Protocol for the construct of xylR+PxylA in pSB1C3

By WANG Yuqi, Cosmos – 2012 HKUST iGEM

I. The first mutagenesis of EcoRI illegal cutting site.

(i) The primary PCR of the EcoRI mutagenesis for xylR+PxylA.

Designed primers:

Primer name	Primer sequence
Primer 1	GTTTCTTCCTGCAGCGGCCGCTACTAGAGTACCCTTTGATTTAAGTGAACAAGT *Prefix*
Primer 4	GTTTCTTCGAATTCGCGGCCGCTTCTAGGAGCTCCTAACTTATAGGGGTAACAC TTAAAAAAGAATC Yellow Part: *Suffix*
Primer 2E	CTAAGTCGGTTAGAATGCCGTTAAGATAGTCG *Mutation site*
Primer 3E	CGACTATCTTAACGGCATTCTAACCGACTTAG *Mutation site*

PCR set-up:

Reagents	Fragment 1	Fragment 2
ddH_2O	Add to 20uL	Add to 20uL
Phusion® HF Buffer (5X)	4uL	4uL
Template – pAX01	200ng	200ng
Phusion® polymerase	0.5uL	0.5uL
Primer 1 (10uM)	0.5uL	-
Primer 2E (10uM)	0.5uL	-
Primer 3E (10uM)	-	0.5uL
Primer 4 (10uM)	-	0.5uL
dNTP	0.5uL	0.5uL
Reaction volume	20uL	20uL

PCR program:

Steps	Temperature (Celsius)	Duration (minute : second)
1.Initial Denaturation	95	08:00
2.Denaturation	95	00:30
3.Annealing	71	00:30
4.Extension	72	01:00
5.Goto step 2 for 20 cycles	-	-
6.Final extension	72	08:00
7.Storage	8	infinite

(ii) Gel purification of primary PCR product.

1. Prepare a 40mL 0.8% agarose gel with CyberSafe as the stain.
2. Load the PCR product of fragment 1 and fragment 2.
3. Run the gel under 130 V for 60 minutes.
4. Cut the bands of fragment 1 (nearly 550bp) and fragment 2 (nearly 850bp).
5. Purify them by Favorgen gel purification Kit.

(iii) Secondary (overlapping) PCR of the EcoRI mutagenesis for xylR+PxylA.

PCR set-up:

Reagents	Volume
ddH_2O	Add to 20uL
Phusion® HF Buffer (5X)	4uL
Fragment 1	76.9ng
Fragment 2	123.1ng
Phusion® polymerase	0.5uL
Primer 1	0.5uL
Primer 4	0.5uL
dNTP	0.5uL
Reaction volume	20uL

Note that the total mass of fragment 1 and fragment 2 is 200ng. Their molar ratio is 1:1.

PCR program:

Steps	Temperature (Celsius)	Duration (minute : second)
1.Initial Denaturation	95	08:00
2.Denaturation	95	00:30
3.Annealing	71	00:30
4.Extension	72	01:30
5.Goto step 2 for 20 cycles	-	-
6.Final extension	72	08:00
7.Storage	8	infinite

(iv) PCR clean-up.

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

(v) Digestion of secondary PCR product and pSB1C3.

Digestion set-up:

Reagents	Secondary PCR product	pSB1C3
ddH_2O	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.

(vi) 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.

(vii) Ligation of digested secondary PCR product and pSB1C3.

Ligation set-up:

Reagent	Volume
ddH_2O	Add to 10uL
10X Invitrogen ligase buffer	1uL
Invitrogen T4 ligase	0.5uL
Insert (Digested secondary PCR product)	100ng
Backbone (Digested pSB1C3)	50ng

Incubate under 37 degree Celsius for 2 hours.

(viii) 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.

(ix) Plasmid extraction of xylR+PxylA (EcoRI mutated) in pSB1C3.

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

II. The second mutagenesis of the first XbaI illegal cutting site.

(i) The primary PCR of the first XbaI mutagenesis for xylR+PxylA.

Designed primers:

Primer name	Primer sequence
Primer 1	GTTTCTTCCTGCAGCGGCCGCTACTAGAGTACCCTTTGATTTAAGTGAACAAGT *Prefix*
Primer 4	GTTTCTTCGAATTCGCGGCCGCTTCTAGGAGCTCCTAACTTATAGGGGTAACAC

	TTAAAAAAGAATC Yellow Part: *Suffix*
Primer 2XI	CCGCCTCTTGATTGCCCTG *Mutation site*
Primer 3XI	CAGGGCAATCAAGAGGCGG *Mutation site*

PCR set-up:

Reagents	Fragment 1	Fragment 2
ddH_2O	Add to 20uL	Add to 20uL
Phusion® HF Buffer (5X)	4uL	4uL
Template – xylR+PxylA (EcoRI mutated) in pSB1C3	200ng	200ng
Phusion® polymerase	0.5uL	0.5uL
Primer 1 (10uM)	0.5uL	-
Primer 2XI (10uM)	0.5uL	-
Primer 3XI (10uM)	-	0.5uL
Primer 4 (10uM)	-	0.5uL
dNTP	0.5uL	0.5uL
Reaction volume	20uL	20uL

PCR program:

Steps	Temperature (Celsius)	Duration (minute : second)
1.Initial Denaturation	95	08:00
2.Denaturation	95	00:30
3.Annealing	71	00:30
4.Extension	72	01:10
5.Goto step 2 for 20 cycles	-	-
6.Final extension	72	08:00
7.Storage	8	infinite

(ii) Gel purification of primary PCR product.

