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**CLONING OF AN OLFACTORY RECEPTOR AND A MEMBRANE
TRAFFICKING PROTEIN HOMOLOG**

A Thesis

Presented to

The Faculty of the Department of Chemical and Materials Engineering

San Jose State University

In Partial Fulfillment

of the Requirements for the Degree

Master of Science

by

Jaquelina do Rosario Lee

August 2004

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A handwritten signature in black ink, appearing to read "Daryl K. Eggers", written over a horizontal line.

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ABSTRACT

CLONING OF AN OLFACTORY RECEPTOR AND A MEMBRANE TRAFFICKING PROTEIN HOMOLOG

By Jaquelina do Rosario Lee

Employing a RecA-mediated cloning method, a mouse olfactory receptor gene, mOR37, was successfully cloned with unknown specificity from a mouse epithelium cDNA library. It was confirmed that the cloned mOR37 is a full-length gene and perfectly matches an olfactory receptor (OR) published in GenBank, except that the 5' end of the cloned mOR37 gene is 45 nucleotides longer. Certain ORs cannot be functionally expressed by localization in the plasma membrane but have been shown to be trafficked to the cell membrane by the *C.elegans* protein ODR-4 (ceODR-4). A human homolog of the ceODR-4 gene, hODR-4 was successfully cloned and found to possess an open reading frame that is longer than a gene with unknown function reported in Genbank. By expressing the hODR-4 with the OR gene, the membrane trafficking function of the hODR-4 gene can be determined.

ACKNOWLEDGMENTS

I deeply appreciate Dr. Ron Caspi and Dr. Chris Lehman for doing the bioinformatics analysis for this research. I specially would like to thank my thesis committee, Dr. Claire Komives, Dr. Chris Lehman and Dr. Daryl Eggers, for advising my thesis research. I greatly appreciate Dr. Richael Axel (Columbia University) who donated the mouse olfactory epithelium cDNA library to us. I also want to thank the people in Pangene Corporation, Jeff, Phong and Dr. David Zarling, for all your help, especially Boris, who mass excised the Rat olfactory bulb lambda cDNA library for me. This thesis experiment was supported by funding provided by CSUPERB and Pangene Corporation.

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CHAPTER 1.0 INTRODUCTION

1.1 Olfactory Receptors

Mammals can receive and process a variety of stimuli, and specific receptor proteins are most often the initial point of recognition. The signal is then processed and received by the brain. One of these signal transduction systems is the olfactory system [1]. The mammalian olfactory system is able to recognize and distinguish among over a thousand different odorant molecules. These are detected by olfactory receptor (OR) proteins, which are members of the G-protein coupled superfamily. Olfactory receptors belong to the superfamily of seven-transmembrane, G-protein-coupled receptors (GPCR's), along with receptors for taste, light and several others [2]. The specific nature of olfactory receptors remained unknown until 1991, when Buck *et al.* cloned and characterized 18 different members of an extremely large multigene family and discovered that they all traverse the membrane seven times and contained sequence similarity to other members of the G-protein coupled receptors [2]. In 1998, Firestein *et al.* overexpressed olfactory receptors in rat olfactory epithelial cells to study the physiological function of these olfactory receptors [3]. In recent years, the understanding of olfactory perception has grown impressively, however, the regulation of OR expression still remains open [4].

1.2 G-Proteins

G-proteins were named because they bind Guanosine Triphosphate (GTP). They are composed of three subunits, alpha (α), beta (β) and gamma (γ), and these three subunits function so as to mediate an intracellular signal when there is an extracellular signal. At rest, the alpha subunit binds with Guanosine DiPhosphate (GDP). When a ligand is bound to the olfactory receptor, the G-protein is activated and the GTP will replace the GDP in the alpha subunit. The alpha subunit then dissociates from the beta and gamma subunits and activates an enzyme, adenylyl cyclase. The alpha subunit rejoins the beta and gamma subunits once the GTP hydrolyzes to GDP [5].

1.3 G-Protein Coupled Receptors (GPCRs)

G-protein coupled receptors (GPCR) are composed of a single polypeptide. GPCR's contain seven membrane-spanning α -helical segments that wrap around back and forth through the membrane, therefore, GPCRs are also known as seven transmembrane receptors. There are two fundamental elements in the transmembrane signaling systems: one that distinguishes an extracellular signal and the other that generates an intracellular signal. When the receptor binds a transmitter, such as an odorant, it undergoes a conformational change, and then it will bind and activate the G protein [1]. There are as many as 1000 members for olfactory receptors in the mammalian genome, making it by far the largest known group of GPCRs [4].

Figure 1. Structure of G Protein Coupled Receptor. GPCRs is comprised of seven transmembrane domains. Three loops of amino acids are extracellular and three loops are intracellular. The NH_2 terminus of the receptor is extracellular and the COOH terminus is intracellular.

1.4 Signal Transduction

Odor detection occurs in the olfactory region located in the nasal cavity.

Odorants are carried by inhaled air to the olfactory epithelium, where the olfactory receptor neurons are located. Once the odorant is bound to the receptor, the activated G-protein will activate adenylyl cyclase (AC), which initiates a cascade of intracellular signals by catalyzing the production of cyclic adenosine monophosphate (cAMP) as a second messenger from adenosine triphosphate (ATP). The cAMP activates the cyclic nucleotide-gated (CNG), cation-permeable channel, thus causing an influx of sodium and calcium ions. The cell generates an action potential which propagates along the olfactory

neuron axons to the olfactory bulb. The brain then translates the signal and recognizes the smell [2, 5].

Figure 2. A Pathway of Olfactory Signal Transduction. After the odorant binds to the receptor (R), it activated the G protein and the G_{α} -GTP stimulates adenylate cyclase (AC) which releases cAMP. The increase in the concentration of cAMP opens the cyclic nucleotide gated channel, generating a membrane potential.

There is evidence that another second messenger, Inositol TriPhosphate (IP_3) is also produced in olfactory transduction in many species. Activated G-protein will activate an intracellular membrane-bound enzyme phospholipase C (PLC). The PLC hydrolyses phosphatidylinositol-4,5-bisphosphate (PIP_2) in the plasma membrane to produce IP_3 and diacyl glycerol (DAG) [5]. IP_3 will then open the calcium channels in the endoplasmic reticulum and raise the calcium ion concentration in cytoplasm [6]. The role of the second messenger, IP_3 -mediated signal transduction remains unclear. There is suggestion that cAMP and IP_3 might influence the production of each other or anti effects of each others, therefore, further study needs to be continued [5, 6].

1.5 DNA Cloning

DNA cloning is defined as the copying of a piece of DNA from one organism into another. The process of DNA cloning starts with the isolation of a specific gene of interest from a source, whether a chromosome or other stretch of DNA and inserting it into a cloning vector. A vector is a circular piece of DNA that has signals for replication and possibly other signals that can be recognized by the organism to receive the DNA. The gene can be inserted into the vector once the vector has been cleaved with restriction endonucleases, which are enzymes that can cut specific sequences of DNA. The vector is then put into a host cell, such as *Escherichia coli* (*E. Coli*), where it can be replicated during normal cell division. Therefore, the DNA segment of interest can be copied millions of times and easily purified for other uses [7].

Figure 3. DNA Cloning. Segment of DNA can be inserted into a cloning vector by opening the cloning vector with restriction endonucleases and the fragments can be ligated together. The recombinant DNA can then be transformed into a host cell.

Currently, polymerase chain reaction (PCR) is the most common method to clone genes that share DNA sequence homology. However, there are limitations for this cloning method. Random or nonspecific primer binding is one of the limitations for PCR

cloning. Also, a high misincorporation rate can occur when using *Taq* or other thermostable DNA polymerases and mutations can be introduced into the amplified DNA when using PCR. Moreover, without prior information of the gene's complete sequence, PCR amplification is not an efficient technique for isolating full-length genes [8].

1.6 Cloning Vectors

Plasmids are circular, double-stranded DNA molecules, which are replicated within a cell independently of the chromosomal DNA molecules. Plasmids can serve as vehicles for DNA cloning and they have three common features [7]. First, the cloning vector needs an origin of replication (*ori*) that provides a DNA sequence recognized by the replication enzymes in the cell, and it is the origin site where the double stranded DNA denatures as a first step in replication [9]. Second, the cloning vector needs a selectable marker. These allow the cells that have been transformed by the cloning vector to be resistant to an antibiotic and thus able to grow in the presence of the antibiotic. Third, the cloning vector is designed to have multiple restriction endonuclease cleavage sites. Restriction endonucleases are enzymes that are isolated from bacteria and are able to cleave double stranded DNA at specific sequences. The cleavage points occur exactly on the axis of symmetry and can result in blunt ends or staggered cuts on the two DNA strands, leaving one to many nucleotides on one strand unpaired, which are referred to as sticky ends. The sticky ends can base pair with complementary sticky ends of the foreign DNA fragments that are being amplified [10]. An example of a cloning vector is shown in Figure 4 on page 7.

Source: Pangene Corporation

Figure 4. An Example of a Cloning Vector, pEAK8. This plasmid is 6164 base pairs long; it contains an origin of replication (*ori*), restriction sites and genes encoding resistance to the drugs of ampicillin (*amp^r*) and puromycin (*puro^r*). It also contains a eukaryotic promoter (EF-1 α) and poly A/splice consensus sequences to facilitate expression of the gene insert in mammalian cells for downstream study.

1.7 cDNA Libraries

Particular genes can be isolated from a DNA library. A DNA library is a collection of DNA fragments derived from a genome of a particular organism. There are two types of DNA libraries, genomic DNA libraries and complementary DNA (cDNA) libraries. Genomic DNA libraries contain DNA fragments from the entire genome of an organism. cDNA libraries contain complementary DNA molecules synthesized from mRNA molecules derived from a particular tissue. Since most eukaryotic mRNA contains 3'-poly(A) tails, mRNA can be selected by specific binding to oligo(dT)-cellulose. The mRNA can be eluted by using high ionic strength buffers to disrupt the hydrogen bonding. Then an enzyme, reverse transcriptase, is used to synthesize a DNA strand, copying RNA as the template. DNA polymerase is then used to convert the single-stranded DNA (ssDNA) into double-stranded DNA (dsDNA) molecules. To assist cloning, oligonucleotide linkers which contain suitable restriction sites are then added to the dsDNA molecule and the cDNA is cloned into a suitable vector. In cDNA library, all the introns are spliced out, therefore, it is less complex and it contains only the transcribed and processed DNA sequences that normally code for a protein [7, 10].

1.8 Functional Expression of GPCRs

Once the gene is cloned, it needs to be expressed in a microorganism or mammalian cell in order to make the gene product by transfection. Transfection is the act of getting exogenous DNA into a cell (analogous to transformation for prokaryotic cells). However, in order to have a functional expression system for odorant receptors, the

cloned OR's have to be able to couple with a G-protein and an enzyme, such as adenylate cyclase, for the production of a second messenger. In this fashion, a measurable signal in response to ligand stimulation is produced [3]. The functional expression of olfactory receptors in heterologous host cells is not trivial. Studies have shown that olfactory receptors are normally maintained in the endoplasmic reticulum of cells and they do not traffick well to the plasma membrane *in vitro* when expressed in non-olfactory cell lines or even cell lines derived from olfactory epithelium [3, 11-14]. Therefore, the analysis of odorant binding to the receptors *in vitro* cannot as yet be fully investigated [3].

1.9 Olfactory Receptor Database

Currently the chemosensory receptor protein and gene sequence information are available from public databases. In 1995, the Olfactory Receptor DataBase (ORDB) was created in order to provide all the known olfactory receptors' sequence information to the public. Besides information on gene size, chromosomal location information and the specific tissue from which the cloned olfactory receptor (OR) was isolated, the ORDB directly links to the PubMed and/or GenBank databases. Moreover, all users can compare any OR sequence to the public database by performing a BLAST search [15]. The ORDB currently contains nearly 1750 OR sequences from more than 30 species [16]. A selection of the numerous GPCR's of interest can be narrowed down by a thorough study from these databases.

The Human Genome Project has increased our knowledge of human olfactory receptors. There are more than 1000 OR genes in mouse and rat, and about 750 OR

genes in human, however, 60% of human OR genes are pseudogenes and less than 350 are intact olfactory receptor [16]. On the other hand, only 20% of the mouse olfactory receptor genes are pseudogenes [17] and fewer than 5% of the sequences are pseudogenes in rats [6]. Pseudogenes are defective sequences of genomic DNA which have a similar sequence to a nonallelic functional gene [10]. This enormous degeneration of olfactory receptor genes in humans might be due to evolutionary development, namely, that people depend on visual and auditory senses much more than olfaction [18-19].

1.10 Artificial Nose Biosensor

The chemosensory receptor protein and gene sequence information that is available from public databases has increased our knowledge of human olfactory receptors [6]. Because the mammalian olfactory receptors are able to recognize and distinguish among over a thousand different odorant molecules, isolation and functional expression of a particular OR would produce a cell responsive to the ligand for that OR. Taking this concept further, by using gene sequence information from public databases and the advancement of cloning technology, it is feasible to generate a biosensor based on olfactory receptors to detect odorant molecules.

