PROLUNG

BIOCONTAINMENT

TRYPTOPHAN REGULATORY SYSTEM

LAB BOOK 2

iGEM Stockholm

Tryptophan system cloning

Objective

The aim is to clone the tryptophan system into the pSB1C3 backbone. The samples that will be digested are the tryptophan system and the biobrick BBa_M30011. BBa_M30011 contains RFP, which enables quick screening of the colonies after ligation.

Procedure

Digestion

Keep all reagents on ice and add enzymes last.

Tango buffer	2ul
EcoRI-HF	1.1ul
PstI	1.1ul
DNA (20ng/ul)	5ul
RNase-free water	To 25ul

Incubation: 37C/1h
Heat-deactivation: 80C/20min

Ligation

For ligation of AsR + RBS, three different molar ratios of vector and insert were used: 1:3,1:5 and 1:7.

Calculations are performed with the help of the online calculator provided by University of Dusseldorf: http://www.insilico.uni-duesseldorf.de/Lig_Input.html

Thaw T4 ligase buffer on ice. Mix all reaction components. Add enzyme last. Keep on ice.

	Volume (20ul)
T4 buffer	2
T4 ligase	1
Vector	12.5
Tryptophan	12.5
RNase-free water	To 20

Incubation: 16C/overnight
Heat-deactivation: 65C/20min

Transformation

Performed according to standard transformation protocol.

The transformation did not yield any colonies. Therefore, the procedures were repeated.

Digestion

Since the previous experiment was unsuccessful, it was repeated using more DNA and a different protocol.

Keep all reagents on ice and add enzymes last.

	Tryptophan	BBa_M30011 (RFP backbone)
10XCutSmart	5ul	5ul
EcoRI-HF	1ul	1ul
PstI-HF	1ul	1ul
DNA (1ug)	35ul	17.6ul
RNase-free water	To 50ul	To 50ul

Incubation: 37C/1h

PCR purification
Performed according to the QiAquick PCR Purification Kit protocol provided by Qiagen.

Ligation
For ligation of AsR + RBS, three different molar ratios of vector and insert were used: 1:3,1:5 and 1:7.
Calculations are performed with the help of the online calculator provided by University of Dusseldorf: http://www.insilico.uni-duesseldorf.de/Lig_Input.html

Thaw T4 ligase buffer on ice. Mix all reaction components. Add enzyme last. Keep on ice.

1 indicates PCR purified vector was used and 2 indicates heat-inactivated vector.

Component	**Trp + pSB1C3 1**	**Trp + pSB1C3 2**
T4 ligase buffer	3ul	2ul
T4 ligase	1.5ul	1ul
Insert DNA	12.5ul	12.5ul
Vector DNA	12.5ul	6.25ul
RNase-free water	0.5ul	6.25ul
Total	**30**	**21.75**

Incubate at 16C/overnight
Heat-inactivate 65C/20min

Transformation
Performed according to standard transformation protocol.

Colony PCR cumate system + pSB1C3
Determine which colonies contain the insert of interest.

Obtaining DNA from colonies.

With a sterile pipette tip, pick a single colony from the respective plate and thoroughly swirl it in 20ul of RNase-free water. Discard the tip. 1ul of this water will be used for the PCR reaction. The rest is plated on an agar plate with the appropriate antibiotic in order to receive whole colonies.

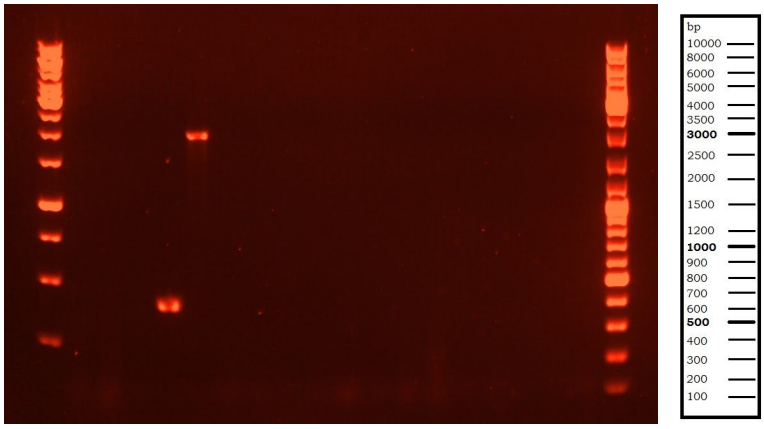
Reaction setup

Component	Volume (ul)
Q5 Hi-Fi Master Mix	25
Primer mix	1.2ul
Colony DNA	1ul
RNase-free water	23ul
Total	50ul

Set conditions according to Table 2.

Table 2. PCR conditions

Step	Temperature/Time
Initial denaturation	94C/5min
25 cycles:	
Denaturation	98C/10s
Annealing	69C/15s
Extension	72C/2min
Final extension	72C/5min
Hold	4C/-



Ladder: M; Lanes 10-17: tryptophan colony PCR

Discussion

No band was detected in the tryptophan-containing wells. Therefore, the picked colonies did not contain the insert.

Amplification of Im2

Objective

Amplify the Im2 fragment from gBlock 2.

Procedure

PCR

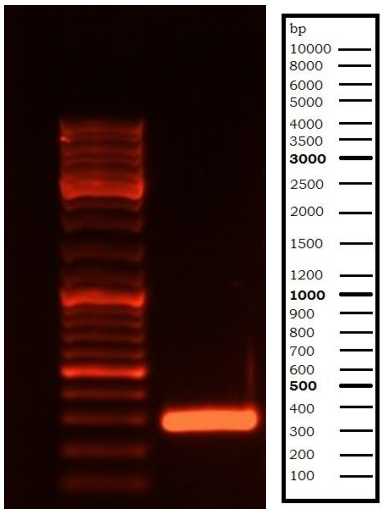
Primer solution: 1ul of FP+XmaI + 1ul of RP+MfeI + 8ul of RNase-free H2O
Keep reaction components on ice. Put Q5 in last.

50ul rxn:

Q5 2X MM	25ul
gBlock 2	1ul
Primer solution	1.2ul
RNase-free H2O	22.8ul

Conditions:

Initial denaturation	94C/1min
25 cycles:	
Denaturation	98C/15s
Annealing	64C/15s
Extension	72C/22s
Final extension	72C/3min
Hold	4C/-



Ladder: M

Amplification of tryptophan regulatory system

Objective

Amplify the tryptophan system gBlock for further use

Procedure

PCR

Primer solution: 1ul of prefix+scar + 1ul of suffix+scar + 8ul of RNase-free H2O
50ul rxn:

Q5 2X MM	25ul
Trp gBlock	1ul
Primer solution	1.2ul
RNase-free H2O	22.8ul

Keep all on ice. Put Q5 in last.

Conditions:

Initial denaturation	98C/1:30min
25 cycles:	
Denaturation	98C/10s
Annealing	69C/15s
Extension	72C/1:20min
Final extension	72C/2min
Hold	4C/-

Tryptophan system was not run on a gel.

PCR purification of Im2 and Trp

Performed according to the QiAquick PCR Purification Kit protocol provided by Qiagen.