Lab plan for construct of *ydjM+Bmp2*

1. Final construct:

Signal peptide *ydjM* + Mature mouse *BMP2* in PSB1C3

2. Lab plan

2.1 PCR of amplification of *ydjM*

1. prepare a master mixture as follow:

dd H2O (μ l)	81
10X Buffer (μ l)	12
Forward primer (μ l)	3
Reserve primer (μ l)	3
Taq polymerase (μ l)	3
dNTP (μ l)	3
Template (Genomic DNA of B. *subtilis*) (20ng/ μ l)	15
Total volume (μ l)	120

2. Separate the master mix in 6 tubes, each 20 μ l.

PCR program

Process	Temperature (℃)	Time (s)
1. initial Denaturation	95	300
2. Denaturation	95	15
3. Annealing	60	15
4. Extension	68	15
5. Repeat cycle 29 times		
6. Final extension	68	300

Sequence of forward primer:

5” GATCATTCTAGAGTCCTAGATGTTAACAAACAGGGGG 3”

Sequence of reverse primer:

5” CCGCTGTTTGTGTTTGGCTTGCGCACTGGCATCTGATGAAAAAC 3”

2.2 Gel checking

1. prepare 2% agarose gel
2. Correct band size: 147 b.p.

2.3 PCR of amplification of *BMP2*

1. prepare a master mixture as follow:

dd H2O (μ l)	94.8
10X Buffer (μ l)	12

Forward primer (μ l)	3
Reserve primer (μ l)	3
Taq polymerase (μ l)	3
dNTP (μ l)	3
Template (*BMP2* with PBS)	1.2
Total volume (μ l)	120

2. Separate the master mix in 6 tubes, each 20 μ l.

PCR program

Process	Temperature (℃)	Time (s)
1. initial Denaturation	95	300
2. Denaturation	95	30
3. Annealing	60	30
4. Extension	68	60
5. Repeat cycle 29 times		
6. Final extension	68	300

Sequence of forward primer:

5” AGTTTTTCATCAGATGCCAGTGCGCAAGCCAAACACAAACAGCGG3”

Sequence of reverse primer:

5” ATGATCACTAGTATTATTAACGACACCCGCAGCCC3”

2.4 Gel checking

1. prepare 2% agarose gel
2. Correct band size: 386 b.p.

2.5 Overlapping PCR

1. Set up the mixture as follow.

Dd H2O (μ l)	60
10X Buffer (μ l)	12
dNTP	3
Template 1 *BMP2* (μ l)	36
Template 2 *YdjM* (μ l)	6
Taq polymerase (μ l)	3
Total volume (μ l)	120

2. Separate the master mix in 6 tubes, each 20 μ l.

PCR program

Process	Temperature (℃)	Time (s)
1. initial Denaturation	95	300

2. Denaturation	95	30
3. Annealing	57	30
4. Extension	68	60
5. Repeat cycle 29 times		
6. Final extension	68	300

Size of PCR product: 487 b.p.

3. Add 0.5 μl (forward primer for amplifying *YdjM*) and 0.5 μl (new reverse primer for amplifying *BMP2*) into each PCR tube.

4. Do PCR again.

PCR program

Process	Temperature (℃)	Time (s)
1. initial Denaturation	95	300
2. Denaturation	95	30
3. Annealing	72	30
4. Extension	68	60
5. Repeat cycle 15 times		
6. Final extension	68	300

Sequence of forward primer:

5” GATCATTCTAGAGAAAGGAGGTTGTTTGCATGGTG 3”

Sequence of reverse primer:

5” ATGATCCTGCAGCGGCCGCTACTAGTATTATTAACGACACCCGCAGCCC3”

2.6 Gel checking

1. prepare 2% agarose gel
2. Correct band size: 501 b.p.

2.7 gel purification

1. Cut the gel which contains DNA band
2. Use DNA purification kit from Favorgen.

2.8 Double digestion

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

2.9 Dephosphorylation

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

2.10 Heat kill enzyme

Heat digestion mixture with 80℃ / 20 minutes

2.11 Gel check of backbone and insert

1. Gel check of insert
    1. Prepare 2% agarose gel

2. Correct band size: ~485b.p.

2. Gel check of backbone
    1. Prepare 1% agarose gel
    2. Correct band size: ~2070 b.p.

2.12 Purify digested insert and backbone

1. Cut gel and use kit to purify insert and backbone.

2.13 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.

2.14 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.

2.15 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.

2.16 Single digestion check of plasmid extracted from bacteria

1. Set up the digestion mixture. Digest the plasmid with StyI.
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: 473b.p. and 2058b.p.

2.17 Sequencing of plasmid

Sequence of the insert in the biobrick

GAATTCGCGGCCGCTTCTAGAGTCCTAGATGTTAACAAACAGGGGGACGAA**atg**TTGAAGAA
AGTCATTTTAGCCGCTTTTATCTTAGTAGGAAGTACTTTGGGAGCTTTTAGTTTTTCATCAGATG
CCAGTGCGCAAGCCAAACACAAACAGCGGAAGCGCCTCAAGTCCAGCTGCAAGAGACACCC
TTTGTATGTGGACTTCAGTGATGTGGGGTGGAATGACTGGATCGTGGCACCTCCGGGCTATCA
TGCCTTTTACTGCCATGGGGAGTGTCCTTTTCCCCTTGCTGACCACCTGAACTCCACTAACCAT
GCCATAGTGCAGACTCTGGTGAACTCTGTGAACTCCAAAATCCCTAAGGCATGCTGTGTCCCC
ACAGAGCTCAGCGCAATCTCCATGTTGTACCTAGATGAAAATGAAAAGGTTGTGCTAAAAAAT
TATCAGGACATGGTTGTGGAGGGCTGCGGGTGTCGTtaataaTACTAGTAGCGGCCGCTGCAG