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Quorum Sensing in Vibrios and Cross-Species Activation of Bioluminescence Lux Genes by Vibrio Harveyi LuxR in an Arabinose-Inducible Escherichia Coli Expression System

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QUORUM SENSING IN *VIBRIOS* AND CROSS-SPECIES ACTIVATION OF BIOLUMINESCENCE *LUX* GENES BY *VIBRIO HARVEYI* LUXR IN AN ARABINOSE-INDUCIBLE *ESCHERICHIA COLI* EXPRESSION SYSTEM

by

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ABSTRACT

QUORUM SENSING IN *VIBRIOS* AND CROSS-SPECIES ACTIVATION OF BIOLUMINESCENCE *LUX* GENES BY *VIBRIO HARVEYI* LUXR IN AN ARABINOSE-INDUCIBLE ESCHERICHIA EXPRESSION SYSTEM

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Anne Marie Wannamaker

The University of Wisconsin-Milwaukee, 2013
Under the Supervision of Dr. Charles Wimpee

Bacterial bioluminescence is observed in over twenty known species, primarily in the family *Vibrionaceae*. However, only *Vibrio fischeri* and *Vibrio harveyi* bioluminescence expression mechanisms are well studied. In *V. harveyi,* expression of the *lux* operon is activated by the transcription factor LuxR ($LuxR_{VH}$), resulting in bioluminescence. Homologs of $LuxR_{VH}$ in other *Vibrio* species have been shown to regulate transcription of a variety of genes. Three parallel quorum sensing pathways co-regulate the expression of $LuxR_{VH}$. The first objective was to assess possible quorum sensing regulation of *lux* operon expression in *V. cholerae*, *V. chagasii, V. orientalis*, and *V. vulnificus* using *V. harveyi* as the control. Secondly, cross-species induction of bioluminescence by $LuxR_{VH}$ was tested on the aforementioned *Vibrio lux* operons using an *Escherichia coli* dual vector expression system. This was accomplished by first generating a plasmid with the *V. harveyi luxR* gene (*$luxR_{VH}$)* driven by an arabinose promoter. Secondly, individual *E. coli* systems had one of the five *Vibrio* species-specific *lux* operons cloned on a separate plasmid. Luminescence was assayed qualitatively on plates, and quantitatively using a

luminometer. Relative light per cell was calculated by dividing measured light by OD_{600}. Relative light assays showed quorum sensing regulation of the *lux* operon in all five *Vibrio* species. Quantitative *V. harveyi lux* operon expression assays in the *E. coli* dual vector systems showed significant increase in light production in samples provided with arabinose. This demonstrated that induction of $luxR_{VH}$ by arabinose resulted in upregulation of *lux* genes. The level of $LuxR_{VH}$ activation of other *Vibrio lux* operons reflects the distance of evolutionary relationships to *V. harveyi.* The most induction was seen with the *V. vulnificus lux* operon followed by *V. orientalis*, *V. chagasii*, and finally *V. cholerae*. This implies conservation of the *lux* operon regulatory mechanism between closely related species and suggests that the studied *Vibrios* utilize $LuxR_{VH}$-type transcription factors. The known $LuxR_{VH}$ homologs, SmcR (*V. vulnificus*) and HapR (*V. cholerae*) were suspected *lux* activators. In addition, other $LuxR_{VH}$-type regulators are now implicated in the other *Vibrio* species. Furthermore, mechanistic conservation of these transcription factors implies regulation by *V. harveyi*-type quorum sensing.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF ABBREVIATIONS

Abbreviation	Meaning	Page
$luxR_{VF}$	*Vibrio fischeri* regulatory gene *luxR*	6
$LuxR_{VF}$	*Vibrio fischeri* LuxR protein	6
$luxR_{VH}$	*Vibrio harveyi* regulatory gene *luxR*	6
$LuxR_{VH}$	*Vibrio harveyi* LuxR protein	6
GCG	Genetics Computer Group	19
BLAST	Basic Local Alignment Search Tool	20
MCMC	Markov Chain Monte Carlo	23
LB	Luria Bertani	24
SWC	Sea water complete	24
OD	Optical density	26
PCR	Polymerase chain reaction	27
araluxR	*araC/ara* promoter region fused to *Vibrio harveyi luxR* gene	28
$pLUX_{VH}$	Plasmid pLUX containing *Vibrio harveyi lux* operon	31
$pLUX_{VCha}$	Plasmid pLUX containing *Vibrio chagasii lux* operon	31
$pLUX_{VCho}$	Plasmid pLUX containing *Vibrio cholerae lux* operon	31
$pLUX_{VO}$	Plasmid pLUX containing *Vibrio orientalis lux* operon	31
$pLUX_{VV}$	Plasmid pLUX containing *Vibrio vulnificus lux* operon	31
$pLUX_{Species}$	Collective term for pLUX plasmids: pLUX containing either the *Vibrio harveyi*, *Vibrio chagasii, Vibrio cholerae, Vibrio orientalis*, or *Vibrio vulnificus lux* operons.	31

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Introduction

Bioluminescence

Bioluminescence is the natural ability for an organism to enzymatically produce light and can be used as a unique phenotype for studying gene regulation. Light production is highly regulated as a result of the process being energetically demanding; using up to 10% of the total cellular energy (52). This energy is utilized in order to produce enzymes required for light production. It is an investment for an organism to produce light; however, in relation to other cellular functions, such as growth or respiration rate, bioluminescence is well integrated and does not negatively affect these functions (82).

Bioluminescence being energetically expensive suggests that this process would occur under conditions that would be advantageous physiologically or ecologically to the organism. For example, a light organ is a mutually beneficial symbiotic relationship between luminescent bacteria and marine organisms like fish or squid. The host utilizes the light for finding and/or attracting prey or for predator evasion. The bacteria produces light for greater integration into the host and possibly for dispersal or survival; allowing a nutrient rich environment that is ideal for propagation before leaving the host (22, 46, 58, 65, 73, 83). Bioluminescence is thought to be a by-product of an enzymatic reaction utilized for removal of oxygen by anaerobic bacteria while atmospheric concentrations of oxygen rose during the Precambrian Great Oxygenation Event. The basis of the hypothesis is the high oxygen consumption of the

bioluminescence reactions, which consumes up to 20% of the cellular oxygen, and is thought to help maintain redox balance (23, 25, 64, 113). Later, the maintenance of bioluminescence would be encouraged by symbiotic relationships with higher organisms that developed with the evolution of light sensing organs. However, very few bioluminescent species form light organ symbiosis; the vast majority being planktonic. In this case, there should be little selective pressure towards conservation of functional bioluminescence genes, yet the genes are highly conserved (53, 110, 117, 122). According to Czyż, Wróbel and Węgrzyn, another possible theory is that the bioluminescence reactions arose in order to provide an internal, readily available light source for photoreactivation; the process where light is utilized to repair UV damaged DNA (18). An internal light source would be beneficial to DNA repair as it ensures that at least one DNA repair system will always be available. Ultimately, the specific function of bioluminescence is still under investigation.

Bioluminescent Bacteria

The most well studied bioluminescent bacteria are *Vibrio fischeri* and *Vibrio harveyi*; however, bioluminescent bacteria can be found in a variety of marine, freshwater, and terrestrial habitats living either as planktonic free-living organisms, saprophytic organisms, pathogenic parasites, gut or light organ symbionts (23, 44, 82). By far, the most luminescent species are found in the family Vibrionaceae, but species are found scattered throughout the γ-Proteobacteria class which includes genera such as *Vibrio*,

Photobacterium, *Photorhabdus*, and *Shewanella* (21). These genera have a common presence of conserved *lux* genes which are expressed in high levels during light production. In the majority of cases, the genes appear to be vertically inherited; however, there are some cases of lateral gene transfer (117, 53). Phylogenetic analysis of *luxA*, a bioluminescence gene, shows a *Vibrio* clade segregated from a *Photobacterium* clade. *Vibrio fischeri luxA* is not included in the *Vibrio* clade; it is more closely related to the *Photobacterium luxA* clade. The *Vibrio* clade includes all the major species used in this study (Figure 1).

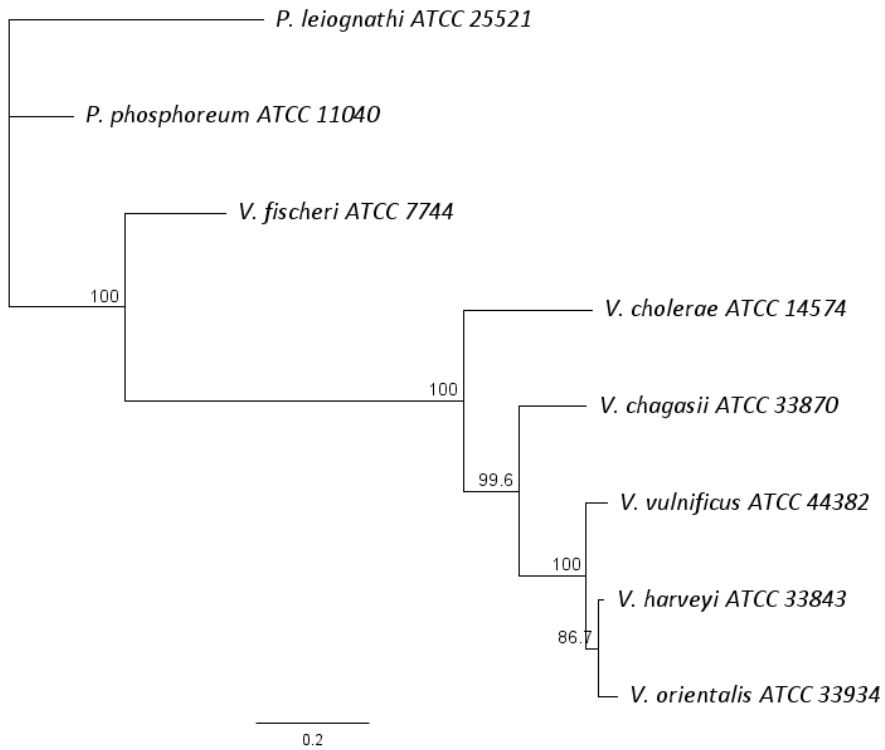


Figure 1: Phylogenetic analysis of bioluminescence gene *luxA*. The *luxA* tree was constructed using Bayesian Markov Chain Monte Carlo statistics with the parameters of General Time Reversible nucleotide substitution cost matrix (Geneious). *Photobacterium leiognathi* is specified as the outgroup. Consensus support percentage is listed at each node.

Sequence analysis investigating the family *Vibrionaceae* showed remnants of *lux* genes indicating non-luminous bacteria are more likely due to multiple losses than acquisition through lateral gene transfer (38, 53, 90, 91, 117). Additionally, the regulation of functional *lux* genes in the majority of bioluminescent bacteria remains unknown.

The *lux* operon

The *lux* genes can be divided into three categories: the core, accessory and regulatory genes (Figure 2, 3). The *lux* operon core genes always consist of five structural genes, *lux*CDABE, that encode enzymes required for light production (6, 23, 26-27, 66, 70, 73-74, 77, 84). The genes *luxA* and *luxB* encode the α and β subunits of the enzyme luciferase, respectively (3-4, 27). The genes *lux*C, lux*D,* and *luxE* respectively encode the reductase (r), acyl-protein synthetase (s), and acyl-transferase (t) polypeptides of a fatty acid reductase complex (9-10). Luciferase acts as an oxidative catalyst that drives the bioluminescent reaction in which oxidation of a reduced flavin ($FMNH_2$) and a long chain aldehyde (RCHO) result in a photon emission; the fatty acid reductase complex supplies the aldehyde for oxidation by luciferase (70, 72).

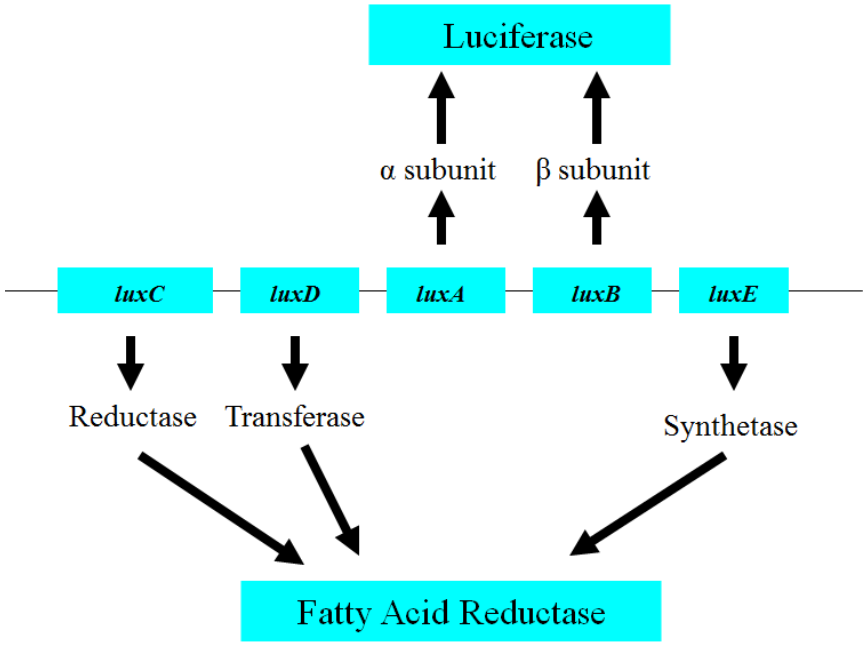


Figure 2: The *lux* operon core genes. The *lux* operon encodes the enzymes luciferase and a fatty acid reductase that are required for light production (89).

The accessory genes, which can be present or absent depending on the bacterial strain, are redundant genes that produce enzymes that can be found in other metabolic systems. In some species, such as *V. fischeri*, *V. harveyi* and *Photobacterium phosphoreum*, accessory genes are found as part of the *lux* operon following the *luxE* gene (108, 23). For example, LuxG, an enzymatic homodimer, supplies $FMNH_2$ to luciferase during the bioluminescent reactions and is homologous to the NAD(P)H-flavin oxidoreductase Fre of *Escherichia coli* (87, 128). LuxH, a 3,4-dihydroxy-2-butanone 4-phosphate synthetase (DHBP synthetase), is another accessory enzyme that is homologous to RibB of *E. coli* and involved in the biosynthesis of riboflavin; a crucial cofactor of FMN (97).

The regulatory genes control the expression of the *lux* operon (core genes). In *V. fischeri*, the gene *luxR* (*luxR*$_{VF}$), which is divergently transcribed and located upsteam of the *lux* operon, encode the transcriptional activator LuxR (LuxR$_{VF}$). LuxR$_{VF}$ is directly regulated by the signal molecule generated by LuxI. LuxI is a member of the core *lux* operon in *V. fischeri*; found upstream of *luxC* (20, 25-26, 37, 102, 105-106). In *V. harveyi*, the *luxR* gene (*luxR*$_{VH}$) is not located near the *lux* operon (NCBI Accession Number: NC_009784). The *V. harveyi* transcriptional activator LuxR (LuxR$_{VH}$) is not homologous to LuxR$_{VF}$ and is a member of the TetR protein family, whereas LuxR$_{VF}$ is the basis of the LuxR-LuxI protein family (33-34, 94-95, 104, 109). Unlike *V. fischeri*, LuxR$_{VH}$ is not directly regulated by a single signaling molecule, but is instead regulated by three parallel signaling protein cascades (48, 104, 107).

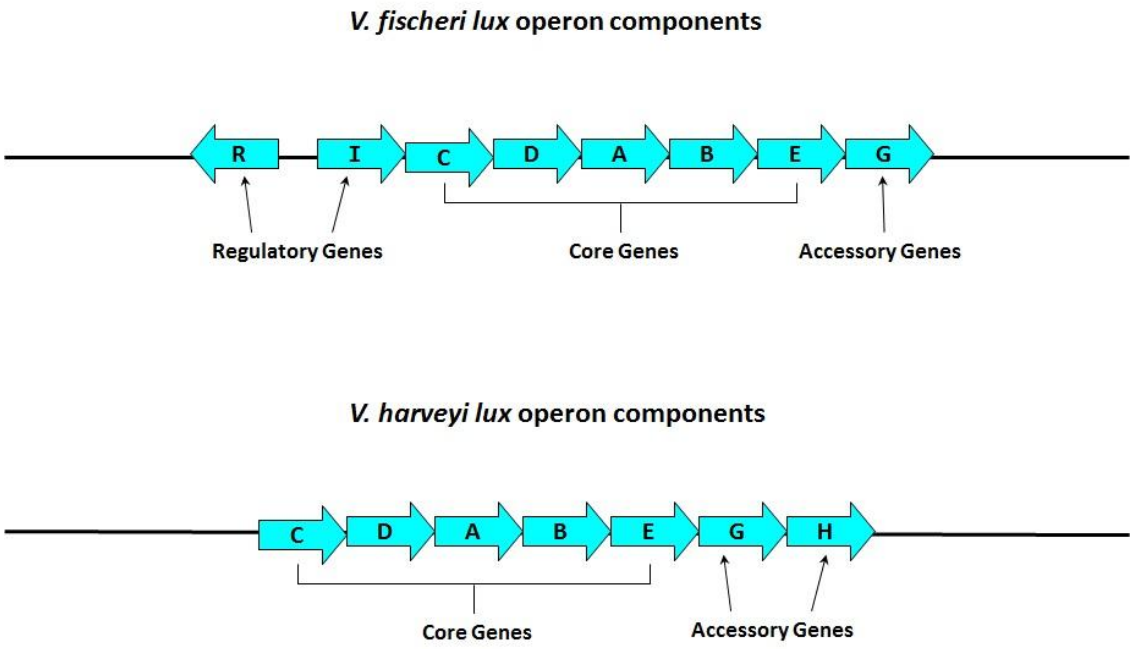


Figure 3: *Vibrio fischeri* and *Vibrio harveyi lux* genes. The *V. fischeri* and *V. harveyi lux* operons are shown with core, accessory, and regulatory genes as indicated.

Biochemistry of bioluminescence reaction

The fatty acid reductase is a multi-subunit complex consisting of multiple copies of LuxC, LuxD and LuxE (120). LuxE, the acyl-transferase subunit, shuttles fatty acids, specifically tetradecanoic acid that was intended for lipid biosynthesis, from the lipid pathway into the bioluminescent pathway. In the bioluminescence pathway, the tetradecanoic acid is first activated by LuxD, the synthetase subunit, forming a fatty acyl-AMP intermediate in an ATP-dependent reaction. This intermediate remains bound to the complex where it is reduced by LuxC, the reductase subunit, to an aldehyde, oxidizing one NADPH

molecule. This aldehyde is then recycled back into the system through oxidation by luciferase (10, 71-73, 83).

Luciferase is a heterodimeric (αβ) flavin monooxygenase that acts as a catalyst; driving the light-emitting reaction (3, 29). Luciferase acts on three substrate molecules: molecular oxygen, reduced flavin ($FMNH_2$) and an aliphatic long chain aldehyde (tetradecanal). Luciferase first binds to $FMNH_2$, then to O_2 and finally to the long chain aldehyde resulting in FMN, a fatty acid, and water as well as emission of a 490 nm blue-green photon. $FMNH_2$ is regenerated by a flavin oxidoreductase in the presence of NADPH (71-73). The bioluminescence reactions are illustrated in Figure 4.

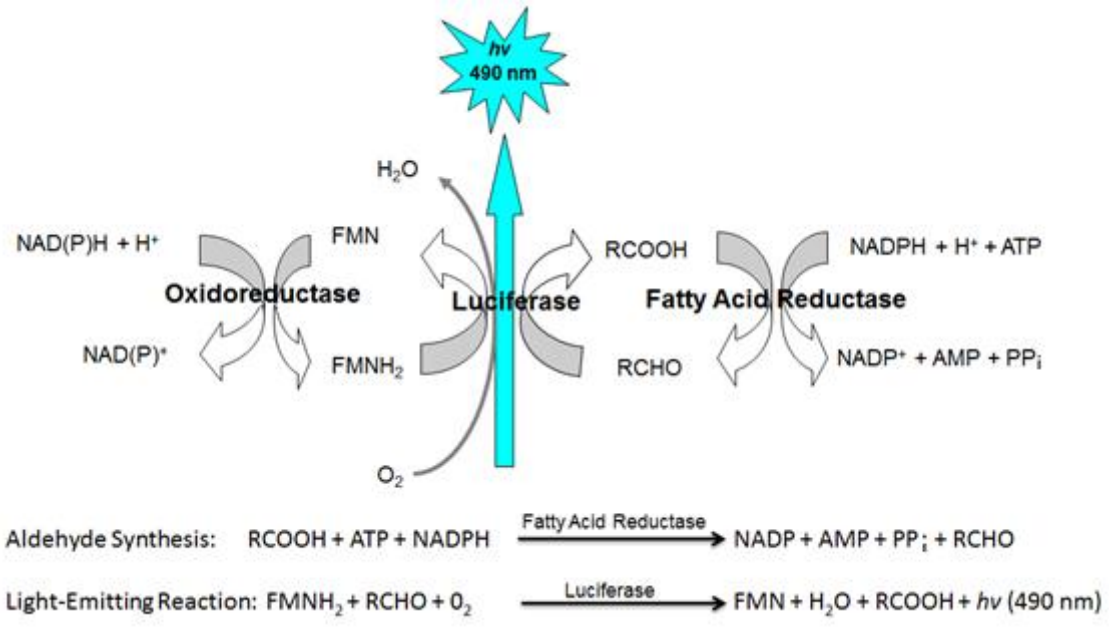


Figure 4: Bioluminescence reduction-oxidation reaction. Figure is adapted from B. Wimpee (125) and shows the aldehyde synthesis reaction on the right side, the light-emitting reaction in the middle, and the restoration of $FMNH_2$ on the left side. Overall enzymatic reactions are shown at the bottom.

Quorum Sensing: Regulation of the *lux* operon

Organisms rely on differential gene expression in response to their surrounding conditions. Quorum sensing is an auto-inductive regulatory mechanism that controls gene expression based on local cellular populations. Cellular populations are sensed indirectly by bacteria using small diffusible molecules called autoinducers. Autoinducers are made by the bacteria themselves and can be species-specific or used for interspecies communication (5-6, 48). The concentration of autoinducers controls gene regulation by acting as the population signal for the cell (24, 51, 75, 81, 83, 124, 127). Bacteria utilize quorum sensing to regulate a large variety of functions including biofilm formation, virulence factors, type III secretion factors, motility, sporulation, competence, fruiting body formation, and bioluminescence (2, 19, 42, 45, 47, 55, 57, 76, 80, 88, 92-93, 96, 105, 123, 129).

Vibrio fischeri Quorum Sensing

V. fischeri utilizes the quorum sensing regulation mechanism to control bioluminescence *lux* gene expression. $LuxR_{VF}$ is a 250 amino acid protein with an active domain containing a helix-turn-helix DNA binding domain and a regulatory domain that contains a binding site for a 3-oxo-hexanoyl homoserine lactone autoinducer, produced by LuxI (20, 43, 51, 99). The regulatory domain blocks the helix-turn-helix DNA binding domain until the autoinducer binds and allows a conformational change that exposes the

binding site. $LuxR_{VF}$ acts as an activator for the *lux* operon and a repressor for the *luxR*$_{VF}$ gene (20, 25, 34, 43, 102).

At low cellular density (Figure 5a), autoinducer concentration is low and $LuxR_{VF}$ is not bound by autoinducers. The *lux* operon is not transcribed and *luxR*$_{VF}$ is not repressed. As cell density increases there is an accumulation of $LuxR_{VF}$ and autoinducer concentration reaches a threshold. At high cellular density (Figure 5b), the autoinducers bind to the N-terminus of $LuxR_{VF}$. Once bound, the regulatory domain of $LuxR_{VF}$ no longer interferes with the active domain, freeing up the helix-turn-helix DNA binding domain. $LuxR_{VF}$, along with σ^{70}-RNA polymerase, binds to a consensus sequence called the *lux* box (ACCTGTAGGATCGTACAGGT) located upstream of *luxI*, and to the *luxI* promoter. The *lux* operon is expressed which increases LuxI, thereby increasing autoinducer concentration, forming a positive feedback loop. Bound $LuxR_{VF}$ also acts as a repressor for *luxR*$_{VF}$, creating a negative feedback loop and equalizing the system (21, 25, 37, 43, 75, 105-106).

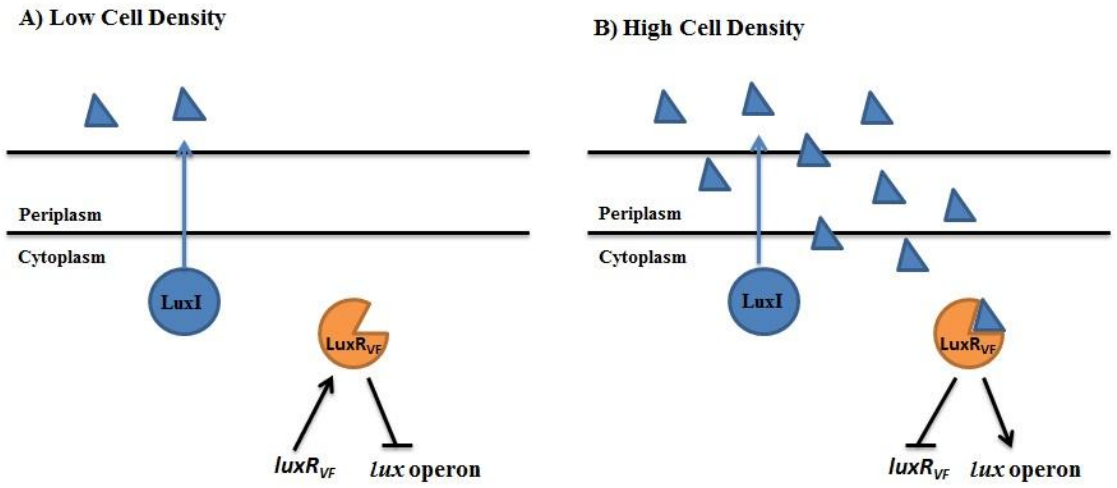


Figure 5: *Vibrio fischeri* quorum sensing system. A) At low cell density, $LuxR_{VF}$ does not repress or activate genes. B) At high cell density, $LuxR_{VF}$ acts as a repressor for $luxR_{VF}$ and an activator for the *lux* operon.

Vibrio harveyi Quorum Sensing

In *V. harveyi*, bioluminescence is under the regulation of three parallel quorum sensing pathways (7, 21, 48). The concentration of autoinducers, AI-1, AI-2 and CAI-1, indirectly regulates the translation of $luxR_{VH}$ mRNA through signaling cascades (6, 12, 25, 48, 81). The strongest autoinducer signal AI-1, N-(3-hydroxybutanoyl) homoserine lactone, is synthesized by LuxM and utilized only for intraspecific population sensing (6, 13, 99, 112). The second strongest autoinducer signal is AI-2, (2S,4S)-2-methyl-2, 3, 3, 4-tetrahydroxytetrahydro furan borate, and is an interspecies autoinducer synthesized by LuxS (14, 85, 100, 126). CAI-1, named after *Vibrio cholerae*, is a (S)-3-hydroxytridecan-4-one synthesized by CqsA and is the weakest signaling autoinducer in *V. harveyi* (49, 76). Extracellular concentrations of AI-1 and CAI-1 are recognized by the transmembrane

spanning sensor receptors LuxN and CqsS, respectfully. LuxP acts as the receptor for AI-2 and transduces the signal to the transmembrane LuxQ protein (14, 48, 76, 85). LuxN and LuxQ are hybrid two-component proteins that have the ability to bind ATP and autophosphorylate at a conserved histidine residue, ultimately passing the phosphate to a conserved aspartate residue. However, if bound by an AI-1 or AI-2, respectively, LuxN and LuxQ alternate to phosphatase activity using the same residues (32, 85, 112). The activity of the catalytic residues of all three sensing systems converges at the LuxU protein. The LuxU protein has a conserved histidine residue that is used to transfer phosphate between the sensing systems and a conserved aspartate residue of the downstream response regulator, LuxO (7-8, 30-32, 48, 78). LuxO is a σ^{54}- binding protein that regulates the transcription of small regulatory RNA molecules (sRNAs) (8, 36, 78). In *V. harveyi,* there are five quorum regulatory sRNAs, referred to as Qrr1-5. With the aid of the chaperone protein Hfq, Qrr1-5 form complementary base pairs with $luxR_{VH}$ mRNA blocking translation (60-61, 78, 114, 116).

At low cell densities (Figure 6a), autoinducer concentrations are low and binding sites on LuxN, CqsS and LuxP are unoccupied. Under this condition LuxN, CqsS and LuxQ bind ATP and autophosphorylate their conserved histidine residues; then pass the phosphate to the conserved aspartate on each respective protein. Any of these sensing systems can transfer the phosphate to LuxN at a histidine residue. LuxN activates LuxO by relaying its phosphate to an aspartate residue on LuxO, allowing LuxO to recruit σ^{54}. The LuxO- σ^{54} complex binds DNA, allowing transcription of Qrr1-5. Qrr1-5 sequester $luxR_{VH}$ mRNA by binding the ribosome binding site in the 5' untranslated region; blocking

translation. Without the transcriptional activator $LuxR_{VH}$, the *lux* operon is not transcribed and no light is produced (7, 48, 61, 107, 114).

At high cell densities (Figure 6b), the increased autoinducer concentration leads to greater binding frequency of each autoinducer with LuxN, CqsS and LuxP, respectively. When autoinducer is bound, these proteins cease binding ATP to perform autophosphorylation, leaving the aspartate residue unoccupied. Without the bound phosphate, this aspartate residue will remove the phosphate from the histidine of LuxN. In turn, LuxN dephosphorylates LuxO leaving it in an inactive form. Without active LuxO Qrr1-5 is no longer transcribed and not available to interfere with translation of $luxR_{VH}$. $LuxR_{VH}$ is now free to bind to the promoter of the *lux* operon expressing the enzymes needed for the bioluminescence reactions; thus, light is produced. $LuxR_{VH}$ is also thought to have a negative feedback loop on itself and through upregulation of Qrr2-4 transcription (115).

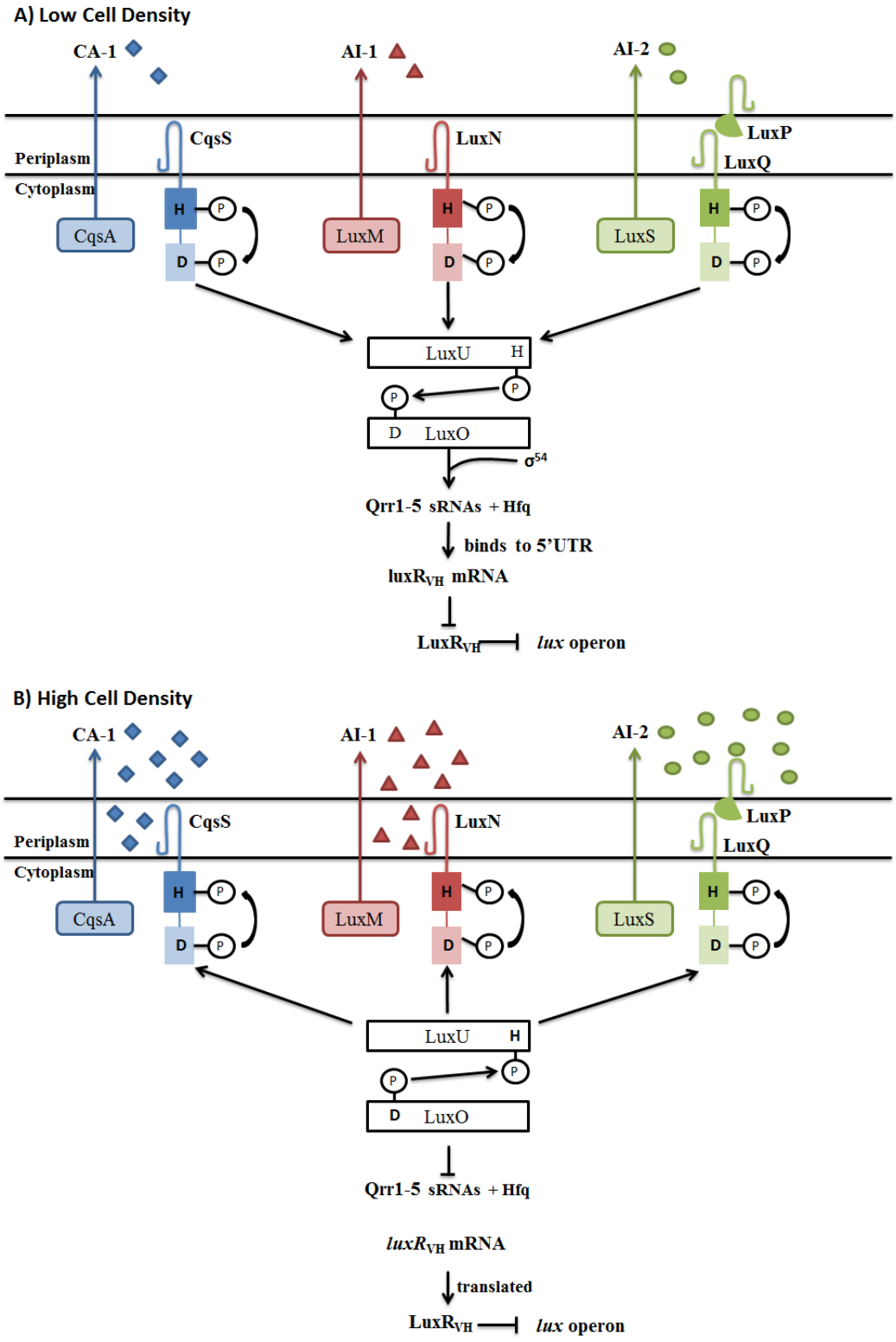


Figure 6: *Vibrio harveyi* quorum sensing. A) At low cellular density, kinase cascades repress the *lux* operon; no light production occurs. B) At high cellular density, phosphatase cascades allow activation of *lux* operon; light is produced.

Vibrio cholerae Quorum Sensing

An analogous quorum sensing regulation that shares many regulatory components with *V. harveyi* is found in *V. cholerae*. The CqsA/CqsS sensor system, originally discovered in *V. cholerae*, and the AI-2/LuxPQ/LuxS sensor system, both have analogous function to the systems of *V. harveyi*. LuxU and LuxO in either *V. harveyi* or *V.* cholerae also show analogous function. There is no AI-1/LuxN/LuxM sensor system found in *V. cholerae*; however, as in *V.* harveyi, a third parallel quorum sensing system seems to be present and acts directly on LuxO (49, 76, 86, 121).

