 minutes at room temperature. The sample was then centrifuged at full speed for 5 minutes, also at room temperature and the supernatant transferred to a new 1.7 microcentrifuge tube.

To the transferred supernatant 0.1 volumes of 3M sodium acetate and 2.5 volumes of 100% ethanol were added and incubated at -20°C for 15 minutes. The sample was then centrifuged at full speed for 10 minutes at 4°C and the supernatant discarded. The remaining pellet was then washed with 500µl ice cold 70% ethanol before being centrifuged at full speed for 5 minutes at 4°C. The supernatant was then completely discarded and the pellet left to air dry for 5minutes at room temperature before being resuspended in 20µl TE buffer. The DNA concentration and quality were measured on both nanodrop and agarose gel. The sample was then either used directly in downstream applications or stored at -20°C.

3.1.12 Zyppy™ Plasmid Miniprep Kit

The Zyppy™ Plasmid Miniprep Kit was used to isolate plasmid as a comparison to the longer method outlined above. The kit was used according to the manufacturer's instructions. Briefly 600 µl of bacterial culture grown in LB medium to was added to a 1.5 ml microcentrifuge tube and 100 µl of 7X Lysis Buffer (Blue)¹ was added. Tube was mixed by inverting the tube 4-6 times, and the solution changing from opaque to clear. 350 µl of cold Neutralisation Buffer (Yellow) was added and mixed

thoroughly. The sample will turned yellow when the neutralization was complete and formed a yellowish precipitate. The sample was inverted an additional 2-3 times to ensure complete neutralisation before being centrifuged at full speed for 4 minutes. The supernatant was transferred into the provided Zymo-Spin™ IIN column. The column was placed into a provided Collection Tube and centrifuged for 15 seconds and the flow-through discarded and the column placed back into the same tube. 200 µl of Endo-Wash Buffer was added to the column and sample was centrifuged for 30 seconds. 400 µl of Zyppy™ Wash Buffer was then added to the column and centrifuged for 1 minute. The column was then transferred to a clean 1.5 ml microcentrifuge tube, and 30 µl of Zyppy™ Elution Buffer 2 was added directly to the column matrix and let stand for one minute at room temperature before a final centrifugation for 30 seconds to elute the plasmid DNA.

3.1.13 Restriction Digest

The restriction digest was carried out using restriction enzymes and their reagents sourced from New England Biolabs.

1µL of DNA was added to a mixture of 1-2µl each of the restriction enzyme (2µL if only one enzyme used, or 1µL each of two enzymes), 10xBSA, and buffer 4 and made up to a 10µL reaction with 5µL of mQH₂O. This was then incubated overnight at 37°C in a thermomixer at 400rpm. A negative control was prepared for each sample also, where the enzyme was replace with 2µL of mQH₂O.

3.1.14 Nanodrop and DNA Agarose Gel Electrophoresis

The quality and concentration of DNA can be determined through both varying wavelengths of light with Nanodrop and comparing to a 1kb ladder, but on a 2% agarose gel.

TE buffer was used as the Nanodrop blank. 2 μ L was placed on the stage after washing with double distilled H₂O and a Kimberly-Clark® Kimwipe, and the arm placed down. Blank measurements were set, then using a Kimwipe the TE buffer was carefully removed from the stage and replaced with 2 μ L of DNA sample. The arm was placed down again, and the sample recorded. Of special note are the absorbance readings of 230nm, 260nm and 280nm. These measure organic solvents, nucleic acids and proteins, specifically the aromatic side chains, respectively. The ratios comparing these three wavelengths are used to assess purity. The 260:280 ratio for RNA should be 2.0-2.2 to be confident of a pure sample. A lower ratio however would indicate protein contamination. The 260:230 ratio should also be very close to 2, and deviations lower than this would indicate the presence of organic solvents such as phenol or GITC. The perfect print out of a pure sample on the nanodrop would show a single sharp peak at 260nm and little else.

Agarose gel electrophoresis is also very similar for DNA as it is RNA, but should appear as one distinct band at a size predicted by the number of bases you expect there to be.

3.2 Immunohistochemistry

Immunohistochemistry involved the detection of protein expression through the use of fluorescent antibodies using tissue sections on microscope slides and western blot analysis. All tissue sections were of *Mus musculus* (mouse) tissue, donated from the Animal Facility at University of Waikato, New Zealand. The animals used were euthanised prior to our obtaining them for dissection, and so no Animal ethics approval was required from the University of Waikato. The mouse tissue section analysis was carried out in order to validate the GTX106683 VMO1 antibody, new to our lab, sourced from GeneTex Inc., United States of America. The GTX106683 VMO1 antibody was compared to controls of β -actin, lysozyme, surfactin and a negative control with no primary antibody to demonstrate that both that the protocol is working and the ratio of signalling to noise. The western blot analysed the protein lysates of mouse lung, liver, kidney, and ear tissue, run alongside a standard of 10mM bovine serum albumen (BSA) on a SDS-PAGE gel. In addition, sonicated solutions of commercial human cell lines THP-1 and BRCA1 were also loaded, Once the protein samples was separated on the SDS-PAGE gel, the protein was stained using Commassie blue or transferred to a membrane and VMO1 antibodies were applied and detected using chemiluminescence.

3.2.1 General Materials and Methods

Unless stated otherwise these statements hold true for methods outlined below. All microscope slides were sourced from Fronine. All 15mL falcon

tubes were sourced from All falcon tubes were Cellstar® branded supplied by Greiner Bio-one. Oxford labware Paraplast® paraffin was used in the Thermo Scientific™ HistoStar™ Embedding Workstation. SDS-PAGE gels were bought from Genscript, and we used the 12 well 4-20% Genscript express PAGE gels running in a Bio-Rad Laboratories Inc. Mini Protean® tetra system with MOPS buffer. Transfer of protein onto a nitrocellulose membrane for Western Blot was carried out using the eBlot® protein transfer system supplied by GenScript. Chemilumescence was detected using the Fujifilm LAS1000 with Intelligent Dark Box II and the accompanying LAS1000 lite V1.5 software.

3.2.2 Slide Preperation

In order for tissue sections to be retained on histological microscope slides during staining and washing steps, the slides being used needed to be coated with an adhesive compound such as gelatine.

Before starting the process the 75x25mm single frosted Corning microscope slides (Corning Inc.) were first placed in 25 slide swing handle glass racks and soaked in hot tap water containing detergent for 1 hour before being rinsed for a further hour under running tap water to get rid of all detergent and soaked in distilled water for at least 15 minutes. They were then allowed to air dry.

To prepare the gelatine solution, 1L of RNase free mQH₂O (in case the slides were used for RNA work in the future) was brought to the boil, and then removed to the bench to cool. When the temperature reached 60°C, 2g of porcine gelatine type A was added, and the solution allowed to cool

further. At 50°C, 0.1g potassium chrome III sulphate (KCr(III)SO₄) was added, and the solution allowed to cool further again. When the temperature reached 35°C the solution was poured into glass tanks and the swing racks containing the cleaned, dried microscope slides were dipped into the gelatine mix for 1 minute, then dried on their side with frosted portion down for 5 minutes. The dip and dry process was repeated once for every rack. The microscope slides were then left in the swing racks to fully dry at 35°C. The coated slides were stored in the swing racks wrapped in tinfoil, with care taken not to touch the clear portion of the sides.

3.2.3 Tissue Preparation for Paraffin Embedding

Mouse tissue samples were dissected using sterilised instruments from freshly euthanised specimens and placed into 15mL falcon tubes containing 4% paraformaldehyde (made in 1xPBS) and left to fix overnight at 4°C on a shaking bed. The fixing ensures the preservation of cell morphology and tissue architecture

Once fixed with 4% paraformaldehyde the tissue was then dehydrated through change of solutions outlined in Table 7 below, in preparation for embedding in paraffin.