CHAPTER 2.0 LITERATURE REVIEW

2.1 Olfactory Receptors

As explained in *1.0 Introduction*, the olfactory transduction system is involved in the coupling of an odorant with a G-protein, which will activate an adenylyl cyclase and produce a second messenger like cAMP. The release of cAMP will open ion channels and generate an action potential, and the signals will be sent to the brain. In 1991, Buck *et al.* found that the olfactory receptors belong to the seven transmembrane protein family [2]. Studies have shown that a single odorant can interact with multiple receptors and a single receptor can recognize multiple odorants, however, different odorants are distinguished by different sets of receptors. Different ligand affinities and stimulation of OR combinations by odorants, allows our olfactory systems to distinguish thousands of odorants [20].

2.2 Signaling Cascades in Odorant Detection

Previous work has shown the capacity of using olfactory receptors to sense an odorant over several orders of magnitude of concentration *in vitro*. Studies have shown that isolating the cilia from frog and rat olfactory epithelia can provide an *in vitro* system to study the mechanism of odor recognition. It has been shown that odorants caused fast and transient elevations of cAMP, and also that odorant stimulation of the adenylyl cyclase is the primary mechanism in the olfactory transduction and is GTP-dependent. In the absence of GTP, the odorants increased adenylyl cyclase activity about 10% above

the basal level in frog cilia and 5% over the basal level in rat cilia. In the presence of GTP, the odorants increased adenylate cyclase activity 30-55% in frog cilia and 30-65% in rat cilia [21].

Previous work at San Jose State University (SJSU) has provided additional evidence showing that cAMP was the secondary messenger in odorant detection. The rate of cAMP production was measured and a mathematical model was also developed demonstrating the significance of the ratio of guanosine triphosphate (GTP)/guanosine diphosphate (GDP). The impact of GDP on the sensor response was identified in the model by the ratio of concentrations of GTP/GDP. At different odorant concentration, the rate of production of cAMP was measured at different GTP/GDP ratios by holding GTP constant. The results indicated that the ratio of concentration of GTP/GDP could control the sensitivity of the detection of a ligand. The range of concentration detection is low when the GTP/GDP ratio is high; on the other hand, the range of concentration detection is high at low GTP/GDP ratio. It has shown that the olfactory system is able to quantify the odorant concentration over several orders of magnitude. It also demonstrated how to maximize the dynamic range of a sensor based on olfactory receptors [22, 23].

2.3 Cloning Technology

RecA is a protein from *Escherichia coli* (*E.coli*) discovered by Clark and Margulies in 1965, where RecA protein is indispensable for *E.coli* recombination *in vivo*. Many additional functions of RecA proteins have been discovered since then [24]. One

of the biological functions for RecA includes damaged DNA repair [24], however, RecA also promotes homologous pairing of single-stranded DNA with double-stranded DNA *in vitro* [8, 24-26]. Studies have shown that ssDNA oligonucleotides that were coated with RecA protein can form a stable nucleo-protein filament and bind to homology within target duplex DNA sequences; in addition, after removal of the RecA, the multi-stranded DNA hybrids are very stable to manipulation [26]. Research has shown that using RecA-mediated processes to capture homologous DNA sequence from a cDNA library is an alternative gene cloning method. This method allows the cloning of a gene from any desired plasmid library. By using the RecA-mediated cloning method, errors induced by PCR can be avoided. Also, a full-length gene can be isolated even if the gene's complete sequence information is unknown [8].

2.4 Functional Expression of Odorant Receptor

Studies have shown that olfactory receptors are able to traffic to the plasma membrane in mature olfactory receptor neurons, however, olfactory receptors are difficult to express functionally in heterologous cells [12]. Murrell *et al.* created a cell line derived from a perinatal rat olfactory sensory neuron (OSN) called *odora* (olfactory-derived, odorant receptor activatable cells), and showed that differentiated *odora* cells traffic olfactory receptors to the plasma membrane *in vitro* and the receptors have the ability to participate in olfactory signal transduction [14].

Dwyer *et al.* isolated and cloned a protein from *C.elegans*, ODR-4. Their study indicated that ODR-4 is required in facilitating the surface delivery of an olfactory

receptor, ODR-10 to the cilia in olfactory neurons [27]. Gimelbrant *et al.* showed that when expressing olfactory receptors in heterologous cell systems, olfactory receptors are retained within the endoplasmic reticulum. In undifferentiated *odora* cells, olfactory receptors traffic into the Golgi and endosomes. In a differentiated state, olfactory receptors traffic to the plasma membrane. They have also shown that *C.elegans* ODR-4 increases the plasma membrane trafficking of rat olfactory receptor U131 in *odora* and Chinese hamster ovary (CHO) cells lines. In contrast, U131 is retained within the endoplasmic reticulum when expressed in CHO cells in the absence of ODR-4 expression. This study demonstrated that *C.elegans* ODR-4 is able to improve membrane trafficking not only of *C.elegans* olfactory receptors, but also of mammalian olfactory receptors [11].

2.5 Summary

To mimic human olfaction or taste senses electronically is a great challenge. Specifically, an electronic nose should be able to distinguish odorants like natural biological noses do. Most of the biosensors that have been developed do not have the sensitivity, trainability or dynamic range of a biological nose. Currently, the majority of biosensors can be classified into four categories, electrochemical, spectroscopic, electrodes and optical methods; however, they all have limitations. For instance, they can only operate in a controlled environment, the properties and characteristics of many volatile chemicals will change with different concentration levels, and the sensor cannot accurately detect the odorants under complex backgrounds. Most importantly, most of the biosensors lack an appropriate dynamic range. Therefore, there is a need to develop a

biosensor based on olfaction, so that it will have the ability, sensitivity and selectivity that mammalian noses do [28]. With current cloning technology, along with the availability of the olfactory receptor sequence information from the public databases, it is possible to clone the G-protein coupled receptors and transfect a mammalian cell line with genes in order to construct a biosensor. In addition, olfactory system uses a combinatorial receptor coding system to encode odor identities and the pattern is specific to the odorant, if this recognizable pattern is placed into a computer device, this biosensor can be trained to recognize and identify the patterns. Given our understanding of how GTP, GDP and their ratio effects the olfactory signal transduction, it is now feasible to develop a trainable biosensor based on olfactory receptors, and this biosensor will have the ability to detect a large dynamic range of odorants' concentration, as well as different odorants and sensing environments.

CHAPTER 3.0 RESEARCH OBJECTIVE

In order to construct a biosensor based on olfactory GPCR, the first step is to clone the olfactory GPCR genes. Olfactory GPCR genes can be cloned from a cDNA library. In addition, cloning of the human ODR-4 homolog and co-expression with the olfactory receptors may aid in plasma membrane localization of olfactory receptors. With the availability of the olfactory receptor sequence information from public databases, the objective of this research is to clone a mouse olfactory receptor and a human ODR-4 gene homolog.

CHAPTER 4.0 MATERIALS AND METHODS

4.1 Primer

4.1.1 Primer Design

The sequence information of the *C.elegans* ODR-4 (ceODR-4) and twenty-eight human, mouse and rat olfactory receptors were obtained from the olfactory receptor database (ORDB) and GenBank and four primers (A, B, C and D) for each gene of interest were designed using primer design software Primer 3. Primer sets should be nested in order to produce an internal primer pair, (BC, probe primer) and an external primer pair (AD, screening primer). At least one primer used for PCR screening should be outside the probe primers in order to avoid a false positive result. Primers were purchased from QIAGEN Operon. The sequence information for ceODR-4 was obtained from GenBank and is shown in Table 1. The primer set for ODR-4 was not designed against ceODR-4 but was designed against a human partial gene sequence that was homologous at the level of amino acids to ceODR4. The protein alignment of the human homolog to ceODR-4 is shown in Table 2. The sequence information for GenBank entry with unknown function whose translation product has homology to ceODR4 is shown in Table 3. The set of primers for hODR-4 are shown in Table 4. The sequence information for the mouse olfactory receptor, mOR37, was obtained from olfactory receptor database and is shown in Table 5. The set of primers for mOR37 are shown in Table 6. Primers were designed against many other OR genes and are summarized in Table 7.

Table 1. Sequence Information for ceODR-4. GenBank accession number is AF055911. Bold letters indicate start and stop codons.

1	ttacagaatc	catg attctg	tttgacgtcc	aactccagga	atgggtgacc	aagtcagcga
61	agaatcacga	atttgtttta	tccgataaag	gaattccggc	gtcagcctac	tttttgctcg
121	gatcattctg	ctcggatggt	gacatccacg	tggcatatgc	ttccaaatgt	ccggttcata
181	gcagtgcatt	ggaagagaat	gctacagaat	catcaaagat	gctggaagac	gaatggatgt
241	cagatcacgc	agaacgtctt	ctccgaatgc	ttcccgggtg	aattcatgtg	gttggaattg
301	cctgggttctc	agataagaag	actttctcag	atcggaaatc	acatattcac	aagacattgg
361	gtcgtattca	gaaaatgaat	aatcaaatca	caacggctaa	tgtggatgac	tcgatttcgg
421	ataatatgat	cacagtgttc	ttcgagaccc	catccaccac	ccccatcgg	gccataattg
481	atgtgaccaa	tcgtggcaac	gatagtgcc	aaaaagtcca	atttcaaaaa	cttgaatgga
541	tttctttggg	gacaaacgca	tctgctcgaa	ttgtgcacaa	tgttcagtt	gacacaggac
601	gccaactga	cttctactct	gacttggtag	tcgccacgaa	aaactttgtg	aataatttat
661	tccaatgtga	atttacactt	cttgatgggtg	agatccgtga	tgataaggag	ccactgatca
721	aagatatcaa	gaagaataaa	aagacaacga	ttgaggcaca	attgttcttg	aatcctcttt
781	ttctagataa	ccgagagctc	ggagctatcg	atgatattgc	gtccaacatg	cacgaagttc
841	tttttgacat	tgaagtctgc	gcagctgttc	caatcagatc	aacagtgaag	gacgcaatcc
901	gagcaatcaa	gcatcattta	gtcaggaatc	tgtttgcaag	agttgaactt	cactaogaat
961	cgatggaagt	tggtgaagag	gaacgatcac	caaaaactgg	aatcacctgc	catcaactac
1021	ctcgtccagc	tactacagta	ctctacacac	atccagcaat	tctcatcaat	gatttctctt
1081	ttgaggctga	taatgtagaa	gatgcacaga	agaattttga	tgacatgatg	gatttacaga
1141	caagcattga	acacgtggat	gaaggctggg	aacgcgcgct	gacaccagaa	gagatggaag
1201	ctgtgcgaac	accgattgag	gatcttcatt	tcgttgactt	cgatggatca	agcgattcat
1261	ggtgcacaac	gaaaaccata	ctgattacta	ttgcattgat	tatcggactc	ttggcatcca
1321	ttattttattt	cactgtagca	cattcct tgag	ctagttttcc	ccgagcatta	tgacgtaatt
1381	ttgcatattt	tccaagtatt	tatatatttt	ttctctgtga	taaaatactt	catttaaaaa
1441	aa					

Table 2. Protein Alignment of Human Homolog to ceODR4. BLASTP program was used to identify sequence alignments and bold letters represent sequence identity (21% have identical residues in the two sequences). “+” represent sequence similarity (additional 65 positions have chemically similar amino acids). Test sequence (query sequence, “Q”), database sequence (subject sequence, “S”).

Identities = 66/314 (21%), Similarities = 131/314 (41%)	
Q: 35	LLGSFCSDGDIHVAYASKCPVHSSALEENATESSKM--LEDEWMSDHAERLLRMLPGGIH 92
	LL CS +V A++ P E +K+ L++EW ++HA ++ RMLPGG+
S: 29	LLIGQCSSQKDYVILATRTPPKKEEQSENLKHPKAKLDNLDEEWATEHACQVSRMLPGGLL 88
Q: 93	VVGIAWFSDKKTFSDRKSHIHKT LGRIQKMNQITTANVDDSIDNMITVFFETPSTTPI 152
	V+G+ + + +D ++ + + + ++K N+ N + +T+ +
S: 89	VLGVFIITTTLELANDFQNALRRLMFAVEKSINRKRLWNFTEEVSESVTLHICASTKKIF 148
Q: 153	GAIIDVTNRGNDSAQKVQFQKLEWISLVTNASARIVHNPVDTGRPTDFYSDLVVATKNF 212
	D+ + SA+ ++ +S + VH ++ P S KN
S: 149	CRTYDIHDP-KSSARPADWKYQSGLSSSWLSLECTVH---INIHPLSATSVSYTLEKNT 204
Q: 213	VNNL-----FQCEFTLLDGEIRDDEPLXXXXXXXXXXXXXEA-----QLFLNP 255
	N L + L++G+++D+ L +A QL LN
S: 205	KNGLTRWAKEIENG VYLINGQVKDEDCDLEGGQKKSSRGNTQATSHSFDVRVLTQLLLN 264
Q: 256	LFLDNRELGAIDDIASNMHEVLFDIEVRAAV-PIRSTVKDAIRAIKHHLVRNLFARVELH 314
	D+R + I S + ++ RA + + VKDA++A+K ++ + R E+
S: 265	---DHRSTATV-QICSGSVNLKGAVKCRAYIHSSKPKVKDAVQAVKRDILNTVADRCEML 320
Q: 315	YESM---EVVEEER 325
	+E + E+ E++R
S: 321	FEDLLLNEIPEKKR 334

Table 3. Sequence Information for Human Homolog to ceODR-4. GenBank accession number is AK000512. Bold letters indicate start and stop codons.