HapR, a homolog of $LuxR_{VH}$, acts as a transcription factor downsteam of LuxO for virulence genes such as cholera toxin genes and genes involved in biofilm formation (42, 49, 54-55, 129). However, unlike $LuxR_{VH}$, HapR acts as a repressor not an activator for these virulence genes. HapR has been shown to bind to the *lux* promoter of *V. harveyi* activating *lux* gene expression (50, 121, 129); however, it has not yet been shown to be the activator of the *V. cholerae lux* operon. HapR is also regulated by Qrr sRNA molecules, however; only four are found in *V. cholerae* (76*)*.

As in *V.* harveyi, under low cellular density (Figure 7a) the autoinducer concentration is low and they are not found bound to either CqsS or LuxP. This allows CqsS and LuxQ to autophosphorylate and act on LuxU. LuxU then activates LuxO by transferring a phosphate and LuxO in turn will activate the transcription of the Qrr1-4 sRNAs genes. These sRNAs will sequester HapR and virulence genes are expressed. Under high

cellular density (Figure 7b), autoinducers are more available to bind to the proper receptors and autophosphorylation does not occur. The signal transduction is reversed where LuxU inactivates LuxO and CqsS/LuxQ inactivates LuxU. With LuxO inactive the Qrr1-4 sRNAs are not synthesized and therefore unable to bind and block translation of HapR. HapR is now available to act as a repressor at promoters of virulence genes; thus, blocking their transcription (49, 76, 86).

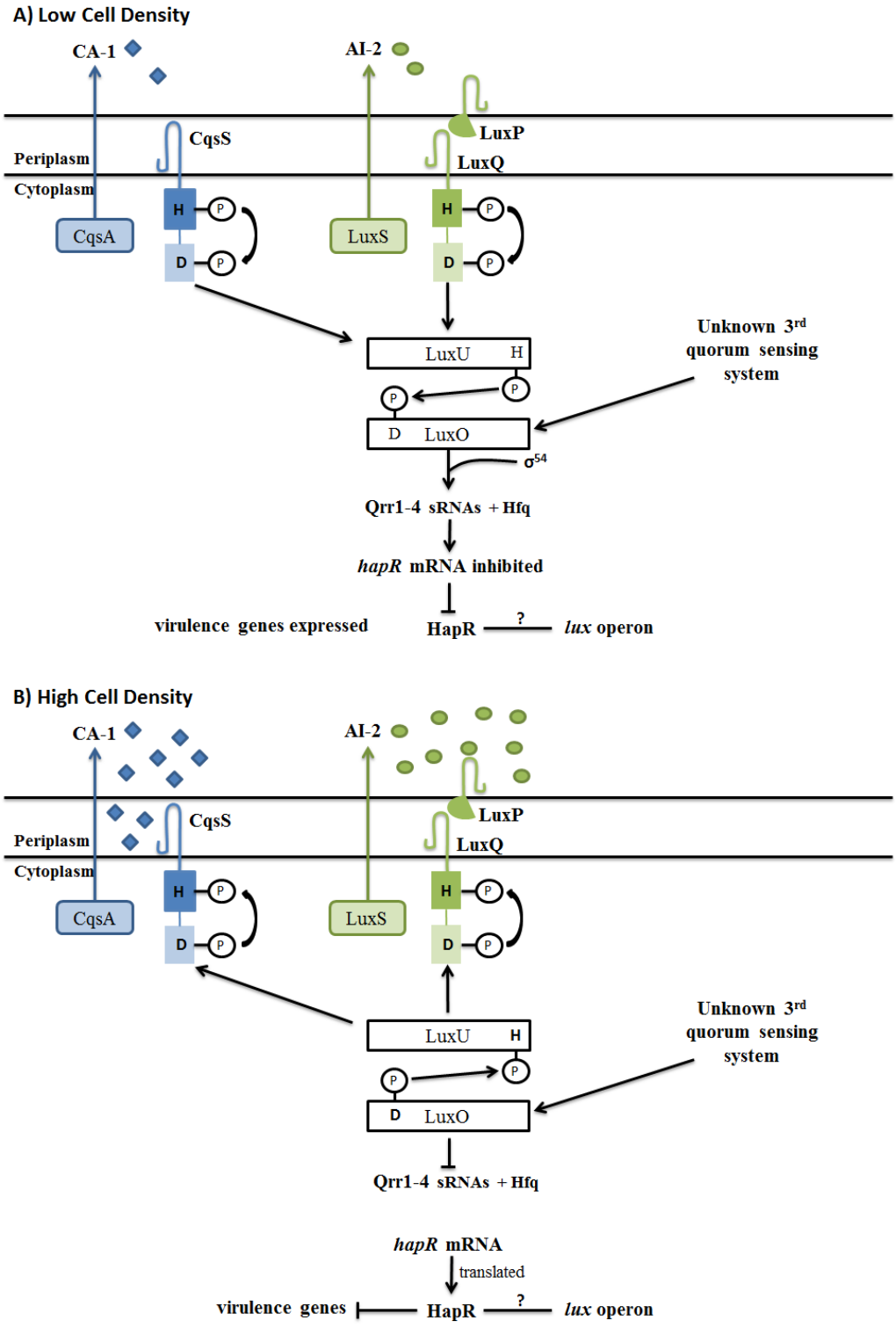


Figure 7: *Vibrio cholerae* Quorum Sensing. A) Virulence gene regulation cascades under low cell density. B) Virulence gene regulation cascades under high cellular density. Effects of HapR on lux operon are unknown.

LuxR$_{VH}$ Induction

Previously, a construct was made to bypass the quorum sensing systems of *V. harveyi* in order to directly induce transcription of *luxR*$_{VH}$ using the *lac* promoter. This construct, along with a second non-competitive vector containing a species-specific *lux* operon, were cloned into *E. coli*; however, using the *lac* promoter to drive transcription of *luxR*$_{VH}$, led to light production in some uninduced samples. The arabinose promoter along with the AraC protein is a more strongly repressible system (Figure 8) that is utilized for the current study to regulate *luxR*$_{VH}$ gene expression (41, 104).

In the absence of arabinose, the AraC proteins form a dimer that binds to the O_2 and I_1 DNA sites, physically bending the DNA, and blocks the access of RNA polymerase; thus, blocking transcription of the downstream gene (11, 59, 62, 101). When arabinose is present, it binds to the N-terminals of the AraC dimer, resulting in a conformational change that opens up a higher binding affinity site for I_2. The dimer then binds to this site and acts as a transcriptional activator for the gene by promoting RNA polymerase binding (11, 59, 101). For this study, the downstream gene that will be regulated by the *ara* promoter is the *luxR*$_{VH}$ (Figure 8).

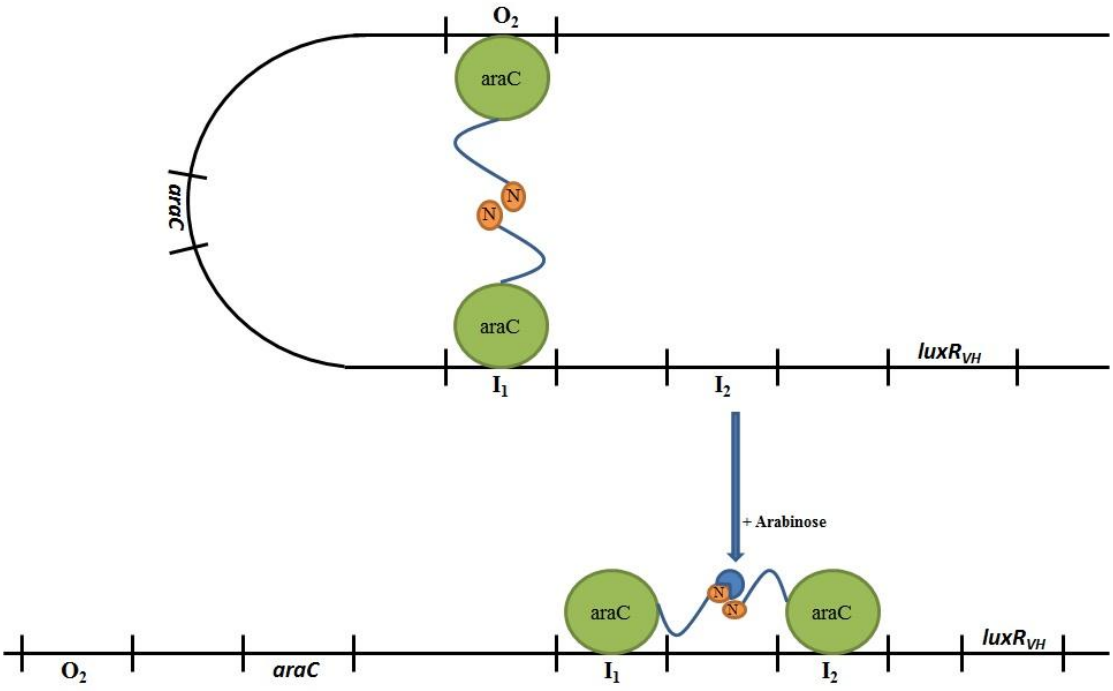


Figure 8: Arabinose promoter control of *luxR$_{VH}$*. The *ara* promoter shown in the repressed conformation at top. The regulon, AraC, alternates binding to activate lux expression in presence of arabinose.

Homologs of LuxR$_{VH}$

It is known that the homologs of LuxR$_{VH}$, HapR (*Vibrio cholerae*), OpaR (*Vibrio* parahaemolyticus), SmcR (*Vibrio vulnificus*), VanT (*Vibrio anguillarum)* and LitR (*Vibrio fischeri*), upregulate transcription of a variety of genes (17, 29, 50, 68-69, 103). McCarter explored whether OpaR of *V. parahaemolyticus*, a nonluminous species, was a homolog of LuxR$_{VH}$ (68). Using Genetics Computer Group (GCG) BestFit analysis, McCarter found that OpaR, a regulator for opacity genes, showed a 96% identity to LuxR$_{VH}$ and a 72% identity to HapR, indicating homology. Croxatto and colleagues also used GCG BestFit analysis to determine if VanT, a regulator for a variety of genes such as

metalloprotease and biofilm formation, was also a homolog of $LuxR_{VH}$ and found an 81% identity to $LuxR_{VH}$, a 74% identity to HapR, and an 82% identity to OpaR, indicating homology (17). LitR of *V. fischeri* has an unknown role as a transcriptional activator for bioluminescence as well as symbiotic colonization and was found to also be a homolog for $LuxR_{VH}$. Using Basic Local Alignment Search Tool (BLAST), DNA Strider and Vector NTI Suite software, Fidopiastis and colleagues aligned the sequences of homologs and showed that LitR has 58-60% identity to $LuxR_{VH}$, HapR, OpaR, and SmcR (28).

Jobling and Holmes investigated HapR, a regulator for hemagglutinin protease, using BLAST to compare open reading frames to $LuxR_{VH}$, and found that HapR was 71% identical to $LuxR_{VH}$ and the DNA binding domain was 95% identical to DNA binding domain of $LuxR_{VH}$ (50). This study also demonstrated that HapR and $LuxR_{VH}$ are functionally interchangeable. Experiments showed that $LuxR_{VH}$ will bind the *hap* promoter and express the gene and HapR will bind to the *lux* promoter and express the operon genes of *V. harveyi*. Shao and Hor investigated another potential $LuxR_{VH}$ homolog, SmcR, which regulates a metalloprotease gene (103). Using BLAST and GenBank database, Shao and Hor found a 93% identity to $LuxR_{VH}$, a 93% identity to OpaR, and a 78% identity to HapR, also indicating homology. Shao and Hor were also able to show functional similarity between SmcR and $LuxR_{VH}$ by using SmcR to bind and activate the *lux* operon of *V. harveyi* in a $LuxR_{VH}$ deficient *E. coli* surrogate system.

Collectively, these studies show homology between *Vibrio* transcription factors, which indicates that there may be conservation of *lux* operon activation in bioluminescent

species (5, 17, 29, 50, 68-69, 103). Some of these studies demonstrated that the homologs of $LuxR_{VH}$, specifically HapR and SmcR, will bind and activate *V. harveyi lux* operon expression in *E. coli*. To further support the idea of activation conservation of the *lux* operon, this study will use inducible $LuxR_{VH}$ to activate the *lux* operon of five different species from the *Vibrio* genus: *harveyi*, *chagasii*, *cholerae*, *orientalis*, and *vulnificus.*

Hypothesis

This study investigates whether *V. harveyi*-type quorum sensing is found in other *Vibrio* species including: *V. chagasii, V. cholera, V. orientalis,* and *V. vulnificus*. To test this I will first determine whether these *Vibrio* species undergo quorum sensing regulation of their *lux* operons. Secondly, I will test cross-species induction of bioluminescence by $LuxR_{VH}$ transcriptional activation of other *Vibrio* species *lux* operons. Mechanistic conservation will indicate that *V. harveyi*-type quorum sensing may be taking place which will demonstrate specific gene conservation among *Vibrio* species.

Methods and Materials

Phylogenetic Analysis and Sequence Alignment

The *luxA* DNA and LuxR homolog amino acid sequences were imported into Geneious version 6.1.2 bioinformatics software application for analysis (35). The phylogenetic tree based on the *luxA* gene in Figure 1 was generated using the MrBayes plugin which performs Markov Chain Monte Carlo (MCMC) probabilistic tree generation (1, 98). The parameters specified are the General Time Reversible nucleotide substitution cost matrix and a chain length of 1,100,000 with an initial burn-in period of 100,000 (110). Three replicates were run using these parameters, in addition to three replicates using Maximum Likelihood with the PhyML plugin (39-40). All runs resulted in the same phylogenetic topography. Amino acid sequence alignments were performed using the ClustalW plugin within Geneious (56). Amino acid alignment used a BLOSUM 62 substitution cost matrix with a gap open cost of 10 and gap extension cost of 1. BLAST was used to identify a potential $LuxR_{VH}$-type homolog in the sequenced *V. orientalis* ATCC 33934 genome (79).

Bacterial Strains

All bacterial strains used in this study are found in Table 1. *Vibrio harveyi* ATCC 33843 was the positive control for this study and the strain used for the *$luxR_{VH}$ gene*. The *lux* operons used in this study were previously cloned into plasmid pGEM-3Z and propagated in *Escherichia coli* Top 10 cells (Dr. Charles Wimpee laboratory,

unpublished). The *lux* operons used in the cloning were derived from the following *Vibrio strains*: *V. harveyi* ATCC 33843*, V. chagasii* ATCC 33870, *V. cholerae* ATCC 14547, *V. orientalis* ATCC 33934, and *V. vulnificus* ATCC 43382. The same *Vibrio* strains were used for the quorum sensing assays. Top 10 *E. coli* was used for cloning as well as *E. coli* qualitative plate and *E. coli* maximum light output assays in this study.

Media

E. coli strains as well as *Vibrio cholerae* were grown in Luria Bertani (LB) medium either as broth [per Liter: 10g bacto-tryptone, 5g yeast extract, 10g NaCl; with aeration at 37°C, or on plates (addition of 15g/L of Agar)] incubated at 37°C. Media used for induction of *luxR*$_{VH}$ expression also contained 0.2% arabinose (L(+)-Arabinose 99+%; ACROS Organics; NJ) (0.4g/100ml media). Chloramphenicol was made in a 1000x stock solution at 25mg/ml in 100% ethanol and stored at -20°C. Ampicillin was made in 1000x stock solution at 100mg/ml in sterile water stored at -20°C. For plate and broth assays the appropriate antibiotic(s) were added to final concentrations of 25µg/ml (chloramphenicol) and 100µg/ml (ampicillin).

All other *Vibrios* were grown in Sea Water Complete (SWC) broth [per Liter: 375ml 2x Artificial Sea Water (per Liter: 58.44g NaCl, 10.15g $MgCl_2$, 12.3g $MgSO_4 \cdot 7H_2O$, 1.49g KCl, 5g peptone, 3g yeast extract, 3ml glycerol, 622 ml dH_2O)] with aeration at room temperature, or on plates (addition of 15g/L of Agar to SWC) kept at room temperature.

NZY recovery medium [per Liter: 5g NaCl, 2g $MgSO_4 \cdot 7H_2O$, 5g yeast extract, 10g NZ amine (Sigma; St. Louis, MD, adjusted to pH 7.5 using NaOH] was used after *E. coli* transformation for cell recovery.

Gel Electrophoresis and Imaging

All DNA electrophoresis gels contained 1% agarose in 1x TAE buffer (0.04M Tris-acetate, 0.001M EDTA) with 1µg/10ml Ethidium Bromide (Promega; Madison, WI). All gels were loaded with a 1KB ladder (Promega) for size comparison. Gels were photographed using a Kodak Gel Logic 100 imaging system (Eastman Kodak Company; Rochester, New York).

All photographs taken of plates to capture bioluminescence were achieved by using GeneSnap 7.12 (SynGene Image Acquisition Software; Fredrick, MD). The settings for pictures taken in the light were: 55 msec exposure, high resolution, no filter, with upperwhite light. The settings for pictures taken in darkness were: 30 min exposure, high resolution, no filter, with no light.

Table 1: Bacterial strains used in this study

Species	Strain
Escherichia coli	Top 10
Vibrio harveyi	ATCC 33843
Vibrio chagasii	ATCC 33870
Vibrio cholerae	ATCC 14547
Vibrio orientalis	ATCC 33934
Vibrio vulnificus	ATCC 43382

Quorum Sensing *Vibrio* Assays

Vibrios were inoculated into 3ml of SWC broth (LB for *V. cholerae*) and allowed to grow in a shaker at 200 rpm overnight at 25°C. Cultures were diluted 1:50 into fresh 50ml of SWC, or LB broth (*V. cholerae*), and kept in the shaker at 200 rpm throughout the experiment. An aliquot was removed at hourly intervals and measured for optical density at 600nm (OD_{600}) and light output using BioPhotometer plus (Eppenndorf; Hamburg, Germany) and Lumac Biocounter M 2010 (3M; St Paul, MN), respectively. Growth and light curves were generated for each sample and approximate light per cell (Relative Light) was calculated. Relative light was calculated by dividing Light by OD_{600} for each hour time point. Relative light was graphed against time for quorum sensing graphs. Individual growth and light curves were generated using Excel (Microsoft Office 2010).

PCRs and Construct Generation

Each polymerase chain reaction (PCR) was performed using a Bio-Rad DNA Engine Thermal Cycler (Bio-Rad Laboratories; Hercules, CA). All PCR products were purified using a QIAquick PCR Purification Kit per manufacturer's protocol (QIAGEN; Hilden, Germany). For amplification of the *araC/ara* promoter region from pBAD-GFP_{uv} (Figure 11) and $luxR_{VH}$ from the *V. harveyi* genome, amplification PCRs were performed.

Amplification PCRs were carried out using the following reaction mix:

1 µl of template DNA

1 µl of forward primer

1 µl of reverse primer

7 µl of dH_2O

10 µl of 2X Phusion Master Mix HF (Finnzymes; Vantaa, Finland)

Crossover PCR was performed to fuse amplification PCR products using:

1 µl of amplification PCR product 1 (*araC/ara* promoter region)

1 µl of amplification PCR product 2 ($luxR_{VH}$ gene)

1 µl of forward primer

1 µl of reverse primer

6 µl of dH_2O

10 µl of 2X Phusion Master Mix HF

Colony PCR was carried out using a small amount of a colony and the following reaction mix:

1 µl of forward primer

1 µl of reverse primer

8 µl of dH_2O

10 µl of GoTaq Green 2x Master Mix (Promega)

All PCRs used the same parameters:

95°C	5 min	
98°C	30 sec	30 cycles
50°C	10 sec	
72°C	1 min	
72°C	7 min	

All PCR products were verified by gel electrophoresis. Crossover PCR product (*araluxR* insert) was additionally verified by sequencing through the University of Chicago CRC DNA Sequencing Facility (Chicago, Illinois).

The plasmid pLS6 (Figure 16), a pACYC- type plasmid that confers chloramphenicol resistance, was first linearized by a blunt end restriction digest at a Sma1 restriction site within the *lacZ* cassette using the following protocol (119):

5 µl of purified pLS6

2 µl of 10X buffer

11 µl of dH_2O

2 µl of Sma1 (Promega)

The cut pLS6 plasmid was then dephosphorylated using temperature sensitive alkaline phosphatase (Promega) and ligated with the *araluxR* insert using the following ligation mix:

1 µl pLS6 plasmid

5 µl 2X buffer

1 µl T4 DNA ligase (Promega)

3 µl *araluxR* insert

The ligation of pLS6 with the *araluxR* insert results in the pARA-LUXR plasmid.

Transformation

Top 10 *E. coli* cells were made competent by the $CaCl_2$ method (15). Transformation of pARA-LUXR plasmid was performed using the following protocol:

Mixed 5 µl of the above ligation mix with 50 µl of competent *E. coli* cells.

Immediately incubated on ice for 30min

Incubated in 42^{O}C water bath for 30 sec

Incubated on ice again for 2 min

Added 250 µl of NZY recovery medium

Incubated at 37°C for 1 hr

To confirm successful transformation, blue and white screening was carried out by plating 100µl of recovered cells, along with 50 µl each of the inducer isopropyl β-D-1-thiogalactopyranoside and the indicator x-gal (5-bromo-4-chloro-indolyl-β-D-galactopyranoside) (Promega), onto LB agar plates containing chloramphenicol. Plates were incubated overnight at 37°C.

White resulting colonies were selected for colony PCR. Colony PCR products were verified by gel electrophoresis for presence of pARA-LUXR. The chosen colony containing pARA-LUXR was re-streaked for isolation onto LB agar plates with chloramphenicol and allowed to grow overnight at 37°C. For further transformation with a second plasmid, cells were made competent by the $CaCl_2$ method (15).

The non-competitive pLUX plasmid containing one of five species-specific *lux* operons was isolated from Top 10 *E. coli* (Dr. Charles Wimpee Laboratory). The species-specific plasmid confers ampicillin resistance and contains the *lux* operon from either *V. harveyi*, $pLUX_{VH}$, *V. chagasii,* $pLUX_{VCha}$, *V. cholerae*, $pLUX_{VCho}$, *V. orientalis*, $pLUX_{VO}$, or *V. vulnificus*, $pLUX_{VV}$. Collectively, this group of plasmids will be referred to as $pLUX_{Species}$. Each of the five $pLUX_{Species}$ plasmids were purified from *E. coli* using Qiaprep Spin Miniprep per manufacturer's protocol.

Transformation of individual $pLUX_{Species}$ into competent cells containing the pARA-LUXR plasmid was performed using the protocol described above, with the exception of replacing the initial 5 µl ligation mix for 1µl of a 1:10 dilution of a purified $pLUX_{Species}$ miniprep product.

Top 10 *E. coli* were made competent and transformed with one of the five $pLUX_{Species}$ plasmids using the protocols described above to generate a $pLUX_{Species}$ control for individual *lux* operons. For the $pLUX_{Species}$/pLS6, Top 10 *E. coli* were made competent and transformed with one of the five $pLUX_{Species}$ plasmids and the purified pLS6 using the previously cited protocols. Test and control samples are found in Table 2.

Table 2: Test and Control Samples

Sample	Contents
Test Strains pARA-LUXR/pLUX$_{Species}$	pLUX$_{Species}$ and pARA-LUXR plasmids
pLUX$_{Species}$ control	pLUX$_{Species}$ plasmid
pLUX$_{Species}$ /pLS6 control	pLUX$_{Species}$ and pLS6 plasmids

E. coli Plate Assays

The five sets of transformed pARA-LUXR/pLUX$_{Species}$ test strains and pARA-LUXR/pLS6 controls were grown overnight at 37°C on LB agar plates containing both chloramphenicol and ampicillin with or without 0.2% arabinose. The five individual pLUX$_{species}$ controls were grown on LB agar plates in the presence of only ampicillin. Induced plates had arabinose present while uninduced plate were absent of arabinose. *E. coli* was streaked onto an induced and an uninduced plate and grown overnight at 37°C. Plates were checked for the presence of light in a dark room and photographed in order to capture light production.

E. coli Maximum Light Output Assays

Maximum light output assays were performed on triplicate samples of *E. coli* for each of the six conditions: induced and uninduced pARA-LUXR/pLUX$_{Species}$, induced and

uninduced $pLUX_{Species}$ control, and induced and uninduced $pLUX_{Species}$/pLS6 control. *E. coli* was inoculated into 3ml of LB broth and shaken at 200 rpm overnight at 37°C. LB contained both ampicillin and chloramphenicol antibiotics for the pARA-LUXR/$pLUX_{Species}$ and $pLUX_{Species}$/pLS6 control conditions and only ampicillin for the $pLUX_{Species}$ control condition

All samples of overnight cultures were diluted 1:50 into 50ml of fresh LB broth and shaken at 200 rpm at 37°C throughout the duration of the experiment. Growth and light readings were taken over a period of six hours using the same procedure as the quorum sensing *Vibrio* assays. The maximum light output for each sample was determined by Relative Light (light/OD_{600}) at hour four. Individual light and growth curves were generated as well as average maximum light output graphs.

Results

Quorum Sensing *Vibrio* Assays

V. harveyi, *V. chagasii*, *V. orientalis*, and *V. vulnificus* naturally produced light when grown on SWC plates as did *V. cholerae* when grown on LB plates (Figure 9).

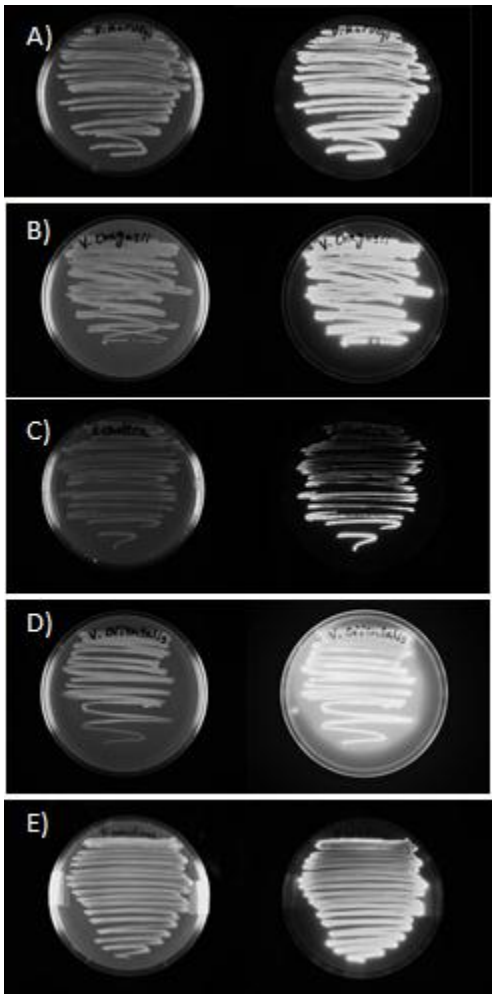


Figure 9: *Vibrio* Bioluminescent Plates. Frames of photographed plates are of the following *Vibrios*: A) *harveyi* B) *chagasii* C) *cholerae* D) *orientalis* E) *vulnificus*. Photographs taken with white light are on the left and photographs taken in darkness with a 30 min exposure time are on the right.

Relative light calculations for individual samples are found in Appendix A. Relative light versus time graphs resulted in a "U" shaped curve for each species in all replicate samples. *V. harveyi* showed the highest overall relative light followed by *V. cholerae*, *V. orientalis*, *V. chagasii,* and *V. vulnificus* (Figure 10).

