luxOU and AI-2: Regulating the response in Biobrick format

Jamie Feng[*], Thane Kubik[*] and Sonja Georgijevic[*‡]

[*]Faculty of Medicine and [‡]Faculty of Education, The University of Calgary, Canada.

Cell-to-cell bacterial communication is achieved by exchanging signal molecules such as autoinducer-2 (AI-2). The AI-2 system is present in gram-negative and gram-positive bacteria and improves efficacy of cellular processes such as motility, genetic competence and biofilm formation. The system involves binding of AI-2, a furanosyl-borate-diester, to a receptor protein followed by a series of phosphorylation steps catalyzed by kinases, phosphatases and phosphorelay proteins. The signal results in the binding of the luxO protein to a promoter, initiating downstream transcription of genes. To establish the *Vibrio harveyi* AI-2 signalling pathway in a non-pathogenic organism, *luxOU* is cloned into Biobrick vector for subsequent construction of the signaling pathway with *luxPQ*. Additionally, *V. harveyi* AI-2 reporter strains identify a lack of AI-2 activity in *Escherichia coli* DH5α supernatant, allowing it to act as a suitable chassis for our cloned *V. harveyi* AI-2 system.

Keywords: quorum sensing, autoinducer-2, *Vibrio harveryi*, Biobrick, *luxO*, *luxU*, signaling,

Abbreviations:

AHL Acylated homoserine lactones
AI-2 Autoinducer-2
QS Quorum sensing
iGEM International Genetically Engineered Machines competition

Microbial species are capable of interacting with other microorganisms by using pheromones as a means of communication in a process called quorum sensing (QS)[1]. QS is used by bacteria to monitor inter- and intra-species population density and regulate population-related cellular processes[2]. The QS molecules accumulate to a minimal stimulatory threshold level which initiates a signalling cascade resulting in modified gene transcription and bacterial behaviour. QS associated processes include bioluminescence, biofilm formation, virulence factor expression, swarming behaviour and conjugation[3]. One of the pheromones used by microbial species is autoinducer-2 (AI-2), a furanosyl-borate-diester[4]. Over 70 bacterial species are capable of producing AI-2 via the synthetase *luxS*[5], examples include *Vibrio harveyi*, *Salmonella typhimurium, Streptococcus pneumoniae* and *Escherichia coli*. The AI-2 system has been identified in both gram-negative (such as and gram-positive bacteria[6]. Another heavily studied quorum sensing system involves the class of compounds known acylated homoserine lactones (AHLs). The AHL system has thus far only been identified in gram-negative bacteria[6]. As such, it has been proposed that AI-2 is a universal medium for bacterial inter-species communication[6].

For the International Genetically Engineered Machines (iGEM) competition the University of Calgary 2009 team has chosen to focus on the construction and characterization of the AI-2 system present in *Vibrio harveryi*. As an annual competition held at the Massachusetts Institute of Technology, the iGEM competition focuses in on an emerging scientific community called Synthetic Biology, an intertwining of principles from Biology and Engineering. Crucial cornerstones of iGEM are the Registry of Standard

Biological Parts (parts.mit.edu) and the concept of Biobricks. Both enable the construction of larger parts capable of being used in subsequent constructions[7]. Calgary aims to add a second QS system to the Registry; the only available one is the AHL system (BBa_F1610 and BBa_F2620[8]). It has been shown that the AHL system in *V. harveryi* induces expression of downstream genes at a lower cell density than the AI-2 system[9]. A second QS system would allow for coordination of bacterial behaviour in environments with a higher degree of control.

AI-2's elaborate signalling pathway (outlined in Figure 1) presents drawbacks in the form of difficulty characterizing multiple proteins and their interactions (i.e. *luxP* and *luxQ*); however this attribute is beneficial as it presents multiple regulation points. Modification to the AI-2 system can take place at receptor proteins LuxP and LuxQ or the response regulator proteins LuxO and LuxU. For example, mutants of LuxO (D47E and D47A) can be used to change transcription of downstream genes[8]. Conversely, in the AHL system, *luxR* acts as both the receptor and response protein, thereby it is the sole possible regulatory mechanism[2].

Characterization of the AI-2 has potential applications in the healthcare setting; disrupting aspects of QS reduces microbial pathogenicity and can be possible novel treatments for bacterial infections. Examples include eukaryotes such as *Delisea pulchra* which possess autoinducer inhibitor systems as a means to prevent bacterial colonization[10]. Furthermore, *Pseudomonas aeruginosa* in environments lacking AHL are less virulent with decreased biofilm formation and pyocyanin release[11]. Targeting a bacterial communication system is beneficial as there is a decreased selection pressure for resistance development compared to antibiotic usage[12]. Antibiotics directly decrease the number of bacteria present via killing; the problem arises when the small percentage of the population resistant to the antibiotic proliferates and becomes pathogenic. This renders the antibiotic therapy useless. Conversely, targeting a bacterial communication system does not target cell growth; instead it prevents populations from coordinating their behaviors to induce virulence. This effectively creates a situation where bacterial pathogenicity is targetted, a much more favorable environment to prevent or eliminate microbial infection.

Introducing the AI-2 signalling system into a non-pathogenic model to characterize for the Registry of Standard Biological Parts would provide a new platform for future iGEM teams to further study the system and investigate different applications of QS as they apply in the context of bacterial pathogenesis. The AI-2 system has the potential to spawn many applications, similar to the AHL system which has been instrumental in projects such as the University of Calgary's iGEM 2008 project titled "Quorum-coupled bacteriocin release"[13]. Construction of the *V. harveyi* AI-2 signalling pathway into a non-pathogenic model requires (1) DNA assembly of required parts and (2) selection of an appropriate model organism. Proteins involved in the signaling pathway include LuxPQ and LuxOU (see Figure 2 for DNA project schematic). Establishing controls for the project (such as mutant LuxO proteins and a luminescent reporter circuit) are described elsewhere[14].

Here we describe the construction of the *luxOU* operon from *V. harveryi* with a promoter (BBa_R0040[15]) and flanking terminators (BBa_B0015[16]). Both *luxO* (GenBank accession no. **P0C5S5**) and *luxU* (Genbank accession no. **AAD12737**) were cloned from cosmid pBB147[17]. With regards to chassis identification, it is hypothesized that *E. coli* DH5α will act as a suitable model organism for cloning the AI-2 system because its genome has a frameshift mutation whereby the *luxS* protein is knocked-out[18]. By using *Vibrio harveyi* AI-2 reporter strains to test for AI-2 activity in various bacterial strains we will investigate the aforementioned hypothesis.

MATERIAL AND METHODS

Revelant bacterial strains, plasmids and primers used in cloning the *luxOU* construct are listed in Table 1.

Plasmid/Primer	Comments	Reference
Bacteria		
TOP10	F⁻ *mcr*A Δ(*mrr-hsd*RMS-*mcr*BC) φ80*lac*ZΔM15 Δ*lac*X74 *rec*A1 *ara*D139 Δ(*ara-leu*) 7697 *gal*U *gal*K *rps*L (Str^R) *end*A1 *nup*G λ-	Laboratory strain
Plasmid		
pCR-BLUNT-II-TOPO	Clone *luxOU* from cosmid Kan^R	Invitrogen, CA
psB1AC3	General construction plasmid Amp^R and Cm^R	iGEM Registry
psB1AK3	B0015 insert Construct B0015+R0040 Amp^R and Kan^R	iGEM Registry
psB1A2	R0040 insert Amp^R	iGEM Registry
Primer		T_m
Sp6	ATTTAGGTGACACTATAG	57
T7	TAATACGACTCACTATAGGG	61
VR	ATTACCGCCTTTGAGTGAGC	52
VF2	ATTACCGCCTTTGAGTGAGC	60
BBK-CP-F	CACCTGACGTCTAAGAAACC	60
BBK-CP-R	AGGAAGCCTGCATAACGCG	60
luxOU-F	ACAGAAGGTCAAAAGTCTCGT	60
luxOU-R	CCCATTTCAAATCTCCTCATG	60
luxOU-RS-F	GAATTCGCGGCCGCTTCTAGAACAGAAGGTCAAAAGTCTCGT	69
luxOU-RS-R	CTGCAGCGGCCGCTACTAGTCCCATTTCAAATCTCCTCATG	71
R0040-R	TGCTCAGTATCTCTATCACTG	60

Table 1. Plasmid backbones and primers used during cloning of genes. Both pCR-BLUNT-II-TOPO (TOPO BL) and psB1AC3 have the ccdB gene incorporated to act as a positive selection factor[19]. All of the plasmids are high-copy number. Sp6, T7, VR and VF2 are commercially available used to sequence our gene of interest in TOPO BL and Biobrick vectors. BBK-CP-F and BBK-CP-R (Thane Kubik, 2009) are custom primers used for sequencing and general PCRs that anneal approximately 100 bp upstream and downstream of the multiple cloning site. l*uxOU*-F and *luxOU*-R are gene-specific primers used to clone our gene of interest in PCR reactions. *luxOU*-RS-F and *luxOU*-RS-R are gene-specific primers with the Biobrick prefix (*EcoR*I, *Not*I and *Xba*I) and suffix (*Spe*I, *Not*I and *Pst*I) respectively; these primers were used to amplify *luxOU* as an insert for Biobrick vectors. R0040-R was used for sequencing *luxPQ*-BBa_B0015-BBa_R0040-*luxOU*-BBa_B0015.

Media and antibiotics – Liquid cultures and agar plates were made with Luria-Bertani(LB) broth. Ampicillin, chloramphenicol and kanamycin were used in final concentrations of 100ng/μL (286mM), 35ng/μL (108mM) and 50ng/μL (103mM) respectively.