Solution	Temperature	Time
1xPBS	4°C	60 minutes
0.85% NaCl	4°C	60 minutes
50% ethanol	Room temperature	30 minutes
70% ethanol	Room temperature	30 minutes
70% ethanol	Room temperature	30 minutes
85% ethanol	Room temperature	60 minutes
95% ethanol	Room temperature	60 minutes
100% ethanol	Room temperature	60 minutes
100% ethanol*	Room temperature	60 minutes
Xylene	Room temperature	30 minutes
Xylene	Room temperature	30 minutes
Xylene	Room temperature	30 minutes
1:1 xylene:paraffin	60°C	45 minutes
Triple filtered paraffin	60°C	20 minutes
Triple filtered paraffin	60°C	20 minutes
Triple filtered paraffin	60°C	20 minutes

Table 7: Dehydration series for paraffin embedding. *Tissue can be stored at 4°C if

there is not enough time to finish whole process.

The tissue was then embedded in triple filtered paraffin using the embedding station. The embedded tissue can then be stored at room temperature until sectioned on a microtome.

3.2.4 Paraffin Section Treatment

10 μ M sections were obtained and floated onto the pre-cleaned gelatine coated microscope slides using 0.2% ethanol in MQH₂O. The sections were fixed to the slides by overnight dehydration on a 37°C hotplate. Table 8 below outlines the protocol used to coat tissue sections with fluorescent antibodies.

Solution	Time
Xylene	10 minutes
Xylene	10 minutes
100% ethanol	2 minutes
100% ethanol	2 minutes
95% ethanol	2 minutes
70% ethanol	2 minutes
50% ethanol	2 minutes
30% ethanol	2 minutes
1x PBS	5 minutes
Antigen Revival Buffer (in glass jar, 700W microwave)	2 minutes
Antigen Revival Buffer(in glass jar, 700W microwave)	2 minutes
Cool slides to room temperature	
1x PBS	5 minutes
1x PBS	5 minutes
0.5% Triton X-100	30 minutes

1x PBS	5 minutes
1x PBS	5 minutes
0.9% H ₂ O ₂ (in 1x PBS)	30 minutes
1x PBS	5 minutes
1x PBS	5 minutes
1x PBST	5 minutes
Block (5% Goat Serum)	30 minutes
1:500 Primary Antibody in 5% goat serum. Used a PapPen.	Overnight in a humidity chamber at 4°C
1x PBST	5 minutes
1x PBST shaking	5 minutes
1x PBST Shaking	5 minutes
1:200 Anti Rabbit Ig Fluroscein in 1x PBS in dark	30 minutes
1x PBS	5 minutes
1x PBS	5 minutes
1x PBS	5 minutes
Dry and mount coverslips	

Table 8: Antibody protocol for paraffin sections

Slides were stored in the dark until photographed using a microscope (see 3.2.7), ideally the same day and no longer than a week post protocol to avoid any loss of fluorescent signal.

3.2.5 Tissue Preparation for OCT

As for paraffin sectioning, mouse tissue samples were dissected using sterilised instruments from freshly euthanised specimens and placed into a

6 well plate containing 4% paraformaldehyde (made in 1xPBS) and left to fix overnight at 4°C on a shaking bed.

Once fixed with 4% paraformaldehyde the tissue was then dehydrated through change of solutions outlined in Table 9.

Remove formaldehyde and wash tissue quickly with 1XPBS	Not applicable
wash tissue with 1XPBS	10 minutes
wash tissue with 1XPBS	10 minutes
wash tissue with 1XPBS	10 minutes
Wash in 30% sucrose quickly	n/a
Wash in 30% sucrose	10 minutes
Wash in 30% sucrose	10 minutes
30% sucrose at	4°C rocking gently overnight
Remove 1/2-1/3 of the sucrose and replace with OCT at	4°C rocking gently for 1 hour
Fill one cryomold container with OCT and place one tissue specimen in using sterile tweezers. Take care to get as little sucrose/OCT mix into the OCT as possible.	Not applicable

Table 9: Dehydration protocol for OCT

Embedded tissue was stored at -80°C until sectioned using a Leica DMR Microscope with a Nikon Digital Camera attached.

3.2.6 OCT Section Treatment

Sections were prepared (10µm) using the Leica CM1850 UV cryostat (Leica Biosystems) and collected onto gelatine coated pre-cleaned microscope slides (kindly donated by Patrick Wightman). Slides had been stored in a slide box at -20°C and thawed overnight before continuing with the antibody protocol. Table 10 below outlines the antibody protocol used with these OCT slides.

Precooled -20 C acetone (in fumehood)	10 minutes
1x PBS (pH 7-7.5)	5 minutes
1x PBS	5 minutes
1x PBS	5 minutes
0.5 Triton X	30 minutes
1x PBS	5 minutes
1x PBS	5 minutes
1x PBS	5 minutes
0.9% H ₂ O ₂ (in 1x PBS)	30 minutes
1x PBS	5 minutes
1x PBS	5 minutes
1x PBST	5 minutes
Block with blocking buffer	30 minutes
Wipe excess fluid with tissue; encircle tissue with pap pen and let dry	2 minutes
1x PBS	5 minutes

1:500 primary antibody in blocking buffer	Overnight in a humidity chamber at 4°C
1x PBS	15 minutes
1x PBS shaking (orbital shaker speed 4)	15 minutes
1x PBST shaking	15 minutes
1:200 FITC conjugated goat anti-rabbit IgG (H+L) polyclonal antibody (in 1x PBS)	30 minutes
1x PBS	15 minutes
1x PBS	15 minutes
1x PBST	60 minutes
1x PBST	60 minutes
Dry in dark	1 hour
Mount using fluroshield with DAPI	
Dry in dark	Overnight
Photograph and store	

Table 10: Antibody protocol for OCT

Slides were stored in the dark until photographed using a Leica DMR Microscope with a Nikon Digital Camera attached microscope, ideally the same day and no longer than a week post protocol to avoid any loss of fluorescent signal.

3.2.7 Microscopy

Immunohistochemistry stained slides were visualised on a Leica DMR Microscope with a Nikon Digital Camera attached. The FITC secondary

antibody was conjugated to the VMO1, lysozyme, surfactin and β -actin primary antibodies and was visualised with UV light and a 460-490nm cube filter.

3.2.8 Bradford Assay

The Bradford assay was used to estimate the protein concentration of samples extracted from mouse tissue and human cells prior to the western blot analysis. BSA standard dilutions were prepared from a 10mM stock to final concentrations of 0mM, 0.1mM, 0.25mM, 0.5mM, 1mM, 2mM, 4mM, 6mM, 8mM, and 10mM in mQH₂O. The Bradford standard can be found in Appendix 1. Protein lysates for the mouse ear, kidney, liver and lung were thawed from -20°C stocks supplied by the Department of Biological Sciences, University of Waikato. Cell samples were prepared by adding 200 μ L of PhosSTOP (Roche) to cell pellets to prevent protein degradation, then sonicating them with three bursts of 30seconds. 1 μ L of samples and standards were then pipetted into a 96well plate and 100 μ L of Bradford's reagent added. The plate was then stored in the dark for 5minutes before analysed on a Thermo Fisher Scientific Inc. Multiskan™ GO Microplate Spectrophotometer and its accompanying software to gain an indication of the protein concentration in each sample.

3.2.9 Western Blot analysis

Protein samples were aliquoted into tubes containing Laemmli buffer (Appendix 1). This buffer contains 2-mercaptoethanol which reduces the intra and inter-molecular disulfide bonds, SDS detergent which denatures the proteins and subunits and gives each an overall negative charge so

that each will separate based on size, bromophenol blue serves as a dye front that runs ahead of the proteins so you can see the sample during loading and the glycerol increases the density of the sample so that it will layer in the sample well. To ensure equal sample loading, 10 μ L of Laemmli buffer was mixed with 20 μ L protein sample for the mouse ear, and human cell lines, 20 μ L buffer to 10 μ L protein sample for the other mouse tissues, and 45 μ L buffer to 5 μ L of BSA 10mM standard (only 10 μ L was loaded). These were then run on a 12% Genscript PAGE gel as outlined above, using MOPS buffer and running at 200mV for approximately half an hour. The gel was then rinsed with distilled water before being transferred to the eBlot[®] protein transfer system, which was set up according to manufacturer's instructions. The eBlot[®] machine was then run for 7 minutes, to transfer across all protein to the provided nitrocellulose membrane.