Figure 10A: *Vibrio harveyi* Quorum Sensing Graphs

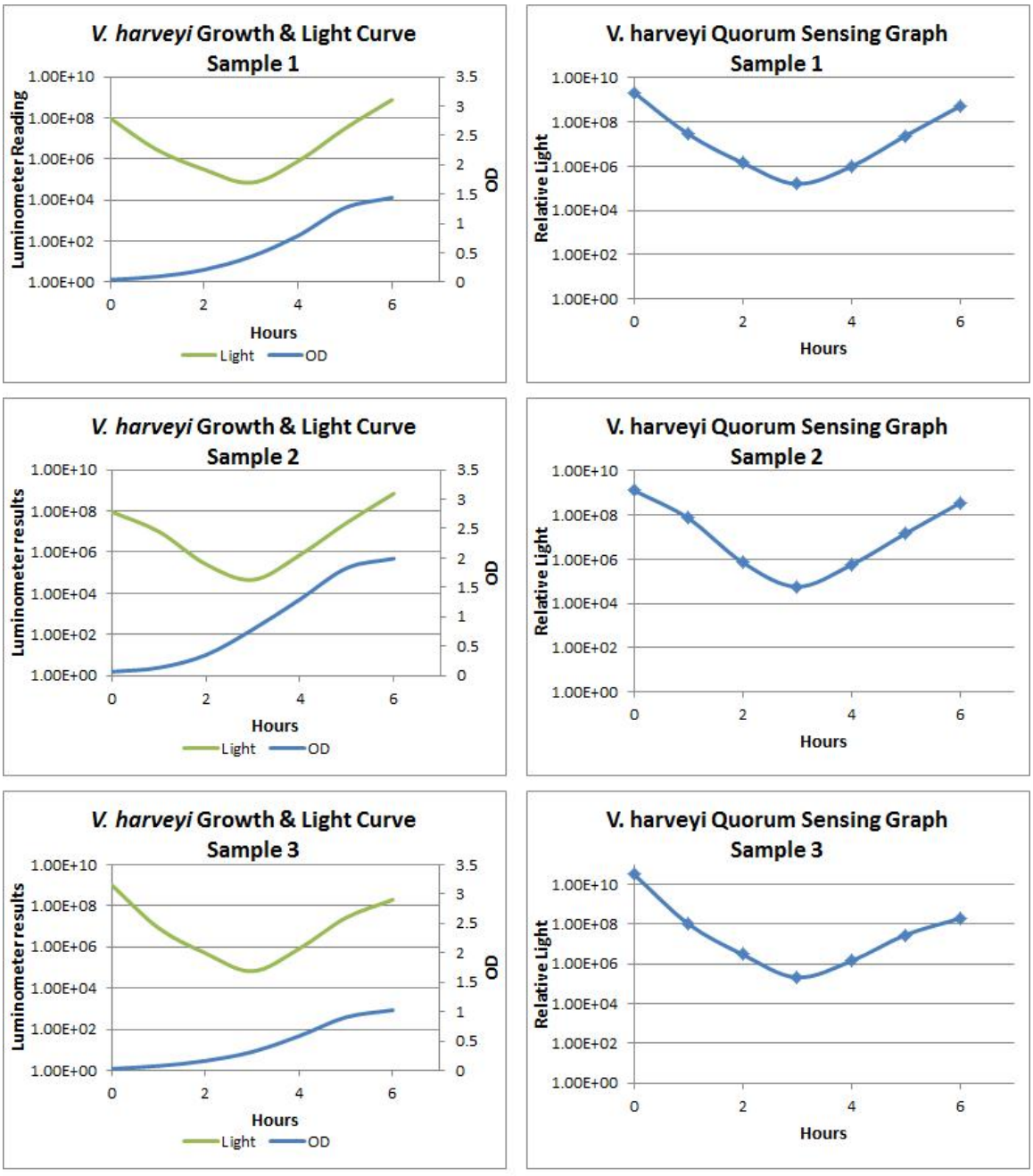


Figure 10B: *Vibrio chagasii* Quorum Sensing Graphs

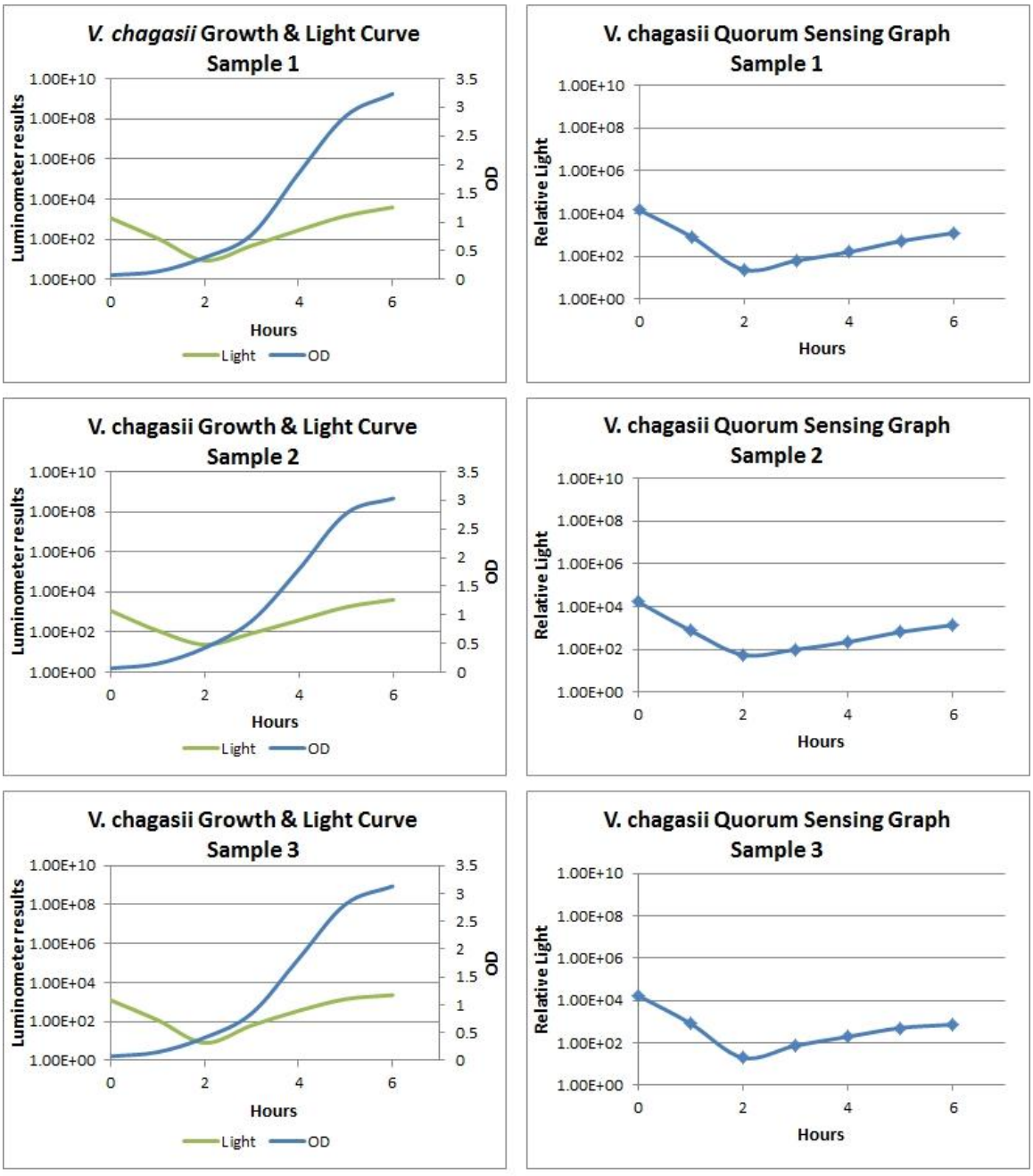


Figure 10C: *Vibrio cholerae* Quorum Sensing Graphs

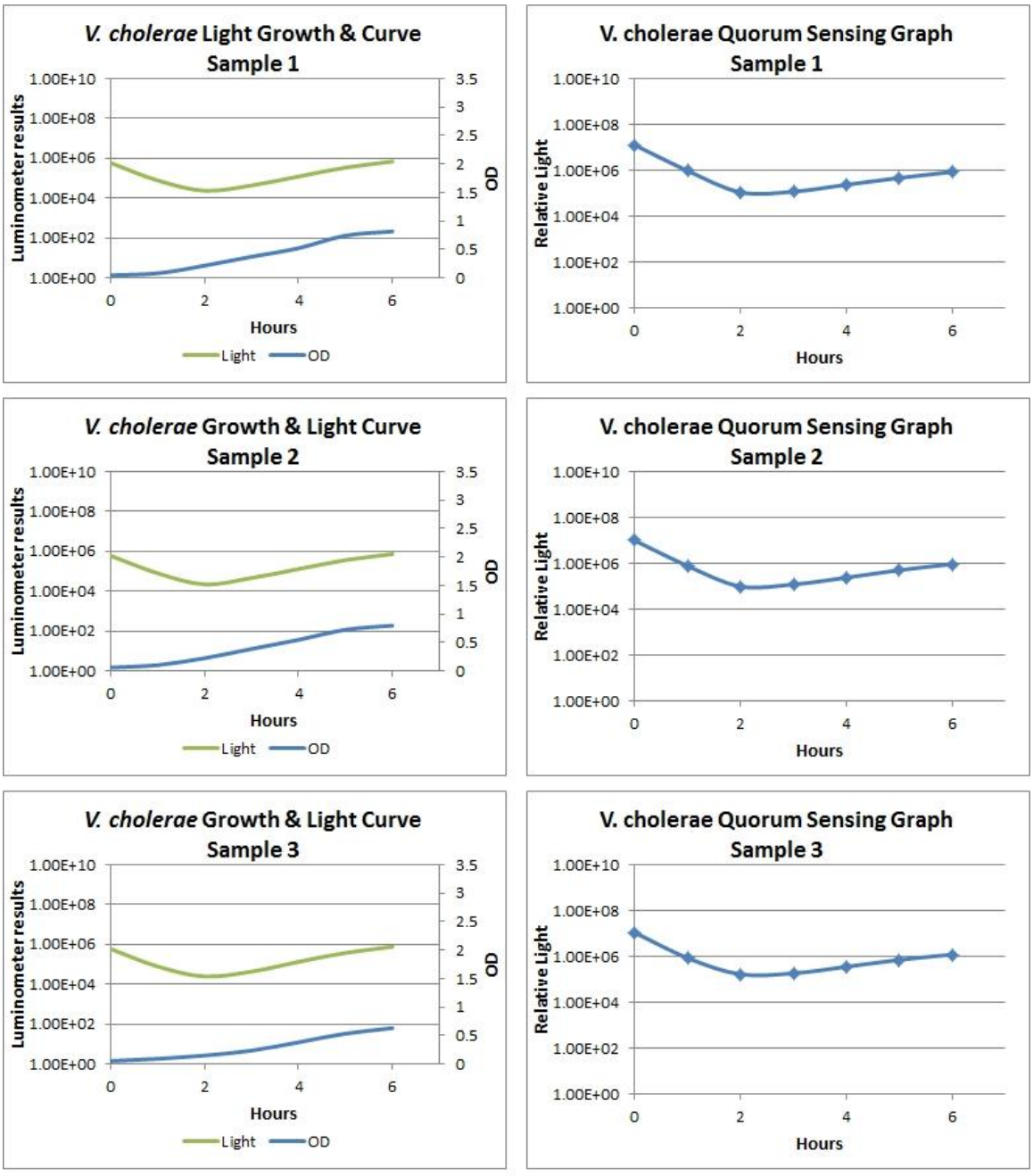


Figure 10D: *Vibrio orientalis* Quorum Sensing Graphs

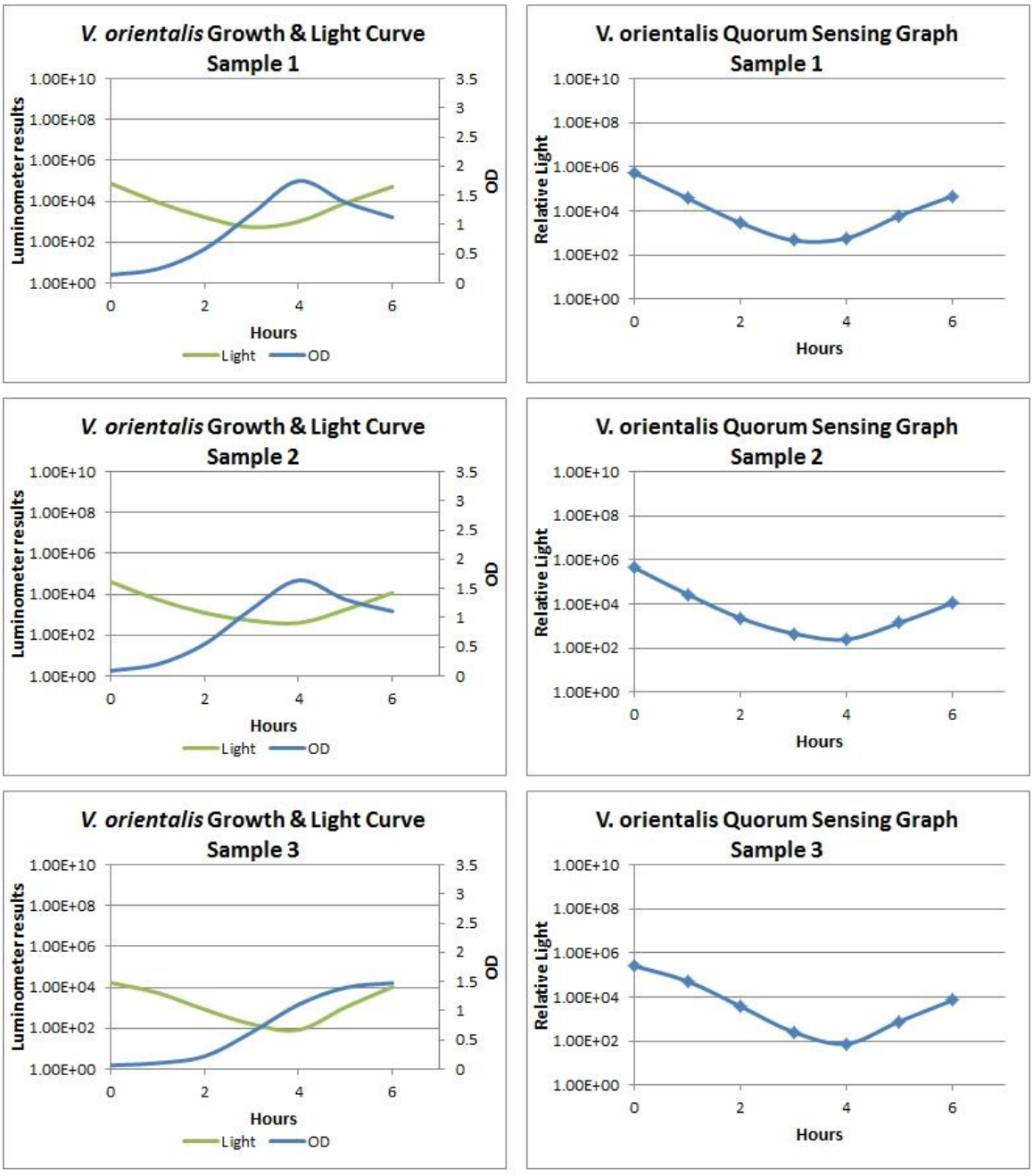


Figure 10E: *Vibrio vulnificus* Quorum Sensing Graphs

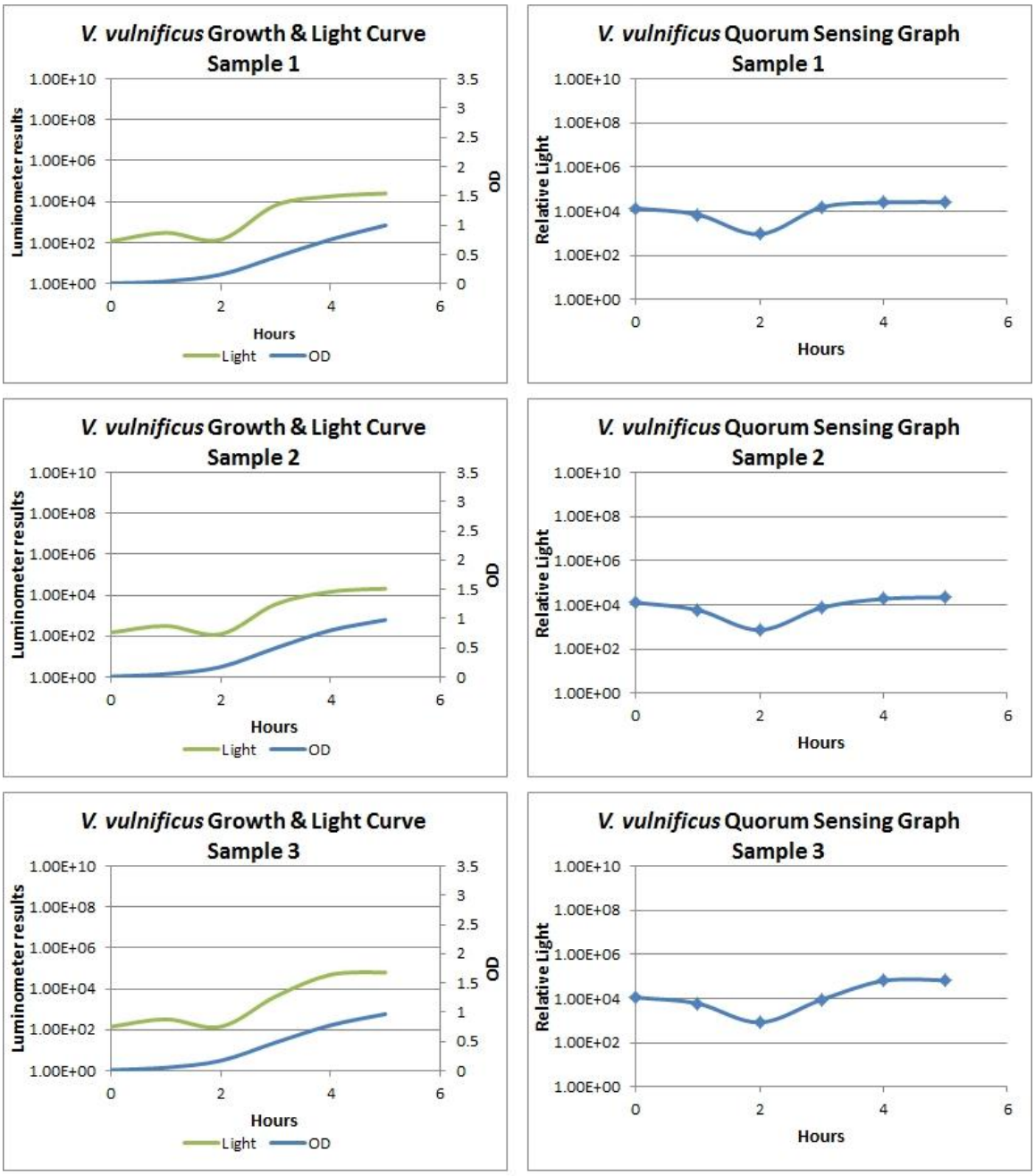


Figure 10: *Vibrio* Quorum Sensing Graphs. Individual growth and light curves are located on the left. Relative light quorum sensing curves are located on the right. Relative light was calculated by dividing light output by OD_{600} at hourly time points.

PCRs and Construct Generation

All plasmids are found in Table 3 and all PCR primers used to generate the pARA-LUXR construct are found in Table 4.

Table 3: List of plasmids used in this study

Plasmid	Description	Reference
pBAD-GFPuv	5371bp plasmid containing *araC* gene and *ara* promoter sequence	Bio-Rad;16
pLS6	5485bp plasmid containing chloramphenicol resistance cassette and *lacZ* cassette	119
pGEM-3Z	2743bp plasmid containing ampicillin resistant cassette and LacZ cassette	Promega
pARA-LUXR	7350bp plasmid with *araLuxR* insert cloned into pLS6 at the Sma1 restriction site of the multi-cloning region	This Study
$pLUX_{VH}$	pGEM-3z + *V. harveyi lux* operon insert interrupting LacZ cassette and ampicillin resistance	This Study
$pLUX_{VCha}$	pGEM-3z + *V. harveyi lux* operon insert interrupting LacZ cassette and ampicillin resistance	This Study
$pLUX_{VCho}$	pGEM-3z + *V. harveyi lux* operon insert interrupting LacZ cassette and ampicillin resistance	This Study
$pLUX_{VO}$	pGEM-3z + *V. harveyi lux* operon insert interrupting LacZ cassette and ampicillin resistance	This Study
$pLUX_{VV}$	pGEM-3z + *V. harveyi lux* operon insert interrupting LacZ cassette and ampicillin resistance	This Study
$pLUX_{Species}$	Collectively referral of $pLUX_{VH}$, $pLUX_{VCha}$, $pLUX_{VCho}$, $pLUX_{VO}$, $pLUX_{VV}$	This Study

Table 4: Oligonucleotide primer sequences used in this study

Primer Name	**Sequence: 5'->3'**
Crossover *araluxR* Forward	5' CTTTAAGAAGGAGATATACATATGGACTCAATTGCAAAGAG 3'
luxR Reverse-phosphorylated	5' TTAGTGATGTTCACGGTTGTAG 3'
luxR Reverse	5' AGTGATGTTCACGGTTGTAG 3'
ara Forward-phosphorylated	5' TTATGACAACTTGACGGCTACATC 3'
ara Forward	5' ATGACAACTTGACGGCTACATC 3'
ara Reverse	5' ATGTATATCTCCTTCTTAAAG 3'

The $luxR_{VH}$ gene was amplified out of the *V. harveyi* genome by PCR using Crossover *araluxR* Forward primer and *luxR* Reverse-phosphorylated primer (Table 4). A second PCR was performed using the *ara* Forward-phosphorylated and *ara* Reverse primers (Table 4) in order to amplify the *araC/ara* promoter region from the pBAD-GFPuv plasmid (Figure 11) obtained from Bio-Rad. Both the PCR products, 618bp $luxR_{VH}$ gene (Figure 12a) and 1247bp *araC/ara* promoter region (Figure 12b), were verified by gel electrophoresis.

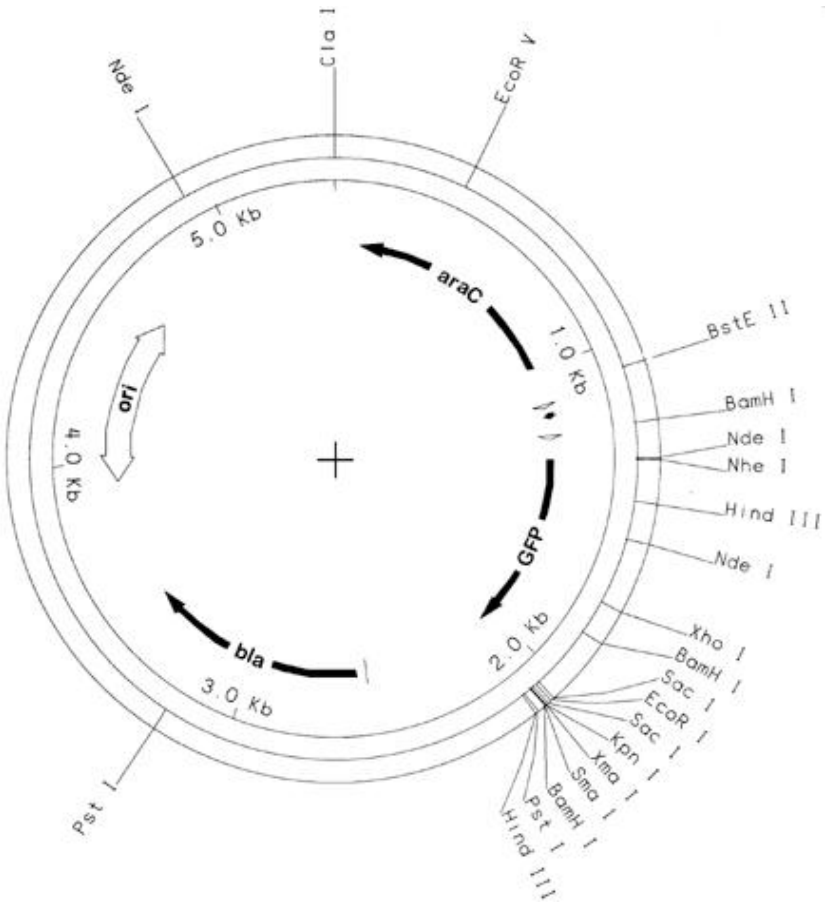


Figure 11: Map of pBAD-GFPuv (pGLO) plasmid. Figure acquired from Bio-rad at http://www.bio-rad.com/ under “teaching resources.”

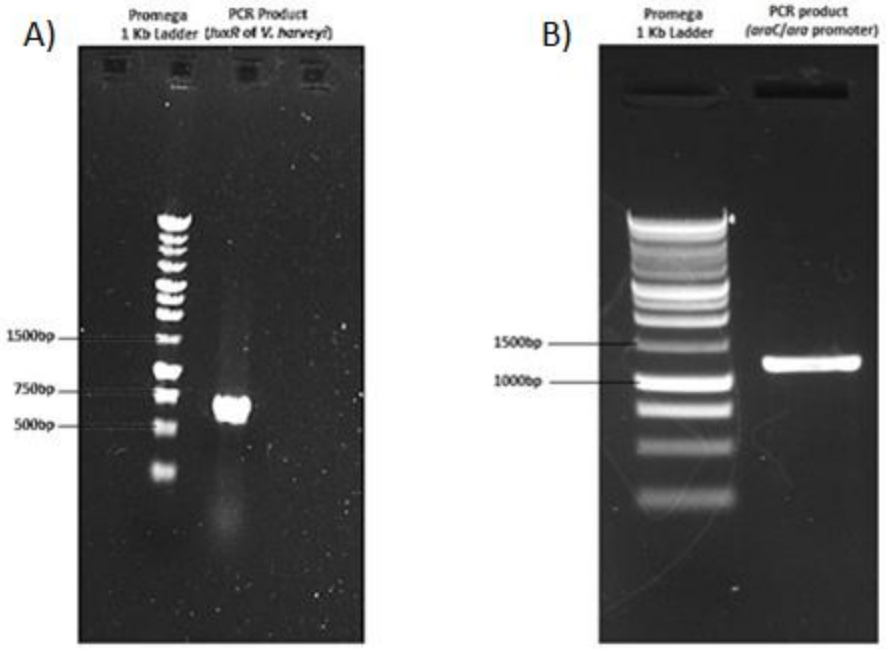


Figure 12: Gel electrophoresis of PCR products *luxR*$_{VH}$ and *araC/ara* promoter. A) Results of *luxR*$_{VH}$ PCR amplification from the *V. harveyi* genome. B) Results of *araC/ara* promoter PCR amplification from pBAD-GFPuv plasmid.

In order to fuse the *araC/ara* promoter region to the *luxR*$_{VH}$ gene, crossover PCR (Figure 13) was performed using Crossover *araluxR* Forward primer and *luxR* Reverse-phosphorylated primer (Table 4). The resulting 1,865bp crossover PCR product (*araluxR* insert) was verified by PCR using the *ara* Forward and *luxR* Reverse primers (Table 4), gel electrophoresis (Figure 14) and sequencing (Figure 15).

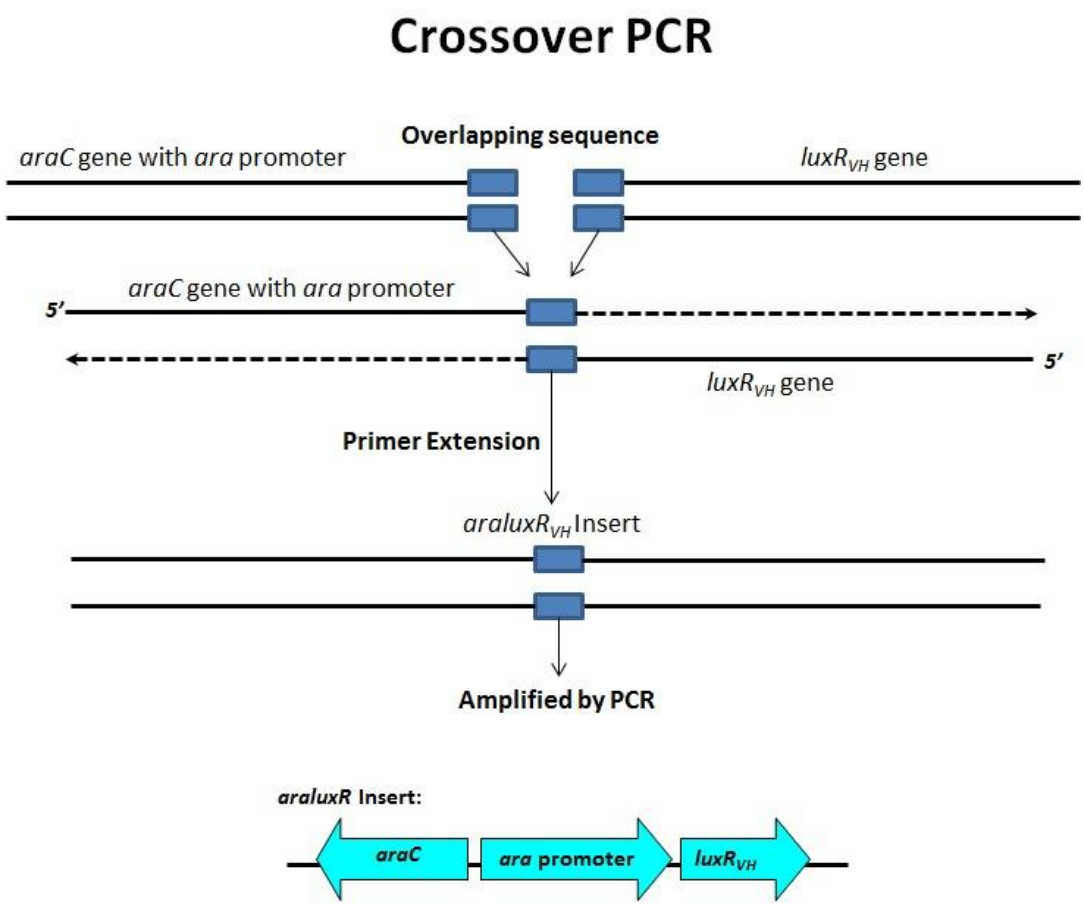


Figure 13: Crossover PCR and *araluxR* insert. Figure shows the process of cross over PCR to generate *araluxR* insert. Gene order and transcription direction is depicted at the bottom.

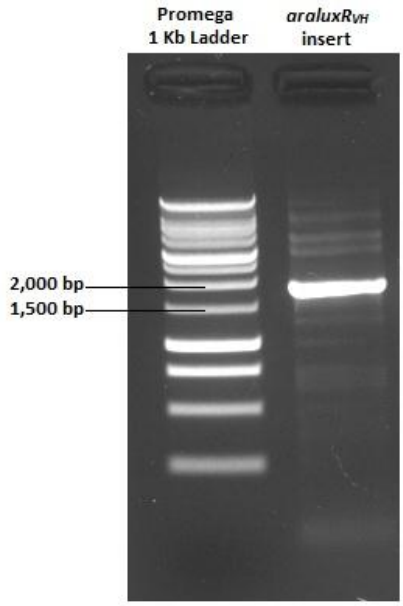


Figure 14: Gel electrophoresis of crossover PCR product. Gel electrophoresis was used to verify size of Crossover PCR product to identify *araluxR* fusion insert.

TTATGACAACTTGACGGCTACATCATTCACTTTTTCTTCACAACCGGCACGGAACTCGCTCGGGCTGGCCCCGGTGC
ATTTTTTAAATACCCGCGAGAAATAGAGTTGATCGTCAAAACCAACATTGCGACCGACGGTGGCGATAGGCATCCGG
GTGGTGCTCAAAAGCAGCTTCGCCTGGCTGATACGTTGGTCCTCGCGCCAGCTTAAGACGCTAATCCCTAACTGCTG
GCGGAAAAGATGTGACAGACGCGACGGCGACAAGCAAACATGCTGTGCGACGCTGGCGATATCAAAATTGCTGTCTG
CCAGGTGATCGCTGATGTACTGACAAGCCTCGCGTACCCGATTATCCATCGGTGGATGGAGCGACTCGTTAATCGCT
TCCATGCGCCGCAGTAACAATTGCTCAAGCAGATTTATCGCCAGCAGCTCCGAATAGCGCCCTTCCCCTTGCCCGGC
GTTAATGATTTGCCCAAACAGGTCGCTGAAATGCGGCTGGTGCGCTTCATCCGGGCGAAAGAACCCCGTATTGGCAA
ATATTGACGGCCAGTTAAGCCATTCATGCCAGTAGGCGCGCGGACGAAAGTAAACCCACTGGTGATACCATTCGCGA
GCCTCCGGATGACGACCGTAGTGATGAATCTCTCCTGGCGGGAACAGCAAAATATCACCCGGTCGGCAAACAAATTC
TCGTCCCTGATTTTTCACCACCCCCTGACCGCGAATGGTGAGATTGAGAATATAACCTTTCATTCCCAGCGGTCGGT
CGATAAAAAAATCGAGATAACCGTTGGCCTCAATCGGCGTTAAACCCGCCACCAGATGGGCATTAAACGAGTATCCC
GGCAGCAGGGGATCATTTTGCGCTTCAGCCATACTTTTCATACTCCCGCCATTCAGAGAAGAAACCAATTGTCCATA
TTGCATCAGACATTGCCGTCACTGCGTCTTTTACTGGCTCTTCTCGCTAACCAAACCCGGTAACCCCGCTTATTAAA
AGCATTCTGTAACAAAGCGGGACCAAAGCCATGACAAAAACGCGTAACAAAAGTGTCTATAATCACGGCAGAAAAGT
CCACATTGATTATTTGCACGGCGTCACACTTTGCTATGCCATAGCATTTTTATCCATAAGATTAGCGGATCCTACCT
GACGCTTTTTATCGCAACTCTCTACTGTTTCTCCATACCCGTTTTTTTGGGCTAGAAATAATTTTGTTTAACTTTAA
GAAGGAGATATACATATGGACTCAATTGCAAAGAGACCTCGTACTAGGCTTTCCCCTCTAAAACGTAAACAGCAACT
AATGGAAATCGCTCTTGAAGTGTTTGCTCGTCGCGGCATTGGTCGTGGTGGTCACGCTGATATTGCAGAGATTGCTC
AAGTTTCTGTTGCGACAGTATTTAACTACTTCCCAACTCGCGAAGACTTGGTGGATGAAGTTCTGAATCACGTGGTT
CGTCAATTCTCGAACTTCTTATCAGATAACATCGACTTAGACATTCATGCACGCGAGAACATCGCTAACATCACTAA
CGCAATGATTGAACTAGTAAGCCAAGATTGCCATTGGTTGAAAGTTTGGTTCGAGTGGAGTGCATCAACACGTGATG
AAGTGTGGCCACTATTCGTAACGACAAACCGCACCAACCAACTGCTGGTGCAAAACATGTTCATCAAAGCTATCGAA
CGTGGGGAAGTTTGTGACCAACATGAACCGGAACACTTGGCGAATCTGTTCCATGGTATTTGTTACTCTATCTTCGT
ACAAGCAAATCGCTCTAAGAGCGAAGCCGAGCTATCAACACTCGTTAGTGCTTACCTAGATATGTTGTGCATCTACA
ACCGTGAACATCACTAA

Figure 15: Sequence of *araluxR* insert. Sequence results of *araC/ara* promoter region and *luxR*$_{VH}$ crossover PCR. The *araC* gene (green) is followed by the *ara* promoter (yellow) fused to LuxR$_{VH}$ gene (blue).

Transformation

The *araluxR* insert was cloned into pLS6 (Figure 16) resulting in plasmid pARA-LUXR.

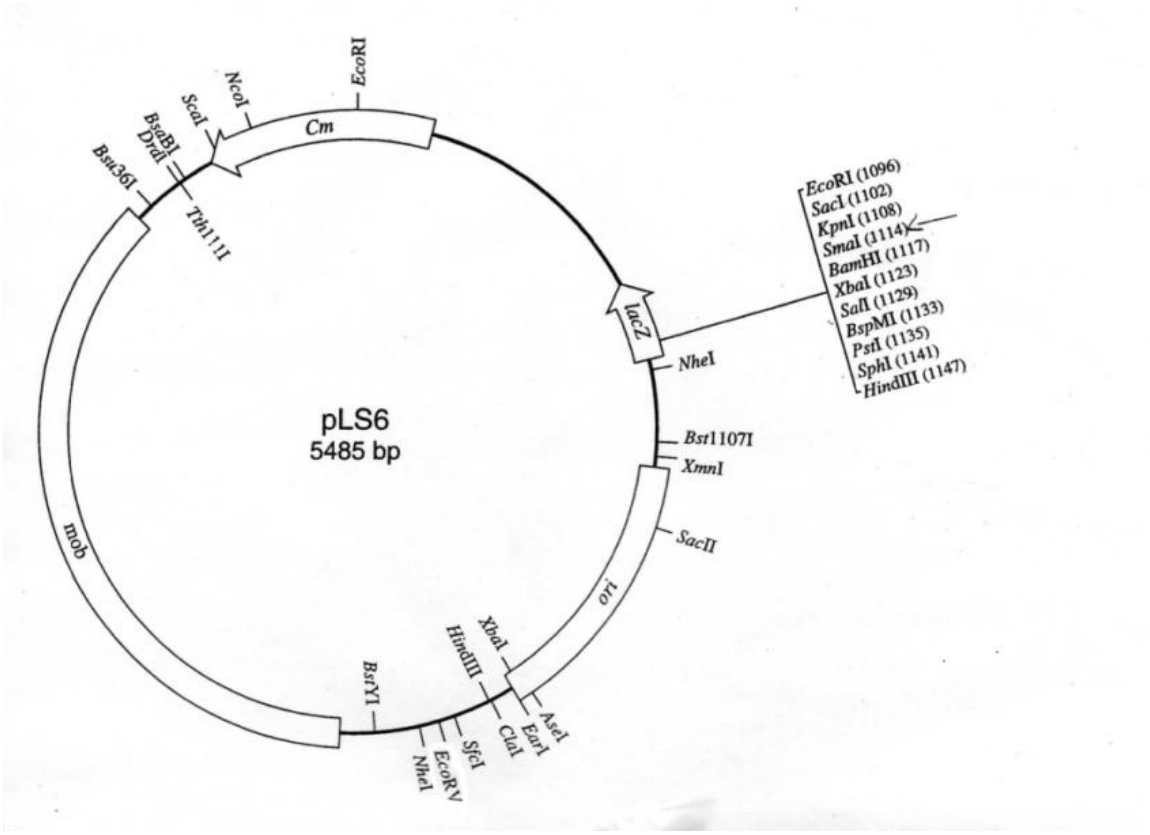


Figure 16: Map of pLS6 plasmid. The *araluxR* insert was cloned at the Sma1 restriction site located within the *lacZ* cassette.

Transformation of pARA-LUXR was tested by blue and white screening and verified by colony PCR using primers *ara* Forward and *luxR* Reverse (Table 4, Figure 17). Lanes 2 and 11 contained the *araluxR* insert DNA generated by cross-over PCR. The presence of the insert was confirmed by size as seen in Lanes 3, 6, 8, and 10. Partial length fragments in lanes 4, 5, 7, and 9 were not further investigated.

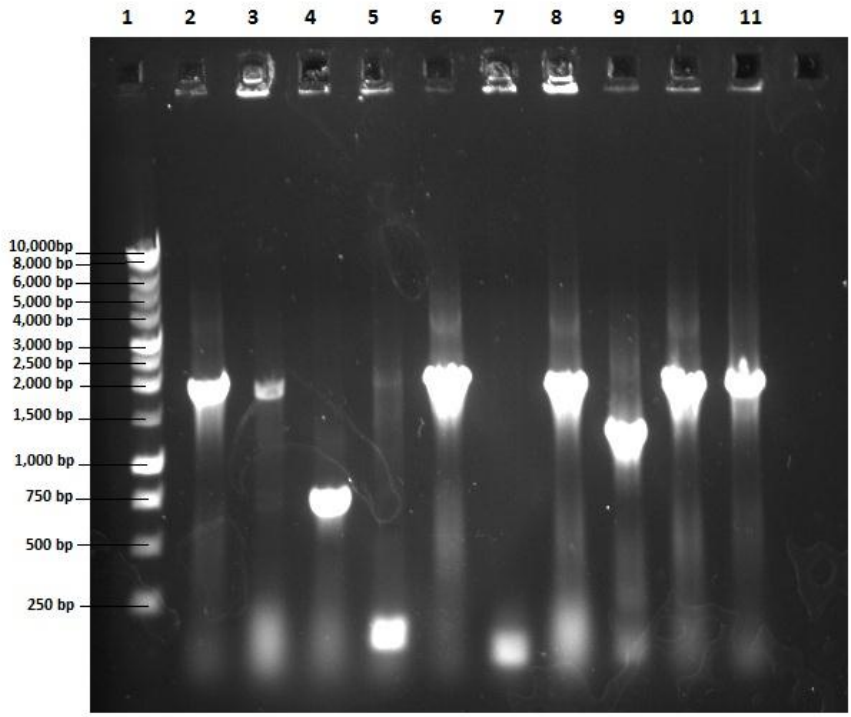


Figure 17: Colony PCR gel electrophoresis. Lane 1 contains a 1Kb DNA ladder from Promega. Lane 2 and Lane 11 contained the control *araluxR* fragment DNA. Lane 3, Lane 6, Lane 8 and Lane 10 contain the *araluxR* insert.

