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Cloning Vomeronasal Type-2 Receptors for Expression and Analysis in a Cell Culture Model System

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Abstract

The vomeronasal organ (VNO) is an olfactory sense organ in the nose of mice that detects pheromone signals through ligand binding to G-protein coupled receptors. There are three families of VNO receptors, V1R, V2R, and FPR. V2Rs in mice primarily serve to bind large molecules like the major urinary proteins (MUPs), proteins secreted in urine that trigger contextual behaviors in the recipient. Through combinatorial coding, multiple combinations of MUPs can activate multiple V2Rs in different ways, leading to complex signals based on a small library of ligands. However, VNO receptors are orphaned, it is not known which MUP ligand binds with which VNO receptor. This research set out to deorphanize V2Rs and pair them with their cognate ligands to create a library of receptor-ligand pairings. Receptor deorphanization will involve cloning V2Rs into mammalian cells, then analyzing them using patch clamp to measure membrane voltage changes when exposed to MUP ligands. Sequences coding for V2Rs were amplified through PCR, visualized on a gel, relevant amplicons were extracted and purified, then TOPO cloned into bacterial plasmids and transformed into JM109 *E. coli* cells for mass growth. Plasmids from *E. coli* were subjected to restriction digests to verify insert sequence length, then ligated into mammalian pEGFP vectors for eventual transfection into eukaryotic cells. To date, receptors have been cloned, visualized, extracted, purified, and transformed for V2Rs 34, 60, 92, 121, 122, 81, and 83. One sample (122-1) indicated a full length sequence, and has been ligated and sent for sequencing. If the results indicate a full length sequence inside the mammalian vector, the plasmid will be transfected for surface expression. This experiment is an important first step to being able to better understand and map the exact neural pathways activated by an environmental chemical stimulus, and how it produces a response in the host.

1. Introduction

Many animals rely on a complex set of sensory systems to understand and adapt to the world around them. In particular, many mammals have specialized sensors to detect pheromones, chemical signals released into the environment that trigger learned or innate behaviors with stereotyped responses. The vomeronasal organ (VNO), an olfactory sense organ located in the nose of mice, is the main detector of pheromone signals, where signals bind to and activate GPCRs. The VNO has three families of G-coupled protein receptors, V1R, V2R, and FPR, and V2Rs detect pheromone ligands, but are specifically tuned to reception of MUPs, or major urinary proteins.[1] MUPs are protein pheromones secreted in urine that trigger many different behaviors based on situational context, and sex of receiving animal. MUPs can be used to incite aggression and mating, and V2Rs can bind foreign MUPs that signal the presence of nearby predators.[2] The mouse genome has 21 distinct MUP genes, with 21 other nonfunctional pseudogenes, as well as a large repertoire of V2R genes to assist in identifying MUPs.[3] A given mouse only expresses 4-12 of these genes. How can such a small library of MUP ligands lead to a wide range of responses? Through combinatorial coding, multiple MUPs activate multiple receptors, and each receptor can detect multiple MUPs, leading to complex signals based on a small library of ligands.[4] This greatly increases the breadth and complexity of signals the V2Rs can receive.

Much of the research on MUPs has been done using mouse MUPs, as mice have a wider array of MUP genes as compared to other mammals, and interpret MUPs extensively in everyday life.[4] One of the problems with MUP research is that many of the cataloged MUP receptors are orphaned; it is not known which ligands they pair with.

As compared to other rodents and mammals, the mouse genome contains a very large library of V1R (308) and V2R (305) genes, allowing for greater receptor, and by extension, signal diversity. Both mice and rats have a large quantity of V2R pseudogenes (182 vs. 186), but mice still have a higher functional amount.[3] It is unknown why mice have such a large repertoire of V2R genes, as rats have only 101 functional V2R genes, and most other mammals have less than 50, if any.[5] The abundant pseudogenes may be holdovers from ancestral species.[6] It has been hypothesized the expansion of V2R genes in mice could be a functional response to the need for greater variety and complexity in signals, as mouse migration into foreign environments would favor mice that have the capacity to bind and respond to a variety of new ligands.[6]

Mice use MUPs to dictate certain social interactions and identifications. When female mice reach their first estrus, they become responsive to the male specific MUP20, and seek out mates based on this pheromone. Detection of MUPs is necessary to induce ovulation in females, as mice who fail to detect MUPs or have no VNO either ovulate late or not at all.[7, 8] After birthing pups, females use this same MUP to identify males intruding on the brood, and attack those who carry this MUP on them. As testosterone is critical for MUP expression, castrated males who cannot produce MUPs cannot produce aggression promoting pheromones, and are attacked at a far smaller rate. Here, the same MUP produces two different responses depending on the female's maturity, indicating that responses to MUP reception can change throughout life. Similarly, virgin female mice do not respond to MUPs produced by pups that induce male aggression behavior. Mothers actually become receptive to these MUPs in part through suckling by pups, meaning an outside mechanical motion can actually induce the response to an activated MUP receptor.[9]