Once the protein was transferred to the membrane, the membrane was carefully transferred to a container and washed three times with TBS-T for 15minutes per wash, then once for 15minutes with TBS before being washed with 5% blocking buffer. The membrane was left in the blocking buffer for 1 hour to prevent the nonspecific binding of the antibodies before being washed three times with TBS-T for 15minutes per wash, then once for 15minutes. 1:1000 VMO1 antibody was then applied to the membrane and left at 4°C overnight. The membrane was then washed again before application of the secondary 1:200 FITC conjugated goat anti-rabbit IgG (H+L) polyclonal antibody. This was left to incubate for an hour before draining and developing the membrane.

The chemiluminescence was developed using Thermo Scientific™ SuperSignal™ West Dura Substrate mixed 1:1 as per manufacturer's instructions, before being analysed using the Fujifilm LAS1000 and its accompanying software.

4 Results

4.1 VMO1 RNA

4.1.1 RNA Extraction

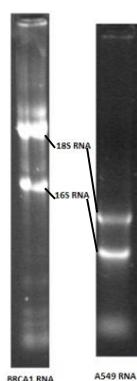


Figure 4: rRNA bands extracted from BRCA1 and A549 RNA

RNA was extracted from A549 (lung carcinoma), THP1 (monocytic cell line), and BRCA1 (breast cancer) cell lines and visualised on a 1% native agarose gel to determine quality as shown in Figure 4. The Two expected rRNA bands were observed, indicating the integrity of RNA was high. Therefore, the RNA extracted was used as a template for cDNA synthesis.

4.1.2 Primer Design

Primers were designed using NCBI Primer-BLAST based on the transcript variant 1 of human *VMO1* mRNA (NM_182566.2) sourced from the NCBI Nucleotide database and designed to be exon spanning to be able to distinguish between cDNA that has been made from the extracted mRNA (see Figure 5) and the *VMO1* gene from genomic DNA. Three primers were designed, two forward (F) and one reverse (R) (Table 4), that hit nothing else except human *VMO1* when using NCBI BLAST analysis .

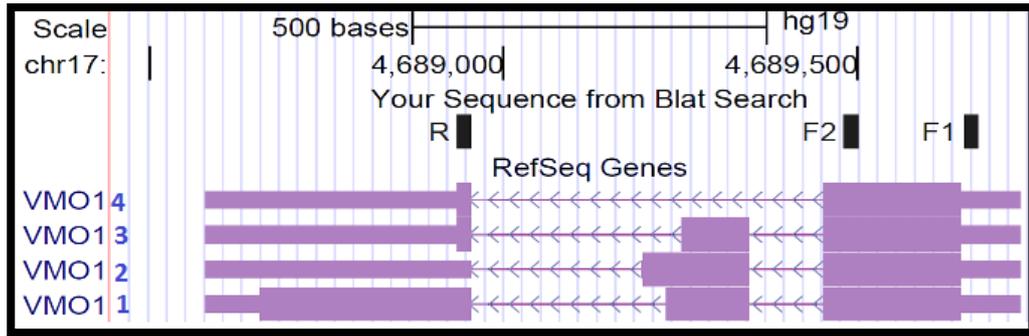


Figure 5: Exon spanning primer design. Diagram showing where each primer hits against all four transcripts of *VMO1*.

4.1.3 PCR Amplification of *VMO1*

PCR amplification was carried out on the cDNA of three samples that were synthesised from RNA extracted from A549, THP1 and BRCA1 cell lines. These were run against a house keeping gene of *GAPDH* for which the primers have been previously published [24]. Figure 6 shows the amplification of the *VMO1* transcripts from these tissues, with A549 in lane three of (1) being the only cell line to positively amplify *VMO1* of the three. In addition, results show suggest the identification of at least three of the four transcript variants. The remaining two cell lines did not amplify *VMO1*, and were run alongside the housekeeping gene *GAPDH* to show that the failure of amplification was due to there being no transcript present rather than because there was an issue with the quality of cDNA template. All PCR was free of contamination as shown by the clear lanes for the negative controls.

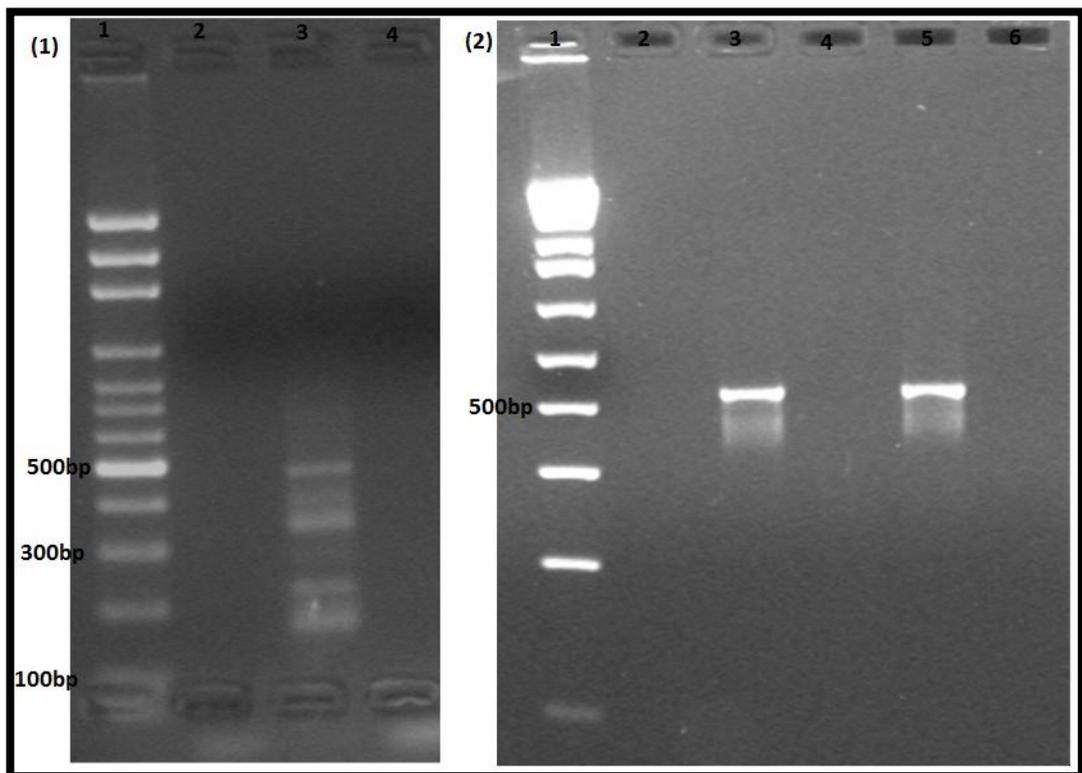


Figure 6: PCR amplification of VMO1. (1) 1% Agarose Gel Electrophoresis from left to right- 1KB ladder (1), THP1 cDNA with *VMO1* primer set one (2), A549 cDNA with *VMO1* primer set one (3), negative control (4). (2) 1% Agarose Gel Electrophoresis from left to right- 1KB ladder (1), BRCA1 cDNA with *VMO1* primer set one (2), BRCA1 cDNA with *GAPDH* primer set (3), negative control (4), THP1 cDNA with *VMO1* primer set one (5), THP1 cDNA with *GAPDH* primer set(6), negative control.

4.2 Plasmid

4.2.1 Nanodrop

4.2.2 Restriction Digest of Transformed Plasmid

Extracted plasmid was digested using EcoR1 and Xba1. Two different extraction methods were used to compare efficiency and all samples were run on a 2% gel with undigested and digested samples side by side. The Zyppy™ kit was far superior in its quality even though the nanodrop showed it had lower concentrations of DNA. The colony that gave rise to sample five was selected to be grown overnight in 6mL of LB at 250rpm and frozen into glycerol stocks for future expression work.