Cells transformed with a second pGEM-3Z based plasmid (Figure 18), $pLUX_{Species}$, containing one of five species-specific *lux* operons resulted in five individual dual vector expression systems for arabinose-inducible bioluminescence (Figure 19).

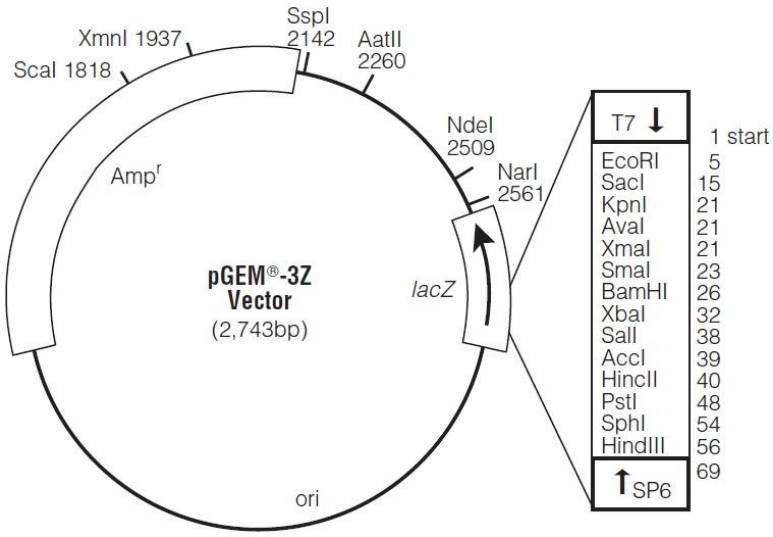


Figure 18: Map of pGEM-3z plasmid. Map acquired from Promega pGEM-3Z Vector instructions for use of product p2151 manual.

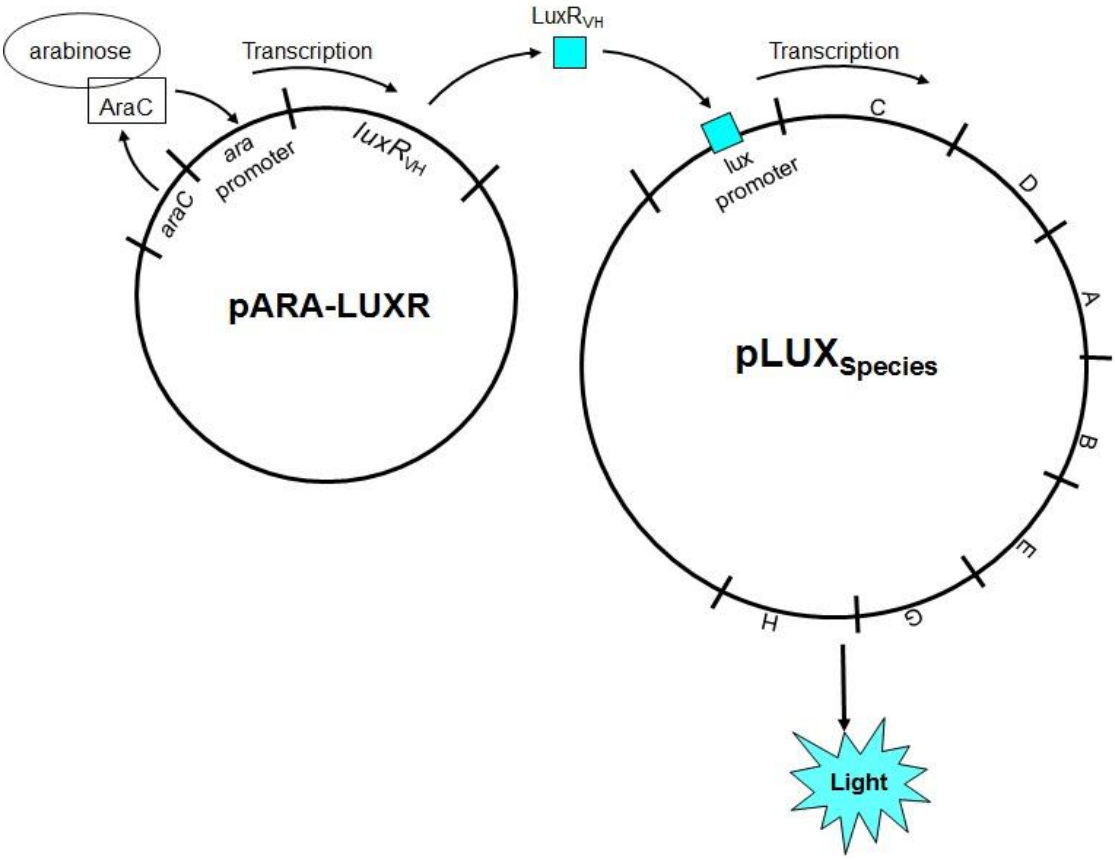


Figure 19: Dual vector expression system for arabinose-induced bioluminescence. In the presence of arabinose, AraC binds to arabinose and then to the *ara* promoter activating the *luxR$_{VH}$* gene. LuxR$_{VH}$ is then able to activate the *lux* operon and light is produced. Five individual systems were generated for each of the five *Vibrio lux* operons.

E. coli Plate Assays

The dual vector expression systems for arabinose-induced bioluminescence were tested by plating transformed cells onto arabinose-induced and uninduced plates. Plates were photographed in the light and in darkness (Figure 20).

Figure 20: *E. coli* Plate Assays

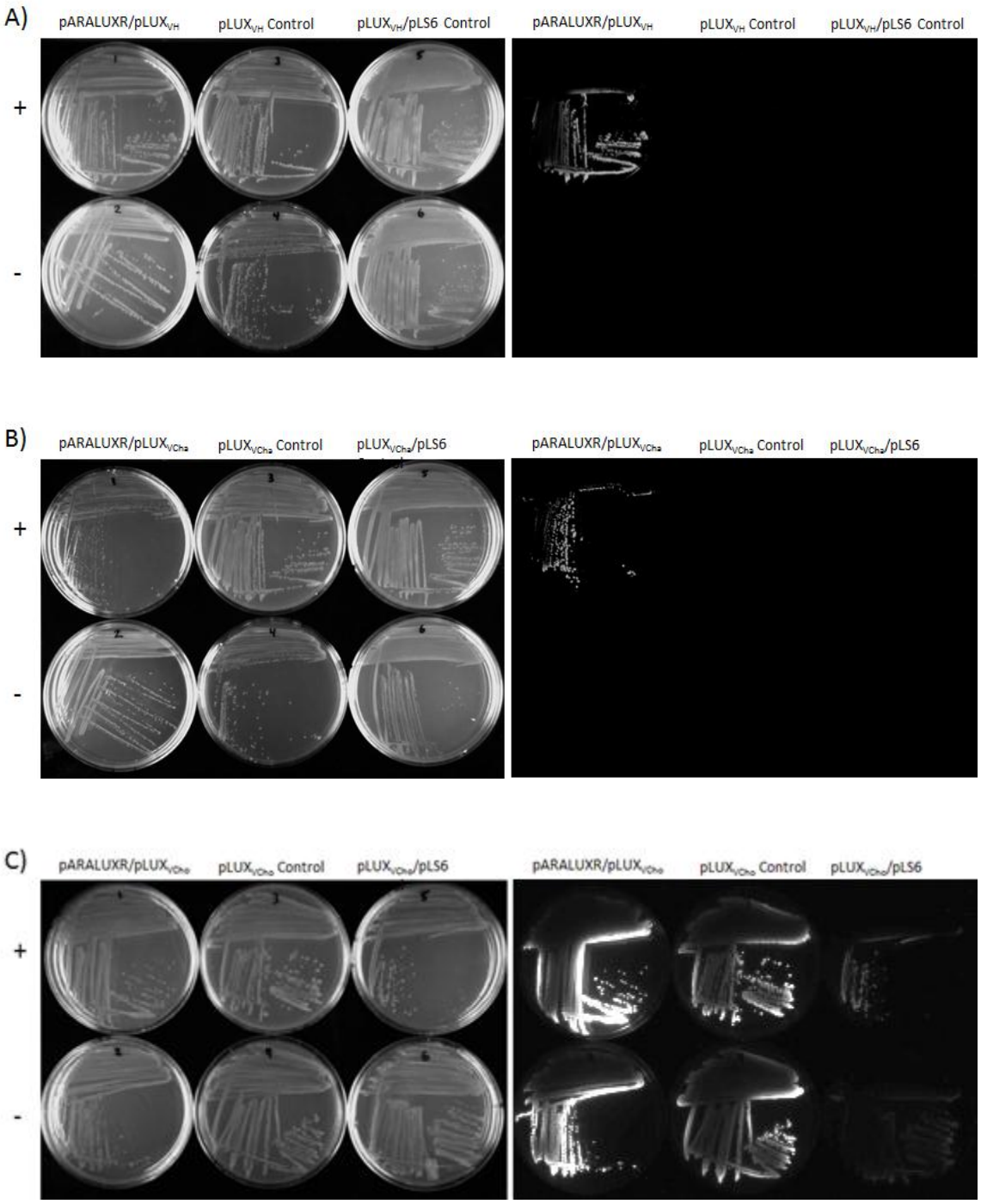


Figure 20 continued: *E. coli* Plate Assays

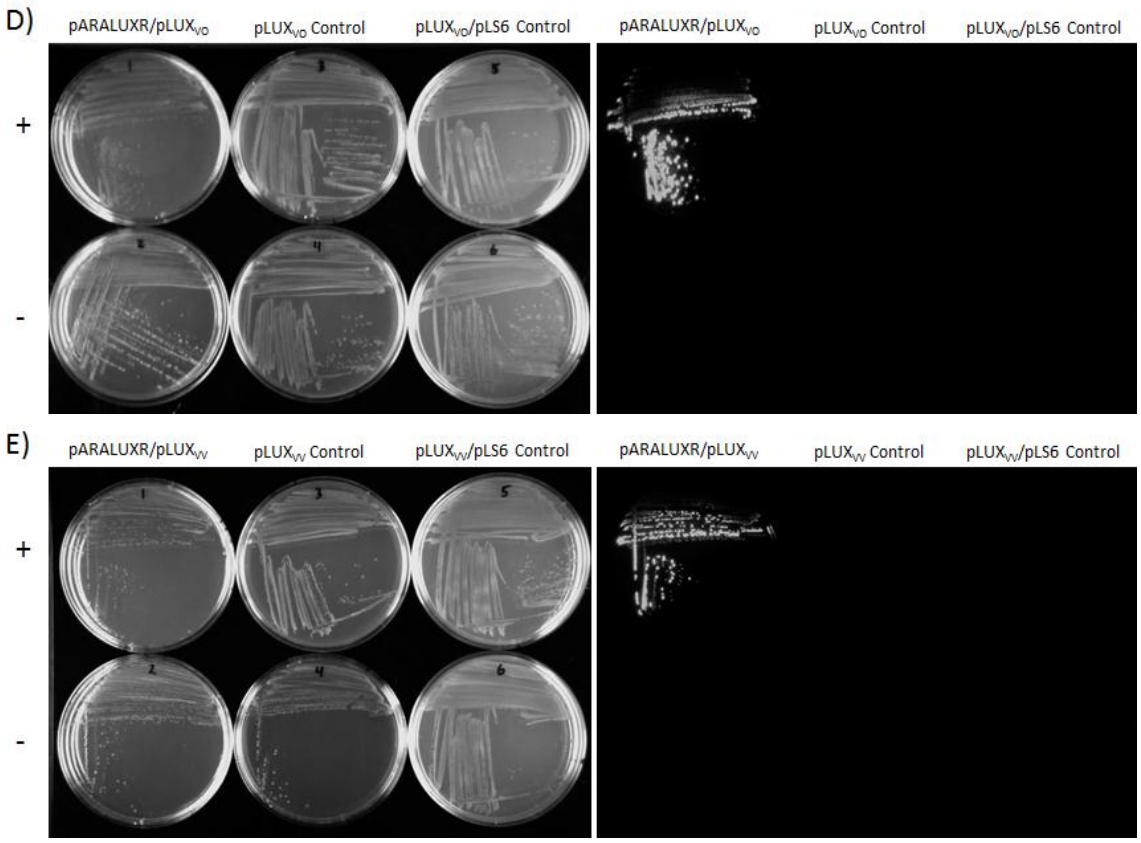


Figure 20: *E. coli* Plate Assays. *E. coli* strains tested under variable conditions for bioluminescence. The *lux* operons under examination are of the following *Vibrios*: A) *harveyi* B) *chagasii* C) *cholerae* D) *orientalis* E) *vulnificus*. Test strains and controls are labeled above picture. Induced plates are the top row labeled "+" and uninduced plates are the bottom row labeled "-". The first column of frames with white light and the second column of frames are photographed in darkness with 30 min exposure.

E. coli containing the *lux* operons of either *V. harveyi*, *V. chagasii*, *V. orientalis*, or *V. vulnificus* each produced light when induced. Additionally, no light production was observed for the uninduced pARA-LUXR/ $pLUX_{Species}$ condition or in either of the induced or uninduced $pLUX_{Species}$ or $pLUX_{Species}$/pLS6 controls. The *V. cholerae lux* operon, however, showed some degree of light production in all induced and uninduced samples. In all samples, induced pARA-LUXR/ $pLUX_{Species}$ conditions showed greater light output than the rest of the conditions.

E. coli Maximum Light Output Assays

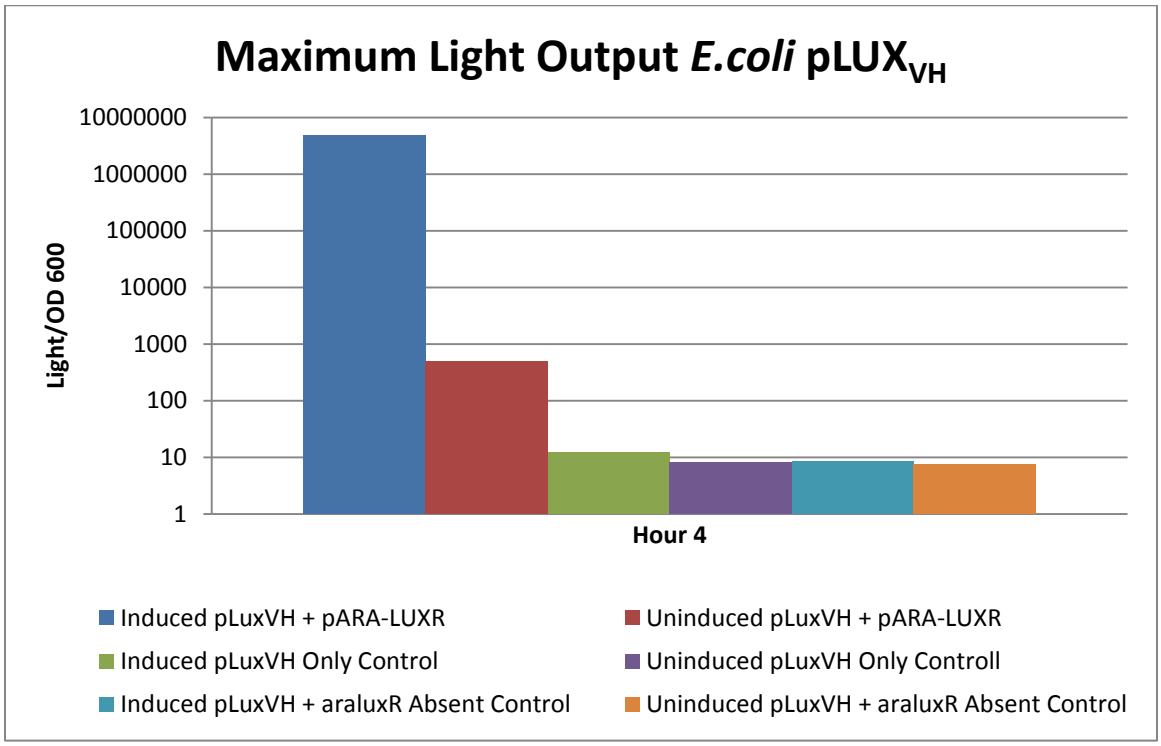
For each species-specific *lux* operon, three replicates were tested under each of the six conditions. Individual growth and light curves are found in Appendix B. All relative light calculations (light /OD_{600}) for each species-specific *lux* operon tested are located in Appendix C. Averages of the maximum light output for each species-specific *lux* operon are found in Table 5.

Table 5: Average maximum light output for *E. coli* samples

Operon tested & Condition	Average Induced	Average Uninduced	Induced vs Uninduced
pARA-LUXR/pLUX$_{VH}$	4872792.78	498.28	9779.226098
pLux$_{VH}$	12.14	8.23	1.47509113
pLUX$_{VH}$/pLS6 control	8.54	7.46	1.144772118
pARA-LUXR/pLUX$_{VCha}$	4658118.07	30593.44	152.2587218
pLux$_{VCha}$	53.87	44.13	1.220711534
pLUXV$_{Cha}$/pLS6 control	57.76	52.46	1.101029356
pARA-LUXR/pLUX$_{VCho}$	782885.56	6970.33	112.3168573
pLux$_{VCho}$	3229.42	2570.76	1.256212171
pLUXV$_{Cho}$/pLS6 control	7313.13	6371.066	1.147865993
pARA-LUXR/pLUX$_{VO}$	1570005.51	1342.44	1169.516336
pLux$_{VO}$	56.63	32.84	1.724421437
pLUX$_{VO}$/pLS6 control	17.31	13.31	1.30052592
pARA-LUXR/pLUX$_{VV}$	4271138.31	1072.1	3983.899179
pLux$_{VV}$	36.95	24.27	1.522455707
pLUX$_{VV}$/pLS6 control	36.49	26.13	1.396479143

E. coli containing the *lux* operons of *V. harveyi*, *V. vulnificus*, and *V. orientalis* yielded the highest light production, exhibiting a 9,800, 4,000, and 1,200 fold increase in light production on average with the induced pARA-LUXR/pLUX$_{Species}$ condition. *E. coli* containing the *lux* operons of *V. chagasii* and *V. cholerae* showed lower light production for the induced pARA-LUXR/pLUX$_{Species}$ condition; however, both still averaged at least a 100 fold increase. For each species-specific *lux* operon tested, all induced and uninduced samples for the pLUX$_{Species}$ control and the pLUX$_{Species}$/pLS6 condition conditions showed on average less than a 2-fold induction. For each species-specific *lux* operon tested, average maximum light output results across all conditions were graphed for comparisons (Figure 21).

Figure 21: *E. coli* sample average maximum light output for all conditions.



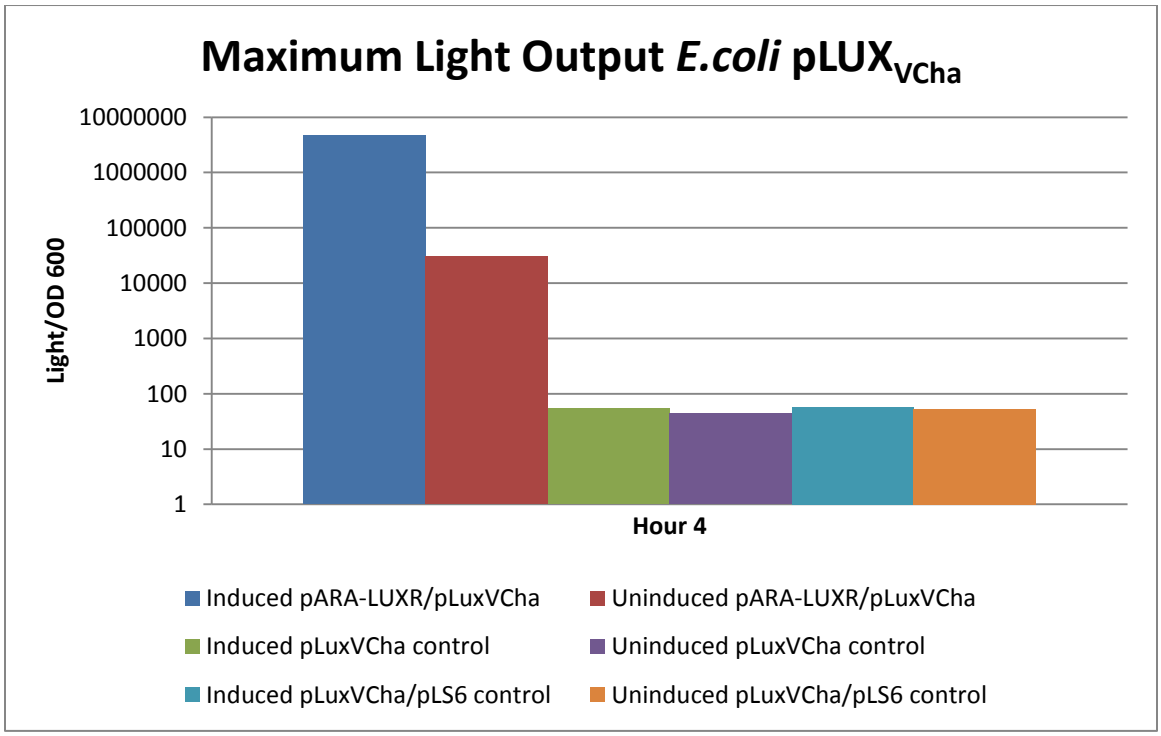
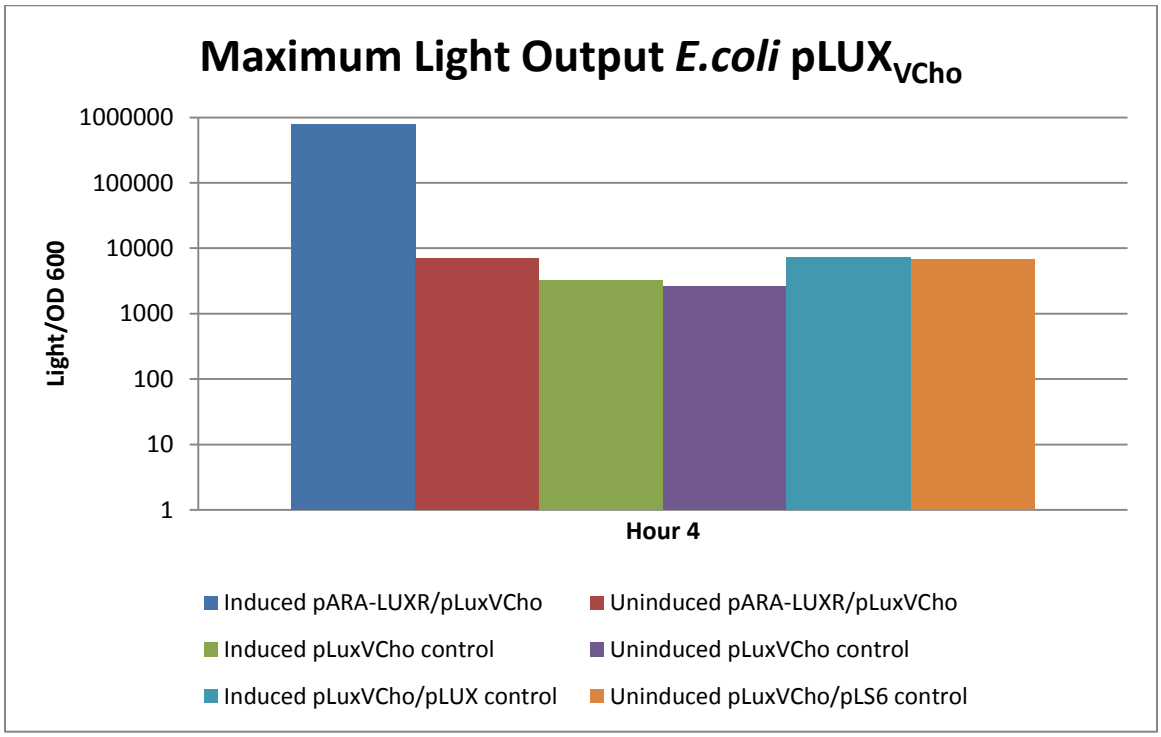


Figure 21 continued: *E. coli* sample average maximum light output for all conditions.



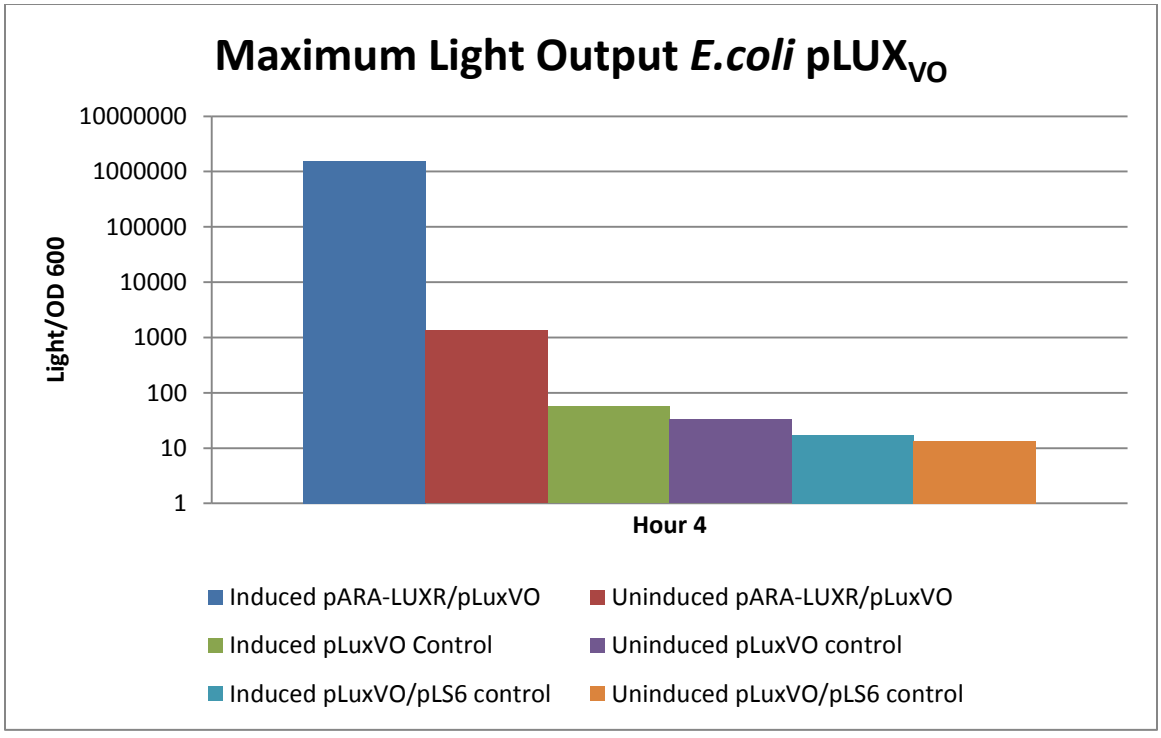


Figure 21 continued: *E. coli* sample average maximum light output for all conditions.

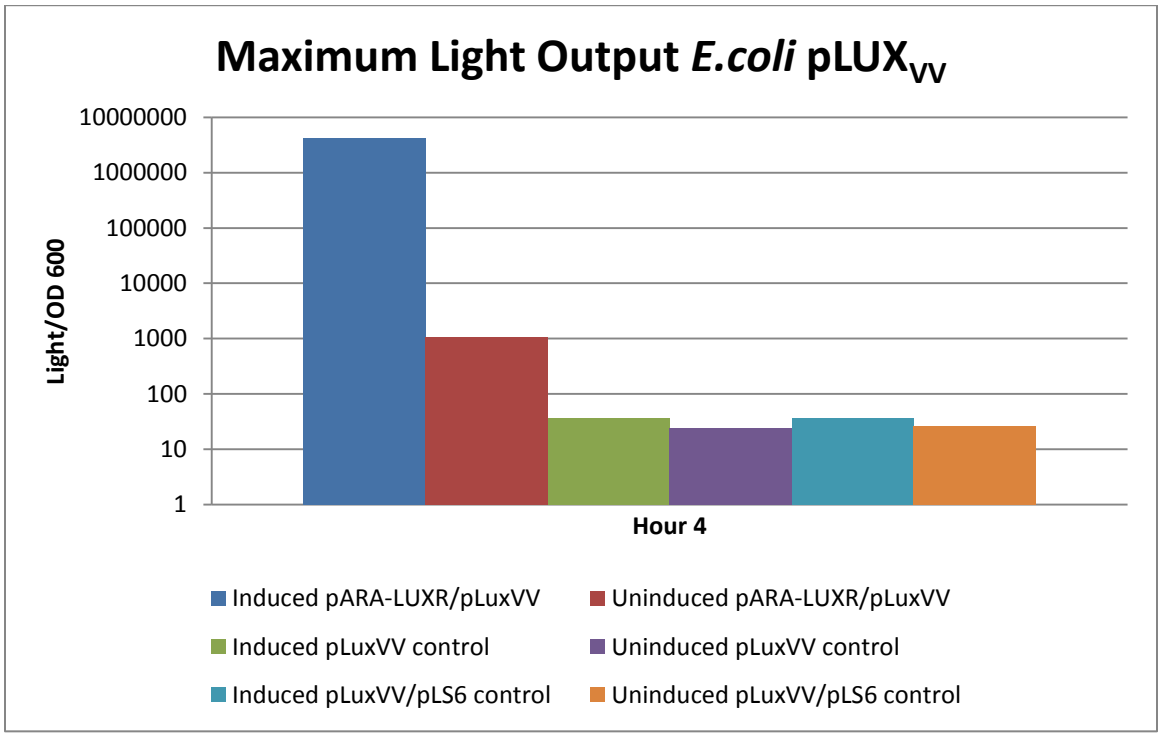


Figure 21: Average Maximum Light Outputs for *E. coli* Quorum Sensing Assay Conditions. Average maximum light output from samples of all five *Vibrio lux* operons tested for each of the six conditions.

Sequence Analysis of $LuxR_{VH}$ Homologs

Results of the sequence alignment showed high identity percentages for *Vibrio parahaemolyticus* OpaR (95.1%), *Vibrio vulnificus* SmcR (92.2%), *Vibrio anguillarum* VanT (81%), *Vibrio cholerae* HapR (70.4%), *Vibrio fischeri* LitR (58.7%) against $LuxR_{VH}$; only a 16.4% identity for TetR of *Salmonella enterica* is observed (Figure 22; Table 6). Additionally, sequence alignment showed the hypothetical protein (H.P.) $LuxR_{VH}$ homolog for *Vibrio orientalis* with 81.9% identity (Figure 22; Table 6).

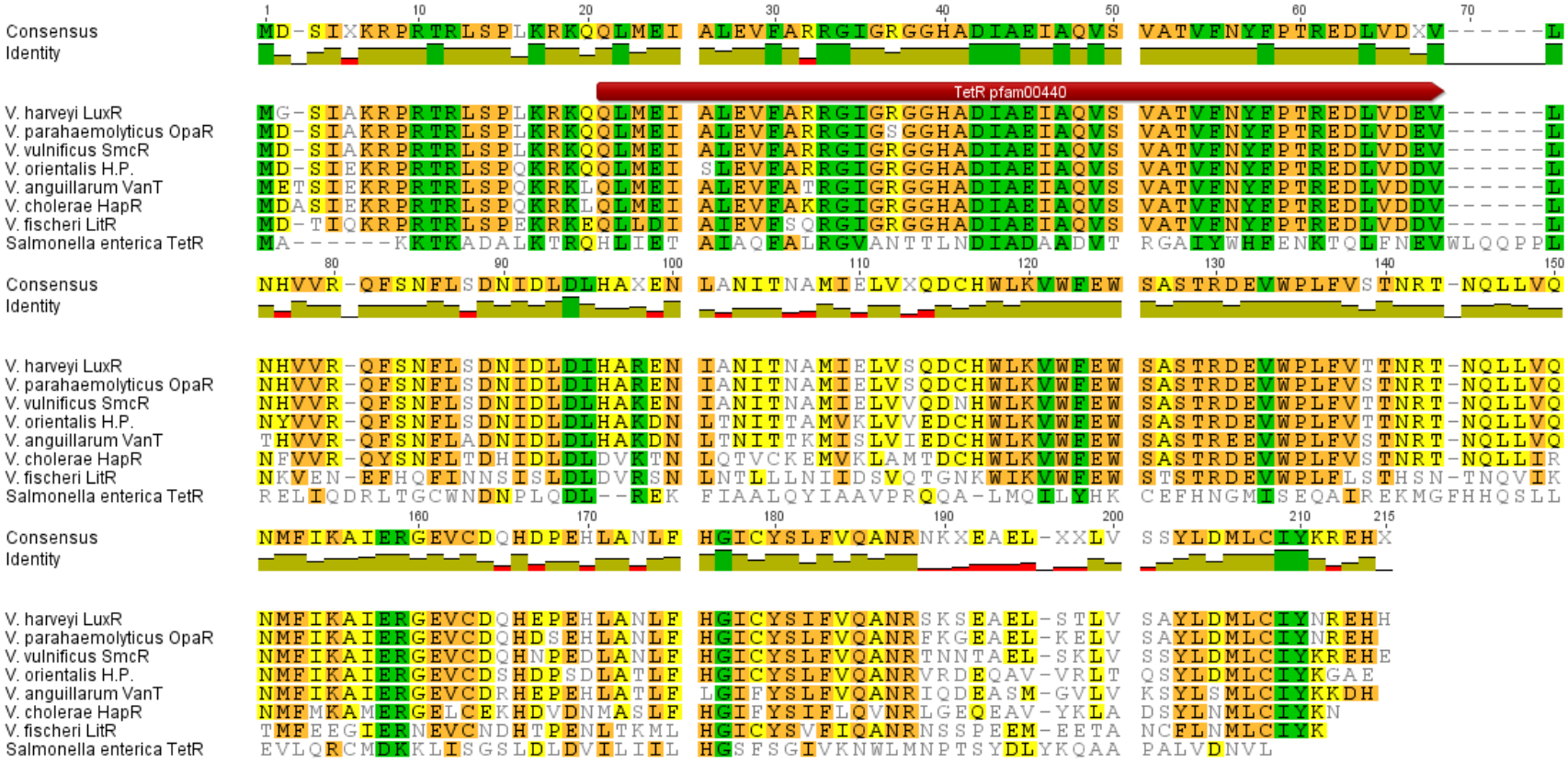


Figure 22: LuxR$_{VH}$ Sequence Alignment of Homologs. Proteins are organized in descending order of homology from LuxR$_{VH}$. The TetR family helix-turn-helix DNA binding domain is annotated in red. Color scheme is as follows: Green 100% similar amino acids, Orange >80% similar, Yellow is >60% similar.