1	gatgaattca	gtggcagtga	attgagaccg	gagggaaatct	ggccccctaga	ggctggtact
61	tgggccccgaa	acccccatct	ccggcggaga	gaccgtccga	ggtaattgtc	tgccacgagt
121	gcacattctg	aaaacaggag	attttagttc	ctaaaa atgg	gaagaacctt	cattgtagaa
181	gagactgttg	gccagtatct	ttcaaacata	aatctccaag	gaaaggcttt	tgtctctggc
241	cttttaatag	gacagtgttc	gtcacaaaag	gattatgtga	ttcttgccac	tagaacgcca
301	cccaaagagg	agcaaagtga	gaacctcaaa	catcccaaag	ctaagttgga	taacttggat
361	gaagaatggg	ccacagaaca	tgcttgccag	gtatccagaa	tgctaccagg	gggactttta
421	gttcttggag	tatttattat	tactacttta	gaactggcaa	atgattttca	aaatgccctg
481	cgtagactaa	tgtttgtgtg	ggaaaagtct	ataaatagaa	agagattgtg	gaatttcaca
541	gaggaggaag	tctcagaacg	agtgcactt	cacatttgtg	cttctacaaa	aaaaatattt
601	tgtcgaactt	atgatatcca	tgatccaaag	agttcagcaa	gaccagcaga	ttggaagtat
661	caaagtggat	tatcatcctc	atggctttct	ttagagtgtg	cagttcacat	taatattcac
721	atcccacttt	ctgctacttc	tgtcagctat	actctggaga	aaaatacaaa	gaatggactt
781	acacgctggg	ccaaggaaat	agaaaatggt	gtttatttga	ttaatggaca	agttaaagat
841	gaagattgtg	acctattaga	aggacagaaa	aaatcttcta	gaggaaatac	tcaagcaact
901	agtcattctt	ttgatgtcag	agtgttaacg	cagttgctcc	tgaattcaga	ccacagatcc
961	acagccacag	tccagatatg	tagcggttct	gtaaacctta	aggggtgctg	gaaatgcaga
1021	gcttatatcc	acagcagtaa	acccaaagt	aaagatgctg	tgcaggcagt	aaagagggat
1081	atattgaaca	cagttgtgtg	tcgttgtgaa	atgctatttg	aggatctgct	tttgaatgaa
1141	attccagaaa	aaaaaagatt	ctg aaaaaga	gttcacgctc	ctcccttata	gagtccttgt
1201	tccccttctc	ggatccactg	taatgttgtg	tgattataaa	tttgacgatg	agtcagctga
1261	agaaatcagg	gaccatttta	tggagatgtt	ggatcacaca	attcaaatag	aagatttggg
1321	aattgcagag	gaaacaaaca	cagcttgtat	gagttcttct	atgaatagtc	aagcttcatt
1381	ggacaacaca	gatgatgaac	aacccaaaaca	accaattaaa	actacaatgt	tattgaaaat
1441	tcagcaaaac	ataggtgtga	ttgcagcatt	tacagttgca	gtccttgctg	cgggtatctc
1501	ctttcattac	ttcagtgtat	agggtgaggg	acaaagagtt	tcttgatcat	ccagagaaca
1561	ttgacagaca	attatgaata	ataaagatgt	taacaatcca	tctgtattta	aaacactagc
1621	agccagatct	gctgccatga	tgcctatttg	gtgtgtttct	gattaaaatg	aaatcacaag
1681	ctgcaaaaaa	aaaaaaaaaa	aaaaaaaaaa	aaaaaaaaaa		

Table 4. ODR-4 Primer Set

Primers	Primer Name	Sequence 5' to 3'
Screening primer forward (A)	ODR4-12	TGGCAGTGAATTGAGACC
Probe primer forward (B)	ODR4-87	GAGAGACCGTCCGAGGTA
Probe primer reverse (C)	ODR4-371R	GCCCATTCTTCATCCAAG
Screening primer reverse (D)	ODR4-487R	GTCTACGCAGGGCATT

Table 5. Sequence Information for mOR37. GenBank accession number is AY073936. The letter “n” means “no call” when it is unable to call a base at a specific position and this may be due to poor quality sequencing.

1	atggetctta	gcaattctag	ctggaggcag	ccccagcccc	ctttcttcct	agtaggtgtt
61	ccaggcttgg	aggaaagtca	gcactggata	gcattgcccc	tgggtatcct	ttacctcttt
121	gctctagtgg	gcaatgtgac	tatcatcttc	attatctgga	ctgattcatc	cttacaccaa
181	cctatgtacc	tcttcctggc	catgcttgct	gctattgacc	tggctcctggc	ctcttcact
241	gcacccaaag	ccctcactgt	gctcctggct	catgcccatg	agattgggta	cattgtatgc
301	ctgactcaga	tgttctttat	tcatgccttc	tcctctatgg	agtcaggcat	acttgtagcc
361	atggctctgg	atcgctatgt	tgccatctgc	cacctctgc	gtcattccac	catcctgcat
421	ccagggatta	tagggcgcat	tgggctggta	gtactgggtgc	ggggattggg	ccttcttttc
481	ccattcccca	ttctgttgca	gaatgttgct	ttctgcagag	caactgtcat	aagccatgcc
541	tattgtgagc	atatggctgt	ggtaaaactt	gcctgttctg	aaaccacagt	gaatcgagct
601	tatggctctg	cannnnnnnn	nnnnnnnnnn	nnnnnnnnnn	nnnnnnnnnn	nnnnnnnnnn
661	nnnnnnnnnn	nnnnnnnnnn	nnnnnnnnnn	nnnnnnnnnn	nnnnnnncat	tggcatttcc
721	tatgccctca	tcctccaggc	agtgtgaag	gtgccagggg	gtgaagccag	acttaaagcc
781	ttcagtagat	gtgggtctca	tgtttgtgtt	attctcatct	tctatgtccc	tggaaatgttc
841	tccttctctc	ctcaccgctt	tggccaccat	gttcccatc	atgtccatgt	tctgctggcc
901	actctctatc	tccttgtgcc	acctgccctg	aatcctcttg	tttatggggg	gaagactcga
961	cagattcgcc	agcgagtgtc	caggggtgtc	tacacaaaag	catcgatttg	a

Table 6. mOR37 Primer Set

Primers	Primer Name	Sequence 5' to 3'
Screening primer forward (A)	mOR37-62	CAGGCTTGGAGGAAAGTC
Probe primer forward (B)	mOR37-119	TTGCTCTAGTGGGCAATG
Probe primer reverse (C)	mOR37-421R	GATGCAGGATGGTGGAAAT
Screening primer reverse (D)	mOR37-483R	TGGGAAAAGAAGGACCAA

Table 7. Olfactory Receptors and Primer Set. The forward primers A and B are introduced from the 5' terminal end of a target DNA strand. The reverse (R) primers C and D are introduced from the 3' terminal end of a target DNA strand.

	Gene name	Organism	Primer set (A, B, C, D)
1	671	Human Brain	671-162, 671-222, 671-504R, 671-712R
2	ORL146	Mouse	146-88, 146-164, 146-478R, 146-560R
3	ORL451	Mouse Olfactory Epithelium	451-46, 451-226, 451-450R, 451-502R
4	ORL849	Mouse olfactory bulb	849-1, 849-35, 849-246R, 849-472R
5	ORL452	Mouse olfactory epithelium	452-6, 452-30, 452-315R, 452-420R
6	ORL464	Mouse olfactory neuron	464-36, 464-103, 464-305R, 464-574R
7	ORL828	Mouse olfactory neuron	828-18, 828-165, 828-395R, 828-448R
8	ORL280	Rat brain	280-201, 280-244, 280-537R, 280-764R
9	ORL1008	Rat brain	1008-62, 1008-101, 1008-343R, 1008-526R
10	ORL338	Rat olfactory bulb	338-1, 338-41, 338-251R, 338-302R
11	ORL284	Rat olfactory bulb	284-2, 284-24, 284-235R, 284-305R
12	ORL300	Rat olfactory bulb	300-1, 300-45, 300-379R, 300-454R
13	ORL297	Rat olfactory bulb	297-2, 297-25, 297-252R, 297-417R
14	ORL285	Rat olfactory bulb	285-26, 285-60, 285-357R, 285-622R
15	ORL286	Rat olfactory bulb	286-1, 286-45, 286-268R, 286-552R
16	ORL287	Rat olfactory bulb	287-33, 287-179, 287-382R, 287506R
17	ORL288	Rat olfactory bulb	288-42, 288-130, 288-361R, 288-491R
18	ORL289	Rat olfactory bulb	289-16, 289-88, 289-288R, 289-607R
19	ORL290	Rat olfactory bulb	290-6, 290-49, 290-320R, 290-424R
20	ORL291	Rat olfactory bulb	291-4, 291-87, 291-364R, 291-491R
21	ORL292	Rat olfactory bulb	292-33, 292-179, 292-382R, 292-506R
22	ORL293	Rat olfactory bulb	293-1, 293-96, 293-313R, 293-553R
23	ORL11	Rat olfactory epithelium	11-4, 11-269, 11-527R, 11-579R

4.1.2 Resuspend Primer

Primers were resuspended in TE buffer (10 mM Tris-HCl, pH 8, 1 mM EDTA) and maintained at concentrations of 100 and 5 μ M for working stock. Primers were stored at -20°C freezer for long-term storage.

4.2 Probe

4.2.1 Library Validation

The twenty-four primer sets for mouse and rat olfactory receptors from section 4.1 were validated with several different cDNA libraries, as shown in Table 8. The rat olfactory bulb cDNA library was purchased from Stratagene (#936520). The mouse olfactory epithelium (OE) cDNA library was donated by Dr. Richard Axel (Columbia University). The human brain, umbilical, placenta, fetal brain, mouse brain and fetal brain, rat brain and fetal brain cDNA libraries were obtained from Pangene Corporation. The human homolog ODR-4 gene primers were validated with the human fetal brain library of Pangene. The PCR reaction mix is per reaction and PCR cycle parameters are described in Tables 9 and 10. *Taq* DNA polymerase and Deoxyribonucleoside triphosphates (dNTP) were obtained from Invitrogen. PCR thermal cycler was either a MJ research PTC-200 or Mastercycler gradient (Eppendorf).

Table 8. Olfactory Receptors

	Gene name	Animal	Tested cDNA Library
1	671	Human	Human brain, umbilical, placenta and human fetal brain
2	mOR37	Mouse	Mouse brain, mouse fetal brain & mouse olfactory epithelium
3	ORL146	Mouse	Mouse brain, mouse fetal brain & mouse olfactory epithelium
4	ORL451	Mouse	Mouse brain, mouse fetal brain & mouse olfactory epithelium
5	ORL849	Mouse	Mouse brain, mouse fetal brain & mouse olfactory epithelium
6	ORL452	Mouse	Mouse brain, mouse fetal brain & mouse olfactory epithelium
7	ORL464	Mouse	Mouse brain, mouse fetal brain & mouse olfactory epithelium
8	ORL828	Mouse	Mouse brain, mouse fetal brain & mouse olfactory epithelium
9	ORL280	Rat	Rat brain & rat fetal brain
10	ORL1008	Rat	Rat brain & rat fetal brain
11	ORL338	Rat	Rat olfactory bulb
12	ORL284	Rat	Rat olfactory bulb
13	ORL300	Rat	Rat olfactory bulb
14	ORL297	Rat	Rat olfactory bulb
15	ORL285	Rat	Rat olfactory bulb
16	ORL286	Rat	Rat olfactory bulb
17	ORL287	Rat	Rat olfactory bulb
18	ORL288	Rat	Rat olfactory bulb
19	ORL289	Rat	Rat olfactory bulb
20	ORL290	Rat	Rat olfactory bulb
21	ORL291	Rat	Rat olfactory bulb
22	ORL292	Rat	Rat olfactory bulb
23	ORL293	Rat	Rat olfactory bulb
24	ORL11	Rat	Rat olfactory bulb

Table 9. PCR Master Mix for Library Validation

10x PCR Buffer	2.5 μ l
50 mM MgCl ₂	0.75 μ l
50% Glycerol	5.0 μ l
10 mM dNTP mix	0.5 μ l
5 μ M Forward Primer	1.0 μ l
5 μ M Reverse Primer	1.0 μ l
5 U/ μ l <i>Taq</i> DNA polymerase	0.25 μ l
Deionized water (dH ₂ O)	13.0 μ l
cDNA library (100 ng/ μ l)	1.0 μ l

Table 10. PCR Program for Library Validation

Step	Temperature	Time
1. Denature	95 °C	3 min.
2. Denature	95 °C	45 sec.
3. Anneal	55 °C	45 sec.
4. Extend	72 °C	45 sec.
<i>5. Repeat step 2 thru 4 thirty four times.</i>		
6. Extend	72 °C	7 min.
7. Store	4 °C	forever

4.2.2 Template Purification

The bands of the amplified PCR product were analyzed by running 12 µl of product on a 1 % agarose-TAE gel containing Ethidium Bromide (EtBr, 1 µg/ml). Each PCR product was mixed with 12 µl loading buffer (2x) prior to loading the sample into each well of the agarose gel. The PCR product bands were purified by gel purification using Qiagen QIAquick Gel Extraction Kit (Qiagen, Cat# 28704), and quantified by using a UV spectrophotometer (Beckman DU 530 or Hewlett Packard 8452A) to check for the DNA concentration. The purified DNA template was stored at –20°C for long-term storage. The protocol for measuring the DNA concentration is described in section 4.5.