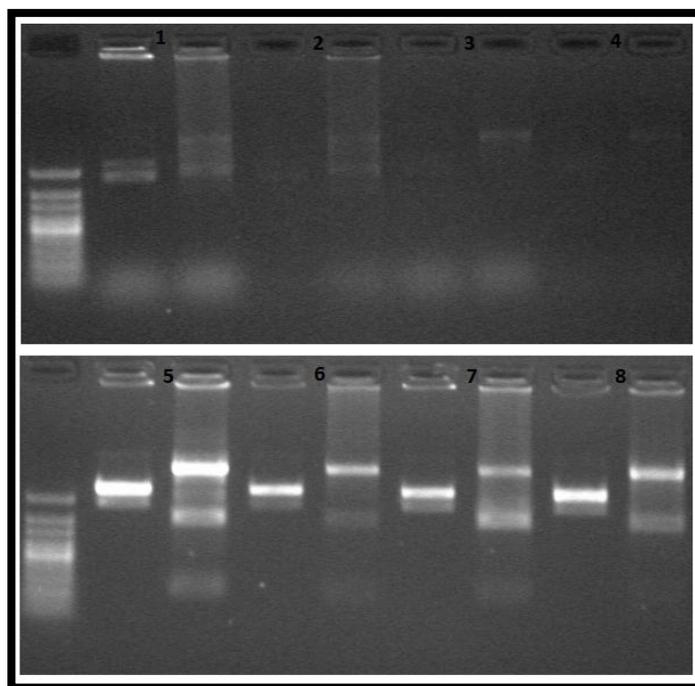


Figure 7: Restriction digest of VMO1 plasmid. Top shows original extraction protocol. Low concentration of extracted DNA. Bottom shows DNA extracted from kit. All samples were loaded in pairs of undigested then digested. Run on a 2% agarose gel against a 100bp ladder.

Sample five was sent for sequencing post cleanup, and was sequenced using the M13F and M13R primers supplied with the vector. The sequences were edited using Geneious version 7.0.06. The sequences were aligned using NCBI BLAST with VMO1 transcript variant 2 with 100% sequence homology indicating that VMO1 is indeed ligated into our clone. Sequences are shown in Appendix four.

4.3 Immunohistological Analysis

4.3.1 Tissue Section Analysis

Analysis of P5 mouse tissue sections was carried out on both paraffin and OCT sections. The mouse ear (Figure 8) is important for the validation of VMO1 as it contains the Reissner's membrane which is the only area that VMO1 is known to be expressed in the mouse, and we could only obtain sections through the use of OCT as there were issues with the decalcification of the head samples used for paraffin sectioning.

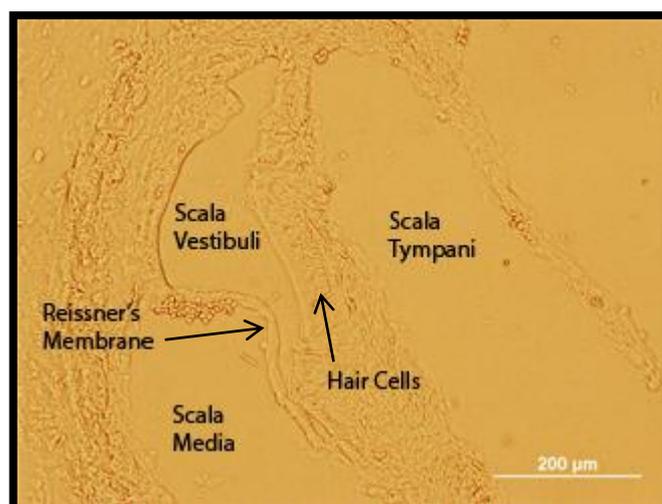


Figure 8: Histological cochlea cross section. Shows main structures of the

Fluorescent microscopy was used to visualise antibody activity with VMO1. Signal (green) was detected in Reissner's membrane and within the inner ear. For the mouse inner ear, we used lysozyme as a house keeping antibody, which showed some activity, and then with no primary antibody, to show background levels of fluorescence. This was then compared to the lung tissues to determine that the VMO1 antibody does not show any nonspecific binding. In addition, this was compared to the house keeping surfactin antibody, which is used as a marker of type 2 cells in the lung.

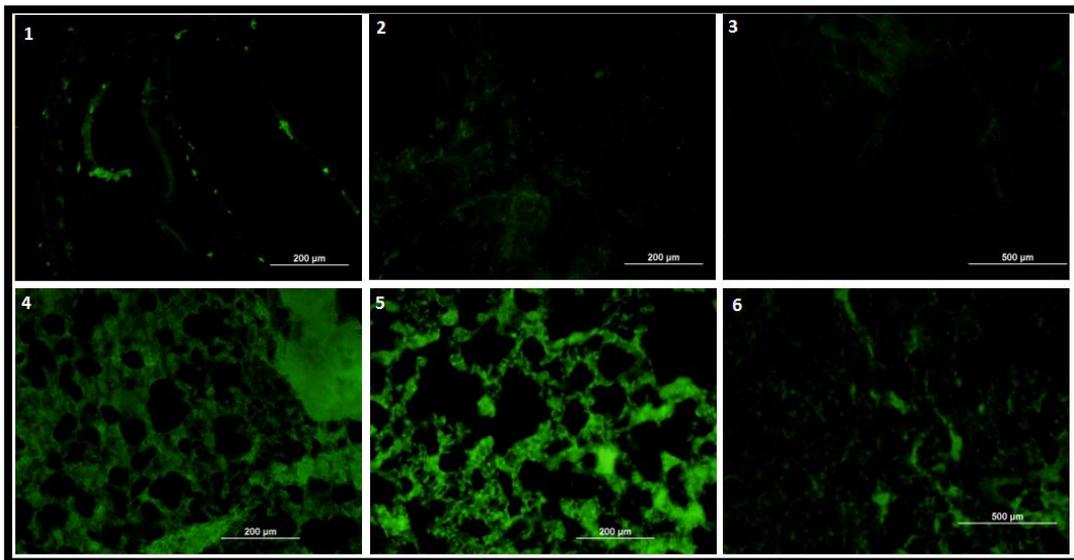


Figure 9: Fluorescent antibody analysis. Top row shows mouse inner ear sections with 1) treated with VMO1 1:500, 2) treated with lysozyme 1:500, and 3) treated with no primary antibody. Bottom row shows mouse lung sections with 4) treated with VMO1 1:500, 5) treated with surfactin 1:1000, and 6) with no primary antibody.

Brighter green spots on panel 5 in Figure 9 were observed which indicated type 2 cells. This was again compared to a section with no primary antibody to show background noise, which would appear to be the source of any fluorescence seen in panel 4 (Figure 9) showing VMO1 antibody binding in the lung.

4.3.2 Western Blot

Western blot analysis showed non-specific binding of VMO1 protein, with multiple banding in all lanes, as well as being detected in other tissues besides the ear, even though there is data to indicate this should not be the case. The protein marker ladder was overlaid from fluorescent imaging, and the membrane was left for an exposure of 80seconds for the chemiluminescence to be captured.

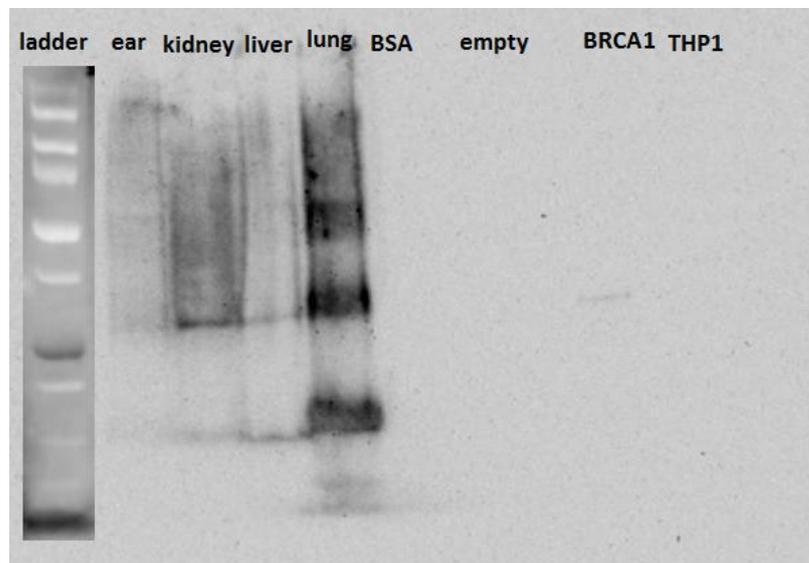


Figure 10: Western blot of tissues. Ladder from fluorescent detection overlaid using Microsoft paint.

No protein was shown in the BSA lane or the THP1 lane, although there is a faint band in the BRCA1 lane, which contradicts the findings of the PCR analysis of mRNA extracts. The absence of protein was not because of lack of protein, but rather that it did not bind to the VMO1 antibody. This was checked by staining the membrane with PonceauS stain for an hour.