Cloning of luxOU

luxOU in pCR-BLUNT-II-TOPO (TOPO BL) was gradient PCR amplified using *luxOU*-RS-F and *luxOU*-RS-R, thereby creating a linear strand of *luxOU* with Biobrick prefix and suffix. Platinum *Pfx* polymerase (Invitrogen, CA) gradient PCR was ran with the following parameters: 94°C for 5 minute, 36 cycles of 94°C (15sec), range of annealing temperatures (58-66°C for 30 sec) and 68°C for 2 minutes, and a final extension at 68°C for 10 minutes. The linear fragment was purified using the QIAquick PCR Purification kit (Qiagen, MD), eluting with 40μL ddH_2O. The two construction techniques chosen: *EcoR*I

with *Pst*I and *Xba*I with *Pst*I (Invitrogen, CA), are explored in the discussion section. ~600ng of linear insert (*luxOU*) and ~200ng of Biobrick vector were digested for 2 hours at 37°C. The vector was treated with Antarctic phosphatase (New England Biolabs, ON) for 30 minutes at 37°C. 5µL each of insert and recipient DNA were ligated with 9µL of 2x QuikLigase buffer and 1µL of QuikLigase for 5 minutes. 20µL of the ligation reaction was transformed into TOP10 chemically competent cells using standard protocols[20]. 25µL and 50µL were plated on 108mM LB agar-chloramphenicol plates.

Construction verification – To verify integration of our sequence of interest, a general three step process was used: (1) colony PCR, (2) restriction digest and (3) sequencing. Colony PCR with platinum *Taq* polymerase (Invitrogen, CA) initially screened colonies using *luxOU*-F and l*uxOU*-R (see Figure 2 for primer annealing sites). The initial denaturing step was extended to 6 minutes. Overnight cultures were used to isolate plasmid using both the QIAprep Spin Miniprep kit (Qiagen, MD) and GenElute Plasmid Miniprep kit (Sigma-Aldrich, MO). Plasmid DNA (~200ng) was *Not*I digested at 37°C for 2 hours according to manufacturer specifications. 10µL of plasmid was sent to sequencing such that there was 100ng/1kb. Sequencing used BBK-CP-F and BBK-CP-R primers.

Addition of BBa_B0015 to luxOU – Constructed using standard Biobrick technique (see *luxOU* into Biobrick vector) and transformed into TOP10 cells. BBa_B0015 is a double terminator used to insulate the *luxOU* and *luxPQ* circuit. psB1AK3 with BBa_B0015 insert was digested with *Xba*I and *Pst*I; psB1AC3 with *luxOU* insert was digested with *Spe*I and *Pst*I. Enzyme selection for the digest of the insert and recipient was based on a schematic created by Sonja Georgijevic (Figure 4).

Creating BBa_B0015-BBa_R0040 construct – Constructed using standard Biobrick technique (see *luxOU* into Biobrick vector) and transformed into TOP10 cells. BBa_R0040 is a tetracycline repressible promoter used to initiate transcription of *luxOU*. psB1A2 with BBa_R0040 insert was digested with *Xba*I and *Pst*I; psB1AK3 with BBa_B0015 was digested with *Spe*I and *Pst*I.

Assembling BBa_B0015-BBa_R0040-luxOU-BBa_B0015 construct – Constructed using standard Biobrick technique (see *luxOU* into Biobrick vector) and transformed into TOP10 cells. psB1AK3 with BBa_B0015-BBa_R0040 insert was digested with *EcoR*I and *Spe*I; psB1AC3 with *luxOU*-BBa_B0015 insert was digested with *EcoR*I and *Xba*I. For use in subsequent constructions, a plasmid switch with *Xba*I and *Pst*I moved BBa_B0015-BBa_R0040-luxOU-BBa_B0015 from psBAC3 (for sequence of construct see Figure 3) into psB1AK3.

Constructing the luxPQ and luxOU signalling circuit[21] *– luxPQ* was cloned into psB1AC3 with a missing *Pst*I site. As such, *luxPQ* was digested as the insert with *EcoR*I and *Spe*I and B0015-R0040-*luxOU*-B0015 in psB1AK3 was digested with *EcoR*I and *Xba*I. TOP10 transformants were plated on LB-kanamycin 103mM agar plates. From there, the completed signalling circuit was cloned into pCS26[22] allowing for the assembly of a promoter library.

AI-2 testing

Isolating AI-2[18] – AI-2 was isolated from overnight cultures of bacterial cultures by centrifugation at 14 000 rpm for 10 minutes. Supernatant was filtered using 0.2µm filters and the cell-free (CF) supernatants are stored at -20°C.

Identifying a suitable reporter strain and AI-2 producing strains – To test for the presence of AI-2 in the CF supernatants, reporter strains are needed. Mutants of *V. harveyi* act as suitable luminescent reporters because of their modified sensitivity to autoinducers. The reporter strain used is MM32. Bacterial strains tested for AI-2 activity in their supernatant include *V. harveyi* BB120 and BB152, *Salmonella typhimurium* 14028 and SS007 and *E. coli* DH5α. See Table 2 for relevant genotypes.

Strain	Relevant genotype	Relevant phenotype			
		Sensor 1	Sensor 2	AHL	AI-2
***V. harveyi* MM32**[23]	(*BB7*) *luxN*::Tn5 *luxS*::Tn5	-	+	+	-
***V. harveyi* BB120**	Wildtype	+	+	+	+
***V. harveyi* BB152**[24]	(*BB7 or BB120*) *luxLM*::Tn5	+	+	-	+
***Salmonella typhimurium* 14028**[25]	Wildtype (	+	+	+	+
***Salmonella typhimurium* SS007**[26]	(14028) *luxS*::T-POP	+	+	+	-
***E. coli* DH5α**[18]	(MG1655) frameshift in *luxS*				-

Table 2. Bacterial Strains used in Isolating and Testing AI-2. MM32 is a reporter strain used which is only luminescent in the presence of AI-2. BB120 produces both AHL and AI-2. BB152 is deficient in AHL production, making it a candidate to isolate AI-2 from. 14028 is another possible candidate for AI-2 isolation as it is also known for AI-2 production. SS007 and DH5α are negative controls which do not produce AI-2 and are candidates for a model organism to house our QS system in. Sensor 1 corresponds to AHL response, sensor 2 corresponds to AI-2 response, AHL corresponds to AHL production and AI-2 corresponds to AI-2 production.

VICTOR and microplate setup[18] – A 96 well microplate was used to test the AI-2 activity in supernatant. UV-sterilized microplates contained 90μL of diluted (1:5000) MM32 overnights in auto-inducer bioassay (AB) media and 10μL of CF supernatant. Controls used in the experiment included plate controls, reporter controls and CF supernatant controls. Plate controls are wells of plain AB media to test for sterility of the plate. Reporter controls consist of dilute reporter strain with sterile LB as the CF supernatant, testing for self-stimulating reporter strains and serving as a baseline for background noise. CF supernatant controls consisted of sterile LB in place of reporter cells and various CF supernatants, controlling for cells in the CF supernatant. Mineral oil was added on top of the aqueous experiments. A VICTOR microplate reader was used, taking OD_{620} and luminescence readings every 20 minutes with shaking at 30°C.

RESULTS

luxOU in TOPO BL, psB1AC3 with a ccdB insert[27] and psB1AK3 with a ccdB insert[28] were isolated with a GenElute Plasmid Miniprep Kit. Presence of restriction sites in Biobrick vectors and *luxOU* in TOPO BL were confirmed with restriction enzyme digest (Figure 5). To clone *luxOU* into Biobrick vector, *luxOU* in TOPO BL was PCR amplified with Biobrick suffix and prefix (Figure 6). PCR products #2 and #8-12 were pooled and purified using a QIAquick PCR Purification Kit. Construction of *luxOU* in psB1AC3 with *Xba*I and *Pst*I was done parallel to construction using *EcoR*I and *Pst*I. Initial transformation into TOP10 competent cells yielded no colonies. A transformation control was used in the following transformation with pBluescript (Stratagene, CA) as a positive control at temperatures of 37°C for 5 minutes and 42°C for 40 seconds (Table 3).

Transformation	Colonies
*Xba*I and *Pst*I / 37°C / 25µL	10
*Xba*I and *Pst*I / 37°C / 50µL	7
*Xba*I and *Pst*I / 42°C / 25µL	1
*Xba*I and *Pst*I / 42°C / 50µL	6
*EcoR*I and *Pst*I / 37°C / 25µL	1
*EcoR*I and *Pst*I / 37°C / 50µL	5
*EcoR*I and *Pst*I / 42°C / 25µL	2
*EcoR*I and *Pst*I / 42°C / 50µL	1

Table 3. Colony yield for transformation of *luxOU* and psB1AC3 construct into TOP10 competent cells. Six colonies were picked from each construction technique and restreaked on LB-chloramphenicol 35 plates.

Verification of *luxOU* in psB1AC3 involved colony PCR and *Not*I restriction digest (Figure 7). Colonies *luxOU* (*Xba*I) C2 and luxOU (*EcoR*I) C1 were selected for continued construction. The overall goal of building the construct of BBa_B0015-BBa_R0040-*luxOU*-BBa_B0015 was segmented into building (1) BBa_B0015-BBa_R0040 and (2) *luxOU*-BBa_B0015. BBa_B0015-BBa_R0040 was constructed in psB1AK3 and *luxOU*-BBa_B0015 was constructed in psB1AC3. Two TOP10 transformants for each construction technique were screened via *Not*I digest (Figure 8) and confirmed with sequencing. Colonies BBa_B0015-BBa_R0040 C1 and luxOU(*XbaI*)-BBa_B0015 C4 were sent for sequencing with BBK-CP-F and BBK-CP-R as sequencing primers. Sequencing results were checked for vector contamination using "VecScreen", protein sequences using "Blastx" and sequence conformation using "Blast2" (National Center for Biotechnology Information, MD).