4.2.3 Probe Synthesis

The purified DNA product from section 4.2.2 was used as a probe template for a PCR reaction. The PCR reaction mixture and the PCR cycle parameters are described in Table 11 and 12. Two reactions per DNA template were performed and pooled. Biotin-14-dATP was obtained from Invitrogen. The extension time was increased to allow for efficient Biotin-14-dATP incorporation.

Table 11. PCR Master Mix (per reaction) for Probe Synthesis.

10X PCR Buffer	5.0 μ l
50 mM MgCl ₂	1.5 μ l
50% Glycerol	10.0 μ l
Biotin-dNTP mix	7.25 μ l
5 μ M Forward Primer	2.0 μ l
5 μ M Reverse Primer	2.0 μ l
5 U/ μ l <i>Taq</i> DNA polymerase	0.5 μ l
dH ₂ O	20.75 μ l
Gel purified DNA template	1.0 μ l

Table 12. PCR Program for Probe Synthesis.

Step	Temperature	Time
1. Denature	95 °C	3 min.
2. Denature	95 °C	45 sec.
3. Anneal	55 °C	45 sec.
4. Extend	72 °C	105 sec.
5. Repeat step 2 thru 4 thirty four times.		
6. Extend	72 °C	7 min.
7. Store	4 °C	forever

4.2.4 Probe Purification

Qiagen Qiaquick Purification Kit (Qiagen, Cat# 28104) was used to purify the amplified DNA probe. After purification, the DNA probe was quantified by using a UV spectrophotometer (Beckman DU 530 or Hewlett Packard 8452A) to check for the DNA concentration and diluted to 25 ng/ μ l with 1x TE. The size of the DNA probe was determined by running 2 μ l of the DNA probe with 2 μ l loading buffer (2x) on a 1%

agarose gel with EtBr. The DNA probe was stored at -20°C for long-term storage. The protocol for measuring the DNA concentration is described in section 4.5.

4.3 DNA Enrichment

4.3.1 Recombinase Coating of Probe

50 ng of the double-stranded DNA (dsDNA) probe from section 4.2.4 was heat denatured with dH_2O by boiling for 3 minutes. Consequently, the single-stranded DNA (ssDNA) probe was coated with 5x Coat mix (1x concentrations are 10 mM Tris-acetate, 50 mM sodium acetate, 2 mM magnesium acetate, 1 mM dithiothreitol), 2.0 mM nucleotide cofactor (ATP- γ -S) and RecA protein at a ratio of one RecA protein monomer per three DNA bases at 37°C for 15 minutes. RecA protein and ATP- γ -S were obtained from Roche Molecular Biochemicals.

4.3.2 Paramagnetic Bead Preparation

Streptavidin coated paramagnetic beads were washed with binding buffer (10 mM Tris-HCL, pH 8.0, 1 M NaCl, 1 mM EDTA) and mixed with undiluted competitor DNA, (salmon sperm DNA can be used to prevent nonspecific binding of DNA from the library) and binding buffer on a rotator for 30 minutes. Streptavidin coated paramagnetic beads (1% wt/vol) were obtained from Seradyn (part #30152104040350). 30 μl of a washed 1% solid (10 mg/ml) beads were used in all the enrichment steps.

4.3.3 Probe Target Hybrid Formation

A 1:100 dilution of competitor DNA in 1x TE (1 mM Tris-HCL pH 8, 0.1 mM EDTA) was prepared and denatured by boiling for 3 minutes. The denatured 1:100

competitor DNA was added to the mixture from section 4.3.1, along with targeting additive (200 mM Magnesium acetate) and 1-5 µg of double-stranded cDNA library and incubated for 20 minutes at 37 °C. After the stable double D-loop hybrids are formed, RecA proteins were removed from the probe-target hybrids by adding 10% SDS solution followed by incubation at 37 °C for 10 minutes.

4.3.4 Hybrid Binding to Magnetic Beads

The washed magnetic beads from section 4.3.2 and the probe target hybrid from section 4.3.3 were mixed and incubated on a rotator at room temperature for 30 minutes. The beads were then washed four times with STE buffer (10 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1 mM EDTA). The salt was removed by washing with 0.1x TE buffer. The capture DNA was eluted from the magnetic beads by adding 10 µl 0.1x TE and incubating at 65 °C for 5 minutes. The eluent, which contains the enriched capture DNA, was transferred into a new tube and stored on ice for the next step. Extra captured DNA was stored at -20 °C for future use.

4.3.5 Transformation for Clone Amplification

Transformation of DH10B cells (Pangene) or ElectroMAX DH10B T1 Phage-Resistant cells (Invitrogen, Cat# 12033-015) with the captured target DNA was achieved by electroporation with an electroporator (Eppendorf Electroporator 2510 or BTX-ECM 600 electroporator). After the electroporation, SOC media was added and the bacteria mixture was transferred into a culture tube and incubated in a shaker at 37 °C for 1 hour. The transformed bacteria were spread onto Luria-Bertani (LB) plates containing ampicillin (*amp*) and incubated at 37 °C overnight.

4.3.6 Blue/White Control

Blue/white control enrichment was performed for each enrichment process in order to test for efficiency of the procedure. The steps were the same as the enrichment procedure described in section 4.3.1-4.3.5, except that a blue/white control biotinylated probe (Bio-HPRT) and 1 μ g of the control blue/white plasmid mix were used. After electroporation, the transformed bacteria were spread onto LB plate containing ampicillin, X-Gal (5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside) and IPTG (isopropyl- β -D-thiogalactopyranoside) and incubated at 37 °C overnight. The control plasmid mix is composed of 10,000 parts pUC19 plasmid, 1 part pUC19 with an HPRT gene insert. The pUC19 plasmid contains a gene called LacZ, which will produce an enzyme called *β -galactosidase*. If an insert is within the *β -galactosidase* coding region, it will disrupt and inactivate the enzyme activity. *β -galactosidase* converts X-Gal into a colored product due to an enzymatic reaction. IPTG serves as an inducer, allowing the gene to be actively transcribed resulting in active *β -galactosidase* enzyme, which will cleave the X-Gal and form a blue color [7, 10]. Bacterial colonies containing the pUC19 plasmid become blue, those containing the inserts (pUC19-HPRT) will disrupt the *β -galactosidase* gene inactivating the enzyme activity; therefore, remain white when plated on IPTG/X-Gal/Amp plates. Ideally, more than 60% of white colonies should be obtained from enrichment.

4.3.7 Plasmid DNA Purification

For performing more than one round of enrichment, transformed cells were scraped off of the agar plates and Qiagen Plasmid Midi Kit (Qiagen, Cat# 12143) was

used to purify the plasmid DNA. This plasmid DNA was compared to the DNA from the starting cDNA library for enrichment by PCR with the screening primers (described in section 4.1.1) and analyzed on a 1% agarose gel in order to compare the band intensities. A second and third round of cDNA capture was performed in order to achieve a satisfactory degree of enrichment of the desired gene by repeating the steps described in section 4.3.1 through 4.3.6. In the second capture, the purified plasmid DNA from the first capture was used as the DNA template instead of the cDNA library. In the third capture, the purified plasmid DNA from the second capture was used as the DNA template. In some cases, plasmid DNA was isolated from scraped cells with the QiaPrep Spin Miniprep Kit (Qiagen, Cat# 27104).

4.4 Validation

4.4.1 Colony Screening

After the third capture enrichment, single colonies were randomly picked from the LB/amp plate and inoculated in LB/*amp* broth. After incubation at 37°C for 3 hours, 1 µl of single colony inoculate was mixed with 9 µl of ddH₂O in a PCR tube. The mixture was then denatured and added to the PCR cocktail listed in Table 13. PCR cycle parameters are the same as section 4.2.1. The size of PCR products were compared to the predicted size from a PCR reaction with the screening primers by mixing 10 µl PCR product with 10 µl loading buffer (2x) and running it on a 1 % agarose gel with EtBr.

Table 13. PCR Master Mix for Colony Screening

10X PCR Buffer	2.5 μ l
50 mM MgCl ₂	0.75 μ l
50% Glycerol	5.0 μ l
10 mM dNTP mix	0.5 μ l
5 μ M Screening Forward Primer	1.0 μ l
5 μ M Screening Reverse Primer	1.0 μ l
5 U/ μ l <i>Taq</i> DNA polymerase	0.25 μ l
dH ₂ O	4.0 μ l
Single colony inoculate	10.0 μ l

4.4.2 Positive Clone Amplification

5 μ l of the colony inoculations that are determined to contain the gene of interest by PCR from section 4.4.1 were added into 3 ml of Terrific Broth (TB) with ampicillin. The culture was shaken overnight at 37 °C. The next day, QIAprep spin Miniprep kit was used to purify the plasmid DNA. The concentration of the purified plasmid DNA was quantified by using an UV spectrophotometer. Also, to confirm that the purified DNA contains the gene of interest, 1 μ l of the purified DNA was added to the PCR cocktail listed in Table 14 and a PCR reaction was performed by using the PCR cycle parameter as described in section 4.2.1.

Table 14. PCR Master Mix for Colony Validation

10X PCR Buffer	2.5 μ l
50 mM MgCl ₂	0.75 μ l
50% Glycerol	5.0 μ l
10 μ M dNTP mix	0.5 μ l
5 μ M Forward screening Primer	1.0 μ l
5 μ M Reverse screening Primer	1.0 μ l
5 U/ μ l <i>Taq</i> DNA polymerase	0.25 μ l
dH ₂ O	13.0 μ l
Miniprep DNA plasmid	1.0 μ l

4.4.3 Plasmid DNA Analysis

The insert size of the gene of interest was determined by using restriction enzymes. Both a single restriction digest and a double restriction digest for each purified DNA was performed and analyzed in order to determine which plasmid preparation contained the largest insert fragments. Enzymes flanking the cloning site release the insert and facilitate insert size determination. DNA plasmids that contained the largest insert fragments were sequenced (Sequetech). Restriction enzymes and buffers were obtained from New England Biolabs.

4.4.4 Full-Length Confirmation

The sequencing result was analyzed by computer software (Contig Express and Vector NTI), and a BLAST search was performed, in order to verify that the cloned DNA sequence matches the predicted gene sequence. Open Reading Frame (ORF) analysis was also performed.

4.5 Summary for Cloning Procedure

A summary flow chart for the cloning method is shown in Figure 5.

A. PRIMER

B. PROBE

C. CAPTURE

D. VALIDATE

Source: Pangene Corporation
Figure 5. DNA Cloning Work Flow Diagram

4.6 Quantifying DNA

1. The UV lamp in the UV/VIS spectrophotometer (Beckman DU 530 or Hewlett Packard 8452A) was turned on an hour prior to use.
2. A 1:60 dilution was made by adding 1 µl of purified DNA into 59 µl of TE.
3. 60 µl of TE was added to a clean quartz cuvette and serves as a blank for the spectrophotometer.
4. The diluted DNA was added to a clean cuvette.
5. The OD value at 260 and 280 nm and the ratio was recorded.
6. Equation (1) was used to calculate the concentration

$$C = 50 \text{ ng/}\mu\text{l} \times A \times D \quad \text{Equation 1}$$

Where C is the concentration of the DNA (ng/µl), A is the OD value at 260nm and D is the dilution factor.

4.7 Library Mass Excision and Amplification

4.7.1 Mass Excision of Mouse Olfactory Epithelium cDNA library

The mouse olfactory epithelium cDNA library was originally cloned into the *Xho* I and *Eco*R I sites of Lambda ZAP II vector. The Lambda ZAP vector has a genome size close to 50 kb in length and it is essentially linear; therefore, Lambda libraries do not work well in the RecA cloning method. Also, cDNA libraries contained in plasmid vectors are more stable and easy to use. DNA Fragments cloned into the Lambda ZAP vector can be excised with helper phage to generate subclones in a phagemid vector. *In vivo* mass excision was done to obtain inserts in the pBluescript SK(-) phagemid from the

Lambda ZAP II vector. The number of infectious particles per volume of stock for the helper phage and lambda library will drop over time; therefore, titering a phage stock is necessary in order to determine the number of infectious particles per volume of stock. The ratio of phage to bacterial cells should be optimized to ensure that there is enough host cells to support the viral production. After mass excision, the titer for the excised phagemids was calculated, and the phagemids were combined with a bacteria host strains in large scale in order to amplify the cDNA libraries (described in section 4.7.5). The mass excision protocol was obtained from Stratagene and summarized as follow.