5 Discussion and Future Directions

5.1 VMO1 expression

5.2 VMO1 expression

VMO1 has an unknown expression profile in human tissues, and the research aim of this thesis was to provide some insight as to where it is or is not expressed. The EST data generated from Ensembl indicated a number of tissues where we would expect to find VMO1 expression, although due to set backs and cell line availability we were only able to analyse three cell lines.

Firstly, hela cells were cultured using aseptic cell techniques to 100% confluency and then the RNA was extracted. However, our RNA extraction protocol was the first set back, having tried one method which using the manufacturer's instructions for Trizol® Reagent supplied by Life Technologies™. This technique yielded low yields of poor quality RNA determined through agarose gel electrophoresis and nanodrop readings, which were unable to continue downstream to cDNA synthesis and PCR. This could be due to the RNA pellet no being fully solubilised, or that the cells remained adhered to the plates. Possibly there was not enough Trizol reagent added, or perhaps the sample was not homogenised enough. Therefore, an alternative method was sourced. Using GITC yielded the observation of two ribosomal bands on the agarose gel, indicating the

RNA was of high integrity. Therefore, the RNA could then be used for the downstream applications.

PCR conditions were another hurdle to the determination of VMO1 expression. Initially, running the PCR conditions with annealing at 55°C ended with no amplification, indicating that the primers were not working.. New reactants were used to no avail, and a gradient of Mg⁺ concentrations ranging from 1.5-2.5 mM also had no effect. Gradient PCR was then used initially between 50°C and 55°C to no effect, and then over a gradient of 50°C to 60°C, which finally yielded a result.

Analysis of the PCR results from cDNA synthesised from mRNA transcripts from the three different human cell types showed that there was *VMO1* expression in the lung (A549 cells) but not in the breast (BRCA1 line SUM149PT carrying the 2288delT mutation) or white blood cells (THP1). This result is in accordance to the EST data which lists the lung as a potential area for the expression, and not the white blood cells, although there is a discrepancy in that the PCR does not show any expression of VMO1 in the BRCA1 cell line, although there is EST data available for VMO1. Interestingly, VMO1 and BRCA1 are both expressed on chromosome 17. In addition, we have used only one of the 43 BRCA1 cell lines available on the commercial market. Possibly, our designed primers did not target a VMO1 isoform in the breast.

5.3 Development of Recombinant VMO1 DNA

Creating a plasmid construct containing VMO1 was the other primary objective of this research in order to be used for further studies of the

protein. This was ultimately successful with a transformed *E. coli* glycerol stock being frozen and stored at -80°C.

Initially, the vector pBluescript (2958bp) was transformed into electrocompetant cells to proliferate before being extracted using , digested with EcoR1 and t-tailed for ligation. T-tailing is required to insert mRNA transcripts as Taq DNA polymerase adds a poly-adenine tail to the 3' of all transcripts, and this then base pairs with the many thymine residues added during t-tailing. pBluescript is a commercial vector that offers extensive multiple cloning site with 21 unique restriction enzyme recognition sites.

The DNA extraction method was carried out on approximately 1mL of culture and used TENS (Tris-Cl, EDTA, NaCl, SDS) buffer which yielded low quantities of DNA which were inadequate for further downstream use. The low yield could be due to having too many steps where DNA could be lost along the way, or that pH of solutions was incorrect, poor culturing conditions (although cultures were not used unless the media was cloudy which would indicate growth) or that the cells were insufficiently lysed. The nanodrop readings were initially very high, around 1000ng/μL, but when run on a agarose gel there was little plasmid DNA observed but a high density RNA cloud, which was subsequently removed by the addition of RNase A to the protocol (results not shown).

To improve the cloning methodology, a new commercial plasmid was obtained; pLUG vector, which required no preparation before ligation. However, the application of this vector upon ligation with the purified

VMO1 lung DNA yielded no colonies on the agar plate when used in conjunction with electroporation. Following review over the manufacturers guidelines, colonies developed when ligated samples were transformed using chemically competent cells (*E. coli*) rather than electrocompetent bacterial cells.

A new DNA extraction protocol (included in section 3.1.11) was used to extract the plasmid from these cells for downstream use, and proved successful as bands of the expected size were obtained and the nanodrop reading was more accurate to what was seen on the gel. The pLUG vector with the *VMO1* insert was also used in conjunction with the mix & go kit, resulting in the highest transformation efficiency of the three transformation protocols used in this study. The Zyppy™ Plasmid Miniprep Kit extraction was also compared to the alkaline lysis method used previously. The Zyppy™ kit yielded lower concentrations of plasmid DNA but of a much higher quality and is much quicker.

The final extracted product was verified as *VMO1* through colony PCR and restriction digest using XX and XX. In addition, the DNA was sent to Waikato DNA Sequencing Facility for sequencing using the M13 sequencing primers. Following data analysis,

Glycerol stocks were also made and stored for future experimentation.

5.4 Antibody Validation

The validation of the VMO1 antibody was a secondary objective of the work of this thesis. VMO1 expression is known to be limited to the Reissner's membrane in mice [1] and so should be the only structure to show activity with the antibody.

Using OCT sections we were able to verify this, with the Reissner's membrane and nearby structures showing a stronger green signal than any background. The fact that nearby structures were also showing a positive reaction to the VMO1 antibody would suggest that VMO1 is a secreted protein. It was also not picked up in the lung sections, which shows that there is little non-specific binding, if any, from this antibody although the surfactin antibody that served as a positive control worked well, and the type II cells were observed as expected.

It would have been ideal if we could have validated the antibody using paraffin sections alongside the OCT sections; however we had no slides of the mouse ear to verify on. This is due to the decalcification protocol not being stringent enough, which left too much bone intact. This meant that any sections would tear on the blade, no matter how much the section thickness was increased. There was no opportunity to gain another mouse head to test another decalcification method on within the time frame of this thesis, and without the ear slides there could be no positive validation of this antibody.

On the western blot analysis, the antibody seemed to have significant nonspecific binding, as it was detected not only in the mouse ear, but also

the lung, liver and kidney. The lung may express VMO1 in low levels, that could have been missed on the histological slides as not all of the lung was sectioned, but the whole lung lysate was used on the western. There was also a band at the right size in the BRCA1 lane. Being a very faint band this could be a false positive, due to the other lanes all having VMO1 bands even though there should be no VMO1 present in those tissues, or else the western was able to detect low levels of VMO1 in the BRCA1 cells where the PCR could not. However cancerous cell lines may not be the best model to represent normal tissue as there are a number of mutations inherent within the cancerous genome.

I would worry then that the results seen in the studies of camel tear film [16,17] may not be entirely accurate as this is the same antibody used in their papers. Otherwise there may be a problem with our antibody perhaps due to shipping or production conditions. The antibody we obtained was immediately aliquoted into 5 μ L aliquots to minimise the need to freeze and thaw the antibody more than needed and stored at -20°C.

The non-specific binding seen could be at least partially due to the possibility that the VMO1 protein could have multiple modified forms in vivo such as acetylation, methylation, myristylation, phosphorylation, glycosylation etc. that make it run as multiple bands. The use of an agent to dephosphorylate, de-glycosylate, and otherwise counteract post transcriptional modification of the protein could be recommended in the future to bring the protein to the correct size. Another reason for the multiple banding pattern is that VMO1 may bind tightly to other proteins to form a complex, which could be counteracted by boiling the sample for longer.

Changing the blocking buffer from milk powder to the blocking buffer we used for the molecular probes on the slides might also help to avoid non-specific binding, as we know that there was none when looking at VMO1 antibody on the slides.

In conclusion, further research is required on the validation of the VMO1 antibody. An investigation into more stringent decalcification methods to improve the integrity of the paraffin ear sections would be beneficial. We used postnatal day (P5) five ear tissue for the ease of dissection and sectioning. It would be desirable to use adult ears (at least P28) and organ tissue at P5 for the immunohistochemical comparisons.

5.5 Future Directions

There are two main directions for this thesis to continue in regards to investigating the function of VMO1. Firstly, sampling a wider variety of human cell lines to extract RNA and test for expression of *VMO1*. In particular, obtaining alternative BRCA1 cell lines and human biopsy breast tissue samples would be useful to clarify VMO1 expression in this tissue.