In male mice, MUP3 is specially hardwired to promote aggression upon first encounter with other males. However, the same MUP can induce countermarking in individuals based on past experience with the MUP and situational context (the presence of another mouse). This means that a target response to a given MUP can depend on other sensory inputs. Countermarking behavior is also dependent on reading the entire library of MUPs in the nearby environment, meaning that a mouse's response to MUP3 is influenced by the blend of MUPs that are being detected, creating a combinatorial strategy of MUP reading by receptors.[4]

Studies are ongoing in this area, with the goal of pairing receptors with their cognate ligands. Previously, microarrays have been used to document V2R gene expression, [3] and ratiometric calcium imaging with fluorescent fura-2 have been used to image VNO neurons.[4, 10] In this group, the main objective is to clone V2 receptors from mouse tissue into expression vectors, which can then be placed into cultured cells where they will be analyzed with patch clamp to look for pairs and deorphanize the receptors. Once receptors and cognate ligands are matched up, the data can be used to better understand and map the exact neural pathways activated by an environmental chemical stimulus, and how it produces a response in the host.

3. Methods

3.1 Primer Design

V2R gene sequences were found on Ensembl.org using the GRCm38.p5 Mouse reference genome. The introns and flanking sequences were deleted, then the modified sequence was plugged into NCBI Primer-BLAST. Primers were BLASTed using the NCBI nr nucleotide database, with *Mus musculus* (House Mouse) as the organism, outputting forward and reverse primers for PCR. Primers were designed to bind within the untranslated region (UTR) on the flanking ends of the sequence, as this region does not code for the receptor proteins and, as such, these primers allowed us to amplify the complete protein-coding sequence.

3.2 DNA Amplification

Designed primers were ordered via Eurofins Genomics, and receptor sequences were amplified using PCR for gel analysis. V2R forward and reverse primers were combined on ice with a C57BL/6j adult mouse VNO cDNA template made from crushed mouse VNOs, Phusion HF buffer, dNTPs, Phusion High-Fidelity DNA Polymerase, and Nuclease free dH_2O in 1.5mL PCR tubes. PCR tubes were centrifuged, then run on a thermocycler for denaturation, annealing, and elongation of V2R gene sequences. GAPDH primers acted as a control.

3.3 Gel Visualization

After PCR, amplified sequences were visualized on an agarose gel at 110 Volts for 60 minutes. As many V2R sequences are typically ~3k base pairs in length, a 1.0% agarose gel was used. Sequences were combined with loading dyes, and run against a 1kb+ base pair gel ladder (500 base pairs to 12000 base pairs) and visualized with UV light.

3.4 Gel Extraction and DNA Quantification

Amplified sequences were gel extracted by cutting the bands out of gels, then purified using a PureLink Wizard SV Gel and PCR Cleanup System. Purified sequences were eluted using warmed TE buffer. DNA concentration of purified sequences was then quantified using a nanodrop spectrophotometer.

3.5 Blunt-End TOPO Cloning, Cell Transformation, and Plating

Purified sequences were cloned into expression vectors using Blunt-End TOPO Cloning. Plasmid expression vectors accept sequences and incorporate them next to a promoter region on the plasmid. Plasmids were then transformed into JM109 competent *E. coli* cells, then cells were spread on antibiotic kanamycin (KAN) plates and grown at 37° C. Plates were then incubated overnight to allow for colony growth.

3.6 Overnight Culture Minipreps

Ten colonies were picked from each KAN plate, and grown for 12-15 hours at 37° C with shaking. Cells were then harvested, then mini prepped using a PureLink Plasmid Miniprep Kit. DNA concentration was quantified via NanoDrop.

3.7 Restriction Digest

To check for correct incorporation of sequences into Plasmids, a restriction digest was performed by combining purified plasmids with a restriction enzyme to separate sequence and plasmid. Digested products were visualized on a 1% agarose gel against a 1kb+ ladder and controls containing undigested plasmids. Plasmids with the expected restriction pattern were subjected to step-wise Sanger sequencing. Sequence alignments from each amplification were added together to verify the entire sequence

3.8 DNA Ligation

Both a plasmid vector containing a full length sequence and a pEGFP vector were digested with a restriction enzyme overnight that produced a stable reading frame after ligation. The digested products were run on an agarose gel, then the DNA bands for the receptor sequence and the recipient pEGFP plasmid were extracted and purified. The purified products were first treated with a phosphatase to remove the phosphate group from 5’ end of the DNA, which ensures correct insert orientation during ligation. The sample was then combined along with DNA ligase to facilitate binding of the sequence to the plasmid, then incubated overnight. The resulting plasmids were sequenced to confirm correct orientation of insert.

