Construction of *Vibrio Harveyi* Autoinducer-2 Signalling System in *Escherichia Coli* Using Biobrick Methodology

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Abstract

Microorganisms use pheromones to monitor their own population density as well as to detect and interact with other microbial species in a process known as quorum sensing (QS). We, the University of Calgary 2009 iGEM team, have engineered the *Vibrio harveyi* AI-2 signalling system in *Escherichia coli*. This has been done by constructing the signalling circuit using the 'Biobrick' molecular cloning technique used in the International Genetically Engineered Machines (iGEM) competition. This circuit has been cloned into a BioBrick plasmid and verified for submission to the Registry of Standard Biological Parts. The signalling circuit was also cloned into and verified in pCS26, a plasmid that will allow the cloning of a library of $\Delta\sigma^{70}$ promoters to control levels of LuxPQ, periplasmic proteins in the AI-2 signalling cascade. Optimal AI-2 signalling will be dependent on these levels. Further, this system will be coupled with the expression of expression of *aiiA*, a gene that codes for an AHL-degrading enzyme.

Keywords:

Autoinducer-2   Signalling   *Escherichia coli*   Biobrick   Quorum Sensing

Introduction

Bacteria are able to communicate by producing and releasing chemical signal molecules termed autoinducers in a process called Quorum Sensing (QS)[1]. An increase in local population density of bacteria results in the accumulation of autoinducers until a minimal threshold concentration is reached, whereby bacteria are able to organize their behaviour by coordinating their gene expression. Such coordinated behaviour includes virulence induction, swarming, biofilm formation and genetic competence[2].

QS was first observed in the bioluminescent bacteria *Vibrio fischeri*[3], where light was emitted only at high population densities, but could be induced in low population densities with the presence of an extracellular substance, later identified as the autoinducer N-acylhomoserine[4] (AHL). An AHL signalling system is already present in the Registry of Biological Parts.

Further research in QS led to the discovery of the universal signalling molecule[5] autoinducer-2 (AI-2), which has been characterized in the gram-negative, bioluminescent marine bacterium *Vibrio harveyi*[1]. AI-2 binds to the periplasmic protein LuxP forming an AI-2-LuxP complex that interacts with LuxQ, a membrane bound histidine kinase[6]. At low population density corresponding to low AI-2 levels, LuxQ autophosphorylates and then subsequently phosphorylates the cytoplasmic protein LuxU[5]. LuxU then passes its phosphate to LuxO, and phospho-LuxO complexes with transcription factor σ^{54} to activate the transcription of the genes encoding five regulatory small RNAs (sRNAs) termed Qrr1-5[7] (Figure 1a). These sRNAs bind and destabilize the mRNA of *luxR*[8], a transcriptional activator of the luciferase operon *luxCDABE*[9]. As the mRNA of *luxR* is

degraded in the presence of low levels of AI-2 and low cell density, *V. harveyi* will not express bioluminescence.

In high population densities and thus high AI-2 levels, LuxQ changes from a kinase to a phosphotase, and thus removes the phosphate of LuxU, which subsequently removes the phosphate of LuxO[1] (Figure 1b). Unphosphorylated LuxO does not complex with σ^{54}, and therefore does not produce sRNAs. This leads to unblocked *luxR* mRNA allowing its translation that drives the expression of bioluminescence via luciferase.

We have engineered the *Vibrio harveyi* AI-2 signalling system in *Escherichia coli* using the molecular cloning techniques used in the International Genetically Engineered Machines (iGEM) competition. This AI-2 signalling system will be coupled with the expression of *aiiA*, a gene that encodes an AHL-degrading enzyme partaking in quorum quenching, allowing us to target biofilm maintenance. This has been done by engineering a genetic circuit encoding the AI-2 signalling cascade (termed the signalling circuit) and a circuit containing the qrr4 promoter followed by Registry-available inverter (BBa_Q04510) and *aiiA* (BBa_C0160) (collectively termed the response circuit).

The construction of the AI-2 signalling system in *E. coli* will add a second cell-to-cell communication system into the Registry of Standard Biological Parts, quite different from the AHL system already present. AHL signalling requires fewer proteins and is produced by the gene *luxI* and can freely diffuse out of the cell and into another[10] where it complexes with LuxR, activating transcription of the luciferase operon[11]. Relative to the AI-2 system, the AHL system does not use secondary messengers, and as a result, one AHL molecule cannot be amplified into multiple signals. The AI-2 system, however, allows for signal amplification due to its phosphorylation cascade. Moreover, AI-2

signalling is considered a universal signalling molecule because it is used by both gram positive and gram negative species, while AHL is merely used by gram negative species[12]. Thus the gram negative *E. coli* housing the AI-2 system will be able to respond to AI-2 from both gram positive and negative species.

Furthermore, the construction of the AI-2 signalling system in *E. coli* will allow for the fine tuned coordination of bacterial behaviour because any gene can be expressed by a highly dense population if it is simply cloned downstream of the signalling cascade. Just as important, the set-up of this system in the laboratory strain of *E. coli* will serve as an important and effective means to study AI-2 signalling used by pathogenic bacteria to induce virulence, and to develop drugs that may inhibit such pathogenecity by blocking QS.

This paper discusses the construction of the AI-2 *V. harvey* signalling circuit (Δ*luxPQ* followed by a terminator, tetracycline-repressible promoter, *luxOU* and a terminator) (Figure 2) in *E. coli.* Using BioBrick methodology, this circuit was constructed in a BioBrick vector and was then cloned into the pCS26 vector. The latter will allow the cloning of a library of 256 different promoters (termed $\Delta\sigma^{70}$ promoters) in front of the Δ*luxPQ* operon with the goal of optimizing AI-2 signalling by altering expressions levels of these periplasmic proteins.

Materials and Methods

BioBrick Cloning and Verification

The iGEM BioBrick (BBK) cloning technique allows for the simple and quick assembly of genetic circuits, and was employed here to construct the signalling circuit. Each genetic part is standardized with flanking endonuclease restriction sites: the BBK prefix (*EcoR*I, *Not*I, *Xba*I) and suffix (*Spe*I, *Not*I, *Pst*I). Once a genetic part (gene, ribosome binding site, promoter, etc…) acquires these sites by PCR amplification and has been cloned into a BBK vector, the construction of genetic circuits (ie $\geq$ 2 genetic parts) involves the restriction digest of the "insert" and the "recipient" (Figure 3). Once cut and ligated, the *Xba*I and *Spe*I restriction sites overlap to form a "scar" in which the BBK restriction sites between the assembled parts disappear. This method also allows for cloning of one part either upstream or downstream of another part while conserving the BBK prefix and suffix that flank the assembled parts, facilitating subsequent cloning. The three methods of verifying BioBrick construction include (1) Restriction Digest, (2) PCR and (3) DNA sequencing. The assembly of the signalling circuit is depicted in Figure 4.

Site-Directed Mutagenesis of ∆luxPQ

luxPQ in a cosmid form was initially obtained from Bonnie Bassler, from which it was amplified using the LuxPQ-F and LuxPQ-R primers (Table 1, Figure 5) and cloned into a pCR-BLUNT-II-TOPO vector (Invitrogen, CA) (Figure 6a). The QuikChange XL Site-Directed Mutagenesis Kit (Stratagene, CA) was used to make specific non-random point mutations to remove the following BBK restriction sites from *luxPQ*: *EcoR*I (from *luxP)* and *EcoR*I and *Xba*I (from *luxQ)*. This was done by synthesizing oligonucleotide primers

specific to these sites (LuxPQ-M1F, LuxPQ-M2F and LuxPQ-M3F; Table 1, Figure 7a) to introduce the silent mutations. This was then sequenced at the University of Calgary DNA Sequencing Facility (University Core DNA Services, Calgary, AB, Canada) with the LuxPQ-F and LuxPQ-S1 primers to verify the elimination of these sites.

Cloning of ΔluxPQ into BBK Vector

Δ*luxPQ* was amplified from pCR-BLUNT-II-TOPO vector using LuxPQ-RS-F and LuxPQ-RS-R primers (Table 1, Figure 5) and platinum *Pfx* (p*Pfx*) (Invitrogen, CA) according to the manufacturer's specifications. The cycling conditions were as follows: 94ºC for 5 minutes; 36 X (94ºC for 30 seconds; 62ºC for 30 seconds; 68ºC for 4 minutes); 68ºC for 10 minutes; the reaction was held at 4ºC. PCR products were run on a 0.8% Agarose gel after which the four products were pooled and purified using the QIAquick PCR Purification Kit (QIAGEN; Hilden, Germany). DNA concentration and purity were measured using the NanoDrop 1000 Spectrophotometer (NanoDrop, DE). 1 μg of linear DNA and the psB1AC3 (Figure 6b) backbone were then both digested with *EcoR*I and *Pst*I overnight at 37ºC. Phosphates were removed from the vector backbone using Antarctic phosphatase (New England Biolabs, ON) for 30 minutes at 37ºC, followed by enzyme inactivation at 65ºC for 10 minutes. Ligation was performed with QuickLigase (New England Biolabs, ON) at room temperature for 5 minutes. The ligate was transformed into chemically-competent TOP10 cells as described by Sambrook & Russell[13], and plated on Luria Bertani(LB)-chloramphenicol [35mg/L] and LB-ampicillin [100mg/L] agar plates, and incubated at 37ºC overnight.

Screening was done using colony PCR with LuxPQ-F, LuxPQ-R, BBK-CP-F and BBK-CP-R primers (Table 1, Figure 5) and platinum *Taq* (p*Taq*) (Invitrogen, CA) according to the manufacturer's specification. Cycling conditions were as follows: 94ºC for 6 minutes; 36 X (94ºC for 30 seconds; 55ºC for 45 seconds; 72ºC for 4 minutes); 72ºC for 10 minutes; held at 4ºC. The products were then run on a 0.8% agarose gel. LB-broth with chloramphenicol [35mg/L] and ampicillin [100mg/L] were used for overnight cultures. Plasmids were isolated using the GenElute Plamid Mini Prep kit (Sigma, MO), and DNA purity and concentration were measured using the NanoDrop 1000 Spectrophotometer (NanoDrop, DE). Δ*luxPQ* in psB1AC3 was sequenced at the University of Calgary DNA Sequencing Facility using BBK-CP-F/R primers (University Core DNA Services, AB).

Construction of Signalling Circuit in BBK Vector

Sequencing revealed the loss of the *Pst*I site on the BBK suffix, and thus a plasmid switch of Δ*luxPQ* from psB1AC3 to psB1AK3 (Figure 6c) and back into psB1AC3 was performed to recover the suffix following protocols described above. The other components of the signalling circuit (terminator-Tetracycline repressible promoter-*luxOU*-terminator; BBK: B0015-R0040-*LuxOU*-B0015) were constructed in psB1AK3[14] and this construct was cut with XbaI and PstI and cloned downstream of *Spe*I and *Pst*I-cut Δ*luxPQ* (Figure 4 a-f). This was done following the protocols described above. Screening of Δ*luxPQ*-B0015-R0040-*LuxOU*-B0015 (hereafter called signalling circuit) in the psB1AC3 vector was done by colony PCR using the following sets of primers: BBK-CP-F/R and LuxPQ-F, LuxOU-R. The circuit was subsequently sequenced with BBK-CP-F, BBK-CP-R and R0040-R (Table 1) primers.

Moving Signalling Circuit into pCS26

The signalling circuit was cloned from psB1AC3 into pCS26, a plasmid containing a Kanamycin resistance gene (Figure 6d). This was done by digesting the signalling circuit insert and pCS26 vector with NotI, and then following the protocols for ligation, transformation and verification as described above. The presence and directionality of the signalling circuit in pCS26 was verified by PCR, pairing the pCS26-S-F primer (Table 1) separately with LuxPQ-R and LuxOU-R primers. The signalling circuit in the pCS26 vector was also sequenced with pCS26-S-F and LuxOU-R primers.