Secondly, once a VMO1 antibody has been validated to recognise 22 kDa protein on the western blot, you could use this antibody to determine function of VMO1 by investigating the protein-protein interactions. This could be achieved by using the digested the VMO1 insert and cloning into a protein expression vector using define restriction enzymes or resigning primers with restriction enzymes to amplify of lung cDNA. By knowing what VMO1 interacts with, a pathway could then be inferred and a potential function elucidated. This could then be combined with enzymatic assays

using a range of saccharide substrates, as it is thought that the binding domain is similar to other proteins that do work on saccharides [2]. Overexpression, and mutation studies could also be conducted to analyse whether the VMO1 protein is toxic to the cell, or if any change occurs in the natural function of the cell.

Finally, no animal model has been published for VMO1 to date. However, mutant embryonic stem cells are available purchase for the development of a VMO1 knockout mouse (<https://www.mousephenotype.org>). It would be interesting to determine if these animals had a hearing loss phenotype.

5.6 Conclusion

RNA was extracted from three human cell lines (A549, THP1 and BRCA1) and converted to cDNA using reverse transcriptase. PCR primers were designed to amplify the *VMO1* coding regions that covered the open reading frame (exons 1-3) to ligated and clone into pLUG vector. Following PCR optimisation, an annealing temperature of 55°C was identified and resulted in the amplification of cDNA synthesised from lung (A549). The PCR product was purified and ligated into the pLUG vector, transformed using XXX. Following colony PCR and restriction digestion analysis, a positive clone was sent to DNA sequencing. Analysis of the consensus sequence confirmed that *VMO1* was amplified from lung cDNA. The next step is to use this data to clone into a protein expression vector. This will aid future studies on VMO1.

The immunohistochemistry data suggests that the VMO1 protein is a secreted protein since signal was detected in the P5 mouse inner ear and

mouse adult lung. However, multiple bands were observed in the western blot. Further investigation is required to validate the VMO1 antibody to demonstrate that it is indeed specific to recognising its target epitope.

With so little known about the VMO1 protein, especially outside of the chicken, there are a myriad of possibilities to be examined and many routes to be discovered that lie outside the bounds of this thesis. For example, the crystal structure of mouse and human VMO1 needs to be determined by X-ray crystallography, and compared to both the chicken VMO1 structure and other human and mouse proteins that could be homologous. Protein partners are another crucial step in elucidation, if not a function, then the pathway in which it is involved. Considering the localisation of *VMO1* in the mouse inner ear, a *VMO1* knockout mice could be developed to discover a potential hearing loss phenotype, and with human ethics approval human samples could be taken and sequenced to see if there is any single nucleotide polymorphisms (SNP's) involved that could lead to a hearing loss phenotype.

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Appendix One: Solutions and Buffers

6X Agarose gel loading buffer

- 3mL Glycerol
- 25mg Bromophenol Blue
- 20µl Xylene Cyanole
- Make up to 10mL with sterile mQH₂O

Antigen revival buffer – sodium citrate buffer

- 1.47g Tri-sodium citrate (dehydrate)
- Make up to 500mL with mQH₂O and autoclave
- 250µl Tween 20

Blocking solution for Immunohistochemistry

- 200µL Goat serum
- 1mL 1X PBS

Blocking solution for Western Blot

- 5g Low fat milk powder
- 50mL 1X TBS-T

Bradford Reagent

- 1part Dye reagent concentrate
- 1 part mQH₂O

- Filter through a Whatman #1 filter paper to remove particulates

10X cOmplete Protease Inhibitor Cocktail

- 1mL 1X PBS
- 1 cOmplete Protease Inhibitor Cocktail Tablet (Roche)

0.5M EDTA pH 8.0

- 93.05g EDTA
- Make up to 500mL with mQH₂O and autoclave

2X Freezing medium

- 6.30g K₂HPO₄
- 0.45g Sodium citrate
- 0.09g MgSO₄·7H₂O
- 1.80g (NH₄)₂SO₄
- 44.00mL Glycerol
- Make up to 500mL with mQH₂O and autoclave

IPTG – Isopropylthiogalactoside

- 200mg IPTG
- 1mL dH₂O

2x Laemmli Dye

- 4mL 10% SDS
- 2mL Glycerol
- 1.2mL 1M Tris pH6.8

- 200 μ L Bromophenol Blue
- 2.6mL H₂O
- Just before use, take 475 μ L of 2x dye and add 25 μ L β -mercaptoethanol

LB broth – Luria Base broth pH 7.0

- 10g Bactotryptone
- 5g Bacto yeast extract
- 10g NaCl
- Make up to 1L with mQH₂O and autoclave

LB plates – Luria base agar plates

- 1L LB broth
- 15g Bactoagar
- Autoclave and add antibiotics at 50°C before pouring

1x MOPS running buffer

- 6.06g Tris base
- 10.46g MOPS
- 1g SDS
- 0.3g EDTA
- To 1L with deionised H₂O

1X PBS – phosphate buffered saline pH 7-7.4

- 8g NaCl
- 0.25g KCl

- 0.2g KH₂PO₄
- 1.15g Na₂HPO₄
- Make up to 1L with mQH₂O.

4% PFA - paraformaldehyde

- 4.0g PFA
- 10.0µl 10M NaOH
- Make up to 50mL with mQH₂O and heat in 65°C water bath to dissolve

1X PBS-T – phosphate buffered saline + Tween-20 pH 7-7.4

- 1L PBS
- 0.5mL Tween-20.

10X PhosSTOP Phosphatase Inhibitor Cocktail

- 1mL 1X PBS
- 1 PhosSTOP Phosphatase Inhibitor Cocktail Tablet (Roche)

Ponceau S stain

- 0.1g Ponceau S
- 0.1g Acetic acid
- Make up to 1L with mQH₂O

Protein lysis buffer

- 2.5mL 1M Tris
- 1.9mL 4M NaCl

- 200µl 0.5M EDTA
- 250µl Triton-X 100
- 0.5mL 1X PhosSTOP Phosphatase Inhibitor Cocktail
- 0.5mL 1X cOmplete Protease Inhibitor Cocktail
- Make up to 50mL with sterile mQH₂O

10% SDS

- 100g SDS in 800mL sterile mQH₂O
- Up to 1L with sterile mQH₂O

Solution I (Lysis buffer I) - 50mM Glucose, 10mM EDTA, 25mM Tris pH8

- 0.9g Glucose.
- 2mL 0.5 EDTA.
- 2.5mL 1M Tris pH8.
- Make up to 100 mL with H₂O.
- Autoclave and store at room temperature.

Solution II (Lysis buffer II) - 0.2N NaOH, 1% SDS.

Prepare fresh for each extraction.

- 0.2mL 10M NaOH.
- 1mL 10% SDS.
- Make up to 10mLs.

Solution III (Lysis buffer III) - 3M KOAc pH6

- 60mL 5M potassium acetate (49.07g potassium acetate in 100mL H₂O.)

- 11.5mL glacial acetate.
- 28.5mL H₂O.

