LabBook :

4 july 2016 :

- ✓ Bottles of LB and LB-Agar (200ml and 400ml):
  LB-Agar: 14g for 400ml of water
  LB: 8g for 400ml of water
  Autoclave

- ✓ Resistance test of *P.putida* :
  2 plates of LB-Agar with either Kanamycin (50µg/ml) or Spectinomycin (50µg/ml).

- ✓ Preparation of HEPES solution 0,5M (for electroporation):
  11g of HEPES (solid) in 64ml of water
  NaOH (0,1M) for pH7

- ✓ Refresh *P.putida* (the Petri dish got old)

- ✓ *E. coli C118* with plasmids pSEVA2311 (KanR) and pSEVA224 (KanR) were separately spread in LB.

  → cells with pSEVA2311 were observed

  → cells with pSEVA224 were not observed!! → Jonathan asked for another sample

5 july 2016 :

- ✓ LB culture of *E. coli C118*
- ✓ HEPES filtration
- ✓ Check the inventory of the fridge
- \- Plate of *P.putida* at 30°C and 37°C → We didn't do this step
- ✓ Liquide culture (5ml of preculture) of *P.putida* to have competent cells

6 july 2016 :

- ✓ Preculture for transformation (5ml) of E. coli DH5 alpha…

7 july 2016 :

- ✓ <u>Miniprep of E. coli C118 with pSEVA2311</u>
  Protocol "Midiprep" with 50ml of culture
  C= 80ng/µl in 200µl of elution buffer
  OD260= 1,616
  OD280= 0,8

- ✓ Transformation of Pseudomonas with pSEVA2311
  40ml of culture (competent cell):
  When OD=0,6 :
  - 20ml for Heat Shock
  - 20ml for Electroporation

- ✓ After electroporation and heat-shock spread on Amp-Plates

8 july 2016 :

- ✓ The transformation of 07/07/2016 didn't work because the plasmid given by Robert didn't match with Pseudomonas→ Second transformation (Heat shock and electroporation)

- ✓ Preparation of plates (LB+Antibiotics):
  - 10 plates LB-Agar, Kanamycin (50 µg/ml)
  - 10 plates LB-Agar, Chloramphenicol (25 µg/ml)
  - 10 plates LB-Agar, Ampicillin (100 µg/ml)
  - 10 plates LB-Agar, without antibiotic

12 july 2016 :

- ✓ Preparation of M9-media (10X, V= 600ml) with different kind of C-sources:
  - Glucose → DONE
  - Glycerol → DONE
  - Fructose → NO YET
  - Benzoate → NO YET
  - Lactic acid → NO YET
  - Methan → NO YET

Compounds	**Cf**	**Mass (g) or volumes (ml)**
CaCl2.H2O	1 mM	0,0882 g
MgSO4	20 mM	1,44 g
FeSO4.H2O	0,1 mM	0,01668 g
M9 (salt)	10 X	67,68 g

Glycerol (100%) *	8% v/v	60,48 g
Casamino acid	2% w/v	12 g
Thiamine 10mg/ml **	10 µg/ml	(0,6 g)
Uracil	200 µg/ml	0,12 g
Leucine	300 µg/ml	0,18 g
NaOH	pH 6,6	-

*Glucose : 59,22g;
** Thiamine has not been added but it has to be before the preculture of *P.putida*.
→ problem: precipitation

13 july 2016

- ✓ Preparation of the deletion process – Transformation of E. coli Mach1 competent cells (CaCl2 treatment) by Heat Shock:

  - Plasmids pKD46, pCP20 and pKD4
  - 3 tubes where 25µl of cells were mixed with 1,5µl of each plasmid (pKD4, pCP20 or pKD46)
  - Incubation on ice 20 min
  - Heat at 42°C (thermocycler) 1min
  - 120µl of LB added to each tube
  - Incubation: 30min at 37°C for the plasmid pKD4, 30°C for the plasmids pCP20 and pKD46
  - Spread on plates (Ampicilline 100 µg/mL)
  - Incubation overnight at 37°C for pKD4, 30°C for pCP20 and pKD46

- ✓ Preparation for the deletion process – Preparation of E. coli DH5 alpha competent cells:
  - 5ml of preculture of DH5 alpha were prepared
  - Incubation overnight at 37°C

14 july 2016 :

- ✓ pKD46 and pCP20 transformation properly worked
- ✓ pKD4 didn't succeed
- ✓ Preparation of 5ml culture of pKD46 and pCP20 to purify plasmid
- ✓ pKD4 since it is not needed for transformation will be used w/o amplification

✓ DH5 alpha competent cells were prepared and stored

15 july 2016 :

✓ Midiprep of pCP20 and pKD46:
- pCP20: 65ng/µl
- pKD46: 50ng/µl

✓ Transformation of P.putida KT2440 with pKD46 plasmid to start deletion process
Incubation overnight at 30°C (Amp Plates)

✓ Spread KT2440 WT and check growth at 37°C

16 july 2016 :

✓ Check transformation results → cells grew on Ampicillin at 30°C

✓ Pick 4 clones from previous plate and spread them in a new plate (Amp with proper concentration) and incubate it at 30°C.

***Pseudomonas Putida* : Working concentration of Antibiotics**

Kan (50µg/ml) → 1,7 µl/ml

Amp (100µg/ml) → 5 µl/ml

Spec (50µg/ml) →1 µl/ml

✓ KT2440 grew well at 37°C

18 july 2016 :

✓ Electroporation of *P.putida* : pKD46
- Electro-competent cells have been prepared

- 1,5 µl of DNA were added to ~ 50µl of electro-competent cells (all the tube)
- 1ml of LB was transfer in a 1,5ml tube
- 500µl of the LB were prepare in the pipette-tip and put rapidly in the electro-cuve after electroporation (at 1800V)
- All the cells+LB of the cuve were transferred to the 1,5 ml tube containing LB
- Incubation + Shaking 1h at 30°C
- Centrifugation 3min 90rpm
- Cells were plated in LB Agar Ampicillin (100 µg/µL) And incubated at 30°C

✓ Spread C118 (pSEVA224) on plates with Kanamycin

19 july 2016 :

✓ The transformation (Electroporation of 18/08) didn't work → maybe the replicon doesn't match with P.Putida

✓ Colony PCR: to check the transformation of P.Putida with p2311

1. Tick one colony and with the same tip, spread a little area on LB plate and dissolve the rest in 10 uL ddH2O

1. Gently vortex and briefly centrifuge DreamTaq Green PCR Master Mix (2X) after thawing.

1. Place a thin-walled PCR tube on ice and add the following components for each 50 µL reaction:

DreamTaq Green PCR Master Mix (2X)	1X	25 µL
Forward primer	0.5 µM	0.25 µL
Reverse primer	0.5 µM	0.25 µL
Colony water	----	2 µL
Water, nuclease-free	----	22.5 uL

1. Gently vortex the samples and spin down.

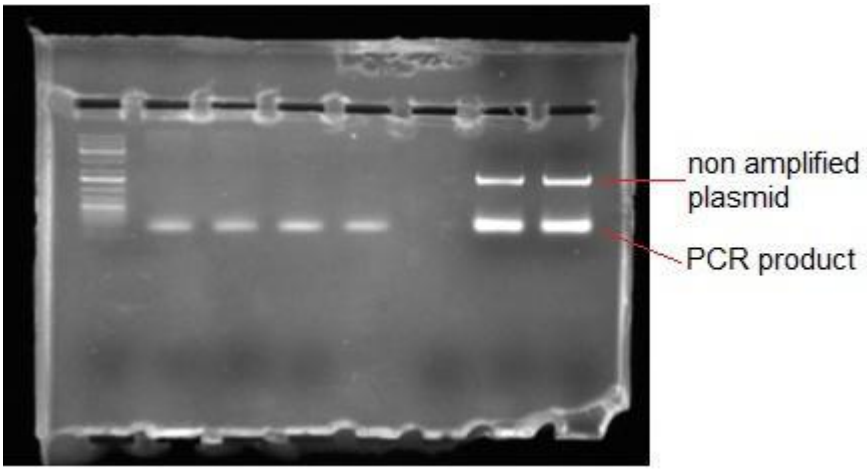
1. Perform PCR using the recommended heat cycling conditions outlined below:

Step	Temperature °C	Time	Number of cycles
Initial denaturation	95 °C	5 min	1
Denaturation	95 °C	30 s	30
Annealing	55(Tm)-5 °C	30	
Extension	72 °C	1 min/kb	
Final extension	72 °C	10 min	1
Pause	4 °C	“00”	---

1. Load 5-15 µL of PCR mixture directly on a gel.

✓ Electrophoresis to check the transformation
- 1% of agarose
- Duplicatas have been done

PCR Colony					PCR		
Marker	**Pseudo. HeatShock 1**	**Pseudo. HeatShock 2**	**Pseudo. Electropor. 1**	**Pseudo. Electropor. 2**	**__**	**PCR product 1**	**PCR product 2**



✓ PCR of pKD4:

- Mix of 100µl

Reaction buffer	20 µl
dNTP mix	2 µl
Primers (reverse and forward)	0,5 µl
Template (pKD4)	1 µl
Q5 polymerase	1 µl
H2O	Qs 100 µl (75,5 µl)

- Cycles

Temperature	**Time**	**Number of cycles**
98 °C	30”	1
90 °C	30”	32
60 °C	30”	
72 °C	1’	
72 °C	5’	1
4 °C	“00”	---

✓ Culture of E. coli C118 pSEVA for midi prep’

20 july 2016 :

✓ Midiprep’ of pSEVA224 (from E. coli)
C=59,9 ng/µl in 200µl of elution buffer

➔ DO260/DO280 = 1,97

✓ Alternative to delete gene:
Transformation of Pseudomonas by a PCR product: Kanamycin resistance cassette + homologous region 5' and 3' using Heat Shock and Electroporation.

→ See protocols for both transfo'

→ We used 10µl of DNA

✓ Preparation of 7 plates for *Pseudomonas* (1,7µl/ml of kanamycin)

✓ Liquid culture of E. coli pSEVA224 (2ml) :

- 2ml of LB
- 3,4µl of Kan → We made a mistake: we should have put 2µl of Kan (since we used E. coli)

✓ Preculture to amplify the vector given by Cyril
(BBa.R0010 pLac)

- E. coli G3A
- 50ml of LB
- 50µL of Amp

21 july 2016 :

!!! The fridge and freezer were off.

✓ The transformation of the PCR product (for the deletion process) didn't worked
  - ➔ We will focus on the construction of the plasmid

✓ Store the culture (in glycerol (1vol of glycerol+1vol of the culture)
✓ Midiprep of the vector R0010
  - ➔ 325ng/µl
  - ➔ DO260/DO280=1,8

✓ Preparation of Ampicillin solution stock 50mg/ml in H2O (Helix) aliquoted in 5 tubes of 2ml.

In sterile condition:

0,5g of Amp + 10 ml of H2O

Filtration (0,2µm)

Storage away from light

✓ Digestion of:

- the vector (with Ba.R0010 pLac from Cyril) by EcoRI (E) and SpeI (S)
- the vector (with Ba.R0010 pLac from Cyril) by EcoRI and PstI (P)
- the phaC sequence by EcoRI and SpeI
- the PropionylCoA synthase sequence by EcoRI and PstI

- By the way, we tried to remove the promoter (BBa.R0010 pLac) from the vector.
- 100µl of Water were added to the phaC sequence and the PropionylCoA synthase sequence.

	Vector (E/S)	**phaC (E/S)**	**Vector (E/P)**	**propCoA(E/P)**
DNA	2µl	10µl	2µl	10µl
Buffer	Cutsmart 10X: 3µl	Cutsmart 10X: 3µl	2.1: 3µl	2.1: 3µl
Enzymes	EcoRI: 1µl SpeI: 1µl	EcoRI: 1µl SpeI: 1µl	EcoRI: 1µl PstI: 1,5µl	EcoRI: 1µl PstI: 1,5µl
H2O	23µl	15µl	22,5µl	14,5µl

➔ 1h at 37°C

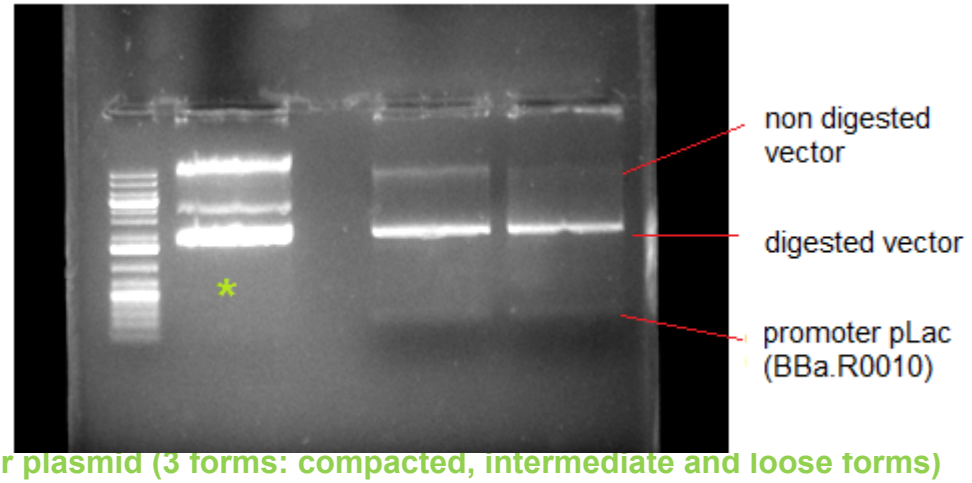
✓ Electrophoresis

- 1% of Agarose

Wells:	**Marker (8µl)**	**Control vector with pLac (5µl DNA+1µl loading buffer)**	**Vector E/S (30µl DNA +6µl loading buffer)**	**Vector E/S (30µl DNA +6µl loading buffer)**
Expected results	__	1 band (2270 bp)	2 bands (2070 bp + 200bp)	2 bands (2070 bp + 200bp)

- Vector (with pLac): 2270 bp

- Promoter pLac ~ 200 bp
- Results



*circular plasmid (3 forms: compacted, intermediate and loose forms)

- We get rid of the promoter and we kept the empty vectors

✓ Purification of the DNA sequences phaC and prop-CoA synth
- Zymoclean Gel DNA Recovery Kit
- Elution with 6µl of water
- Wait 1-2min before the last spin
- The check at the spectrophotometer nanodrop showed a pic around 240nm
  - ➔ Maybe: problem with the wash buffer reminded in the column
  - ➔ Inversion of the 2 columns before the second spin

22 july 2016 :

✓ Ligations

Empty vector (R0010)	10µl
Insert **phaC**	20µl
T4 buffer 10X	4µl
T4 ligase	2µl
H2O	5µl

Empty vector (R0010)	10µl
Insert **propCoA synth.**	20µl
T4 buffer 10X	4µl

T4 ligase	2µl
H2O	5µl

- Incubation 1h at RT

✓ Transformation of E. coli DH5 alpha, Top 10 and BL21 by Heat Shock
- Same protocol
- We didn't recover enough DNA, insert from previous midiprep, purification.
- These strains were spread on labeling plates:

22/07/16 E. coli phaC DH5 alpha	22/07/16 E. coli phaC Top10	22/07/16 E. coli phaC BL21
22/07/16 E. coli propCoA DH5 alpha	22/07/16 E. coli propCoA Top10	22/07/16 E. coli propCoA BL21

- Incubation overnight at 37°C

23 july 2016 :

✓ "Transformed" clones were observed.
✓ Colony PCR.