Final construction of the *luxOU* construct was carried out using BBa_B0015-BBa_R0040 C1 in psBAK3 and luxOU(*XbaI*)-BBa_B0015 in psB1AC3 C4. Eight colonies were picked from the BBa_B0015-BBa_R0040-*luxOU*-BBa_B0015 (50µL) plate for screening. Colony PCR was not used as a means to verify the construction because if BBa_B0015 sequence specific primers were used, multiple bands would be apparent due to the nature of intrinsic BBa_B0015 terminators in the vector backbone[18]. Therefore to confirm successful construction of the *luxOU* construct, *Not*I verification digest was performed on C1, C7 and C8 (Figure 9). C1 and C7 were sent for sequencing with BBK-CP-F and BBK-CP-R as sequencing primers.

BBa_B0015-BBa_R0040-*luxOU*-BBa_B0015 C1 and C7 were used for the plasmid switch of the construct into psB1AK3. Construction of the signalling circuit used Δ*luxPQ* C7 in psB1AC3 and BBa_B0015-BBa_R0040-*luxOU*-BBa_B0015 psB1AK3 C1. Verification for the cloning of *luxPQ*-

BBa_B0015-BBa_R0040-*luxOU*-BBa_B0015 into psB1AK3 was done with colony PCR and verification digest using *Xba*I and *Pst*I showing bands at approximately 6kb (0.7% agarose gels not shown). Sequencing with R0040-R, BBK-CP-F and BBK-CP-R indicated the signalling circuit was successfully cloned into psB1AK3.

To test the now constructed signalling circuit, AI-2 was isolated and tested with the MM32 reporter strain. In Figure 10, it is seen that MM32 is responsive (i.e. luminescent) to the AI-2 present in the supernatant of *V. harveyi* BB120, a wildtype strain, and BB152, a strain deficient in AHL production. *Salmonella typhimurium* 14028 CF supernatant is also seen to cause luminescence in MM32, however the levels are much lower than that of the *V. harveyi* strains. *S. typhimurium* SS007 and *E. coli* DH5α produce a minimal (SS007) or zero (DH5α) response in the MM32 reporter strain. Figure 11 shows growth in the reporter control, but no luminescence; as expected growth occurs (OD_{620} curve in red), but there is minimal luminescence (blue curve). In the plate control, there is no luminescence or absorbance visible. The results from the CF supernatant controls are interesting in that there is growth present in the unfiltered CF supernatants and in one of the filtered CF supernatants. There is limited luminescence in the contaminated CF supernatants (except for BB120). Notable results include the presence of luminescence in BB120 filtered supernatant but now growth occurs.

DISCUSSION

During construction of the biobrick plasmid with *luxOU*, two different construction techniques were chosen (*Eco*RI with *Pst*I and *Xba*I with *Pst*I). The comparison was made due to the nature of the linear fragment and the need to add extra base pairs for *Eco*RI to cut efficiently. The linear PCR-amplified *luxOU* fragment contains an *Eco*RI site at the 5' end. *Eco*RI requires additional bases outside of its recognition sequence for efficient cleavage[29]. Figure 7a allows for comparison of the two approaches. Lanes 8-13 (*Xba*I with *Pst*I) are more uniform in brightness compared to lanes 2-7 (*Eco*RI with *Pst*I). This may be indicative of the range of construction efficiencies present when a *Eco*RI site at the end of a linearized fragment is used in construction. Through this experience, the importance of factoring in biological limitations and considerations during experimental design is reinforced.

Experimental design also became important when antibiotic selection pressures had not been taken into account. A plasmid switch had to be done with the *luxOU* construct so that the signaling circuit could be cloned into pCS26. If the original *luxOU* construct was used, the *luxPQ* and *luxOU* circuit in psB1AK3 would have been cloned from a plasmid with kanamycin resistance into pCS26 (which carries the kanamycin resistance gene). Without the selection pressure of different antibiotics, the efficacy of the cloning process would most likely decrease.

The size difference of the *luxOU* constructs (Figure 9) suggests successful addition of the promoter BBa_R0040 and the terminator BBa_B0015. The *luxOU*-BBa_B0015 construct is 129bp larger compared to the *luxOU* construct; the BBa_B0015-BBa_R0040-*luxOU*-BBa_B0015 construct is 183bp longer than *luxOU*-BBa_B0015. Despite the fact that colony PCR was not used to verify the cloning, colony PCR in fact could have been used for this purpose. The concern with colony PCR was the need to use BBa_B0015 primers which would results in multiple bands from the presence of terminators in the vector backbone; however, primers R0040 forward and *luxOU* reverse could have been used in the PCR. R0040-F and *luxOU*-R could have been used because the regions they anneal to are from two different plasmids (i.e. they are the two separate parts being cloned). While restriction digest is useful in determining sizes of inserts in plasmids, it is unable to discern the order of specific parts in the cloned product. This fact is highlighted by the need to sequence the construct before definitively affirming the successful cloning of the circuit.

The next step was cloning of the signaling pathway consisting of: *luxPQ*-BBa_B0015-BBa_R0040-*luxOU*-BBa_B0015 for subsequent cloning into pCS26. The construction of the signaling circuit was done such that the signaling circuit was cloned into both psB1AC3 and psB1AK3. *luxPQ*-BBa_B0015-BBa_R0040-*luxOU*-BBa_B0015 in psB1AC3 proved problematic in cloning, yielding inserts with sizes ranging from 500bp to 1.5kb (data not shown). This may have been a result of moving *luxPQ* (~4kb) into the *luxOU*(~2.3kb) construct and the problems present when cloning larger pieces of DNA into vectors with shorter genes of interest. The solution to this problem would have been to clone *luxOU* into *luxPQ*, however because the *luxPQ* circuit originally lacked a *PstI* site this was not possible. It was not until *luxPQ* was cloned successfully according to Biobrick specifications (i.e. with Biobrick prefix and suffix) that the signaling circuit was successfully constructed in psB1AC3[21].

Overall, while the Biobrick standard is a useful technique in cloning DNA, it is definitely not the most user-friendly. An improvement over the Biobrick methodology is TOPO cloning (Invitrogen, CA). TOPO cloning removes the necessity to digest and ligate products; instead offering users the capability to

readily ligate linear DNA into the plasmid in one simple step. Another similar methodology is the In-Fusion biobrick assembly method which uses Clontech In-Fusion PCR Cloning Kit (Clontech, CA). This method uses an enzyme which fuses linear fragments with overlapping regions together with linearized vectors. This ability would have been particularly useful in cloning *luxOU* and *luxPQ* given the difficulties with antibiotic pressure and the need for plasmid switches; in short the above technologies would have provided us with the flexibility of conducting a plasmid switch in a much shorter period of time. The obvious advantage of the above methods is the time saved by reducing the need for a restriction digest step and a ligation step, instead combining the two; however, a drawback is the higher costs associated with the enzymes and the custom primers needed for each unique construction. Within the context of a student-based research competition such as the iGEM, where funds are of the essence, the alternatives to the Biobrick method provide an illustrious but costly choice which can be avoided with careful planning and consideration.

The AI-2 isolation results looks favorably on using *E. coli* DH5α as the model organism for our signaling system. The lack of luminescence, and therefore presence of AI-2, in the supernatant of *E. coli* DH5α can be seen in Figure 10. In comparision with SS007, which is also another theoretical negative control, we can see the differences in AI-2 production between *E. coli* DH5α and *S. typhimurium SS007*. Whereas the knocked-out gene in SS0077 still appears to produce AI-2, *E. coli* DH5α is completely deficient in any AI-2 production. By using *E. coli* DH5α as a chassis for the *V. harveyi* AI-2 system, it can be ensured that our system is responsive solely to exogenously added AI-2, such as AI-2 derived from BB152. An interesting observation is the differences in luminescence between *V. harveyi* BB152/BB120 and *S. typhimurium* 14028. Whereas both bacterial species are known to produce AI-2, the amount of luminescence in 14028 is over 4 times less than that of *V. harveyi*. A possible explanation of this is the differences in boration between the molecules produced by each strain. The differences in boration[23] can result in changes in specificity, thereby highlighting the usage of AI-2 in both inter- and intra- species communication.

Refering back to Figure 10, it appears filtering the supernatant does not seem to have an effect on luminescence (i.e. AI-2 is not removed by the filter). The levels of luminescence are comparable between the two situations (filtered vs. unfiltered). However, the importance of filtration is demonstrated by looking at the controls (Figure 11). CF supernatant is controlled for by using sterile AB media in place of dilute reporter strain and adding 10uL of said supernatant to the sample. If CF supernatants are indeed cell-free no growth should occur. The importance of filtering can be visualized here: the filtered supernatants (indicated by an 'F') show no growth (save for BB152). Comparing this to the unfiltered CF supernatants, it is apparent the presence of cells in the CF supernatants. If there are indeed cells present in the CF supernatants, the assay could be affected by the inter-species competition between the two different bacteria. An anomaly present in the CF supernatant controls is the BB120 F well, where there is luminescence but no cell growth is detected. Moving forward, it is imperative that the supernatant controls be conducted again to ensure the validity of the initial results, especially for the BB120 filtered CF supernatant.

The selection of the appropriate reporter strain is validated by looking at the reporter control consisting of dilute reporter cells and sterile LB media. The fact that there is growth in the well, but negligible luminescence indicates that AI-2 is not being produced by our reporter strain. Additionally, we can

conclude that the UV sterilization method if appropriate as there is no growth in wells supplemented with sterile LB.