50X TAE - Tris-acetate EDTA buffer

- 242g Tris base dissolved in 800 mL mQH₂O
- 57.1mL Glacial acetic acid
- 100mL 0.5M EDTA (pH 8.0)
- Make up to 1L with mQH₂O

1X TAE running buffer

- 20mL 50X TAE
- 980mL mQH₂O
- 0.1% DEPC treated water - diethyl pyrocarbonate
- 2mL DEPC
- Make up with 2L mQH₂O, mix overnight using a magnetic stirrer, then

1X TBS - Tris buffered saline

- 50mL 1M Tris
- 30mL 5M Sodium chloride
- Make up to 1L with mQH₂O and autoclave

1X TBS-T - Tris buffered saline + Tween-20

- 999mL TBS
- 1mL Tween-20

TE buffer – Tris EDTA pH 8.0

- 10mL 1X Tris-HCl
- 2mL 0.5M EDTA
- 0.9% H₂O₂
- 16.7mL H₂O₂
- Make up to 500mL with 1X PBS

1M Tris HCL pH 8.0

- 500mL H₂O
- 60.5g Tris
- Autoclave

XGAL - 5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside

- 20mg Xgal
- 1mL Dimethyl formamide

Appendix Two: Plasmid Vectors

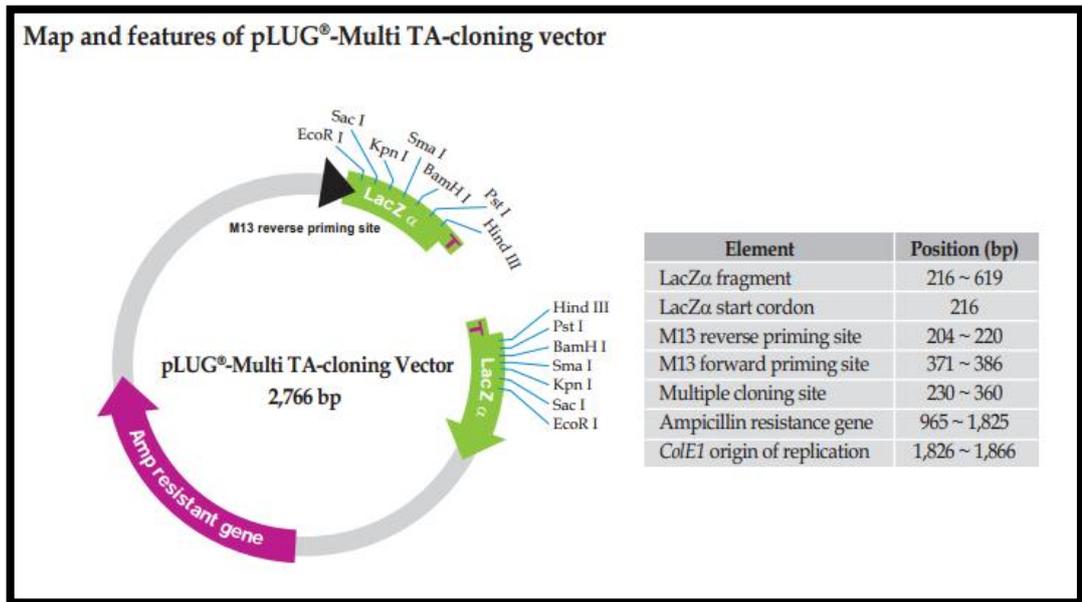


Figure 11: map of pLUG[®]-multi TA cloning vector

Appendix Three: VMO1 mRNA Alignments

Line one= transcript one (NM_182566.2). Line two= transcript two (NM_001144939.1). Line three= transcript three (NM_001144940.1). Line four= transcript four (NM_001144941.1).

>Exon one

```
AGGCCCTTCCGCAATCGGAGCCCTCACAGAGGCCAAACTGATATAAATCTGCTTAGGAGGCCGTGATTCACAGACGCTACAGGATGGAGCGGGGCGCAGGAGCCAAGCTGCTGCCGC
AGGCCCTTCCGCAATCGGAGCCCTCACAGAGGCCAAACTGATATAAATCTGCTTAGGAGGCCGTGATTCACAGACGCTACAGGATGGAGCGGGGCGCAGGAGCCAAGCTGCTGCCGC
AGGCCCTTCCGCAATCGGAGCCCTCACAGAGGCCAAACTGATATAAATCTGCTTAGGAGGCCGTGATTCACAGACGCTACAGGATGGAGCGGGGCGCAGGAGCCAAGCTGCTGCCGC
AGGCCCTTCCGCAATCGGAGCCCTCACAGAGGCCAAACTGATATAAATCTGCTTAGGAGGCCGTGATTCACAGACGCTACAGGATGGAGCGGGGCGCAGGAGCCAAGCTGCTGCCGC
TGCTGCTGCTTTCGCGGGCGACTGGTTTTCACATGTGCACAGACAGATGGCCGGAACGGCTACACGGCGGTCATCGAAGTGACCAGCGGGGGTCCCTGGGGCGACTGGGCCTGGCCT
TGCTGCTGCTTTCGCGGGCGACTGGTTTTCACATGTGCACAGACAGATGGCCGGAACGGCTACACGGCGGTCATCGAAGTGACCAGCGGGGGTCCCTGGGGCGACTGGGCCTGGCCT
TGCTGCTGCTTTCGCGGGCGACTGGTTTTCACATGTGCACAGACAGATGGCCGGAACGGCTACACGGCGGTCATCGAAGTGACCAGCGGGGGTCCCTGGGGCGACTGGGCCTGGCCT
TGCTGCTGCTTTCGCGGGCGACTGGTTTTCACATGTGCACAGACAGATGGCCGGAACGGCTACACGGCGGTCATCGAAGTGACCAGCGGGGGTCCCTGGGGCGACTGGGCCTGGCCT
GAGATGTGTCCCGATGGATTCCTCGCCAGCGGGTTCCTCGCTCAAG
GAGATGTGTCCCGATGGATTCCTCGCCAGCGGGTTCCTCGCTCAAG
GAGATGTGTCCCGATGGATTCCTCGCCAGCGGGTTCCTCGCTCAAG
GAGATGTGTCCCGATGGATTCCTCGCCAGCGGGTTCCTCGCTCAAG
```

>Exon two

```
GTGGAGCCTCCCCAAGGCATTCCTGGCGACGACACTGCAC TGAATGGGATCAGGCTGCAC TGC GCGCGCGGGAAACGTCC TAGGCAATACGCACGTGGTAGAGTCCCAGTCTGGAA-
GTGGAGCCTCCCCAAGGCATTCCTGGCGACGACACTGCAC TGAATGGGATCAGGCTGCAC TGC GCGCGCGCGGGAAACGTCC TAGGCAATACGCACGTGGTAGAGTCCCAGTCTGGAA-
GTGGAGCCTCCCCAAGGCATTCCTGGCGACGACACTGCAC TGAATGGGATCAGGCTGCAC TGC GCGCGCGCGGGAAACGTCC TAGGCAATACGCACGTG-----
-----
GTGGGGCGCAGGGGTCGAGGATCCCTTGGGGTGAT
-----
-----
```

>Exon three

```
GCTGGGGCGAATGGAGTGAGCCGCTGTGGTGTGCGGGCGGCGCCTACCTAGTGGCTTTCTCGCTTCGCGTGGAGGCACCCACGACCTCGGTGACAACACAGCAGCGAACAACGTG
```

GCTGGGGCGAATGGAGTGAGCCGCTGTGGTGTTCGCGGGCGCGCTACCTAGTGGCTTTCTCGCTTCGCGTGGAGGCACCCACGACCCTCGGTGACAACACAGCAGCGAACAACGTG
-CTGGGGCGAATGGAGTGAGCCGCTGTGGTGTTCGCGGGCGCGCTACCTAGTGGCTTTCTCGCTTCGCGTGGAGGCACCCACGACCCTCGGTGACAACACAGCAGCGAACAACGTG
-CTGGGGCGAATGGAGTGAGCCGCTGTGGTGTTCGCGGGCGCGCTACCTAGTGGCTTTCTCGCTTCGCGTGGAGGCACCCACGACCCTCGGTGACAACACAGCAGCGAACAACGTG
CGCTTCCGCTGTTTCAGACGGCGAGGAAC'TGCAGGGGGCC'TGGGC'TGAGC'TGGGGAGAC'TTTGGAGACTGGAGTGACCATTGCCCCAAGGGCGCGTGCGGGCTGCAGACCAAGATCCA
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GGGACCTAGAGGGCTCGGGCGATGACACTGCGCTGAACGACGCGCGCTTATTTCTGCTGCCGAGTTGAACGGCGCCGCCGCCGCCGCTCTCTCCCGGGCCAGGAGGCTAGTCCCACC
TCTTGCTATTTAAAGCTTCTCTGAGTTGAAAAAAAAAAAAAAAAAAAA
TCTTGCTATTTAAAGCTTCTCTGAGTTGAAAAAAAAAAAAAAAAAAAA
TCTTGCTATTTAAAGCTTCTCTGAGTTGAAAAAAAAAAAAAAAAAAAA
TCTTGCTATTTAAAGCTTCTCTGAGTTGAAAAAAAAAAAAAAAAAAAA

Appendix Four: DNA Sequence

>M13F primer (supplied with plasmid kit)