4.7.2 Bacterial Preparation

1. The host strains cells, BB4 (stratagene, Cat. # 200269) and XL1-Blue MRF' (Stratagene, Cat. # 200158), were streaked onto two different LB with tetracycline (LB-Tet) [25mg/L] agar plates and incubated overnight at 37 °C.
2. A single colony was picked from each plate into two separate flasks containing 50 ml LB broth with supplements [0.2% (w/v) maltose and 10 mM MgSO₄] and incubated overnight at 30 °C.
3. The BB4 and XL1-Blue MRF' cells were spun down at [1000 x gravity (g)] and the cell pellets were resuspended in 25 ml of 10 mM MgSO₄. The concentration of the cells was adjusted with 10 mM MgSO₄ to an OD₆₀₀ of 1.0.

4.7.3 Library Titering Protocol

1. The BB4 cells from section 4.7.2 were diluted to 0.5 OD₆₀₀ with 10 mM MgSO₄.

2. The amplified lambda phage library was diluted in SM buffer (0.1 M NaCl, 8 mM MgSO₄, 50 mM Tris-Cl, pH 7.5, 0.01% Gelatin) by the following ratios: 1:1000, 1:10,000, 1:100,000 and 1:1,000,000.
3. 200 µl of BB4 cells from step 1 and 1 µl of each dilution of lambda phage library from step 2 were mixed and incubated at 37 °C for 15 minutes.
4. 3 ml of NZY top agar (melted and cooled to 48 °C), 15 µl of 0.5M IPTG and 50 µl of X-gal (250 mg/ml) were added to each of the mixtures from step 3.
5. The mixtures from step 4 were immediately poured onto dry, prewarmed NZY agar plates. After the agar had solidified, the plates were incubated at 37 °C overnight.
6. White plaques (recombinant plaques) were counted and used in equation 2 to determine the library titer in recombinant plaque forming units per milliliter (pfu/ml). Blue colonies were the plaques that contained no insert. The white plaques should be 10-100 fold above the blue plaques.

$$\frac{[\text{pfu} \times \text{dilution factor}] \times 1000 \text{ } \mu\text{l/ml}}{[\text{volume plated (}\mu\text{l)}]} \quad \text{Equation 2}$$

4.7.4 Mass Excision Protocol

1. A portion of the amplified lambda phage library was combined with BB4 cells at a multiplicity of infection (MOI) of 1:10 lambda phage-to-cell ratio. This low MOI was used to ensure that cells are not superinfected with more than one lambda phage. A helper phage, R408 (Stratagene, Cat.#200252), was added at a 10:1 helper phage-to-cell ratio to ensure that the helper phage infects every cell in

the mixture. In order to ensure statistical representation of the excised clones, 10- to 100- fold above the primary library size was excised. Below is a summary of the amount of cells that are needed.

- 10^7 pfu of the lambda phage library (10- to 100- fold above the primary library size)

Mouse Olfactory Epithelium cDNA Library

Titer = 2×10^{10} pfu/ml on 12/2000

Average insert size = 1.8 kb

Complexity = 1.65×10^6

- 10^8 BB4 cells (1:10 lambda phage-to-cell ratio, BB4 at OD₆₀₀ of 1 corresponds to 8×10^8 cells/ml)

- 10^9 pfu of R408 helper phage (10:1 helper phage-to-cells ratio)

Titer for R408 helper phage $> 7.5 \times 10^{10}$ pfu/ml

Actually volume used is shown in Table 15.

Table 15. Mass Excision Calculated Number of Cells or Phage. Titer for the lambda library was five fold dropped from the original concentration.

	Cells Ratio needed	Original Concentration	Actual volume used
Lambda phage Lib.	8×10^7 pfu	4×10^9 pfu / ml	21 μ l
BB4 cells	8×10^8 cells	8×10^8 cells / ml	1 ml
R408 helper phage	8×10^9 pfu	7.5×10^7 pfu / ml	107 μ l

2. After incubation of the mixture at 37 °C for 15 minutes, 20 ml of LB plus supplement was added to the mixture and incubated in a shaker at 37 °C for 3 hours. In this step, the lambda Zap II vector was infected with the host strains bacteria (BB4 cells) and a filamentous helper phage (R408 helper phage). The

filamentous helper phage caused replication of the central portion of the lambda clone and yielded an ssDNA product which was packaged in the filamentous phage coat [10].

3. The mixture was then incubated at 65-70 °C for 20 minutes in order to lyse the lambda phage particles and the cells.
4. The mixture was then spun for 10 minutes at (1000 x g) and the supernatant was saved for the next step and stored at -80 °C for future use.
5. The phagemid lysate is then titered as follows:
 1 µl of the supernatant from step 4 and 200 µl of the XL1-Blue MRF' cells from step 3 in section 4.7.2 was combined and incubated at 37 °C for 15 minutes. In this step, the excised phagemids were mixed with a bacterial host strain (XL1-Blue cells) which is resistant to lambda phage but sensitive to the filamentous phage. This bacterial host strain will take up the filamentous phage and convert the ssDNA to dsDNA [10].
6. 100 µl of the cell mixture with several different dilutions was plated onto LB/*amp* agar plates and incubated overnight at 37 °C. Since pBluescript SK plasmid contains the *amp* resistance gene, colonies result from phagemid infection.
7. The agar plate that achieved single colony isolation was used to count the number of colonies. Equation 3 was used to determine the titer [in colony forming units per milliliter (cfu/ml)] of the excised phagemids.

$$\frac{[\text{number of colonies (cfu)} \times \text{dilution factor}] \times 1000 \text{ } \mu\text{l/ml}}{[\text{volume plated (}\mu\text{l)}]} \quad \text{Equation 3}$$

4.7.5 Library Amplification

After calculated the titer (cfu/ml) for the excised phagemids from section 4.7.4, a portion of the supernanant (step 7 in section 4.7.4) was combined with XL1-Blue MRF' cells (step 3 in section 4.7.2) at a 1:10 phagemid-to-cell ratio. The mixture was incubated at 37 °C for 15 minutes and then mixed with 500 ml of LB/amp broth. The mixture was then incubated in a shaker overnight at 30 °C. The cell culture was spun down and the phagemid DNA was purified with the Qiagen Plasmid Midi Kit (Qiagen, Cat# 12143). The DNA concentration was measured as described in section 4.6.2.

CHAPTER 5.0 DATA AND ERROR ANALYSIS

5.1 Analysis of the Size of DNA

To verify the size of the PCR products from library validation, probe synthesis, each enrichment step and positive colony screening, the size of purified PCR products was determined by running the DNA with loading buffer and a DNA ladder on a 1% agarose-TAE gel with EtBr. The DNA ladder served as a reference marker. By comparing to the DNA ladder, the actual PCR fragment size was estimated. Although the prediction of the size of the DNA fragment can not be precise (because predicted gene sequences are not always correct); the gel electrophoresis image's result allows for confirmation of the expected product sizes.

5.2 Validate the Capture Enrichment

An enrichment validation was performed after each enrichment step by using a semi-quantitative PCR. This was done by PCR amplification of different dilutions (0.5 and 100 ng) of the original cDNA library, along with the first and second capture with the screening primers. The level of enrichment was determined by comparing the amplification product signal for each sample to that generated from the starting library on an agarose gel.

5.3 Sequence Analysis of Cloned Gene

DNA plasmids that contained the largest insert fragments from the restriction digest were sequenced (Sequetech, Mountain View, CA). 100ng of DNA plasmid and 5 μ mole x volume of 5 μ M concentration of the primers are the minimal amount required by Sequetech for sequencing. Initially an internal primer (usually C) was used to confirm a match to predicted gene sequences. Following this, vector primers flanking the cloning site were used to confirm the full-length nature of the insert. The insert was then fully double-pass sequenced by primer walking.

CHAPTER 6.0 RESULTS AND DISCUSSION

6.1 Cloning Result for Trafficking Protein, ODR-4

6.1.1 Library Validation

Human brain, fetal brain, placenta and umbilical cDNA library were initially used as the DNA template in the PCR reaction to validate the presence of the target gene. The set of four designed primers (A, B, C, and D) were tested with each cDNA library in four different possible pair combinations (AC, AD, BC, and BD). Positive and negative controls were used to see if there is any contamination in the PCR mix and to ensure effective PCR. The PCR product size was compared to the predicted band size for the appropriate primer pair combination. 10 μ l of PCR product was mixed with 10 μ l loading buffer (2x) and run on a 1% agarose gel with EtBr. The expected PCR fragment sizes were observed for all the primer combinations from the human fetal brain cDNA library but not the other libraries tested. The band sizes for all four primer combinations are shown in Table 16 and the agarose gel picture is shown in Figure 6. By visualizing the bands on an agarose gel and comparing to the DNA ladder, the actual PCR fragment size was estimated.

Table 16. Library Validation Result for ODR-4.

Primers pair	Expected Band size (bp)	Actual Band size (bp)	Lane
AC	359	~370	2
AD	475	~490	3
BC	284	~300	4
BD	400	~400	5

Figure 6. Human Fetal Brain Library Validation Analysis for ODR-4. Lane 1: 1Kb plus DNA ladder, lane 2: AC primer set, lane 3: AD primer set, lane 4: BC primer set, lane 5: BD primer set.

6.1.2 Probe Synthesis

All four primer combinations showed the presence of the ODR-4 gene in the human fetal brain cDNA library, therefore, BC was picked to use as probe primers and AD for screening primers in order to eliminate the possibility where a small amount of probe would carry over and lead to a false positive in the later steps. The product resulting from the BC PCR was used as a template for the preparation of the biotinylated probe after extraction from the agarose gel as described in section 4.2.2, 4.2.3, and 4.2.4. The size of the DNA probe was confirmed by running the PCR probe on a 1% agarose gel. The agarose gel picture for the BC probe is shown in Figure 7. The actual probe's size matched the expected size (284bp) very closely.

Figure 7. Probe Analysis for ODR-4. Lane 1: 1 Kb PLUS DNA ladder. Lane 2 and 3: BC biotinylated probe.

6.1.3 Capture

After purifying and quantifying the DNA probe in section 6.1.2, the enrichment procedures were performed as described in section 4.3. However, problems were experienced in the first three attempts. Many factors could result in a lack of enrichment or complete loss the target gene.

The first step in enrichment is the denaturation and RecA coating of the biotinylated dsDNA probe. The amount of RecA and dsDNA bioprobe being used is critical because the ssDNA bioprobe is coated with RecA at a ratio of one RecA protein monomer per three DNA bases to form two complementary nucleoprotein helices. Excess RecA can bind other ssDNA molecules resulting in background hybrid formation. 5 μ g of human fetal brain ds-cDNA library was added in the first capture for ODR-4 and incubated at 37 °C. This step allowed the nucleoprotein filament to invade the dsDNA

library to form a probe-target hybrid in order to isolate the gene from the library. After mixing and incubation of the probe-target hybrids and magnetic beads, the beads were then washed four times in order to remove any non-hybridized DNA, followed by a wash with 0.1x TE to remove the salt. The last step of the enrichment was to elute the captured target DNA [28]. This cloning technology is utilizing the ability of RecA protein to form a nucleoprotein filament with ssDNA and allow the nucleoprotein complex to invade the dsDNA library to isolate the gene from the library. The DNA inside the nucleoprotein filament is completely coated with RecA protein and protected from digestion by phosphodiesterases and nucleases [29]; therefore, the amount of bioprobe, RecA, reaction buffer and nucleotide cofactor directly affects the ability of the nucleoprotein filament to efficiently mediate the search for homologous sequences and participate in DNA strand exchange to form a stable probe-target hybrid.

After three enrichments were attempted and unsuccessful, the bioprobe's mass was re-quantified. It was concluded that less than 50 ng of the bioprobe was used in the first three attempts. Therefore, a new bioprobe was made and diluted to 25 ng/ μ l. An enrichment validation was performed after the first enrichment step. This was done by PCR amplification of two different dilutions, 100 ng/ μ l and 0.5 ng/ μ l, of the original human fetal brain cDNA library and first capture DNA with the screening primers. The level of enrichment was determined by comparing the amplification signal from the original library. The agarose gel for the first enrichment is shown in Figure 8. Lanes 2 and 3 are the first capture and the cDNA library at 100 ng/ μ l, respectively. Lanes 4 and 5

are the first capture and the cDNA library at 0.5 ng/μl, respectively. Both concentrations of the captured DNA showed enrichment of the target gene at the correct predicted size.