In general, BB152 appears to be a suitable candidate to isolate AI-2 from and *E. coli* DH5α should be a suitable model organism for our AI-2 signalling pathway. Further work is needed in repeating trials for the CF supernatant controls. The majority of further work should be put towards implementing the Biobricked *V. harveyi* AI-2 signalling pathway. *luxOU* has been cloned alongside *luxPQ* into pCS26; one more additional step needed before the system can be introduced into *E. coli* is the creation of synthetic promoters to streamline expression levels of *luxPQ*[21]. Finally, the fransformation of the system into *E. coli* DH5α is needed for further characterization of the pathway.

FIGURES

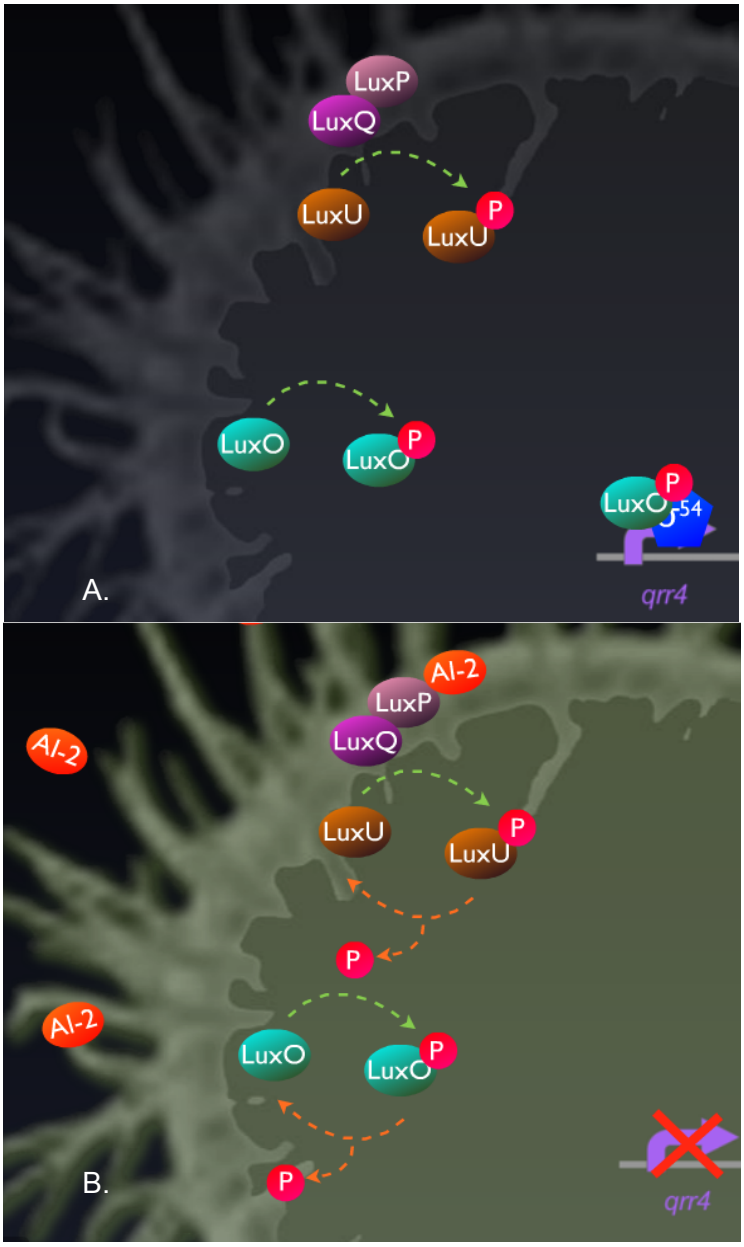

Figure 1. Schematic diagram of the signaling pathway for the AI-2 QS system. LuxP and LuxQ are membrane-bound proteins which act as sensors or receptors for the AI-2 molecule. LuxU is a phosphorelay protein, which, in conjuction with the DNA-binding response regulator protein LuxO control AI-2 related transcription[3]. **A.** AI-2 signalling pathway in the absence of appreciable levels of AI-2 in the environment. LuxQ acts as a kinase in this situation, autophosphorylating and transferring the phosphate to LuxU; in turn, LuxU phosphorylates LuxO. LuxO-P binds with σ^{54} to Pqrr, activating transcription of downstream genes. In *V. harveryi*, LuxO-P and σ^{54} activate transcription of quorum regulating RNAs (qrr). The qrr interact with a RNA chaperone (Hfq) to destabilize the *luxR* mRNA and hence terminate any subsequent translation of *luxR*[3]. LuxR is required for processes such as bioluminescence. **B.** AI-2 signalling pathway with high cell density (resulting in appreciable levels of AI-2 in the environment). LuxQ acts as a phosphatase and removes the phosphate from LuxO via LuxU. LuxO is unable to complex with σ^{54} to bind to Pqrr. In *V. harveryi*, no qrr are produced and LuxR is translated, initiating bacterial behaviour such as bioluminescence. Figures reprinted with permission[30].

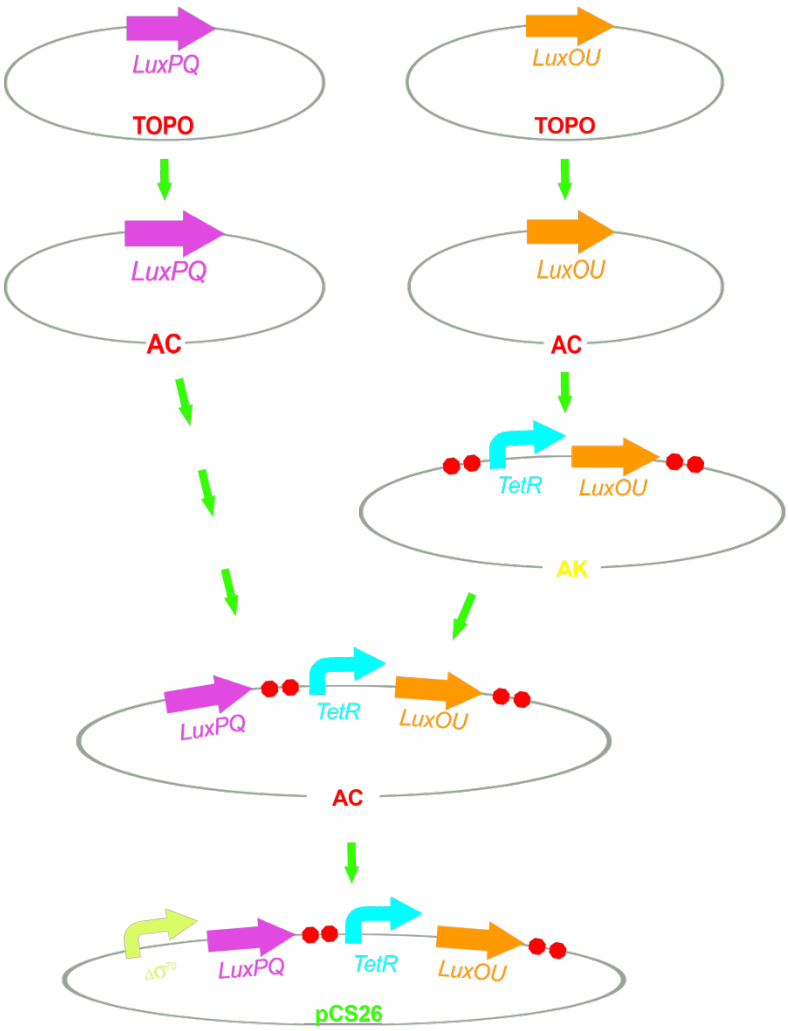

Figure 2. Project schematic for establishing *V. harveyi* AI-2 signalling system in *E. coli*. This paper will focus on the construction of the *luxOU* construct (consisting of a tetracycline repressible promoter and *luxOU* flanked by double terminators). *luxPQ* cloning with the *luxOU* construct is detailed in another paper[18]. TOPO stands for the TOPO Blunt vector, AC stands for psB1AC3 and AK stands for psB1AK3. Figures reprinted with permission[21].