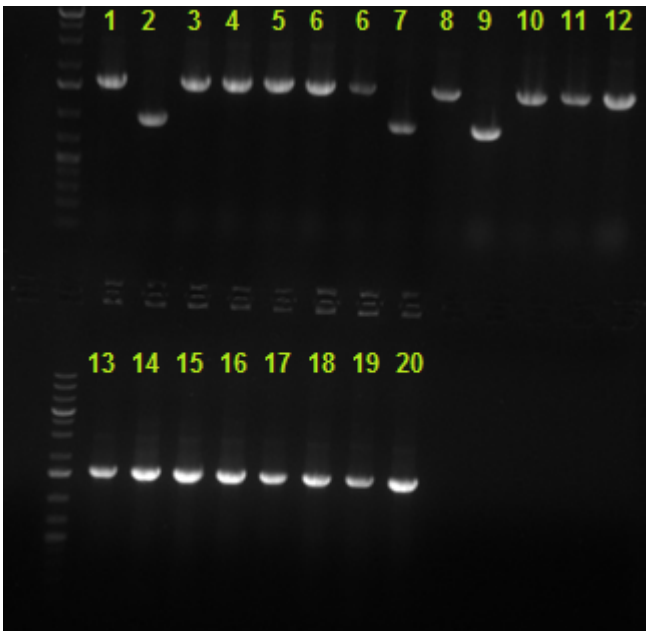
25 july 2016 :

✓ Media :
- 5 bottle of LB:
  - 3 x 400 ml
  - 2 x 200 ml
- 5 bottle of LB-Agar:
  - 3 x 400 ml
  - 2 x 200 ml

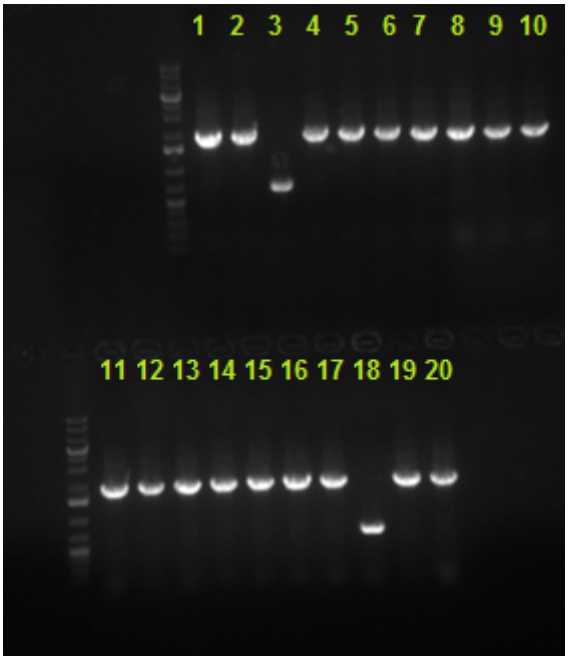
✓ Previous transformations (22/07) worked.

✓ Electrophoresis (PCR colony 23/07)

- Gel 1%
- propCoA (in Cyril's vector):



- phaC (in Cyril's vector) :



✓ Precultures:
- 2 ml of LB
- Amp (1µl/ml)

- A good colony:
  - phaC : 1, 2, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 19, 20.
  - propCoA :1, 3, 4, 5, 6, 8, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20.
- Incubation overnight at 37°C

26 July 2016 :

✓ Miniprep' kit Sigma-Aldrich : extraction of the plasmids from the precultures of the 26th of july
- For the propCoA transferase : problem with the 8th tube we lost a lot of bacteria*

Colonies (with the phaC)	1	2	4	5	6	7	8	9	10
Concentrations of DNA (ng/µl)	282.3	170.4	174.6	176.5	188.9	167.8	185.9	191.4	173.4
Colonies (with the phaC)	11	12	13	14	15	16	17	19	20
Concentrations of DNA (ng/µl)	160.9	173.0	170	185.3	162	189.5	193.6	183	163

Colonies (with the propCoA)	1	3	4	5	6	8	10	11	12
Concentrations of DNA (ng/µl)	150.5	143.4	156	151.8	234.2	29.4*	194.5	167.2	162.8
Colonies (with the propCoA)	13	14	15	16	17	18	19	20	-
Concentrations of DNA (ng/µl)	169.4	282.8	192.3	226	203.3	175.1	256.5	181.7	-

✓ M9 medium 2 x 50 ml of 2,5 X
- Without C-sources, thiamine.

→ Problem: precipitation (the same than previous M9 media)

27 july 2016 :

- ✓ Stock solutions:
  - CaCl2 1 M: 27,745 g in 250 ml of milliQ
  - MgSO4 1 M: 30,1 g in 250 ml of milliQ
  - FeSO4 10 mM: 0,379 g in 250 ml of milliQ

- ✓ Preculture of Pseudomonas putida KT2440 (2ml)

27 july 2016 :

- ✓ Aliquots for sequencing V= 20 µl

- ✓ Medium M9 1X without Carbone source and filtred (2µm) in 2 falcon of 50 ml:
  - Casamino acid : 1,2 g
  - Leucine: 3 mg
  - Uracil: 2 mg
  - Thiamine 10 mg/ml: 10 µl
  - M9 salt 5X: 20 ml
  - CaCl2 1 M: 10 µl
  - MgSO4 1 M: 100 µl
  - FeSO4 10 mM: 1 µl
  - Water milliQ qs 100 ml
  - NaOH for pH between 6 and 7,4 → no need since the pH was already at 6,8
    - ➔ **No precipitation**

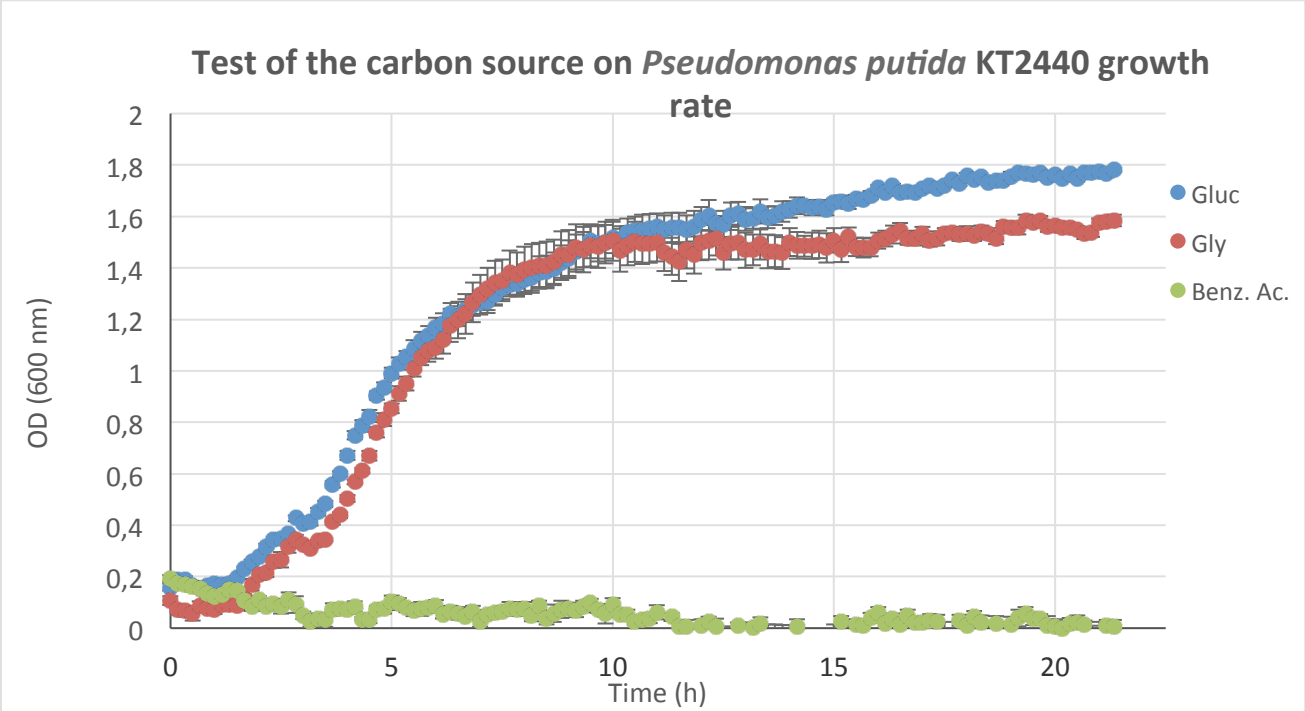
- ✓ 10 ml of Media with C-source:
  - 0, 10037 g of Glycerol (100%) *(0,16 put)* in 10 ml of milliQ water
  - 0, 0982 g of Glucose in 10 ml of milliQ water
  - 0, 057 g of Benzoic acid in 10 ml of milliQ water

- ✓ Preparation for plate (96 wells) and Growth rate with the CLARIO star machine:
  - 26, 7 µl of the overnight preculture of Pseudomonas in medium M9 (with either Benzoic acid, Glucose or Glycerol) qs 1 ml to have around OD= 0,005.

	1	2	3	4
A	-	Benzoic acid M9 medium	Glucose M9 medium	Glycerol M9 medium

B	Benzoic acid M9 medium	BE-M9 with cells	BE-M9 with cells	BE-M9 with cells
C	Glucose M9 medium	GLU-M9 with cells	GLU-M9 with cells	GLU-M9 with cells
D	Glycerol M9 medium	GLY-M9 with cells	GLY-M9 with cells	GLY-M9 with cells

- Plate mode, slow kinetic, DO 600 nm, T=30°C, shaking (200rpm) before cycle of 10 min



Growth rate of Pseudomonas putida in M9 minimal medium supplemented with 3 different carbon source.

2 August 2016

Resolubilisation of IDT gBlocks in 100 µL water (Cf=10ng/µL)

Digestion

Mix:

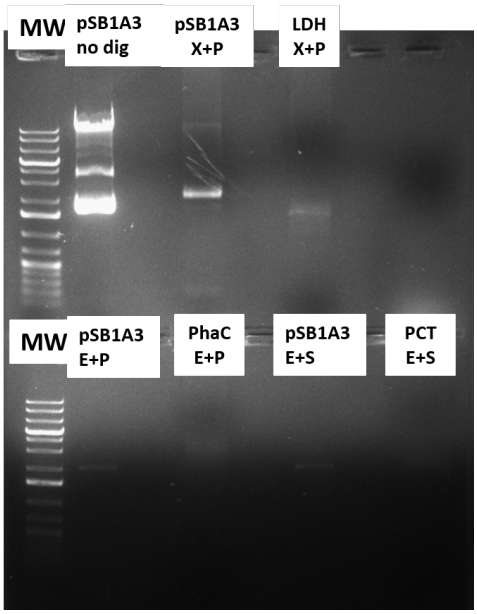
	PhaC2	PCT1	LDH1	pSB1A3
GBlock gene or plasmid	10 µL	10 µL	10 µL	2 µL (≈600ng)
Buffer NEB	3 µL (2.1)	3 µL (CutSmart)	3 µL (2.1)	3 µL (CutSmart or 2.1)*
Enzyme 1	1 µL (EcoRI)	1 µL (EcoRI)	1 µL (XbaI)	1 µL (EcoRI or XbaI)

Enzyme 2	1 µL (PstI)	1 µL (SpeI)	1 µL (PstI)	1 µL (PstI or SpeI)
H2O (qs 30µL)	15 µL	15 µL	15 µL	23 µL

*CutSmart when we don't digest by PstI and 2.1 when PstI is used

Incubation 1h 37°C

Agarose gel 1%



➔ Not enough gene on the gel to detect it!