Results

Verifying luxPQ mutation and cloning ΔluxPQ into BBK Vector

We reasoned that *E. coli* could serve as a suitable chassis to house the AI-2 signalling system; however, certain modifications to the operons found in *V. harveyi* had to be made before BBk construction in *E. coli. luxPQ* was obtained in a cosmid, cloned into pCR-BLUNT-II-TOPO, and silently mutated at specific nucleotides to remove BBK restriction sites with the operon. Sequencing revealed successful nucleotide base pair changes and conservation of amino acid sequence (Figure 7). Δ*luxPQ* was then cloned from pCR-BLUNT-II-TOPO into the psB1AC3 vector. This was done using LuxPQ-RS-F and LuxPQ-RS-R primers to add the BBK prefix and suffix to the operon. Subsequent verification by PCR revealed the expected band size of Δ*luxPQ* at around 3.9kb for each of the products (Figure 8).

These products were pooled and the concentration of linear PCR product was measured to be 316.9ng/μL. Linear BBK-flanked Δ*luxPQ* was then cloned into psB1AC3 and transformed into TOP10 cells. Subsequent screening by colony PCR revealed a band at ~3.9kb and isolated plasmid was sequenced and found to be missing the *Pst*I restriction site on the BBK suffix (results not shown).

To recover the *Pst*I site, a plasmid switch of Δ*luxPQ* from psB1AC3 to psB1AK3 was performed. Colonies were screened by PCR using BBK-CP-F/R primers and had desired band sizes of ~3.9kb (results not shown) once run on an agarose gel. Δ*luxPQ* was then moved back into psB1AC3 to allow for the antibiotic selection while constructing the signalling circuit in psB1AK3. Similar to the first plasmid switch, a colony PCR was set up with LuxPQ-F/R and BBK-CP-F/R primers to verify the presence of Δ*luxPQ* in

psB1AC3. Both sets of primers confirmed the desired band size for most of the screened colonies (Figure 9).

Construction of Signalling Circuit in BBK Vector

The B0015-R0040-*LuxOU*-B0015 construct was then successfully cloned downstream of Δ*luxPQ* in psB1AC3 using the BBK construction technique. This was verified by colony PCR with BBK-CP-F/R and LuxPQ-F/LuxOU-R primers where one colony had the single expected band size of around 6.1 kb (Figure 10). The signalling circuit was then sequenced with BBK-CP-F, BBK-CP-R and R0040-R primers and verified the presence of desired parts in the circuit (Figure 11).

Moving Signalling Circuit from BBK Vector to pCS26

The signalling construct was cloned from psB1AC3 into pCS26 by *Not*I digest. This was verified by plasmid PCR by pairing the pCS26-S-F primer separately with LuxPQ-R and LuxOU-R primers to verify the presence and directionality of the circuit. Two colonies revealed expected sizes, respectively, for both sets of primers: 4.0kb and 6.1kb (Figure 12). One colony of the signalling circuit in pCS26 was then sequenced with the pCS26-S-F primer and the LuxOU-R primer, which verified the presence of Δ*luxPQ* and *luxOU*, respectively (Figure 13). As the construct was cloned into pCS26 by *Not*I digest, the *EcoR*I site of the BBK prefix and the *Pst*I site of the BBK suffix were no longer present.

Discussion

AI-2 signalling is an effective means by which bacteria such as *V. harveyi* are able to communicate and coordinate their gene expression. Our aim was to engineer the *V. harveyi* AI-2 signalling system in *E. coli* by constructing a signalling circuit (Δ*luxPQ*-B0015-R0040-*luxOU*-B0015) using BBK methodology. This circuit was cloned and verified in the BBK and pCS26 plasmids.

Although using Biobricks ensures the standardization of genetic parts and circuits to allow for further construction, we found it quite difficult to clone large pieces (>3.0 kb) using this method. A method such as TOPO cloning would potentially provide a quicker means to attempt construction, however, would not necessarily be of easy use to synthetic biologists compared to using Biobricks.

Construction of Signalling Circuit in BBK

After numerous trials, Δ*luxPQ* was cloned into a BBK vector, only to reveal the loss of the *Pst*I restriction site on the BBK suffix. Cloning of Δ*luxPQ* into psB1AC3 was done with *Xba*I and *Pst*I and because the vector and insert were successfully ligated, the mutation in the *Pst*I site must have been spontaneous and have occurred after ligation, yet before sequencing.

The B0015-R0040-*LuxOU*-B0015 construct was subsequently cloned downstream of Δ*luxPQ* (Figure 4). The signalling circuit was constructed in this manner because cloning Δ*luxPQ* from the TOPO vector into the BBK vector took several attempts, whereas *luxOU* was cloned from TOPO into BBK on the first trial, allowing construction to continue around the latter operon. Δ*luxPQ* was more difficult than *luxOU* to clone into

the BBK vector most likely because of the size difference in these constructs (3.8kb compared to 1.9kb). Similarly, several attempts were made to clone Δ*luxPQ* (as the insert) upstream of B0015-R0040-*LuxOU*-B0015 in psB1AK3 because the absence of the *Pst*I site would have no effect on this cloning method (Figure 3). These attempts, however, were unsuccessful as the gels run after PCR revealed wrong sized bands. This construction approach was unsuccessful again, probably because of the size difference between the constructs.

With these unsuccessful attempts, it was reasoned that the B0015-R0040-*LuxOU*-B0015 construct should be inserted downstream of Δ*luxPQ*. This required the recovery of the *Pst*I site on the BBK suffix of Δ*luxPQ* in psB1AC3. This was done by performing a plasmid switch of Δ*luxPQ* into psB1AK3. As the other part of the signalling circuit (B0015-R0040-*LuxOU*-B0015) was already present in the psB1AK3 vector, Δ*luxPQ* was switched back into psB1AC3 to allow for antibiotic selection when cloning these pieces together. These two plasmid switches were successful (Figure 9) and allowed for the B0015-R0040-*LuxOU*-B0015 construct to be cloned downstream of Δ*luxPQ*. This was verified to be successful by PCR (Figure 10) and sequencing (Figure 11).

No ribosome binding sites were cloned into this circuit because both the *luxPQ* and *luxOU* operons have internal ribosome binding sites. Although terminators also exist within these operons, Registry-available terminators were cloned downstream of both operons for further insulation, particularly for *luxPQ* to ensure that *luxOU* stays solely under control of the R0040 promoter.

Importance of Signalling Circuit in pCS26

After the signalling circuit was constructed in psB1AC3, it was then cloned into pCS26 (Figure 4). This was done by *Not*I digest and did not ensure directional cloning; therefore, PCR was done with gene specific and vector primers not only to verify the presence of certain elements within the construct, but to identify the colonies where the insert was cloned in the right direction. The direction of the signalling circuit in pCS26 was most crucial because the promoter region of Δ*luxPQ* lies between the *Xho*I and *BamH*I cloning site on the vector, just upstream of the *Not*I multiple cloning site (Figure 6d). This region will allow for the cloning of a library of variable strength $\Delta\sigma^{70}$ promoters in order to alter levels of LuxPQ. These protein levels are important because luxP and luxQ are found in the periplasm, an area of limited space in the cell, and if they are under constitutive control, the cell will most likely be overloaded. The promoters will be constructed with primers with four degenerate bases in the promoter consensus sequence allowing for 256 possible promoters. These will be separately cloned into the pCS26 vector between the *Xho*I and *BamH*I sites upstream of Δ*luxPQ*. The purpose of this procedure is to identify promoters capable of optimizing AI-2 signalling by producing a certain amount of LuxPQ. This will be done by transforming a reporter circuit (P_{qrr4} followed by GFP) that will provide functional data for AI-2 signalling, whereby the brightest colony in the absence of AI-2 and the darkest colony in the presence of AI-2 will be identified. This reporter will simultaneously be used to test whether the signalling circuit is functional. It should be noted that *luxOU* is under constitutive control of the TetR (R0040) promoter because the LuxU and LuxO proteins are found in the cytoplasm, an area of considerably greater space than the periplasm.

Signalling Circuit in Overall Goal

Although the construction of a reporter circuit will be crucial to optimize AI-2 signalling and ensure that the circuit is functional, there are many other important facets to achieving this goal. The reporter circuit itself must be functional, something that will be tested with mutant LuxO proteins that will either mimic the phosphorylated and active or unphosphorylated and inactive forms of LuxO. Moreover, once the signalling circuit is functional, we seek to couple this QS system with a desired response to demonstrate how AI-2 signalling can be used. This requires the construction of a response circuit, again tested with the mutant LuxO proteins and then coupling this circuit with the signalling circuit. This entire project is depicted in Figure 14.

Response Circuit

The response circuit that has been envisioned and is currently under construction[15] comprises the qrr4 promoter followed by the registry-available c1λ inverter (BBa_Q04510) and *aiiA* (BBa_C0160). If coupled with the signalling circuit and in the presence of AI-2 (or in high population density), the engineered *E. coli* will produce aiiA, an enzyme that will degrade AHL, another QS signal molecule used by bacteria. This will target biofilm maintenance as bacteria such as *Pseudomonas aeruginosa* rely on this molecule for biofilm formation[16]. In this sense, the AI-2 produced by the bacteria in the biofilm will serve as the input to our system, resulting in the degradation of AHL. Further, it may be useful to clone another gene downstream of the inverter called *dspB* from *Actinobacillus actinomycetemcomitans* that encodes an enzyme that can hydrolyze the polysaccharide matrix present in a biofilm and thus cause biofilm detachment[17].

Significance of AI-2 Signalling System in E. Coli

Once complete, the power of the AI-2 signalling system coupled with a response circuit in a laboratory strain of *E. coli* lies within the principle of QS and coordinating bacterial behaviour. The *E. coli* will be able to express a gene with a desired function at high population densities simply if this gene is cloned downstream of the inverter on the response circuit. This engineered system will prove quite versatile will respect to the output from AI-2 signalling.

The usefulness of AI-2 signalling in *E. coli* stretches far beyond the ability to clone in a gene of interest to express in high population densities. This safe, non-pathogenic, laboratory *E. coli* model of AI-2 signaling will contribute to the understanding of QS systems used by pathogenic bacteria to induce virulence. Periplasmic protein LuxP has been identified as a ribose-binding protein and its crystal structure has been determined along with important amino acids for AI-2 binding[18]. Although this allows for the investigation of other LuxP-binding molecules *in silico*, the presence of AI-2 signalling in *E. coli* will allow for *in vitro* investigations of activating and inactivating ligands. With this comes the development of novel therapeutics aimed at attenuating QS and thus virulence induction in pathogenic bacteria by, for example, synthesizing a drug capable of competitively inhibiting the AI-2 binding site on LuxP. Targeting and preventing QS is particularly appealing considering the emergence of increasingly antibiotic resistant bacteria[19].

