Identifying the *Galleria mellonella* (Wax Moth) Pheromone Biosynthesis Activating Neuropeptide Gene

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Galleria mellonella (wax moths) pose a threat to the honey bee population because they destroy the hives. We plan to combat this threat by designing a wax moth specific trap. To do so, we will use the Pheromone Biosynthesis Activation Neuropeptide (PBAN) to induce the production of pheromones in the wax moths. We will accomplish this by putting the PBAN in sugar water so that when the wax moths drink the water, the PBAN will induce them to produce pheromones that will attract other wax moths to the trap. Unfortunately, the wax moth PBAN gene has not yet been identified. Therefore, we used the Consensus-Degenerate Hybrid Oligonucleotide Primer (CODEHOP) design strategy to identify and sequence the wax moth gene based on comparisons with similar genes that have been identified in other insects. The CODEHOP primers appear to have amplified the gene based on gel electrophoresis and sequence analysis. Based on these results, we are confident that we can use the gene to produce the PBAN protein in *Escherichia coli*. We will then design a trap and place a mixture of the PBAN protein and sugar water inside. When the protein is ingested by wax moths, it will induce pheromone production, attracting more moths to the trap. This method should reduce wax moth populations and allow for more prosperous honeybee hives.

Keywords: Pheromone Biosynthesis Activating Neuropeptide (PBAN), Polymerase Chain Reaction (PCR), COnsensus-DEgenerate Hybrid Oligonucleotide Primer Strategy (CODEHOP), *Galleria mellonella*

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Honeybee hives are victims of intruders. Wax moths enter hives and destroy them from the inside out. They consume the nectar, kill the larva, and lay thousands of eggs in the decaying aftermath. Beekeepers from around the world have been observing the mysterious and sudden disappearance of bees and honey bee colonies since the 1990s. As this parasite continues to decimate bee populations, the world faces a serious problem: one in every three bites of food eaten worldwide is dependent on pollinators, specifically bees (Klein et al. 2007).

To help solve this problem, our team plans to create a biological trap that targets only wax moths, so that honeybees will be protected from this threat and their populations can stabilize.

At first the entire concept seemed ridiculous; a trap that discriminates all but one species of insect seemed impossible. We first imagined a light that would only attract wax moths and quickly concluded such a thing would only remain a dream. But perhaps scent would work instead? All animals create their own pheromones to attract mates of the same species. If we could force moths to create these pheromones, it would be as if an invisible light bulb really did exist! Only wax moths would detect and respond to the scent and be lured into the trap where they would continue to create more pheromones. We researched this idea and found that a previous iGEM team, Formosa, had already used this to trap a different type of insect (Wei et al. 2014). After contacting them and getting permission, we used their research as a stepping stone to achieve our goal.

Unlike the Formosa team's experience, the sequence of Pheromone Biosynthesis Activation Neuropeptide (PBAN) has unfortunately not been published for wax moths. However, it has been discovered for other species of insects, so we tackled this dilemma by comparing the genomes of similar moths. We identified similarities, specifically the PBAN portion.

Materials and Methods

OpenPCR Thermocycler Assembly. In order to run polymerase chain reaction (PCR) on our experiments, our team needed a PCR machine. Unfortunately, many PCR machines were thousands of dollars beyond our budget, so we built our own PCR machine using the OpenPCR kit (Chai Biotechnologies, Inc., Santa Clara CA).

DNA Extraction. In order to extract the DNA from *Escherichia coli* (Carolina Biological Supply Company, Burlington, NC), silkworm (Extra small silkworms; The Silkworm Shop, San Diego, CA), and wax moth (Bug Co., Ham Lake, MN) cells, the boil preparation method was used. This entailed heating the cell or tissue sample to 95°C for 10 min. in the thermocycler prior to initiating PCR. When this did not work for the insect cells, we extracted the DNA using the Promega Wizard SV Tissue Purification Kit (Promega Corporation, Madison WI) following the manufacturer's protocol

Standard PCR Amplification. PCR was used to extract and amplify genes. We accomplished this using our newly assembled open PCR machine. We combined 12.5 µL Q5® High-Fidelity 2X Master Mix (New England Biolabs, Ipswich MA), 1.25 µL 10 µM Forward Primer, 1.25 µL 10 µM Reverse Primer, 1.0 µL genomic DNA, and 9 µL nuclease free water in a PCR tube. We ran the thermocycler with the following cycling conditions: 30 s at 98°C, 10 s at 98°C, 20 s at 65°C, 20 s at 72°C, and cycle 35 times. Table 1 shows the primers we used in this study. These cycling conditions were used for all of the primers.

