PROLUNG

BIOCONTAINMENT

FIRST GBLOCKS

LAB BOOK 5

iGEM Stockholm

Assembly of gBlocks with pSB1C3

Objective

Assemble gBlocks with the plasmid vector using the NEBuilder MM from NEB.

Procedure

According to NEBuilder HiFi DNA Assembly protocol

Set up the reaction on ice according to the table below:
Vector:insert ratio: 1:2

	25ng vector	50ng vector
gBlock 1	1.5ul	3ul
gBlock 2	2.5ul	5ul
Master Mix	10ul	10ul
RNase-free water	6ul	2ul
Total	20ul	20ul

Incubate samples in a thermocycler at 50C for 15 minutes.

Note: Extended incubation up to 60 minutes may help to improve assembly efficiency in some cases
Store samples on ice or at -20C for subsequent transformation.

Transformation

Performed according to standard transformation protocol.

Results

No colonies observed on the plates.

Im2 PCR

Primer resuspension:
Spin down dried oligos for 4-5s.

Add appropriate volume of RNase-free water to each (according to IDT datasheet received with said primers)

For forward primer Im2: for 100uM add 242ul

For reverse primer Im2: for 100uM add 191ul

Make a working solution of the primers by mixing 1ul of each primer and adding them to 8ul of RNase-free water.

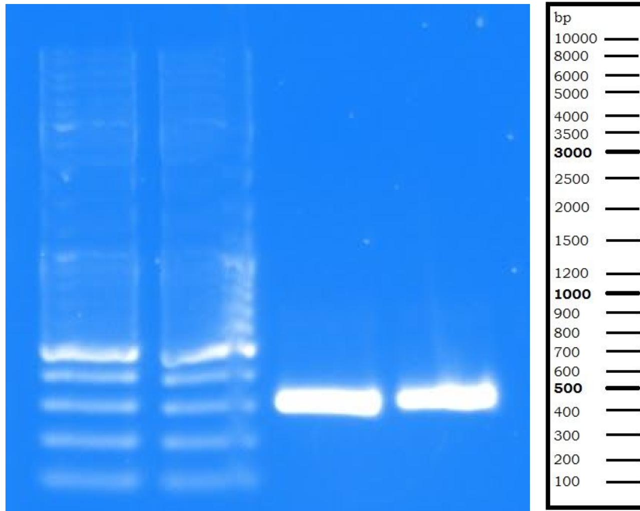
Reaction setup:
2 samples were prepared
Keep reaction mix on ice.

Component	Volume (ul)
Q5 Hi-Fi Master Mix	25
Primer mix	1.2
gBlock 2 (DNA)	0.8
RNase-free water	23
Total	50ul

Table 2. PCR conditions

Step	Temperature (℃)	Time
Initial denaturation	94	60s
25 cycles:		
Denaturation	98	15s
Annealing	62	15s
Extension	72	22s
Final extension	72	3min
Hold	4	-

Gel electrophoresis: 5ul PCR product + 1ul 6xLD, 2.5ul ladder; 110V for 45min.



Ladder: M

PCR purification of PCR Im2
Performed according to the QiAquick PCR Purification Kit protocol provided by Qiagen.

NanoDrop:
Abs: 0.766
260/280: 1.89
260/230: 1.85
70.8 ng/uL

Transformation with Bba_K880005

Procedure
Resuspension of dried down DNA followed by transformation

Add 10uL to Distribution plate 7, Well 3F → Bba_K880005, 2125 bp size
Store at -20C after use

Transformation
Performed according to standard transformation protocol.

Cloning of Im2 and pSB1C3

Objective

Clone Im2 into the pSB1C3 plasmid backbone.

Im2 purified from previous entry (70.8 ng/uL)
pSB1C3 (25ng/ul)

Procedure

Digestion

Keep all components on ice. Add enzymes last.

	Im2	MasterMix
NEBuffer 2	2.5	5
EcoRI-HF	0.5	0.5
PstI	0.5	0.5
BSA	0.5	0.5
DNA	5	-
RNase-free water	11	18.5
Total	20	25

For the digestion of the backbone mix 4ul of the MAsterMix with 4ul of the plasmid DNA to a total volume of 8ul.
Incubate for 20 minutes at 37C.
Heat inactivation for 20 minutes at 80C.

Ligation

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.

	1:1	1:3
T4 ligase buffer	1	1
T4 ligase	0.5	0.5
Vector (pSB1C3)	4	2
Insert (Im2)	0.5	1
RNase-free water	4	5.5
Total	10	10

Incubate for 20 minutes at room temperature.
Chill on ice.

