Supplementary Material For:

Overlap extension PCR cloning: a simple and reliable way to create recombinant plasmids

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Standard molecular techniques

All primers used in the study (Supplementary Table S3) were purchased from IDT (Coralville, IA, USA). DNA for PCR amplification of *luxA*, *luxB*, *luxC*, *luxD*, and *luxE* genes was from *Photorhabdus luminescens* (ATCC number 29999; Manassas, VA, USA) . *Escherichia coli* K12 DNA for PCR amplification of the *lacZ* and *gusA* genes was purified by standard methods (1). pCA24N (2) vector was used as a DNA template to PCR-amplify the *gfp* gene. DNA fragments and PCR mixtures were analyzed on 0.8% Seakem LE agarose gels (Lonza Rockland, Rockland, ME, USA) using 1 kb DNA ladder (New England BioLabs, Ipswich, MA, USA) as molecular size markers. Restriction enzymes and DNA modification enzymes were from New England BioLabs, and reactions were carried out under the recommended conditions. All other chemicals used in the study were of molecular biology grade. When necessary, DNA fragments were purified using agarose gels followed by purification using QIAquick-gel extraction kits from QIAGEN (Valencia, CA, USA). DNA sequencing was performed by Macrogen (Rockville, MD, USA); BioBricks were assembled as directed in the BioBrick Assembly Manual (New England BioLabs).

Bacterial strains, plasmids, growth conditions and transformation procedures.

All strains and plasmids used in the study are listed in Supplementary Table S2. Chemically competent *E. coli* cells were prepared according to Inoue et al. (3). For each transformation, 1 µL overlap extension PCR reaction (Figure 1C) was used to transform 20 µL competent cells. The transformants (250 µL) were spread on Luria Bertani medium (LB) agar plates containing appropriate antibiotic and 100 µM isopropyl β-D-1-thiogalactopyranoside (IPTG; if necessary). The constructs produced in this study conform to the BioBricks standard [Knight, T. Draft Standard for Biobrick Biological Parts (OpenWetWare, MIT, Cambridge, MA, USA, 2007). http://hdl.handle.net/1721.1/45138], but we emphasize that the cloning technique described here can be applied to any insert and vector without regard to restriction sites.

A custom BioBrick accepting vector (pIMBB) was created by cloning a synthetic multiple cloning site (*Eco*RI-*Not*I-*Xba*I-*Sph*I-ribosome binding site-*Nco*I-*Eco*RV-*Hin*dIII-*Spe*I-*Not*I-*Pst*I) into the *Dra*III and *Afl*III sites of pSL1180 (Amersham Pharmacia Biotech, Piscataway, NJ, USA). This plasmid retains its ColE1 origin and β-lactamase gene and has restriction sites *Eco*RI, *Not*I, *Xba*I, *Spe*I, or *Pst*I present only in the multiple cloning site, which is crucial for BioBricks assembly. Desired BioBricks cloned into this plasmid can subsequently be combined with others (see BioBrick Assembly Manual at http://ginkgobioworks.com/support for details. A series of reporter BioBricks (*gfp*, *gusA*, *lacZ*, *luxA*, *luxB*, *luxC*, *luxD*, *and luxE*) was produced by PCR amplification with appropriate primers (Supplementary Table S3) and subsequent cloning into the pIMBB vector. Each BioBrick was then combined with the ribosomal binding site (rbs) BioBrick (oligos 213–214; Supplementay Table S3). After that, rbs-fused *luxA, luxB, luxC, luxD and luxE* BioBricks were combined to create a *luxAluxBluxCluxDluxE* BioBrick (synthetic LUX operon). Reporter BioBricks were then separately combined with a BioBrick containing the T5-promoter and two *lac* operators (oligos 221–222; Supplementary Table S3) to enable regulated expression in the presence of the *lacI* repressor.