Figure 8. 1st Capture Enrichment Validation for ODR-4. Lane 1: 1Kb Plus DNA ladder. Lane 2 and 3: 1st Capture DNA and human fetal brain cDNA library at 100 ng/μl. Lane 4 and 5: 1st Capture DNA and human fetal brain cDNA library at 0.5 ng/μl.

6.1.4 Target Validation

In most cases, the enrichment obtained by the first capture is not sufficient for an efficient screening. After the second enrichment, single colonies were picked and screened for the presence of the target gene, however, no positive clones were found. Therefore, a third capture was pursued. Ninety one colonies were picked into a 96 well plate; two wells contained a positive and a negative control. These were used as DNA templates for PCR reactions using the screening primers. A subset of these samples is shown in the gel image in Figure 9. Eight positive PCR products were obtained.

Figure 9. Validation of Colony Screening. Lane 1 and 12: 1Kb Plus DNA ladder. Lane 2: negative control. Lane 3: Positive control. Lane 4-11: PCR positive colonies A-H.

6.1.5 Plasmid DNA Analysis

The insert size of the gene of interest was determined by using restriction enzymes. A single restriction digest with *EcoRI* and a double restriction digest with *EcoRI* and *NotI* for each purified DNA was performed and analyzed in order to determine which contain the largest insert fragments. An agarose gel image for the restriction analysis is shown in Figure 10. Lane 1, 10 and 19 is the 1 Kb plus ladder. Lane 2-9 is the single digest for clone A-H, the letter S in front of each clone's letter represent single digest. Lane 11-18 is the double digest for clone A-H, the letter D in front of each clone's letter represent double digest.

Figure 10. Agarose Gel Image for Restriction Analysis. Lane 1, 10 and 19 is the 1 Kb plus ladder. Lanes 2-9: Single digest for clone A-H. Lanes 11-18: Double digest for clone A-H. S represents single digest. D represents double digest.

6.1.6 Full Length Confirmation

From the restriction digest analysis, Clone C had the largest insert size, 4 Kb. Therefore, the purified plasmid DNA for Clone C was sequenced with an internal primer (C), along with the forward (pEAK SF-1) and reverse (pEAK SR-1) vector primers. After analysis, it was concluded that a human full-length gene sequence was cloned, which has homology to the previously characterized *C.elegans* ODR-4 gene. By primer walking, both strands of the full insert were sequenced and bioinformatics analysis was performed. It was concluded that the open reading frame (ORF) for the human ODR-4 homolog (hODR-4) is longer than a purported full-length gene of unknown function reported in Genbank (1362 bp vs 1005 bp). This was because the Genbank entry has a stretch of 9 A's just before its stop codon, and the clone C sequence has only 8 A's, this shifted the reading frame on the Genbank entry, resulting in a premature termination

codon. We are confident in our sequence read of 8 A's because this region was sequenced with 4 different primers and each produced high quality sequence traces. Other than that there is a perfect match between the two sequences within the coding sequence. The hODR4 also has an extended 3' untranslated region when compared to the GenBank entry. Figure 11 shown the double strand primer walks in the 8 A's region for hODR4 sequence and the contig map of all sequencing for hODR4 is shown in Figure 12. Figure 13 shown gene sequence alignment for ODR4.

	1101	1150
» ODR-2631	(1)	
» ODR4_2227	(1)	
» ODR4_1591	(1)	
« ODR4_2081R	(1)	
» ODR4_1191	(1)	
« ODR4_1654R	(87)	TGCTTTTGAATGAAATTCAG <u>AAAAAAAA</u> GATTCTGAAAAAGAGTTCCAC
» ODR4_821	(259)	TGCTTTTGAATGAAATTCAG <u>AAAAAAAA</u> GATTCTGAAAAAGAGTTCCAC
« ODR4_1219R	(539)	TGCTTTTGAATGAAATTCAG <u>AAAAAAAA</u> GATTCTGAAAAAGAGTTCCAC
» ODR4_401	(703)	TGCTTTTGAATGAAATTCAG <u>AAAAAA</u>
« ODR4_901R	(•706)	
» ODR4_87	(•647)	
» PEAK SF-1	(•604)	
« ODR4_487R	(•422)	
Contig 1	(1101)	TGCTTTTGAATGAAATTCAG <u>AAAAAAAA</u> GATTCTGAAAAAGAGTTCCAC

Figure 11. Double Strand Primer Walks for hODR4 Sequence (8 A's region).

“»” and “«” represent forward and reverse primers introduced from the 5' and 3' terminal end of the target DNA strand, respectively. Contig is the contiguous sequence information for both strand of the region of interest. Bold and underlined letters represent the 8 A's region.

Figure 12. Contig Map of All Sequencing for hODR4. Double stand primer walks provided contiguous sequence information for both strands of the region of interest.

ODR-4 Genbank	(1)	GATGAATTCAGTGGCAGTGAATTGAGA	CGG	
ODR4 contig	(1)	-----	TCA	
Consensus	(1)		C	AGGGAATCTGGCCCCTAGA
		51		100
ODR-4 Genbank	(51)	GGCTGGTACTTGGGCCCCGAAACCCCATCTCCGGCGGAGAGACCGTCCGA		
ODR4 contig	(24)			
Consensus	(51)			
		101		150
ODR-4 Genbank	(101)	GGTAATTGTCTGCCACGAGTGCACATTCTGAAAACAGGAGATTTAGTTC		
ODR4 contig	(74)			
Consensus	(101)			
		151		200
ODR-4 Genbank	(151)	CTAAAATG		
ODR4 contig	(124)			
Consensus	(151)	CTAAAATGGGAAGAACCTACATTGTAGAAGAGACTGTTGGCCAGTATCT		
		201		250
ODR-4 Genbank	(201)	TTCAAACATAAAATCTCCAAGGAAAGGCTTTTGTCTCTGGCCTTTTAATAG		
ODR4 contig	(174)			
Consensus	(201)			
		251		300
ODR-4 Genbank	(251)	GACAGTGTTCGTCACAAAAGGATTATGTGATTCTTGCCACTAGAACGCCA		
ODR4 contig	(224)			
Consensus	(251)			
		301		350
ODR-4 Genbank	(301)	CCCAAAGAGGAGCAAAGTGAGAACCTCAAACATCCCAAAGCTAAGTTGGA		
ODR4 contig	(274)			
Consensus	(301)			
		351		400
ODR-4 Genbank	(351)	TAACTTGGATGAAGAATGGGCCACAGAACATGCCTGCCAGGTATCCAGAA		
ODR4 contig	(324)			
Consensus	(351)			
		401		450
ODR-4 Genbank	(401)	TGCTACCGGGGGACTTTTAGTTCTTGGAGTATTTATTATTACTACTTTA		
ODR4 contig	(374)			
Consensus	(401)			
		451		500
ODR-4 Genbank	(451)	GAACTGGCAAATGATTTTCAAATGCCCTGCGTAGACTAATGTTTGCTGT		
ODR4 contig	(424)			
Consensus	(451)			
		501		550
ODR-4 Genbank	(501)	GGAAAAGTCTATAAATAGAAAGAGATTGTGGAATTTACAGAGGAGGAAG		
ODR4 contig	(474)			
Consensus	(501)			
		551		600
ODR-4 Genbank	(551)	TCTCAGAACGAGTGACACTTCACATTTGTGCTTCTACAAAAAATATTT		
ODR4 contig	(524)			
Consensus	(551)			
		601		650
ODR-4 Genbank	(601)	TGTCGAACTTATGATATCCATGATCCAAAGAGTTCAGCAAGACCAGCAGA		
ODR4 contig	(574)			
Consensus	(601)			
		651		700
ODR-4 Genbank	(651)			
ODR4 contig	(624)			

Consensus	(651)	TTGGAAGTATCAAAGTGGATTATCATCCTCATGGCTTTCTTTAGAGTGTA	
		701	750
ODR-4 Genbank	(701)	CAGTTCACATTAAATATTCACATCCCACCTTTCTGCTACTTCTGTCAGCTAT	
ODR4 contig	(674)	CAGTTCACATTAAATATTCACATCCCACCTTTCTGCTACTTCTGTCAGCTAT	
Consensus	(701)	CAGTTCACATTAAATATTCACATCCCACCTTTCTGCTACTTCTGTCAGCTAT	800
		751	
ODR-4 Genbank	(751)	ACTCTGGAGAAAAATACAAAGAATGGACTTACACGCTGGGCCAAGGAAAT	
ODR4 contig	(724)	ACTCTGGAGAAAAATACAAAGAATGGACTTACACGCTGGGCCAAGGAAAT	
Consensus	(751)	ACTCTGGAGAAAAATACAAAGAATGGACTTACACGCTGGGCCAAGGAAAT	850
		801	
ODR-4 Genbank	(801)	AGAAAATGGTGTATTATTGATTAATGGACAAGTTAAAGATGAAGATTGTG	
ODR4 contig	(774)	AGAAAATGGTGTATTATTGATTAATGGACAAGTTAAAGATGAAGATTGTG	
Consensus	(801)	AGAAAATGGTGTATTATTGATTAATGGACAAGTTAAAGATGAAGATTGTG	900
		851	
ODR-4 Genbank	(851)	ACCTATTAGAAGGACAGAAAAAATCTTCTAGAGGAAATACTCAAGCAACT	
ODR4 contig	(824)	ACCTATTAGAAGGACAGAAAAAATCTTCTAGAGGAAATACTCAAGCAACT	
Consensus	(851)	ACCTATTAGAAGGACAGAAAAAATCTTCTAGAGGAAATACTCAAGCAACT	950
		901	
ODR-4 Genbank	(901)	AGTCATTCTTTTGTATGTCAGAGTGCTAACGCAGTTGCTCCTGAATTCAGA	
ODR4 contig	(874)	AGTCATTCTTTTGTATGTCAGAGTGCTAACGCAGTTGCTCCTGAATTCAGA	
Consensus	(901)	AGTCATTCTTTTGTATGTCAGAGTGCTAACGCAGTTGCTCCTGAATTCAGA	1000
		951	
ODR-4 Genbank	(951)	CCACAGATCCACAGCCACAGTCCAGATATGTAGCGGTTCTGTAAACCTTA	
ODR4 contig	(924)	CCACAGATCCACAGCCACAGTCCAGATATGTAGCGGTTCTGTAAACCTTA	
Consensus	(951)	CCACAGATCCACAGCCACAGTCCAGATATGTAGCGGTTCTGTAAACCTTA	1050
		1001	
ODR-4 Genbank	(1001)	AGGGTGCTGTGAAATGCAGAGCTTATATCCACAGCAGTAAACCCAAAGTT	
ODR4 contig	(974)	AGGGTGCTGTGAAATGCAGAGCTTATATCCACAGCAGTAAACCCAAAGTT	
Consensus	(1001)	AGGGTGCTGTGAAATGCAGAGCTTATATCCACAGCAGTAAACCCAAAGTT	1100
		1051	
ODR-4 Genbank	(1051)	AAAGATGCTGTGCAGGCAGTAAAGAGGGATATATTGAACACAGTTGCTGA	
ODR4 contig	(1024)	AAAGATGCTGTGCAGGCAGTAAAGAGGGATATATTGAACACAGTTGCTGA	
Consensus	(1051)	AAAGATGCTGTGCAGGCAGTAAAGAGGGATATATTGAACACAGTTGCTGA	1150
		1101	
ODR-4 Genbank	(1101)	TCGTTGTGAAATGCTATTTGAGGATCTGCTTTTGAATGAAATTCCAGAAA	
ODR4 contig	(1074)	TCGTTGTGAAATGCTATTTGAGGATCTGCTTTTGAATGAAATTCCAGAAA	
Consensus	(1101)	TCGTTGTGAAATGCTATTTGAGGATCTGCTTTTGAATGAAATTCCAGAAA	1200
		1151	
ODR-4 Genbank	(1151)	AAAAAGATTCTGAAGAGCTTCCACGTCCTCCCTTATCGAGTCTTGT	
ODR4 contig	(1124)	AAAAAGATTCTGAAGAGCTTCCACGTCCTCCCTTATCGAGTCTTGT	
Consensus	(1151)	AAAAAGATTCTGAAGAGCTTCCACGTCCTCCCTTATCGAGTCTTGT	1250
		1201	
ODR-4 Genbank	(1201)	TCCCCTTCCTGGATCCACTGTAATGTTGTGTGATTATAAATTTGACGATG	
ODR4 contig	(1173)	TCCCCTTCCTGGATCCACTGTAATGTTGTGTGATTATAAATTTGACGATG	
Consensus	(1201)	TCCCCTTCCTGGATCCACTGTAATGTTGTGTGATTATAAATTTGACGATG	1300
		1251	
ODR-4 Genbank	(1251)	AGTCAGCTGAAGAAATCAGGGACCATTTTATGGAGATGTTGGATCACACA	
ODR4 contig	(1223)	AGTCAGCTGAAGAAATCAGGGACCATTTTATGGAGATGTTGGATCACACA	
Consensus	(1251)	AGTCAGCTGAAGAAATCAGGGACCATTTTATGGAGATGTTGGATCACACA	1350
		1301	
ODR-4 Genbank	(1301)	ATTCAAATAGAAGATTTGGAAATTGCAGAGGAAACAAACACAGCTTGTAT	
ODR4 contig	(1273)	ATTCAAATAGAAGATTTGGAAATTGCAGAGGAAACAAACACAGCTTGTAT	
Consensus	(1301)	ATTCAAATAGAAGATTTGGAAATTGCAGAGGAAACAAACACAGCTTGTAT	1400
		1351	
ODR-4 Genbank	(1351)	AGTTCAGCTGAAGAAATCAGGGACCATTTTATGGAGATGTTGGATCACACA	
ODR4 contig	(1323)	AGTTCAGCTGAAGAAATCAGGGACCATTTTATGGAGATGTTGGATCACACA	