1. Prepare a 40mL 0.8% agarose gel with CyberSafe as the stain.
2. Load the PCR product of fragment 1 and fragment 2.
3. Run the gel under 130 V for 60 minutes.
4. Cut the bands of fragment 1 (nearly 450bp) and fragment 2 (nearly 950bp).
5. Purify them by Favorgen gel purification Kit.

(iii) Secondary (overlapping) PCR of the EcoRI mutagenesis for xylR+PxylA.

PCR set-up:

Reagents	Volume

ddH_2O	Add to 20uL
Phusion® HF Buffer (5X)	4uL
Fragment 1	64.3ng
Fragment 2	135.7 ng
Phusion® polymerase	0.5uL
Primer 1	0.5uL
Primer 4	0.5uL
dNTP	0.5uL
Reaction volume	20uL

Note that the total mass of fragment 1 and fragment 2 is 200ng. Their molar ratio is 1:1.

PCR program:

Steps	Temperature (Celsius)	Duration (minute : second)
1.Initial Denaturation	95	08:00
2.Denaturation	95	00:30
3.Annealing	71	00:30
4.Extension	72	01:30
5.Goto step 2 for 20 cycles	-	-
6.Final extension	72	08:00
7.Storage	8	infinite

(iv) PCR clean-up.

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

(v) Digestion of secondary PCR product and pSB1C3.

Digestion set-up:

Reagents	Secondary PCR product	pSB1C3
ddH_2O	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.

(vi) 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.

(vii) Ligation of digested secondary PCR product and pSB1C3.

Ligation set-up:

Reagent	Volume
ddH_2O	Add to 10uL
10X Invitrogen ligase buffer	1uL
Invitrogen T4 ligase	0.5uL
Insert (Digested secondary PCR product)	100ng
Backbone (Digested pSB1C3)	50ng

Incubate under 37 degree Celsius for 2 hours.

(viii) 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.

(ix) Plasmid extraction of xylR+PxylA (EcoRI mutated, first XbaI mutated) in pSB1C3.

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

III. The second mutagenesis of the first XbaI illegal cutting site.

(i) The primary PCR of the first XbaI mutagenesis for xylR+PxylA.

Designed primers:

Primer name	Primer sequence
Primer 1	GTTTCTTCCTGCAGCGGCCGCTACTAGAGTACCCTTTGATTTAAGTGAACAAGT *Prefix*
Primer 4	GTTTCTTCGAATTCGCGGCCGCTTCTAGGAGCTCCTAACTTATAGGGGTAACAC TTAAAAAAGAATC Yellow Part: *Suffix*
Primer 2XII	CTAAATGAGAATGGACTCTTGAAGAAACTTCG *Mutation site*
Primer 3XII	CGAAGTTTCTTCAAGAGTCCATTCTCATTTAGAC *Mutation site*

PCR set-up:

Reagents	Fragment 1	Fragment 2
ddH_2O	Add to 20uL	Add to 20uL
Phusion® HF Buffer (5X)	4uL	4uL
Template – xylR+PxylA (EcoRI & first XbaI mutated) in pSB1C3	200ng	200ng
Phusion® polymerase	0.5uL	0.5uL
Primer 1 (10uM)	0.5uL	-
Primer 2XII (10uM)	0.5uL	-
Primer 3XII (10uM)	-	0.5uL
Primer 4 (10uM)	-	0.5uL
dNTP	0.5uL	0.5uL
Reaction volume	20uL	20uL

PCR program:

Steps	Temperature (Celsius)	Duration (minute : second)
1.Initial Denaturation	95	08:00
2.Denaturation	95	00:30
3.Annealing	71	00:30
4.Extension	72	01:30 for fragment 1 00:30 for fragment 2
5.Goto step 2 for 20 cycles	-	-
6.Final extension	72	08:00
7.Storage	8	infinite

(ii) <u>Gel purification of primary PCR product.</u>

1. Prepare a 40mL 2% agarose gel with CyberSafe as the stain.
2. Load the PCR product of fragment 1 and fragment 2.
3. Run the gel under 130 V for 60 minutes.
4. Cut the bands of fragment 1 (nearly 1250bp) and fragment 2 (nearly 150bp).
5. Purify them by Favorgen gel purification Kit.

(iii) <u>Secondary (overlapping) PCR of the EcoRI mutagenesis for xylR+PxylA.</u>

PCR set-up:

Reagents	Volume
ddH_2O	Add to 20uL
Phusion® HF Buffer (5X)	4uL
Fragment 1	178.6 ng
Fragment 2	21.4 ng
Phusion® polymerase	0.5uL
Primer 1	0.5uL
Primer 4	0.5uL

dNTP	0.5uL
Reaction volume	20uL

Note that the total mass of fragment 1 and fragment 2 is 200ng. Their molar ratio is 1:1.

PCR program:

Steps	Temperature (Celsius)	Duration (minute : second)
1.Initial Denaturation	95	08:00
2.Denaturation	95	00:30
3.Annealing	71	00:30
4.Extension	72	01:30
5.Goto step 2 for 20 cycles	-	-
6.Final extension	72	08:00
7.Storage	8	infinite

(iv) PCR clean-up.

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

(v) Digestion of secondary PCR product and pSB1C3.

Digestion set-up:

Reagents	Secondary PCR product	pSB1C3
ddH_2O	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.

(vi) 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.

(vii) Ligation of digested secondary PCR product and pSB1C3.

Ligation set-up:

Reagent	Volume
ddH_2O	Add to 10uL
10X Invitrogen ligase buffer	1uL
Invitrogen T4 ligase	0.5uL
Insert (Digested secondary PCR product)	100ng

Backbone (Digested pSB1C3)	50ng

Incubate under 37 degree Celsius for 2 hours.

(viii) 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.

(ix) Plasmid extraction of xylR+PxylA (fully mutated) in pSB1C3.

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