4. Results

4.1 Primer design and Gel visualization

To date, primers have been designed for all V2Rs that have UTR regions flanking the main sequence, including those that only have one set of UTRs either at the beginning or end of the sequence. V2R sequences have been amplified, and visualized for V2R 34, 60, 81, 83, 92, 121, and 122. All have shown shown successful PCR expression at ~2500-3000 base pairs, although some smearing is present with 81 (Fig. 1E).

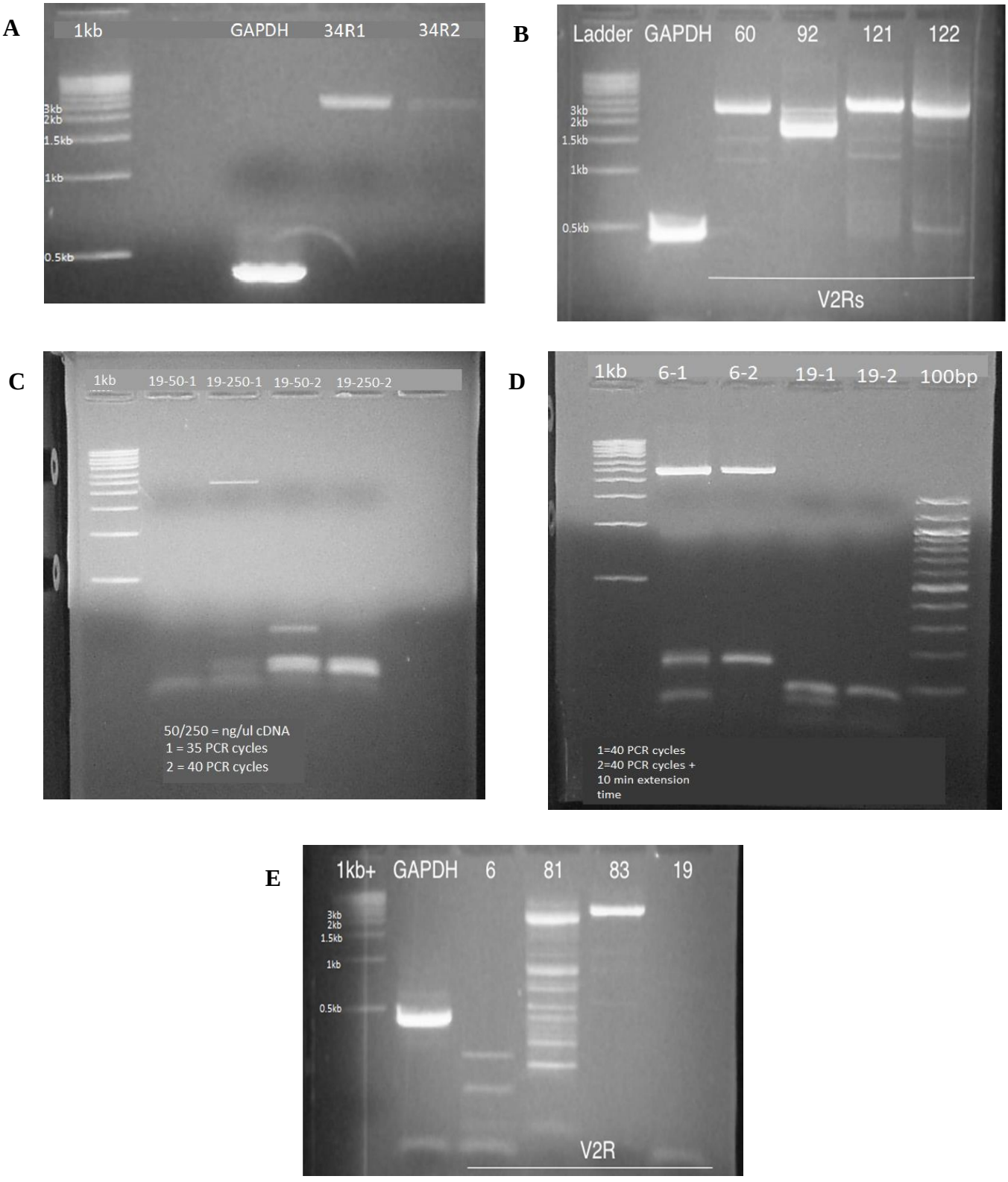


Figure 1. Gel visualization of PCR-amplified V2Rs

Figure 1. Amplification of V2Rs 34 using two possible reverse primers (**A**), 60, 92, 121, 122 (**B**), 19 (**C**), 6 (**D**), 81, and 83 (**E**) from C57Bl/6j adult male VNO cDNA. Bands for 19 only appeared during one PCR, and require further optimization to be consistent, while 81 shows heavy smearing, indicating possible problems with thermocycler protocol. All visualized sequences show strong bands between 2500 - 3000 base pairs.

