Construction of Pveg promoter + spoVG RBS + lytC + linker + RPMrel + GFP + double terminator (K733007)

STAGE 1

1) PCR amplification of [consensus RBS + GFP + double terminator] region using BBa_E0840 as the template.

Forward primer design

5' – [17bp prefix region, including XbaI restriction site] [11bp B. subtilis consensus RBS] [22bp overlap with GFP including 3bp spacer preceding GFP start codon] – 3'

Forward primer sequence

5' – CGCGGCCGCTTCTAGAGAAAGGAGGTGTTAGATGCGTAAAGGAGAAGAAC – 3' (50bp)

Reverse primer design

5' – [18bp overlap with pSB1A2 plasmid downstream of suffix] – 3'

Reverse primer sequence

5' – TACCGCCTTTGAGTGAGC – 3' (18bp)

PCR reaction set-up

Reagents	Quantity per reaction
ddH_2O	Add up to total 20µL volume
Phusion® HF Buffer (5X)	4µL
DNA Template - BBa_E0840	10ng
Forward Primer (10µM)	0.5µL
Reverse Primer (10µM)	0.5µL
dNTP (10µM)	0.5µL
Phusion® Polymerase	0.5µL

PCR program

Step Number	Step Name	Temperature (ºC)	Duration (minutes:seconds)
1	Initial Denaturation	95	05:00
2	Denaturation	95	00:30
3	Annealing	56	00:30
4	Extension	72	01:00
5	Goto step 2 for 30 cycles	-	-
6	Final Extension	72	05:00
7	Storage	8	∞

2) Reaction clean-up of above PCR.

Purification of the PCR products using the materials and protocols packaged in the FavorPrep™ GEL/PCR Purification Kit.

3) Digestion of [consensus RBS + GFP + double terminator] PCR product and pSB1C3.

Digestion reaction set-up

Reagents	Quantity per reaction	
	PCR Product	**pSB1C3**
ddH_2O	Add up to total 20µL volume	Add up to total 20µL volume
10X NEBuffer 4	2µL	2µL
DNA	1µg	1µg
XbaI	0.5µL	0.5µL
PstI-HF	0.5µL	0.5µL

Reaction incubation at 37ºC for 2 hours.

4) Gel purification of digested pSB1C3.

Run the digestion product in 1% agarose gel for an appropriate time. Identify the band resolved at 2070bp and cut it from the gel. This is the pSB1C3 backbone. Purify the gel cutting using the materials and protocols packaged in the FavorPrep™ GEL/PCR Purification Kit.

5) Reaction clean-up of PCR product digestion reaction.

Purify the digestion products using the materials and protocols packaged in the FavorPrep™ GEL/PCR Purification Kit.

6) Ligation of PCR product into standard backbone pSB1C3.

Ligation reaction set-up

Reagents	Quantity per reaction	
	Ligation	**(-) Control**
ddH_2O	Add up to total 10µL volume	Volume of insert, then add up to total 10µL volume
10X T4 Ligase Buffer	1µL	1µL
Insert (mass ratio value)	3	-
Backbone (mass ratio value)	1	1
T4 Ligase	0.5µL	0.5µL

Reaction incubation at room temperature for 2 hours.

7) Transformation of above ligation product.

We transformed using competent *Escherichia coli* DH10B cells. We are also selecting for chloramphenicol resistance. The following steps take these factors into account.

- Add whole ligation product to 100µL competent cells.
- Place mixture on ice for 10 minutes.
- Heat shock mixture at 42ºC for 90 seconds.
- Place mixture on ice for 2 minutes.
- Add 1ml LB to the mixture.

- Recover the cells by incubation at 37ºC for 1 hour.
- Centrifuge mixture at 16.1rcf for 90 seconds.
- Remove majority of the supernatant.
- Resuspend pellet in an appropriate volume of LB.
- Transfer suspension to 25µg/ml chloramphenicol LB plate and spread.
- Incubate at 37ºC overnight.

8) Plasmid extraction of successfully transformed colonies.
Extract [consensus RBS + GFP + double terminator] construct in pSB1C3 using the materials and protocols packaged in the FavorPrep™ Plasmid DNA Extraction Mini Kit.