Table 1. PCR primers used in the study

Name	Sequence
BioBrick Primers: The BioBrick primers were used as a control in Figure 1. The primers should effectively attach to their predetermined location on the pSB1C3 plasmid in *E. coli* and result in a successful amplification.	
VF2	TGCCACCTGACGTCTAAGAA
VR	ATTACCGCCTTTGAGTGAGC
Silkworm Primers: The Silkworm primers are simply the primers used to consolidate the PBAN gene in silkworms.	
SWP-BAN-FP	GATACTTCTCGCCCAGGC
SWP-BAN-RP	TGTCTTGTTAACGCTTCTGGC
CODEHOP Primers: see CODEHOP Primer Design	
F-PBAN	ACAGTGACAAAAAAAGT-GATCTTCACNCCNAARCT
R-PBAN	CTTTTACCGTATGACAGTTCTCTNCCNAGNC-GNGG
R-PBAN2	CTGTACATTTCTTGGTCAGCNGGNGTNGCNGG
Sequencing Primers: These were constructed from the consensus regions of the CODEHOP primers and used for product amplification and sequencing.	
FP01	ACAGTGACAAAAAAAGTGATCTTC
RP01	CTGTACATTTCTTGGTCAGC

Gel Electrophoresis. Gel electrophoresis was performed using the FlashGel dock and 1.2% FlashGel cassettes (both from Lonza Group, Basel, Switzerland). Generally, 4 µL aliquots of PCR product were mixed with 1 µL FlashGel Loading Buffer and added to the wells. The gels were run at 50 V for about 15 min. and photographed with a cell phone camera.

Consensus-Degenerate Hybrid Oligonucleotide Primer (CODEHOP) Primer Design. We decided to take on the difficult and interesting challenge of trying to identify an unknown sequence with the following understandings and methods. The CODEHOP primers are split into two parts. The first is known as a 5' consensus clamp, which contains mostly conserved regions of the wax moth genome. The second region contains a 3' degenerate core, which consists of less conserved regions and contains randomized nucleotides in places where the genome is unknown. The primer mix in our experiments contains primers with all possible random nucleotides. During transcription, the best primer will line up with the DNA sequence and begin transcription. Rose et al. (2003) provides a detailed description of the CODEHOP design process that we followed.

DNA Recovery. To recover the CODEHOP amplicon to be sequenced, we used the FlashGel recovery system. This required

Table 2. Original CODEHOP primer design

F-PBAN Codon Selection											
Consensus Clamp*	Degenerate Core										
T	V	T	K	K	V	I	F	T	P	K	L
ACA	GTG	ACA	AAA	AAA	GTG	ATC	TTC	ACN	CCN	AAR	CTN
R-PBAN Codon Selection											
Degenerate Core	Consensus Clamp*										
P	R	L	G	R	E	L	S	Y	G	K	R
CCN	CGN	CTN	GGN	AGA	GAA	CTG	TCA	TAC	GGT	AAA	AGA

*The codons in the consensus clamp region were chosen using the Bombyx mori (silkworm) codon usage table (Wei et al. 2014)

using a FlashGel with wells in the middle so we could pull the band of genomic material out of it. We followed the manufacturer's protocol when extracting the silkworm and wax moth amplicons.

Sequencing. We sent our silkworm and wax moth samples, recovered using the FlashGel system, to GeneWiz (South Plainfield, NJ) for custom sequencing along with a 5 µM sample of the FP01 sequencing primer, as recommended by the company.

Results

We Successfully Assembled and Tested the OpenPCR Thermocycler. We needed an affordable thermocycler in order to amplify and sequence the wax moth PBAN gene. To accomplish this, we purchased a cheap OpenPCR Thermocycler kit from Chai Biotechnologies.

We bought a kit online and started assembling parts in the thermocycler (Figure 1). It took us roughly 6 h to assemble. We verified that the thermocycler worked by amplifying the Cellulose Binding Domain-Green Fluorescent Protein (CBD-GFP) coding region from a BioBrick sample supplied by the Tuscarora High School Bioengineering Club, using the universal BioBrick forward (VF2) and reverse (VR) primers. The PCR machine successfully amplified the target DNA as shown in Figure 2.