4.2 Bacterial Transformation

All sequences were TOPO cloned and transformed into JM109 *E. coli* cells on kanamycin or ampicillin plates. The TOPO XL-2 Vector sequences codes for both kanamycin and ampicillin resistances, so only cells that took up a vector would be able to grow on antibiotic selective plates (Fig 2.). Furthermore, cells that took up empty vectors were terminated through a genetic kill switch on the vector expressed when the vector itself had not integrated a sequence.

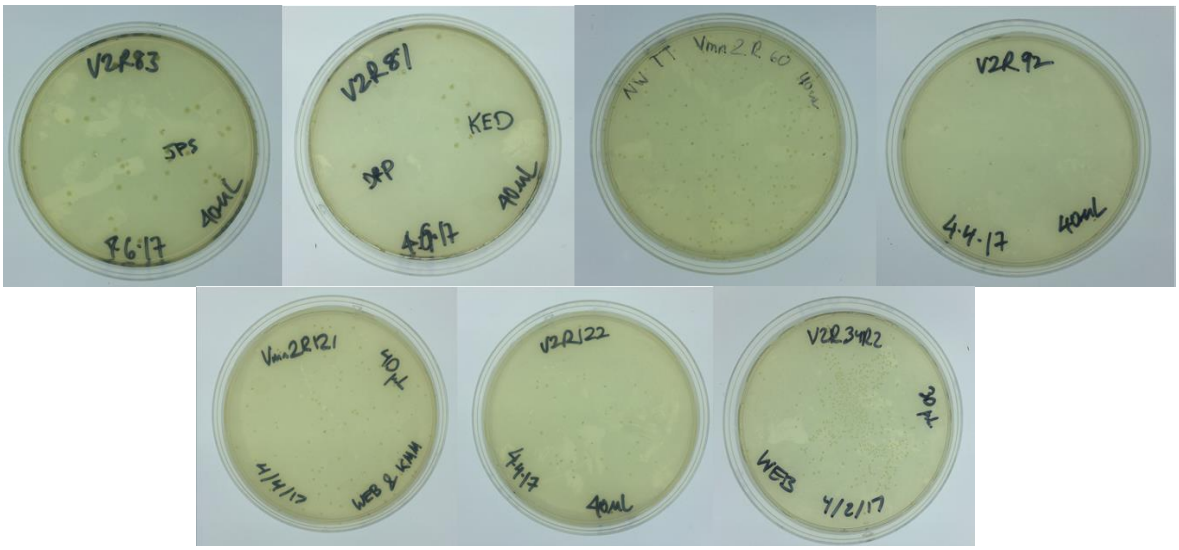


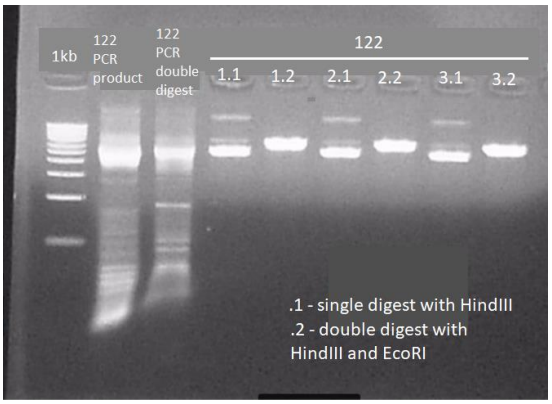
Figure 2. Colonies of cells transformed with TOPO-cloned V2R sequences

Figure 2. Colonies of JM109 cells transformed with TOPO-cloned V2R sequences on kanamycin plates. Amplified sequences were gel extracted and TOPO-cloned into expression vectors. Individual colonies denote potentially genetically distinct *E. coli*.

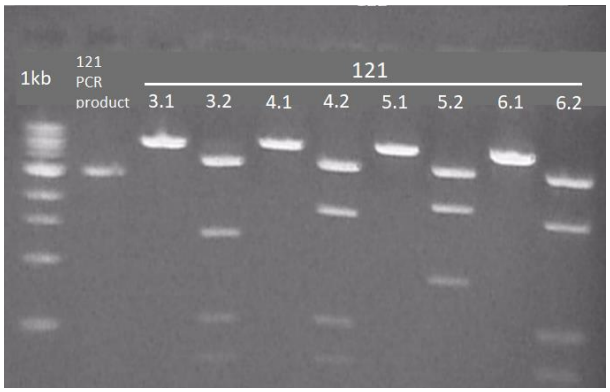
4.3 Restriction Digests

EcoRI restriction digests were performed on TOPO cloned cells from all plates. Cells are digested with a single control digest of HindIII to cut and linearize the plasmid, and a EcoRI and HindIII double digest to linearize and separate TOPO cloned sequences. Successful digests showed strong bands at ~7kb for controls, and ~4kb and ~3kb respectively for separated vector + insert (Fig. 3A). Sequences with EcoRI or HindIII cut sites in-sequence should display further splitting (Fig. 3B), only lanes with the correct number of band splits for a given sequence indicate success.