Digestion

Mix:

	PhaC2	PCT1	LDH1
GBlock gene	10 µL	10 µL	10 µL
Buffer NEB	3 µL (2.1)	3 µL (CutSmart)	3 µL (2.1)
Enzyme 1	1 µL (EcoRI)	1 µL (EcoRI)	1 µL (XbaI)**
Enzyme 2	1 µL (PstI)	1 µL (SpeI)*	1 µL (PstI)
H2O (qs 30µL)	15 µL	15 µL	15 µL

* PCT1 has a PstI restriction site in its sequence so the digestion was done by SpeI
** LDH1 has a EcoRI restriction site site in its sequence so the digestion was done by XbaI

Incubation 1h 37°C

DNA clean-up → kit Monarch PCR and DNA cleanup (#T1030G) NEB

DNA extraction from gel

→ DNA Gel extraction kit (#T1020G) NEB

Incubation at 55°C to dissolve gel

400 µL buffer for 100 mg of agarose gel

DNA elution in 40 µL water

Ligation

10 µL Vector (pSB1A3 digested)

20 µL Insert digested

4 µL 10X T4 DNA ligase buffer

2 µL T4 DNA ligase

5 µL H2O

Incubation RT 1h

Transformation

- Thaw 25 µL of competent DH5α *E. coli*
- Add 10 µL of ligated DNA
- 20 min incubation in ice
- 1 min 42 °C
- Add 120 µL of LB medium
- Incubation 40 min 37°C
- Spread on plates LBA (Ampicillin)
- Incubation 37°C overnight

3 August 2016

Colonies PCR

Mix (50 µL total volume reaction):

- 25 µL DreamTaq PCR Mastermix 2X (ThermoScientific)
- 5 µL 10X DreamTaq Green Buffer (ThermoScientific)
- 0.25 µL Forward Primer (0.5 µM) iG001
- 0.25 µL Reverse Primer (0.5 µM) iG002
- 19.5 µL H2O filtrated (qs 50 µL)

Mix prepared for 15 reactions (plus one reaction prepared without Green buffer)
+ clones

PCR program:

- 95°C 5 min
- 95°C 30s
- 50°C 30s
- 72°C 2 min

×30

- 72°C 10 min

- 10°C ∞

Nanodrop

PhaC2 3.5ng/µL

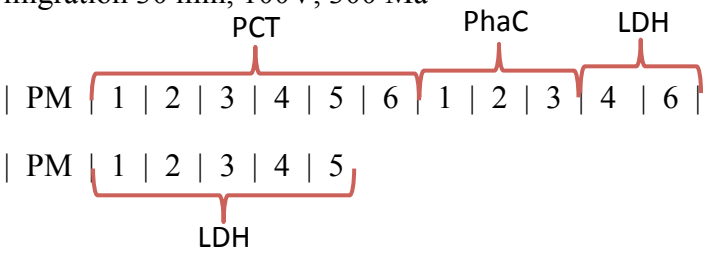
PCT1 3.8 ng/µL

LDH1 8.7 ng/µL

pSB1A3 (digested by X+P) 3.3 ng/µL

gel electrophoresis

migration 30 min, 100V, 300 Ma



⇨ The results were not conclusive

4 August 2016

Colonies PCR

Mix (25 µL total volume reaction):

- 12.5 µL DreamTaq PCR Mastermix 2X (ThermoScientific)
- 2.5 µL 10X DreamTaq Green Buffer (ThermoScientific)
- 0.25 µL Forward Primer (0.5 µM) iG001
- 0.25 µL Reverse Primer (0.5 µM) iG002
- 9.5 µL H2O filtrated (qs 25 µL)

Mix prepared for 7 reactions
+ clones

PCR program:

- 95°C 5 min
- 95°C 30s
- 50°C 30s
- 72°C 2 min

×30

- 72°C 10 min
- 10°C ∞

⇨ The results were not conclusive

8 august 2016

Ligation with:

- 50 ng vector
- 5µL T4 DNA ligase Buffer 10X
- 3 µL T4 DNA
- H2O qs for 50 µL
- DNA

PhaC 2 (3.5ng/µL)	11.7 µl	Vector E/P (6.4 ng/µL)	7.8 µl	H2O 22.5 µl
PCT 1 (3.8 ng/µL)	30.4 µl	Vector E/S (12.2 ng/µL)	4.1 µl	H2O 7.5 µl
LDH 1 (8.7 ng/µL)	13.9 µl	Vector X/P (5.9 ng/µL)	8.5 µl	H2O 19.6 µl

We put 27,5 µl of Pct1 DNA because there was not enough.

Transformation:

Thaw DH5-alpha competent bacteria in the ice.
Using the protocol processing by heat shock.
25µl of bacteria + Mix of ligation (2µl each time)
Incubation 20 minutes in ice and then 1 min at 42 ° C
Adding 120µl LB and incubate at 37 ° for 1 hour
Spread on LB Ampicillin with the öse.
Incubation overnight 37°C.

9 August 2016

Colonies PCR

Mix PCR with 25 µL of total volume reaction prepared for 12 reactions prepared as before (DreamTaq #K1071 Thermo Scientific).

PCR program:

- 95°C 5 min
- 95°C 30s
- 50°C 30s
- 72°C 1 min

×30

- 72°C 10 min
- 10°C ∞

⇨ No bands on the gel (maybe problems with the enzyme)

10 August 2016

Digestion

Mix:

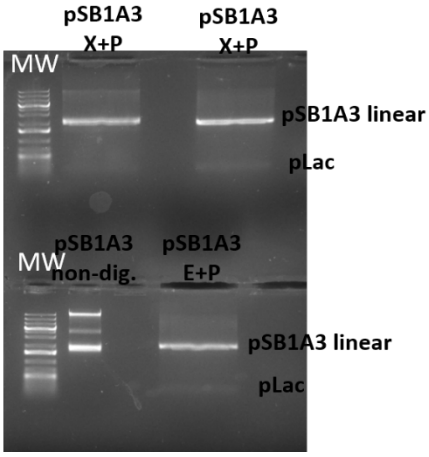
	PhaC2	PhaC3	PCT2	LDH1
GBlock gene	20 µL	20 µL	20 µL	20 µL
Buffer NEB	3 µL (2.1)	3 µL (2.1)	3 µL (CutSmart)	3 µL (2.1)
Enzyme 1	1 µL (EcoRI)	1 µL (EcoRI)	1 µL (EcoRI)	1 µL (XbaI)*
Enzyme 2	1 µL (PstI)	1 µL (PstI)	1 µL (PstI)	1 µL (PstI)
H2O (qs 30µL)	5 µL	5 µL	5 µL	5 µL

* LDH1 has a EcoRI restriction site site in its sequence so the digestion was done by XbaI

	pSB1A3	pSB1A3
Vector DNA	2 µL (≈600ng)	6 µL (≈2µg)
Buffer NEB	3 µL (CutSmart or 2.1)*	9 µL (CutSmart or 2.1)*
Enzyme 1	1 µL (EcoRI)	3 µL (XbaI)
Enzyme 2	1 µL (PstI)	3 µL (PstI)
H2O (qs 30µL)	23 µL	69 µL
		Divided in 3 reaction tubes

Incubation 1h 37°C

1% agarose gel



▲2 trash nucleotides after the PstI restriction site in the suffix were forgotten so the digestion risks to fail →

▲ PhaC genes doesn't match the gene in our reference publications but it corresponds to the gene used by the YALE IGEM team in 2013 (Réf.)

11 August 2016

Extraction of digested vectors (pSB1A3 X+P and pSB1A3 E+P) from agarose gel (kit Zymoclean gel DNA Recovery kit #D4001S)

Concentration of the samples:
pSB1A3 digested by X+P → 3.9 ng/µL
pSB1A3 digested by E+P → 7.2 ng/µL

Digestion by SpeI

Mix:

- 20 µL genes digested by E+P or X+P or vector digested by X+P
- 3 µL buffer NEB CutSmart
- 1 µL enzyme SpeI
- 6 µL H20 (qs 30 µL)

Or mix:

- 50 µL genes digested by E+P or X+P or vector digested by X+P
- 6 µL buffer NEB CutSmart
- 2 µL enzyme SpeI
- 2 µL H20 (qs 60 µL)

DNA purification

DNA clean & concentrator-5 kit (#D4003S Zymo Research)

For the vector: 2 volumes of binding buffer add to DNA (ratio 2:1)

For the genes: 5 volumes of binding buffer add to DNA (ratio 5:1)

Elution with 20 µL of water and incubation 2 min before centrifugation for DNA elution

Ligation

Mix:

	PhaC2	PhaC3	PCT2
Vector pSB1A3	4.5 µL	4.5 µL	4.5 µL
Gene	14 µL	11 µL	10 µL
T4 DNA ligase Buffer	3 µL	3 µL	3 µL
T4 DNA ligase	1 µL	1 µL	1 µL
H2O (qs 30µL)	7.5 µL	10.5 µL	11.5 µL

Incubation 1h RT

Sample frozen at -20°C until transformation

12 August 2016

Transformation

Thaw DH5-alpha competent bacteria in the ice.
Using the protocol processing by heat shock.
25µl of bacteria + 5 µL ligation mix
Incubation 20 minutes in ice
1 min at 42 ° C
3 min in ice
Adding 120µl of LB

Incubation at 37 ° for around 50 min
Spread on LB Ampicillin plates
Incubation overnight 37°C

13 August 2016

Results transformation:

We got clones from the transformation. We took 3 clones from pct(v2), 2 from phaC(v2) and 2 from phaC(v3) and performed PCR colony.

PCR Colonies:

20 µL mix per sample with:

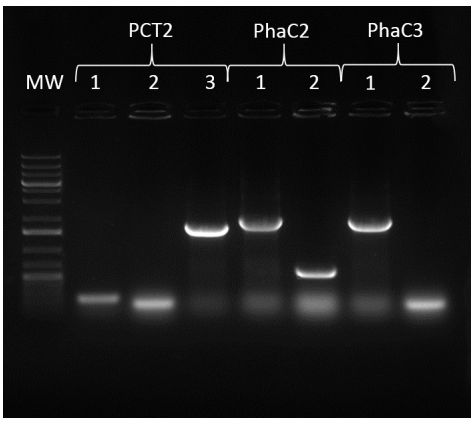
- 10 µL Green Taq Master Mix 2X (#K1081 Thermo Scientific)
- 0.25 µL Forward primer iG001 (10 µM)
- 0.25 µL Forward primer iG002 (10 µM)
- 9.5 µL H2O (qs 20 µL)

Mix for 9 reactions
+ clones

PCR program:

1) 95°C 5 min
2) 95°C 30s
3) 50°C 30s
4) 72°C 2 min
   30 repeats of the steps bloc from 2) to 4)
5) 72°C 10 min
6) 10°C infinite

Agarose electrophoresis (90 V, 111 mA, 40 min):



➔ Result of PCR Colony: 1 clone of each was good

Precultures

3 mL LB medium + positive clones for each gene (PhaC2, PhaC3, PCT2)

Incubation 37°C with shaking (around 16 h incubation)

➔ New transformation with the rest of ligation mix

Transformation (E. coli DH5-alpha with pSB1A3 ligated with gene) by heat shock

Thaw DH5-alpha competent bacteria in ice
100 µl of bacteria + 10 µL ligation mix
Incubation 20 minutes in ice
45s at 42 ° C
3 min in ice
Adding 900 µl of LB
Incubation 1h, 37 °C
Spread on LB Ampicillin plates
Incubation overnight 37°C

14 August 2016

➔ No colony for PCT2 transformation!

Miniprep from precultures

Transformed bacteria were stored at -80°C in 50% glycerol (2 aliquotes for each preculture)

GenElute Plasmid Miniprep Kit (Sigma, PLN350-1KT)
Incubation 1 min for elution of DNA before centrifugation

pSB1A3-PhaC2 144 ng/µL
pSB1A3-PhaC3 96.1 ng/µL
pSB1A3-PCT2 143.3 ng/µL

Digestion

Mix:

	LDH1 gBlock gene (10 ng/µL)	pSB1A3 (X+P)
DNA	20 µL (200 ng)	50 µL
10X Buffer NEB CutSmart	3 µL	6 µL
enzyme XbaI	1 µL	2 µL
enzyme SpeI	1 µL	-
H2O (qs 30 µL)	5 µL	2 µL (qs 60 µL)

Incubation 1h 37°C

Dephosphorylation

Add 1 µL alkaline phosphatase (FastAP #EF0654 Thermo Scientific) to the digestion mix (for LDH1 digestion or vector pSB1A3 digestion)
Incubation 30 min 37°C
Inactivation of the enzyme: incubation 75°C, 10 min

Recombinant vector digestion

Mix:

	pSB1A3-PhaC2	pSB1A3-PhaC3	pSB1A3-PCT2
DNA (around 200 ng or 600 ng)	2 µL	2.5 µL	6 µL (around 600 ng)
Buffer NEB CutSmart 10X	3 µL (2.1)	3 µL (2.1)	3 µL (CutSmart)
Enzyme 1	1 µL (EcoRI)	1 µL (EcoRI)	1 µL (EcoRI)
Enzyme 2	1 µL (SpeI)	1 µL (SpeI)	1 µL (XbaI)
H2O (qs 20µL)	14 µL	13.5 µL	10 µL

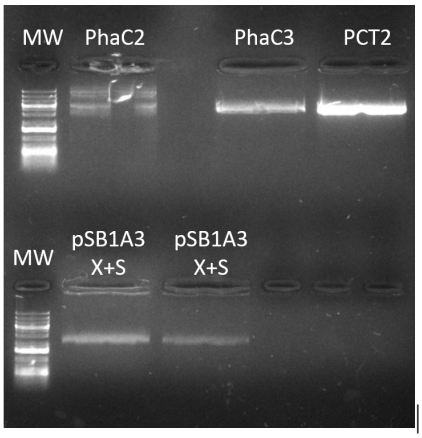
Incubation 1h 37°C

DNA purification (LDH gene)

DNA clean & concentrator-5 kit (#D4003S Zymo Research)
Ratio 5:1 of DNA binding buffer for LDH gene fragment
Elution 30 µL water with incubation 3 min before centrifugation
LDH1 → 6.9 ng/µL

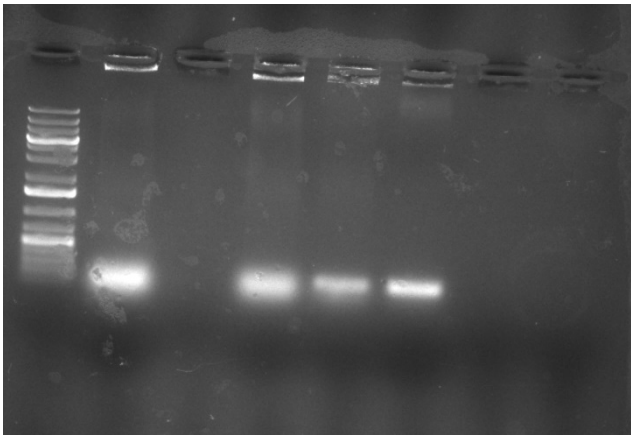
Agarose gel for digested recombinant vectors

All the digested samples were charged on the gel.