Table 6: Percent Identity Comparison of $LuxR_{VH}$ Homologs.

		V. harveyi	*V. parahaemolyticus*	*V. vulnificus*	*V. orientalis*	*V. anguillarum*	*V. cholerae*	*V. fischeri*	*S. enterica*
V. harveyi **ATCC 33843**	**LuxR**		95.1	92.2	81.9	81.0	70.4	58.7	16.4
V. parahaemolyticus **10329**	**OpaR**	95.1		91.7	81.9	80.5	70.9	58.7	16.9
V. vulnificus **ATCC 44382**	**SmcR**	92.2	91.7		84.8	81.5	72.4	59.7	15.5
V. orientalis **ATCC 33934**	**Hyp. Prot.**	81.9	81.9	84.8		83.9	76.4	58.2	13.5
V. anguillarum **ATCC 68554**	**VanT**	81.0	80.5	81.5	83.9		73.9	57.9	12.0
V. cholerae **ATCC 14547**	**HapR**	70.4	70.9	72.4	76.4	73.9		59.4	14.4
V. fischeri **BAA-1741**	**LitR**	58.7	58.7	59.7	58.2	57.9	59.4		14.5
S. enterica **R6-199**	**TetR**	16.4	16.9	15.5	13.5	12.0	14.4	14.5	

Discussion

Quorum Sensing Among *Vibrios*

Among the bioluminescent *Vibrios* tested in this study, only *V. harveyi* and *V. cholerae* have been previously shown to utilize quorum sensing regulation for various genes (48, 76). Since light production is utilized in this study as an indicator of quorum sensing in *Vibrios*, qualitative light production was confirmed by plating (Figure 9). For the quorum sensing assays, a positive quorum sensing graph would be indicated by a "U" shaped curve of relative light production over time (76). The initial high relative light output is a remnant of residual light production from the high-density parent cultures that are diluted back as the original inoculum. Relative light production then decreases as the cells react to the lowered cell density. Finally, the relative light output increases as culture cell density rises. Thus, the change in relative light is due to the dependence of light production on the cell population, indicating quorum sensing regulation.

Observed light output is strain-specific and varies between strains as seen in Appendix A. Since *V. harveyi* bioluminescence is specifically known to be regulated by quorum sensing (48, 76), this species serves as the positive control in the quorum sensing assays. Each of the replicate trials for the five tested *Vibrio* species: *harveyi*, *chagasii*, *cholerae*, *orientalis*, and *vulnificus* resulted in the expected "U" shaped relative light curve (Figure 10). The results indicate that *V. cholerae, V. chagasii*, *V. orientalis*, and *V. vulnificus* undergo quorum sensing regulation of bioluminescence similar to that of *V. harveyi*.

Dual vector system for arabinose-induced bioluminescence

To determine if $LuxR_{VH}$ is capable of upregulating *lux* operon expression in the other *Vibrio* species, a method of directly inducing $luxR_{VH}$ was needed. Quorum sensing regulation is bypassed by using *E. coli* surrogates with a plasmid containing the *araC* gene and the *ara* promoter linked to the $luxR_{VH}$ gene. Successful amplification of the $luxR_{VH}$ from the *V. harveyi* genome by PCR is confirmed by the 600bp band seen on the electrophoresis gel shown in Figure 12a. The *araC* gene and linked *ara* promoter were amplified from pBAD-GFPuv using PCR and confirmed by the resulting 1250bp band on gel electrophoresis (Figure 12b). Successful fusion of the *araC/ara* region to $luxR_{VH}$ was accomplished by crossover PCR. The 1865bp *araluxR* crossover PCR product was confirmed by size using gel electrophoresis (Figure 14) as well as by sequence (Figure 15). Successful transformation of the first plasmid, pARA-LUXR, was confirmed by colony PCR amplification of *araluxR* and gel electrophoresis. Results show that four of eight colonies had successful transformation, indicated by the bands between the 2000bp and 1500bp markers (Figure 17). Second, individual $pLUX_{Species}$ plasmids containing a species-specific *lux* operon driven by their native promoter were transformed into the *E. coli* containing the pARA-LUXR to generate the five individual dual vector systems for arabinose induced bioluminescence.

Each of the six testing conditions for *E. coli* plate assays and *E. coli* maximum light production assays include: induced and uninduced pARA-LUXR/$pLUX_{species}$, induced and uninduced $pLUX_{species}$/pLS6 control, and induced and uninduced $pLUX_{Species}$ control. *E.*

coli was first plated in order to confirm light production and photographed (Figure 20A-E). Light production was seen for each of the species-specific *lux* operons tested, confirming that the *E. coli* dual vector system for arabinose-inducible bioluminescence successfully promotes light production when induced with arabinose. Additionally, no light production was observed in the absence of arabinose or in any controls for all strains except *V. cholerae*. In the case of *V. cholerae* there is low level light production in the uninduced samples as well as both induced and uninduced controls (Figure 20C). This possibly indicates a promoter recognized by *E. coli* without induction. Incidentally, the *lux* operon of *V. cholerae* is shown to be more phylogenetically distant from the other four studied *Vibrio lux* operons (Figure 1); this is also shown in other studies (111, 118). Overall, there is clear activation of transcription at the *lux* promoter of other *Vibrio lux* operons by $LUXR_{VH}$, which suggests regulation conservation of the *lux* operons. The results presented in this study clearly show cross-species induction by $LUXR_{VH}$ in all *Vibrio* species tested. Furthermore, the results show this dual vector system is useful for bypassing quorum sensing to directly express a regulator.

Cross Species Induction of Bioluminescence by *V. harveyi* LuxR

During exponential growth phase, light production reaches a maximum as the culture growth exits exponential growth phase then plateaus throughout stationary growth

phase. In *Vibrios*, this is possibly due to autoinducer saturation at their receptors as cell population reaches stationary phase and/or the $LuxR_{VH}$ feedback loop on the Qrr sRNAs 2-4 (115). However, in the *E. coli* dual vector system used in this study, the decrease in light production may be attributed to cell resource or arabinose depletion. Maximum light output for each species-specific *lux* operon was determined in order to quantify the amount of $LuxR_{VH}$ activation of each respective *lux* operon. Since light production drops around hour five and cell density continues to increase, maximum light output was calculated at hour four of cellular growth phase. For each condition, replicate samples have individual light and OD_{600} over time graphs located in Appendix B. Average results were utilized for inter-specific comparisons of *lux* operon activation by $LuxR_{VH}$ (Figure 21, Table 5).

Each sample tested had light production for the arabinose-induced pARA-LUXR/$pLUX_{species}$ condition as well as varying amounts of reduced light production for uninduced pARA-LUXR/$pLUX_{species}$ conditions (Figure 21, Appendix B). Some light production seen in uninduced samples may result from arabinose promoter leakage or a *lux* promoter recognized by *E. coli*. As seen in Figure 21, this higher light production for the uninduced trials for the *lux* operons of *V. chagasii* and *V. cholerae* is likely to be due to more distant evolutionary relationships compared to *V. harveyi* (111, 117). Light production was not seen in uninduced samples for the plate assay, but identified here because of greater instrumental sensitivity. Except for the *lux* operon of *V. cholerae*, all control conditions had little to no light production compared to the pARA-LUXR/$pLUX_{species}$ conditions. All *lux* operon control samples had no more than a 2-fold

difference between induced and uninduced samples (Figure 21, Table 5). These results demonstrate that bioluminescence is due to activation of a *lux* operon by $LuxR_{VH}$ recognizing a binding site and activating some level of *lux* operon transcription for each of the species-specific *lux* operons.

Next to the positive control *V. harveyi lux* operon, *V. vulnificus* and *V. orientalis* had the highest maximum light output increase when induced with arabinose. This was seen by comparing the light production for induced versus uninduced pARA-LUXR/$pLUX_{species}$ conditions (Figure 21, Table 5). This indicates that $LuxR_{VH}$ is recognizing a binding site near the *lux* promoter of the *V. vulnificus* and *V. orientalis lux* operons well. As seen in Figure 1, *V. vulnificus* and *V. orientalis* are the most phylogenetically similar to *V. harveyi* of the tested *Vibrios* (111, 117) and this level of *lux* operon activation by $LuxR_{VH}$ was expected.

V. vulnificus had the most significant increase with a 4,000 times greater relative light for induced versus uninduced pARA-LUXR/$pLUX_{species}$ conditions. Current studies suggest that *V. vulnificus* acquired the *lux* operon through lateral gene transfer from *V. harveyi*, which appears well supported by the results (117). SmcR of *V. vulnificus* was shown to activate the *lux* operon of *V. harveyi* as well as having a 93% amino acid identity to $LuxR_{VH}$ (103). Taken together, the Shao and Hor study along with the results shown here, suggest that SmcR may be the transcriptional activator for the *lux* operon of *V. vulnificus.* Furthermore, this similarity may indicate that SmcR is regulated by *V. harveyi*-type quorum sensing regulation.

The transcriptional activator for the *V. orientalis lux* operon is not yet confirmed; however, BLAST results indicate a hypothetical quorum sensing regulator protein. Homolog sequence alignment showed an 81.9% identity between the hypothetical protein of *V. orientalis* and LuxR of *V. harveyi* (Figure 22, Table 6). This study shows that $LuxR_{VH}$ upregulates transcription of the *V. orientalis lux* operon, further supporting the argument of a possible $LuxR_{VH}$-type transcription factor. Combining these results with the quorum sensing *Vibrio* assay suggests that *V. orientalis* may utilize a *V. harveyi*-type quorum sensing mechanism.

The *lux* operon of *V. chagasii* had the next closest maximum light output to the *V. harveyi lux* operon after *V. vulnificus* and *V. orientalis,* respectfully. The *lux* operon of *V. chagasii* showed less induction by $LuxR_{VH}$ for induced pARA-LUXR/$pLUX_{species}$ samples than the positive *V. harveyi lux* operon control (Figure 21, Table 5). The phylogenetic relationship of *V. chagasii* to *V. harveyi* is further removed than *V. vulnificus* or *V. orientalis*, suggesting a possible reason for the reduced light output (111, 117). Like *V. orientalis*, the *lux* operon transcriptional activator for *V. chagasii* is unknown; however, sequence data for the *V. chagasii* strain in this study is not available for $LuxR_{VH}$ homolog identification. These results similarly indicate that regulation may be performed by a possible $LuxR_{VH}$-type transcription factor in a *V. harveyi*-type quorum sensing system.

As expected, the expression of the *lux* operon of *V. cholerae* increased in the pARA-LUXR/$pLUX_{species}$ induced sample; however, the change is much less than the other studied *lux* operons. Uninduced pARA-LUXR/$pLUX_{species}$ and the other control samples

produced significantly higher levels of light than other *lux* operons under study (Figure 21). *V. cholerae* is more distantly related than the other *Vibrios* tested in this study (111, 117), possibly explaining the lower level of light induction by $LuxR_{VH}$ in the *E. coli* induced pARA-LUXR/$pLUX_{species}$ condition and the higher baseline light production in other trials. The $LuxR_{VH}$ homolog in *V. cholerae*, HapR, is also 71% identical to $LuxR_{VH}$ and accordingly, some cross species induction of *V. cholerae lux* operon by $LuxR_{VH}$ was shown. This is most likely due to $LuxR_{VH}$ recognizing a binding site near the *lux* promoter as the DNA binding domain of HapR is a 95% identical to $LuxR_{VH}$ (50). *V. cholerae* also has similar quorum sensing to *V. harveyi* (Figure 6, 7), yet may differ enough to explain the reduced small induction compared to the other *Vibrio* species under study. The observed induction of the *V. cholerae lux* operon does, however, indicate that HapR may be a $LuxR_{VH}$-type transcription factor for the *lux* operon in *V. cholerae*. This implicates that *V. cholerae*-type quorum sensing regulates bioluminescence in a manner similar to that of *V. harveyi*.

Conclusion

The research presented in this thesis is critical for understanding the regulation of quorum sensing transcription factors. In *V. harveyi*, $LuxR_{VH}$ controls over 600 genes that cover virulence factors, biofilm formation, and bioluminescence (118). Additionally, other studies indicate that high cellular density increases lateral gene transfer among species, an important topic as more bacteria acquire clinical antibiotic resistance (2, 54, 63, 67). This research may provide additional insight into preventing the spread of antibiotic resistance through greater understanding of gene regulation by quorum sensing. Overall, this research generates a greater understanding of gene regulation, phylogenetic relationships, and diversity in bioluminescent bacteria.

Future research may focus on confirmation of the proposed *V. orientalis* transcriptional factor as the *lux* operon regulator. Additionally, a *V. chagasii* transcriptional factor homologous to $LuxR_{VH}$ should be identified. Also, further testing of known $LuxR_{VH}$ homologs in *V. vulnificus* (SmcR) and *V. cholerae* (HapR) should be tested to confirm transcriptional activation of each respective *lux* operon. Additionally, known and yet to be determined transcriptional activators of *V. chagasii*, *V. cholerae*, *V. orientalis* and *V. chagasii* need to be further investigated to elucidate activator binding sites. Finally, quorum sensing regulation mechanisms of the transcriptional regulators in these *Vibrios* need to be identified to confirm *V. harveyi*-type quorum sensing.

This study indicates that these *Vibrio* species: *harveyi*, *chagasii*, *cholerae*, *orientalis*, and *vulnificus*, undergo quorum sensing regulation of bioluminescence. Additionally, the

arabinose-induced increase in light production shows that $LuxR_{VH}$ successfully activates the *lux* operons of each of the *Vibrio* species in the dual vector *E. coli* system. This $LuxR_{VH}$ activation implies that $LuxR_{VH}$ recognizes a binding site near the *lux* promoter of different *Vibrio lux* operons. Additionally, the fact that $LuxR_{VH}$ induces *lux* operons of *V. chagasii, V. cholerae*, V. *orientalis* and *V. vulnificus* implies conservation of the regulatory mechanism among these *Vibrios,* suggesting that the other *Vibrios* may use a $LuxR_{VH}$-type transcription factor for their respective *lux* operon regulation. The extent of activation likely depends on the similarity of the other *Vibrios lux* operons to that in *V. harveyi* (Figure 1). The increased *lux* gene upregulation by $LuxR_{VH}$ seen for the *lux* operons of *V. orientalis*, and *V. vulnificus* likely reflects their closer evolutionary relationship to *V. harveyi*, followed by *V. chagasii* and *V. cholerae*, which shows less significant induction (Figure 21, Table 5). This research reinforces the argument that *lux* operon regulation is mechanistically conserved among closely related *Vibrio* strains. Collectively, these results show cross species activation of *V. chagasii*, *V. cholerae*, *V. orientalis*, and *V. vulnificus lux* operons by $LuxR_{VH}$ as well as implying that the regulation of the *lux* operon transcriptional factors of *V. chagasii*, *V. orientalis*, *V. vulnificus* as well as *V. cholerae* is of the V. *harveyi*-type quorum sensing.

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Appendix A: Relative Light Calculations for *Vibrio* Samples

V. harveyi

Species Sample	Hour	Light	OD_{600}	Relative Light
Vibrio harveyi Sample 1	0	95355867	0.044	2192088897.00
	1	2776584	0.097	28713381.60
	2	308167	0.213	1448834.04
	3	69685	0.438	159207.22
	4	773217	0.792	975914.43
	5	30436604	1.267	24022576.16
	6	751000008	1.440	521527783.30
Vibrio harveyi Sample 2	0	89634337	0.068	1313323619.00
	1	9971637	0.137	72785671.53
	2	260167	0.353	737017.00
	3	45643	0.788	57959.37
	4	725593	1.296	559871.14
	5	27015407	1.830	14762517.49
	6	697009472	1.990	350256016.10
Vibrio harveyi Sample 3	0	1003358492	0.031	32896999738.00
	1	8237444	0.079	104935592.40
	2	500896	0.166	3026561.93
	3	66852	0.319	209896.39
	4	822174	0.586	1403027.30
	5	26088719	0.906	28795495.58
	6	197846630	1.027	192645209.30

V. chagasii

Species Sample	Hour	Light	OD_{600}	Relative Light
Vibrio chagasii Sample 1	0	1127	0.078	14448.72
	1	112	0.142	788.73
	2	9	0.386	23.32
	3	49	0.783	62.58
	4	289	1.848	156.39
	5	1466	2.844	515.47
	6	3933	3.232	1216.89
Vibrio chagasii Sample 2	0	1156	0.069	16753.62
	1	117	0.154	759.74
	2	24	0.430	55.81
	3	89	0.903	98.56
	4	398	1.802	220.87
	5	1796	2.772	647.91
	6	4128	3.032	1361.48
Vibrio chagasii Sample 3	0	1248	0.074	16979.59
	1	120	0.148	810.81
	2	8	0.408	19.61
	3	62	0.843	73.55
	4	352	1.825	192.88
	5	1406	2.808	500.71
	6	2251	3.132	718.71

V. cholerae

Species Sample	Hour	Light	OD_{600}	Relative Light
Vibrio cholerae Sample 1	0	580604	0.046	12621826.09
	1	76573	0.081	945345.68
	2	23655	0.217	109009.22
	3	43453	0.374	116184.49
	4	122604	0.523	234424.47
	5	343291	0.742	462656.33
	6	686582	0.816	841193.34
Vibrio cholerae Sample 2	0	594116	0.060	9901933.33
	1	78762	0.103	764679.61
	2	21980	0.226	97256.64
	3	46477	0.387	120095.61
	4	130135	0.547	237906.76
	5	364378	0.724	503284.53
	6	728756	0.796	915062.78
Vibrio cholerae Sample 3	0	584405	0.053	11026509.43
	1	77270	0.092	839891.30
	2	24748	0.151	164438.53
	3	43368	0.237	182987.34
	4	134799	0.383	352415.69
	5	377437	0.535	705489.72
	6	754874	0.631	1197262.49

V. orientalis

Species Sample	Hour	Light	OD_{600}	Relative Light
Vibrio orientalis Sample 1	0	73951	0.143	517139.86
	1	8901	0.243	36629.63
	2	1660	0.585	2837.61
	3	537	1.181	454.70
	4	1023	1.784	573.43
	5	8244	1.381	5969.59
	6	51741	1.125	45992.00
Vibrio orientalis Sample 2	0	40669	0.094	432648.94
	1	5506	0.206	26728.16
	2	1237	0.551	2245.01
	3	518	1.150	450.43
	4	403	1.640	245.73
	5	1784	1.310	1361.83
	6	12074	1.110	10877.48
Vibrio orientalis Sample 3	0	16887	0.067	252044.78
	1	5373	0.106	50688.68
	2	828	0.223	3713.00
	3	161	0.632	254.75
	4	84	1.107	75.88
	5	1083	1.396	775.79
	6	10458	1.475	7090.17

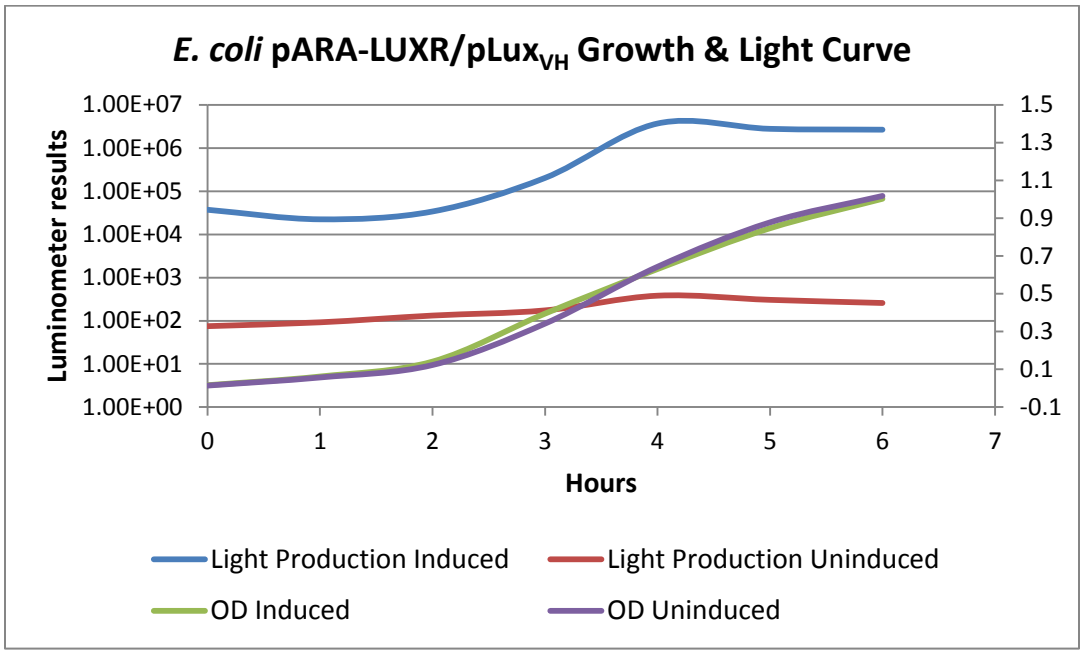
V. vulnificus

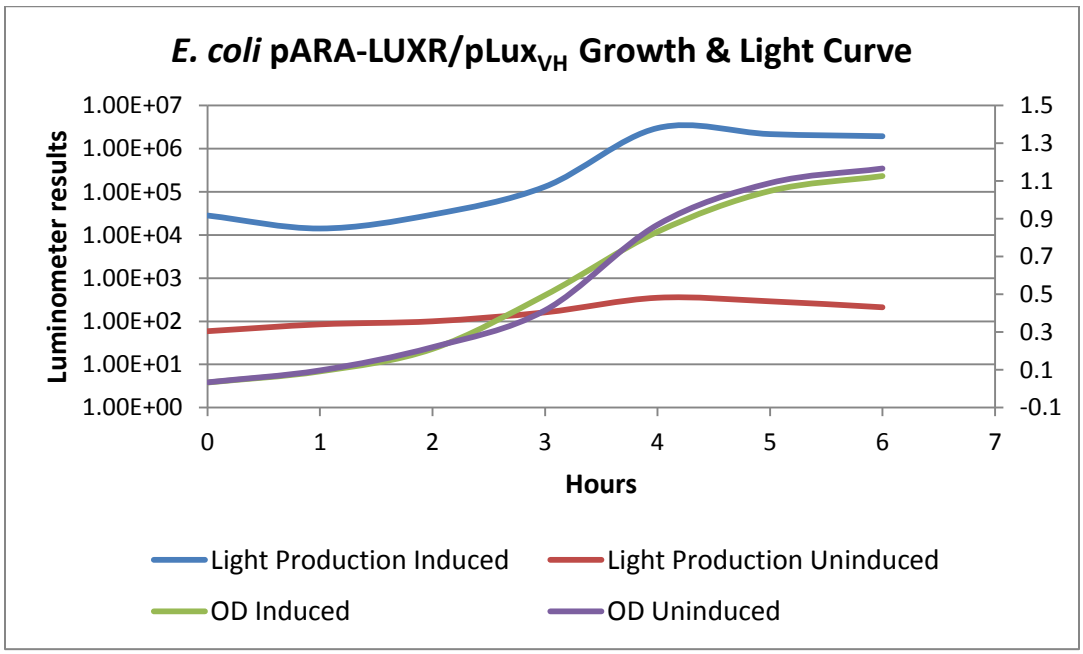
Species Sample	Hour	Light	OD_{600}	Relative Light
Vibrio vulnificus Sample 1	0	121	0.009	13444.44
	1	313	0.045	6955.56
	2	144	0.16	900.00
	3	6829	0.459	14878.00
	4	18502	0.76	24344.74
	5	25560	1	25560.00
Vibrio vulnificus Sample 2	0	152	0.012	12666.67
	1	318	0.055	5781.82
	2	127	0.176	721.59
	3	3678	0.5	7356.00
	4	14795	0.801	18470.66
	5	20927	0.98	21354.08
Vibrio vulnificus Sample 3	0	145	0.013	11153.85
	1	330	0.056	5892.86
	2	143	0.175	817.14
	3	4294	0.485	8853.61
	4	49348	0.78	63266.67
	5	63548	0.97	65513.40

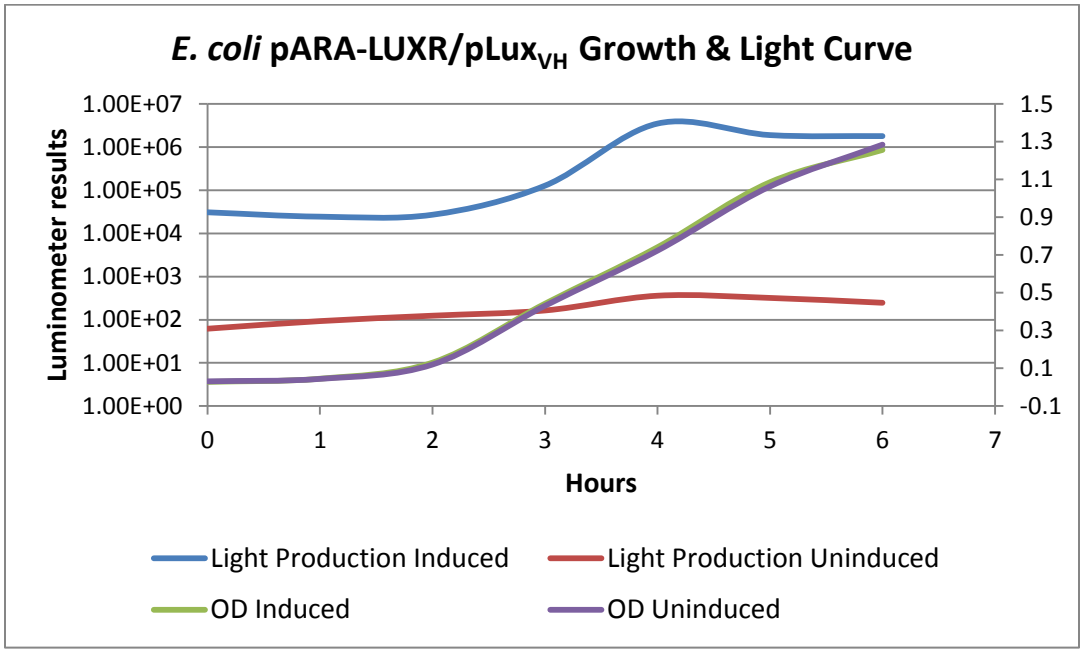
Appendix B: Individual *E. coli* Growth & Light Curves

Test: *Vibrio harveyi lux* Operon

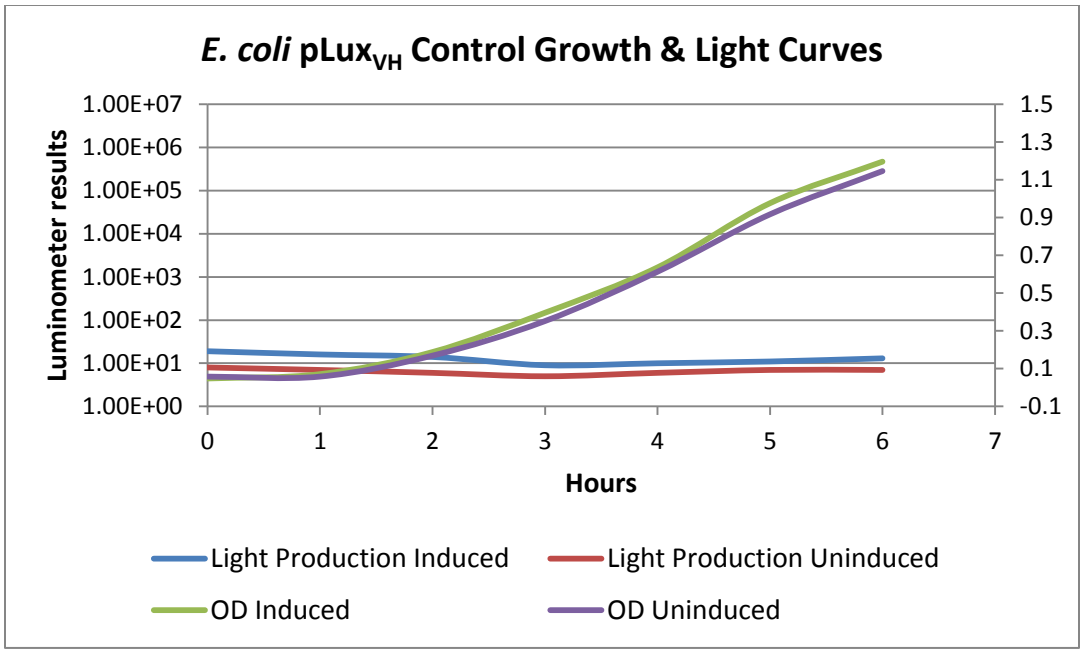
Condition: *E. coli* containing pARA-LUX/pLUX$_{VH}$

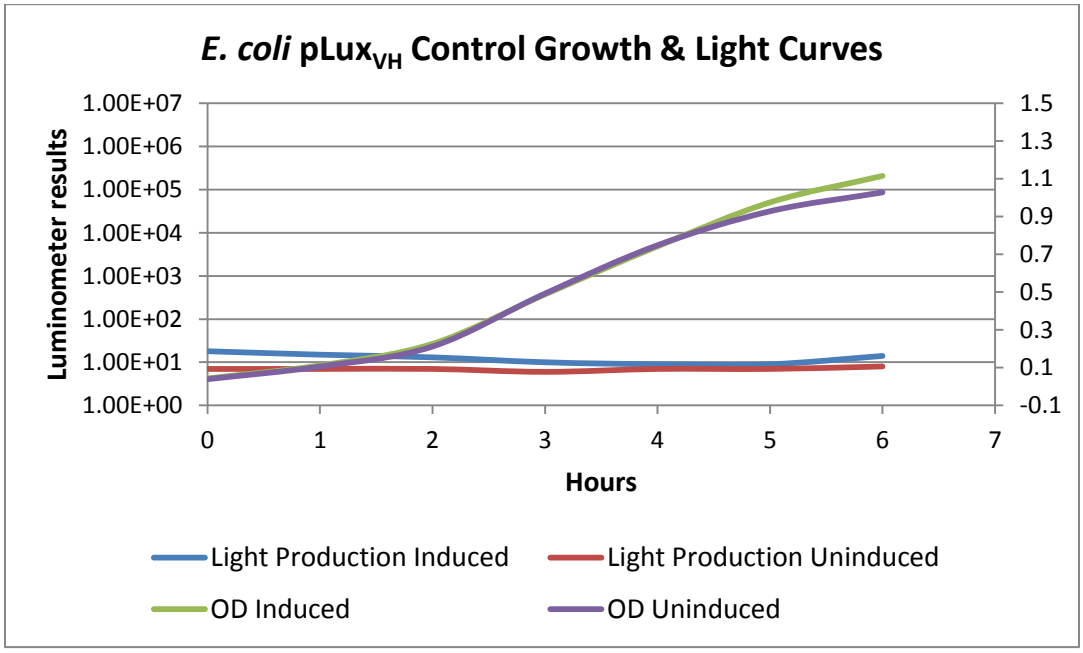


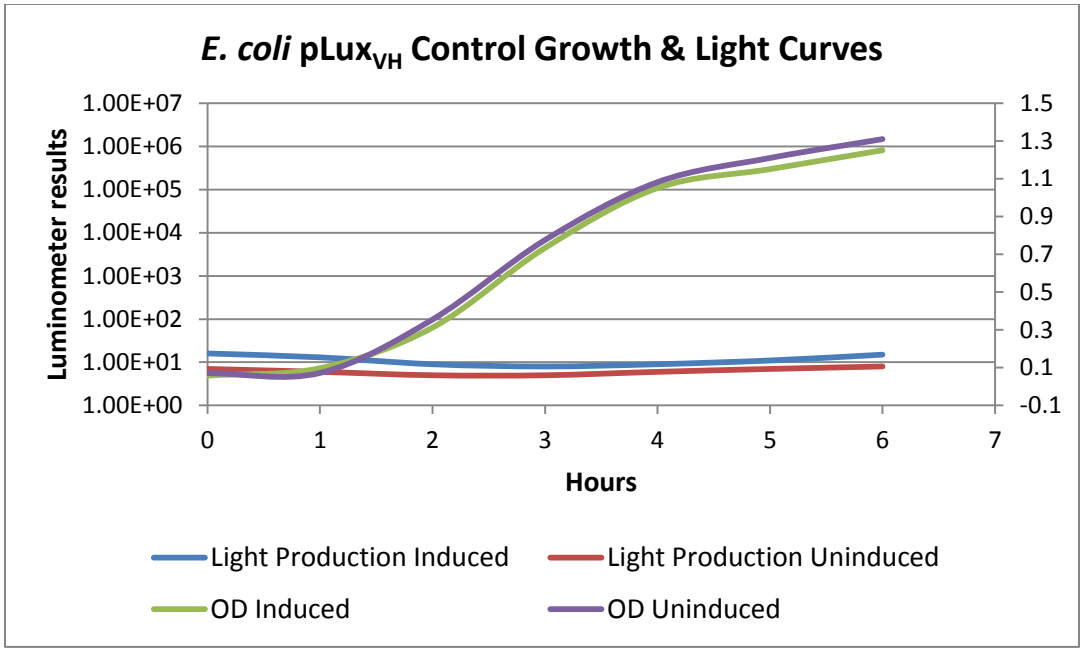




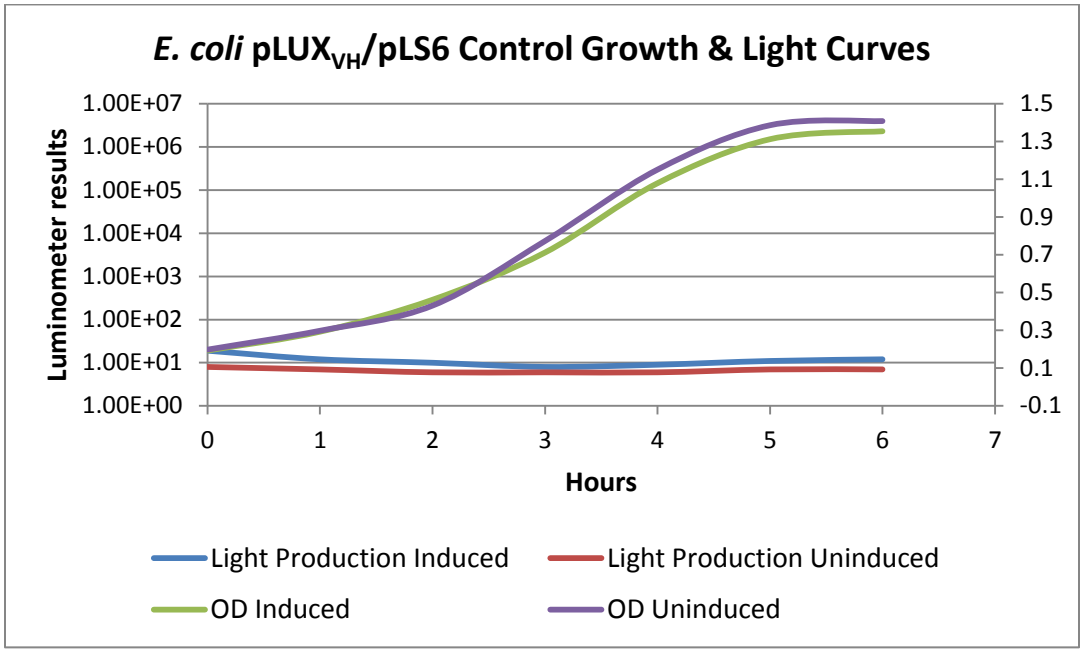
Condition: *E. coli* containing $pLUX_{VH}$ control