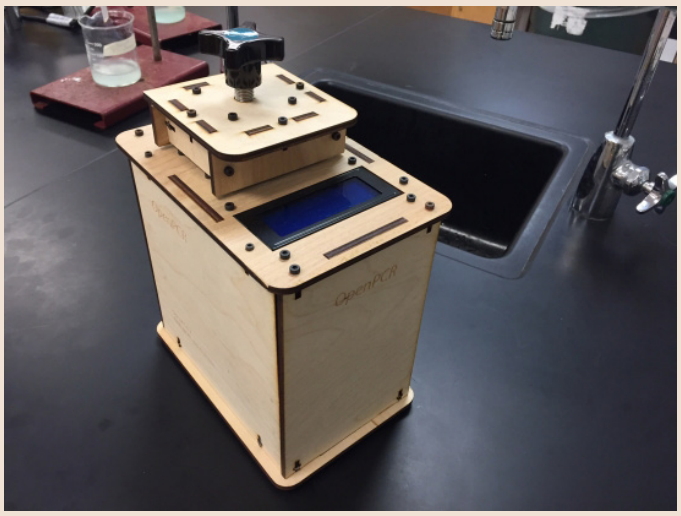

Figure 1. *We assembled the thermocycler over a 3 wk period. Total assembly time was 8 h.*

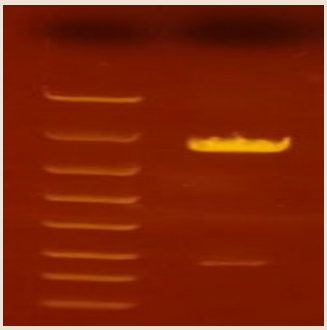

Figure 2. *We used standard procedure for the polymerase chain reaction (PCR) reactions. In Well 1, a 100-4,000 bp ladder was placed to act as a reference for the other well. Well 2 contained the PCR-amplified BioBrick sample and produced a strong band under 2,000 bp and weaker band at around 275 bp. The larger band roughly corresponded with the 1,585 bp sample we would have expected to generate from the BioBrick template. From these results, we can conclude that our thermocycler is functional and effective. We can now begin our experiments with the wax moth genome.*

Insect Cell Lysates Appear to Inhibit PCR Amplification. In order to test whether we could amplify the PBAN gene from silkworms and possibly wax moths, we designed a pair of primers based on the published silkworm sequence for the PBAN gene. These primers were used to amplify a 1,069 bp section of the PBAN gene using the SWPBAN-FP and SWPBAN-RP primers shown in Table 1. Both primers had a melting temperature of 63°C.

Since it is possible to amplify genomic DNA directly from boiled *E. coli* by using the boil prep technique, we tried the same with the silkworm and wax moth tissue. As shown in Figure 3, our silkworm primers were only effective in the absence of insect tissues. The boil preparation method for DNA extraction proved to be unsuccessful with the silkworm and wax moth tissue. We postulated that there may be a PCR inhibitor in the insect cells.

In order to test whether the PCR failed because of inhibitors in the insect cells, we mixed the *E. coli* sample with the wax moth

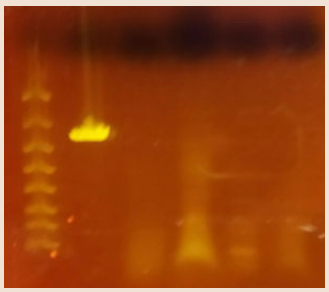

Figure 3. *Boil prep extraction of insect DNA inhibits PCR. This experiment tested whether insect tissue cells contain PCR inhibitors. The PCR reactions using our silkworm primer design produced results only when no insect tissue was present. Well 1 contains the Lonza 100-4,000 bp ladder. Well 2 contains an E. coli culture with BioBrick primers and was the only test to properly amplify DNA. Well 3 contains wax worm tissue with an E. coli culture and BioBrick primers. Well 4 holds silkworm tissue, an E. coli culture, and BioBrick primers. Well 5 holds a wax worm tissue slice with the silkworm primer, and well 6 contains the silkworm tissue with silkworm primers. None of the material in wells 3-6 were successfully amplified because they contained tissue. Because the insect tissue was not amplified with either primer, we can deduce that there is some PCR inhibitor in the silkworm and wax worm cells.*

and silkworm tissue cells and attempted to amplify the *E. coli* cells with the BioBrick *E. coli* primers. Under normal circumstances, the *E. coli* DNA should have amplified with the BioBrick primers. However, when the insect tissue cells are added to the *E. coli*, the primers do not work.

Our results show that lysates in the wax moth and silkworm cells inhibit PCR reactions. We concluded that for future experiments, we would need to use a purification kit to completely separate the DNA from the insect tissue. For this process, we chose the Promega Wizard SV Tissue Purification Kit to successfully separate these elements.

Primers for the Silkworm PBAN Gene Failed to Amplify the Wax Moth Gene. Since something in the insect tissue lysate appears to inhibit PCR, we used Promega's SV Wizard kit to purify the silkworm and wax moth DNA from the tissue samples.