➔ Digestion was a failure for the 2 plasmids containing the PhaC2 and PhaC3 genes. The vector containing PCT2 is open but we have no way to be sure that the digestion was efficient for the 2 enzymes or just for one of them.

Colony PCR on the clones obtained after the 2nd transformation of the ligation mixes:



➔ PCR on colony failed

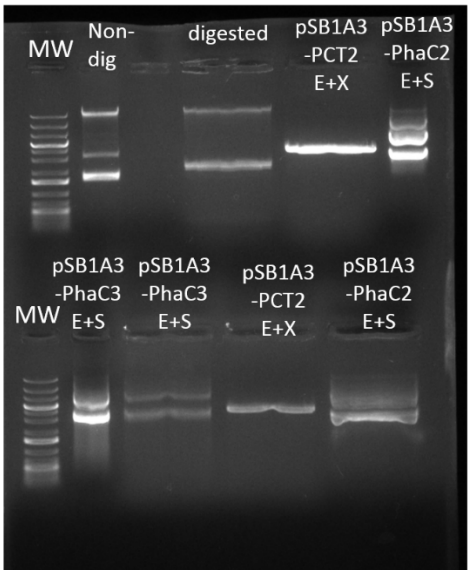
16 August 2016

Digestions

	LDH1 gBlock gene (10 ng/µL)	pSB1A3 (352 ng/µL)
DNA	30 µL (300 ng)	2 µL
10X Fastdigest buffer (ThermoScientific)	4 µL (clear buffer)	2 µL (green buffer)
enzyme XbaI	1 µL	1 µL
enzyme SpeI	1 µL	1 µL
H2O (qs 40 µL)	4 µL	14 µL (qs 20 µL)

	pSB1A3-PhaC2	pSB1A3-PhaC3	pSB1A3-PCT2
DNA (around 200 ng or 600 ng)	2 µL	2.5 µL	6 µL (around 600 ng)
Buffer Fastdigest green 10X (ThermoScientific)	2 µL	2 µL	2 µL
Enzyme 1	1 µL (EcoRI)	1 µL (EcoRI)	1 µL (EcoRI)
Enzyme 2	1 µL (SpeI)	1 µL (SpeI)	1 µL (XbaI)
H2O (qs 20µL)	14 µL	13.5 µL	10 µL

Agarose gel 1%



DNA purification (digested by X + S LDH1 gene)
DNA clean & concentrator-5 kit (#D4003S Zymo Research)
Ratio 5:1 of DNA binding buffer for LDH gene fragment

17 August 2016

- Autoclave
- Purification Gel d'agarose via le kit « Zymoclean Gel DNA Recovery »

- weight of the empty tube: 1 g
- weight of the tube + vector PSB1A3 (X+S) n°1 : 1,1 g is 100 mg
  n°2 : 1,1 is 100 mg
- vector size: 2155 bp
- Step 1: For 100 mg d'agarose we add 300 µl of ADB
- Step 2: Put samples at 55 °C for 10 minutes and centrifuge at 300 rpm
- Step 3: Transfert in the column
- Step 4: Centrifugation while 1 minute at 13 000 rpm then suppression of the flow-through
- Step 5: Add 200µl of DNA Wash Buffer and centrifugation while 30 seconds X2
- Step 6: Centrifugation empty for 2 minutes
- Step 7: Add 10 µl DNA Elution Buffer, wait 2 minutes
- Step 8: Centrifuge 1 minutes at 13 000 rpm

- Nano drop

- DO vector Psb1a3 n°1 : 39 ng/µl
- DO vector Psb1a3 n°2 : 43,2 ng/ µl

18 August 2016

Chloramphenicol stock

50 ng/mL chloramphenicol stock (2.5 g in 50 mL ethanol 100%)
30 aliquots stored at -20°C

pSB1C3-mRFP

Solubilisation of pSB1C3-mRFP (2016 IGEM plate 5 - 1F) with 10 µL water nuclease-free (ThermoScientific)
Incubation 5 min RT
Transformation in E. coli DH5-alpha strain

Transformation

Thaw 25 µl DH5-alpha competent bacteria in ice
Add 2 µL of resolubilized pSB1C3-mRFP DNA from 2016 IGEM plate 5
Incubation 20 minutes on ice
1 min at 42 ° C
3 min in ice
Adding 120 µl of LB
Incubation 1h, 37 °C with shaking
Spread on LB Chloramphenicol plates
Incubation overnight 37°C

Solubilisation of the primers iG051, iG052, iG049 and iG050 and aliquots of 10 times dilutions.

19 August 2016

Precultures

3 mL LB medium + 1.8 µL Chloramphenicol (50 mg/mL) → plates at 30 µg/mL chloramphenicol

- ➔ No conclusive results for PCR on colonies for pSB1C3-mRFP plates so we did precultures with 4 other clones (A, B, C and D)
- ➔ The Mastermix Taq DNA polymerase seems to not work anymore (after freezer problem, the polymerases lost their activities)

Q5 PCR for changing antibiotic resistance in pSEVA 224 plasmid

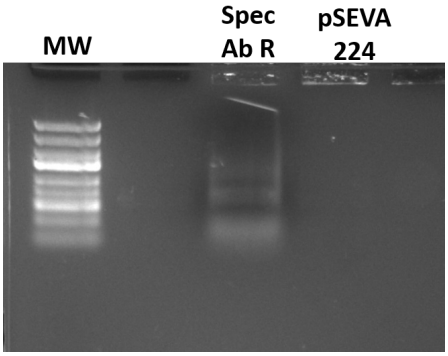
10 µL reaction buffer
1 µL dNTP mix
1 µL 10 times diluted primers (X2)
0.5 µL DNA template
0.5 µL Q5 DNA polymerase
H20 qs 50 µL (=36 µL)

2 different reactions:

1) Amplification of spectinomycin resistance gene with pCDF vector as DNA template (length expected = 1220 bp) primers used: iG050 and iG052 (1 min of DNA polymerization and primers annealing at 60°C)
2) Amplification of pSEVA224 backbone without kanamycin resistance gene (pSEVA224 as DNA template; length expected = 4253 bp) primers used: iG049 and iG051 (3 min of DNA polymerization and primers annealing at 60°C)

PCR program:

1) 95°C 5 min
2) 95°C 30s
3) 60°C 30s
4) 72°C 1 min or 3 min
   35 repeats of the steps bloc from 2) to 4)
5) 72°C 5 min
6) 10°C infinite



➔ Amplification of pSEVA 224 failed → why? Good primers so why!?

20 August 2016

DNA miniprep of pSB1C3-mRFP (clone A, B, C and D)

Stock in 50% glycerol stored at -80°C
GenElute Plasmid Miniprep Kit (Sigma, PLN350-1KT)
Incubation 2 min for DNA elution before centrifugation and centrifugation of 1min30s instead of 1min.
Optional Wash Buffer step performed and use of nuclease-free water for elution.
Clone A: 240.3 ng/µL
Clone B: 150.3 ng/µL
Clone C: 111.0 ng/µL
Clone D: 76.6 ng/µL

PCR on DNA miniprep:

Primers iG001 and iG002.

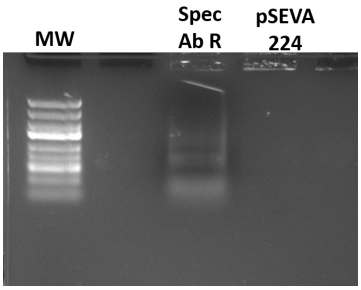
20 µL mix per sample with:

- 10 µL Green Taq Master Mix 2X (#K1081 Thermo Scientific)
- 0.25 µL Forward primer iG001 (10 µM)
- 0.25 µL Forward primer iG002 (10 µM)
- 9.5 µL H2O (qs 20 µL)

PCR program:

7) 95°C 5 min
8) 95°C 30s
9) 50°C 30s
10) 72°C 1 min
    30 repeats of the steps bloc from 2) to 4)
11) 72°C 5 min
12) 10°C infinite

<u>Agarose gel 1% for changing antibiotic PCR</u>



➔ The amplification of pSEVA → new attempt

<u>Digestion vectors pSB1C3-mRFP (on DNA miniprep)</u>

- 10 µL plasmid DNA
- 3 µL Buffer 10X green FD (FastDigest)
- 1 µL EcoRI
- 1 µL SpeI
- 15 µL H20 (qs 30 µL)

Size expected: 2051 bp

Digestion was checked on agarose gel and the band corresponding to pSB1C3 digested by E+S was cut in order to recover the DNA.

22 August 2016

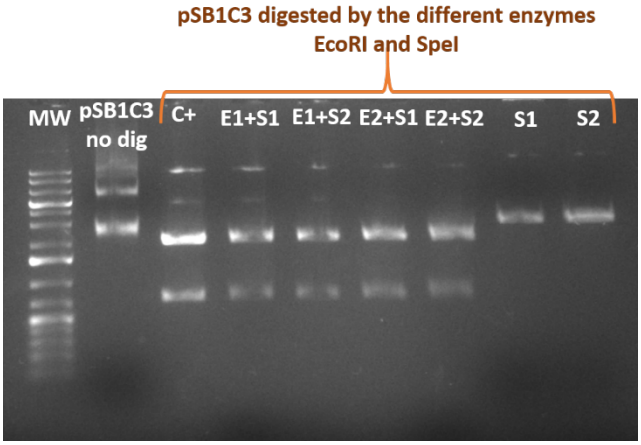
Digestion efficiency test of restriction enzymes EcoRI and SpeI

Mix:

- 4 µL vector pSB1C3 (clone A)
- 3 µL buffer 10X CutSmart or FastDigest Green
- 1 µL EcoRI and/or SpeI
- 21 µL or 22µL H2O

Incubation 1h 37°C

Agarose gel 1%



DNA extraction from agarose gel (linear vector extracted from clones A, B and C)
Zymoclean gel DNA Recovery kit #D4001S
Vector A: 20.9 ng/µL
Vector B: 13.3 ng/µL
Vector C: 13.1 ng/µL

Expected sizes after digestion by E+S:
pSB1C3 2051 bp
mRFP 742 bp

23 August 2016

· Digestion gBlocks by E+S

- 20 µl gBlocks DNA
- 3 µl 10X NEB Buffer (Cutmart)
- 1 µl EcoRI
- 1 µl SpeI

- 5 µl H2O (qs 30µl)

  · Incubation 1h at 37°C

  · Purification Agarose Gel with the kit « Zymoclean Gel DNA Recovery kit » (#D4001S Zymo Research)

- Cf protocole 17 august 2016
- Elution with 20µl H2O

  · Nanodrop 1

- LDH2 : 10,3 ng/µl
- Phac3 : 5,9 ng/µl
- Phac 4 : 8,5 ng/µl
- Pct 2 : 8,9 ng/µl

  too low concentration for ligation so I put at the Speed Vae. Protocole « Vaccum Drive »

  resuspend with 10 µl H2O

  · Nanodrop 2

- LDH2 = 15 ng/µl Ratio 260/280 : 1,92
- Phac 3 = 8,8 ng/µl Ratio 260/280 : 2,20
- PhaC4 = 9,2 ng/µl Ratio 260/280 : 1,05
- Pct2 = 10,8 ng/µl Ratio 260/280 : 1,88

Calculs :

gènes	Longueur of gènes	Amount (ng)	Concentration (ng/µl)	Volume (µl)
Plasmid psB1C3	2051 pb		20,9	2,4
PhaC3	1717 pb	209,3	8,8	23,8
LDH2	1227 pb	149,6	15	10
Phac 4	1720 pb	209,7	9,2	22,8
Pct 2	1612 pb	196,5	10,8	18,2

Calculs of amount of insert: 50 x5 x longueur gBlock / Longueur plasmid

Calculs of volume of insert: amount of insert / concentration of insert

As we suspended in 10µl I do not have sufficient quantities.