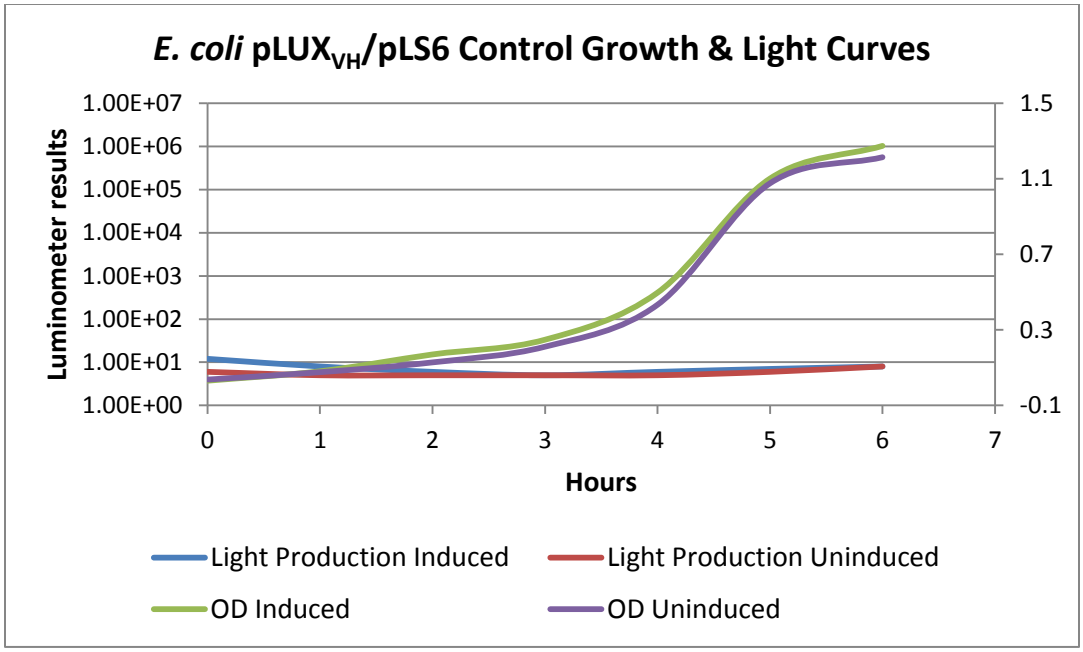


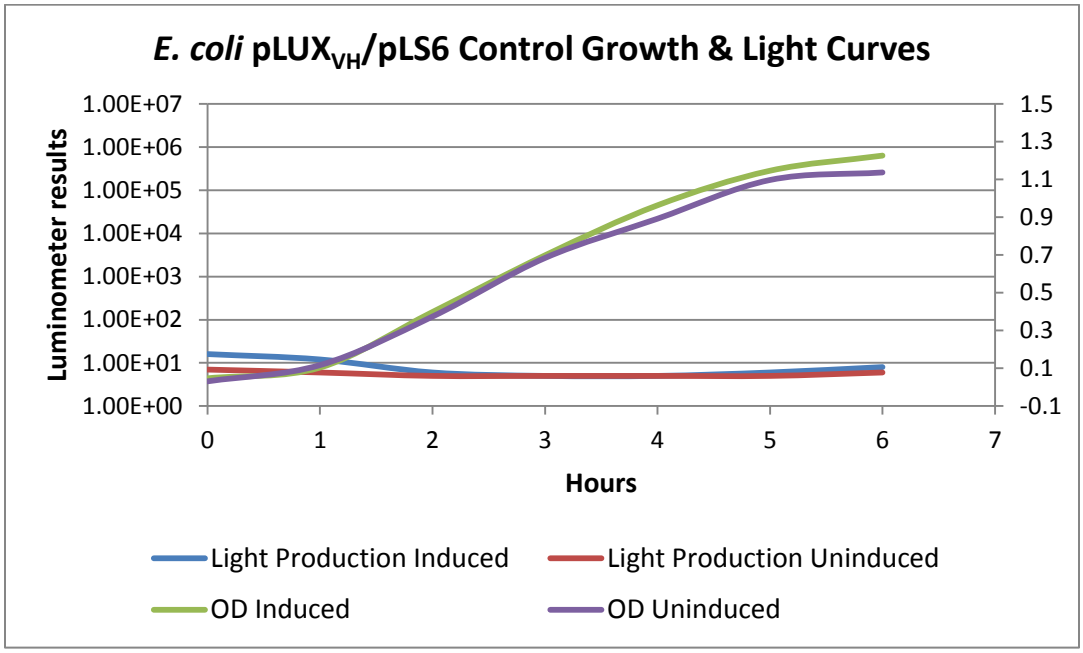

E. coli pLuxVH Control Growth & Light Curves
Luminometer results
1.00E+07
1.00E+06
1.00E+05
1.00E+04
1.00E+03
1.00E+02
1.00E+01
1.00E+00
1.5
1.3
1.1
0.9
0.7
0.5
0.3
0.1
-0.1
0
1
2
3
4
5
6
7
Hours
Light Production Induced
Light Production Uninduced
OD Induced
OD Uninduced


E. coli pLuxVH Control Growth & Light Curves
Luminometer results
1.00E+07
1.00E+06
1.00E+05
1.00E+04
1.00E+03
1.00E+02
1.00E+01
1.00E+00
1.5
1.3
1.1
0.9
0.7
0.5
0.3
0.1
-0.1
0
1
2
3
4
5
6
7
Hours
Light Production Induced
Light Production Uninduced
OD Induced
OD Uninduced

Condition: *E. coli* containing pLUX$_{VH}$/pLS6 control

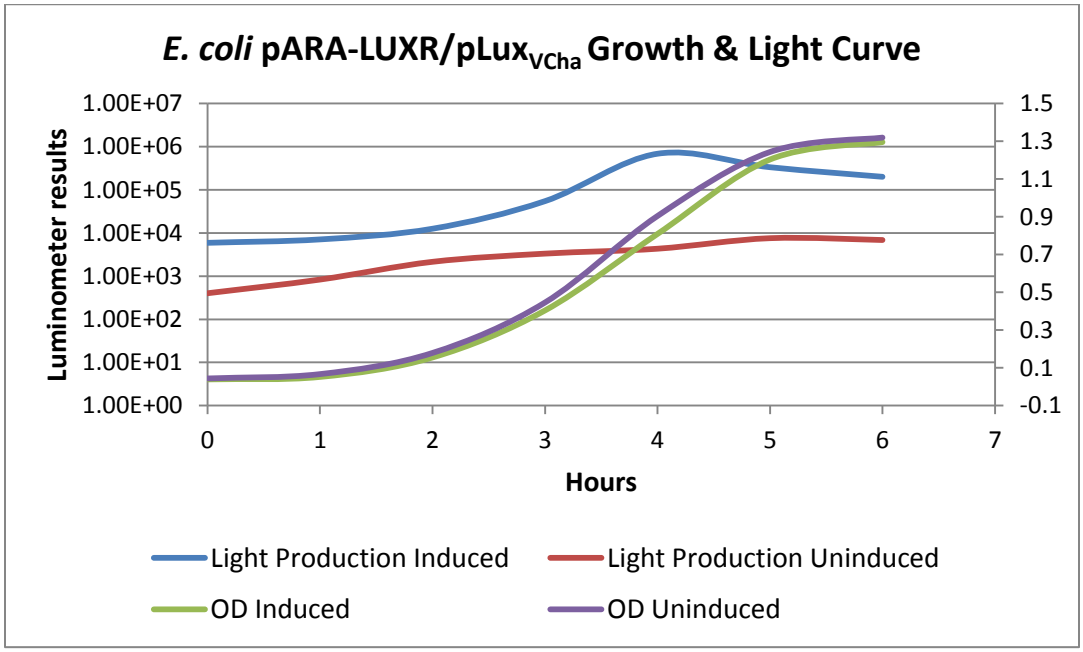


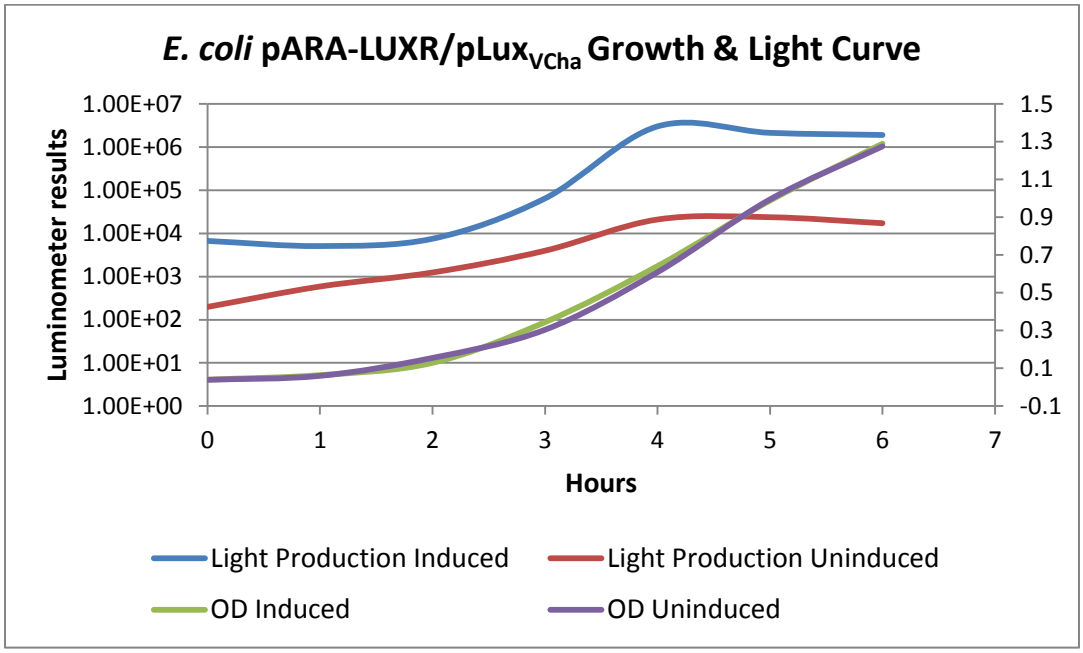


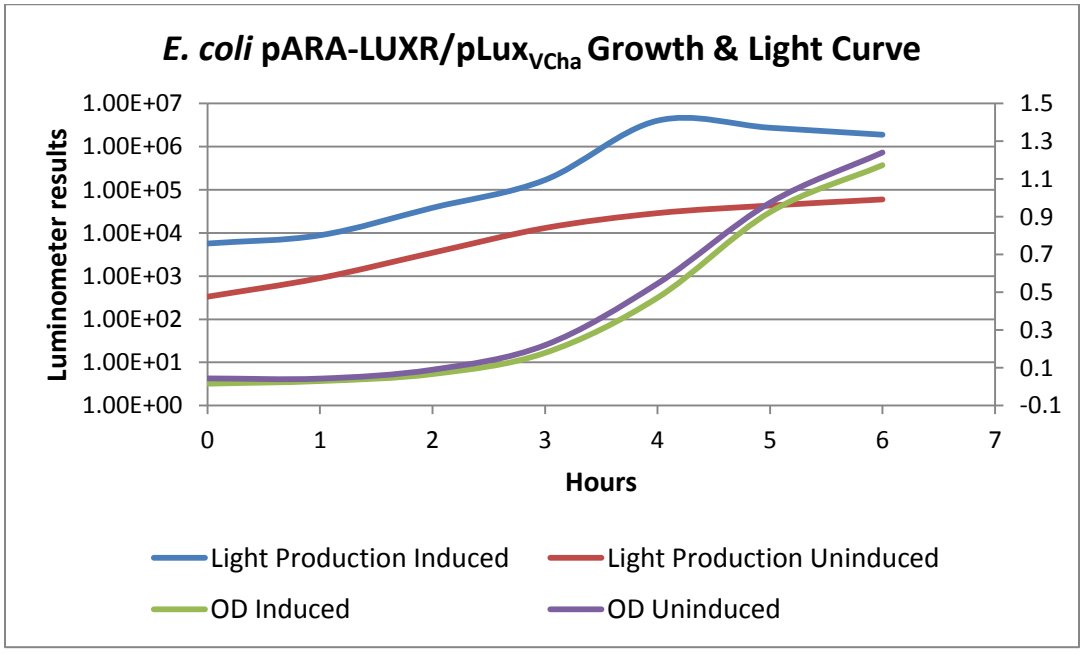


Test: *Vibrio chagasii lux* Operon

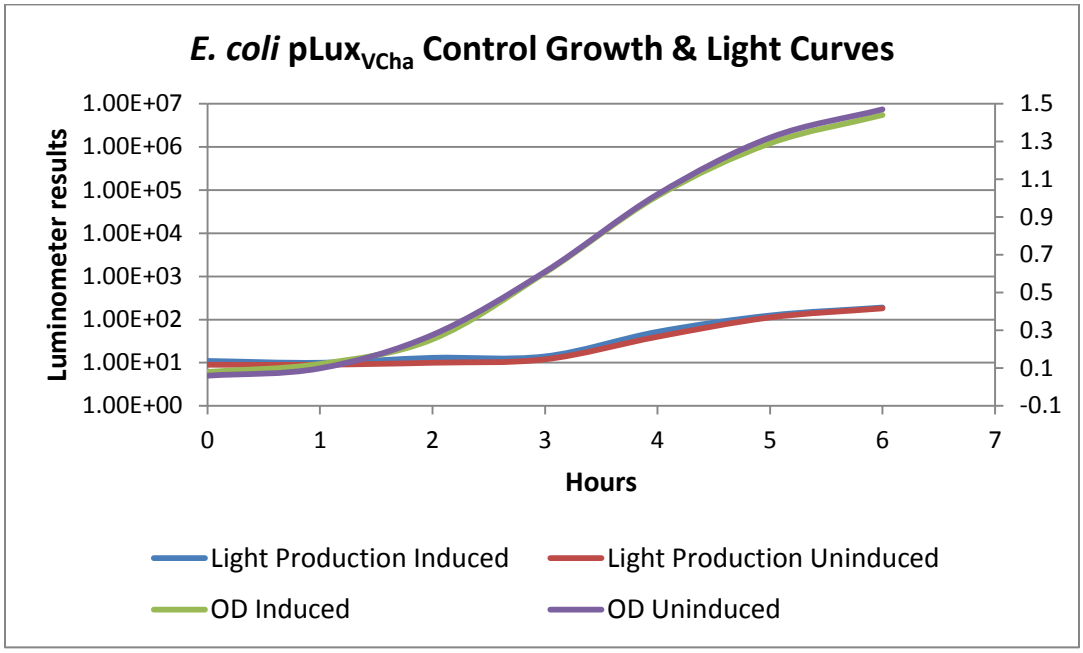
Condition: *E. coli* containing pARA-LUXR/pLUX$_{VCha}$

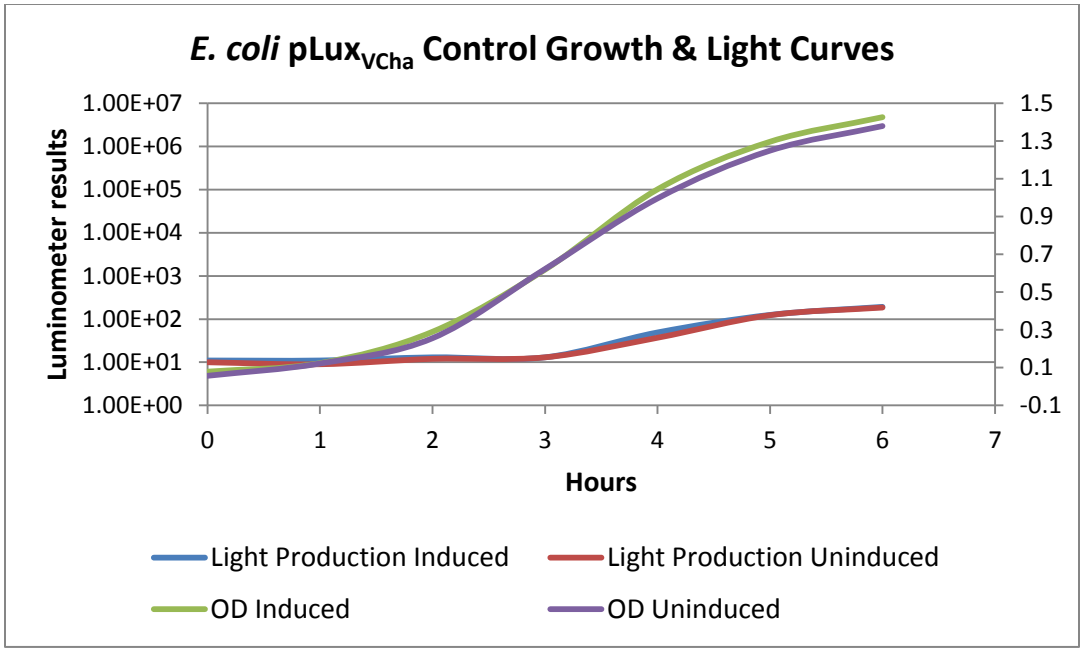


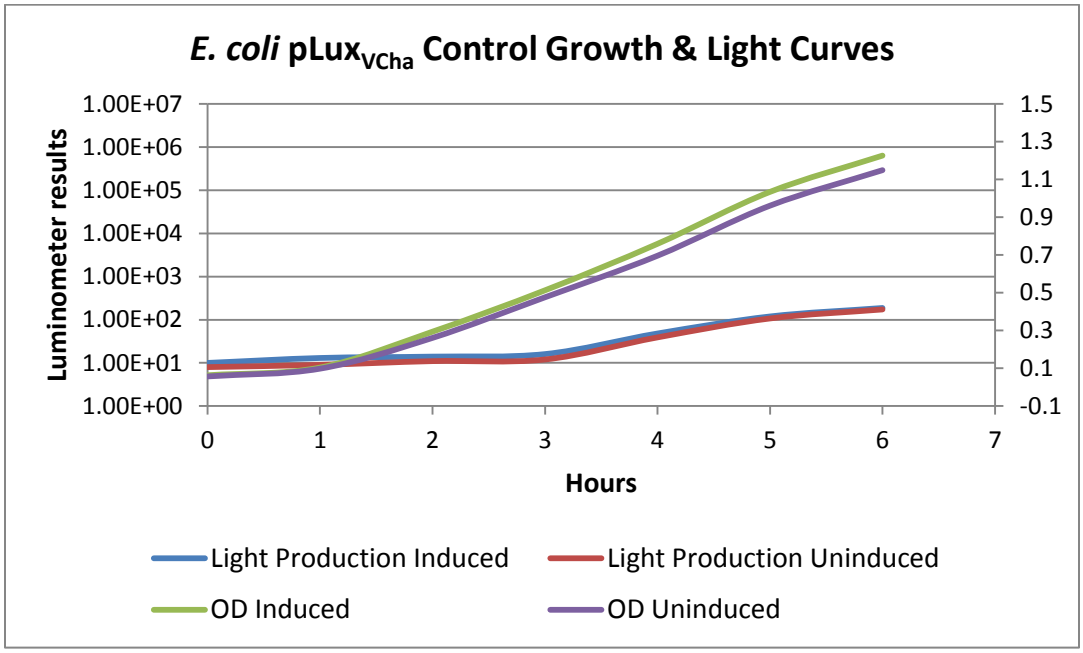

E. coli pARA-LUXR/pLuxVCha Growth & Light Curve
Luminometer results
1.00E+07
1.00E+06
1.00E+05
1.00E+04
1.00E+03
1.00E+02
1.00E+01
1.00E+00
1.5
1.3
1.1
0.9
0.7
0.5
0.3
0.1
-0.1
0
1
2
3
4
5
6
7
Hours
Light Production Induced
Light Production Uninduced
OD Induced
OD Uninduced


E. coli pARA-LUXR/pLuxVCha Growth & Light Curve
Luminometer results
1.00E+07
1.00E+06
1.00E+05
1.00E+04
1.00E+03
1.00E+02
1.00E+01
1.00E+00
1.5
1.3
1.1
0.9
0.7
0.5
0.3
0.1
-0.1
0
1
2
3
4
5
6
7
Hours
Light Production Induced
Light Production Uninduced
OD Induced
OD Uninduced

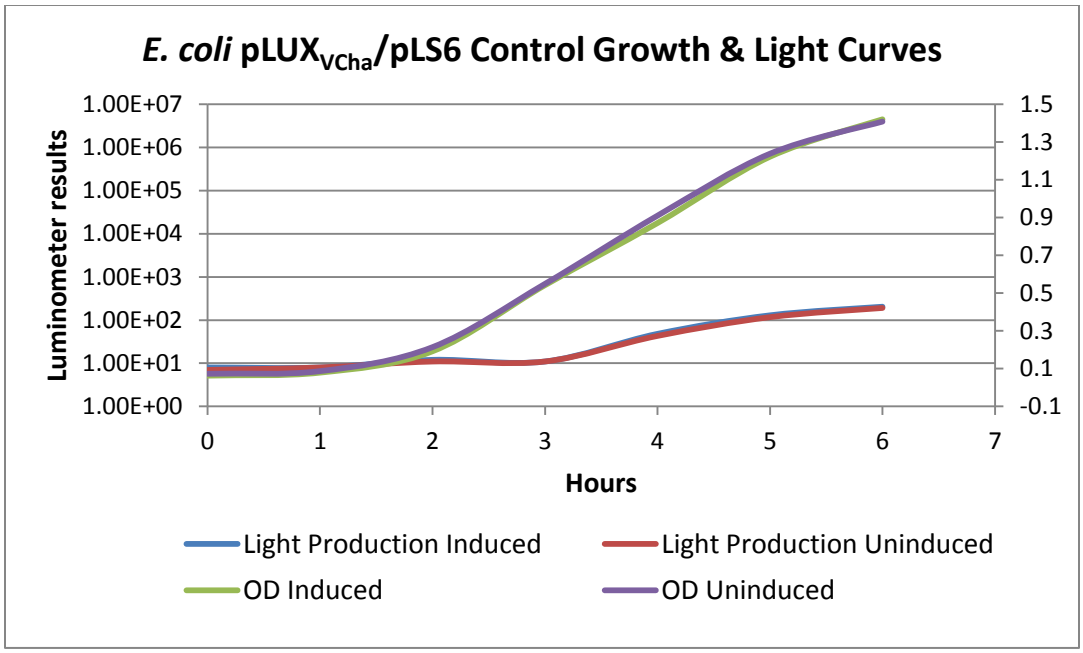
Condition: *E. coli* containing pLUX$_{VCha}$ control

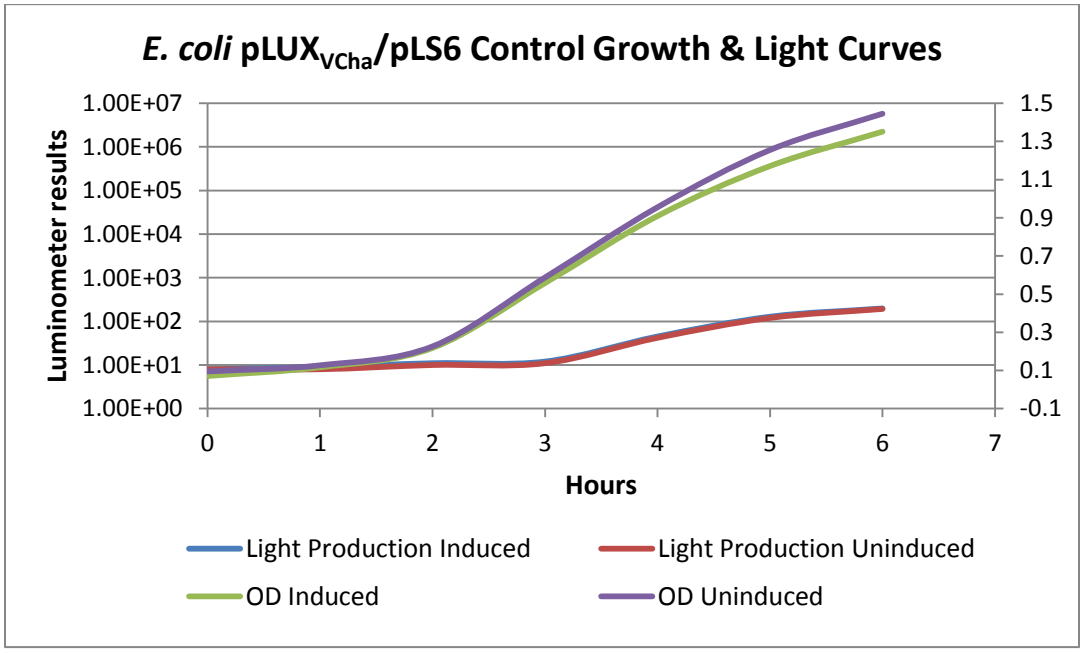


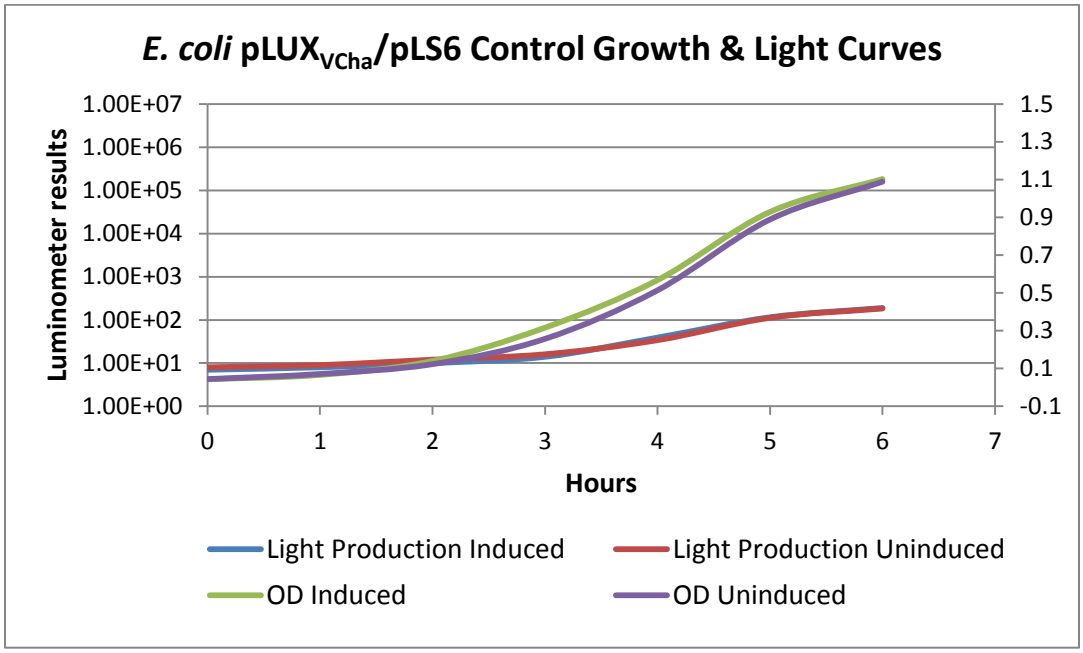




Condition: *E. coli* containing pLUX$_{VCha}$ /pLS6 control

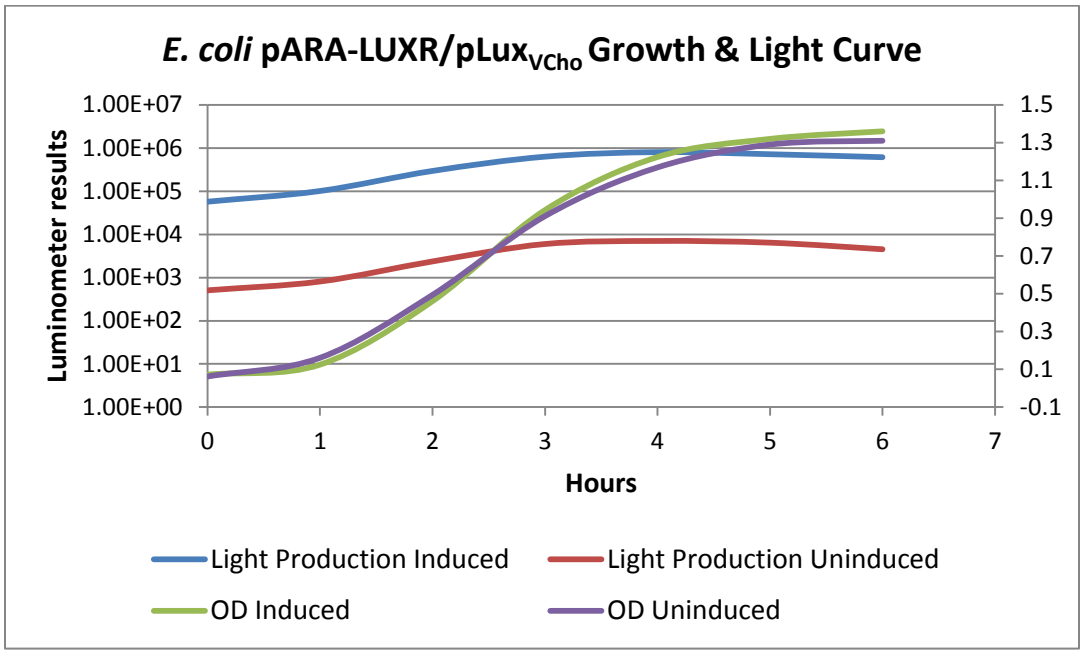


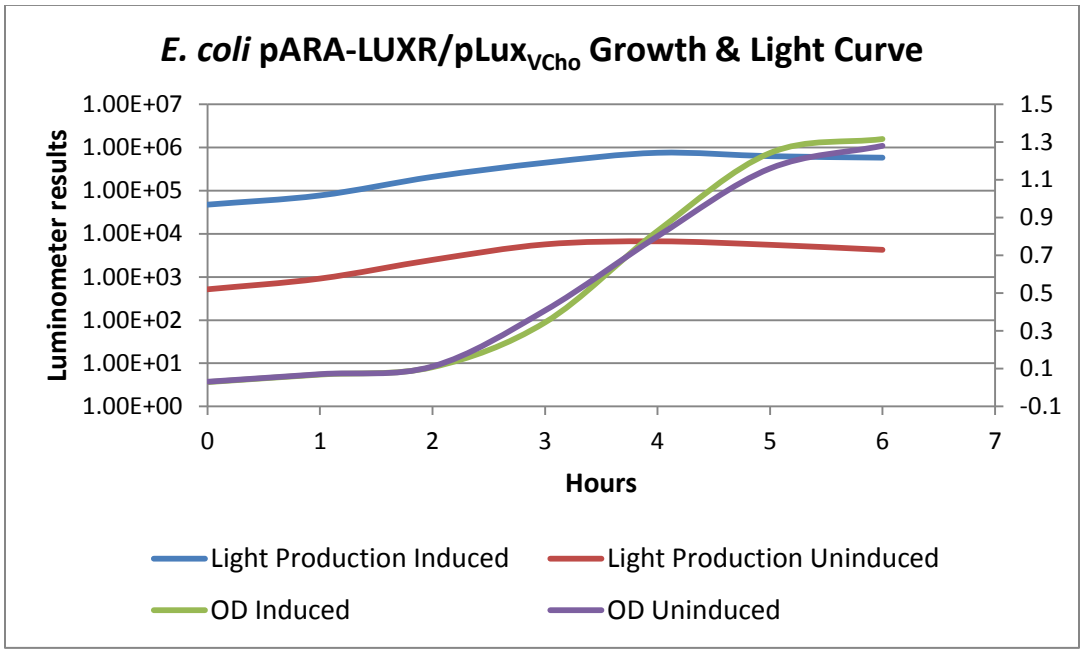

E. coli pLUXVCha/pLS6 Control Growth & Light Curves
Luminometer results
1.00E+07
1.00E+06
1.00E+05
1.00E+04
1.00E+03
1.00E+02
1.00E+01
1.00E+00
1.5
1.3
1.1
0.9
0.7
0.5
0.3
0.1
-0.1
0
1
2
3
4
5
6
7
Hours
Light Production Induced
Light Production Uninduced
OD Induced
OD Uninduced