Acknowledgements

Tables & Figures

Primer	Sequence	GC (%)	Tm (ºC)
LuxPQ-M1F	GTGAATTAGCAACA**GAGTTC**GGAAAGTTCTTCC	42.4	75.4
LuxPQ-M2F	CGCACACACCA**GAGTTC**CGTTTTCTAACG	51.7	76.0
LuxPQ-M3F	CCTCCATTGGT**TCGAGA**CACATGCTCG	55.6	75.6
LuxPQ-S1	CCGTGATAATAACTTTGAGC	40	56
LuxPQ-RS-F	**GAATTCGCGGCCGCTTCTAGA**ATGCTCGATAAAAACTAAAAGAGC	33.3	64.0
LuxPQ-RS-R	**CTGCAGCGGCCGCTACTAGT**CCGATACCCTAGAAAAAACAATGC	41.7	68.0
LuxPQ-F	ATGCTCGATAAAAACTAAAAGAGC	33.3	64.0
LuxPQ-R	CCGATACCCTAGAAAAAACAATGC	41.7	68.0
BBK-CP-F	CACCTGACGTCTAAGAAACC	50.0	60.0
BBK-CP-R	AGGAAGCCTGCATAACGCG	57.9	60.0
R0040-R	TGCTCAGTATCTCTATCACTG	42.9	60.0
LuxOU-R	CCCATTTCAAATCTCCTCATG	42.9	60.0
pCS26-S-F	AGCTGGCAATTCCGACGTC	57.9	60.0

Table 1. List of primers used in the construction of the signalling circuit. Primer name, sequence, GC content and melting temperature are all listed here. Primers LuxPQ-M1F, LuxPQ-M2F and LuxPQ-M3F were designed to incorporate a mutation in the *luxPQ* sequence to eliminate the any BBK sites (shown in pink). The single nucleotide change is underlined. The green nucleotides for LuxPQ-RS-F and LuxPQ-RS-R represent the BBK restriction sites to be incorporated to flank *luxPQ*. Primers designed by Thane Kubik.
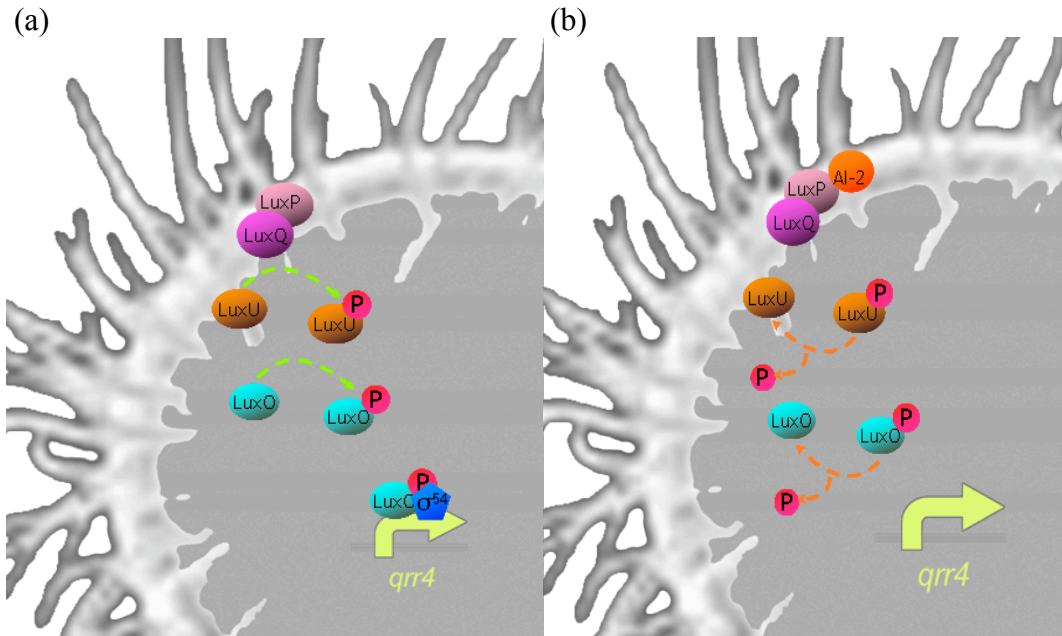
Figure 1. AI-2 signalling cascade in the absence and presence of AI-2. (a) In the absence of AI-2, LuxQ autophosphorylates and subsequently phosphorylates the cytoplasmic protein LuxU, which passes its phosphate to LuxO. Phospho-LuxO complexes with transcription factor σ^{54} to activate the transcription of genes downstream of one of the five qrr4 promoters. The promoter depicted here is qrr4, as it is the one engineered into our system. (b) In the presence of AI-2, LuxQ changes from a kinase to a phosphotase, and thus removes the phosphate of LuxU, which subsequently removes the phosphate of LuxO. Nothing binds to the qrr4 promoter and therefore there is no expression of downstream genes.
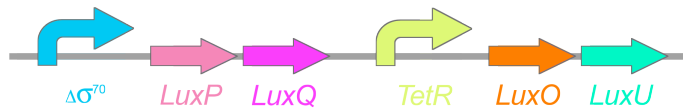
Figure 2. Schematic depiction of AI-2 signalling circuit. This genetic circuit encodes the proteins necessary for the AI-2 signalling cascade (see Figure 1). Curved arrows represent promoters, while straight arrows represent genes. The *luxOU* operon is under constitutive control of the TetR promoter (BBa_R0040), whereas the Δ*luxPQ* operon is under control of the $\Delta\sigma^{70}$ promoter, allowing for control of expression levels. For simplicity, the terminators after each operon are not shown in this circuit.
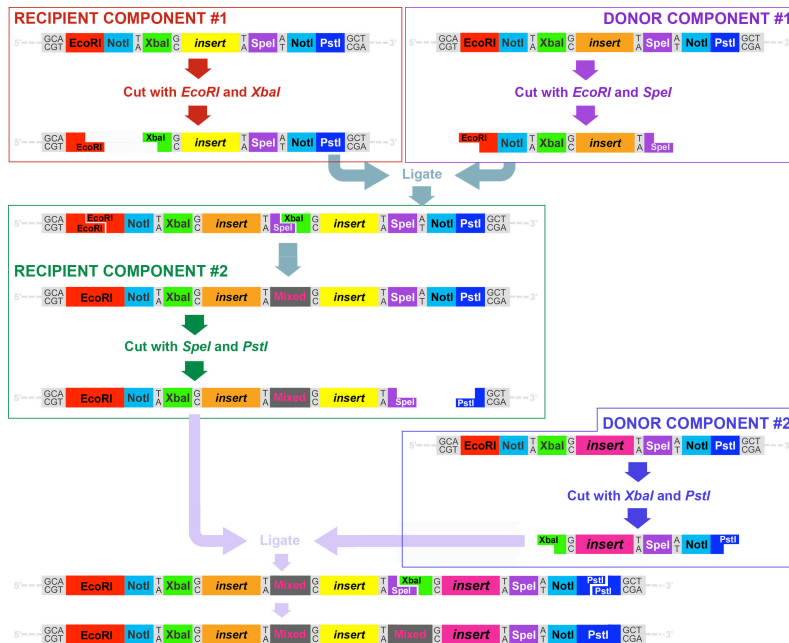
Figure 3. Schematic diagram of BioBrick construction technique created by Sonja Georgijevic. The cloning of a genetic part (donor component #1) in front of another part (recipient component #1) requires digesting with *EcoR*I/*Spe*I and *EcoR*I/*Xba*I respectively. Ligation results in the formation of a 'scar' in which the *Xba*I and *Spe*I restriction sites between the two parts overlap, removing any restriction site present. Similarly, the insertion of a genetic part (donor component #2) behind another part (recipient component #2) requires digesting with *Xba*I/*Pst*I and *Spe*I/*Pst*I respectively. The BBK construction method allows for conservation of the BBK prefix and suffix that flank the entire circuit to allow for subsequent cloning, while ensuring no restriction sites are present between each part. Diagram adapted from Idempotent Vector Design for Standard Assembly of Biobricks (Tom Knight).
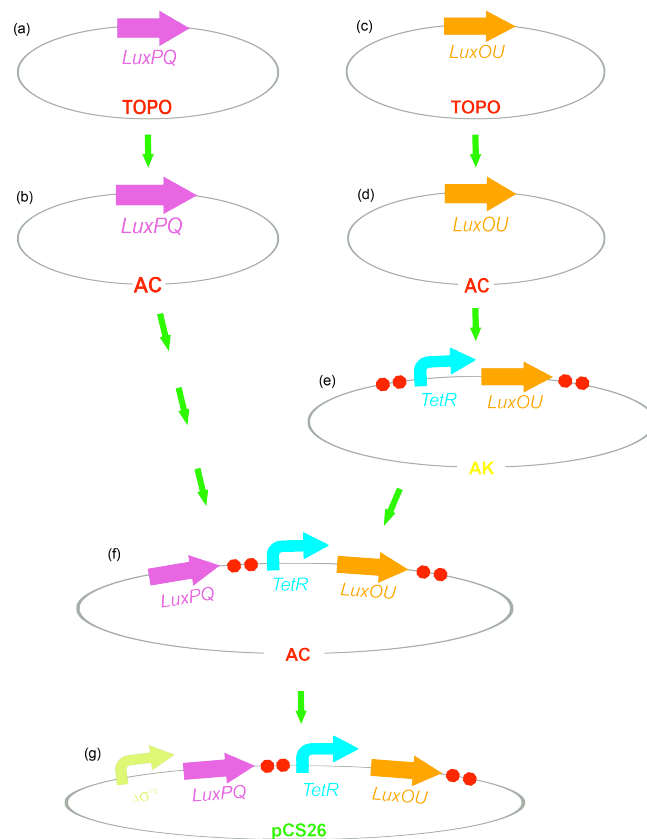
Figure 4. Schematic Diagram of Signalling Circuit Construction. Δ*luxPQ* in TOPO vector after mutagenesis (a) was BBK amplified into the psB1AC3 vector (b), acquiring BBK restriction sites. Similarly, *luxOU* was moved from TOPO (c) into the psB1AC3 vector (d). The B0015-R0040-*luxOU*-B0015 circuit in psB1AK3 was constructed from *luxOU* in psB1AC3 (e). This construct was then cloned downstream of Δ*luxPQ* (f), and the entire construct was then moved into the pCS26 vector (g), which will allow for the cloning of the library of synthetic σ^{70} promoters. (*LuxPQ = ΔluxPQ; LuxOU = luxOU;* TOPO = TOPO vector; AC = psB1AC3; AK = psB1AK3; TetR = Tetracycline repressible promoter (BBa_R0040); red octagon = terminator (BBa_B0015); $\Delta\sigma^{70} = \sigma^{70}$ promoter region).
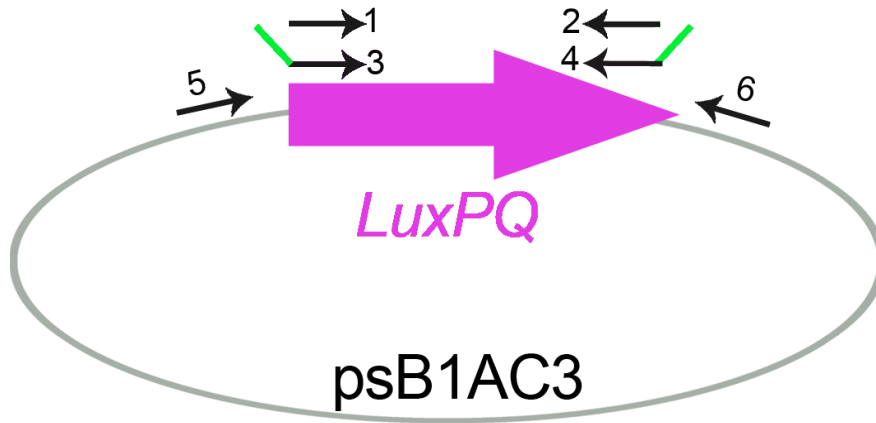
Figure 5. Schematic diagram of annealing regions for primers used with *luxPQ*. Primers are represented by black arrows: (1) LuxPQ-F, (2) LuxPQ-R, (3) LuxPQ-RS-F, (4) LuxPQ-RS-R, (5) BBK-CP-F, (6) BBK-CP-R. Green lines shown attached to primers 3 and 4 represent the BBK prefix and suffix. Primers 1-4 specifically anneal to *luxPQ*, whereas primers 5 and 6 anneal to the BBK vector backbone (psB1AC3 or psB1AK3).