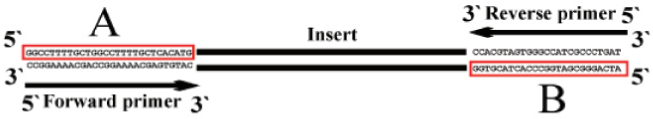
Overlap extension PCR cloning

Phusion DNA polymerase (New England BioLabs) (or Expand Long Template enzyme mix, Roche, Basel, Switzerland) and chimeric primers (5′ end, vector-specific sequences; 3′ end, insert-specific sequences) were used to PCR-amplify the inserts (Figure 1A). In particular, primers 278 and 279 were used to PCR-amplify *gfp*, *gusA*, *lacZ*, and LUX operon from pIMBB for cloning into pQBAV3Cam vector. Primers 278 and 280 were used to PCR-amplify *gfp* from pIMBB-gfp for cloning into pQBAV2ACam. Each PCR was subjected to a temperature regimen similar to the following: initial denaturation at 100°C for 2 min, denaturation at 94°C for 30 s, annealing at 60°C for 30 s, extension at 68°C for 90 s/kb for 30–32 cycles, with a final extension of 68°C for 10 min. All PCR-amplified inserts were gel-purified prior to use as megaprimers in (see Figure 1B).

Primer design for overlap extension PCR follows 3 easy steps presented in Supplementary Figure S1. First, design appropriate primers A and B to PCR-amplify the insert using web-based tools [e.g., Primer3 (http://primer3.sourceforge.net), Primer Design (www.bioinformatics.org/jambw/5/2/index.html), or Primer-Blast (www.ncbi.nlm.nih.gov/tools/primer-blast)]. Second, select the desirable insert points on the plasmid; they can be in close proximity to each other or, preferably, 50 to several hundred base pairs apart. Now select 30–40 bp upstream of the left point of insert on the top strand of the plasmid. Copy this sequence and estimate its Tm using an online tool (Oligo Calculator, www.idtdna.com/analyzer/Applications/OligoAnalyzer/Default.aspx). If Tm parameter (60-65°C; see main text) is satisfied, save the sequence as primer C. Select 30–40 bp downstream of the right point of insert on the bottom strand of the plasmid. Copy the reverse sequence and analyze its Tm using an online tool (Oligo Calculator); If the Tm parameter is satisfied, save the sequence as primer D. Attach the sequence of primer C to the 5' end of the primer A. Attach the sequence of primer D to the 5' end of the primer B.

Overlap extension PCR (Figure 1B) requires a combination of high GC content in the vector-specific part of the chimeric primers (5′ end) and relatively low (as compared with the primers designed Tm) annealing temperature in the thermocycler.