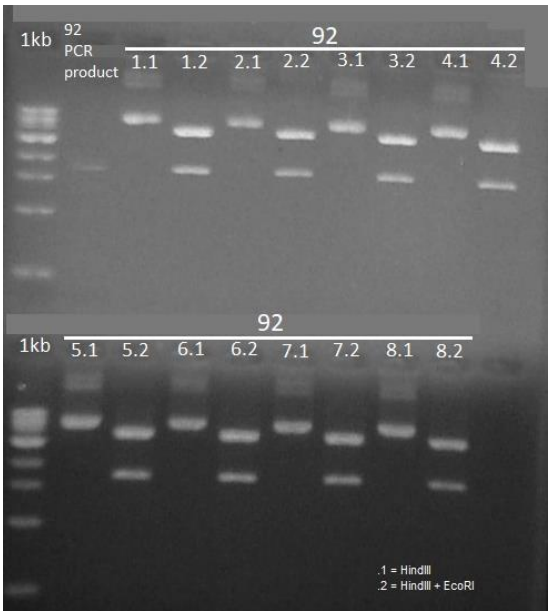
A


1kb
122 PCR product
122 PCR double digest
122
1.1 1.2 2.1 2.2 3.1 3.2
.1 - single digest with HindIII
.2 - double digest with HindIII and EcoRI

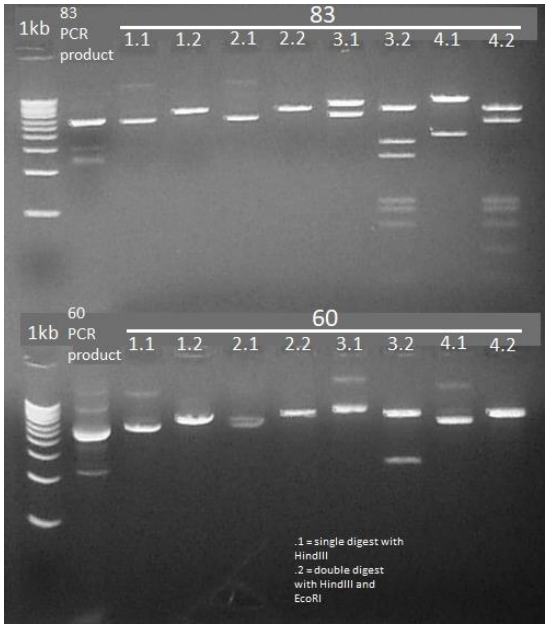
B


1kb
121 PCR product
121
3.1 3.2 4.1 4.2 5.1 5.2 6.1 6.2

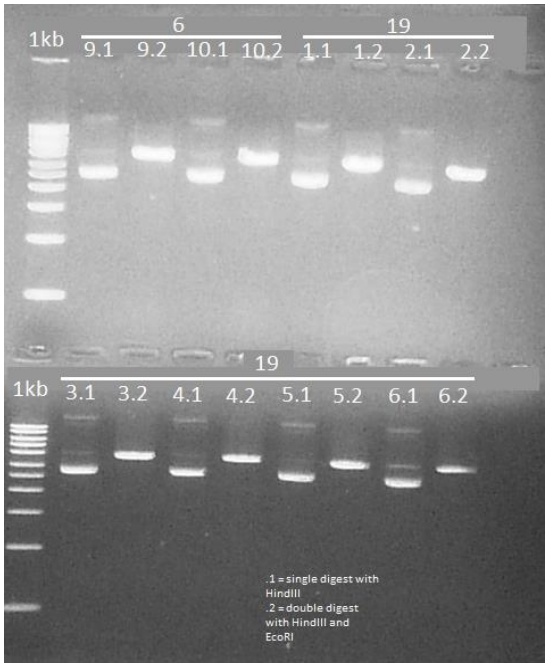
C


1kb
92 PCR product
92
1.1 1.2 2.1 2.2 3.1 3.2 4.1 4.2
1kb
92
5.1 5.2 6.1 6.2 7.1 7.2 8.1 8.2
.1 = HindIII
.2 = HindIII + EcoRI