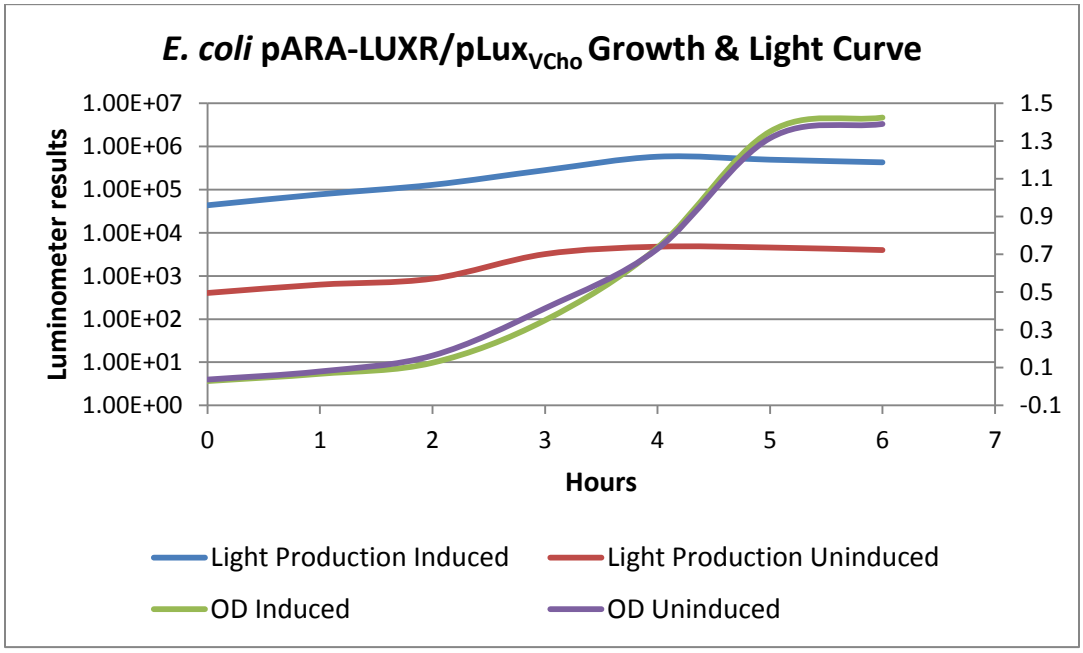

E. coli pLUXVCha/pLS6 Control Growth & Light Curves
Luminometer results
1.00E+07
1.00E+06
1.00E+05
1.00E+04
1.00E+03
1.00E+02
1.00E+01
1.00E+00
1.5
1.3
1.1
0.9
0.7
0.5
0.3
0.1
-0.1
0
1
2
3
4
5
6
7
Hours
Light Production Induced
Light Production Uninduced
OD Induced
OD Uninduced

Test: *Vibrio cholerae lux* Operon

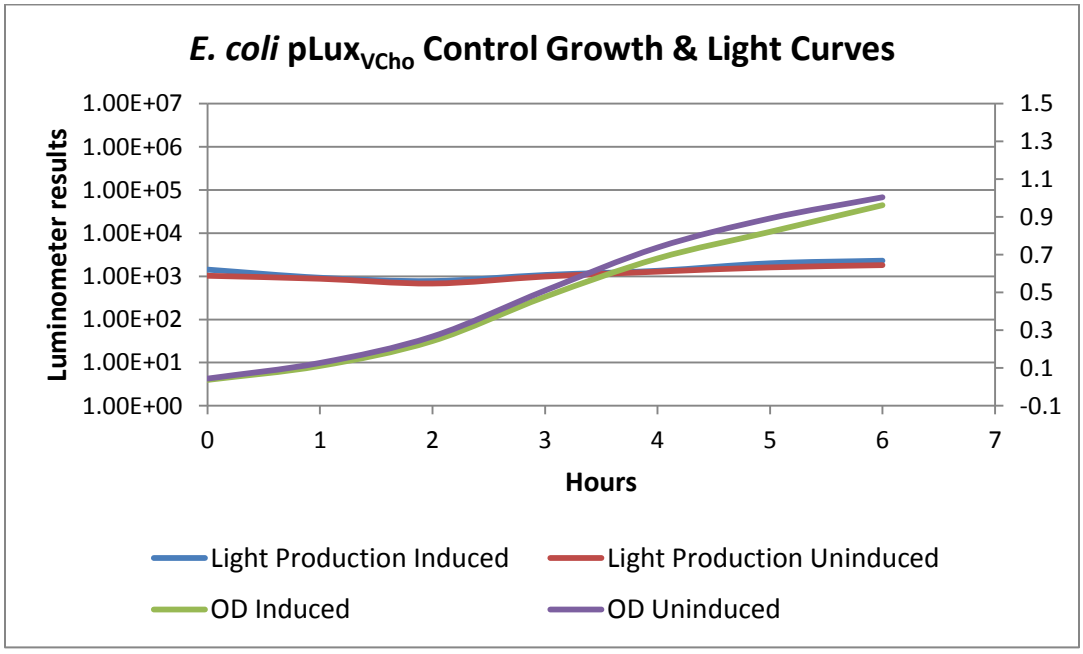
Condition: *E. coli* containing pARA-LUXR/pLUX$_{VCho}$

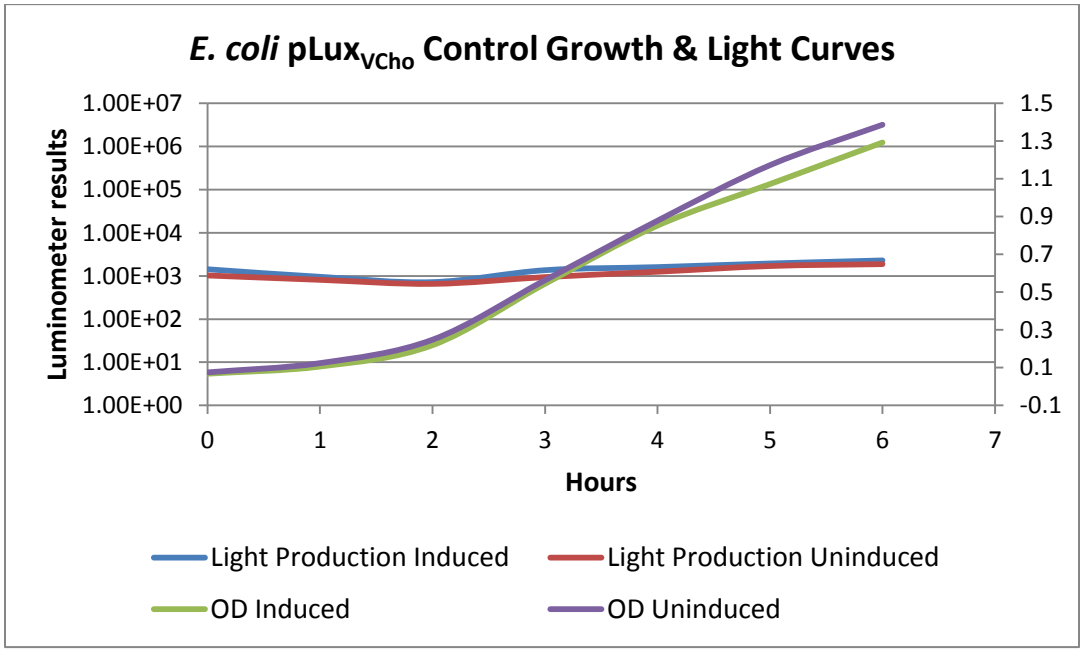


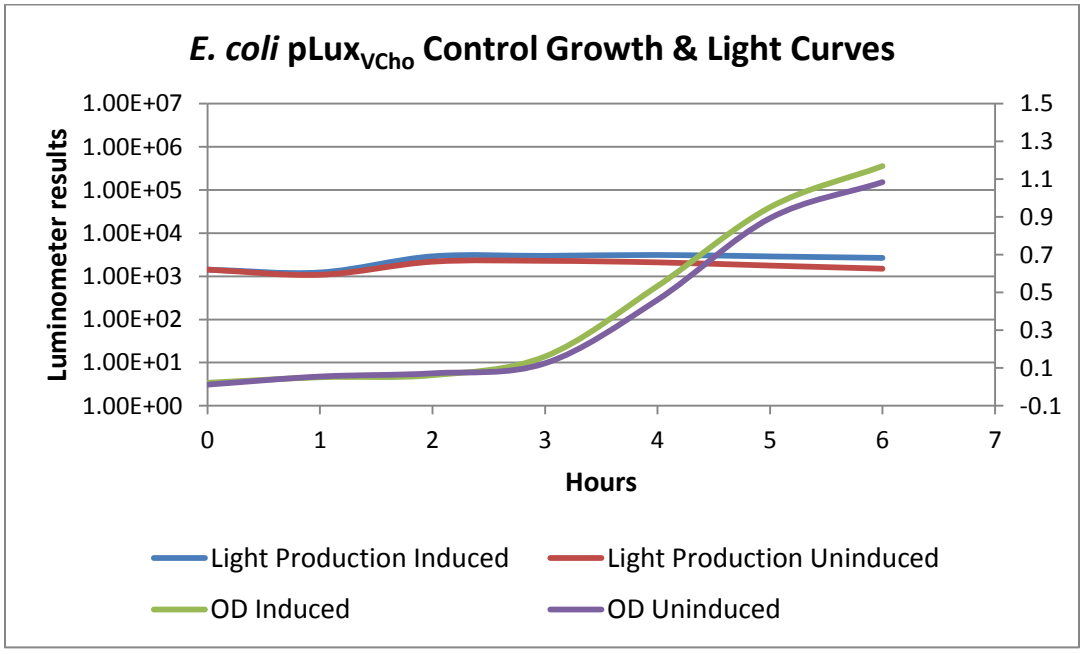




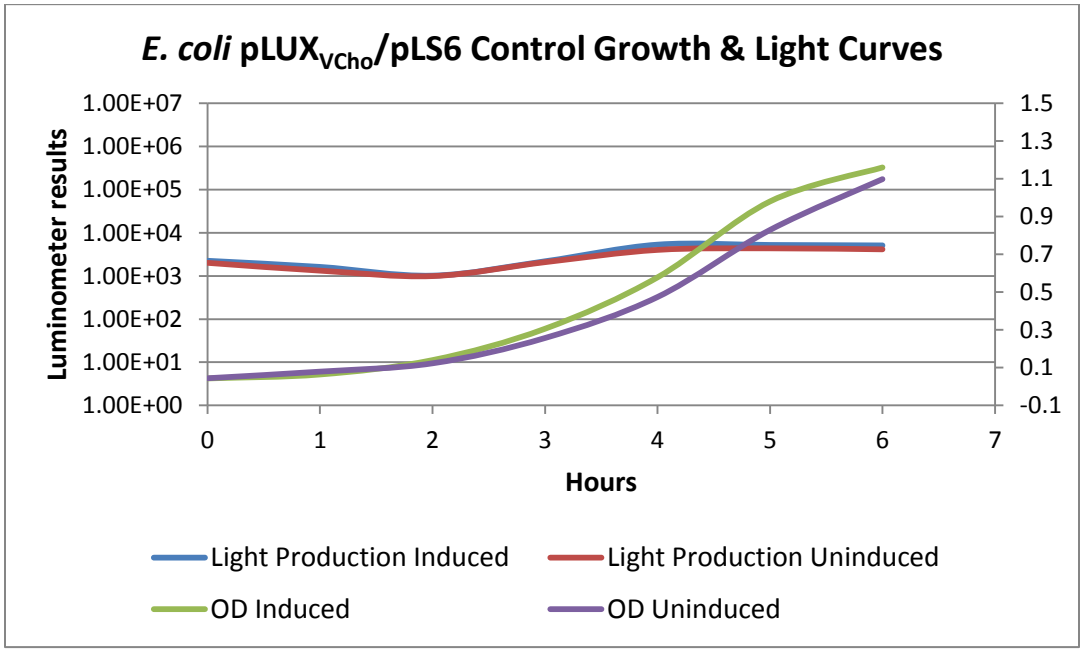
Condition: *E. coli* containing pLUX$_{VCho}$ control

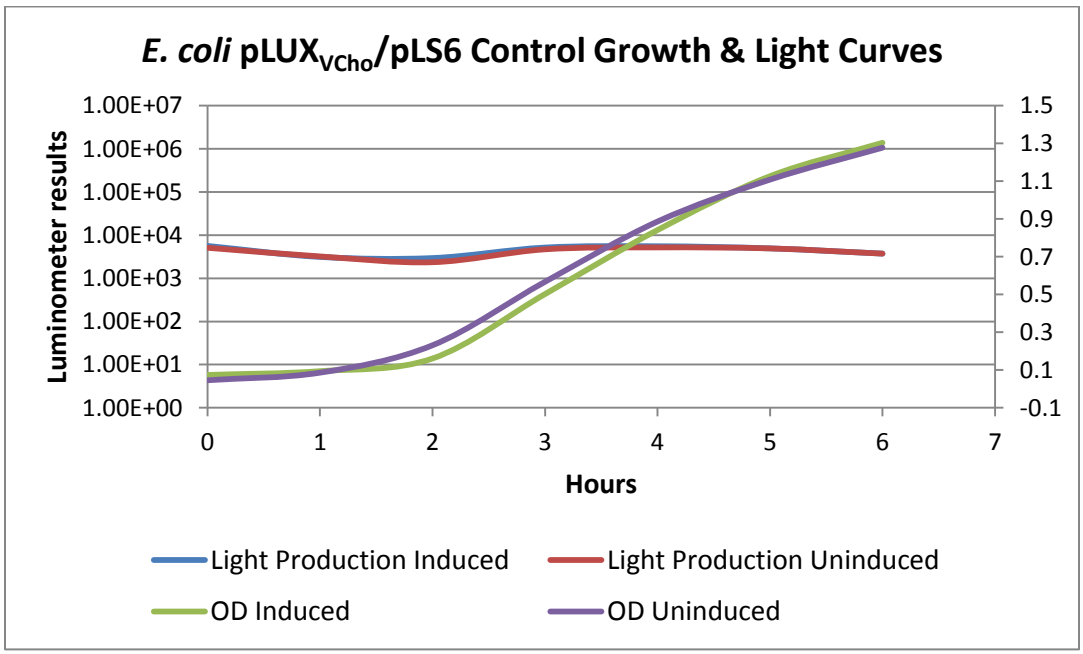


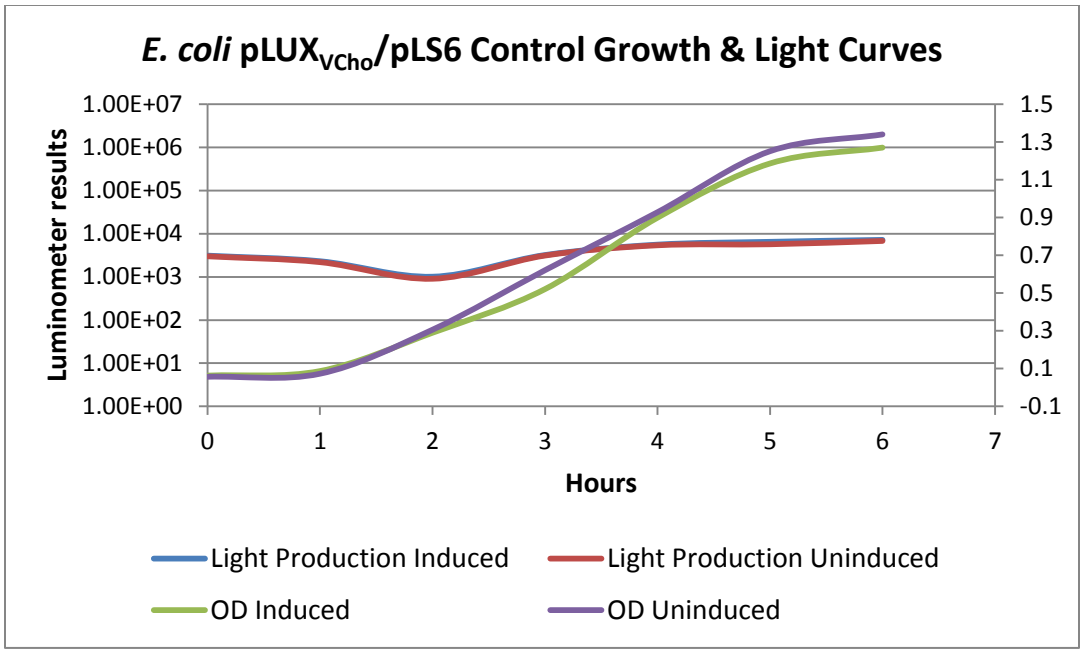

E. coli pLuxVCho Control Growth & Light Curves
Luminometer results
1.00E+07
1.00E+06
1.00E+05
1.00E+04
1.00E+03
1.00E+02
1.00E+01
1.00E+00
1.5
1.3
1.1
0.9
0.7
0.5
0.3
0.1
-0.1
0
1
2
3
4
5
6
7
Hours
Light Production Induced
Light Production Uninduced
OD Induced
OD Uninduced


E. coli pLuxVCho Control Growth & Light Curves
Luminometer results
1.00E+07
1.00E+06
1.00E+05
1.00E+04
1.00E+03
1.00E+02
1.00E+01
1.00E+00
1.5
1.3
1.1
0.9
0.7
0.5
0.3
0.1
-0.1
0
1
2
3
4
5
6
7
Hours
Light Production Induced
Light Production Uninduced
OD Induced
OD Uninduced

Condition: *E. coli* containing pLUX$_{VCho}$ /pLS6 control

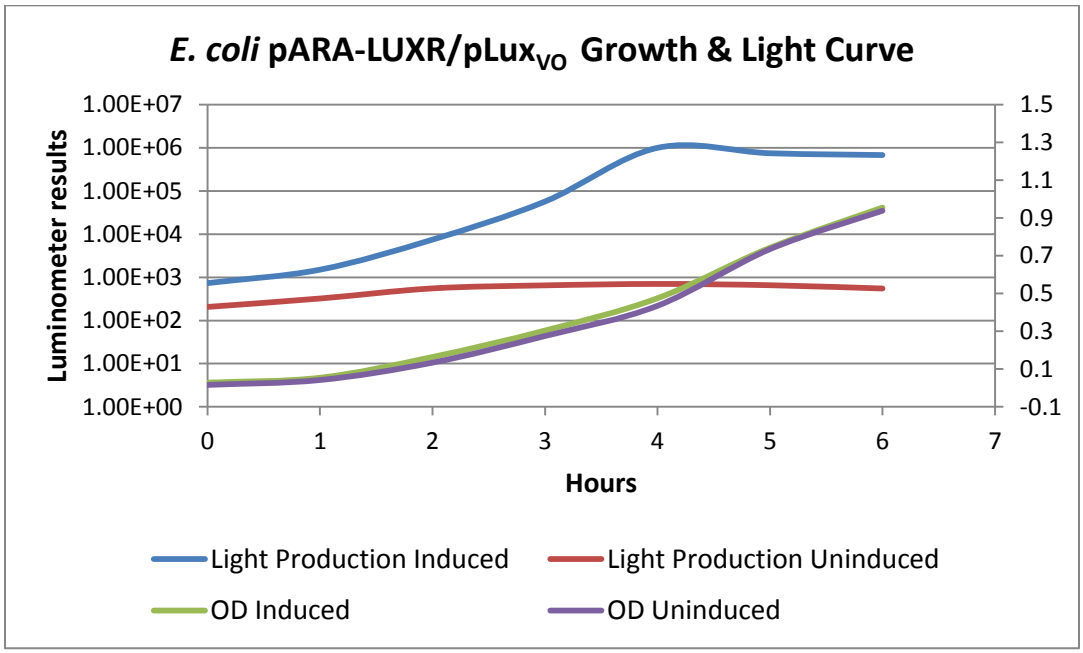


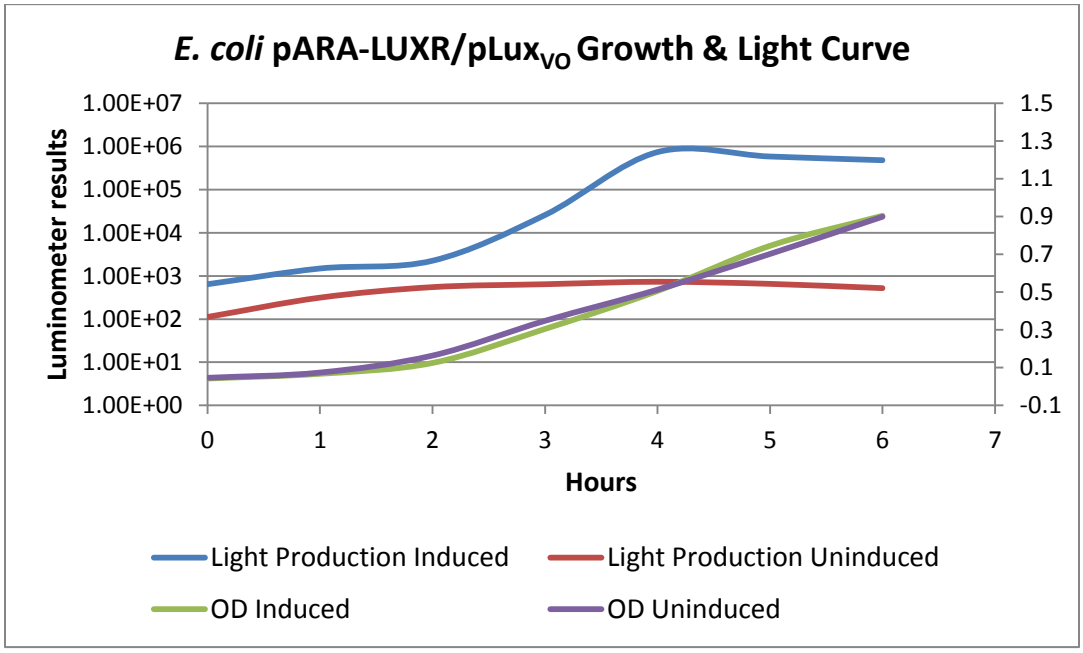


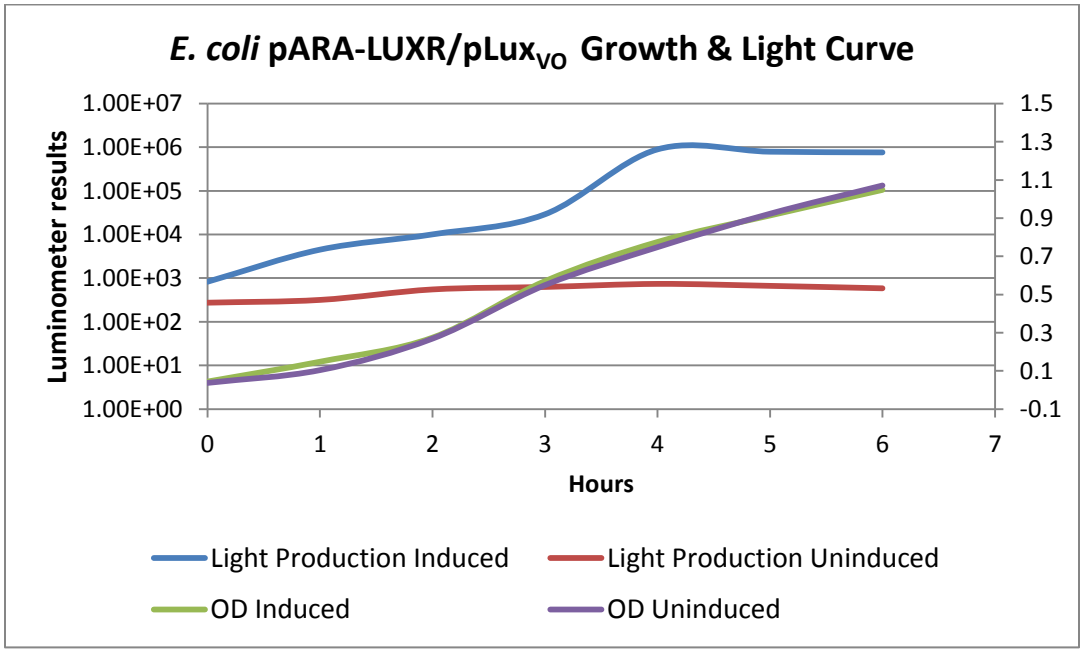


Test: *Vibrio orientalis lux* Operon

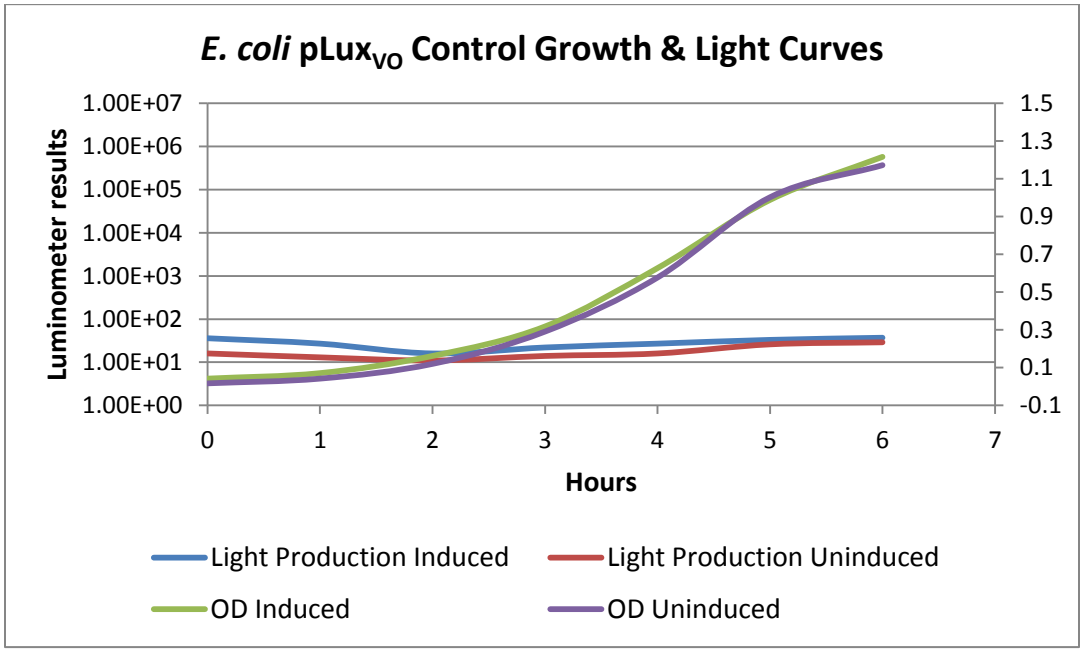
Condition: *E. coli* containing pARA-LUXR/pLUX$_{VO}$

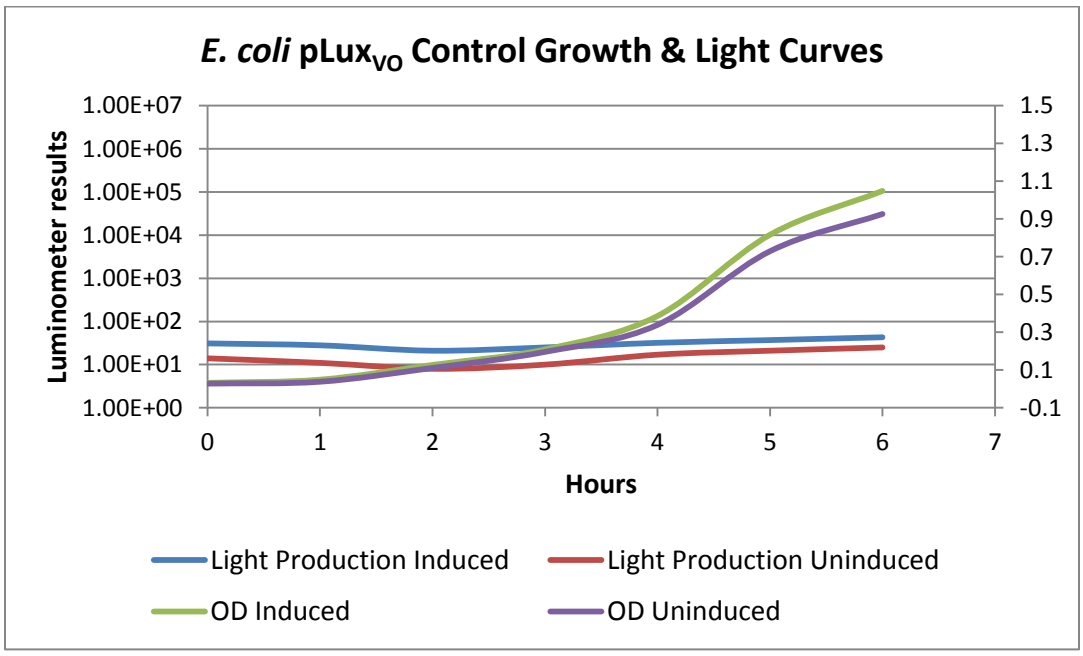


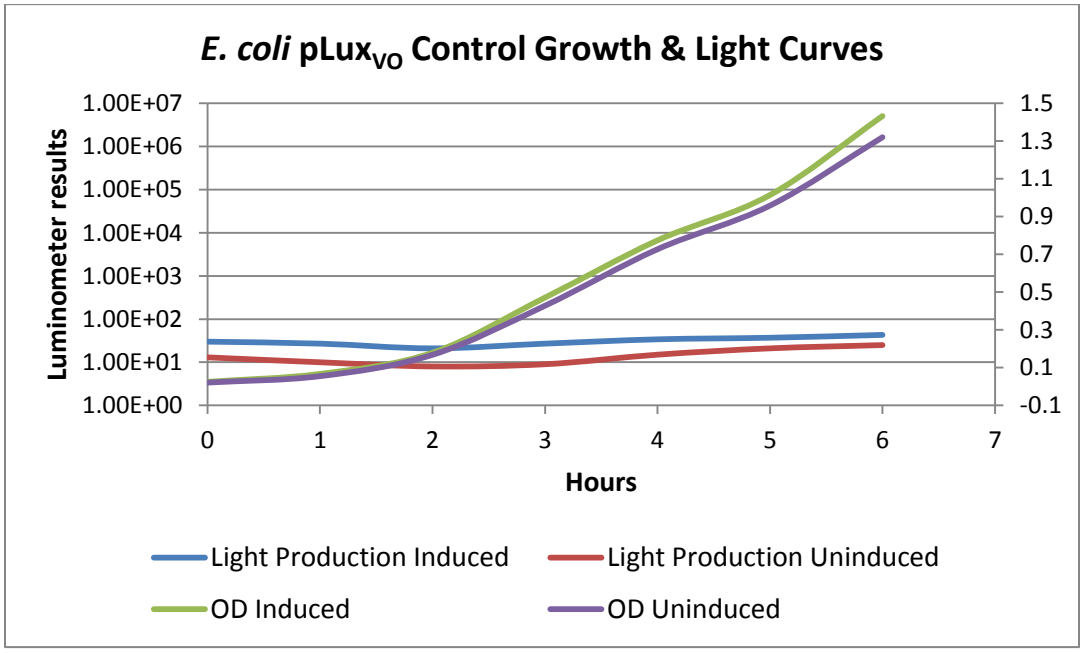

E. coli pARA-LUXR/pLuxVO Growth & Light Curve
Luminometer results
1.00E+07
1.00E+06
1.00E+05
1.00E+04
1.00E+03
1.00E+02
1.00E+01
1.00E+00
1.5
1.3
1.1
0.9
0.7
0.5
0.3
0.1
-0.1
0
1
2
3
4
5
6
7
Hours
Light Production Induced
Light Production Uninduced
OD Induced
OD Uninduced


E. coli pARA-LUXR/pLuxVO Growth & Light Curve
Luminometer results
1.00E+07
1.00E+06
1.00E+05
1.00E+04
1.00E+03
1.00E+02
1.00E+01
1.00E+00
1.5
1.3
1.1
0.9
0.7
0.5
0.3
0.1
-0.1
0
1
2
3
4
5
6
7
Hours
Light Production Induced
Light Production Uninduced
OD Induced
OD Uninduced

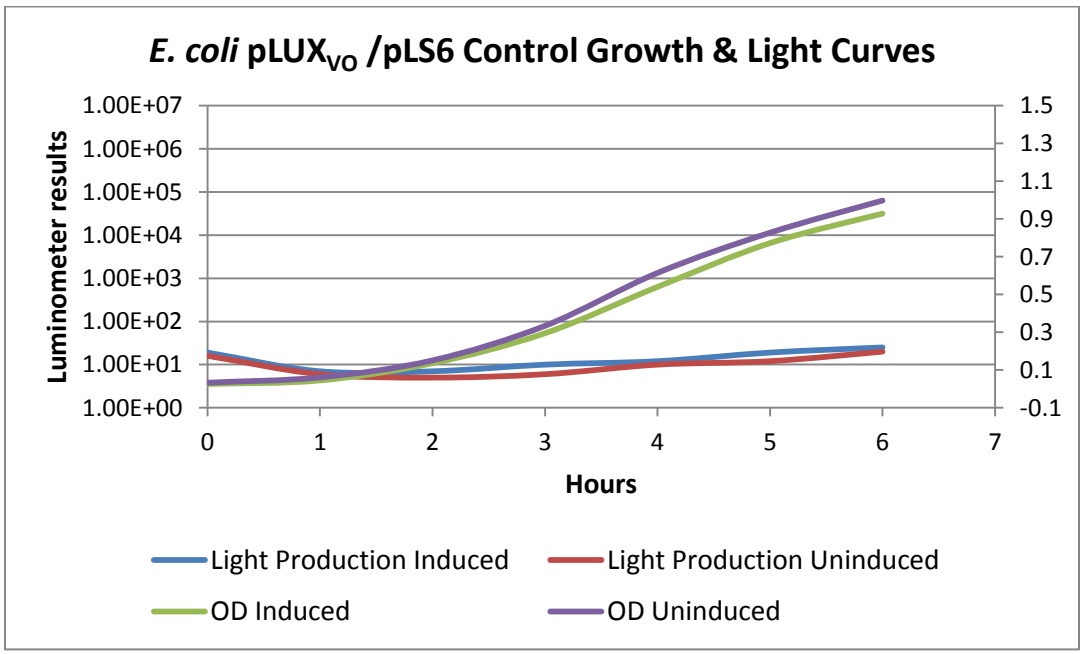
Condition: *E. coli* containing pLUX$_{VO}$ control