· For Ligation Mix:

Insert	DNA (µl)	Vector (µl)	Buffer T4 Ligase 10X (µl)	T4 Ligase (µl)	H2O (qs 20µl)
Pct2	9	0,96	2	0,5	7,54
LDH2	9	1,29	2	0,5	7,21
Phac4	9	0,96	2	0,5	7,54
Phac3	9	0,96	2	0,5	7,54

- I put at 22°C the ligation Mix while 10 min
- I take 5µl for the transformation of 50µl cellule chimical competente

· Transformation

- Use protocol of Bacterial transformation by heat shock

-> Thaw competent cells on ice

-> mixed 50 µl of competent bacteria with 5 µl of DNA

-> incubate on ice for 20 minutes

-> placing the tubes at 42 ° C for 1 min then put 3 min in ice

-> Add 250µl of LB and incubate 1h at 37°C

-> Spread on box LB Chloramphenicol (30 µg/mL)

-> incubation overnight 37°C

- Poored 6 plates of 25 ml of LB agar + chloramphenicol (30 µg/µl)
- Incubation at 37°C

24 August 2016

· Digestion gBlocks by E+S

- 20µl gBlocks DNA
- 3 µl 10X NEB Buffer (Cutmart)
- 1 µl EcoRI
- 1 µl SpeI
- 5 µl H2O (qs 30µl)

· Incubation 1h at 37°C

· Purification Agarose Gel with the kit « Zymoclean Gel DNA Recovery kit » (#D4001S Zymo Research)

Elution with 20 µL nuclease-free water

PhaC3 (E+S) → 12.2 ng/µL

PhaC4 (E+S) → 6.3 ng/µL

LDH2 (E+S) → 16.8 ng/µL

PCT2 (E+S) → 12.4 ng/µL

Concentration of the DNA: around 1h in evaporator

DNA resuspension with 11 µL water

➔ First concentration failed so second concentration of DNA performed in evaporator (1h)

25 August 2016

Resuspension of DNA with 11 µL water

PhaC3 (E+S) → 10.4 ng/µL

PhaC4 (E+S) → 10.6 ng/µL

LDH2 (E+S) → 27.0 ng/µL

PCT2 (E+S) → 20.1 ng/µL

➔ DNA concentration by water evaporation failed!

<u>Ligation genes inside pSB1C3 (ratio 5:1)</u>

	PhaC4	PhaC3	PCT2	LDH2
Buffer T4 DNA ligase (10X)	2 µL	2 µL	2 µL	2 µL
T4 DNA ligase	0.5 µL	0.5 µL	0.5 µL	0.5 µL
Vector	0.96 µL	0.96 µL	0.96 µL	0.96 µL
Insert (gene)	7.9 µL	8.05 µL	3.91 µL	2.2 µL
H20 (qs 20 µL)	8.63 µL	8.49 µL	12.6 µL	14.3 µL

10 min incubation at 22°C

Transformation

- Thaw 50 µL bacteria (E. coli DH5-alpha) chemo-competent
- Add 5 µL DNA (mix ligation)
- Incubation 20 min on ice
- Heat shock: 45s at 42°C
- Incubation 3 min on ice
- Add 950 µL LB
- Incubation 1h at 37°C with shaking
- Centrifugation 1 min at 4000 rpm
- Throw away supernatant
- Resuspension with the rest of supernatant (around 50 µL)
- Spread on plates LB Chloramphenicol (30 µg/mL)

26 August 2016

Preparation plates LBC (30 µg/µL)

Ligation (ratio 1:1)

	PhaC4	PCT2	PhaC3	LDH2
Buffer T4 DNA ligase (10X)	1 µL	1 µL	1 µL	1 µL
T4 DNA ligase	0.5 µL	0.5 µL	0.5 µL	0.5 µL
Vector	2.39 µL	2.39 µL	2.39 µL	2.39 µL
Insert (gene)	3.96 µL	1.95 µL	4.02 µL	1.11 µL
H20 (qs 10 µL)	2.15 µL	4.15 µL	2.08 µL	5.0 µL

Incubation 16h 16°C

27 August 2016

- PCR amplification of LDH_V2, PCT_V2, PHAC_V3 and PHAC_V4

Mix: Vf=50µl

		For 5 reactions
5X Q5 Reaction Buffer	10 µl	50 µl
10 mM dNTP	1 µl	5 µl
10 µM Fw	2,5 µl	12,5 µl
10 µM Rv	2,5 µl	12,5 µl
DNA	1 µl	-
Q5 (0,2 U/µl)	0,5 µl	2,5 µl
H2O	32,5 µl	162,5 µl

- Solubilization of primers

For PR_IG063: Add 167µl H2O for [100µM] → Aliquot 10µl into 90µl H2O [10µM]

For PR_IG064: Add 246µl H2O for [100µM] → Aliquot 10µl into 90µl H2O [10µM]

- Expected length:

  Ldh_V2= 1248 bp ~±2nt

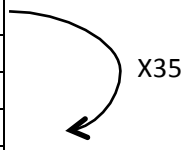
  Pct_V2= 1633 bp

  PhaC_V3= 1738 bp

  PhaC_V4= 1692 bp

- Cycle:

98°C	30 scd
98°C	30 scd
55°C	30 scd
72°C	1 min
72°C	2 min
10°C	∞



28 August 2016

Transformation *E. coli* DH5-alpha

- Thaw 25 µL chemo-competent bacteria
- Add 5 µL DNA (mix ligation) (2 µL of pSB1C3-mRFP from clone A as positive transformation control)
- Incubation 20 min on ice
- Heat shock: 45s at 42°C
- Incubation 3 min on ice
- Add 900 µL LB
- Incubation 1h20 at 37°C with shaking

- Centrifugation 1 min 30s at 5000 rpm
- Throw away all the supernatant
- Resuspension with 100 µL LB medium
- Spread on plates LB Chloramphenicol (30 µg/mL)
- Incubation overnight 37°C

29 August 2016

The GFP gene contained in Gen4_BNR vector (provided by Cécile) was amplified by PCR with Q5 DNA polymerase with primers allowing to add the prefix and suffix regions at both sides of the amplified region.

Mix:

- 10 µL Q5 reaction buffer 5X
- 1 µL dNTP mix
- 2.5 µL 10 µM Forward Primer (iG063)
- 2.5 µL 10 µM Reverse Primer (iG064)
- 1 µL DNA template
- 0.5 µL Q5 DNA polymerase
- 32.5 µL Nuclease-free water (qs 50 µL)

PCR program:

1) 98°C 30s
2) 98°C 30s
3) 55°C 30s
4) 72°C 1 min
   35 repeats of the steps bloc from 2) to 4)
5) 72°C 2 min
6) 10°C infinite

➔ After checking on agarose 1% gel, we determined that the GFP was successfully amplified.

Digestion

The amplified fragment was digested by EcoRI and PstI during 1h at 37°C. The reaction was performed in NEB CutSmart buffer.

The digested mix was purified by using the kit Wizard SV Gel and PCR Clean-Up System (Ref: A9282, Promega).

30 August 2016

Ligation

The GFP fragment was then ligated separately in pSEVA 224 or pSEVA 2311 digested by E+P in a ratio 5:1.

The ligation mix was stored at -20°C until transformation.

1st September 2016

- Gel electrophoresis of the PCR amplification (27 october)

➡ Good length

- PCR Purification with a final volume = 50uL
  - Nanodrop

Gènes	Concentration (ng/µl)	Ratio 260/280
LDH_V2	109,5	1,82
PCT_V2	56,5	1,80
PHAC_V3	63,6	1,82
PHAC_V4	88,1	1,81

2 September 2016

- Digestion

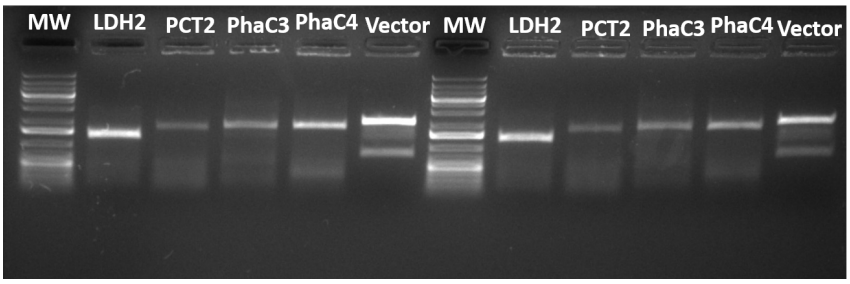
Genes	Quantity of DNA (µl)	Restriction Enzymes (µl)	Buffer Digest NEB 2.1 (µl)	Water Qs 50µl (µl)
LDH2 (109,5 ng/µl)	10	EcoR1 HF : 1µl Pst1 :1µl	5	33
PCT2 (56,5 ng/µl)	20	EcoR1 HF : 1µl Pst1 :1µl	5	23
PhaC3 (63,9 ng/µl)	17,5	EcoR1 HF : 1µl Pst1 :1µl	5	25,5
PhaC4 (88,1 ng/µl)	12,5	EcoR1 HF : 1µl Pst1 :1µl	5	30,5
Vector PsB1C3 (240,3 ng/µl)	4,5	EcoR1 HF : 1µl Pst1 :1µl	5	38,5

Incubation 1h at 37°c and after incubation 20min at 80°C.

- Gel Extraction

50 ml of Agarose 1% with 2.5 µl of midorigreen.

Migration 25 min at 100mv.



*Digestion mixes with EcoRI and PstI were loaded on the gel.

3 September 2016

DNA extraction from agarose gel

Kit: Wizard SV Gel and PCR Clean-Up System (Ref: A9282, Promega)

pSB1C3 → 6.4 ng/µL
LDH2 → 4.8 ng/µL
PCT2 → 2.9 ng/µL
PhaC3 → 4 ng/µL
PhaC4 → 5.8 ng/µL

DNA concentration by water evaporation:

pSB1C3 → 7.1 ng/µL
LDH2 → 6.5 ng/µL
PCT2 → 5.5 ng/µL
PhaC3 → 4.0 ng/µL
PhaC4 → 5.0 ng/µL

➔ DNA concentration still seems not efficient

<u>DNA transformation</u>

- Thaw 50 µL chemo-competent DH5-alpha *E. coli*
- Add 5 µL DNA (mix ligation with promoters from pSEVA 2311 or 224)
- Incubation 25 min on ice
- Heat shock: 45s at 42°C
- Incubation 3 min on ice
- Add 250 µL LB
- Incubation 1h15 at 37°C with shaking
- Spread on plates LB Kanamycin (50µg/mL) (50 µL of bacteria on one plate and 10 µL on another one for each vector)
- Incubation overnight 37°C

➔ The cells didn't express detectable fluorescence. A collaboration was setting up with Paris-Bettencourt IGEM team in order to characterize the IPTG inducible promoter by GFP expression. They also tried to characterize the cyclohexanone inducible promoter by the same way but it was a failure due to reaction between cyclohexanone and polystyrene (PS) from 96 wells plates.

4 September 2016

Ligation in pSB1C3

	LDH2 (6.5ng/µL)	PCT2 (6.5ng/µL)	PhaC3 (4.0ng/µL)	PhaC4 (5.0ng/µL)
Vector	4.2 µL	4.2 µL	4.2 µL	4.2 µL
Insert (gene)	2.8 µL	0.5 µL	0.5 µL	0.5 µL
Buffer T4 DNA ligase (10X)	2.39 µL	2.39 µL	2.39 µL	2.39 µL
T4 DNA ligase	3.96 µL	1.95 µL	4.02 µL	1.11 µL
H20 (qs 10 µL)	2.15 µL	4.15 µL	2.08 µL	5.0 µL

Incubation 1h 16°C

Poor plates LBC (30 µg/mL chloramphenicol)

Transformation

- Thaw 25 µL chemo-competent DH5-alpha *E. coli*
- Add 4 µL DNA (mix ligation)
- Incubation 25 min on ice
- Heat shock: 45s at 42°C
- Incubation 3 min on ice
- Add 225 µL LB
- Incubation 1h at 37°C with shaking
- Spread 120 µL on plates LBC
- Incubation overnight 37°C

7 September 2016

- Digestion of gene

Genes	Quantity of DNA (µl)	Restriction Enzymes (µl)	Buffer Digest NEB 2.1 (µl)	Water Qs 50µl (µl)
LDH2 (109,5 ng/µl)	10	EcoR1 HF : 1µl Pst1 :1µl	5	33
PCT2 (56,5 ng/µl)	20	EcoR1 HF : 1µl Pst1 :1µl	5	23

PhaC3 (63,9 ng/µl)	17,5	EcoR1 HF : 1µl Pst1 :1µl	5	25,5
Ratio 1 : 1				

PhaC4 (88,1 ng/µl)	12,5	EcoR1 HF : 1µl Pst1 :1µl	5	30,5

Incubation 1h at 37°c and after incubation 20min at 80°C.

8 September 2016

- Digestion of backbone IGEM

Mix: Enzyme Master Mix E+P

- 0,5 µL NEB 2.1 Buffer
- 0,5 µL BSA (Bovine Serum Albumin)
- 0,5 µL EcoR1-HF
- 0,5 µL Pst1
- 0,5 µL Dnp1
- 18 µL H2O

Mix of 4µL of linearized backbone and 4 µL of master mix enzyme

incubation of all the solution at 37°C during 30 min and then, heat deactivation during 20 min at 80°C.

- Ligation

Genes	length genes	Volume Final	Concentration Final	amount for 1 µL (Cf/Vf)
PSB1C3	2037 bp	8 µL	100 ng	25 ng =2µL
LDH_V2	1227 bp	50 µL	1095 ng	21,9 ng/µl
PCT_V2	1612 bp	50 µL	1130 ng	22,6 ng/µl
PhaC_V3	1717 bp	50 µL	1118,25 ng	22,365 ng/µl
PhacC_V4	1720 bp	50 µL	1101,25 ng	22,025 ng/µl

Amount calculation of insert for Ligation with NEBio Calculator :

Genes	Amount	Volume for deduced
LDH_V2	15,06 ng	0,7 µL
PCT_V2	19,78 ng	0,9 µL
PhaC_V3	21,07 ng	0,9 µL
PhaC_V4	21,11 ng	0,96 µL

Mix of Ligation:

- 2 µL of PSB1C3
- Volume calculate of digested insert
- 1 µL T4 Ligase Buffer
- 0,5 µL T4 Ligase
- Qs 10 µL

I put the mix at 16°c while 30min and after heat kill for 20 min at 80°c

- Transformation by heat shock

With 2µL of Ligation mix and 25µL of Competent Cells.