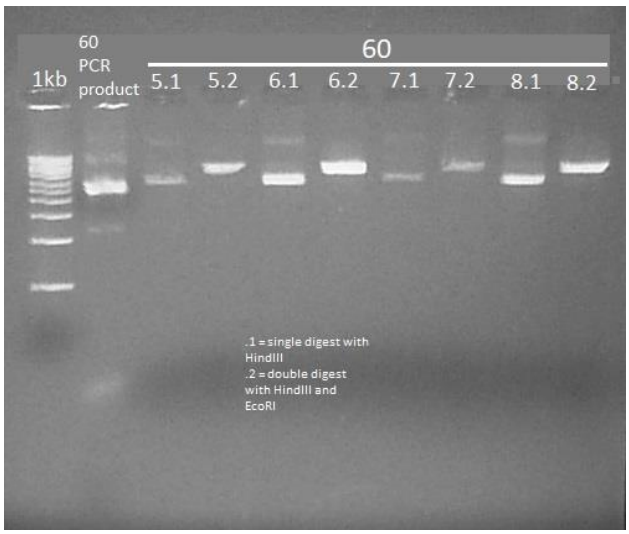
D


1kb
83 PCR product
83
1.1 1.2 2.1 2.2 3.1 3.2 4.1 4.2
1kb
60 PCR product
60
1.1 1.2 2.1 2.2 3.1 3.2 4.1 4.2
.1 = single digest with HindIII
.2 = double digest with HindIII and EcoRI

E


1kb
6
9.1 9.2 10.1 10.2
19
1.1 1.2 2.1 2.2
19
1kb
3.1 3.2 4.1 4.2 5.1 5.2 6.1 6.2
.1 = single digest with HindIII
.2 = double digest with HindIII and EcoRI

F


1kb
60 PCR product
60
5.1 5.2 6.1 6.2 7.1 7.2 8.1 8.2
.1 = single digest with HindIII
.2 = double digest with HindIII and EcoRI

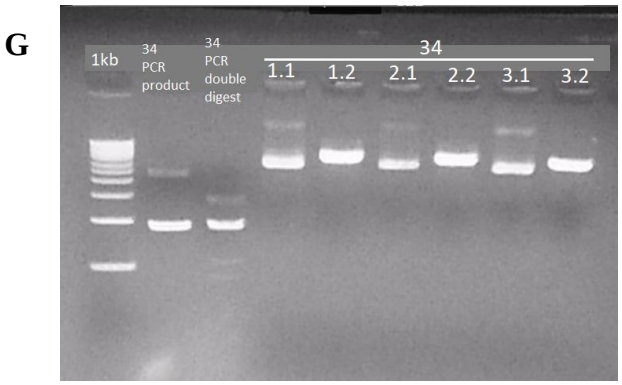
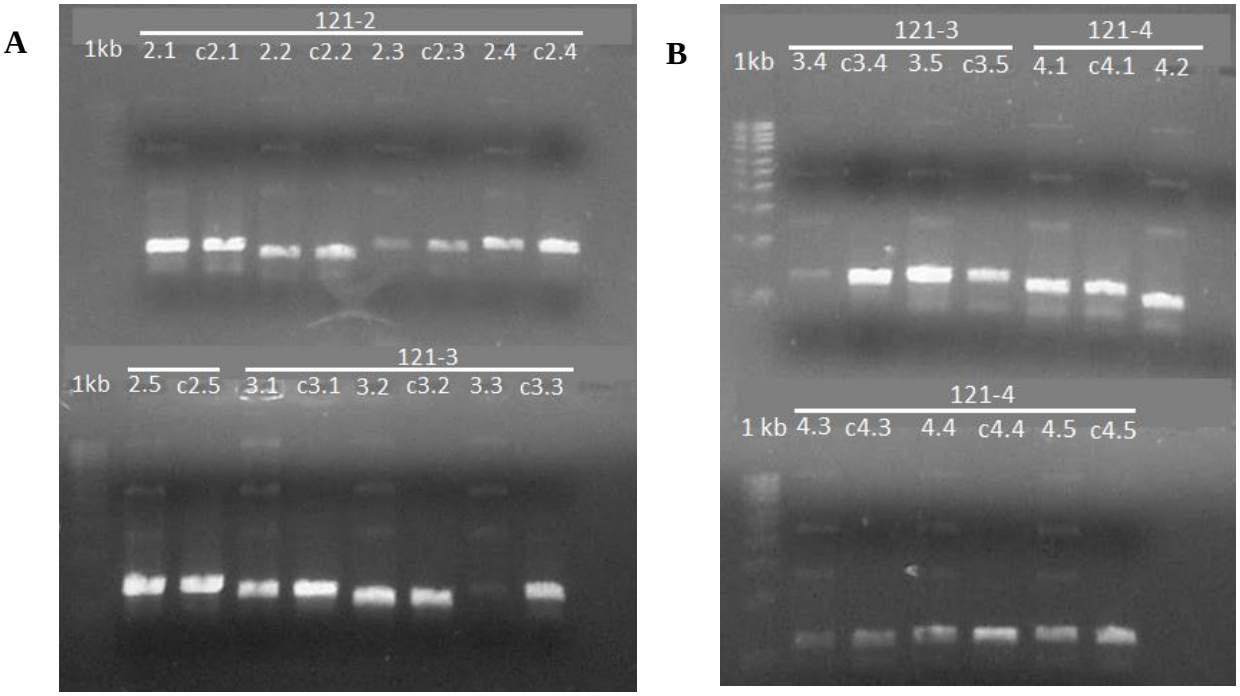