We used our silkworm primers to amplify the PBAN genes from the kit-purified silkworm and wax moth genomes. We prepared several mixes. One mix contained silkworm primers and the silkworm genome, and another contained the silkworm primers and the wax moth genome. As seen in Figure 4, the silkworm primers did not successfully prime the wax moth genome, which could indicate a difference in the regions of the PBAN genes targeted by these primers.

Our Original CODEHOP Design Didn't Work. We used the CODEHOP technique (Rose et al. 2003) to design primers for the unknown sequence of the wax moth PBAN gene.

Table 3. Revised CODEHOP primer design

R-PBAN2 Codon Selection											
Degenerate Core				Consensus Clamp*							
P	A	T	P	A	D	Q	E	M	Y	R	Q
CCN	GCN	ACN	CCN	GCT	GAC	CAA	GAA	ATG	TAC	AGA	CAA

*The codons in the consensus clamp region were chosen using the Bombyx mori (silkworm) codon usage table (Wei et al. 2014).

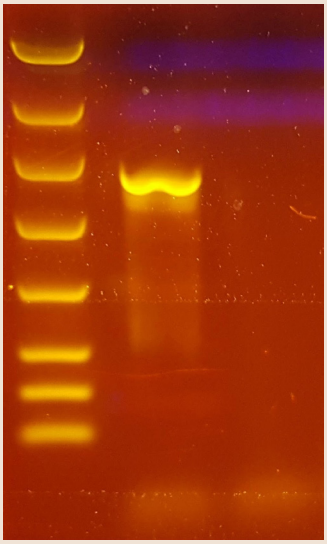

Figure 4. *Silkworm primers amplify the silkworm, but not the wax moth Pheromone Biosynthesis Activation Neuropeptide (PBAN) gene. In the experiment, we were trying to figure out whether or not the silkworm primers were able to amplify the wax worm PBAN. Unfortunately, the silkworm primers do not appear to be capable of amplifying the PBAN out of the wax worm genome, which is to be expected, given the likely differences in the two genes. In the picture of our results above, the single band in well 2 indicated that we successfully amplified the 1,069 bp PBAN gene target from the silkworm sample, but not the wax moth sample, which occupied wells 3.*

CODEHOP works by using primers that have both 5' consensus and 3' degenerate regions. The degenerate region consists of codons for a region of highly conserved amino acids. Each codon has a randomized nucleotide in the third position. This means that each third position could be occupied by G, C, A, or T. As a result, all possible codons are represented in the CODEHOP primer set. This ensures that at least some of the primers can prime the target template and be extended by the DNA polymerase.

The consensus region contains nonrandomized codons unlike the degenerate regions. This ensures that all primers can assist in the creation of additional PCR products once the original templates are copied. When designing the consensus region, we used the silkworm codon table to select the most likely codons, which closely resemble the codon table of wax moths.

To select the amino acids to use, we compared the PBAN regions of many similar insects and chose the most highly conserved amino acid sequences throughout the different species. We then used the previously published silkworm codon table (Wei et al. 2014) to select those codons that were most likely to code for the conserved amino acids in the PBAN sequence alignments. The resulting primer design is shown in Table 2.

In order to test our CODEHOP sequences, we set up a series of PCR reactions in which the CODEHOP primers were added to the silkworm and wax moth DNA samples. Even though we were searching for the wax moth PBAN, we used the silkworm genome as a positive control since we know that gene exists, as well as its exact sequence. We also used our previously designed silkworm primers as a positive control for the PCR primer itself. For a negative control, we ran our CODEHOP PCR reaction without any template DNA.

The results of the gel (Figure 5) indicated that the silkworm PCR primers worked as expected, but the CODEHOP primers did not generate fragments of the expected size. In fact, the bands were the same size as those produced in the reaction without the template DNA. This lead us to reconsider the primer design.

Figure 5. *Our first attempt at Consensus-Degenerate Hybrid Oligonucleotide Primer (CODEHOP) design fails to amplify PBAN gene. In this experiment, we attempted to amplify the wax moth PBAN gene using the CODEHOP primers. The first well shows the 100-4,000 bp DNA markers. Wells 2-4 show the results of the silkworm PBAN primer applied to the silkworm primers, SWPBAN-FP and SWPBAN-RP (not using the CODEHOP strategy). Wells 5-6 show the results of the CODEHOP primers applied to the silkworm and wax moth DNA, respectively. The DNA from the CODEHOP wells appear to be shorter than the regular BioBrick primers on the silkworm genome used before.*

Our Redesigned CODEHOP Primers May Have Hit Their Mark. In the results of our prior experiments, we concluded that our reverse primers might be causing errors in our results, possibly because it is targeting more than one highly conserved region of the PBAN gene. In this experiment, we designed a new CODEHOP reverse primer by looking for a new highly conserved region. Our newly designed reverse CODEHOP primer is shown in Table 3. In order to test our new primers, we prepared three tubes that had the silkworm primers with the silkworm genome, CODEHOP primers with the silkworm genome, and CODEHOP primers with the wax moth genome. We also had tubes that contained new CODEHOP primers with PCR mix, with no controls.