(a)

(b)

(c)

(d)
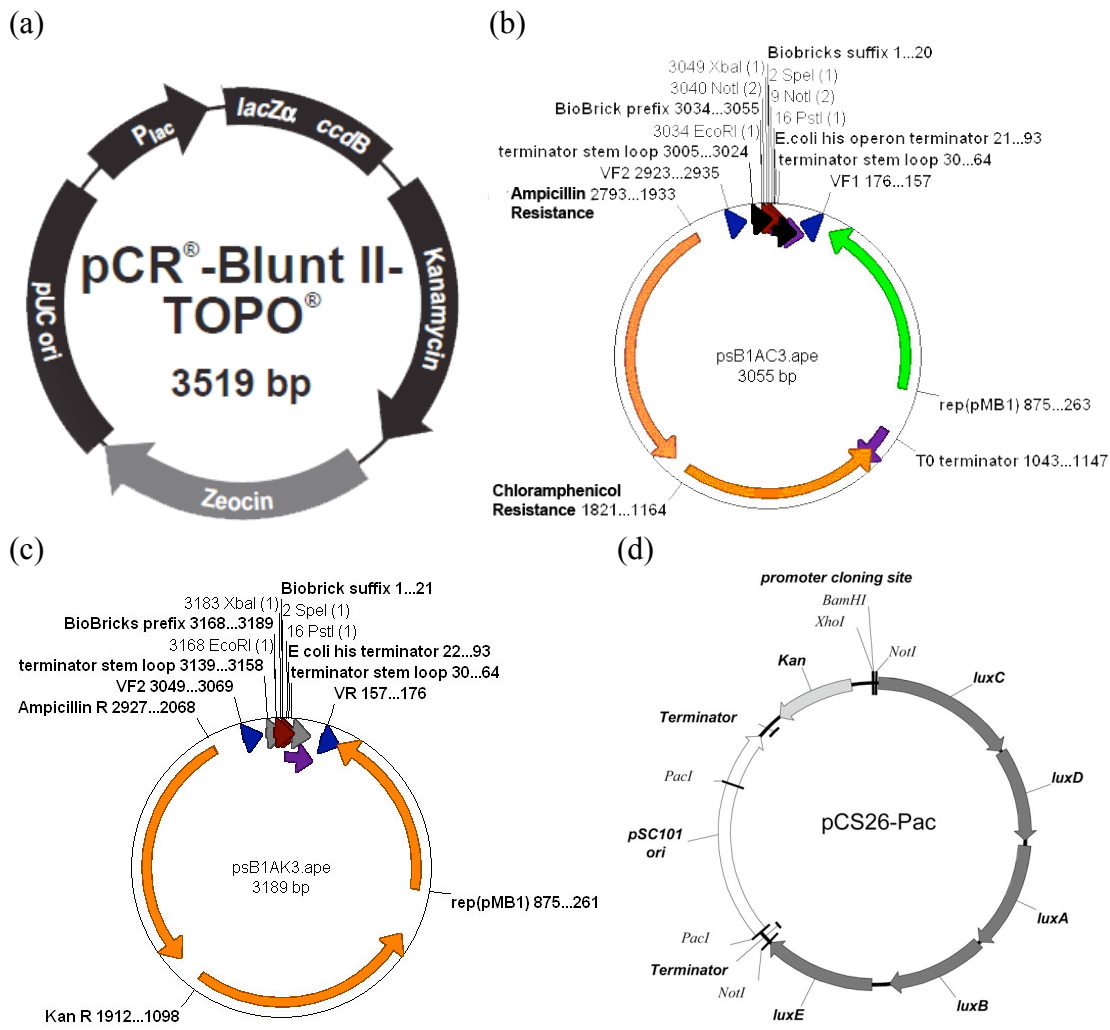
Figure 6. TOPO, BioBrick and pCS26 plasmids. (a) The multiple cloning site is located directly upstream of *lacZα* in the pCR-BLUNY II-TOPO vector (Invitrogen, CA). Genes coding for Zeocin and Kanamycin resistance are also present on this vector with the pUC origin of replication. (b) The salient features of psB1AC3 include ampicillin and chloramphenicol resistance, high copy number and the BBK prefix and suffix for housing genetic BBK parts. Although not depicted, Registry part BBa_P1010 (cell death gene) is initially present within the multiple cloning site to allow for selection after cloning a desired part into the vector. The origin of replication is pMB1 – high copy. (c) The

psB1AK3 (also with Registry insert BBa_P1010) vector is similar to psB1AC3, although it has a gene for kanamycin resistance in place of chloramphenicol. This plasmid (d) The pCS26 vector is low copy and its multiple cloning site is flanked by *Not*I restriction sites. It also contains the *Xho*I and *BamH*I sites for cloning of the $\Delta\sigma^{70}$ promoters, and has a gene for kanamycin resistance.

Plasmid maps adapted from invitrogen.com (a), iGEM Registry (b, c), Genomic Profiling of Iron-Responsive Genes in *Salmonella enterica* Serovar Typhimurium by High-Throughput Screening of a Random Promoter Library (Bjarnason *et al.*, 2003) (d).

(a)

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Query  30    ATGAAGAAAGCGTTACTATTTTCCCTGATTTCTATGGTCGGTTTTTCTCCAGCGTCTCAA  89
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1     ATGAAGAAAGCGTTACTATTTTCCCTGATTTCTATGGTCGGTTTTTCTCCAGCGTCTCAA  60

Query  90    GCAACACAAGTTTTGAATGGGTACTGGGGTTATCAAGAGTTTTTGGACGAGTTTCCCGAG  149
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  61    GCAACACAAGTTTTGAATGGGTACTGGGGTTATCAAGAGTTTTTGGACGAGTTTCCCGAG  120

Query  150   CAACGAAATCTGACCAATGCTTTATCAGAAGCAGTACGAGCACAGCCGGTCCCACTGTCT  209
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  121   CAACGAAATCTGACCAATGCTTTATCAGAAGCAGTACGAGCACAGCCGGTCCCACTGTCT  180

Query  210   AAACCGACACAACGCCCGATTAAAATATCAGTGGTTTACCCAGGACAGCAAGTTTCAGAT  269
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  181   AAACCGACACAACGCCCGATTAAAATATCAGTGGTTTACCCAGGACAGCAAGTTTCAGAT  240

Query  270   TACTGGGTAAGAAATATTGCATCATTCGAAAAACGTTTGTATAAGTTGAATATTAACTAC  329
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  241   TACTGGGTAAGAAATATTGCATCATTCGAAAAACGTTTGTATAAGTTGAATATTAACTAC  300

Query  330   CAACTGAACCAAGTGTTTACTCGTCCAAATGCTGATATCAAGCAACAAAGCTTGTCATTA  389
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  301   CAACTGAACCAAGTGTTTACTCGTCCAAATGCTGATATCAAGCAACAAAGCTTGTCATTA  360

Query  390   ATGGAAGCGCTCAAGAGCAAATCGGATTACTTGATTTTCACGCTTGATACGACAAGACAC  449
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  361   ATGGAAGCGCTCAAGAGCAAATCGGATTACTTGATTTTCACGCTTGATACGACAAGACAC  420

Query  450   CGTAAATTTGTTGAGCACGTTTTGGACTCAACGAACACCAAATTGATCTTGCAAAATATC  509
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  421   CGTAAATTTGTTGAGCACGTTTTGGACTCAACGAACACCAAATTGATCTTGCAAAATATC  480

Query  510   ACTACACCAGTCCGTGAGTGGGACAAACATCAACCGTTTTTATATGTCGGATTTGACCAC  569
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  481   ACTACACCAGTCCGTGAGTGGGACAAACATCAACCGTTTTTATATGTCGGATTTGACCAC  540
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<p align="center">LuxPQ-M1F →</p>

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Query  570   GCAGAAGGCAGTCGTGAATTAGCAACAGAGTTCGGAAAGTTCTTCCCAAAACACACATAT  629
             |||||||||||||||||||||||||||||| ||||||||||||||||||||||||||||||
Sbjct  541   GCAGAAGGCAGTCGTGAATTAGCAACAGAATTCGGAAAGTTCTTCCCAAAACACACATAT  600

Query  630   TACAGTGTGCTCTACTTTTCTGAAGGTTATATTAGCGATGTGAGAGGTGATACTTTTATT  689
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  601   TACAGTGTGCTCTACTTTTCTGAAGGTTATATTAGCGATGTGAGAGGTGATACTTTTATT  660

Query  690   CACCAAGTAAACCGTGATAATAACTTTGAGCTACAATCAGCGTATTACACGAAGGCAACC  749
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  661   CACCAAGTAAACCGTGATAATAACTTTGAGCTACAATCAGCGTATTACACGAAGGCAACC  720

Query  750   AAGCAATCCGGCTATGATGCTGCGAAAGCGAGTTTAGCAAAACATCCAGATGTTGATTTT  809
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  721   AAGCAATCCGGCTATGATGCTGCGAAAGCGAGTTTAGCAAAACATCCAGATGTTGATTTT  780

Query  810   ATCTATGCATGTTCGACCGACGTAGCATTAGGTGCAGTAGACGCACTGGCTGAGTTGGGA  869
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  781   ATCTATGCATGTTCGACCGACGTAGCATTAGGTGCAGTAGACGCACTGGCTGAGTTGGGA  840

Query  870   CGTGAAGATATTATGATCAATGGCTGGGGTGGAGGCTCTGCTGAGTTAGACGCTATCCAG  929
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  841   CGTGAAGATATTATGATCAATGGCTGGGGTGGAGGCTCTGCTGAGTTAGACGCTATCCAG  900

Query  930   AAGGGTGATTTAGACATCACCGTCATGCGTATGAATGATGACACTGGCATAGCCATGGCA  989
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  901   AAGGGTGATTTAGACATCACCGTCATGCGTATGAATGATGACACTGGCATAGCCATGGCA  960


Query  990   GAAGCGATTAAGTGGGACTTGGAAGATAAAACCAGTTCCGACCGTATACTCAGGTGACTTT  1049
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            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct   961 GAAGCGATTAAGTGGGACTTGGAAGATAAACCAGTTCCGACCGTATACTCAGGTGACTTT  1020

Query  1050 GAAATCGTAACAAAGGCAGATTCACCGGAGAGAATCGAAGCGCTGAAAAAGCGCGCATTT  1109
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1021 GAAATCGTAACAAAGGCAGATTCACCGGAGAGAATCGAAGCGCTGAAAAAGCGCGCATTT  1080

Query  1110 AGATATTCAGATAATTGATGACAACAACGCGATCAAACATTAAAAAGCGTCGCTCGCTGG  1169
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1081 AGATATTCAGATAATTGATGACAACAACGCGATCAAACATTAAAAAGCGTCGCTCGCTGG  1140

Query  1170 CGACGCTCATAACAAAGATCATCATTTTAGTTCTTGCCCCAATTATTCTGGGGATTTTCA  1229
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1141 CGACGCTCATAACAAAGATCATCATTTTAGTTCTTGCCCCAATTATTCTGGGGATTTTCA  1200

Query  1230 TTCAGAGCTATTACTTCTCCAAGCAAATCATTTGGCAAGAAGTAGACCGAACCAAACAGC  1289
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1201 TTCAGAGCTATTACTTCTCCAAGCAAATCATTTGGCAAGAAGTAGACCGAACCAAACAGC  1260

Query  1290 AAACCTCTGCACTGATCCACAATATATTTGATAGCCACTTTGCGGCGATCCAGATACATC  1349
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1261 AAACCTCTGCACTGATCCACAATATATTTGATAGCCACTTTGCGGCGATCCAGATACATC  1320

Query  1350 ATGACAGTAATTCCAAGAGCGAAGTCATTCGTGATTTCTACACTGATCGCGACACGGATG  1409
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1321 ATGACAGTAATTCCAAGAGCGAAGTCATTCGTGATTTCTACACTGATCGCGACACGGATG  1380
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                                                        LuxPQ-M2F
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Query  1410 TGCTCAACTTTTTCTTCCTCAGTATCGACCAAAGCGATCCGTCGCACACACCAGAGTTCC  1469
            |||||||||||||||||||||||||||||||||||||||||||||||||||||| |||
Sbjct  1381 TGCTCAACTTTTTCTTCCTCAGTATCGACCAAAGCGATCCGTCGCACACACCAGAATTCC  1440

Query  1470 GTTTTCTAACGGACCACAAAGGCATCATTTGGGACGATGGAAATGCGCATTTCTATGGTG  1529
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1441 GTTTTCTAACGGACCACAAAGGCATCATTTGGGACGATGGAAATGCGCATTTCTATGGTG  1500

Query  1530 TGAACGACCTTATCCTTGATAGCCTTGCCAATCGGGTCAGTTTCAGTAACAACTGGTATT  1589
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1501 TGAACGACCTTATCCTTGATAGCCTTGCCAATCGGGTCAGTTTCAGTAACAACTGGTATT  1560
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Query  1590 ACATTAATGTCATGACCTCCATTGGTTCGAGACACATGCTCGTGCGCCGTGTGCCGATCC  1649
            ||||||||||||||||||||||||||||| ||||||||||||||||||||||||||||||
Sbjct  1561 ACATTAATGTCATGACCTCCATTGGTTCTAGACACATGCTCGTGCGCCGTGTGCCGATCC  1620

Query  1650 TAGACCCTTCAACAGGAGAGGTGCTTGGTTTCTCATTTAATGCCGTCGTCTTAGACAACA  1709
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1621 TAGACCCTTCAACAGGAGAGGTGCTTGGTTTCTCATTTAATGCCGTCGTCTTAGACAACA  1680