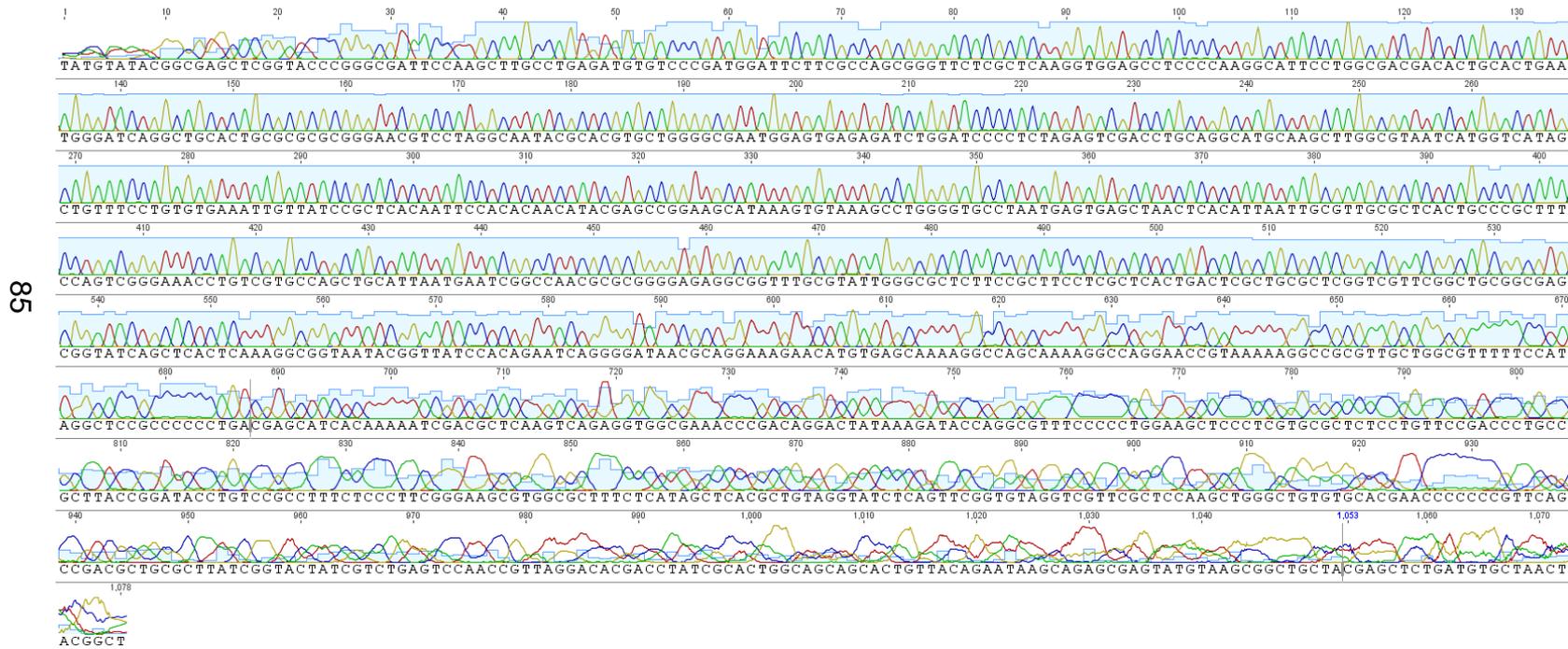
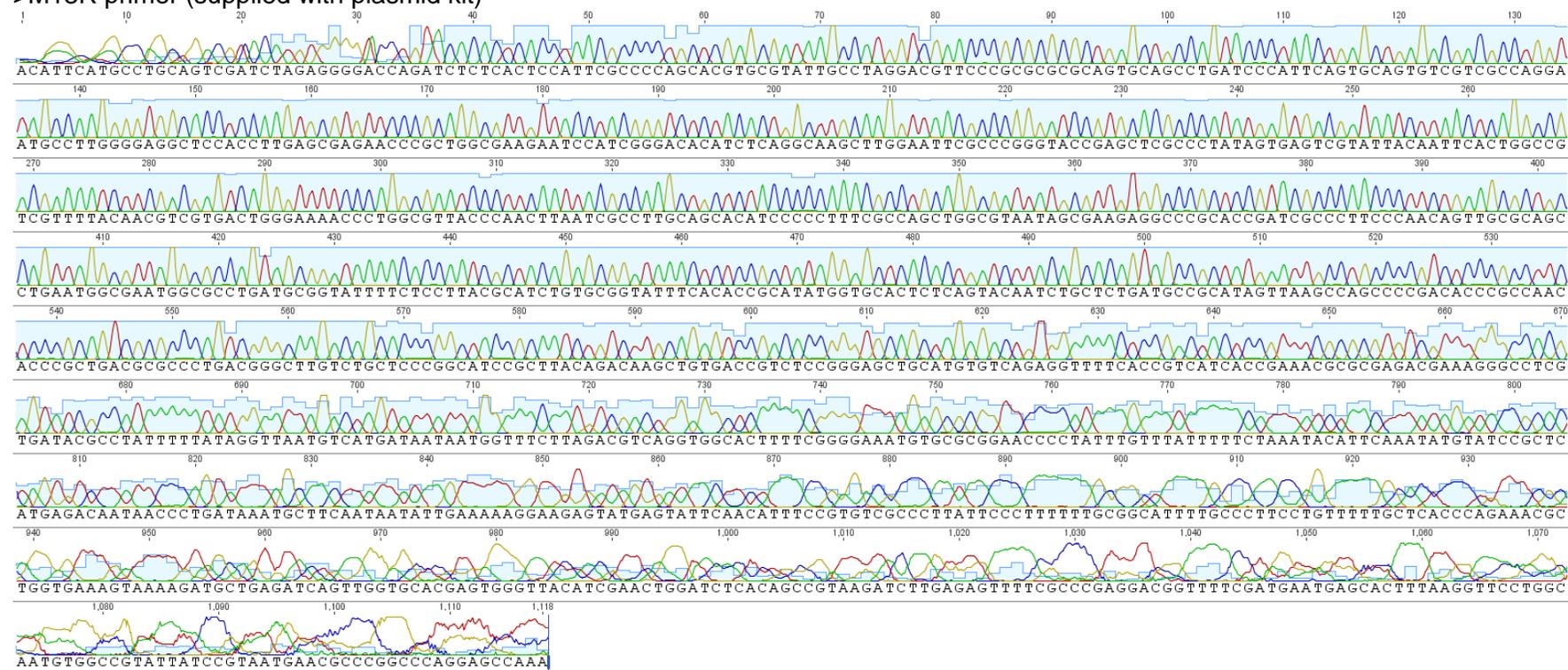


Figure 12: Restriction digest sequence with M13F primer edited on Geneious 7.0.06

>M13R primer (supplied with plasmid kit)



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Figure 13: Restriction digest sequence with M13F primer edited on Geneious 7.0.06

Appendix Five: Genetically Modified Organisms

C2 Laboratories, Biological Sciences Department, UoW

Containment Manual, Version 6.5 issued on 17-02-12

Form for Lab records of New Organisms Register of GMOs

Developed in Facility Number 759

HSNO ACT APPROVAL No. APP201152 UOW Appl. No: GMD101146

PROJECT: Characterisation of VMO1 in Human Tissues

P.C.LEVEL: 1

Additional Controls:

Researcher: Hannah Crossan

***Project Leader/Supervisor:** Dr. Linda Peters

Host Species and Strain: *E. coli* strain DH5 α

Vector: pLUG

Insert DNA: Human *Vmo1*

Species of donor of nucleic acids: *Mus musculus*

Note: all records must be dated and initialled. Continue on a new page if necessary.

DATE NAME OF GMO Assign a code and number	STORAGE DETAILS Specify fridge or freezer	TRANSFER DETAILS Reg. No. of sending/receiving facility	DISPOSAL DETAILS Specify method used
30.1.13 HC001		Not applicable	Autoclave
27.2.13 HC002		Not applicable	Autoclave
14.3.13 HC003		Not applicable	Autoclave
6.7.13 HC004		Not applicable	Autoclave
13.11.13 HC005	4°C fridge plates	Not applicable	
29.11.13 HC006	4°C fridge plates -80°C glycerol stock	Not applicable	