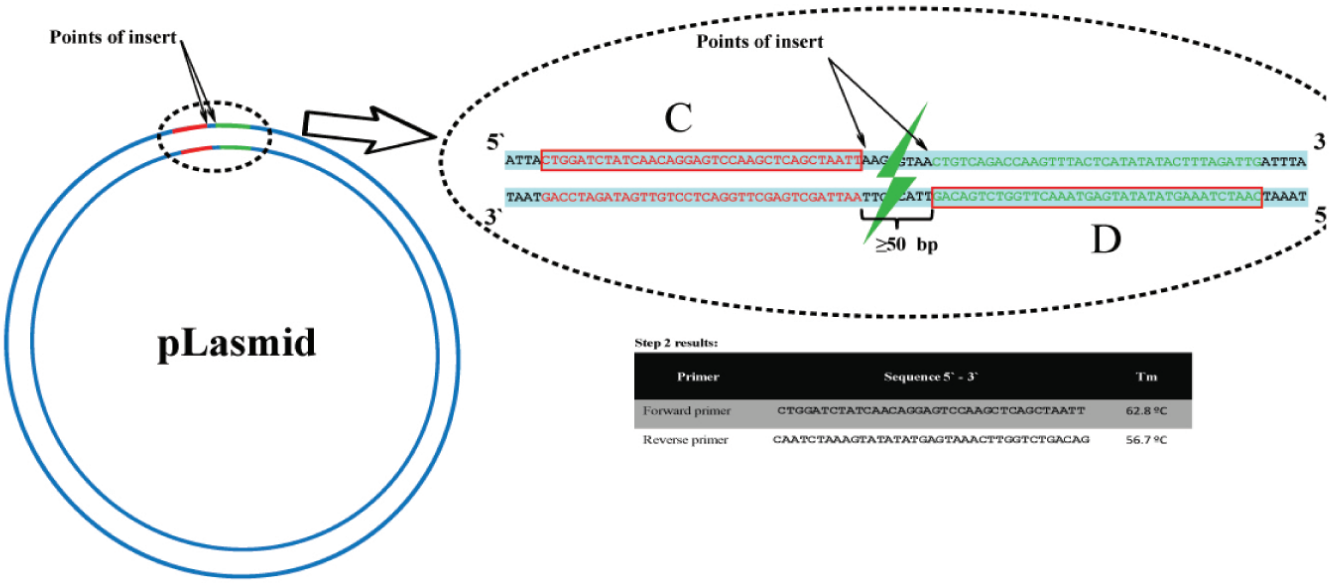
1



Step 1 results:

Primer	Sequence 5` - 3`	Tm
Forward primer	GGCCTTTTGCTGGCCTTTTGCTCACATG	64.4 ºC
Reverse primer	ATCAGGGCGATGGCCCACTACGTGG	66.6 ºC

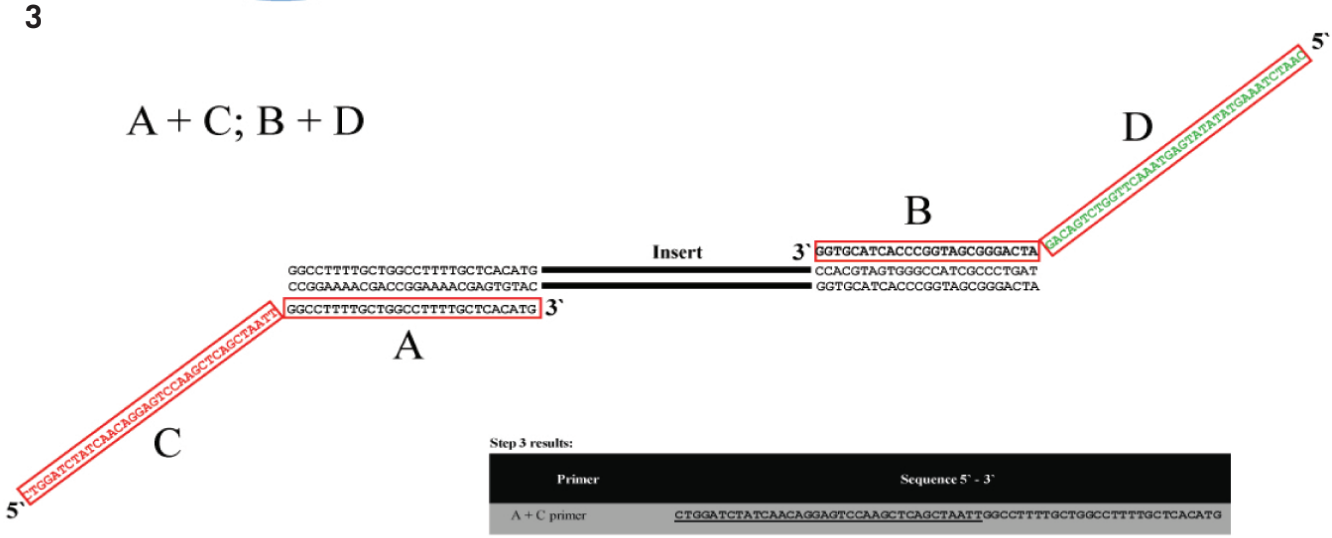
2



Step 2 results:

Primer	Sequence 5` - 3`	Tm
Forward primer	CTGGATCTATCAACAGGAGTCCAAGCTCAGCTAATT	62.8 ºC
Reverse primer	CAATCTAAAGTATATATGAGTAAACTTGGTCTGACAG	56.7 ºC

3



Step 3 results:

Primer	Sequence 5` - 3`
A + C primer	CTGGATCTATCAACAGGAGTCCAAGCTCAGCTAATTGGCCTTTTGCTGGCCTTTTGCTCACATG
B + D primer	CAATCTAAAGTATATATGAGTAAACTTGGTCTGACAGATCAGGGCGATGGCCCACTACGTGG