Query  1710 ACTTCGCTTTGATGGAAAAGCTCAAGAGTGAAAGTAACGTCGACAATGTGGTGCTGGTTG  1769
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1681 ACTTCGCTTTGATGGAAAAGCTCAAGAGTGAAAGTAACGTCGACAATGTGGTGCTGGTTG  1740

Query  1770 CTAATAGCGTTCCTTTAGCAAACTCTTTGATTGGTGATGAGCCATATAACGTTGCTGATG  1829
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1741 CTAATAGCGTTCCTTTAGCAAACTCTTTGATTGGTGATGAGCCATATAACGTTGCTGATG  1800

Query  1830 TATTGCAGCGTAAAAGTTCAGACAAAAGACTCGATAAGCTGTTGGTAATAGAAACGCCAA  1889
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1801 TATTGCAGCGTAAAAGTTCAGACAAAAGACTCGATAAGCTGTTGGTAATAGAAACGCCAA  1860

Query  1890 TCGTCGTAAATGCAGTGACTACCGAGCTTTGCTTGTTGACGGTACAAGACAATCAGAGTG  1949
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1861 TCGTCGTAAATGCAGTGACTACCGAGCTTTGCTTGTTGACGGTACAAGACAATCAGAGTG  1920


Query  1950 TGGTGACATTACAAATCCAACATATTCTAGCCATGCTTGCATCGATCATCGGTATGATCA  2009
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              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1921   TGGTGACATTACAAATCCAACATATTCTAGCCATGCTTGCATCGATCATCGGTATGATCA   1980

Query  2010   TGATTGCCTTAATGAGTAGGGAATGGATTGAGAGTAAAGTTTCGGCGCAGTTAGAATCTT   2069
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1981   TGATTGCCTTAATGAGTAGGGAATGGATTGAGAGTAAAGTTTCGGCGCAGTTAGAATCTT   2040

Query  2070   TGATGTCTTACACCCGCTCTGCTCGTGAGGAAAAAGGGTTTGAACGATTTGGCGGTTCGG   2129
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  2041   TGATGTCTTACACCCGCTCTGCTCGTGAGGAAAAAGGGTTTGAACGATTTGGCGGTTCGG   2100

Query  2130   ATATTGAAGAGTTTGATCACATCGGTTCAACCCTTGAAAGTACATTCGAAGAGCTTGAAG   2189
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  2101   ATATTGAAGAGTTTGATCACATCGGTTCAACCCTTGAAAGTACATTCGAAGAGCTTGAAG   2160

Query  2190   CGCAGAAGAAGTCGTTCCGAGATCTGTTTAATTTTGCCTTATCACCCATCATGGTTTGGT   2249
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  2161   CGCAGAAGAAGTCGTTCCGAGATCTGTTTAATTTTGCCTTATCACCCATCATGGTTTGGT   2220

Query  2250   CTGAAGAGAGTGTCCTGATTCAGATGAACCCTGCCGCGCGCAAAGAATTAGTGATCGAAG   2309
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  2221   CTGAAGAGAGTGTCCTGATTCAGATGAACCCTGCCGCGCGCAAAGAATTAGTGATCGAAG   2280

Query  2310   ACGATCATGAAATCATGCATCCGGTCTTCCAAGGCTTTAAAGAGAAATTGACCCCACACC   2369
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  2281   ACGATCATGAAATCATGCATCCGGTCTTCCAAGGCTTTAAAGAGAAATTGACCCCACACC   2340

Query  2370   TCAAAATGGCGGCTCAAGGTGCGACGTTGACTGGGGTGAACGTGCCTATTGGTAATAAGA   2429
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  2341   TCAAAATGGCGGCTCAAGGTGCGACGTTGACTGGGGTGAACGTGCCTATTGGTAATAAGA   2400

Query  2430   TCTACCGATGGAACTTGTCGCCAATTCGTGTTGATGGCGATATCAGTGGCATTATTGTGC   2489
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  2401   TCTACCGATGGAACTTGTCGCCAATTCGTGTTGATGGCGATATCAGTGGCATTATTGTGC   2460

Query  2490   AAGGCCAAGACATTACAACACTTATCGAAGCCGAGAAGCAGAGTAACATTGCGCGTAGAG   2549
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  2461   AAGGCCAAGACATTACAACACTTATCGAAGCCGAGAAGCAGAGTAACATTGCGCGTAGAG   2520

Query  2550   AAGCAGAAAAATCGGCGCAAGCACGTGCAGACTTCCTTGCTAAAATGAGCCATGAAATTC   2609
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  2521   AAGCAGAAAAATCGGCGCAAGCACGTGCAGACTTCCTTGCTAAAATGAGCCATGAAATTC   2580

Query  2610   GTACGCCAATCAACGGCATTTTAGGTGTCGCCCAATTATTGAAAGATTCTGTCGATACAC   2669
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  2581   GTACGCCAATCAACGGCATTTTAGGTGTCGCCCAATTATTGAAAGATTCTGTCGATACAC   2640

Query  2670   AAGAGCAGAAGAATCAAATCGACGTCCTGTGCCACAGTGGCGAGCACTTGCTTGCAGTAC   2729
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  2641   AAGAGCAGAAGAATCAAATCGACGTCCTGTGCCACAGTGGCGAGCACTTGCTTGCAGTAC   2700

Query  2730   TGAACGATATTCTCGATTTCTCAAAGATAGAGCAGGGCAAGTTCAATATTCAGAAACACC   2789
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  2701   TGAACGATATTCTCGATTTCTCAAAGATAGAGCAGGGCAAGTTCAATATTCAGAAACACC   2760

Query  2790   CGTTCTCCTTCACCGATACCATGCGTACATTGGAAAATATTTATCGTCCGATTTGCACAA   2849
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  2761   CGTTCTCCTTCACCGATACCATGCGTACATTGGAAAATATTTATCGTCCGATTTGCACAA   2820

Query  2850   ATAAGGGGGTGGAGTTGGTCATCGAGAATGAGCTTGACCCGAATGTTGAAATCTTCACCG   2909
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  2821   ATAAGGGGGTGGAGTTGGTCATCGAGAATGAGCTTGACCCGAATGTTGAAATCTTCACCG   2880

Query  2910   ATCAAGTCCGCTTGAATCAGATTCTATTTAACTTAGTGAGTAATGCCGTTAAGTTCACGC   2969
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  2881   ATCAAGTCCGCTTGAATCAGATTCTATTTAACTTAGTGAGTAATGCCGTTAAGTTCACGC   2940

Query  2970   CGATTGGCTCGATTCGACTGCACGCAGAACTTGAACAATTCTATGGTGCGGAGAACAGCG   3029
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  2941   CGATTGGCTCGATTCGACTGCACGCAGAACTTGAACAATTCTATGGTGCGGAGAACAGCG   3000
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Query  3030   TGTTAGTTGTGGAACTGACTGATACTGGCATCGGCATTGAAAGCGATAAGCTCGACCAAA   3089
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  3001   TGTTAGTTGTGGAACTGACTGATACTGGCATCGGCATTGAAAGCGATAAGCTCGACCAAA   3060

Query  3090   TGTTCGAACCTTTTGTGCAAGAAGAGTCGACAACCACACGCGAATATGGCGGTAGCGGCC   3149
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  3061   TGTTCGAACCTTTTGTGCAAGAAGAGTCGACAACCACACGCGAATATGGCGGTAGCGGCC   3120

Query  3150   TAGGTTTGACCATCGTTAAGAACCTAGTCGATATGTTAGAAGGTGATGTTCAGGTCCGCA   3209
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  3121   TAGGTTTGACCATCGTTAAGAACCTAGTCGATATGTTAGAAGGTGATGTTCAGGTCCGCA   3180

Query  3210   GTAGCAAGGGGGGGGGGACAACATTTGTTATAACACTTCCAGTAAAAGATCGTGAGCGTG   3269
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  3181   GTAGCAAGGGGGGGGGGACAACATTTGTTATAACACTTCCAGTAAAAGATCGTGAGCGTG   3240

Query  3270   TCTTAAGGCCTCTGGAGGTCAGTCAACGTATCAAGCCGGAAGCCTTGTTTGATGAAAGTT   3329
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  3241   TCTTAAGGCCTCTGGAGGTCAGTCAACGTATCAAGCCGGAAGCCTTGTTTGATGAAAGTT   3300

Query  3330   TAAAAGTGCTACTGGTGGAAGATAACCATACCAATGCGTTTATCCTTCAGGCTTTCTGTA   3389
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  3301   TAAAAGTGCTACTGGTGGAAGATAACCATACCAATGCGTTTATCCTTCAGGCTTTCTGTA   3360

Query  3390   AGAAGTATAAAATGCAGGTGGATTGGGCGAAAGATGGGCTGGACGCGATGGAGCTCCTTT   3449
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  3361   AGAAGTATAAAATGCAGGTGGATTGGGCGAAAGATGGGCTGGACGCGATGGAGCTCCTTT   3420

Query  3450   CTGATACCACCTACGATCTGATCCTCATGGATAACCAATTACCCCACCTTGGTGGTATTG   3509
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  3421   CTGATACCACCTACGATCTGATCCTCATGGATAACCAATTACCCCACCTTGGTGGTATTG   3480

Query  3510   AGACCACGCACGAGATTCGCCAGAACTTGAGGCTTGGAACGCCAATTTACGCGTGTACAG   3569
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  3481   AGACCACGCACGAGATTCGCCAGAACTTGAGGCTTGGAACGCCAATTTACGCGTGTACAG   3540

Query  3570   CAGACACCGCGAAAGAAACCAGTGATGCGTTTATGGCGGCAGGTGCAAACTATGTCATGC   3629
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  3541   CAGACACCGCGAAAGAAACCAGTGATGCGTTTATGGCGGCAGGTGCAAACTATGTCATGC   3600

Query  3630   TGAAGCCAATTAAAGAGAATGCGTTACATGAGGCGTTTGTCGATTTCAAACAACGTTTCT   3689
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  3601   TGAAGCCAATTAAAGAGAATGCGTTACATGAGGCGTTTGTCGATTTCAAACAACGTTTCT   3660

Query  3690   TGGTAGAAAGAACCTAACGGTTTAATGGCAGTGATAAGTTAGGGGCTGAAGTTAAATAAT   3749
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  3661   TGGTAGAAAGAACCTAACGGTTTAATGGCAGTGATAAGTTAGGGGCTGAAGTTAAATAAT   3720

Query  3750   AAAAGTAAAGAAGGGAGCGTAATGCACTAATGCCGTTCACTTAAGGTGATCGGCATTGTT   3809
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  3721   AAAAGTAAAGAAGGGAGCGTAATGCACTAATGCCGTTCACTTAAGGTGATCGGCATTGTT   3780

Query  3810   TTTTCTAGGGTATCGG   3825
              ||||||||||||||||
Sbjct  3781   TTTTCTAGGGTATCGG   3796
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Query  1127   MTTTRSNIKKRRSLATLITKIIILVLAPIILGIFIQSYYFSKQIIWQEVDRTKQQTSALI   1306
              MTTTRSNIKKRRSLATLITKIIILVLAPIILGIFIQSYYFSKQIIWQEVDRTKQQTSALI
Sbjct  1      MTTTRSNIKKRRSLATLITKIIILVLAPIILGIFIQSYYFSKQIIWQEVDRTKQQTSALI   60

Query  1307   HNIFDSHFAAIQIHHDSNSKSEVIRDFYTDRDTDVLNFFFLSIDQSDPSHTPEFRFLTDH   1486
              HNIFDSHFAAIQIHHDSNSKSEVIRDFYTDRDTDVLNFFFLSIDQSDPSHTPEFRFLTDH
Sbjct  61     HNIFDSHFAAIQIHHDSNSKSEVIRDFYTDRDTDVLNFFFLSIDQSDPSHTPEFRFLTDH   120

