Construction of lytC + linker + FLAG™ (K733008)

This construct was built in an illogical and roundabout manner since issues we encountered led us to change our construction method part of the way through. We record here the exact pathway of construction we followed, but we recommend that any future parties intending to rebuild this construct choose a more direct method.

STAGE 1

1) PCR amplification of [Pveg + spoVG RBS + lytC + linker + FLAG™] 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] [24bp reverse-complementary sequence of codon-optimized FLAG™] [15bp reverse-complementary overlap with linker] - 3'

Reverse primer sequence
5' – GTTTCTTCACTAGTATTATTATTTATCATCATCATCTTTATAATCGGCCGCGGCTTTCGC – 3' (60bp)

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	98	05:00
2	Denaturation	98	00:30
3	Annealing	60	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 product using the materials and protocols packaged in the FavorPrep™ GEL/PCR Purification Kit.

3) Digestion of [Pveg promoter + spoVG RBS + lytC + linker + FLAG™] 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 2	2µL	2µL
DNA	1µg	1µg
EcoRI	0.5µL	0.5µL
SpeI	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 product 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 [Pveg + spoVG RBS + lytC + linker + FLAG™] construct in pSB1C3 using the materials and protocols packaged in the FavorPrep™ Plasmid DNA Extraction Mini Kit.

Notes.
It was discovered following sequencing of this construct and the K316037 plasmid from which it was amplified that the *Escherichia coli* transposon *insAB* was present within the lytC coding region of both. We logically assumed that the plasmid had been mutated by its carrier. *insAB*, which has a length of approximately 700bp, causes the PCR product band to be resolved around 700bp higher than expected when run in agarose gel. When sequenced, the construct was found to have intact promoter, ribosome binding site, linker, FLAG™ peptide and terminator regions, with only the lytC coding region mutated. Thus it was decided to amplify 'safe' copies of the 1-954bp region of lytC and the linker + FLAG™ sequence separately after which overlapping PCR would be used to join them.

STAGE 2

1) PCR amplification of 1-954bp lytC from the *Bacillus subtilis* genome.
Forward primer design
5' – [6bp cap] [7bp XbaI restriction site] [30bp overlap with lytC] – 3'

Forward primer sequence
5' – GATCATTCTAGAGTTGCGTTCTTATATAAAAGTCCTAACAATG – 3' (43bp)

Reverse primer design
5' – [27bp reverse-complementary overlap with lytC] – 3'

Reverse primer sequence
5' – TACAACTGGATTCTTTAGCTGATTAGC – 3' (27bp)

PCR reaction set-up

Reagents	Quantity per reaction
ddH_2O	Add up to total 20µL volume
Phusion® HF Buffer (5X)	4µL
DNA Template - *B. subtilis* 168 genomic DNA	200ng

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	98	05:00
2	Denaturation	98	00:30
3	Annealing	55	00:30
4	Extension	72	01:00
5	Goto step 2 for 25 cycles	-	-
6	Final Extension	72	05:00
7	Storage	8	∞

2) Reaction clean-up of above PCR by gel purification.
Run the PCR product in 1% agarose gel for an appropriate time. Identify the band resolved at 967bp and cut it from the gel. This is the coding region for the cell wall binding domain of lytC with attached 5' cap. Purify the gel cutting using the materials and protocols packaged in the FavorPrep™ GEL/PCR Purification Kit.

3) PCR amplification of [linker + FLAG™] from existing construct.
Forward primer design
5' – [31bp overhang overlapping with lytC] [19bp overlap with linker] – 3'

Forward primer sequence
5' – GGTTGCTAATCAGCTAAAGAATCCAGTTGTAAGCAGAGGCTCACGCGCAC – 3' (50bp)

Reverse primer design
5' – [8bp cap] [6bp BamHI restriction site] [7bp SpeI restriction site] [31bp overlap with FLAG™ and linker sequence] – 3'

Reverse primer sequence
5' – GTTTCTTCGGATCCACTAGTATTATTATTTATCATCATCATCTTTATAATCG – 3' (52bp)
NB. This reverse primer was originally intended to allow PCR addition of a BamHI site to the 3' end of the construct. This would allow it to be ligated into the multiple cloning site of integration vector pDG1661. That function was never pursued and the primer was recycled for use here.

PCR reaction set-up

Reagents	Quantity per reaction
ddH_2O	Add up to total 20µL volume
Phusion®-HF Buffer (5X)	4µL

DNA Template - [Construct in pSB1C3 produced in STAGE 1]	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	98	05:00
2	Denaturation	98	00:30
3	Annealing	62	00:30
4	Extension	72	00:30
5	Goto step 2 for 25 cycles	-	-
6	Final Extension	72	05:00
7	Storage	8	∞

4) Reaction clean-up of above PCR by gel purification.
Run the PCR product in 1% agarose gel for an appropriate time. Identify the band resolved at 154bp and cut it from the gel. This is the coding region for the cell wall binding domain of lytC with attached 5' cap. Purify the gel cutting using the materials and protocols packaged in the FavorPrep™ GEL/PCR Purification Kit.

5) Overlapping PCR linking products from steps 2 and 3.
Forward primer design
5' – [6bp cap] [7bp XbaI restriction site] [30bp overlap with lytC] – 3'

Forward primer sequence
5' – GATCATTCTAGAGTTGCGTTCTTATATAAAAGTCCTAACAATG – 3' (43bp)

Reverse primer design
5' – [8bp cap] [6bp BamHI restriction site] [7bp SpeI restriction site] [31bp overlap with FLAG™ and linker sequence] – 3'

Reverse primer sequence
5' – GTTTCTTCGGATCCACTAGTATTATTATTTATCATCATCATCTTTATAATCG – 3' (52bp)

PCR reaction set-up

Reagents	Quantity per reaction
ddH_2O	Add up to total 20µL volume
Phusion® HF Buffer (5X)	4µL
1-954bp lytC PCR product	430ng

linker + FLAG™ PCR product	70ng
Forward Primer (10µM)*	0.5µL
Reverse Primer (10µM)*	0.5µL
dNTP (10µM)	0.5µL
Phusion® Polymerase	0.5µL

* Primers were only added on the *6th cycle* of denaturation, annealing and extension. See the PCR program below for more details.

PCR program (part annealing, first 5 cycles)

Step Number	Step Name	Temperature (ºC)	Duration (minutes:seconds)
1	Initial Denaturation	98	05:00
2	Denaturation	98	00:30
3	Annealing	55	00:30
4	Extension	72	00:45
5	Goto step 2 for 5 cycles	-	-
6	Final Extension	72	10:00
Add primers at this stage.			
7	Initial Denaturation	98	05:00
8	Denaturation	98	00:30
9	Annealing	68	00:30
10	Extension	72	01:00
11	Goto step 8 for 30 cycles	-	-
12	Final Extension	72	10:00
13	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 [lytC + linker + FLAG™] 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 2	2µL	2µL
DNA	1µg	1µg
EcoRI	0.5µL	0.5µL

SpeI	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.
Purification of 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 + spoVG RBS + lytC + linker + FLAG™] construct in pSB1C3 using the materials and protocols packaged in the FavorPrep™ Plasmid DNA Extraction Mini Kit.