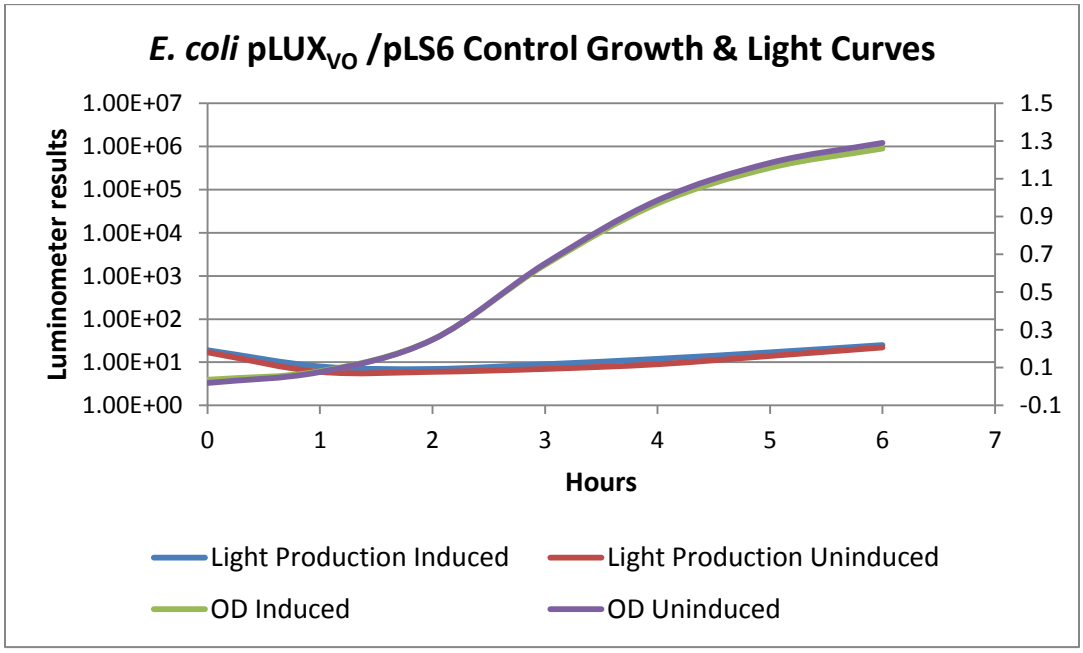


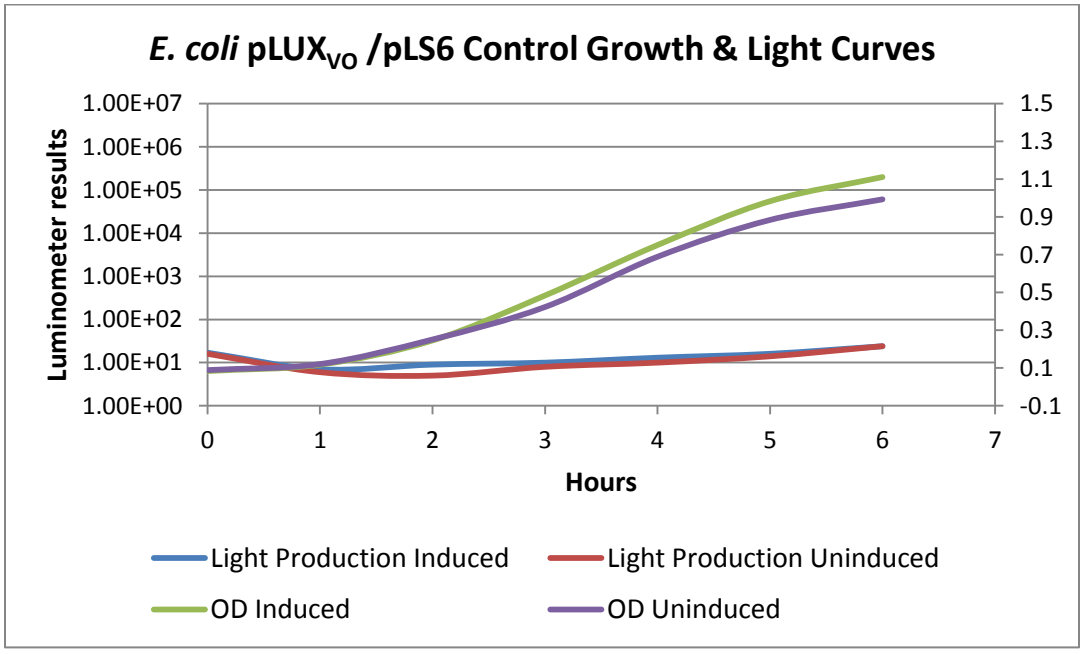




Condition: *E. coli* containing pLUX$_{VO}$/pLS6 control

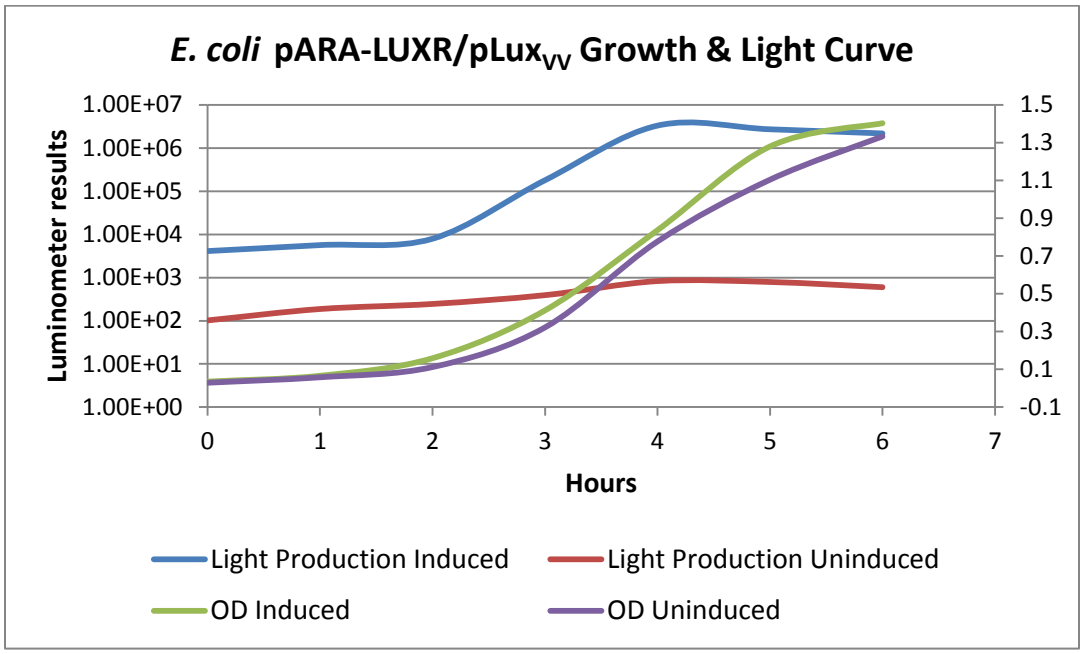


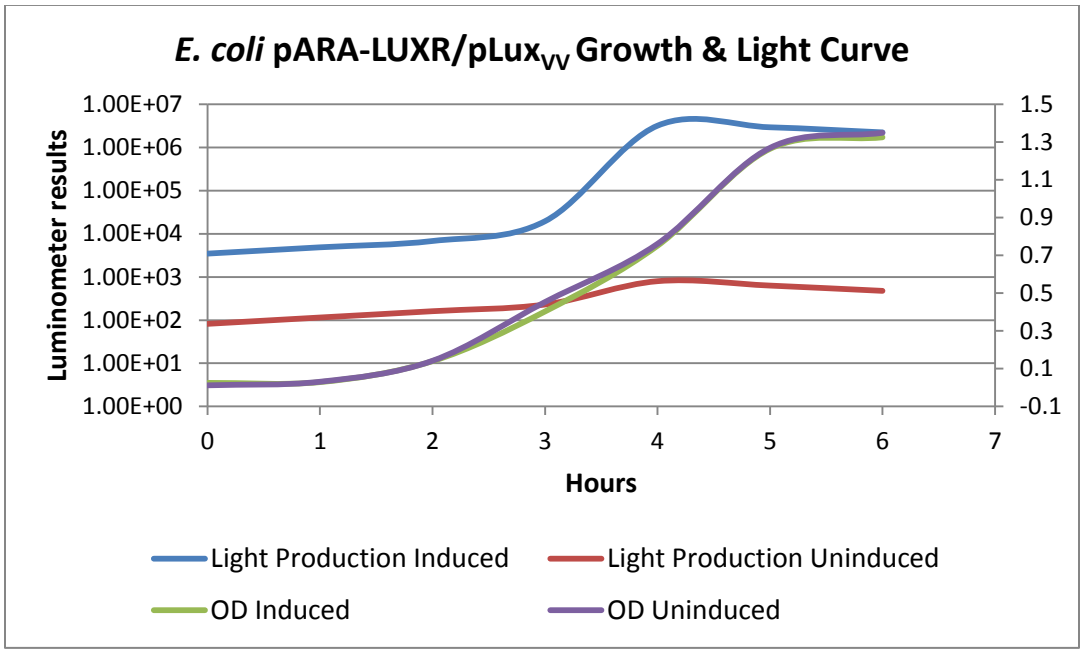

E. coli pLUXVO /pLS6 Control Growth & Light Curves
Luminometer results
1.00E+07
1.00E+06
1.00E+05
1.00E+04
1.00E+03
1.00E+02
1.00E+01
1.00E+00
1.5
1.3
1.1
0.9
0.7
0.5
0.3
0.1
-0.1
0
1
2
3
4
5
6
7
Hours
Light Production Induced
Light Production Uninduced
OD Induced
OD Uninduced

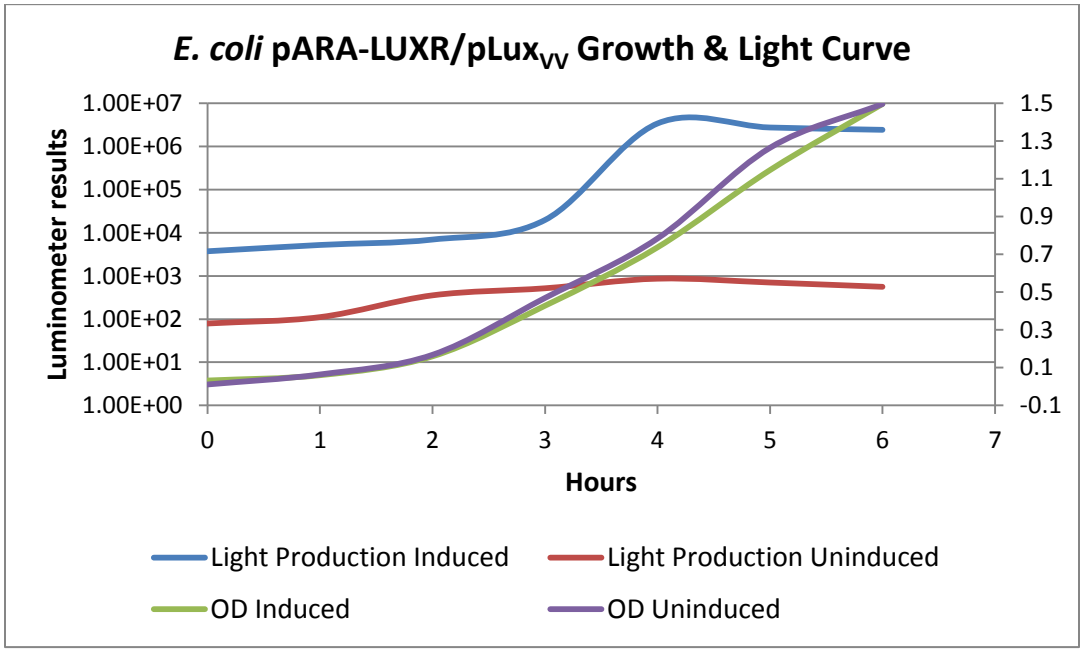

E. coli pLUXVO /pLS6 Control Growth & Light Curves
Luminometer results
1.00E+07
1.00E+06
1.00E+05
1.00E+04
1.00E+03
1.00E+02
1.00E+01
1.00E+00
1.5
1.3
1.1
0.9
0.7
0.5
0.3
0.1
-0.1
0
1
2
3
4
5
6
7
Hours
Light Production Induced
Light Production Uninduced
OD Induced
OD Uninduced

Test: *Vibrio vulnificus lux* Operon

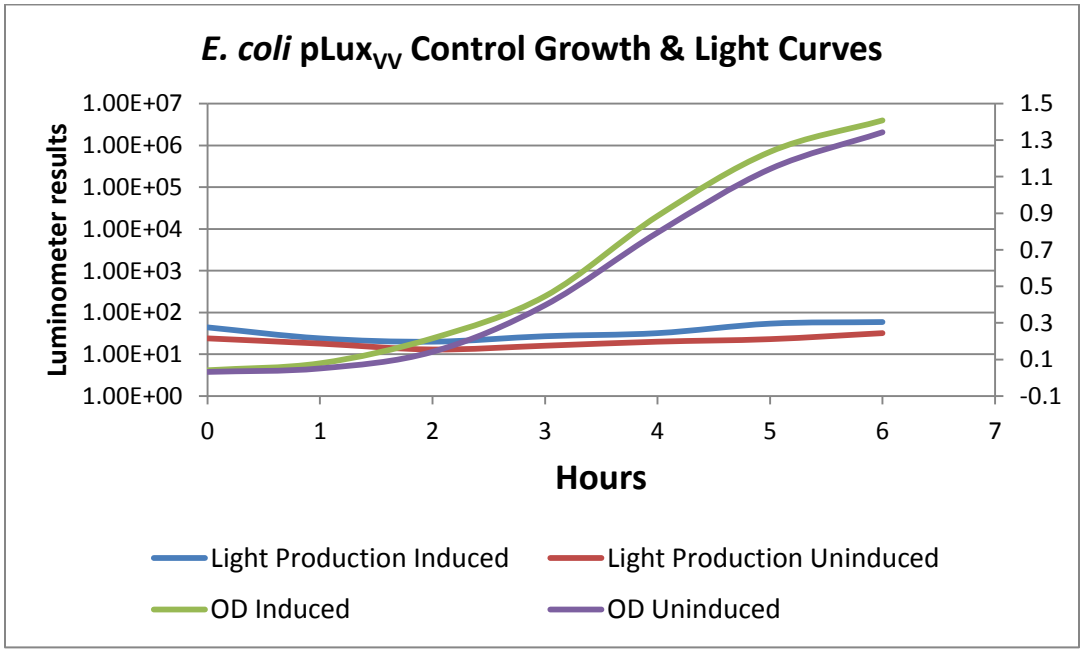
Condition: *E. coli* containing pARA-LUXR/pLUX$_{VV}$

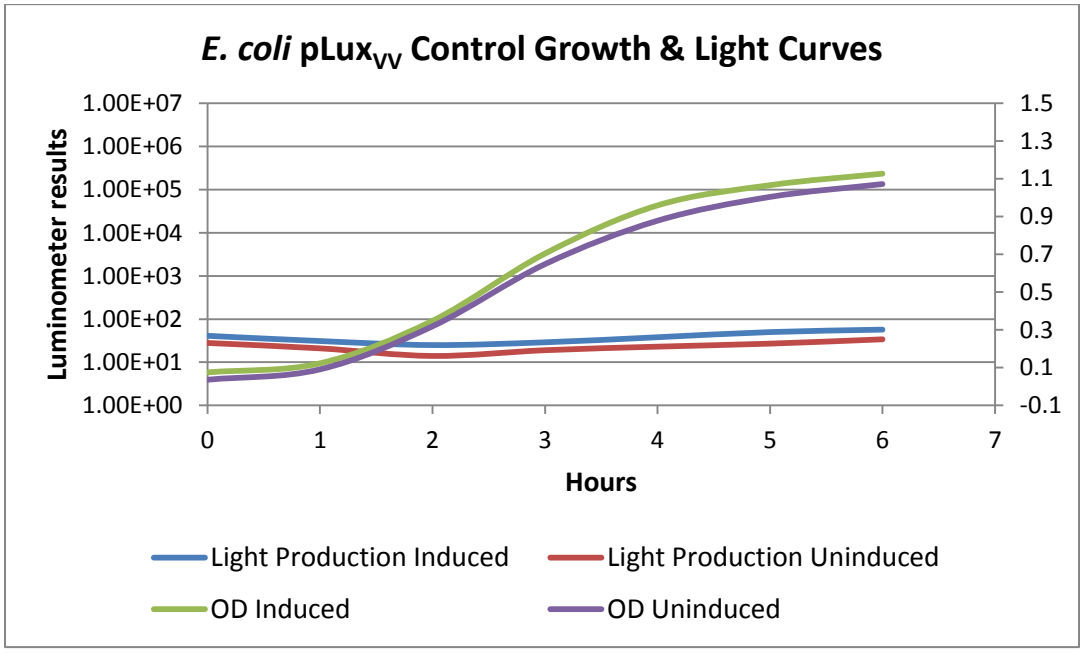


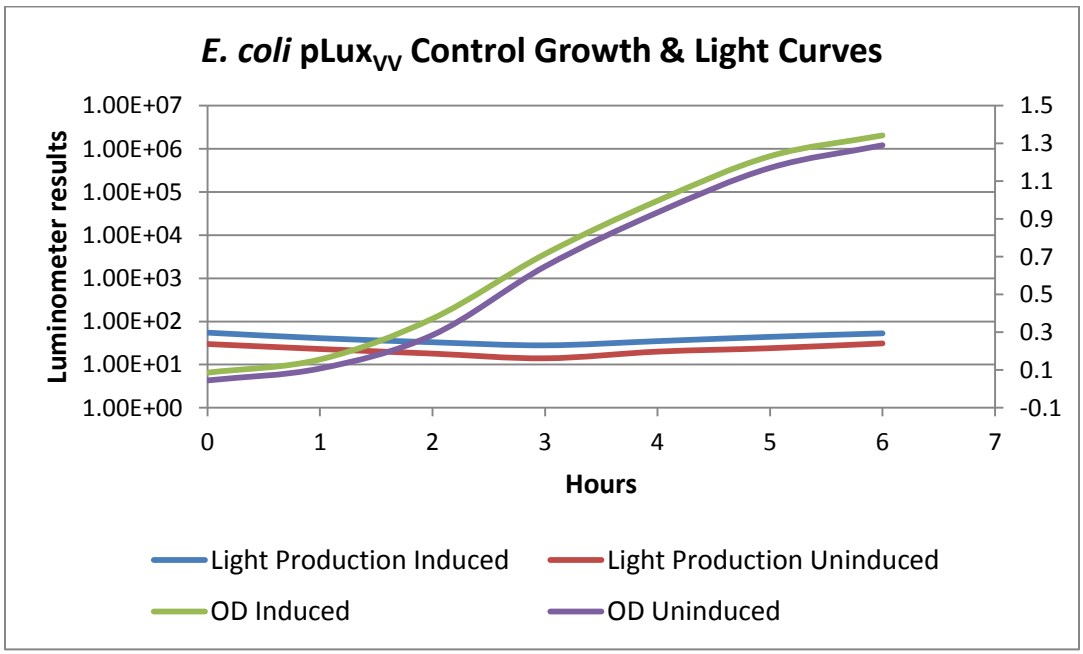




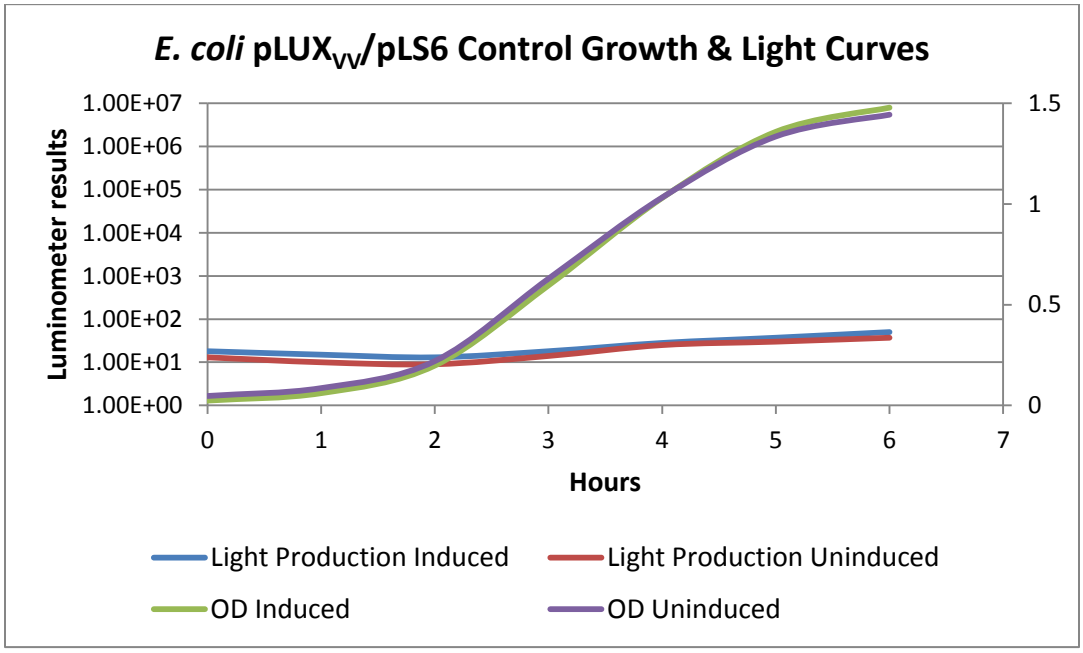
Condition: *E. coli* containing pLUX$_{VV}$ control

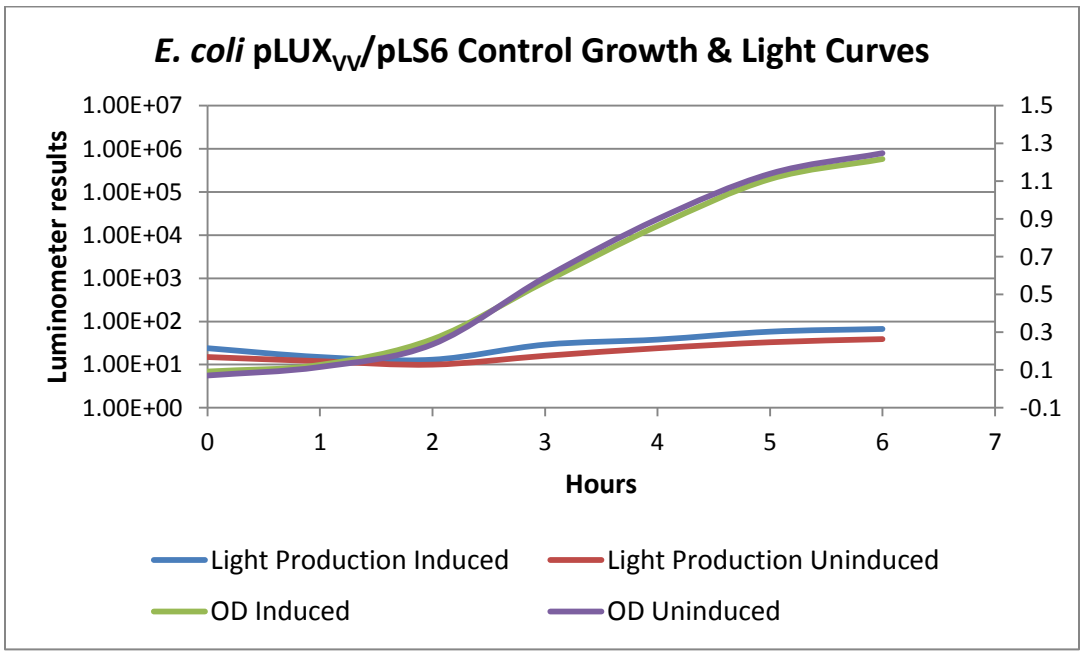


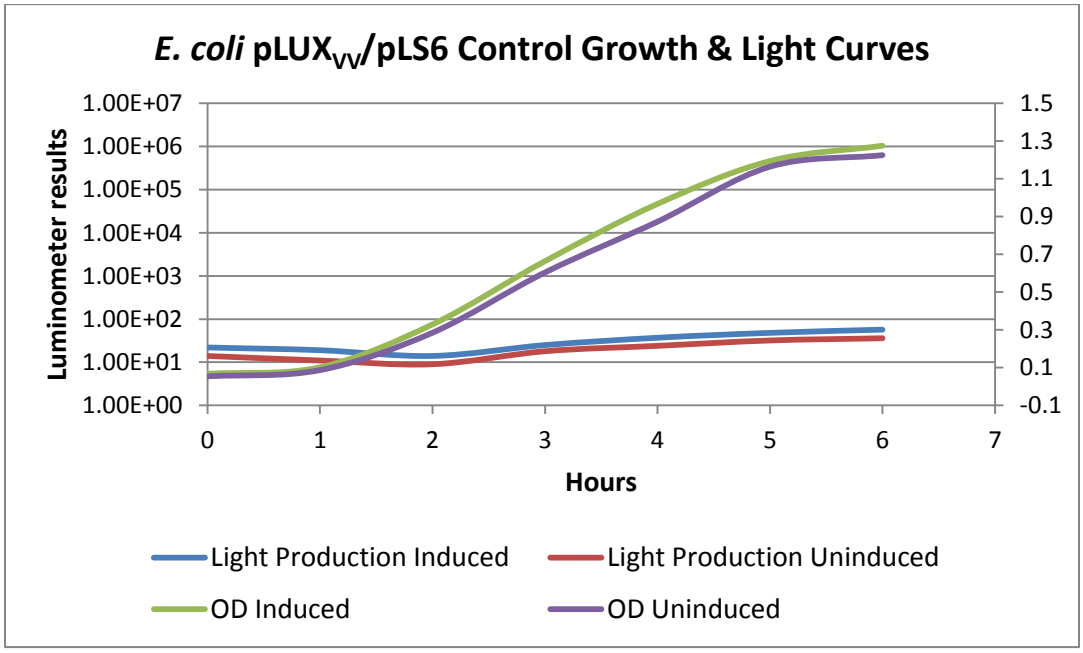

E. coli pLuxVV Control Growth & Light Curves
Luminometer results
1.00E+07
1.00E+06
1.00E+05
1.00E+04
1.00E+03
1.00E+02
1.00E+01
1.00E+00
1.5
1.3
1.1
0.9
0.7
0.5
0.3
0.1
-0.1
0
1
2
3
4
5
6
7
Hours
Light Production Induced
Light Production Uninduced
OD Induced
OD Uninduced


E. coli pLuxVV Control Growth & Light Curves
Luminometer results
1.00E+07
1.00E+06
1.00E+05
1.00E+04
1.00E+03
1.00E+02
1.00E+01
1.00E+00
1.5
1.3
1.1
0.9
0.7
0.5
0.3
0.1
-0.1
0
1
2
3
4
5
6
7
Hours
Light Production Induced
Light Production Uninduced
OD Induced
OD Uninduced

Condition: *E. coli* containing pLUX$_{VV}$ /pLS6 control






E. coli pLUXVV/pLS6 Control Growth & Light Curves
Luminometer results
1.00E+07
1.00E+06
1.00E+05
1.00E+04
1.00E+03
1.00E+02
1.00E+01
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1.5
1.3
1.1
0.9
0.7
0.5
0.3
0.1
-0.1
0
1
2
3
4
5
6
7
Hours
Light Production Induced
Light Production Uninduced
OD Induced
OD Uninduced

Appendix C: Average Relative Light Calculations for *E. coli* Samples

Calculation for individual samples performed at hour four.

Test: The *Vibrio harveyi lux* Operon

Condition	Sample	Light	OD_{600}	Relative Light (Light/OD_{600})
pARA-LUXR/$pLux_{VH}$ Induced	1	3699540	0.586	6313208.19
	2	2986050	0.831	3593321.30
	3	3491480	0.741	4711848.85
	Average	**3392357**	**0.719**	**4872792.78**
pARA-LUXR/$pLux_{VH}$ Uninduced	1	381	0.643	592.53
	2	351	0.868	404.38
	3	360	0.723	497.93
	Average	**364**	**0.745**	**498.28**
$pLux_{VH}$ Control Induced	1	10	0.636	15.72
	2	9	0.742	12.13
	3	9	1.051	8.56
	Average	**9**	**0.810**	**12.14**
$pLux_{VH}$ Control Uninduced	1	6	0.613	9.79
	2	7	0.748	9.36
	3	6	1.082	5.55
	Average	**6**	**0.814**	**8.23**
$pLux_{VH}$/pLS6 Control Induced	1	9	1.079	8.34
	2	6	0.497	12.07
	3	5	0.963	5.19
	Average	**7**	**0.846**	**8.54**
$pLux_{VH}$/pLS6 Control Uninduced	1	6	1.151	5.21
	2	5	0.433	11.55
	3	5	0.892	5.61
	Average	**5**	**0.825**	**7.46**

Test: The *Vibrio chagasii lux* Operon

Condition	Sample	Light	OD_{600}	Relative Light (Light/OD_{600})
pARA-LUXR/$pLux_{VCha}$ Induced	1	683694	0.809	845110.01
	2	3003692	0.641	4685946.96
	3	3976793	0.471	8443297.24
	Average	**2554726**	**0.640**	**4658118.07**
pARA-LUXR/$pLux_{VCha}$ Uninduced	1	4312	0.904	4769.91
	2	20970	0.609	34433.50
	3	28707	0.546	52576.92
	Average	**17996**	**0.686**	**30593.44**
$pLux_{VCha}$ Control Induced	1	52	1.011	51.43
	2	49	1.044	46.93
	3	48	0.759	63.24
	Average	**50**	**0.938**	**53.87**
$pLux_{VCha}$ Control Uninduced	1	40	1.020	39.22
	2	37	0.996	37.15
	3	39	0.696	56.03
	Average	**39**	**0.904**	**44.13**
$pLux_{VCha}$/pLS6 Induced	1	48	0.871	55.11
	2	45	0.909	49.50
	3	39	0.568	68.66
	Average	**44**	**0.783**	**57.76**
$pLux_{VCha}$ /pLS6 Control Uninduced	1	43	0.910	47.25
	2	42	0.955	43.98
	3	34	0.514	66.15
	Average	**40**	**0.793**	**52.46**

Test: The *Vibrio cholerae lux* Operon

Condition	Sample	Light	OD_{600}	Relative Light (Light/OD_{600})
pARA-LUXR/$pLux_{VCho}$ Induced	1	806477	1.224	658886.438
	2	751280	0.829	906248.492
	3	576672	0.736	783521.739
	Average	**711476**	**0.930**	**782885.556**
pARA-LUXR/$pLux_{VCho}$ Uninduced	1	7057	1.169	6036.784
	2	6694	0.801	8357.054
	3	4751	0.729	6517.147
	Average	**6167**	**0.900**	**6970.328**
$pLux_{VCho}$ Control Induced	1	1341	0.680	1972.059
	2	1601	0.852	1879.108
	3	3117	0.534	5837.079
	Average	**2020**	**0.689**	**3229.415**
$pLux_{VCho}$ Control Uninduced	1	1280	0.738	1734.417
	2	1249	0.878	1422.551
	3	2100	0.461	4555.315
	Average	**1543**	**0.692**	**2570.761**
$pLux_{VCho}$ /pLS6 Control Induced	1	5297	0.578	9164.360
	2	5521	0.841	6564.804
	3	5583	0.899	6210.234
	Average	**5467**	**0.773**	**7313.132**
$pLux_{VCho}$ /pLS6 Control Uninduced	1	4005	0.474	8449.367
	2	5182	0.886	5848.758
	3	5390	0.928	5808.190
	Average	**4859**	**0.763**	**6702.105**

Test: The *Vibrio orientalis lux* Operon

Condition	Sample	Light	OD_{600}	Relative Light (Light/OD_{600})
pARA-LUXR/$pLux_{VO}$ Induced	1	997726	0.475	2100475.79
	2	738051	0.506	1458598.81
	3	891980	0.775	1150941.94
	Average	**875919**	**0.585**	**1570005.51**
pARA-LUXR/$pLux_{VO}$ Uninduced	1	702	0.435	1613.79
	2	732	0.513	1426.90
	3	738	0.748	986.63
	Average	**724**	**0.565**	**1342.44**
$pLux_{VO}$ Control Induced	1	27	0.627	43.06
	2	32	0.386	82.90
	3	34	0.774	43.93
	Average	**31**	**0.596**	**56.63**
$pLux_{VO}$ Control Uninduced	1	16	0.577	27.73
	2	17	0.339	50.15
	3	15	0.727	20.63
	Average	**16**	**0.548**	**32.84**
$pLux_{VO}$ /pLS6 Control Induced	1	12	0.540	22.22
	2	12	0.969	12.38
	3	13	0.751	17.31
	Average	**12**	**0.753**	**17.31**
$pLux_{VO}$ /pLS6 Control Uninduced	1	10	0.614	16.29
	2	9	0.986	9.13
	3	10	0.689	14.51
	Average	**10**	**0.763**	**13.31**

Test: The *Vibrio vulnificus lux* Operon

Condition	Sample	Light	OD_{600}	Relative Light (Light/OD_{600})
pARA-LUXR/$pLux_{VV}$ Induced	1	3313547	0.837	3958837.51
	2	3190540	0.751	4248388.81
	3	3394761	0.737	4606188.60
	Average	**3299616**	**0.775**	**4271138.31**
pARA-LUXR/$pLux_{VV}$ Uninduced	1	829	0.777	1066.92
	2	798	0.761	1048.62
	3	863	0.784	1100.77
	Average	**830**	**0.774**	**1072.10**
$pLux_{VV}$ Control Induced	1	32	0.885	36.16
	2	38	0.959	39.62
	3	35	0.998	35.07
	Average	**35**	**0.947**	**36.95**
$pLux_{VV}$ Control Uninduced	1	20	0.794	25.19
	2	23	0.878	26.20
	3	20	0.934	21.41
	Average	**21**	**0.869**	**24.27**
$pLux_{VV}$/pLS6 Control Induced	1	28	1.030	27.18
	2	38	0.863	44.03
	3	37	0.967	38.26
	Average	**34**	**0.953**	**36.49**
$pLux_{VV}$/pLS6 Control Uninduced	1	25	1.032	24.22
	2	24	0.899	26.70
	3	24	0.873	27.49
	Average	**24**	**0.935**	**26.14**