Transformation

Performed according to standard transformation protocol.

Cloning of Im2 and BBa_B0015

Objective

Introduce Im2 into a double terminator in order to create a composite BioBrick.

Procedure

Cloning of Im2 into BBa_B0015 (backbone: pSB1C3)

Im2 PCR purified (70.8 ng/uL, 321bp)
Bba_B0015 (144 ng/uL, 2199 bp)

Digestion

Keep all components on ice and add enzymes last.

	Im2	BBa_B0015
10X CutSmart buffer	5	5
EcoRI-HF	1	1
SpeI	1	-

XbaI	-	1
DNA	10	7
RNase-free water	33	36
Total	50ul	50ul

Incubate for 20 minutes at 37C
Heat inactivation for 20 minutes at 65C

Ligation

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.

	1:3	1:5
T4 ligase buffer	2	2
T4 ligase	1	1
Vector (BBa_B0015)	2.5	2.5
Insert (Im2)	1.7	2.6
RNase-free water	12.8	12
Total	20	20

Incubate for 20 minutes at room temperature
Chill on ice

Transformation

Performed according to standard transformation protocol.

<u>Results</u>

Colonies were found on both plates; 1:3 ratio yields more colonies

MiniPrep and glycerol stocks

Im2+DT, Im2+pSB1C3, K880005;
2x4ml taken from each for MiniPrep.

Performed according to QIAprep Spin Miniprep Kit protocol provided by Qiagen. Note: Half of the samples were eluted with RNase-free water (samples labeled 1), while the rest were eluted with EB buffer (labeled 2).

NanoDrop measurements:
K88 = BBa_K880005

	Im2+DT 1	Im2+DT 2	Im2+pSB 1C3 1	Im2+pSB 1C3 2	K88 1	K88 2
Abs	0.293	0.325	0.247	0.333	0.354	0.385
A-260 10mm path	0.593	0.61	0.523	0.641	0.749	0.795
A-280 10mm path	0.314	0.341	0.263	0.347	0.39	0.427
260/280	1.39	1.79	1.99	1.85	1.88	1.86
260/230	2.03	1.88	2.12	1.93	2.12	2.06
ng/ul	29.7	30.5	26.2	32.1	37.5	39.7

Digestion

Im2+DT, K880005
Im2: 16.4uL used
K880005: 12.6uL used

Reaction setup according to an optimized protocol from Roche. Keep all components on ice. Add enzymes last.

DT = BBa_B0015

	Im2+DT	K88
Buffer H	2.5	2.5

DNA	16.4	12.6
XbaI	0.2	-
PstI	0.2	0.2
SpeI	-	0.2
RNase-free water	6	10
Total	25ul	25ul

Incubation: 37C/1h
Deactivation: 80C/20min

Ligation

Im2+DT (vector)
K88 (insert)

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.