As shown in Figure 6, the CODEHOP primers with both moth genomes produced similar band lengths, indicating that our new primers might have successfully amplified our target.

According to the gel, PCR products are approximately 400 bp in length. This is actually longer that what we would have expected based on the portion of the PBAN amino acid sequence that we're targeting. However, we know that the PBAN gene contains introns, which may account for the longer PCR product.

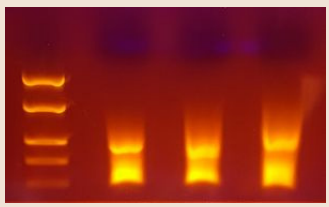

Figure 7. *We conducted this experiment to test whether our primers were creating faulty products that could not be sequenced by repeating our previous DNA recovery experiment. Well 1 contains a 100-1,500 bp ladder. Well 2 contains the gel-purified silkworm genome, and well 3 contains the gel-purified wax moth DNA from our previous amplification experiment. The bands are the expected length. These products were recovered from the gel and sent to GeneWiz for sequencing.*

Sequencing Results Are Inconclusive. Our first attempt at sequencing the genome extracted from the wax moth tissue was inconclusive. We sent our products to GeneWiz, but they could not be sequenced, leading the company to believe there was an issue with our primers. To test this, we repeated the DNA recovery process using our CODEHOP primers. Using a FlashGel, we performed gel electrophoresis, and the bands produced were the expected length (Figure 7). We recovered this amplicon and sent it to GeneWiz to be sequenced, from which we are currently awaiting results.

Discussions

During the initial experiments, one of our first findings was that our DNA needed to be purified before we could run PCR, because we found that our silkworm primers did not work on the wax moth genome, possibly because there was a PCR inhibitor in the wax moth tissue. To fix this problem, we used a purification kit to extract the genome—this was successful.

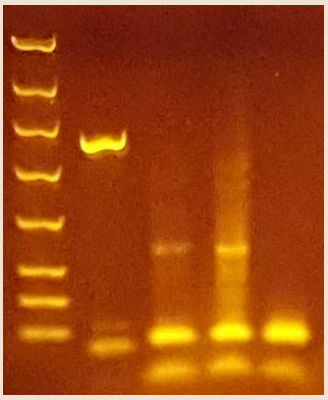

Figure 6. *This experiment tested our new CODEHOP primer design and whether it can successfully amplify the targeted PBAN gene of the wax moth. The first well contains the 100-4,000 bp DNA markers. The second well contains silkworm primers with the silkworm genome, as a positive control. Well 3 holds the silkworm genome with the new CODEHOP primers, and well 4 holds the wax moth genome with the newly designed CODEHOP primers. Well 5 contains the CODEHOP primers with PCR mix only, and well 6 contains the new primers with distilled water as negative controls. The bands of similar length produced by the silkworm and the wax moth indicate success in targeting the PBAN gene in the wax moth with our redesigned CODEHOP primers. The difference in length between the primers with the wax moth and silkworm genome and the primers without a genome present confirms that these new primers are not amplifying themselves, as was suspected with our previous CODEHOP primer design. This proves success in locating and amplifying the PBAN gene of the wax moth.*

Later, we designed CODEHOP primers, which appeared to be successful in amplifying the wax moth PBAN gene.

We recovered our silkworm and wax moth PCR products to be sent to GeneWiz for sequencing. Our first attempt at this produced inconclusive results, so we repeated the DNA recovery process to find what went wrong and sent the new amplicons back for sequencing. Our next step is to determine whether we located the PBAN gene in the wax moth. From there, we can begin to produce PBAN in *E. coli* and design our trap, which will induce wax moths to release pheromones, attracting even more wax moths to the trap and away from honeybee hives. Once we find the wax moth pheromone gene, our plan is to produce the PBAN protein in *E. coli*. The protein will be incorporated into our trap in a sugar water mixture that the moths will consume. This will trigger production of pheromones, attracting more wax moths to the trap. We do not yet know what concentration is needed or how sensitive the moths are to the pheromones, but we will hopefully have these answers soon. Lastly, we will place these traps near existing hives in order to protect the honeybees from the wax moths.

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