CACCTGACGTCTAAGAAACCATTATTATCATGACATTAACCTATAAAAATAGGCGTATCACGAGGCAGAATTTCAGA
BBK-CP forward
TAAAAAAAATCCTTAGCTTTCGCTAAGGATGATTTCTGGAATTCGCGGCCGCTTCTAGAGCCAGGCATCAAATAAAA
Biobrick prefix
CGAAAGGCTCAGTCGAAAGACTGGGCCTTTCGTTTTATCTGTTGTTTGTCGGTGAACGCTCTCTACTAGAGTCACAC
TGGCTCACCTTCGGGTGGGCCTTTCTGCGTTTATATACTAGAGTCCCTATCAGTGATAGAGATTGACATCCCTATCA
Biobrick scar
GTGATAGAGATACTGAGCACTACTAGAACAGAAGGTCAAAAGTCTCGTTATCTACTTATGGTAGAAGACACCG
R0040 reverse
Biobrick scar
luxOU forward
CATCCGTTGCGGCACTTTACCGCTCTTACCTCACGCCACTTGGCATCGATATCAATATTGTTGGAACAGG
CAGAGACGCCATTGAAAGCCTGAACCATCGCATTCCTGATCTTATTCTGCTCGATCTTCGTCTACCTGAT
ATGACGGGGATGGACGTATTGCACGCGGTGAAGAAAAGCCACCCAGACGTGCCAATCATCTTCATGACAG
CCCATGGTTCTATCGATACTGCGGTAGAGGCGATGCGCCACGGTTCTCAAGACTTCCTAATCAAACCATG
TGAAGCAGACCGTTTACGTGTCACGGTGAACAATGCGATCCGTAAAGCAACCAAATTAAAGAATGAAGCT
GACAACCCCGGTAACCAAAATTACCAAGGCTTCATCGGCAGTAGCCAAACGATGCAGCAGGTTTACCGCA
CCATTGACTCGGCAGCGAGCAGTAAAGCGAGTATTTTCATCACGGGTGAAAGTGGTACGGGTAAAGAAGT
GTGTGCCGAAGCGATTCACGCAGCAAGCAAACGCGGTGATAAGCCGTTTATCGCCATCAACTGTGCGGCA
ATCCCGAAAGACCTTATTGAAAGTGAGCTGTTTGGTCACGTAAAAGGTGCGTTTACTGGTGCTGCGAATG
ACCGACAAGGTGCGGCAGAGCTTGCTGATGGCGGCACCTTGTTCCTTGATGAACTCTGTGAAATGGACTT
GGATCTTCAAACTAAGCTATTGCGCTTTATCCAAACGGGTACATTCCAAAAAGTCGGTTCTTCTAAAATG
AAGAGCGTGGATGTGCGCTTTGTGTGTGCAACTAACCGAGACCCTTGGAAAGAAGTGCAAGAAGGCCGTT
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TTCGCACAAGACGTGATTGAAAGATTCAACAGCTACGAATGGCCGGGTAACGTTCGCCAGTTGCAAAACG
TATTGCGTAATATCGTGGTACTGAACAATGGCAAAGAGATCACGCTGGATATGTTACCGCCACCACTGAA
TCAGCCTGTTGTGCGCCAATCGGTAGCAAAATTCATTGAACCTGACATTATGACGGTGTCAGATATTATGCCGCT
TTGGATGACAGAGAAAATGGCTATTGAGCAGGCAATTCAAGCGTGTGAAGGCAACATTCCACGCGCTGCTGGCTATT
TGGATGTTAGTCCATCAACGATTTATCGCAAGTTGCAAGCTTGGAATAGCAAGGACGAAAAACAAAACGTATGAATA
CGGACGTATTAAATCAGCAAAAAATTGAAGAACTGTCTGCGGAAATTGGTAGCGATAATGTTCCTGTTTTGCTTGAT
ATTTTTCTTGGGGAAATGGACTCCTACATTGGCACTTTAACTGAACTTCAGGGCTCAGAGCAGCTGTTGTATTTAAA
AGAGATCAGCCACGCACTGAAAAGTAGTGCTGCCAGCTTTGGCGCAGATCGATTGTGTGAACGAGCGATTGCCATCG

Figure 3. Sequence of BBa_B0015-BBa_R0040-luxOU-BBa_B0015 in psBAC3 indicating sequences where various primers used anneal. Vector backbone is highlighted in black, biobrick suffix and prefix are indicated by blue and the scar created by ligation of *XbaI* and *SpeI* cut products is indicated by green. BBa_B0015 (double terminator) is highlighted in red, BBa_R0040 (tetracycline repressible promoter) is shown in purple and the *luxOU* gene is shown in orange. Primers used for sequencing include BBK-CP forward, R0040 reverse and BBK-CP reverse. The *luxOU* forward and reverse primers were used to amplify *luxOU* in PCRs. Primers not shown include *luxOU*-RS forward and reverse; these primers anneal to the same regions as *luxOU* forward and reverse with an appended sequence of the Biobrick prefix or suffix, respectively.

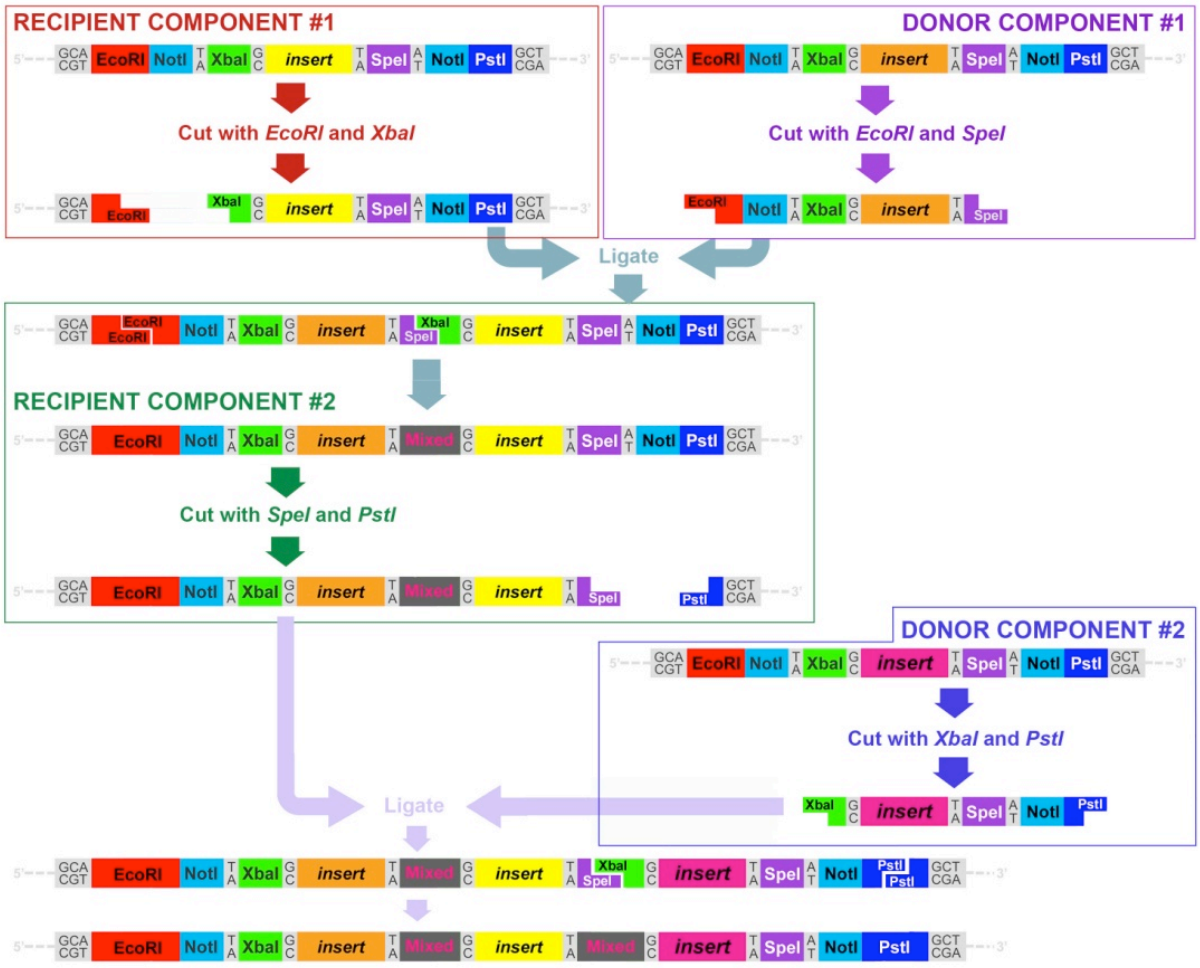


Figure 4. Biobrick construction technique schematic diagram created by Sonja Georgijevic based on Biobrick construction method.