Supplementary Figure S1. Making the primers for overlap extension PCR cloning. (1) Design appropriate primers A and B to PCR-amplify the insert using web-based tools (e.g., Primer3, Primer Design, or Primer-Blast). (2). Select the desirable insert points on the plasmid; they could be in close proximity to each other or, preferably, 50 to several hundred bp apart. Then select 30–40 bp upstream of the left point of insert on the direct strand of the plasmid; copy this sequence. Analyze Tm using online tool (Oligo Calculator); if Tm parameter is satisfied, save the sequence of the primer C. Select 30–40 bp downstream of the right point of insert on the direct strand of the plasmid; copy the sequence. Analyze Tm using online tool (Oligo Calculator); if Tm parameter is satisfied, make reverse complement of the sequence using online tool (Reverse complement) and save the sequence of the primer D. (3) Attach the sequence of the primer C to the 5′ end of the primer A; attach the sequence of the primer D to the 5’ end of the primer B.

Supplementary Table S1. Comparison of the performance of different PCR systems in overlap extension PCR cloning

DNA polymerase	Processivity	Number of colonies expressing GFP/plate	Number of white colonies/plate
KOD DNA polymerase	>300 bases (5)	14	0
Phusion DNA polymerase	>360 bases (6)	417	8
Expand Long Template DNA polymerase mix	50–60 bases for *Taq* DNA polymerase (5)	12	2
Deep Vent DNA polymerase	?	0	0
Pfu DNA polymerase	15–20 bases (5)	9	1
Taq DNA polymerase	50–60 bases (6)	Not tested	Not tested

Phusion DNA polymerase was used to PCR-amplify green fluorescent protein (*gfp*) gene from the pIMBB-gfp plasmid. The PCR products were gel-purified and used in the overlap extension PCR reaction with pQE30 vector. Three nanograms of pQE30 vector was mixed with 500 ng insert in the total reaction volume of 10 µL, and subjected to 25 cycles of PCR with different PCR systems: *KOD*, Phusion, Expand Long Template mix, DeepVent and *Pfu*. The original plasmid was destroyed in restriction digests with *Dpn*I, and the overlap extension PCR products were used to transform competent *E. coli* cells.