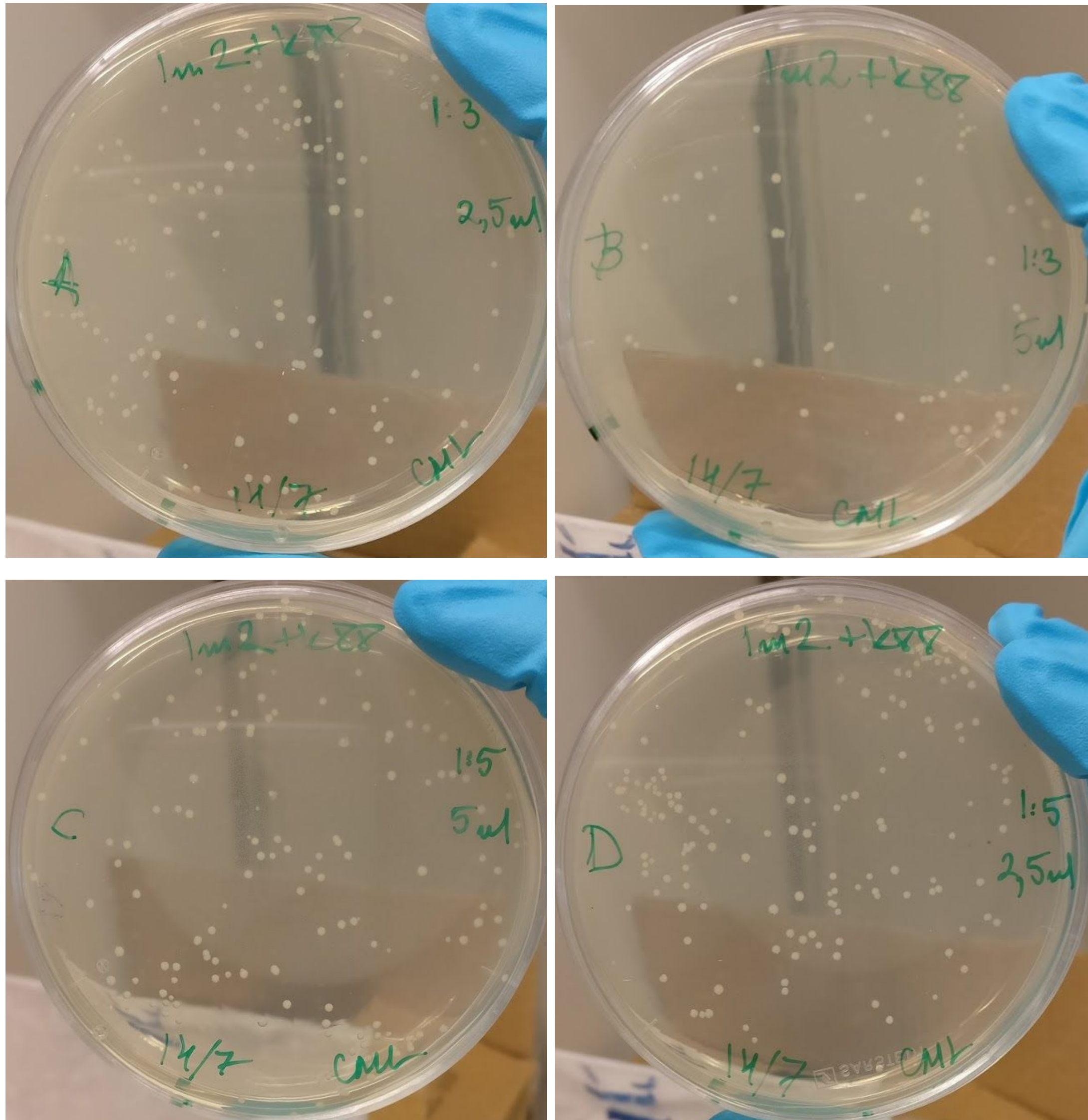
1:3 / 1:5

	1:3	1:5
10X buffer	3	3
T4 ligase	1.5	1.5
Vector (Im2+DT)	2.5	2.5
Insert (K88)	2	3.4
RNase-free water	21	19.6
Total	30ul	30ul

Incubation: RT/1h
Deactivation: 65C/10min

Transformation of Im2+DT and K88

Performed according to standard transformation protocol.



Colony PCR

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 with genomic integration.

Reaction setup x3. Note: Keep all on ice.

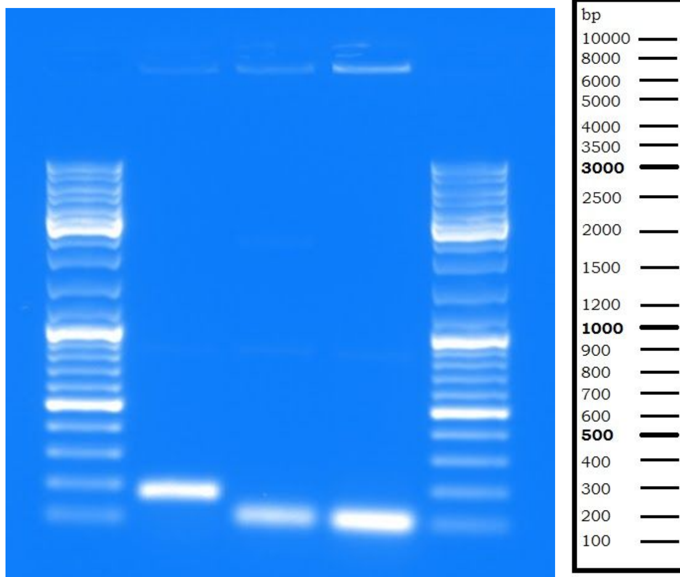
Component	Volume (ul)
Q5 Hi-Fi Master Mix	25
Primer mix	1.2ul
Colony DNA	1ul
RNase-free water	23ul
Total	50ul

Set conditions according to Table 2.

Table 2. PCR conditions

Step	Temperature (℃)	Time
Initial denaturation	98	6min
25 cycles:		
Denaturation	95	10s
Annealing	69	15s
Extension	72	30s
Final extension	72	2min
Hold	4	-

Gel 110V/45 mins



Ladder: M

Bands correspond to Im2 and DT by themselves, meaning that the ligation did not work. There are some very faint bands above, but too faint to be useful.