Lab plan for construct of Pveg + R.B.S.

1. Final construct:

Pveg promoter (BBa_K316001)+ R.B.S. of B.subtilis (BBa_K143021) + PSB1C3

2. Lab plan

2.1 PCR

1. prepare a master mixture as follow:

dd H2O (μ l)	80.5
10X Buffer (μ l)	10
Forward primer (μ l)	2.5
Reserve primer (μ l)	2.5
Taq polymerase (μ l)	2.5
Template (Pveg biobrick (BBa_K316001)) (54.5ng/ μ l)	2
Total volume (μ l)	100

Sequence of forward primer:

5’ggatgatttctggaattcgc3’

Sequence of reverse primer:

5’tgatcactagtattcaccacctttacatttattgtacaacacgagcc3’

PCR program

Process	Temperature (℃)	Time (s)
1. initial Denaturation	95	30
2. Denaturation	95	15
3. Annealing	55	15
4. Extension	68	10
5. Repeat cycle 29 times		
6. Final extension	68	300

2. Separate the master mix in 5 tubes, each 20 μ l.

2.2 Gel checking

1. prepare 2% agarose gel
2. Correct band size: 156 b.p.

2.3 ethanol precipitation

1. Add 90 μ l of PCR product and 10 μ l of 3M sodium acetate pH5.2.
2. Mix with 250 μ l 100% EtOH.
3. Leave it at room temperature for 30 minutes.

4. Centrifuge the solution with 13200 r.p.m. for 10 minutes.
5. Remove the supernatant. Add 200 μl of 75% EtOH. Vortex briefly.
6. Centrifuge the solution with 13200 r.p.m. for 5 minutes. Remove supernatant.
7. Dry the tube for 5 minutes. Resuspend the tube with 40 μl ddH2O.

2.4 Double digestion

1. Digest the PCR product (as insert) and BBa_J04450 (as backbone) with EcoRI and SpeI
2. Incubate 2hours.

2.5 Dephosphorylation

Add 0.5 μl AP and 10X buffer into the tube containing BBa_J04450.

2.6 Heat kill enzyme

Heat digestion mixture with 65℃/ 20 minutes

2.7 Purify digested insert and backbone using ethanol precipitation

1. Follow 2.3, Purify both insert and backbone

2.8 Ligation

1. Mix the insert and backbone with T4 ligase and ligase buffer. Prepare 1:3, 1:4 and 1:5 backbone to insert ratio. Also, prepare one tube without the insert. This is the negative control.
2. Stand the tube in room temperature for 1h.

2.9 Transformation

1. Take out the competent cell from -80 freezer.
2. Cool down the cell in ice for 10 minutes.
3. Add all ligation products into the cells. Leave it for 10 minutes.
4. put the tubes into 42℃ water bath for exactly 90 seconds.
5. Put them back into ice for 2 minutes.
6. Add 1ml LB in all cells. Incubate the tubes in 37℃ for 1 hour.
7. Spin down the cells. Transfer 100 μl of each tube of cells into separate Chloramphenicol (25ng/ μl) plates. Spread the plate. Incubate in 37℃ overnight.
8. Observe the colonies formed on next day.

2.10 Extraction of plasmid from bacteria

1. Pick 8 individual colony into 5ml Chloramphenicol (25ng/ μl) LB medium.
2. Incubate into 37℃ shaker overnight.
3. Extract the plasmid DNA using Favorgen mini prep kit.

2.11 Single digestion check of plasmid extracted from bacteria

1. Set up the digestion mixture. Digest the plasmid with XhoI.
2. Heat kill the enzymes. Load the digested sample into 2% gel.
3. Expected correct band size: 1000b.p. and 1500b.p.

2.17 Double digestion check of plasmid extracted from bacteria

1. Set up the digestion mixture. Digest the plasmid with AatII and SphI.
2. Heat kill the enzymes. Load the digested sample into 2% gel.
3. Expected correct band size: ~500b.p. and ~1500b.p.

2.17 Sequencing of plasmid

Sequence of the insert in the biobrick

gaattcgcggccgcttctagagaattttgtcaaaataattttattgacaacgtcttattaacgttgatataatttaaattttatttgacaaaaatgggctcgtgttgtacaataaatgtaaaggtggtgaatactagtagcggccgctgcag