Supplementary Table S2. Bacterial strains and plasmids used in the study

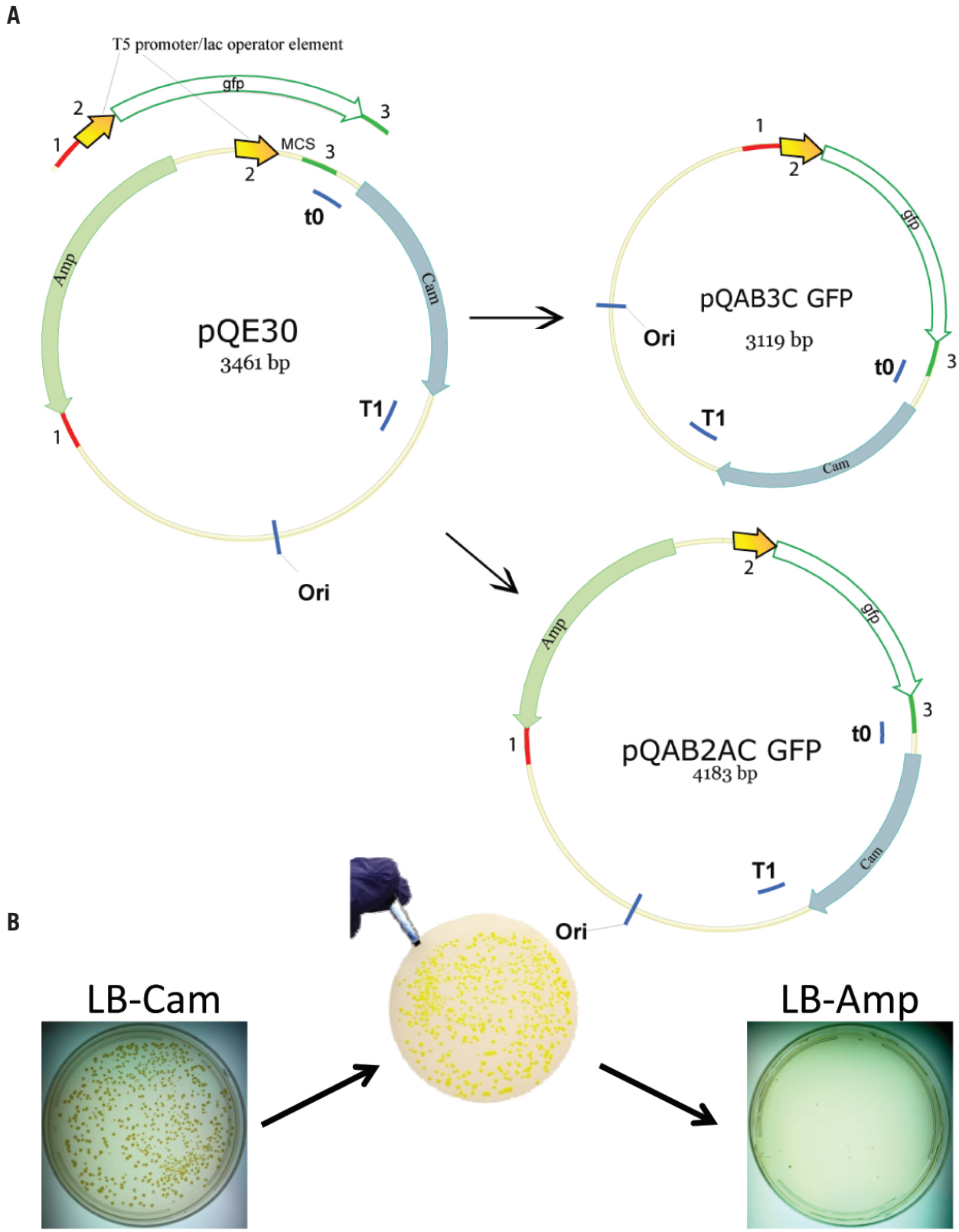
Name	Description	Reference or source
Strains		
Escherichia coli		
AG1	F- recA1 endA1 gyrA96 thi-1 hsdR17(rk- mk+) supE44 relA1	"NBRP (NIG, Japan): *E.coli*"
Photorhabdus luminescens		ATCC number 29999
Plasmids		
pQE30	Expression Vector; T5lacO; ColE1 replicon; Amp^rCam^r	Qiagen
pCA24N	Expression Vector; T5lacO; ColE1 replicon; Cam^r	(2)
IMBB-pSL1180-gfp	Amp^r	This study
pIMBB	BioBrick accepting vector; ColE1 replicon; Amp^r	This study
pIMBB-gfp	Amp^r	This study
pIMBB-gusA	Amp^r	This study
pIMBB-lacZ	Amp^r	This study
pIMBB-luxABCDE	Amp^r	This study
pQBAV3Cam-gfp	Cam^r	This study
pQBAV3Cam-gusA	Cam^r	This study
pQBAV3Cam-lacZ	Cam^r	This study
pQBAV3Cam-luxABCDE	Cam^r	This study
pQBAV2ACam-gfp	Amp^rCam^r	This study

We recommend a molar excess of insert over plasmid template. The concentration of megaprimers (PCR product of Figure 1A) is much lower than the concentration of synthetic primers in regular PCRs; they could be shorter than expected and thus have lower melting temperature. Acceptor plasmid template (3–30 ng) was mixed with 250× molar excess of insert DNA (from Figure 1A) in a 10-µL total volume containing Phusion DNA polymerase reaction mixture containing dNTPs, buffer, and enzyme. The insert and vector underwent denaturation (98°C for 30 s), annealing (60°C for 30 s), and polymerase-catalyzed extension (98°C for 1.5 min per kb according to the length of the longest piece) for 5–30 cycles. We normally added an extra 10-min extension step in the end of the program. For average-sized vectors and inserts, the total reaction time was <2 h. The same PCR parameters and volumes were used for overlap PCR cloning reactions with other polymerases and polymerase mixtures [*KOD* (Merck, San Diego, CA, USA); Expand Long Template mix (Roche); Deep Vent (New England BioLabs); and *Pfu* (Stratagene, La Jolla, CA, USA)], except that the buffer system was substituted with one recommended by each respective manufacturer.