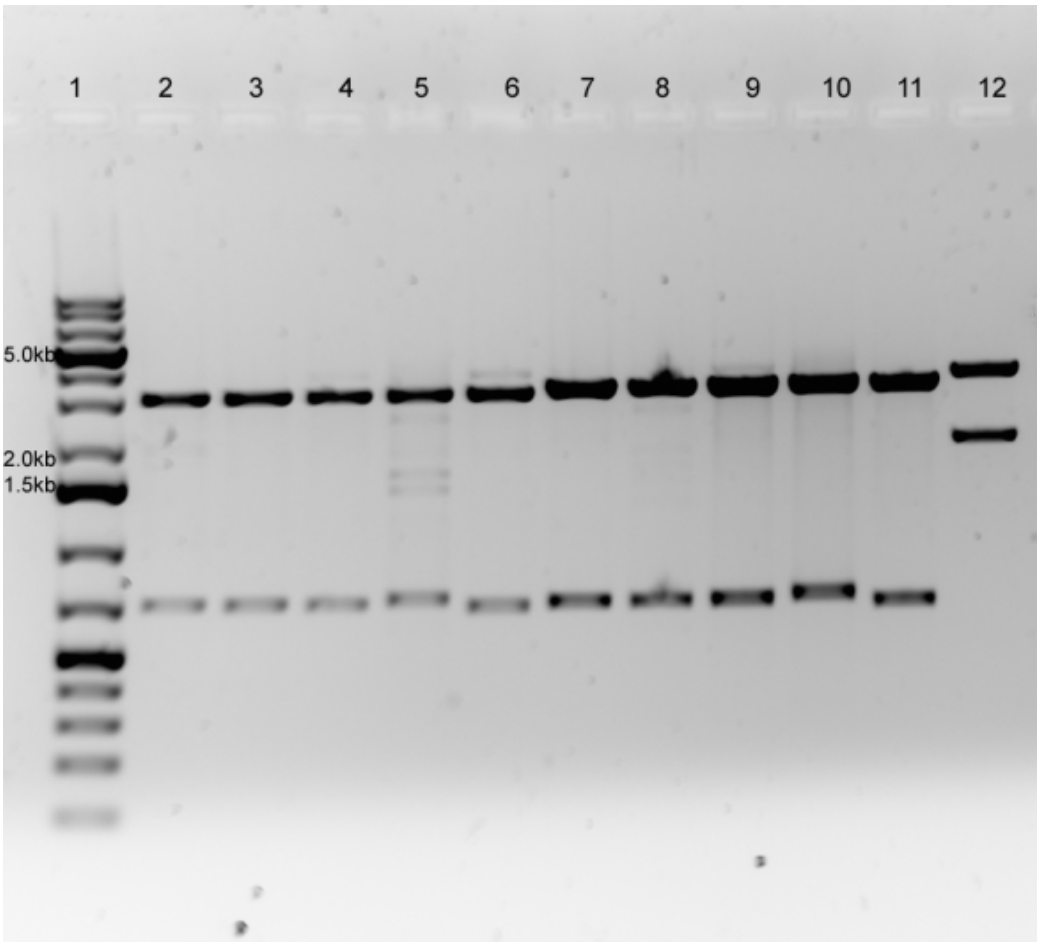


Figure 5. 1% agarose gel (100V) for verification of *luxOU* in TOPO BL and verification of restriction sites in psB1AC3 and psB1AK3 with a ccdB insert. 1000ng of psB1AC3 (lanes 2-6) and psB1AK3 (lanes 7-11) were digested with (A) *Not*I, (B) *EcoR*I and *Spe*I, (C) Xba*I* and *Pst*I, (D) *EcoR*I and *Pst*I and (E) Xba*I* and *Spe*I. 850ng of *luxOU* in TOPO BL (lane 12) was digested with *EcoR*I. Expected band sizes for lanes 2-6 are 3kb (vector backbone) and 700bp (ccdB). Expected band sizes for lanes 7-11 are 3.2kb (vector backbone) and 700bp (ccdB). Expected band sizes for lane 12 is 3.1kb (vector backbone) and 2kb (*luxOU*).5µL of GeneRuler 1kb Plus DNA Ladder was loaded. Digests were left overnight at 37^{o}C and 9µL of each sample was loaded with 1µL 10x Orange G.

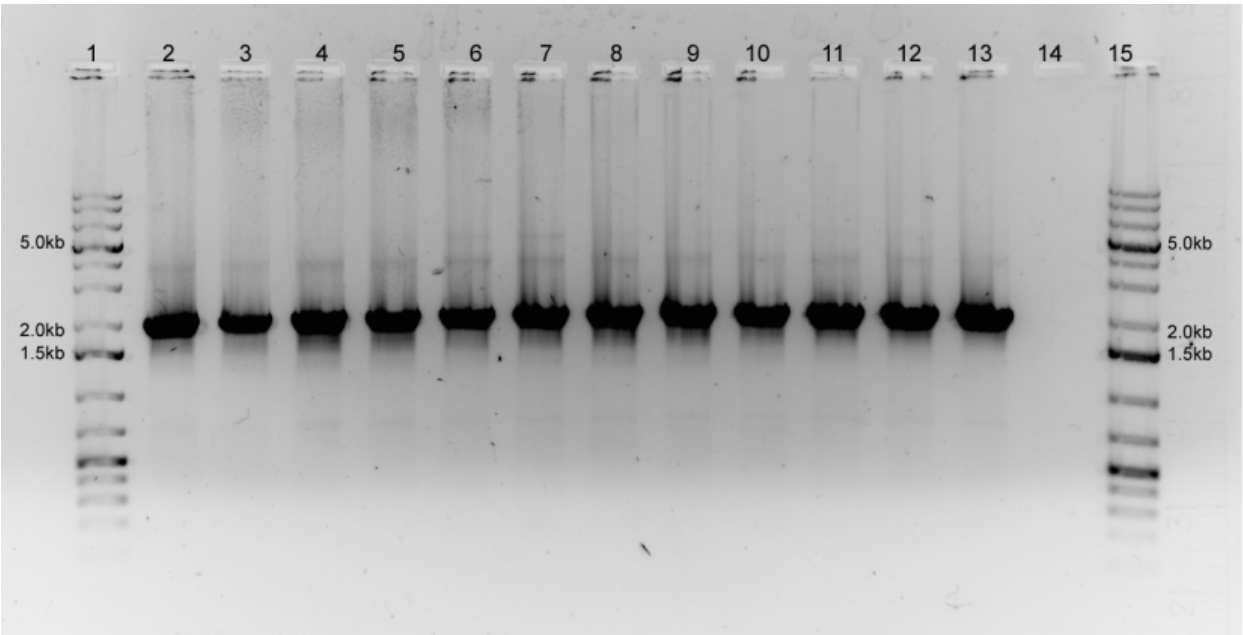
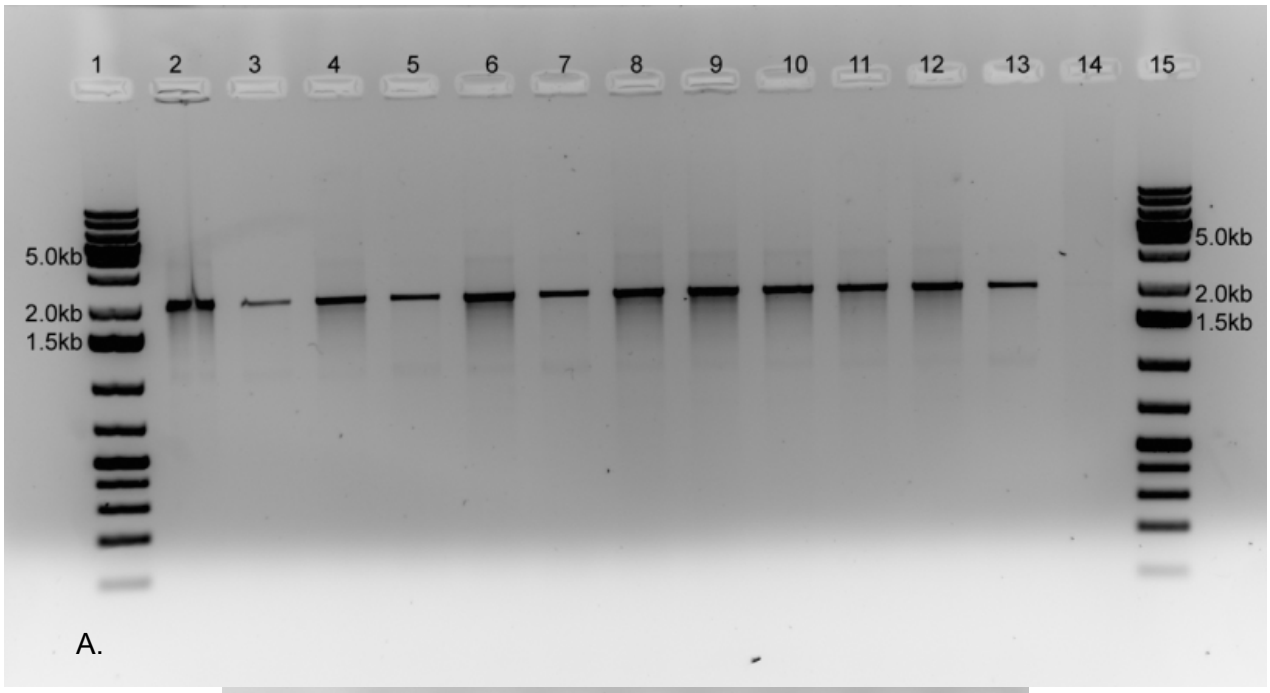


Figure 6. 1% agarose gel (100V) for gradient PCR amplification of *luxOU* with Biobrick suffix and prefix. pPFX amplified 40ng of TOPO BL with *luxOU*. Gene specific primers with flanking Biobrick restriction sites were used at annealing temperatures ranging from 58°C (left) to 66°C (right). Lanes 2-13 are samples from each reaction carried out at different temperatures. Lane 14 is a negative control. Expected band size is 2kb. 5µL of GeneRuler 1kb Plus DNA Ladder was loaded. 3µL of each sample was loaded with 2µL 10x Orange G to a total volume of 20µL.