Query  1487   KGIIWDDGNAHFYGVNDLILDSLANRVSFSNNWYYINVMTSIGSRHMLVRRVPILDPSTG   1666
              KGIIWDDGNAHFYGVNDLILDSLANRVSFSNNWYYINVMTSIGSRHMLVRRVPILDPSTG
Sbjct  121    KGIIWDDGNAHFYGVNDLILDSLANRVSFSNNWYYINVMTSIGSRHMLVRRVPILDPSTG   180
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Query  1667   EVLGFSFNAVVLDNNFALMEKLKSESNVDNVVLVANSVPLANSLIGDEPYNVADVLQRKS   1846
              EVLGFSFNAVVLDNNFALMEKLKSESNVDNVVLVANSVPLANSLIGDEPYNVADVLQRKS
Sbjct  181    EVLGFSFNAVVLDNNFALMEKLKSESNVDNVVLVANSVPLANSLIGDEPYNVADVLQRKS   240

Query  1847   SDKRLDKLLVIETPIVVNAVTTELCLLTVQDNQSVVTLQIQHILAMLASIIGMIMIALMS   2026
              SDKRLDKLLVIETPIVVNAVTTELCLLTVQDNQSVVTLQIQHILAMLASIIGMIMIALMS
Sbjct  241    SDKRLDKLLVIETPIVVNAVTTELCLLTVQDNQSVVTLQIQHILAMLASIIGMIMIALMS   300

Query  2027   REWIESKVSAQLESLMSYTRSAREEKGFERFGGSDIEEFDHIGSTLESTFEELEAQKKSF   2206
              REWIESKVSAQLESLMSYTRSAREEKGFERFGGSDIEEFDHIGSTLESTFEELEAQKKSF
Sbjct  301    REWIESKVSAQLESLMSYTRSAREEKGFERFGGSDIEEFDHIGSTLESTFEELEAQKKSF   360

Query  2207   RDLFNFALSPIMVWSEESVLIQMNPAARKELVIEDDHEIMHPVFQGFKEKLTPHLKMAAQ   2386
              RDLFNFALSPIMVWSEESVLIQMNPAARKELVIEDDHEIMHPVFQGFKEKLTPHLKMAAQ
Sbjct  361    RDLFNFALSPIMVWSEESVLIQMNPAARKELVIEDDHEIMHPVFQGFKEKLTPHLKMAAQ   420

Query  2387   GATLTGVNVPIGNKIYRWNLSPIRVDGDISGIIVQGQDITTLIEAEKQSNIARREAEKSA   2566
              GATLTGVNVPIGNKIYRWNLSPIRVDGDISGIIVQGQDITTLIEAEKQSNIARREAEKSA
Sbjct  421    GATLTGVNVPIGNKIYRWNLSPIRVDGDISGIIVQGQDITTLIEAEKQSNIARREAEKSA   480

Query  2567   QARADFLAKMSHEIRTPINGILGVAQLLKDSVDTQEQKNQIDVLCHSGEHLLAVLNDILD   2746
              QARADFLAKMSHEIRTPINGILGVAQLLKDSVDTQEQKNQIDVLCHSGEHLLAVLNDILD
Sbjct  481    QARADFLAKMSHEIRTPINGILGVAQLLKDSVDTQEQKNQIDVLCHSGEHLLAVLNDILD   540

Query  2747   FSKIEQGKFNIQKHPFSFTDTMRTLENIYRPICTNKGVELVIENELDPNVEIFTDQVRLN   2926
              FSKIEQGKFNIQKHPFSFTDTMRTLENIYRPICTNKGVELVIENELDPNVEIFTDQVRLN
Sbjct  541    FSKIEQGKFNIQKHPFSFTDTMRTLENIYRPICTNKGVELVIENELDPNVEIFTDQVRLN   600

Query  2927   QILFNLVSNAVKFTPIGSIRLHAELEQFYGAENSVLVVELTDTGIGIESDKLDQMFEPFV   3106
              QILFNLVSNAVKFTPIGSIRLHAELEQFYGAENSVLVVELTDTGIGIESDKLDQMFEPFV
Sbjct  601    QILFNLVSNAVKFTPIGSIRLHAELEQFYGAENSVLVVELTDTGIGIESDKLDQMFEPFV   660

Query  3107   QEESTTTREYGGSGLGLTIVKNLVDMLEGDVQVRSSKGGGTTFVITLPVKDRERVLRPLE   3286
              QEESTTTREYGGSGLGLTIVKNLVDMLEGDVQVRSSKGGGTTFVITLPVKDRERVLRPLE
Sbjct  661    QEESTTTREYGGSGLGLTIVKNLVDMLEGDVQVRSSKGGGTTFVITLPVKDRERVLRPLE   720

Query  3287   VSQRIKPEALFDESLKVLLVEDNHTNAFILQAFCKKYKMQVDWAKDGLDAMELLSDTTYD   3466
              VSQRIKPEALFDESLKVLLVEDNHTNAFILQAFCKKYKMQVDWAKDGLDAMELLSDTTYD
Sbjct  721    VSQRIKPEALFDESLKVLLVEDNHTNAFILQAFCKKYKMQVDWAKDGLDAMELLSDTTYD   780

Query  3467   LILMDNQLPHLGGIETTHEIRQNLRLGTPIYACTADTAKETSDAFMAAGANYVMLKPIKE   3646
              LILMDNQLPHLGGIETTHEIRQNLRLGTPIYACTADTAKETSDAFMAAGANYVMLKPIKE
Sbjct  781    LILMDNQLPHLGGIETTHEIRQNLRLGTPIYACTADTAKETSDAFMAAGANYVMLKPIKE   840

Query  3647   NALHEAFVDFKQRFLVERT   3703
              NALHEAFVDFKQRFLVERT
Sbjct  841    NALHEAFVDFKQRFLVERT   859
```

**Figure 7. Nucleotide and amino acid sequence alignment of *luxPQ and ΔluxPQ.*** (a) Nucleotide alignment of Δ*luxPQ* (Query) and *luxPQ* (Subject). The coding sequence for Δ*luxP* on the query sequence is from nucleotide 30 to 1127, whereas nucleotides 1126 to 3706 code for Δ*luxQ*. Three silent mutations were introduced to *luxPQ* [Vibrio harveyi ATCC BAA-1116] by QuikChange XL Site-Directed Mutagenesis in order to remove BBK sites, and thus generate Δ*luxPQ*. This was then sequenced and aligned (BLAST) with *luxPQ* to verify the removal of these sites. Nucleotides 599 (Δ*luxP*) and 1465

(Δ*luxQ*) were mutated from 'A' to 'G' resulting in the loss of the *EcoR*I site, and nucleotide 1618 (Δ*luxQ*) was mutated from 'T' to 'G' to remove the *Xba*I restriction site. The restriction sites are highlighted in green on the *luxPQ* sequence (Subject) whereas the mutated sites are highlighted in yellow on the Δ*luxPQ* sequence (Query). Mutagenic primers (black arrows) are also depicted. Alignment was 99% (3793/3796) with no gaps.

(b) Amino acid alignment of *luxPQ* (Subject) and Δ*luxPQ* (Query). The sequences align 100%, with no gaps, revealing that the mutations present in Δ*luxPQ* were silent and thus did not affect the amino acid sequence.
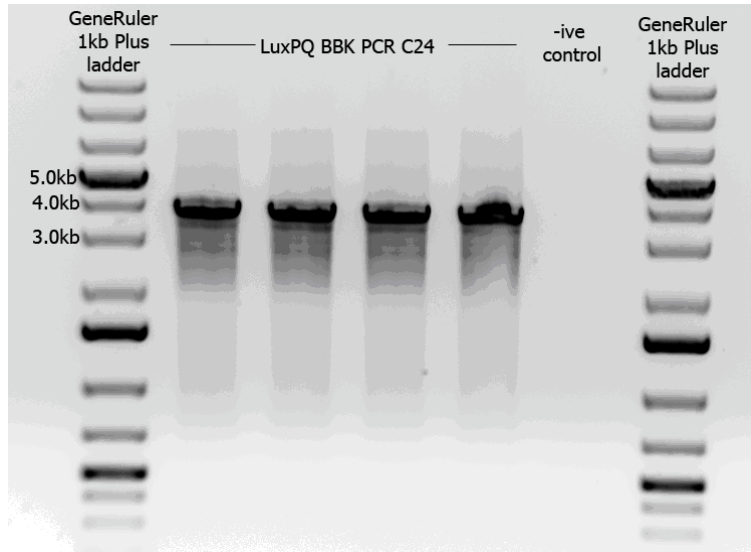
Figure 8. BBK PCR amplification of Δ*luxPQ* from pCR-BLUNT-II-TOPO with *LuxPQ*-RS-F and *LuxPQ*-RS-R primers run on a 0.8% agarose gel (90V). GeneRuler 1kb DNA Ladder Plus (Fermentas, ON) was loaded into wells 1 and 7, PCR products with the same Δ*luxPQ* template in TOPO was loaded into wells 2-5 and the negative control (water) in well 6. BBK PCR amplification of Δ*luxPQ* reveals expected band sizes of just under 4.0kb.
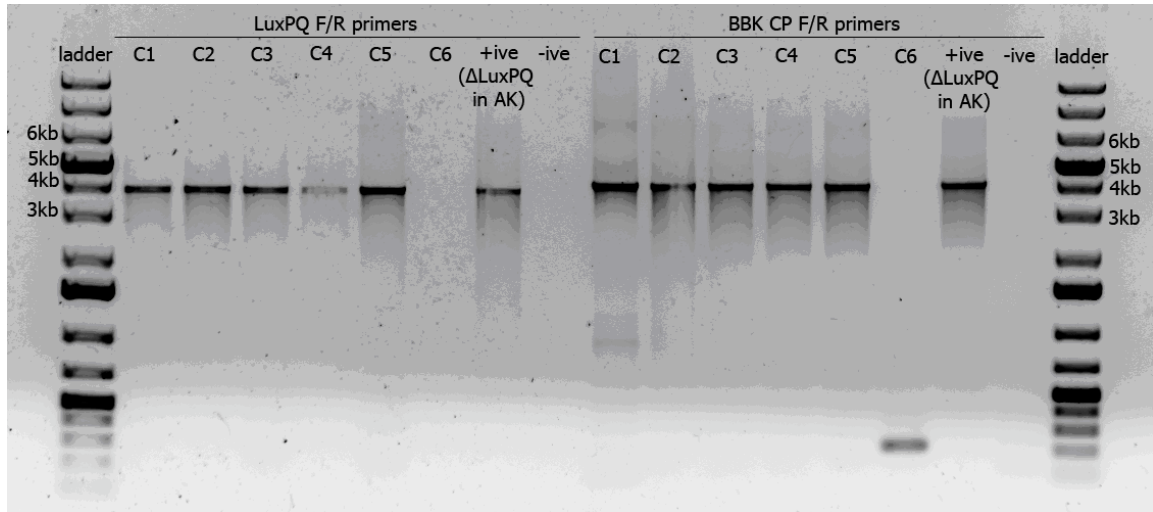
Figure 9. Colony PCR to verify Δ*luxPQ* plasmid switch from psB1AK3 to psB1AC3 using LuxPQ-F/R and BBK-CP-F/R primers on a 0.8% agarose gel (90V). 5μL of GeneRuler 1kb DNA Ladder Plus (Fermentas, ON) were loaded into wells 1 and 18. Six colonies were screened with both sets of primers. Δ*luxPQ* in psB1AK3 was used as the positive control. This gel confirms the presence of Δ*luxPQ* in psB1AC3 for colonies 1, 2, 3, 4 and 5 because of the desired band size of 3.9-4.0kb.
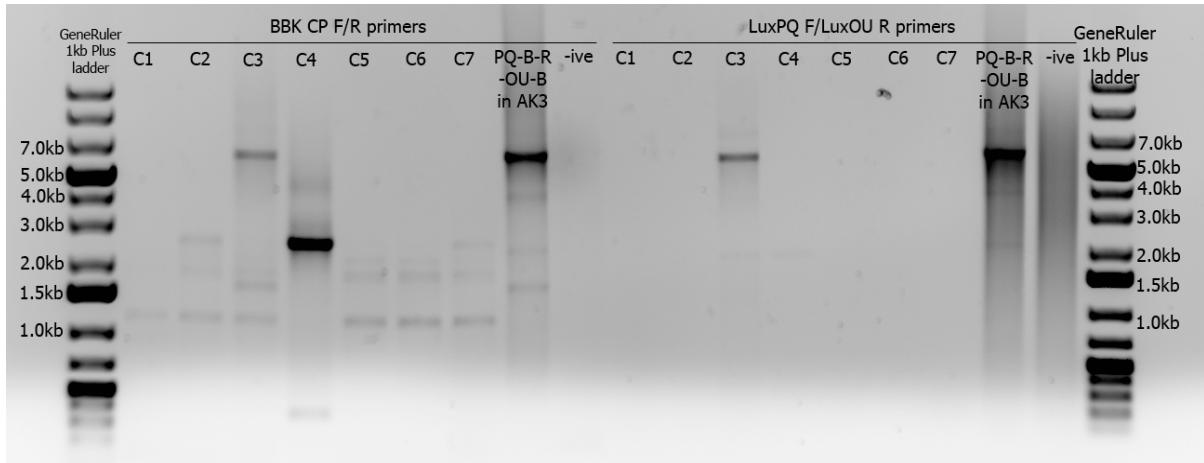
**Figure 10.** Colony PCR to verify presence of signalling circuit (Δ*luxPQ*-B0015-R0040-*LuxOU*-B0015) in psB1AC3 using BBK-CP-F/R and LuxPQ-F/LuxOU-R primers on a 0.8% agarose gel (90V). Seven colonies were screened with two set of primers (lanes 2-8, 11-17) and the signalling circuit in psB1AK3 was used as a positive control. Colony 3 was the only colony with the desired band size of around 6.1kb.

(a)
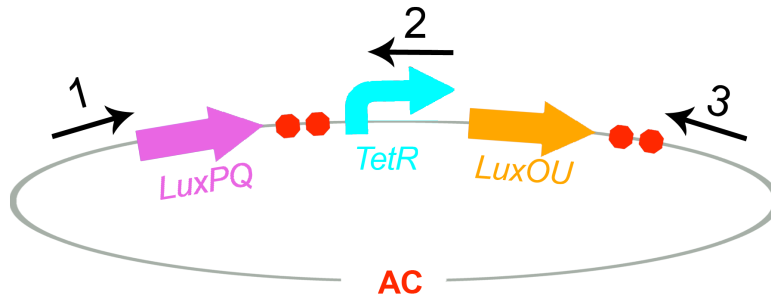
(b) Sequence from primer 1 (BBK-CP-F)