The *Dpn*I endonuclease works well in Phusion HF buffer. We typically add 10 units of the enzyme directly to the PCR tube right after the final extension is done and incubate the reaction for an hour at 37°C (see Figure 1C). Restriction endonuclease *Dpn*I targets methylated DNA sequences and can thereby cleave the DNA template isolated from most *E. coli* strains, but not the PCR product (4).

Additional results

Recombinant plasmids often have repeated elements such as promoters, ribosomal binding sites, terminators, and scar sites. We wondered whether overlap extension PCR cloning was sensitive to the presence of internal sequences in the target plasmid identical to those in the insert. Chimeric primers were used to PCR-amplify a *gfp* gene fused to the T5 promotor/lac operator. A similar T5 promotor/lac operator sequence was present in the



Supplementary Figure S2. Overlap extension PCR cloning of the fragment with internal sequence identity to the plasmid. (A) Outline of the possible outcomes of cloning. (B) Analyses of the overlap extension PCR cloning using antibiotic selection. *E. coli* cells were plated after the transformation on LB-Cam plate. Colonies formed on the plate were transferred using nitrocellulose on the LB-Amp plate to determine an outcome of the cloning reaction.

Supplementary Table S3. Oligonucleotides and PCR primers used in the study

Primer	Sequence	Additional information
201 SP/luxA/Bba_CluxA	GGAATTCGCGGCCGCTTCTAGATGAAATTTGGAAACTTTTTGCTTACATACCA	PCR amplification of *luxA* gene of *Photorhabdus luminescens*
202 ASP/luxA/Bba_CluxA	CTGCAGCGGCCGCTACTAGTATTATTAATATAATAGCGAACGTTGTTTTTCTTTAAGA	PCR amplification of *luxA* gene of *Photorhabdus luminescens*
203 SP/luxB/Bba_CluxB	GGAATTCGCGGCCGCTTCTAGATGAAATTTGGATTGTTCTTCCTTAACTTC	PCR amplification of *luxB* gene of *Photorhabdus luminescens*
204 ASP/luxB/Bba_CluxB	CTGCAGCGGCCGCTACTAGTATTATTAGGTATATTCCATGTGGTACTTCTTAATA	PCR amplification of *luxB* gene of *Photorhabdus luminescens*
205 SP/luxC/Bba_CluxC	GGAATTCGCGGCCGCTTCTAGATGACTAAAAAAATTTCATTCATTATTAACGGCC	PCR amplification of *luxC* gene of *Photorhabdus luminescens*
206 ASP/luxC/Bba_CluxC	CTGCAGCGGCCGCTACTAGTATTATTATGGGACAAATACAAGGAACTTATCTTCTTC	PCR amplification of *luxC* gene of *Photorhabdus luminescens*
207 SP/luxD/Bba_CluxD	GGAATTCGCGGCCGCTTCTAGATGGAAAATGAATCAAAATATAAAACCATCG	PCR amplification of *luxD* gene of *Photorhabdus luminescens*
208 ASP/luxD/Bba_CluxD	CTGCAGCGGCCGCTACTAGTATTATTAAGACAGAGAAATTGCTTGATTTTCAATC	PCR amplification of *luxD* gene of *Photorhabdus luminescens*
209 SP/luxE/Bba_CluxE	GGAATTCGCGGCCGCTTCTAGATGACTTCATATGTTGATAAACAAGAAATTACAGC	PCR amplification of *luxE* gene of *Photorhabdus luminescens*
210 ASP/luxE/Bba_CluxE	CTGCAGCGGCCGCTACTAGTATTATTAACTATCAAACGCTTCGGTTAAGCTTA	PCR amplification of *luxE* gene of *Photorhabdus luminescens*