- 2µL of Ligation with 25µL of competent cells.
- Put the mix 20 min in the ice.
- Put the mix at 42°C while 45 seconds
- 2min on the ice.
- Add 950µL of LB
- Recovery 1h at 37°C with agitation
- 7 000 rpm during 1min
- Suppression of the flow-throw
- Resuspend with the remaining
- Spread on plates (put the simple in the middle of the plates)
- Incubation overnight 37°C

- Plates preparation

1 plates = 25mµL of LB Agar + 15 µL of chloramphenicol [30µg/mL]

$$Vi = \frac{30\mu g\ x\ 25\ mL}{50\ 000\ \mu g} = 15\ \mu g$$

Preculture

3 mL LB liquid medium + 1 µL *E. coli* DH5-alpha bacteria from stock at -80°C

09 September 2016

- Preparation of chemo-competent *E. coli* DH5-alpha by heat shock

- WORK STERILE
- Launch a pre-culture of the strain in LB at 37 ° C

- Inoculate 25 ml of LB 1/50 (500µl) with the pre-culture
- Let it growth at 37 ° C with stirring until OD(600 nm) = 0.5-0.7
- After 1h30, OD=0,9
- For Spectrometer measurements, 1 mL of LB for the blank
- A dilution was performed to obtain 0,5 OD(600 nm) and the culture was split into 2 falcons with 27,5 mL of culture
- Cells are cooled 10 min on ice
- Centrifugate culture 6 min at 4000 rpm at 4°C
- Suppression of the flow-through
- Dilution of the pellet in 1/2 volume of cold CaCl2 0.1M (13,75 mL)
- Cells cooled on ice during 20 min
- Culture centrifugated 6 min at 4000 rpm at 4°C
- Suppression of the flow-through
- Resuspension the pellet in 1 / 50e of volume (550 µl) of cold CaCl2 0.1M + 10% glycerol
- $Vi = \frac{\frac{1}{100} x\ 550}{5/100} = 110 \mu L$
- The mix is 440µL CaCl2+ 110µL glycérol 50%
- 22 aliquots of 50 µL bacteria

11 September 2016

- PCR Amplification of LDH2, PCT2, PHAC3, PHAC4 with Q5 DNA polymerase

For 50µL reaction

- 25 µL of Q5 Master Mix
- 2,5 µL of Forward Primer (IG063)
- 2,5 µL of Reverse Primer (IG064)
- 0,5 of Template DNA
- 19,5 of H2O

Cycle :

Temperature	Time	
98°C	30 secondes	
98°C	30 secondes	X35
55°C	30 secondes	
72°C	1 minute	
72°C	2 minutes	
10°C	Pause	

- PCR Colony LDH2 with One Taq

For 25 µL to 1 Reaction → Vf=125µL

Mix :

- 25 µL of One Taq Standard Reaction Buffer
- dNTPs= 2,5 µL
- 2,5 µL of Forward Primer (IG001)
- 2,5 µL of Reverse Primer (IG002)
- 0,625 µL of One Taq DNA Polymérase
- 91,9 µL of Nuclease Free Water

Cycle:

Temperature	Time	
94°C	30 secondes	
94°C	30 secondes	X30
50°C	1 minute	
68°C	1min 45	
68°C	5 minutes	
10°C	Pause	

12 September 2016

- MiniPrep Colonies LDH

- 3µL of LB
- Take one of the colony in the plates
- Incubated at 37°C overnight
-

- Digestion Backbone IGEM pSB1C3 linearized by E+P

Mix: Enzyme Master Mix E+P for 22 reaction

- 2 µL NEB 2.1 Buffer
- 2 µL BSA (Bovine Serum Albumin)
- 2 µL EcoRI-HF
- 2 µL PstI
- 2 µL DnpI
- 72 µL H2O

16 µL of linearized backbone and 16 µL of the MasterMix enzyme

Incubation at 37°C during 30 min

Enzyme inactivation with 20 min incubation at 80°C.

Precultures

5 mL LB medium

+ 5 µL antibiotic (Kanamycin 50 mg/mL for pSEVA 212S, Spectinomycin 50 mg/mL for pSEVA 424 and Gentamycin 10 mg/mL for pSEVA 628S)

+ some bacteria (sent as bacteria aliquots in LB medium by de Lorenzo)

Incubation 37°C overnight

Ratio 1 : 2		
Genes	Amount	Volume for deduced
LDH_V2	30,12 ng	1,37 µL
PCT_V2	39,57 ng	1,71 µL
PhaC_V3	42,15 ng	1,88 µL
PhaC_V4	42,22 ng	1,92 µL

Ratio 1 : 5		
Genes	Amount	Volume for deduced
LDH_V2	75,29 ng	3,44 µL
PCT_V2	98,92 ng	4,38 µL
PhaC_V3	105,4 ng	4,71 µL
PhaC_V4	105,5 ng	4,79 µL

13 September 2016

Miniprep of pSEVA plasmids

2 aliquots stocks in 50% glycerol stored at -80°C

GenElute Plasmid Miniprep Kit (Sigma, PLN350-1KT)

3 DNA vectors: pSEVA 424 (equivalent to the pSEVA 224 but with spectinomycin resistance gene instead of kanamycin, pSEVA 212S and pSEVA 628S used for genes deletion in genomic DNA in *Pseudomonas putida*)

Digestion of the "old" gene by E+P

Ligation mix:

- 2 µL of PSB1C3
- Volume calculate of digested insert
- 1 µL T4 Ligase Buffer
- 0,5 µL T4 Ligase
- Qs 10 µL

I put the mix at 16°c while 30min and after heat kill for 20 min at 80°c

- Minipreps DNA Purification System kit "Wizard Plus"

- Take 2µl of overnight culture
- Centrifuge at top speed for 1min
- Suppression of the flow-throw
- Resuspend pellet with 250µl of Cell Resuspension Solution
- Add 250µl of Cell Lysis Solution and invert 4 times to mix
- Add 10µl of Alkaline Protease Solution and invert 4 time to mix
- Incubate 5min at room temperature
- Add 350µl of Neutralization Solution and invert 4 times to mix
- Centrifuge at top speed for 10min at room temperature
- Insert Spin Column into Collection Tube
- Decant cleared lysate into Spin Column
- Centrifuge at top speed for 1min
- Discard flow-through
- Add 750µl of Wash Solution and centrifuge at top speed 1 min
- Discard flow-through
- Repeat with 250µl of Wash Solution
- Centrifuge at top speed for 2min
- Transfer Spin Column to 1,5ml tube
- Add 100µl of Nuclease free water to the Spin Column
- Centrifuge at the top speed for 1min
- Discard column

- Purification of Amplification PCR LDH2, PCT2, PHAC3, PHAC4
- Nanodrop

For the gene:

Genes	Concentration (ng/µl)	Ratio 260/280
LDH2	62,7	1,78
PCT2	52,8	1,79
PHAC3	50,3	1,76
PHAC4	69,7	1,78

For the LDH colonies:

Colonies	Concentration (ng/µl)	Ratio 260/280
LDH_n°1	70	1,92
LDH_n°2	31	2,06

- Digestion “New” genes LDH2, PCT2, PHAC3, PHAC4

Digestion by EcoR1-HF+PSt1

Genes	Quantity of DNA (µl)	Restriction Enzymes	Buffer Digest NEB 2.1 (µl)	H2O (µl)
LDH2 (62,7 ng/µl)	16	1 µl EcoR1-HF 1µl Pst1	5	27
PCT2 (52,8 ng/µl)	19	1 µl EcoR1-HF 1µl Pst1	5	24
PHAC3 (63,9 ng/µl)	16	1 µl EcoR1-HF 1µl Pst1	5	27
PhAC4 (69,7 ng/µl)	14,5	1 µl EcoR1-HF 1µl Pst1	5	28,5
Psb1c3 (240,3 ng/µl)	4,5	1 µl EcoR1-HF 1µl Pst1	5	38,5

Other Digestions:

Genes	Quantity of DNA (µl)	Restriction Enzymes	Buffer Digesr NEB 2.1 (µl)	H2O (µl)
PHAC3	16	1 µl Xba1 1 µl Pst1	5	27
PHAC4	14,5	1 µl Xba1 1 µl Pst1	5	28,5
PCT2	19	1 µl EcoR1-HF 1 µl Spe1	5	24

Incubation at 37°c during 30min and then enzymes deactivation by heat kill for 20 min at 80°C.

- PCR Purification
  - Nanodrop Vf=50µl

Gènes	Concentration (ng/µl)	Ratio 260/280
LDH_V2	62,7	1,78
PCT_V2	52,8	1,79
PHAC_V3	50,3	1,76
PHAC_V4	69,7	1,78

14 September 2016

The promoter region of the vectors pSEVA 224 and pSEVA 2311 were amplified by PCR with Q5 DNA polymerase with primers allowing to add the prefix and suffix regions at both sides of the amplified region.

Mix:

- 10 µL Q5 reaction buffer 5X
- 1 µL dNTP mix
- 2.5 µL 10 µM Forward Primer (iG063)
- 2.5 µL 10 µM Reverse Primer (iG064)
- 1 µL DNA template
- 0.5 µL Q5 DNA polymerase
- 32.5 µL Nuclease-free water (qs 50 µL)

PCR program:

1) 98°C 30s
2) 98°C 30s
3) 55°C 30s
4) 72°C 1 min
   35 repeats of the steps bloc from 2) to 4)
5) 72°C 2 min
6) 10°C infinite

➔ After checking on agarose 1% gel, we determined that the fragments were successfully amplified.

Digestion

The amplified fragments were digested by EcoRI and PstI during 1h at 37°C. The reaction was performed in NEB CutSmart buffer.

The digested mixes were purified by using the kit Wizard SV Gel and PCR Clean-Up System (Ref: A9282, Promega).

Design Primer for Gibson Assembly -> PR_IG0075 ->81

15 September 2016

Ligation

The 2 fragments were then ligated separately in linear pSB1C3 provided by IGEM Headquarters in a ratio 5:1.

The ligation mix was used for transformation in *E. coli* DH5-alpha.

Transformation

- Thaw 25 µL chemo-competent DH5-alpha *E. coli*
- Add 3 µL DNA (mix ligation)

- Incubation 20 min on ice
- Heat shock: 45s at 42°C
- Incubation 3 min on ice
- Add 225 µL of LB medium
- Incubation 1h at 37°C with shaking
- Spread 120 µL on plates LBC
- Incubation overnight 37°C

Preculture of clones in 3mL LB Cam. From the ligation.

16 September 2016

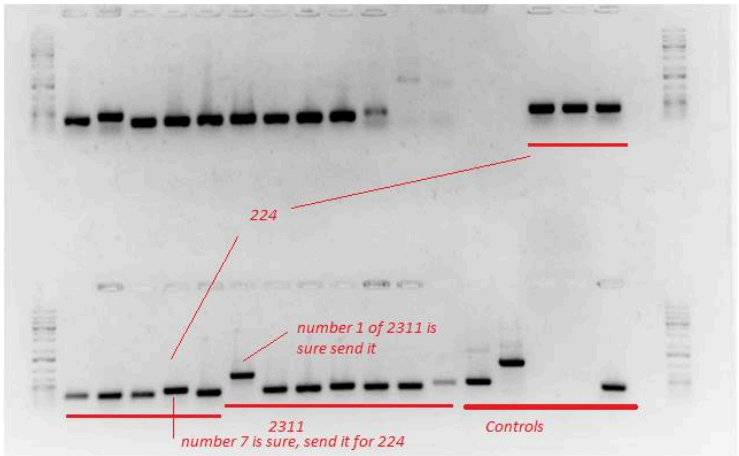
Colonies PCR

Mix (25 µL total volume reaction):

- 12.5 µL DreamTaq PCR Mastermix 2X (ThermoScientific)
- 2.5 µL 10X DreamTaq Green Buffer (ThermoScientific)
- 0.25 µL Forward Primer (0.5 µM) iG001
- 0.25 µL Reverse Primer (0.5 µM) iG002
- 9.5 µL H2O filtrated (qs 25 µL)

+ clones

Agarose gel 1%



Precultures

Positive clones selected from the agarose gel results were picked and used to inoculate 3 mL of LBC (25 µg/mL chloramphenicol) medium.