```
AATAGGCGTATCACGAGGCAGAATTTCAGATAAAAAAAATCCTTAGCTTTCGCTAAGGATGATTTCTGGAATTCGCGGC
CGCTTCTAGAATGCTCGATAAAAACTAAAAGAGCAATAGATGAAGAAAGCGTTACTATTTTCCCTGATTTCTATGGTCG
GTTTTTCTCCAGCGTCTCAAGCAACACAAGTTTTGAATGGGTACTGGGGTTATCAAGAGTTTTTGGACGAGTTTCCCGA
GCAACGAAATCTGACCAATGCTTTATCAGAAGCAGTACGAGCACAGCCGGTCCCACTGTCTAAACCGACACAACGCCCG
ATTAAAATATCAGTGGTTTACCCAGGACAGCAAGTTTCAGATTACTGGGTAAGAAATATTGCATCATTCGAAAAACGTT
TGTATAAGTTGAATATTAACTACCAACTGAACCAAGTGTTTACTCGTCCAAATGCTGATATCAAGCAACAAAGCTTGTC
ATTAATGGAAGCGCTCAAGAGCAAATCGGATTACTTGATTTTCACGCTTGATACGACAAGACACCGTAAATTTGTTGAG
CACGTTTTGGACTCAACGAACACCAAATTGATCTTGCAAAATATCACTACACCAGTCCGTGAGTGGGACAAACATCAAC
CGTTTTTATATGTCGGATTTGACCACGCAGAAGGCAGTCGTGAATTAGCAACAGAGTTCGGAAAGTTCTTCCCAAAACA
CACATATTACAGTGTGCTCTACTTTTCTGAAGGTTATATTAGCGATGTGAGAGGTGATACTTTTATTCACCAAGTAAAC
CGTGATAATAACTTTGAGCTACAATCAGCGTATTACACGAAGGCAACCAAGCAATCCGGCTATGATGCTGCGAAAGCGA
GTTTAGCAAACATCCAGATGTTGATTTTATCTATGCATGTTCGACCGACGTAGCATTAGGTGCAGTAGACGCACTGGCT
GAGTTGG
```

(c) Sequence from primer 2 (R0040-R)

```
CTATCCTGATAGGGACTCTAGTATATAAACGCAGAAAGGCCCACCCGAAGGTGAGCCAGTGTGACTCTAGTAGAGAGCG
TTCACCGACAAACAACAGATAAAACGAAAGGCCCAGTCTTTCGACTGAGCCTTTCGTTTTATTTGATGCCTGGCTCTAG
TCCGATACCCTAGAAAAAACAATGCCGATCACCTTAAGTGAACGGCATTAGTGCATTACGCTCCCTTCTTTACTTTTAT
TATTTAACTTCAGCCCCTAACTTATCACTGCCATTAAACCGTTAGGTTCTTTCTACCAAGAAACGTTGTTTGAAATCGA
CAAACGCCTCATGTAACGCATTCTCTTTAATTGGCTTCAGCATGACATAGTTTGCACCTGCCGCCATAAACGCATCACT
GGTTTCTTTCGCGGTGTCTGCTGTACACGCGTAAATTGGCGTTCCAAGCCTCAAGTTCTGGCGAATCTCGTGCGTGGTC
TCAATACCACCAAGGTGGGGTAATTGGTTATCCATGAGGATCAGATCGTAGGTGGTATCAGAAAGGAGCTCCATCGCGT
CCAGCCCATCTTTCGCCCAATCCACCTGCATTTTATACTTCTTACAGAAAGCCTGAAGGATAAACGCATTGGTATGGTT
ATCTTCCACCAGTAGCACTTTTAAACTTTCATCAAACAAGGCTTCCGGCTTGATACGTTGACTGACCTCCAGAGGCCTT
AAGACACGCTCACGATCTTTTACTGGAAGTGTTATAACAAATGTTGTCCCCCCCCCCTTGCTACTGCGGACCTGAACAT
CACCTTCTAACATATCGACTAGGTTCTTAACGATGGTCAAACCTAGGCCGCTACCGCCATATTCGCGTGTGGTTGTCGA
CTCTTCTTGCACAAAAGGTTCGAACATTTGGTCGAGCTTATCGCTTTCAATGCCGATGCCAGTATCAGTCAGTTCCACA
ACTAACACGCTGTTCTCCGCACCATAGAATTGTTCAAGTTCTGCGTGCAG
```

(d) Sequence from primer 3 (BBK-CP-R)