Consensus	(1351)	GAGTTCCTTCTATGAATAGTCAAGCTTCATTGGACAACACAGATGATGAAC	1401	1450
ODR-4 Genbank	(1401)	AACCAAAACAACCAATTA		
ODR4 contig	(1373)	AACCAAAACAACCAATTA		
Consensus	(1401)	AACCAAAACAACCAATTA	1451	1500
ODR-4 Genbank	(1451)	ATAGGTGTGATTGCAGCATT		
ODR4 contig	(1423)	ATAGGTGTGATTGCAGCATT		
Consensus	(1451)	ATAGGTGTGATTGCAGCATT	1501	1550
ODR-4 Genbank	(1501)	CTTTCATTACTTCAGTGATT		
ODR4 contig	(1473)	CTTTCATTACTTCAGTGATT		
Consensus	(1501)	CTTTCATTACTTCAGTGATT	1551	1600
ODR-4 Genbank	(1551)	CCAGAGAACATTGACAGACA		
ODR4 contig	(1523)	CCAGAGAACATTGACAGACA		
Consensus	(1551)	CCAGAGAACATTGACAGACA	1601	1650
ODR-4 Genbank	(1601)	TCTGTATTAAAACTAGCAG		
ODR4 contig	(1573)	TCTGTATTAAAACTAGCAG		
Consensus	(1601)	TCTGTATTAAAACTAGCAG	1651	1700
ODR-4 Genbank	(1651)	GTGTGTTTCTGATTAAAAT		
ODR4 contig	(1623)	GTGTGTTTCTGATTAAAAT		
Consensus	(1651)	GTGTGTTTCTGATTAAAAT	1701	1750
ODR-4 Genbank	(1701)	GAAGCTGCATGTAACAAAT		
ODR4 contig	(1673)	GAAGCTGCATGTAACAAAT		
Consensus	(1701)	GAAGCTGCATGTAACAAAT	1751	1800
ODR-4 Genbank	(1720)	GAAGCTGCATGTAACAAAT		
ODR4 contig	(1723)	GAAGCTGCATGTAACAAAT		
Consensus	(1751)	GAAGCTGCATGTAACAAAT	1801	1850

Figure 13. hODR4 and GenBank Gene Alignment. Sequences for hODR4 and a GenBank entry with unknown function were aligned using the NCBI BLAST2 sequence alignment program. The GenBank sequence (ODR-4 GenBank) is shown on the top line and the human homolog ODR4 sequence (ODR4 contig) is shown in the middle line. The bold letters indicate start and stop codons for GenBank sequence. The bold and underlined letters indicate stop codons for hODR4 sequence.

6.2 Cloning Olfactory Receptors using Non-Chemosensory cDNA Libraries

In the early stage of this work, ten olfactory receptors were picked from the Olfactory Database and four sets of primers were designed for each receptor. Previous studies have shown that OR genes are expressed not only in chemosensory neurons, but

also in other tissues [30]. Several cDNA libraries which were available from Pangene Corporation were initially used as DNA templates in PCR reactions to validate the presence of the target genes. The organism for the olfactory receptors and the cDNA libraries that were used in the library validation step is shown in Table 17 on the next page. The set of four designed primers (A, B, C, and D) were tested with each cDNA library in four different possible pair combinations (AC, AD, BC, and BD). Positive and negative controls were used to see if there is any contamination in the PCR mix and that PCR was effective. The PCR products were checked for band sizes corresponding to that predicted for the appropriate primer pair combination. Unfortunately, only two of these ten receptors showed the presence of the target gene in the libraries that were tested, ORL280 and 1008. Only the screening primers (AD) for these two receptors showed a positive result in the library screening and there were multiple bands on the agarose gel. The bands at the correct size were cut out and purified to make a bioprobe. The purified DNA probes were then sent to sequence to validate the presence of the target gene. BLAST searches were carried out verifying that both of the DNA probes were not olfactory receptors. Therefore, cloning of OR's from non olfactory libraries was not continued.

Table 17. Library Validation for Olfactory Receptors with Non-Chemosensory cDNA Libraries.

	Gene name	Organism	cDNA Library
1	671	Human brain	Human brain & fetal brain, umbilical & placenta
2	mOR37	Mouse	Mouse brain, mouse fetal brain
3	ORL146	Mouse	Mouse brain, mouse fetal brain
4	ORL451	Mouse Olfactory Epithelium	Mouse brain, mouse fetal brain
5	ORL849	Mouse olfactory bulb	Mouse brain, mouse fetal brain
6	ORL452	Mouse olfactory epithelium	Mouse brain, mouse fetal brain
7	ORL464	Mouse olfactory neuron	Mouse brain, mouse fetal brain
8	ORL828	Mouse olfactory neuron	Mouse brain, mouse fetal brain
9	ORL280	Rat brain	Rat brain & rat fetal brain
10	ORL1008	Rat brain	Rat brain & rat fetal brain

6.3 Cloning Olfactory Receptors using Rat Olfactory Bulb cDNA Library

Since the first ten set of the olfactory receptors were unsuccessful using non-chemosensory cDNA libraries, a rat olfactory bulb Lambda cDNA library was purchased from Stratagene (Cat# 936520). The rat olfactory bulb Lambda cDNA library was cloned into the *EcoR* I site of the Lambda ZAP II vector, therefore, an *in vivo* mass excision was done to obtain inserts in the pBluescript SK(-) phagemid from the Lambda ZAP II vector. Fourteen rat olfactory receptors were picked from the olfactory receptor database and four sets of primers were designed for each receptor. The mass excised rat olfactory bulb cDNA library was initially used as the DNA template in the PCR reaction to validate the presence of the target gene. The fourteen new sets of the olfactory receptors are shown in Table 18. The set of four designed primers (A, B, C, and D) were tested in four different possible pair combinations (AC, AD, BC, and BD).

Table 18. Library Validation for Olfactory Receptors with Rat Olfactory Bulb cDNA Library.

	Gene name		Gene name
1	ORL338	8	ORL288
2	ORL284	9	ORL289
3	ORL300	10	ORL290
4	ORL297	11	ORL291
5	ORL285	12	ORL292
6	ORL286	13	ORL293
7	ORL287	14	ORL11

As determined from the library validation, only 7 genes, ORL 284, 297, 338, 285, 288, 289, and 11, were present in the library. However, all of these genes produced multiple bands on the agarose gel. Because one of the bands was at the expected size, the band was cut out and purified to make a bioprobe. The purified bioprobes were sequenced and a BLAST search was performed to verify the gene sequence. Out of these 7 rat olfactory receptors, only 2 receptors, ORL 285 and 289, matched their predicted sequence. Probe synthesis and enrichment steps were pursued, but the enrichment processes for these two rat olfactory receptors were very problematic and unsuccessful. Olfactory receptors are expressed at a much lower level in the olfactory bulb, which might be the reason why cloning olfactory receptors from an olfactory bulb cDNA library was unsuccessful. In the olfactory receptor database, more olfactory receptors were isolated from olfactory epithelium compared to olfactory bulb. Olfactory epithelial cDNA libraries are not commercially available, but, a mouse olfactory epithelium cDNA library was kindly provided by Dr. Richael Axel (Columbia University).

6.4 Cloning Results for Mouse Olfactory Receptor

6.4.1 Library Validation

The mouse olfactory epithelium cDNA library was originally cloned into the *Xho*I and *Eco*RI sites of Lambda ZAP II vector. The Lambda ZAP II cDNA cloning vector was converted *in vivo* to a pBluescript SK (-) phagemid vector by mass excision. The excision process is summarized in section 4.7. The set of primers for a rat olfactory receptor (hoping for homology) and seven mouse receptors that were tested with different cDNA library in the earlier stage of the experiment were used to do a library validation with this new cDNA library. The mass excised mouse olfactory epithelium cDNA library was initially used as the DNA template in the PCR reactions to validate the presence of target genes. The eight olfactory receptors and the tissue where these gene sequences were initially isolated are shown in Table 19. The set of four designed primers (A, B, C, and D) were tested in four different possible pair combinations (AC, AD, BC, and BD). A negative control was used to see if there is any contamination in the PCR mix.

Table 19. Library Validation for Olfactory Receptors with Mouse Olfactory Epithelium cDNA Library.

	Gene name	Original tissue
1	mOR37	Mouse
2	ORL146	Mouse
3	ORL451	Mouse Olfactory Epithelium
4	ORL849	Mouse olfactory bulb
5	ORL452	Mouse olfactory epithelium
6	ORL464	Mouse olfactory neuron
7	ORL828	Mouse olfactory neuron
8	ORL11	Rat olfactory epithelium

The expected and actual band sizes for all four primer combinations are shown in Table 20 and the agarose gel picture is shown in Figures 14 and 15. Five out of the 8

olfactory receptors were present in the mouse olfactory epithelium cDNA library. All four primer combinations for these five olfactory receptors matched their expected PCR fragment sizes very closely.

Table 20. Gel Electrophoresis Analysis Data for Olfactory Receptor.

Gene Name	Primer Pair	Expected size (bp)	Figure/gel lane	Actual size (bp)
mOR 37	AC	359	Fig.14A / 1	~350
	AD	421	Fig.14A / 2	~400
	BC	302	Fig.14A / 3	~300
	BD	364	Fig.14A / 4	~350
ORL146	AC	390	Fig.14A / 7	no band
	AD	472	Fig.14A / 8	no band
	BC	314	Fig.14A / 9	no band
	BD	396	Fig.14A / 10	~100
ORL451	AC	404	Fig.14B / 1	~400
	AD	456	Fig.14B / 2	~450 + ~800
	BC	224	Fig.14B / 3	~230
	BD	276	Fig.14B / 4	~280
ORL849	AC	245	Fig.14B / 7	~250
	AD	471	Fig.14B / 8	~500
	BC	211	Fig.14B / 9	~200
	BD	437	Fig.14B / 10	~450
ORL452	AC	309	Fig.15A / 1	~300
	AD	414	Fig.15A / 2	~430
	BC	285	Fig.15A / 3	~300
	BD	390	Fig.15A / 4	~400
ORL464	AC	269	Fig.15A / 7	no band
	AD	538	Fig.15A / 8	no band
	BC	202	Fig.15A / 9	no band
	BD	471	Fig.15A / 10	no band
ORL828	AC	377	Fig.15B / 1	~400
	AD	430	Fig.15B / 2	~450
	BC	230	Fig.15B / 3	~250
	BD	283	Fig.15B / 4	~300
ORL11	AC	523	Fig.15B / 7	no band
	AD	575	Fig.15B / 8	no band
	BC	258	Fig.15B / 9	~1000 + multiple
	BD	310	Fig.15B / 10	~200

Figure 14. Gel Electrophoresis Image for Mouse Library Validation. Lanes A6, A12, B6 and B12 are the 5Kb ladder. Lanes A5, A11, B5 and B11 are the negative controls for mOR37, ORL 146, 451 and 849, respectively. Lanes A1-A4 are AC, AD, BC and BD primers for mOR37. Lanes A7-A10 are AC, AD, BC and BD primers for ORL 146. Lanes B1-B4 are AC, AD, BC and BD primer for ORL451. Lanes B7-B10 are AC, AD, BC and BD primers for ORL849.

Figure 15. Gel Electrophoresis Image for Mouse Library Validation (Picture 2). Lanes A6, A12, B6 and B12 are 5Kb ladder. Lanes A5, A11, B5 and B11 are the negative control for ORL 452, 464, 828 and 11, respectively. Lanes A1-A4 are AC, AD, BC and BD primer for ORL 452. Lanes A7-A10 are AC, AD, BC and BD primers for ORL 464. Lanes B1-B4 are AC, AD, BC and BD primer for ORL828. Lanes B7-B10 are AC, AD, BC and BD primers for ORL11.

6.4.2 Probe Synthesis

All four primer combinations showed the presence of the 5 genes in the mouse olfactory epithelium cDNA library; therefore, BC was picked for probe primers and AD for screening primers for all five genes. The BC PCR product was used as a template for the preparation of the biotinylated probe after extraction from an agarose gel as described in section 4.2.2, 4.2.3, and 4.2.4. The agarose gel picture for all five BC probes is shown in Figure 16. The actual probe sizes matched the expected sizes very closely.