Figure 3. Restriction digests to separate vector and insert DNA

Figure 3. EcoRI and HindIII restriction digests of plasmids shows full-length sequence in vector. **(A):** Digests of V2R 122. Successful double digests display bands at 4kb and 3kb, indicating separated plasmid and sequence. Bands appearing higher indicate undigested plasmid. (**B):** Digests of V2R 121. V2R 121 contains two EcoRI cut sites in sequence, and should display four bands. **(C):** Digests of V2Rs 83 and 60. Both contain no cut sites in sequence. **(D):** Digests of V2R 34, 34 contains no cut sites in sequence. **(E):** Digests of V2R 6 and 19. Neither contain cut sites in sequence. **(F):** Digests of V2R 60. Contains no cut sites in sequence. **(G):** Digests of V2R 34, 34 contains no cut sites in sequence

4.4 Sequencing Primers

In order to verify identity of sequences inserted into plasmid, "sequencing primers" were designed for V2R amplicons that had displayed restriction digests suggesting successful cloning and transformation. These primers were designed to amplify 600-800 base pair sections of the V2R sequences, with a degree of overlap to ensure all of the sequence was accounted for (Fig. 4). These primers were visualized on a gel for in-house diagnostic purposes, as well as used by Genewiz for Sanger sequencing. (Fig. 4G-H). Successful sequencing primers should display amplicons ~600-800 base pairs in size across all samples (Fig 4A).