Observation

➔ All the precultures have grown

- PCR colony

Mix:

- Dream Taq MM 2X
- Fw
- Rv
- DNA
- H2O

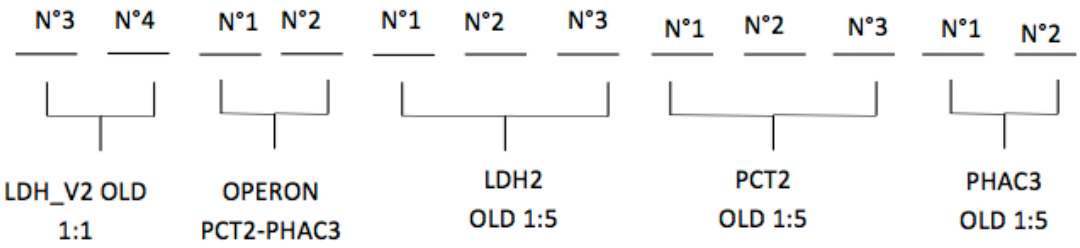
10 µl	280 µl
1,5 µl	42 µl
1,5 µl	42 µl
1 µl	-
6 µl	168

X28

Just to remind:

Position E5: Mix without clone
Position E6: Mix with pLAC into Psb1c3
Position E7: Mix with mRF-histag into Psb1C3

Plan de migration:



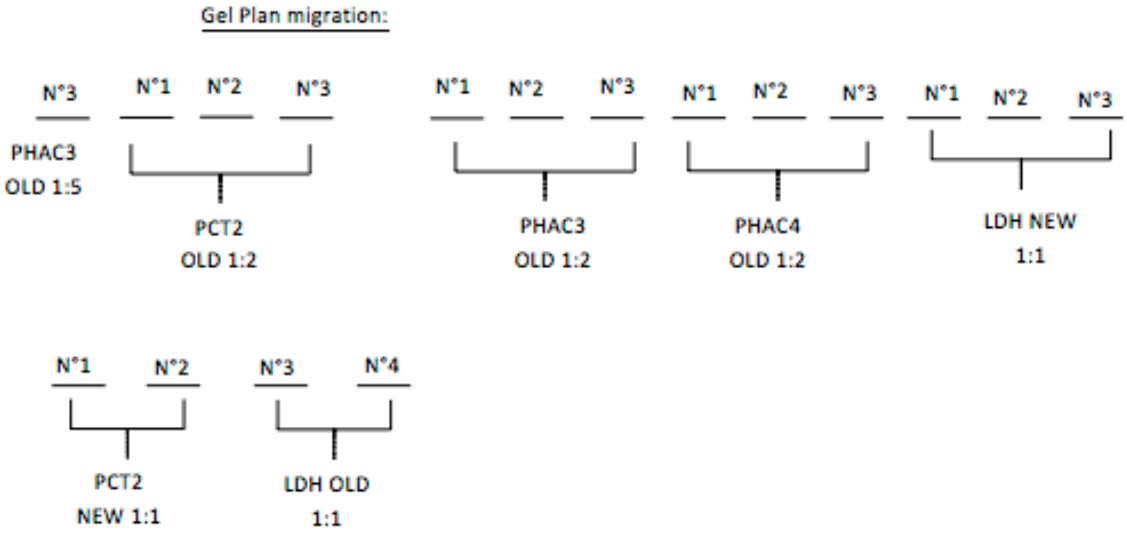
- Expected Bands

LDH_V2= ~1550
PCT_V2=~1950
PHAC_V3=~2050
PHAC_V4=~2000

- Cycle

95°C	3 min
95°C	30 scd
50°C	30 scd
72°C	1,15 min
72°C	5 min
10°C	∞

X30



20 September 2016

Precultures

3 mL LB medium in 15 mL Falcon tube+ 1µL P. putida KT2440 from -80°C stock

Incubation 30°C overnight with shaking.

- PCR Gibson of pct_V2, phac_V3, phac_V4, Psb1b3 HQ, Psb1c36 mRFP-HISTAG

Mix:

Vf=50µl

- 5X Q5 Reaction Buffer	10 µl	70 µl
- 10 mM dNTP	1 µl	7 µl
- 10 µM Fw	2,5 µl	- µl
- 10 µM Rv	2,5 µl	- µl
- DNA	1 µl	-
- Q5 (0,2 U/µl)	0,5 µl	3,5 µl
- H2O	32,5 µl	227 µl

X7

- Solubilization of primers

For PR_IG075: Add 209µl H2O for [100µM]

For PR_IG076: Add 198µl H2O for [100µM]

For PR_IG077: Add 242µl H2O for [100µM]

For PR_IG078: Add 236µl H2O for [100µM]

For PR_IG079: Add 257µl H2O for [100µM]

For PR_IG080: Add 300µl H2O for [100µM]

For PR_IG081: Add 235µl H2O for [100µM]

For PR_IG082: Add 229µl H2O for [100µM]

For PR_IG083: Add 238µl H2O for [100µM]

For PR_IG084: Add 266µl H2O for [100µM]

Aliquot 1/10 of each primer

- Gel purification of PCT2,PHACV3,PHACv4
- Purification

21 September 2016

- Gel Extraction pSb1C3 + mRFP-Histag

Gel weight= 300mg

- PCR clean-up pct2, phac_v3, phac_v4

Nanodrop:

Gènes	Concentration (ng/µl)	Ratio 260/280
PCT_V2	152	1,82
PHAC_V3	130,7	1,84
PHAC_V4	132,9	1,83
Psb1c3 IHQ	108,3	1,80
Psb1c3 Biobrick	14,7	1,74

- Gibson
  - Ratio Calcul

➔ Pct2-phac3 into Psb1c3

Mix:

Psb1c3 IGHQ	0,92 µl
H2O	6,75 µl
PCT2	1,04 µl
PHAC3	1,29 µl

➔ Pct2-phac4 into Psb1c3 IGHQ

Mix:

Psb1c3 IGHQ	0,92 µl
H2O	6,76 µl
PCT2	1,04 µl
PHAC4	1,27 µl

➔ Pct2-phac3 into Psb1c3 Biobrick

Mix:

Psb1c3 Biobrick	6,80 µl
H2O	0,87 µl
PCT2	1,04 µl
PHAC3	1,29 µl

➔ Pct2-phac4 into Psb1c3 Biobrick

Mix:

Psb1c3 Biobrick	6,80 µl
H2O	0,89 µl
PCT2	1,04 µl
PHAC4	1,27 µl

Add 10µl of HIFI DNA Assembly MM in each.
And 10µl of hifi with 10µl of positive control
Keep on ice !

- Incubation: 50°C, 15 minutes then on ice

- Chemically Competent Cells Transformation

➔ Add 2µl of the chilled assembled to 25µl competent cells
➔ Waitting 30min in ice
➔ Heat shock at 42°C for 30scd
➔ Transfer the tube on ice for 2min
➔ Add 950µl LB for each
➔ Incubation: 60min, 37°c, 250 rpm
➔ Spread 100µl on the plate
➔ Incubation 37°c overnight

23 September 2016

- Gibson plate observation

➔ DH5-alpha pct2-phac3-psb1c3 iGHQ 14 clones
➔ DH5-alpha pct2-phac4-psb1c3 iGHQ 13 clones
➔ DH5-alpha pct2-phac3-psb1c3 Biobrick 1 clones

➔ DH5-alpha pct2-phac4-psb1c3 Biobrick 5 clones

- PCR Colony

Mix:

- Dreamtaq Master Mix 2X	10 µl	350 µl
- PR_IG001	1,5 µl	52,5 µl
- PR_IG002	1,5 µl	52,5 µl
- DNA	1 µl	-
- H2O	6 µl	210 µl

→ X35

Cycle:

95°C	3 min
95°C	30 scd
50°C	30 scd
72°C	1,30 min
72°C	2 min
10°C	∞

X25

- Result Gibson

N°2 pct2 phac4 psb1c3 iG HQ

N°1 pct2 phac4

N°5 pct2 phac4 psb1c3 Biobrick

- Préculture in LB+ Cam (10µg/ml)
- Miniprep of the cultures
- Gel Verification of Gibson PCR

➔ Don't work

Growth experiments

Stock in 50% glycerol stored at -80°C

Experiments performed in triplicates on 96 wells plate with LB medium as blank.

The plate was incubated at 30°C with shaking during 20h after inoculating with 1/1000 dilution of preculture.

The OD at 600 nm was read every 20s.

The experiments were repeated 3 consecutive days from fresh precultures started the day before.

The results were sent to Imperial College IGEM team for collaboration along with the results of carbon source tests performed in July.

27 September 2016

- Sequencing

➔ WatchBox 1887819 (27.09.16)

28th September 2016

- Gibson

➔ Calcul for Ratio 1:1

Mix DNA 1:

pct2	0,62 µl
Phac3	0,76 µl
H2O	8,62 µl

Mix DNA 2:

pct2	0,66 µl
Phac3	0,81 µl
H2O	8,63 µl

Mix DNA3:

pct2	0,62 µl
Phac4	0,75 µl
H2O	8,63 µl

Mix DNA 4:

pct2	0,66 µl
Phac4	0,80 µl
H2O	8,54 µl

➔ Calcul for Ratio 1:2

Mix 5:

Diluted pct2	1,04 µl
Diluted Phac3	1,29 µl
H2O	6,75 µl
IGHQ Vector	0,92 µl

➔ Mix 6:

Diluted pct2	1,04 µl
Diluted Phac4	1,27 µl
H2O	6,76 µl
IGHQ Vector	0,92 µl

➔ Mix 7:

Diluted pct2	1,04 µl
Diluted Phac3	1,29 µl
H2O	4,27 µl
Biobrick Vector	3,40 µl

➔ Mix 8:

Diluted pct2	1,04 µl
Diluted Phac4	1,27 µl
H2O	4,29 µl
Biobirck Vector	3,40 µl

➔ Put 15min to 50°C and after at -20°C

29th September 2016

- Transformation

➔ 2µl product + 25µl of Dh5-alpha cells+ 950µl SOC Medium

➔ 1h Recovery

- Culture plate LB Cam (10µg/ml)
- Preparing plate

Ci= 50mg/ml $vi = \frac{10\mu g * 25mL}{50000} = 5\mu l$

Cf= 10µg/ml

Vf= 25ml

- Purification PCR of gblock fragment
- Nanodrop

Nothing ☹

30th September 2016

- Concentration / SpeedVac
- Verification Gel of purifications
- PCR amplification gblock

4 October 2016

Gibson assembly 2nd step

pSB1C3 IGEM Headquarters 110.3 ng/µL

pSB1C3 mRFP 14.1 ng/µL

Mix Gibson from the 1st step giving the operon:

1) PCT2+PhaC3 65.5 ng/µL
2) PCT2+PhaC4 60.8 ng/µL
3) PCT2+PhaC3 63.4 ng/µL
4) PCT2+PhaC4 65.7 ng/µL

Gibson ratio 1:1

Vector amplified from pSB1C3-mFRP	1)	Vector	3.40 µL (50 ng)
		Insert (mix 1)	1.21 µL
		H2O (qs 10 µL)	5.40 µL
	2)	Vector	3.40 µL (50 ng)
		Insert (mix 2)	1.30 µL
		H2O (qs 10 µL)	5.30 µL
	3)	Vector	3.40 µL (50 ng)
		Insert (mix 3)	1.25 µL
		H2O (qs 10 µL)	5.35 µL
	4)	Vector	3.40 µL (50 ng)
		Insert (mix 4)	1.20 µL
		H2O (qs 10 µL)	5.40 µL
Linear vector pSB1C3 provided by IGEM Headquarters	5)	Vector	0.91 µL (100 ng)
		Insert (mix 1)	2.42 µL
		H2O (qs 10 µL)	6.68 µL
	6)	Vector	0.91 µL (100 ng)
		Insert (mix 2)	2.60 µL
		H2O (qs 10 µL)	6.49 µL
	7)	Vector	0.91 µL (100 ng)
		Insert (mix 3)	2.50 µL
		H2O (qs 10 µL)	6.60 µL
	8)	Vector	0.91 µL (100 ng)
		Insert (mix 4)	2.41 µL
		H2O (qs 10 µL)	6.68 µL

Add 10 µL NEB HiFi DNA Assembly Mastermix 2X
Incubation 50°C, 15 min
Cool on ice

Transformation

- Thaw 50 µL chemo-competent DH5-alpha *E. coli*
- Add 2 µL DNA (mix Gibson)
- Incubation 30 min on ice
- Heat shock: 30s at 42°C
- Incubation 2 min on ice
- Add 950 µL NEB medium (#B9020S)
- Incubation 1h at 37°C with shaking
- Centrifugation 7000 rpm 1 min
- Resuspend cells with rest of supernatant after throwing away the most of it
- Spread all the content on plates LBC
- Incubation overnight 37°C

7 October 2016

Solubilisation of IDT gBlocks in 100 µL of nuclease-free water and short centrifugation

Incubation 50°C during 20 min

Vortexing and then, short centrifugation again

-PCR of gBlock genes

Mix:

- 10 µL Q5 reaction buffer 5X
- 1 µL dNTP mix
- 2.5 µL 10 µM Forward Primer (iG063)
- 2.5 µL 10 µM Reverse Primer (iG064)
- 1 µL DNA template
- 0.5 µL Q5 DNA polymerase
- 32.5 µL Nuclease-free water (qs 50 µL)

Mix for 5 reactions

PCR program:

1) 98°C 30s
2) 98°C 30s
3) 55°C 30s
4) 72°C 1 min
   35 repeats of the steps bloc from 2) to 4)
5) 72°C 2 min
6) 10°C infinite

Colony PCR on Gibson transformation

- 12.5 µL DreamTaq Green PCR Master Mix 2X (Thermo Scientific, #K1081)
- 1.25 µL 10 µM Forward Primer (iG001)
- 1.25 µL 10 µM Reverse Primer (iG002)

- 10 µL Nuclease-free water (qs 25 µL)

+ clone

Mix for 23 reactions

PCR program:

1) 95°C 5 min
2) 95°C 30s
3) 50°C 30s
4) 72°C 3 min
   30 repeats of the steps bloc from 2) to 4)
5) 72°C 10 min
6) 10°C infinite

-Nanodrop of PCR genes purification of 3.09.16

Gènes	Concentration (ng/µl)	Ratio 260/280
LDH_V2	138,2	1,88
PCT_V2	115,6	1,92
PHAC_V3	125,4	1,91
PHAC_V4	134,7	1,91

8 October 2016

➔ Problem with several PCR reaction tubes → we do them again

Colony PCR on Gibson transformation

- 12.5 µL DreamTaq Green PCR Master Mix 2X (Thermo Scientific, #K1081)
- 1.25 µL 10 µM Forward Primer (iG001)
- 1.25 µL 10 µM Reverse Primer (iG002)
- 10 µL Nuclease-free water (qs 25 µL)