213 INS_S/RBS/BBa_B0034	AATTCGCGGCCGCTTCTAGAGAAAGAGGAGAAATA	Ribosomal binding site in BioBrick format
214 INS_AS/RBS/BBa_B0034	CTAGTATTTCTCCTCTTTCTCTAGAAGCGGCCGCG	Ribosomal binding site in BioBrick format
221 INS_S/T52lacO/BBa_R0AB2	AATTCGCGGCCGCTTCTAGAGGAAATCATAAAAAATTTATTTGCTTTGTGAGCGGATAA-CAATTATAATAGATTCAATTGTGAGCGGATAACAATTA	T5promoter/lac operator in BioBrick format
222 INS_AS/T52lacO/BBa_R0AB2	CTAGTAATTGTTATCCGCTCACAATTGAATCTATTATAATTGTTATCCGCTCA-CAAAGCAAATAAATTTTTTATGATTTCCTCTAGAAGCGGCCGCG	(T5promoter/lac operator in BioBrick format
252 SP/GFP/1	GGAATTCGCGGCCGCTTCTAGATGCGTAAAGGAGAAGAACTTTTCACTGGAGTTGTCCC	PCR amplification of *gfp* gene from pCA24N vector
253 ASP/GFP/736	CGACTGCAGCGGCCGCTACTAGTATTATTATTTGTATAGTTCATCCATGCCATGTG-TAATCC	PCR amplification of *gfp* gene from pCA24N vector
1363ASP/LacZ wt/3075	CCAGCTGCAGCGGCCGCTACTAGTATTATTATTTTTGACACCAGACCAACTGGTAATG	PCR amplification of *lacZ* gene from K12 *E. coli* genomic DNA
1364SP/LacZ wt/1	GCCGCTTCTAGATGACCATGATTACGGATTCACTGGC	PCR amplification of *lacZ* gene from K12 *E. coli* genomic DNA
1365ASP/UidAwt/1812	CCAGCTGCAGCGGCCGCTACTAGTATTATTATTGTTTGCCTCCCTGCTGCG	PCR amplification of *gusA* gene from K12 *E. coli* genomic DNA
1366SP/UidAwt/1	GCCGCTTCTAGATGTTACGTCCTGTAGAAACCCCAACCCG	PCR amplification of *gusA* gene from K12 *E. coli* genomic DNA
278 INS/IMBB/pQE30	CTGGATCTATCAACAGGAGTCCAAGCTCAGCTAATTGGCCTTTTGCTGGCCTTTT-GCTCACATG*	Overlap extenstion PCR cloning primer
279 INS/IMBB/pQE30S	CAATCTAAAGTATATATGAGTAAACTTGGTCTGACAGATCAGGGCGATGGCCCAC-TACGTGG*	Overlap extenstion PCR cloning primer
280 INS/IMBB/pQE30L	CCTATAAAAATAGGCGTATCACGAGGCCCTTTCGTCTTCATCAGGGCGATGGCCCAC-TACGTGG*	Overlap extenstion PCR cloning primer
281 INS/pQE30/noXbaI	CAAATCCGCCCTCCAGAGCTGCCTCGCGC	To remove *Xba*I restriction site from pQE30
282 INS/pQE30/noXbaI	GCGCGAGGCAGCTCTGGAGGGCGGATTTG	To remove *Xba*I restriction site from pQE30

* 5' addition to the primer complimentary to the vector has been underlined

pQE30 vector; the insert had three regions identical to those on the plasmid (Supplementary Figure S2A). The primers also contained sequences similar to two regions on the plasmid separated by an Amp resistance marker. Successful overlap extension PCR cloning should therefore insert the T5 promoter/gfp cassette and eliminate the Amp marker from the pQE30 vector. Most of the colonies that appeared on the LB-agar plate supplemented with chloramphenicol displayed the "green" phenotype (Supplementary Figure S2B). Replica plating of the plate on LB-Amp resulted in only two surviving colonies (out of >500) that did not have the green phenotype (Supplementary Figure S2B), which probably reflected carryover of the original plasmid.

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