Figure 16. Gel Image of Olfactory Receptor Probes. Lanes 1 and 7 are 5Kb ladder. Lane 2: mOR37 BC bioprobe (302bp). Lane 3: ORL451 BC bioprobe (224bp). Lane 4: ORL849 BC bioprobe (211bp). Lane 5: ORL452 BC bioprobe (285bp). Lane 6: ORL828 BC bioprobe (230bp). Values shown inside the parentheses are the expected size.

6.4.3 Capture

The captured DNA was checked for enrichment by PCR amplification of two different dilutions (100ng/ μ l and 0.5ng/ μ l) of the original mouse olfactory epithelium cDNA library and the first captured DNA with the screening primers (AD). One out of five olfactory receptors, mOR37, showed enrichment as shown in Figure 17.

Figure 17. Gel Image for mOR37 1st Capture Enrichment. Lane 1 and 7 is 5Kb ladder. Lane 2 and 3: 1st Capture DNA and mouse olfactory epithelium cDNA library at 100 ng/μl. Lane 4 and 5: 1st Capture DNA and mouse olfactory epithelium cDNA library at 0.5 ng/μl. Lane 6: negative control.

Because the Seradyn magnetic beads were running low, streptavidin coated MagneSphere particles were ordered from Promega (cat# Z5481), a lower cost supplier. The concentration of the Promega magnetic beads was more dilute (1 mg/ml) compared to the Seradyn beads (10 mg/ml). The enrichment processes were continued for mOR37, with 100 μl beads used. Enrichment of the target gene was not observed in repeated attempts using the Promega beads, because of this, samples enriched with each bead type was tested for the abundance of mOR37 in a PCR reaction using the screening primers. The gel image is shown in Figure 18 and it is apparent that only the Seradyn beads produced enriched samples.

Figure 18. Gel Image for mOR37 2nd Capture Enrichment. Lanes 1 and 7: 5KB ladder. Lane 2: 2nd Capture DNA (Promega). Lane 3: 2nd Capture DNA (Seradyn). Lane 4: 1st Capture DNA. Lane 5: mouse olfactory epithelium library. Lane 6: negative control. All DNA is at 100ng/ μ l.

6.4.4 Blue/White Control Enrichment

Due to several enrichment failures using Promega's magnetic beads, a blue/ white control experiment was performed using the Promega and Seradyn beads in order to test for efficiency. The blue/white control enrichment followed section 4.3. 50 ng/ μ l blue/white control biotinylated probe (Bio-HPRT) was used. Three different volumes, 100 μ l, 200 μ l and 300 μ l, of the Promega magnetic beads were tested, and the beads were resuspended in 30 μ l final binding buffer. 1 μ g of the blue/white control library was used for the enrichment. The captured DNA was eluted and electroporated. The electroporated cells were plated on a LB/amp/X-Gal/IPTG agar plates and incubated overnight. Ideally, more than 60% of white colonies should be obtained from enrichment. The Promega beads, regardless of the volume used, resulted in less the 5% white colonies. The Seradyn beads yielded about 66% white colonies. This demonstrated that for this procedure Seradyn beads were superior to Promega beads. Therefore, Sera-Mag

Magnetic Streptavidin Microparticles were used for all further studies (Cat# 30152104011150).

6.4.5 Target Validation and Positive Clone Amplification

Third capture enrichment was continued using Seradyn magnetic beads, single colonies were picked and screened for the presence of the target gene. Forty-eight colonies were picked and used as DNA template for PCR reactions using the screening primers. Seven positive PCR products were obtained. Positive colony inoculums were amplified in DNA minipreps. The presence of mOR37 sequences was confirmed using the DNA as templates for PCR reactions with screening primers (Figure 19).

Figure 19. Gel image for mOR37 Miniprep PCR Validation. Lanes 1 and 12: 0.5 Kb ladder. Lanes 2-8: Clone A-G purified positive colony DNA (100ng/μl). Lane 9: mouse epithelium cDNA library (100ng/μl). Lane 10: 1st captured DNA (100ng/μl). Lane 11: Negative control.

6.4.6 Plasmid DNA Analysis

The insert size of the gene of interest was determined by using restriction enzymes (Figure 20). All positive plasmids yielded the same 2.8 kb insert size.

Figure 20. Restriction Analysis for mOR37. Capital Letters indicate clones; “1” represent single digest; “2” represents double digest; “L” represents 1Kb PLUS ladder.

6.4.7 Full length Confirmation

The purified plasmid DNA was combined and sequenced with two internal primers, C and D, along with the forward (T3) and reverse (T7) vector primers. A BLAST search showed that the clone is a mouse olfactory receptor similar to the published mOR37 sequence. A bioinformatics analysis shows that there are in-frame stop codons upstream from the start codon demonstrating that the cloned mOR37 is a full-length gene. In addition, the cloned mOR37 is a perfect match to a mouse olfactory receptor published in GenBank (AY317806), except that the 5' end of the cloned mOR37 gene is longer. This

result in an ORF that is 996 bp long, which is 45 nucleotide longer than the mouse olfactory receptor gene published in GenBank. The BLAST search result and the assembled full sequence translation for mOR37 are shown in Figure 21 and 22, respectively. Table 21 shows a summary of the hODR-4 and mOR37 clones compared to the genes published in GenBank.

QUERY: 46 ATGGCTCTTAGCAATTCTAGCTGGAGGCAGCCCCAGCCCCCTTTCTTCTAGTAGGTGTT 105
 ||
 SBJCT: 1 ATGGCTCTTAGCAATTCTAGCTGGAGGCAGCCCCAGCCCCCTTTCTTCTAGTAGGTGTT 60

QUERY: 106 CCAGGCTTGGAGGAAAGTCAGCACTGGATAGCATTGCCCCCTGGGTATCCTTTACCTCTTT 165
 ||
 SBJCT: 61 CCAGGCTTGGAGGAAAGTCAGCACTGGATAGCATTGCCCCCTGGGTATCCTTTACCTCTTT 120

QUERY: 166 GCTCTAGTGGGCAATGTGACTATCATCTTCATTATCTGGACTGATTCATCCTTACACCAA 225
 ||
 SBJCT: 121 GCTCTAGTGGGCAATGTGACTATCATCTTCATTATCTGGACTGATTCATCCTTACACCAA 180

QUERY: 226 CCTATGTACCTCTTCCTGGCCATGCTTGCTGCTATTGACCTGGTCTGGCCTCTTCCACT 285
 ||
 SBJCT: 181 CCTATGTACCTCTTCCTGGCCATGCTTGCTGCTATTGACCTGGTCTGGCCTCTTCCACT 240

QUERY: 286 GCACCCAAAGCCCTCACTGTGCTCCTGGCTCATGCCCATGAGATTGGGTACATTGTATGC 345
 ||
 SBJCT: 241 GCACCCAAAGCCCTCACTGTGCTCCTGGCTCATGCCCATGAGATTGGGTACATTGTATGC 300

QUERY: 346 CTGACTCAGATGTTCTTTATTTCATGCCTTCTCCTCTATGGAGTCAGGCATACTTGTAGCC 405
 ||
 SBJCT: 301 CTGACTCAGATGTTCTTTATTTCATGCCTTCTCCTCTATGGAGTCAGGCATACTTGTAGCC 360

QUERY: 406 ATGGCTCTGGATCGCTATGTTGCCATCTGCCACCCTCTGCGTCATTCCACCATCCTGCAT 465
 ||
 SBJCT: 361 ATGGCTCTGGATCGCTATGTTGCCATCTGCCACCCTCTGCGTCATTCCACCATCCTGCAT 420

QUERY: 466 CCAGGGATTATAGGGCGCATTGGGCTGGTAGTACTGGTGCGGGGATTGGTCCTTCTTTTC 525
 ||
 SBJCT: 421 CCAGGGATTATAGGGCGCATTGGGCTGGTAGTACTGGTGCGGGGATTGGTCCTTCTTTTC 480

QUERY: 526 CCATTCCCCATTCTGTTGCAGAATGTTGTCTTCTGCAGAGCAACTGTCATAAGCCATGCC 585
 ||
 SBJCT: 481 CCATTCCCCATTCTGTTGCAGAATGTTGTCTTCTGCAGAGCAACTGTCATAAGCCATGCC 540

QUERY: 586 TATTGTGAGCATATGGCTGTGGTAAACTTGCCTGTTCTGAAACCACAGTGAATCGAGCT 645
 ||
 SBJCT: 541 TATTGTGAGCATATGGCTGTGGTAAACTTGCCTGTTCTGAAACCACAGTGAATCGAGCT 600

QUERY: 646 TATGGTCTGTGAGTGGCACTGCTGGTGGTAGGACTAGATGTCCTGGCCATTGGCATTTC 705
 ||
 SBJCT: 601 TATGGTCTGTGAGTGGCACTGCTGGTGGTAGGACTAGATGTCCTGGCCATTGGCATTTC 660

QUERY: 706 TATGCCCTCATCCTCCAGGCAGTGCTGAAGGTGCCAGGGGGTGAAGCCAGACTTAAAGCC 765
 ||
 SBJCT: 661 TATGCCCTCATCCTCCAGGCAGTGCTGAAGGTGCCAGGGGGTGAAGCCAGACTTAAAGCC 720

QUERY: 766 TTCAGTACATGTGGGTCTCATGTTTGTGTTATTCTCATCTTCTATGTCCCTGGAATGTTT 825
 ||
 SBJCT: 721 TTCAGTACATGTGGGTCTCATGTTTGTGTTATTCTCATCTTCTATGTCCCTGGAATGTTT 780

QUERY: 826 TCCTTCCTCACTCACCGCTTTGGCCACCATGTTCCCCATCATGTCCATGTTCTGCTGGCC 885
 ||
 SBJCT: 781 TCCTTCCTCACTCACCGCTTTGGCCACCATGTTCCCCATCATGTCCATGTTCTGCTGGCC 840


```

QUERY: 886 ACTCTCTATCTCCTTGTGCCACCTGCCCTGAATCCTCTTGTTTATGGGGTGAAGACTCGA 945
      |||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
SBJCT: 841 ACTCTCTATCTCCTTGTGCCACCTGCCCTGAATCCTCTTGTTTATGGGGTGAAGACTCGA 900

QUERY: 946 CAGATTCGCCAGCGAGTGCTCAGGGTGTTCACACAAAAGCATCGATTGA 996
      |||||||||||||||||||||||||||||||||||||||||||||||||||||||
SBJCT: 901 CAGATTCGCCAGCGAGTGCTCAGGGTGTTCACACAAAAGCATCGATTGA 951

```

Figure 21. mOR37 and GenBank Gene Alignment. mOR37 and GenBank (AY317806) sequences were aligned using the NCBI BLAST2 sequence alignment program. The mOR37 sequence (QUERY) is on top and GenBank sequence (SBJCT) is at the bottom.

Table 21. Summary of Cloned and GenBank Sequence ORFs for mOR37 and hODR4.

Gene	Cloned Gene Sequence (bp)	Published Sequence (bp)
hODR-4	1362	1005
mOR37	996	951

CHAPTER 7.0 CONCLUSIONS AND FUTURE STUDY

7.1 Conclusions

RecA protein catalyzes homologous pairing of ssDNA with dsDNA facilitating the RecA-mediated cloning method used in this work. Twenty-five sets of olfactory receptor genes were probed, due to rarity and wrong tissues of cDNA libraries were used; only one was successfully cloned and found to be a mouse olfactory receptor gene, mOR37, with unknown specificity from the mouse epithelium cDNA library. It was confirmed that the cloned olfactory receptor, mOR37, is a full-length gene and is similar to an olfactory receptor published in GenBank (AY317806), except that the 5' end of the cloned mOR37 gene product is 15 amino acids longer than that published in GenBank. Because the amino-terminus of these GPCRs are found extracellularly, these added 15 amino acids will be located here. This full-length gene will more authentically represent function compared to the truncated published form. In addition, a human gene with homology to the previously characterized *C.elegans* ODR-4 gene was successfully cloned. This sequence differs from a GenBank submission by one nucleotide shifting the reading frame of the GenBank entry, resulting in a premature termination codon. Therefore, the open reading frame of the human form of ODR-4 (hODR-4) is longer than the one reported in GenBank (1362bp vs. 1005bp).

7.2 Future Study

The cloning process for the mouse olfactory receptor genes with known specificity should continue in order to obtain more olfactory receptors. In addition, functionally expressing the mOR37 in mammalian cells needs to be done. Also, co-expression of the hODR-4 and ceODR-4 in mammalian cells to investigate whether the hODR-4 gene is capable of aiding the trafficking of chemosensory receptors to the plasma membrane should be pursued. Furthermore, study of ligand specificity for the mouse olfactory receptor, mOR37, needs to be done once it has been functionally expressed in mammalian cells.

In the future, if more olfactory receptor genes are cloned and functionally expressed in mammalian cell lines, the localization and activation of these receptors can be examined. With this and our understanding of how GTP, GDP and their ratios affects olfactory signal transduction, it may be practical to develop a trainable biosensor based on the olfactory receptor proteins, and these biosensors could have the ability to detect a large dynamic range of odorant concentration, as well as being useful for different odorants and in different sensing environments.

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