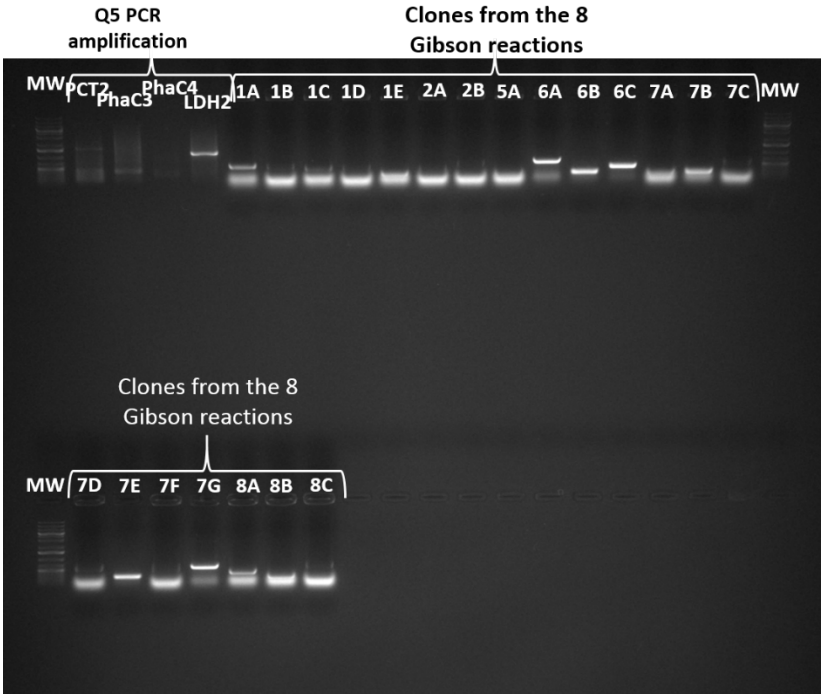
+ clone

Mix for 9 reactions

PCR program:

1) 95°C 5 min
2) 95°C 30s
3) 50°C 30s
4) 72°C 3 min
   30 repeats of the steps bloc from 2) to 4)
5) 72°C 10 min
6) 10°C infinite

Agarose gel 1%



Digestion E+P

Mix: Vf=100µl

DNA	50 µl
Buffer 2.1	10 µl
EcoR1	1,5 µl
Pst1	1,5 µl
H2O	37µl

PCR clean-up

- Nanodrop

7 October		
Gènes	Concentration (ng/µl)	Ratio 260/280
LDH_V2	37,7	2,04
PCT_V2	29,8	2,07
Phac_V3	35,7	2,04
Phac_V4	40,3	2,05

30 September		
Gènes	Concentration (ng/µl)	Ratio 260/280
LDH_V2	8,8	2,49
PCT_V2	7,7	2,32
Phac_V3	11,0	2,68

Phac_V4	8,1	2,46

21 September		
Gènes	Concentration (ng/µl)	Ratio 260/280
LDH_V2	7,9	2,54
PCT_V2	7,8	2,59
Phac_V3	7,0	2,35
Phac_V4	7,3	2,31

3 September		
Gènes	Concentration (ng/µl)	Ratio 260/280
LDH_V2	16,3	2,20
PCT_V2	7,1	2,79
Phac_V3	8,2	2,30
Phac_V4	9,0	2,52

9 October 2016

Pouring of 46 LBC (10 µg/mL chloramphenicol) plates + 5 LBK (50 µg/mL kanamycin) plates + 2 LBS (50 µg/mL spectinomycin) platesx

10 October 2016

Concentration measurements:

LDH 7.10: 37.7 ng/µL
PCT 7.10: 29.8 ng/µL
PhaC3 7.10: 35.7 ng/µL
PhaC4 7.10: 40.3 ng/µL

LDH 3.09: 16.3 ng/µL
PCT 3.09: 7.1 ng/µL
PhaC3 3.09: 8.2 ng/µL
PhaC4 3.09: 9.0 ng/µL

LDH 21.09: 7.9 ng/µL
PCT 21.09: 7.8 ng/µL
PhaC3 21.09: 7.0 ng/µL
PhaC4 21.09: 7.3 ng/µL

LDH 30.08: 8.8 ng/µL
PCT 30.08: 7.7 ng/µL
PhaC3 30.08: 11.0 ng/µL
PhaC4 30.08: 8.1 ng/µL

11 October 2016

- Preparation 1L water+ 0,1% HCOOH
- Test of solubility -> 10µl of standard in 90µl Solvant
    - Anthocyanin

➔ Isobutanol = not soluble
➔ DMSO = not soluble
➔ Ethyl Acétate = not soluble
➔ CH3CN 60% = not soluble
➔ CH3CN 100% = not soluble
➔ ETOH = not soluble
➔ HCL = ~soluble
➔ Methanol + Water + HCL = ~soluble
➔ Methanol+ HCL= ~soluble

- Quercetin

➔ EtOH = soluble

- Maldivin

➔ Soluble in Methanol HCL

Bettencourt Collaboration = we decided to do spectrometry masse instead of HPLC because it is more fast in time and the quercetin will be passed in negative mode but the maldivin and anthocyanin will be passed in negative mode.

12t October 2016

- Solution Stock

➔ At 2mg/ml in EtOH 100%

2mg -> 1ml EtOH

12,3 mg -> 6,15 ml EtOH

- Standard range in H2O mQ

- Quercetin

Isotopic mass = 302,042664 Da

Formule= C15H10O7

Solution Stock: [2000mg -> 1000ml]

- [20 mg/1000 ml]

➔ 6 µl in 594 µl H2O mQ -> Solution 1

- [10mg/L]

➔ 300 µL of 1 + 300 µl H2O -> Solution 2

- [5 mg/L]

➔ 300µl of 2 + 300µl H2O -> Solution 3

- [1mg/L]

➔ 100 µl of 3 + 400 µl H2O -> Solution 4

- [0,5 mg/L]

➔ 250 µl of 4 + 250 µl H2O -> Solution 5

- [0,1 mg/L]

➔ 50µl of 5 + 200 µl H2O -> Solution 6

- Maldivin

Isotopic mass = 330,073955 Da

Formule= C17H15O7

Solution Stock: 50µl + 450µl MeOH-HCl 2M -> [100 mg/L]

- [20 mg/1000 ml]

➔ 100 µl in 400 µl H2O mQ or CH3CN 60% -> Solution 1

- [10mg/L]

➔ 250 µL of 1 + 250 µl H2O mQ or CH3CN 60%-> Solution 2

- [5 mg/L]

➔ 250 µl of 2 + 250µl H2O mQ or CH3CN 60%-> Solution 3

- [1mg/L]

➔ 100 µl of 3 + 400 µl H2O mQ or CH3CN 60%-> -> Solution 4

- [0,5 mg/L]

➔ 250 µl of 4 + 250 µl H2O mQ or CH3CN 60%-> -> Solution 5

- [0,1 mg/L]

➔ 50µl of 5 + 200 µl H2O mQ or CH3CN 60%-> -> Solution 6

- Plaque MS

➔ 75 µl of each dilution + 75 µl Solution C

Solution C = 95% acetate + 5% carbonate ammonium 10mM

- Vial

➔ 200 µl of each dilution in insert into vial for test HPLC

13th October 2016

- Ligation 1:10 With different

Vf= 25µl

Length GB3 = 2038 bp

IGHQ = 2037 bp

DNA Mix:

Vf= 20 µl

7 October 2016

LDH + IGFQ (100ng)

PHAC3 + GB3 (75ng) 1:5

LDH + GB3 (50ng)	
Vector	2,16 µl
H2O	9,77 µl
LDH	8,08 µl

Vector	0,92 µl
H2O	2,92 µl
LDH	16,2 µl

PCT + IGHQ (75ng)	
Vector	0,69 µl
H2O	0,78 µl
PCT	20,09 µl

PCT + GB3(50ng)	
Vector	2,16 µl
H2O	4,46 µl
PCT	13,39 µl

PHAC3 + IGHQ (75ng)	
Vector	0,69 µl
H2O	1,45 µl
PHAC3	17,85 µl

PHAC3 + GB3(50ng)	
Vector	2,16 µl
H2O	5,95 µl
PHAC3	11,90 µl

PHAC4 + IGHQ (75ng)	
Vector	0,92 µl
H2O	2,05 µl
PHAC4	21,12 µl

PHAC4 + GB3(50ng)	
Vector	2,16 µl
H2O	7,29 µl
PHAC4	10,56 µl

3rd September 2016

LDH + GB3 (50ng) 1:10	
Vector	2.16 µl
H2O	0.89 µl
LDH	18.68 µl

21th September 2016

PHAC3 + GB3 (75ng) 1:5	
Vector	2.16 µl
H2O	2.44 µl
PHAC3	25.9 µl

30th September 2016

PHAC3 + GB3 (75ng) 1:5	
Vector	2.16 µl
H2O	2.51 µl
PHAC3	30.33 µl

- Preparation of standard range for MS
  20mg/L; 10mg/L; 5mg/L; 1mgL/L

- Preparation of samples
  I take 75 uL of supernatant of each + 75uL of solution C.

14 October 2016

- Transformation of the ligation product by heat shock
  3uL of ligation product into DH5alpha strain.
  Recovery 1 hour.
  I spread them in LB + cam plates.

15 October 2016

Digestion PhaC3_only and PCT2_only

- 30 µL IDT gBlaock gene
- 5 µL buffer NEB CutSmart 10X
- 1 µL EcoRI
- 1 µL SpeI
- 13 µL nuclease-free water (qs 50 µL)

Incubation 1h 37°C

DNA clean & concentrator-5 kit (Zymo Research, D4003S)
DNA binding buffer added with 5:1 ratio
1 min incubation after adding 20 µL of nuclease-free water before centrifugation for elution

Ligation

	Quick ligase	T4 DNA ligase
pSB1C3 digested by E+S (from clone B → 13.3 ng/µL)	6 µL	6 µL
gene digested by E+S	3 µL	3 µL
Buffer	10 µL Quick ligase buffer 2X (NEB)	2 µL T4 DNA ligase buffer 10X (NEB)
ligase	1 µL Quick ligase (NEB)	1 µL T4 DNA ligase
H2O (nuclease-free water)	-	8 µL

Transformation ligation mixes in pSB1C3

- Thaw 50 µL chemo-competent DH5-alpha *E. coli*
- Add 2 µL DNA (mix ligation)
- Incubation 20 min on ice
- Heat shock: 45s at 42°C
- Incubation 3 min on ice
- Add 450 µL LB medium
- Incubation 1h at 37°C with shaking
- Centrifugation 7000 rpm 3 min
- Throw away supernatant
- Resuspend cells with rest of supernatant (around 50 µL)
- Spread all the content on plates LBC (10 µg/mL chloramphenicol)
- Incubation overnight 37°C

Transformation ligation mixes in pSEVA 224, 424 or 2311

- Thaw 50 µL chemo-competent DH5-alpha *E. coli*
- Add 2 µL DNA (mix ligation)
- Incubation 20 min on ice
- Heat shock: 45s at 42°C
- Incubation 3 min on ice
- Add 450 µL LB medium
- Incubation 1h at 37°C with shaking
- Centrifugation 7000 rpm 3 min
- Throw away supernatant
- Resuspend cells with rest of supernatant (around 50 µL)

- Spread all the content on plates LBK (50 µg/mL kanamycin) for pSEVA 224 or 2311 or LBS (50 µg/mL spectinomycin) for pSEVA 424
- Incubation overnight 37°C

DNA PCR clean-up kit (NEB) for gBlock genes amplified by PCR

16 October 2016

➔ Our strain of DH5-alpha bacteria seems already resistant to spectinomycin → test by preculture in 3 mL LBS (50 µg/mL spectinomycin) liquid medium

Concentration of amplified gBlocks after PCR clean-up

PhaC4: 86.6 ng/µL
PhaC3: 120.6 ng/µL
LDH2: 202.5 ng/µL
PCT2: 154.6 ng/µL

Digestion by E+P of amplified genes

	PhaC3	PhaC4	PCT2	LDH2
DNA genes	7 µL	7 µL	5 µL	4 µL
Buffer NEB CutSmart	5 µL	5 µL	5 µL	5 µL
EcoRI	1 µL	1 µL	1 µL	1 µL
PstI	1 µL	1 µL	1 µL	1 µL
H2O nuclease-free	36 µL	36 µL	38 µL	39 µL

DNA clean & concentrator-5 kit (#D4003S Zymo Research)
Ratio 5:1 of DNA binding buffer
Incubation 1 min with 20 µL of nuclease-free water before centrifugation for elution

Precultures

2 mL LBC (25 µg/mL chloramphenicol) + clones from transformation of DH5-alpha E. coli with ligation mixes of pSB1C3 with PhaC3_only or PCT2_only

17 October 2016

Miniprep

GenElute Plasmid Miniprep Kit (Sigma, PLN350-1KT)

3 clones for PhaC3 and 14 clones for PCT2

Testing methods to detect the interest compound in HPLC

Samples run

- Preparation of samples
  - Vortex and samples centrifugation, 2mn, max speed.
  -Take 120uL of supernatant of each and put them in each 1.5 tube.
  -Add 120 uL acetonitrile 100%.
  -Centrifugation, 1mn, max speed.
  -Load 200uL in the vials for HPLC.

18 October 2016

Colony PCR on the last trials of insertion of PhaC3, PhaC4, PCT2, LDH2, PhaC3_only and PCT2_only inside pSB1C3 and of insertion of PhaC4, PCT2 and LDH2 inside pSEVA224 and pSEVA2311 in process.

HPLC Analysis (see Bettancout collaboration)

Sequencing of 224 and 2311 plasmids

19 October 2016

Characterization of pSEVA2311 in process.