

Protocol for the construct of RBS+*ycdD* in pSB1C3

By WANG Yuqi, Cosmos – 2012 HKUST iGEM

I. PCR of RBS+*ycdD* from *Bacillus subtilis* 168 genomic DNA.

Designed primers:

Primer name	Primer sequence
Forward Primer	GTTTCTTCGAATTCGCGGCCGCTTAG ^{aaa} ggaggtgtatgtttt ^{ttgtctg} standard biobrick prefix RBS for subtilis(aaaggaggtgt)
Reverse Primer	GTTTCTTCCTGCAGCGGCCGCTACTAGATGATTATCCTCCGCTGACTA standard biobrick suffix

PCR set-up:

Reagents	RBS+ <i>ycdD</i>
ddH ₂ O	Add to 20uL
Phusion® HF Buffer (5X)	4uL
Template – <i>Bacillus subtilis</i> 168 genomic DNA	50ng
Phusion® polymerase	0.5uL
Forward primer (10uM)	0.5uL
Reverse primer (10uM)	0.5uL
dNTP	0.5uL
Reaction volume	20uL

PCR program:

Steps	Temperature (Celsius)	Duration (minute : second)
1.Initial Denaturation	95	08:00
2.Denaturation	95	00:30
3.Annealing	55	00:30
4.Extension	72	00:40
5.Goto step 2 for 30 cycles	-	-
6.Final extension	72	08:00
7.Storage	8	infinite

II. PCR clean-up.

1. Purify the PCR product of the secondary PCR by Favorgen PCR clean-up Kit.

III. Digestion of secondary PCR product and pSB1C3.

Digestion set-up:

Reagents	RBS+ <i>ycdD</i>	pSB1C3
ddH ₂ O	Add to 20uL	Add to 20uL
10 X NEB No.4 digestion buffer	2uL	2uL

PCR product	1ug	-
pSB1C3	-	1ug
EcoRI – HF	0.5uL	0.5uL
PstI – HF	0.5uL	0.5uL
Reaction volume	20uL	20uL

Incubate under 37 degree Celsius for 2 hours.

IV. Gel purification of digested pSB1C3.

1. Prepare a 40mL 0.8% agarose gel with CyberSafe as the stain.
2. Load the digested pSB1C3.
3. Run the gel under 130 V for 60 minutes.
4. Cut the bands of pSB1C3 backbone (nearly 2000bp).
5. Purify them by Favorgen gel purification Kit.

V. Ligation of digested secondary PCR product and pSB1C3.

Ligation set-up:

Reagent	Volume
ddH ₂ O	Add to 10uL
10X Invitrogen ligase buffer	1uL
Invitrogen T4 ligase	0.5uL
Insert (Digested RBS+ <i>ycdD</i>)	100ng
Backbone (Digested pSB1C3)	50ng

Incubate under 37 degree Celsius for 2 hours.

VI. Transformation.

1. Add 10uL of the ligation product in (vii) to 100uL of *E. coli DH10 β* competent cells.
2. Put the mixture in 1 on ice for 10 minutes.
3. Heat shock the mixture for 90 seconds under 42 degree Celsius.
4. Chill the mixture on ice for 2 minutes.
5. Add 1mL LB into the mixture.
6. Incubate the mixture in 5 for 1 hour under 37 degree Celsius.
7. Centrifuge the mixture for 90 seconds under 13200 rpm.
8. Remove 1mL supernatant.
9. Resuspend the pellet.
10. Transfer the resuspende solution onto a 25ug/mL chloramphenicol LB plate.
11. Spread the plate.
12. Incubate for overnight.

VII. Plasmid extraction of RBS+*ycdD* in pSB1C3.

1. Use the Favorgen plasmid extraction mini-Kit to extract the intended plasmid.