```
CCTTGCCCTTTTTTGCCGGACTGCAGCGGCCGCTACTAGTATATAAACGCAGAAAGGCCCACCCGAAGGTGAGCCAGTG
TGACTCTAGTAGAGAGCGTTCACCGACAAACAACAGATAAAACGAAAGGCCCAGTCTTTCGACTGAGCCTTTCGTTTTA
TTTGATGCCTGGCTCTAGTCCCATTTCAAATCTCCTCATGGTTGGAGACTTGATCTTATTCTTCGCGCTAGGCGATTAG
AATGCGGGTTTTATGGAGCGCTTACTGTCTAACTAGACGAAAAGCATTGAGAAAGCGGCTTCGATTCGGAGCAAACTCC
CCAAACAAAAAAGCCCTTCCACACCGGAAGGGCTTTGTGTTTTTACGTTGCGCTTAATGTCTTGCTCGAAACGTTAGTT
TGTCCAAGAACGGTAGGCGTCACGAGTGATATGAAGTAAAGCGAGCATTTCGCTCGTCTCCATCCCCTGCTCTTGCAAT
TGATTCGCTTTTGCTTTCTTGTCGATGGCAATCGCTCGTTCACACAATCGATCTGCGCCAAAGCTGGCAGCACTACTTT
TCAGTGCGTGGCTGATCTCTTTTAAATACAACAGCTGCTCTGAGCCCTGAAGTTCAGTTAAAGTGCCAATGTAGGAGTC
CATTTCCCCAAGAAAAATATCAAGCAAAACAGGAACCATTATCGCTACCAATTTCCGCAGACAGTTCTTCAATTTTTTGC
TGATTTAATACGTCCGTATTCATACGTTTTGTTTTTTCGTCCTTGCTATTCCAAGCTTGCAACTTGCGATAAATCGTTGA
TGGACTAACATCCAAATAGCCAGCAGCGCGTGGAATGTTG
```

Figure 11. **Schematic of annealing regions for s****equencing primers and DNA sequences for signalling circuit in psB1AC3.** (a) This schematic diagram depicts the annealing regions of the primers (black arrows) used for sequencing of the signalling

circuit in psB1AC3, shown as 'AC'. Black arrows numbered 1, 2 and 3 represent primers BBK-CP-F, R0040-R and BBK-CP-R respectively. (b) Sequencing results from primer 1 (BBK-CP-F). Colour scheme: grey – BBK vector backbone, green – BBK prefix restriction sites, pink – Δ*luxPQ*. (c) Sequencing results from primer 2 (R0040-R). Colour scheme: cyan – R0040 promoter, red – B0015 terminator, pink – Δ*luxPQ*. The pink Δ*luxPQ* sequence shown in (b) and (c) corresponds to the Δ*luxPQ* that was previously sequenced and aligned with *luxPQ* (Figure 7a). (d) Sequencing results from primer 3 (BBK-CP-R). Colour scheme: grey – BBK vector backbone, green – BBK suffix restriction sites, red – B0015 terminator, orange – *luxOU*.
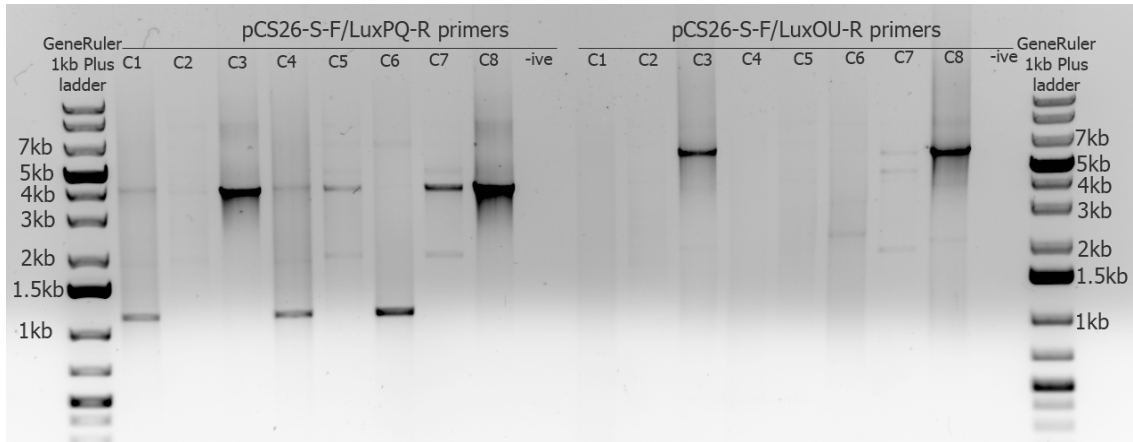
Figure 12. Plasmid PCR to verify presence of signalling circuit in pCS26 by using the pCS26-S-F primer with LuxPQ-R and LuxOU-R primers. Seven colonies were screened with the two sets of primers, with no positive control. The first set of primers verifies the presence of Δ*luxPQ*, whereas the second primer set verifies both (1) the presence of *luxOU* and (2) if a construct of the size of the signalling circuit is present. Colonies 3 and 8 revealed expected sizes for each pair of primers: 4.0kb and ~6.1kb.

(a)

(b) Sequence from Primer 1 (pCS26-S-F)

CTATAAAAATAGGCGTATCACGAGGCCCTTTCGTCTTCACCTCGAGGGGATCCTCTAGTT**GCGGCCGCT**TCTAGAATGC
TCGATAAAAACTAAAAGAGCAATAGATGAAGAAAGCGTTACTATTTTCCCTGATTTCTATGGTCGGTTTTTCTCCAGCG
TCTCAAGCAACACAAGTTTTGAATGGGTACTGGGGTTATCAAGAGTTTTTGGACGAGTTTCCCGAGCAACGAAATCTGA
CCAATGCTTTATCAGAAGCAGTACGAGCACAGCCGGTCCCACTGTCTAAACCGACACAACGCCCGATTAAAATATCAGT
GGTTTACCCAGGACAGCAAGTTTCAGATTACTGGGTAAGAAATATTGCATCATTCGAAAAACGTTTGTATAAGTTGAAT
ATTAACTACCAACTGAACCAAGTGTTTACTCGTCCAAATGCTGATATCAAGCAACAAAGCTTGTCATTAATGGAAGCGC
TCAAGAGCAAATCGGATTACTTGATTTTCACGCTTGATACGACAAGACACCGTAAATTTGTTGAGCACGTTTTGGACTC
AACGAACACCAAATTGATCTTGCAAAATATCACTACACCAGTCCGTGAGTGGGACAAACATCAACCGTTTTTATATGTC
GGATTTGACCACGCAGAAGGCAGTCGTGAATTAGCAACAGAGTTCGGAAAGTTCTTCCCAAAACACACATATTACAGTG
TGCTCTACTTTTCTGAAGGTTATATTAGCGATGTGAGAGGTGATACTTTTATTCACCAAGTAAACCGTGATAATAACTT
TGAGCTACAATCAGCGTATTACACGAAGGCAACCAAGCAATCCGGCTATGATGCTGCGAAAGCGAGTTTAGCAAAACAT
CCAGATGTTGATTTTATCTATGCATGTTCGACCGACGTAGCATTAGGTGCAGTAGACGCACTGGCTGAGTTGGGACGTG
AAGATATTATGATCATGGCTGGGGTGGA

(c) Sequence from Primer 2 (LuxOU-R)

TTCTTCGCGCTAGGCGATTAGAATGCGGGTTTTATGGAGCGCTTACTGTCTAACTAGACGAAAAGCATTGAGAAAGCGG
CTTCGATTCGGAGCAAACTCCCCAAACAAAAAAGCCCTTCCACACCGGAAGGGCTTTGTGTTTTTACTTGCGCTTAATG
TCTTGCTCGAAACGTTAGTTTGTCCAAGAACGGTAGGCGTCACGAGTGATATGAAGTAAAGCGAGCATTTCGCTCGTCT
CCATCCCCTGCTCTTGCAATTGATTCGCTTTTGCTTTCTTGTCGATGGCAATCGCTCGTTCACACAATCGATCTGCGCC
AAAGCTGGCAGCACTACTTTTCAGTGCGTGGCTGATCTCTTTTAAATACAACAGCTGCTCTGAGCCCTGAAGTTCAGTT
AAAGTGCCAATGTAGGAGTCCATTTCCCCAAGAAAAATATCAAGCAAAACAGGAACATTATCGCTACCAATTTCCGCAG
ACAGTTCTTCAATTTTTTGCTGATTTAATACGTCCGTATTCATACGTTTTGTTTTTCGTCCTTGCTATTCCAAGCTTGC
AACTTGCGATAAATCGTTGATGGACTAACATCCAAATAGCCAGCAGCGCGTGGAATGTTGCCTTCACACGCTTGAATTG
CCTGCTCAATAGCCATTTTCTCTGTCATCCAAAGCGGCATAATATCTGACACCGTCATAATGTCAGGTTCAATGAATTT
TGCTACCGATTGGCGCACAACAGGCTGATTCAGTGGTGGCGGTAACATATCCAGCGTGATCTCTTTGCCATTGTTCAGT
ACCACGATATTACGCAATACGTTTTGCAACTGGCGAACGTTACCCGGCCATTCGTAGCTGTTGAATCTTTCAATCACGT
CTTGTGCGAAACGGACGAAACTCTTACCTTCCTCATGAGACATATAACCAAGCAACGAGTATGCAATTTCAATAACGTC
TTTACCACGCTCACGCAGCGGCGGAAG

Figure 13. **Sequencing primers and DNA sequences for signalling circuit in pCS26.**

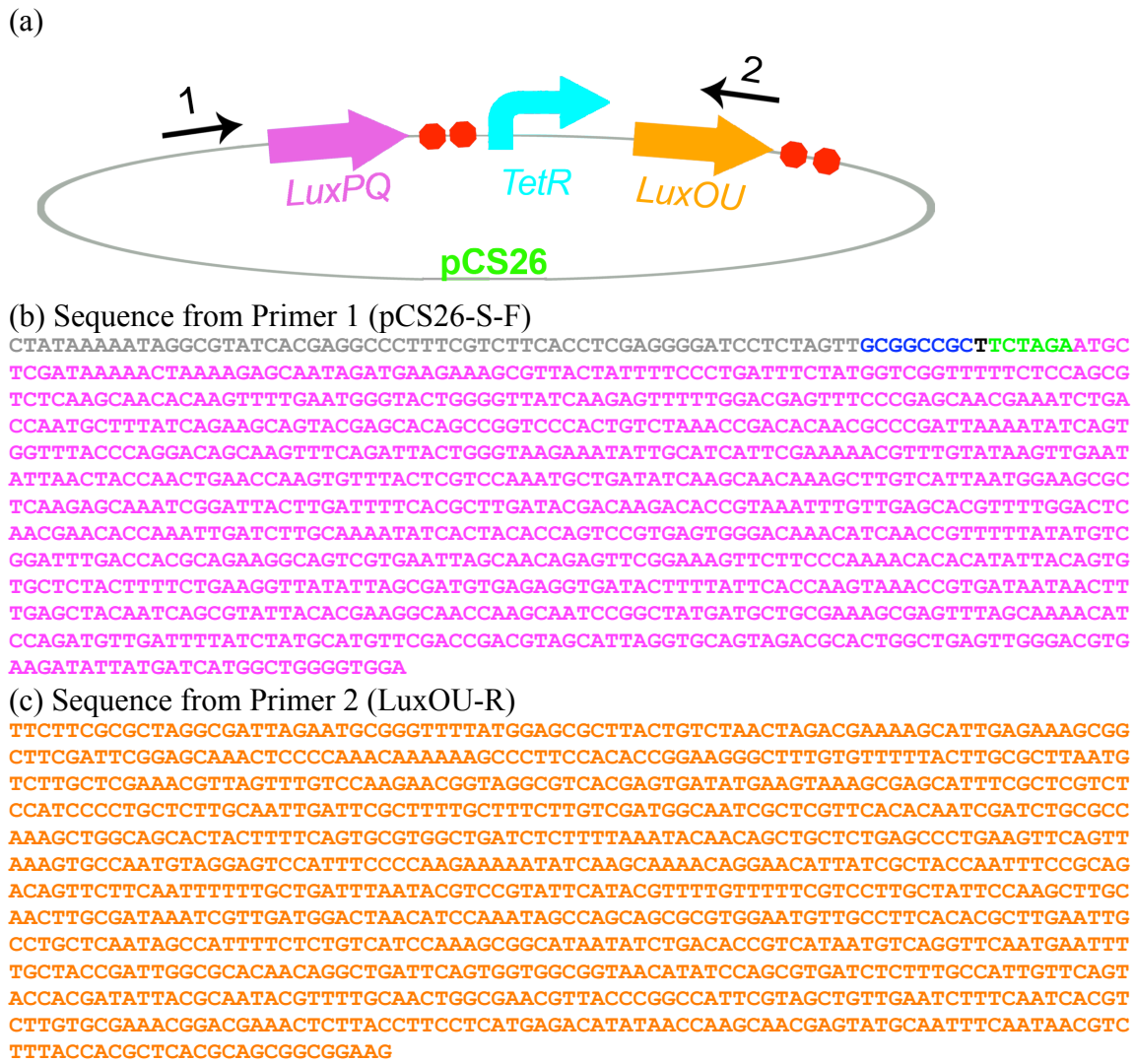(a) This schematic diagram depicts the annealing regions of the primers (black arrows) used for sequencing of the signalling circuit. Arrows numbered 1 and 2 represent pCS26-S-F and LuxOU-R respectively. (b) Sequencing results from primer 1 (pCS26-S-F). Colour scheme: grey – pCS26 vector backbone, blue – *Not*I restriction site, bright green – *Xba*I restriction site, pink – *luxP*. (c) Sequencing results from primer 2 (LuxOU-R). Colour scheme: orange – *luxOU*.
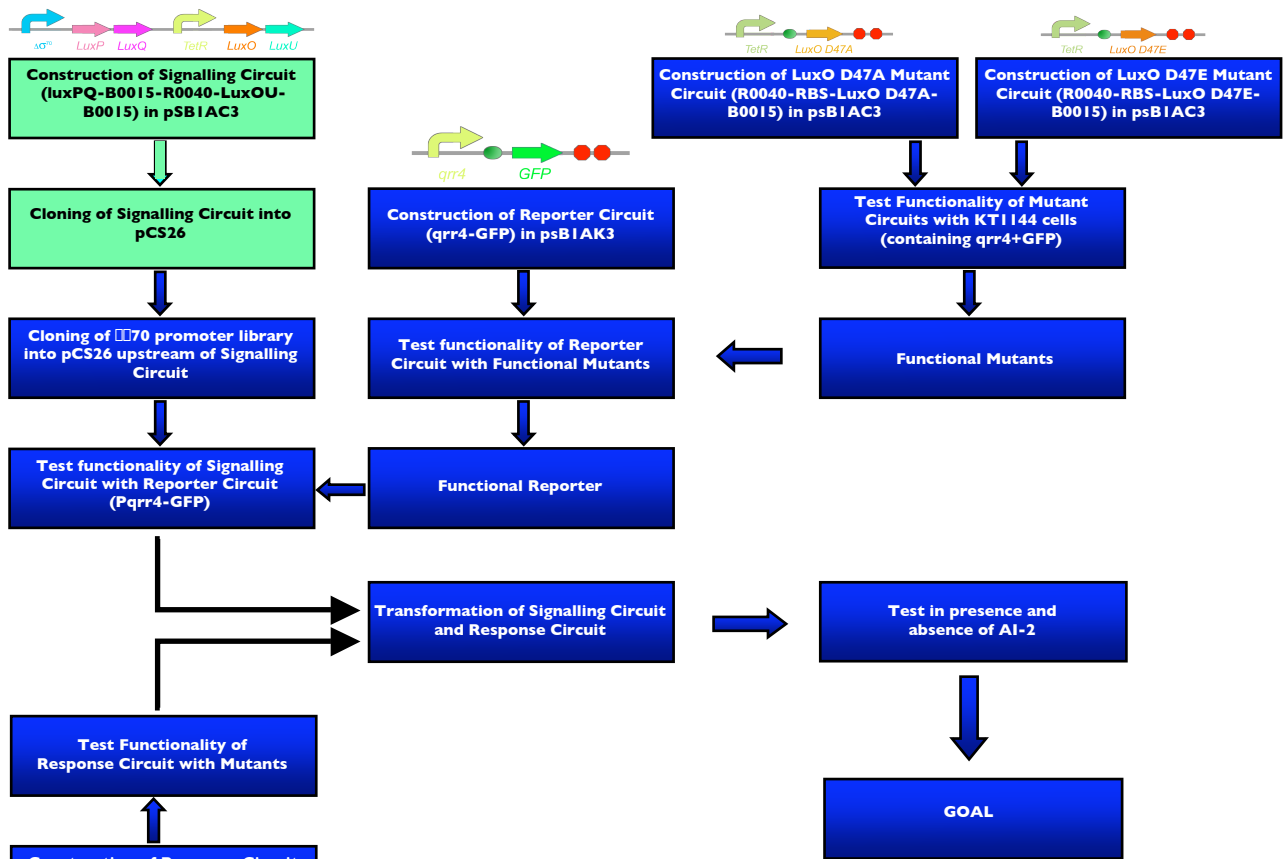
Figure 14. Flow chart of construction and use of mutant, reporter, response and signalling circuits. Each circuit is schematically depicted above or below its description in the blue/green boxes. The mutant circuits will be tested with KT1144 cells and then used to test the reporter and response circuits. The reporter will then be used to test the signalling circuit. The signalling and response circuits will then be coupled to reach the end goal of the AI-2 signalling system in *E. coli.* Boxes in light green depict was has been described in this paper. Accomplished to date include construction of mutant and reporter circuits and partial construction of the response circuit. The mutants are currently being tested.

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