STAGE 2

1) PCR amplification of [Pveg promoter + spoVG RBS + lytC + linker + RPMrel] region using BBa_K316037 as the template.

Forward primer design
5’ – [6bp cap] [20bp overlap with standard prefix] – 3’

Forward primer sequence
5’ – GATCATGAATTCGCGGCCGCTTCTAG – 3’ (26bp)

Reverse primer design
5' - [8bp cap] [7bp SpeI restriction site] [6bp reverse-complementary double stop codon] [27bp reverse-complementary sequence of codon-optimized RPMrel] [15bp reverse-complementary overlap with linker] - 3'

Reverse primer sequence
5’ - GTTTCTTCACTAGTATTATTAACACATCGGGCGATCTTCGATCGGACAGGCCGCGGCTTTCGC - 3’ (63bp)

PCR reaction set-up

Reagents	Quantity per reaction
ddH_2O	Add up to total 20µL volume
Phusion® HF Buffer (5X)	4µL
DNA Template - BBa_K316037	100ng
Forward Primer (10µM)	0.5µL
Reverse Primer (10µM)	0.5µL
dNTP (10µM)	0.5µL
Phusion® Polymerase	0.5µL

PCR program

Step Number	Step Name	Temperature (ºC)	Duration (minutes:seconds)
1	Initial Denaturation	95	05:00
2	Denaturation	95	00:30

3	Annealing	65	00:30
4	Extension	72	01:30
5	Goto step 2 for 25 cycles	-	-
6	Final Extension	72	05:00
7	Storage	8	∞

2) Reaction clean-up of above PCR.
Purify the PCR products using the materials and protocols packaged in the FavorPrep™ GEL/ PCR Purification Kit.

3) Digestion of [Pveg + spoVG RBS + lytC + linker + RPMrel] PCR product and [consensus RBS + GFP + double terminator] construct in pSB1C3.
Digestion reaction set-up

Reagents	**Quantity per reaction**	
	PCR Product	**pSB1C3 Construct**
ddH_2O	Add up to total 20µL volume	Add up to total 20µL volume
10X NEBuffer 2	2µL	2µL
DNA	1µg	1µg
EcoRI	0.5µL	0.5µL
XbaI	-	0.5µL
SpeI	0.5µL	-

Reaction incubation at 37ºC for 2 hours.

4) Reaction clean-up of PCR product and pSB1C3 construct digestion reaction.
Purify the digestion products using the materials and protocols packaged in the FavorPrep™ GEL/PCR Purification Kit.

6) Ligation of PCR product into standard backbone pSB1C3.
Ligation reaction set-up

Reagents	**Quantity per reaction**	
	Ligation	**(-) Control**
ddH_2O	Add up to total 10µL volume	Volume of insert, then add up to total 10µL volume
10X T4 Ligase Buffer	1µL	1µL
Insert (mass ratio value)	3	-
Backbone (mass ratio value)	1	1
T4 Ligase	0.5µL	0.5µL

Reaction incubation at room temperature for 2 hours.

7) Transformation of above ligation product.

Again consider the following steps suitable for transformation of *E. coli* DH10B competent cells for selection with chloramphenicol.

- Add whole ligation product to 100µL competent cells.
- Place mixture on ice for 10 minutes.
- Heat shock mixture at 42ºC for 90 seconds.
- Place mixture on ice for 2 minutes.
- Add 1ml LB to the mixture.
- Recover the cells by incubation at 37ºC for 1 hour.
- Centrifuge mixture at 16.1rcf for 90 seconds.
- Remove majority of the supernatant.
- Resuspend pellet in an appropriate volume of LB.
- Transfer suspension to 25µg/ml chloramphenicol LB plate and spread.
- Incubate at 37ºC overnight.

8) Plasmid extraction of successfully transformed colonies.
Extract [Pveg promoter + spoVG RBS + lytC + linker + RPMrel + consensus RBS + GFP + double terminator] construct in pSB1C3 using the materials and protocols packaged in the FavorPrep™ Plasmid DNA Extraction Mini Kit.