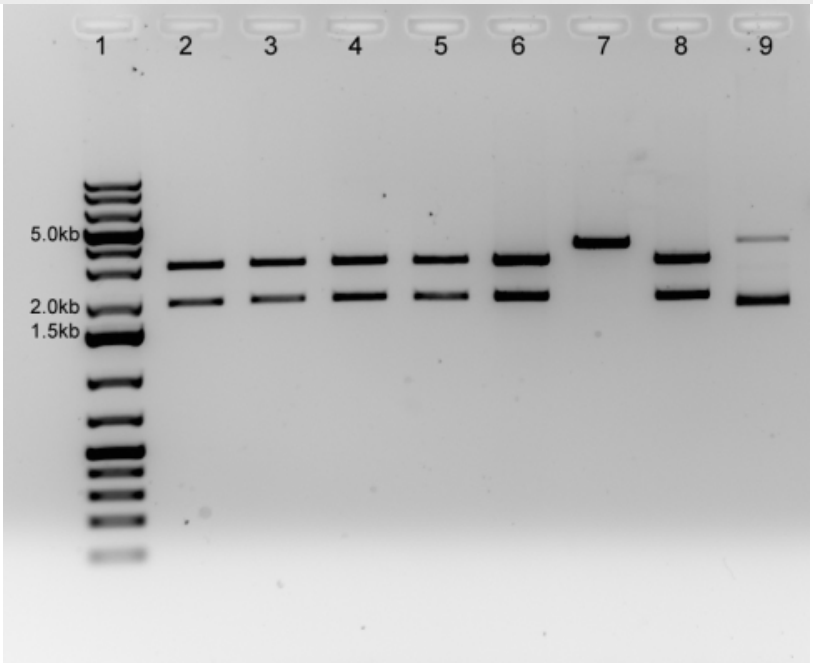


Figure 7. Verification steps to determine whether or not *luxOU* cloning into psB1AC3 was successful involved colony PCR and restriction digest of mini-prepped plasmids. **A.** 1% agarose gel (100V) for colony PCR of TOP10 transformants using pTaq. Six colonies from each construction techniques were screened. Lane 2-7 are transformants from the *EcoR*I and *Pst*I construction. Lane 8-13 are transformants from the *Xba*I and *Pst*I construction. Lane 14 is the negative control. Expected band size was 2kb. Gene specific primers were used. 5µL of GeneRuler 1kb Plus DNA Ladder was loaded. 3µL of each sample was loaded with 2µL 10x Orange G to a total volume of 20µL. **B.**1% agarose gel (100V) for *Not*I verification digest of *luxOU* in psB1AC3 (*Xba*I and *EcoR*I construction). 200ng of plasmid was cut with *Not*I for 2 hours at 37°C. Lanes 2-5 are C1, C2, C4 and C5 respectively from the *Xba*I and *Pst*I construction. Lanes 6-9 are C1-C4 respectively from the *EcoR*I and *Pst*I construction. Expected band sizes were: 3kb (psB1AC3) and 2kb (*luxOU*) 5µL of GeneRuler 1kb Plus DNA Ladder was loaded. 20µL was loaded of the restriction digest reaction mixture with 4µL 10x Orange G to a total volume of 40µL.

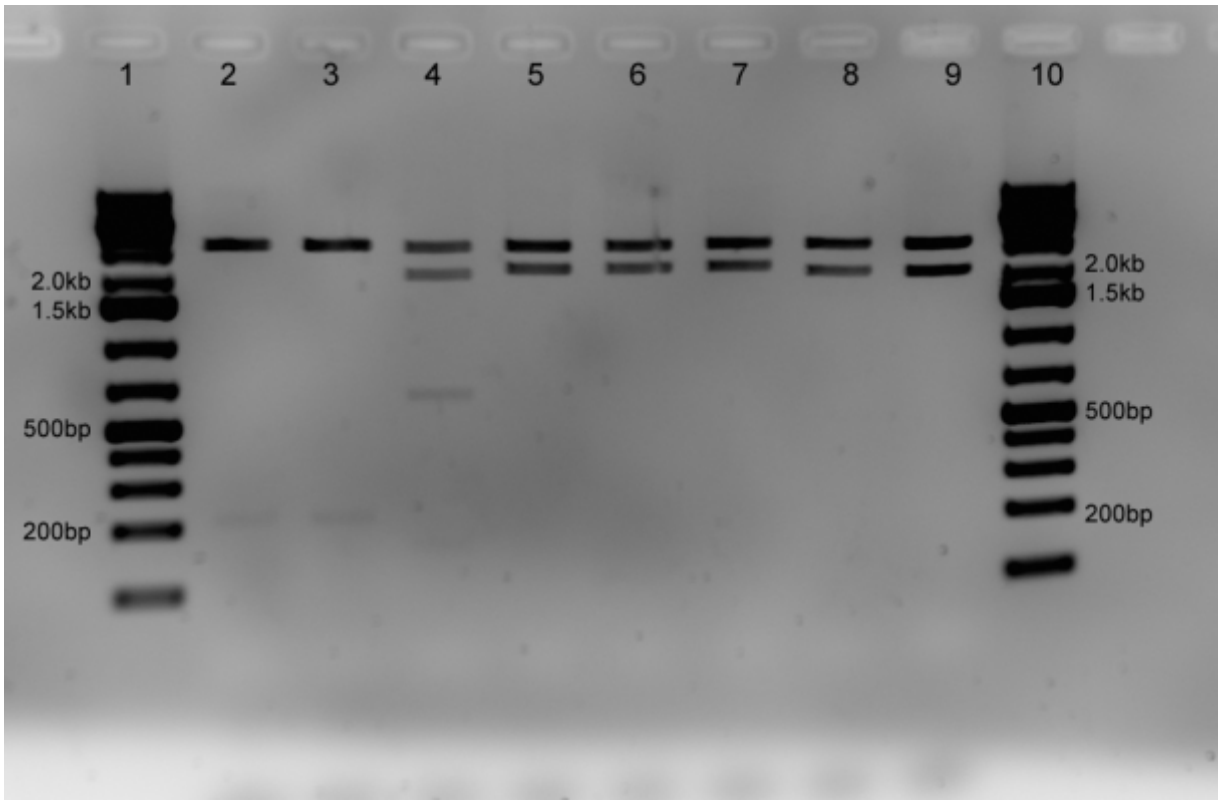


Figure 8. 1% agarose gel (100V) for *Not*I verification digest of construction yielding BBa_B0015-BBa_R0040 and *luxOU*(*Xba*I or *EcoR*I)-BBa_B0015. 200ng of plasmid was cut with *Not*I for 2 hours at 37°C. BBa_B0015-BBa_R0040 C1 is lane 2) and C4 is lane 4. *luxOU*(*Xba*I)-BBa_B0015 C3 is in lane 4 and C4 is in lane 5. *luxOU*(*EcoR*I)-BBa_B0015 C3 is in lane 6 and C4 in lane 7. Uncut *luxOU* in psB1AC3 (*EcoR*I C1 and *Xba*I C2) were used as positive controls in lanes 8 and 9, respectively. Expected band sizes for the BBa_B0015-BBa_R0040 construction are 3.2kb (psB1AK3) and 183bp. Expected band sizes for the *luxOU*-BBa_B0015 construction are 3kb (psB1AC3) and 2.1kb. 5µL of GeneRuler 1kb Plus DNA Ladder was loaded. 2µL of the restriction digest product was loaded with 2µL 10x Orange G to a total volume of 20µL.

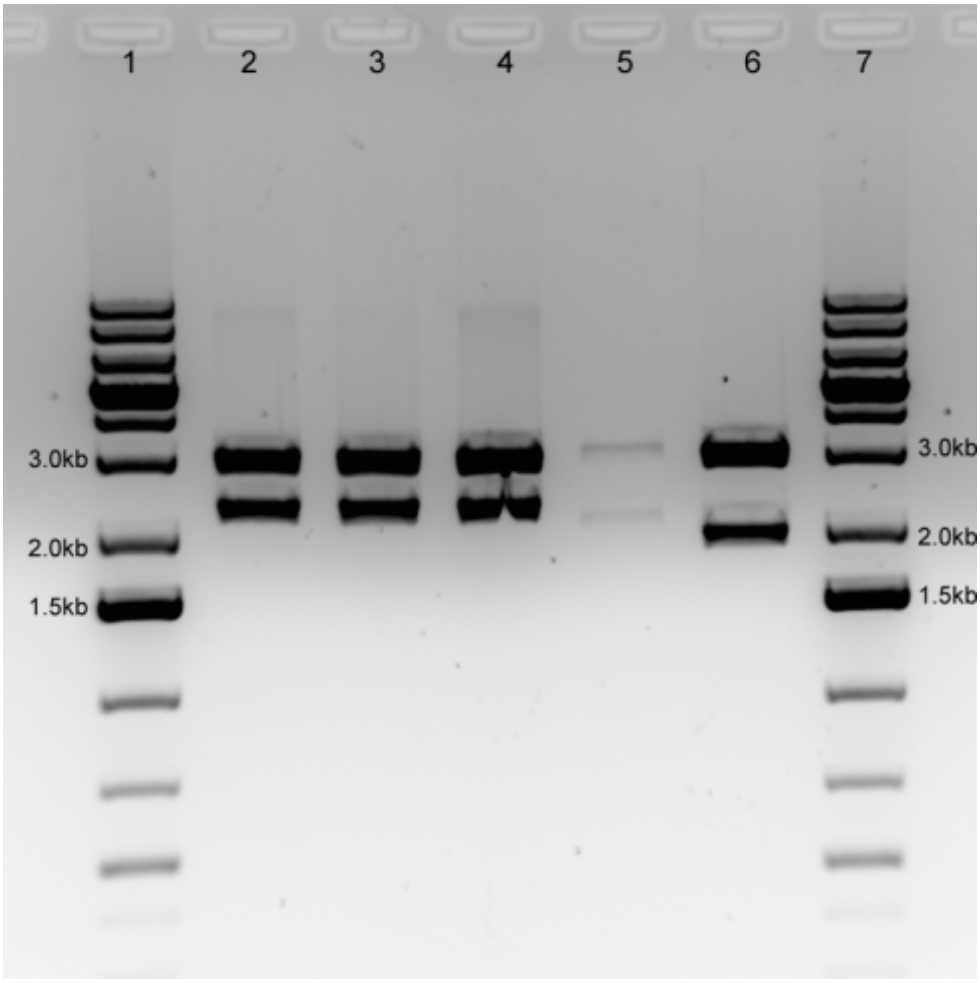


Figure 9. 1% agarose gel (100V) for *Not*I verification digest of construction yielding BBa_B0015-BBa_R0040-*luxOU*-BBa_B0015 in psB1AC3. 200ng of plasmid was cut with *Not*I for 2 hours at 37°C (lanes 2-4). Digested *luxOU*-BBa_B0015 in psB1AC3 (lane 5) and *luxOU* in psB1AC3 (lane 6) were used as positive controls. Expected band sizes for the BBa_B0015-BBa_R0040-*luxOU*-BBa_B0015 construction in psB1AC3 are 3055bp (psB1AC3) and 2257bp. Expected band sizes for the *luxOU*-BBa_B0015 construction are 3055bp (psB1AC3) and 2128bp; expected band sizes for *luxOU* are 3055bp (psB1AC3) and 1945bp. 5µL of GeneRuler 1kb Plus DNA Ladder was loaded. 2µL of the restriction digest product was loaded with 2µL 10x Orange G to a total volume of 20µL.