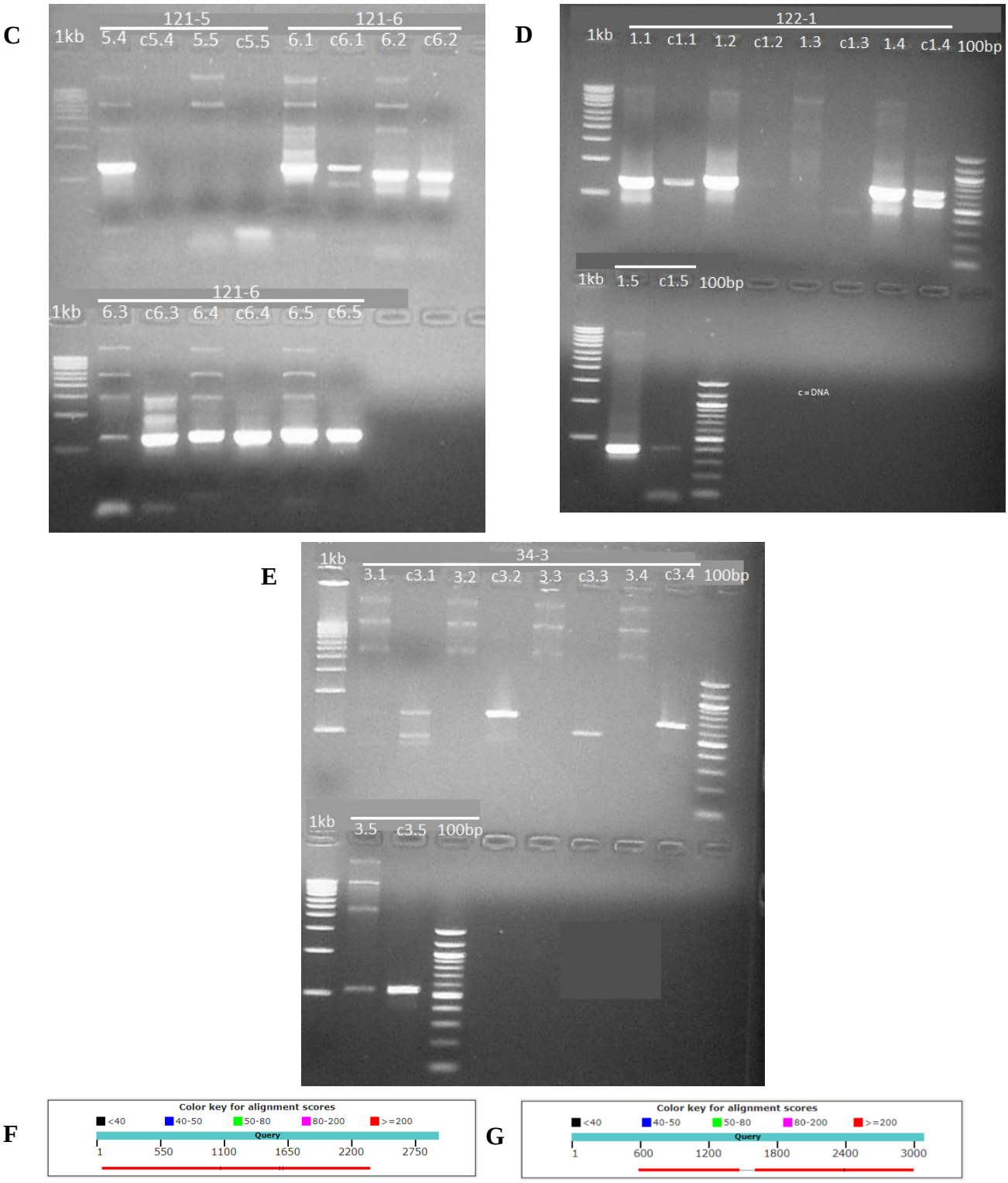


Figure 4. Sequencing primers amplification of vector sequences

Figure 4. Sequencing Primers vs Plasmid and cDNA and Genewiz sequence alignment confirm conserved identity of V2R 122 in-vector. **(A)** - (**C)**: Sequencing primers were used to test for the presence of full length V2R sequences in plasmids labelled 121-2, 121-3, 121-4, 121-5, and 121-6 and a cDNA library control. Successful priming should

produce ~700 bp bands. 121-2 through 121-6 all showed successful priming except 121-5. (**D**) Amplification with sequential primers of sample 122-1. This sample was sent to Genewiz for sequencing despite not seeing a positive amplification using sequencing primer 3. **(E)** Amplification with sequential primers of sample 34-3. This sample failed to show priming, but was submitted for sequencing to probe errors in technique. **(F)** Genewiz sequence alignment of 122-1. High alignment scores from 49 to 2362 indicated a full length sequence inside the plasmid. There was no expression after 2362 bp, however this region consisted of UTR, and was not needed to express the full receptor sequence. **(G)** Genewiz sequence alignment of 121-6. Lack of priming at the beginning and middle sections indicated an incomplete sequence. The results for 34-1 showed no priming overall, and are not included. Samples 121-2 through 121-6 are currently being sequenced, with results pending.

5. Conclusion

To date, receptor sequences for V2R 6, 19, 34, 60, 92, 121, 122, 81, and 83 have been cloned, visualized, extracted, purified, and transformed. Of these, three V2R 34, two 83, eight 92, fifteen 121, and two 122 samples have been sent for sequencing, as the others have not shown full length sequences in TOPO vectors. One sample (122-1) indicated a full length sequence after in-house and Genewiz sequencing, and has been ligated, though unsuccessfully, due to the sequence inserting into the vector backwards. Current ligations involve a phosphatase treatment to ensure correct orientation, the results of these are currently pending. After further ligation if results indicate a full length sequence inside the mammalian vector, the plasmid will be transfected for surface expression. From current results, it is anticipated that with further refinement of procedures 121 will produce a full length sequence in the pEGFP vector given its similarity to 122. All other sequences also require procedure refinement, and have yet to produce full length sequences in the TOPO vector, so further investigation is needed. There is a particular "cluster" of V2R receptors, from V2R 38 to 44 that all share high sequence similarity. Primers designed to amplify these sequences also prime V2R 32 and 26. Including two splice variants for 42, this cluster comprises 9 different sequences, and will be a major topic of further investigation due to the ease of creating primers for these sequences owing to the large UTR regions that flank each of them.

Difficulty exists in creating specific primers for many of the V2R sequences, as they either contain no UTRs or primer BLAST cannot generate primers that do not prime within the sequence itself. It is possible to create primers that prime within the introns, but this strategy is imperfect because if start codons exist within any intronic regions, our coding sequences would not be a faithful amplification of the V2R of interest. Comparisons to previous work are hard to draw due to the novelty of this research. However, significant progress has been made towards our goal to clone full length V2Rs for deorphanization.

As V2Rs produce stereotyped, instinctual behaviors in mice, they should activate the same neural networks during each exposure. This makes them a consistent and useful model system for examining the exact neural pathways activated by an environmental chemical stimulus, and how it produces a response in the host. Study of model systems like this could provide insight into the coding of behavioral information, which could be applied more complex stimulus to response behaviors in other organisms including humans.

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