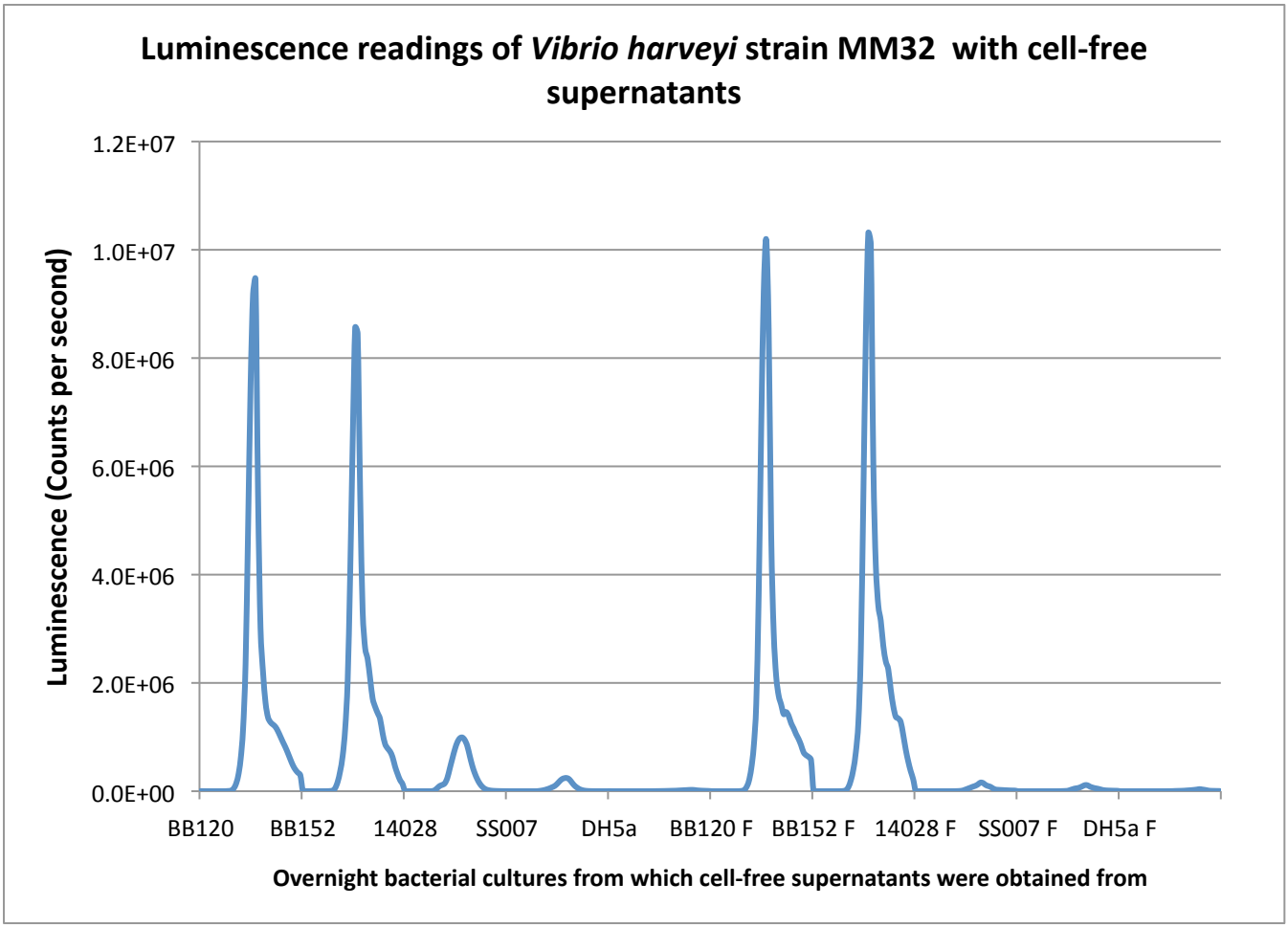


Figure 10. Luminescence readings for AI-2 activity test in cell-free supernatants of various strains of bacteria with *V.harveyi* MM32 reporter strain taken over a 20 hour read period. MM32 is responsive (i.e. luminescent) to the AI-2 present in the supernatant of *V. harveyi* BB120, a wildtype strain, and BB152, a strain deficient in AHL production. *Salmonella typhimurium* 14028 produces AI-2 but due to differences in boration, specificity to the *V. harveyi* reporter strain, and therefore luminescence, decreases. *S. typhimurium* SS007 and *E. coli* DH5α are incapable of producing AI-2 and serves as a negative control. 90µL of freshly diluted (1:5000) MM32 cells in autoinducer bioassay (AB) media was added to 10µL of cell free supernatant. Readings taken with VICTOR every 30 minutes; absorbance readings not shown. 3 trials in total; general trends are consistent (data not shown).

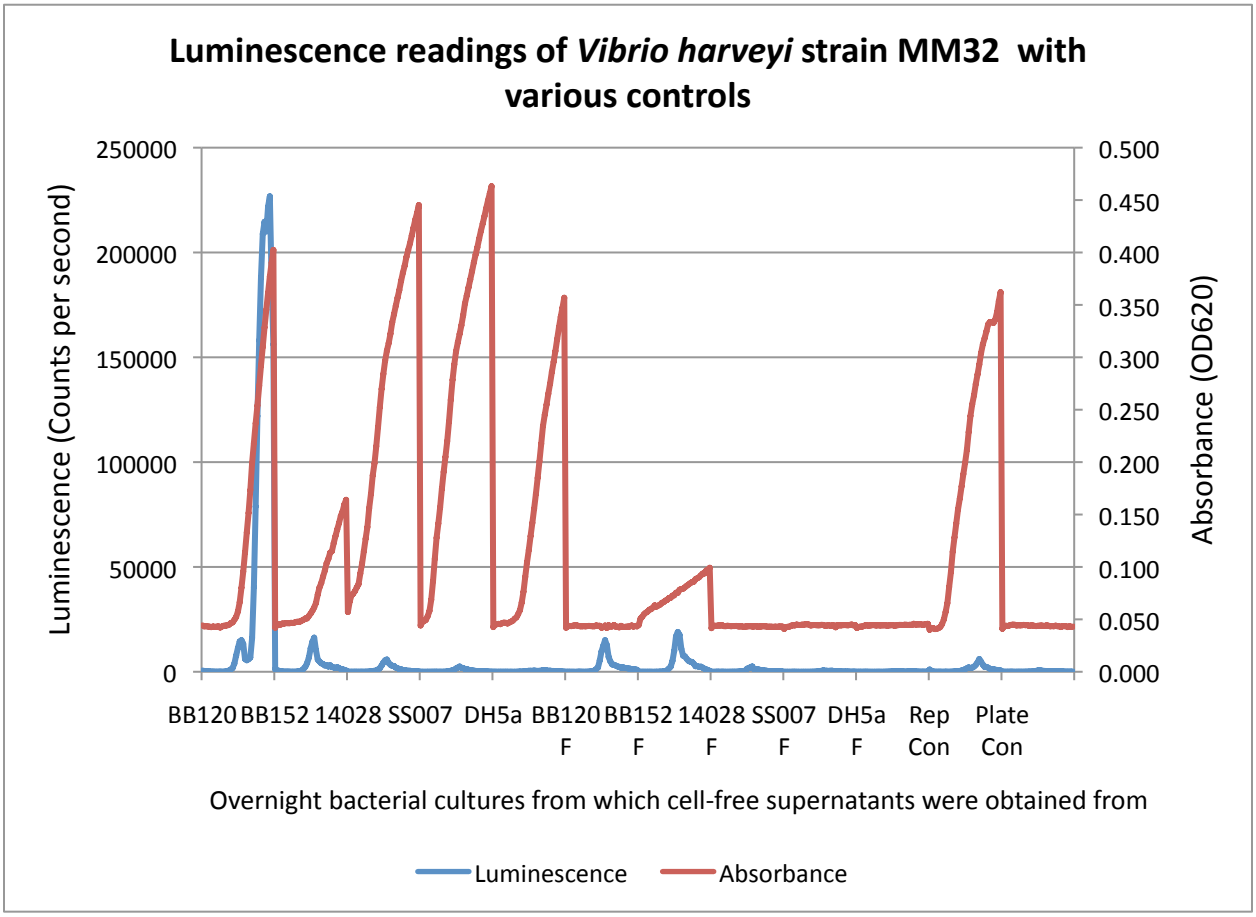


Figure 11. Luminescence readings for controls used in the AI-2 activity test of cell-free supernatants from various strains of bacteria with *V.harveyi* MM32 reporter strain taken over a 20 hour read period. A comparison was made between unfiltered and filtered (indicated by "F") CF supernatants. The presence of growth curves in the unfiltered CF supernatants stresses the importance of filtering supernatants. Presence of cells in CF supernatants explains the luminescence seen in the BB120 and BB152 experiments. The presence of luminescence and growth in BB120 F and BB152 F requires investigation into why cells were still present in the CF supernatant. Growth in the reporter control (dilute reporter cells with sterile LB media) indicates the lack of auto-luminescent cells. Lack of growth and fluorescence in the plate control (sterile LB media) shows the sterile plate. 90µL of sterile autoinducer bioassay (AB) media was added to 10µL of cell free supernatant (or LB, as indicated). Rep con stands for reporter control and Plate con stands for plate control. Readings taken with VICTOR every 30 minutes.

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