Auxotrophic approach

LUNDI 03/07/2017

Made with benchling

RFP expression

Resuspended lyophilised DNA (from M11 well - iGEM) in 10 µL H2O
Electroporation with TOP10 competent cells, recovered in liquid LB for 30 minutes at 37°C.
Cells plated out on LB agar + chloramphenicol (30 µg/mL) and incubated overnight at 37°C.

MARDI 04/07/2017

Uniform cell layer : inoculated liquid LB + Cm, incubated onvernight at 37°C

MERCREDI 05/07/2017

Recovered DNA from several liquid cultures with High Pure Plasmid Isolation Kit (Roche). DNA eluted with 40 µL H2O.
Concentration (NanoDrop):

Table1

	A	B	C
1		Conc (ng/µL)	260/280
2	Tube 1	43.5	1.68
3		197.3	1.52
4	Tube 2	21.7	2.22
5		277.5	1.47
6	Tube 3	170.8	1.59
7	Tube 4	25.7	1.64
8		25.4	1.89

Purification with Monarch Nucleic Acid Purification kit.

Table2

	A	B	C
1		Conc (ng/µL)	260/280
2	Tube 1	50.3	1.69
3		38.7	1.84
4	Tube 2	40.1	1.90
5		54.3	1.72
6	Tube 3	49.3	1.80
7		44.1	1.76
8	Tube 4	58.5	1.70
9		42.5	1.77

Vector preparation

- Biobrick prelevement

- pAra promoter

- T7 promoter + RBS
- RBS
- terminator

- Desalted one RBS sample (sample -)

Electroporation: both samples (- and +) worked
No desalting for the other three other plasmids.

Cells recovered in liquid LB + Cm for 45 minutes at 37°C, plated out on LB agar + Cm overnight at 37°C.

BL21 (DE3) competent cells
10 mL preculture of BL21 cells in liquid LB, incubated overnight at 37°C.

JEUDI 06/07/2017

Uniform cell layer for every plate. Inoculated liquid LB + Cm and replated in the evening on LB agar + Cm.
Glycerol stock: 500 µL of cells + 50 µL glycerol 100%. Stored at -80°C.

BL21 (DE3) competent cells
Inoculated 2x500 mL of liquid LB with 5 mL of preculture. Incubated 2h10 at 37°C with shaking. OD600 = 0.5 and 0.53
Resuspended in 350 mL of chilled glycerol 10% (v/v) and centrifuged at 4000 RPM for 10 minutes. Step repeated with 100 mL and 50 mL.
Resuspended in 4 mL chilled glycerol 10% (v/v) and aliquoted in 100 µL portions. Stored at -80°C (blue-striped tubes).

VENDREDI 07/07/2017

Transformation of pCas plasmid into BL21. Recovered in liquid LB at 37°C for 1h, then plated out on LB agar + Kan (30 µg/mL) and incubated over the weekend at room temperature.

LUNDI 10/07/2017

RBS, terminator and pBAD plates didn't grow: redid from the 05/07 plates.

Antibiotic stocks

Ampiciline	100 mg/mL in H2O
Chloramphenicol	34 mg/mL in EtOH
Kanamycine	30 mg/mL in H2O
Tetracycline	12.5 mg/mL in EtOH

MARDI 11/07/2017

RBS, terminator and pBAD plates didn't grow: precultures in LB + Cm from frozen stock.
Extraction of T7 + RBS DNA (Roche kit) + concentration (Monarch kit).

Table3

	A	B	C
1		Conc (ng/µL)	260/280
2	Tube 1	43.8	1.77
3		33.9	1.77
4	Tube 2	47.9	1.66
5		59.2	1.61

Stored at -20°C.

MERCREDI 12/07/2017

Retransformed RBS, terminator and pBAD in BL21.

JEUDI 13/07/2017

Verification of auxotrophic strain (TyrA- from Keio Collection)
- 2 plates M9 agar
- 2 plates M9 agar + tyrosine (5 mg/L)
- 2 plates M9 agar + thiamine (??)
- 2 plates M9 agar + tyrosine + thiamine

Control: AceK- strain from Keio Collection.

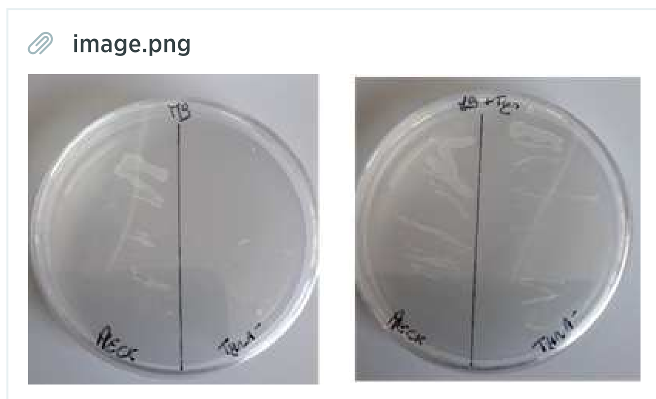
image.png

Plasmids
RBS didn't grow: retransformed in TOP10 cells (from S.W.), recovered in liquid LB for 45 minutes at 37°C and plated out on LB agar + Cm. Incubated overnight at 37°C.

Precultures of T7 + RBS and terminator.

VENDREDI 14/07/2017

Preculture of RBS, incubated at 37°C for the day.
DNA extraction (Roche kit), eluted with 40 µL H2O. Purification with Monarch kit.

Table4

	A	B	C
1		Conc (ng/µL)	260/280
2	pBAD	75.3	1.75
3		67.3	1.80
4	Terminator	50.8	1.97
5		17.6	1.58
6		18.1	1.47
7	RBS	21.6	1.81
8		23.1	1.79

DIMANCHE 16/07/2017

Preculture of terminator.

LUNDI 17/07/2017

DNA extraction (Roche kit).

Table5

	A	B	C
1		Conc (ng/µL)	260/280
2	Terminator	45.6	1.92
3		43.8	1.93

3A assembly
Preparation of master mixes as per iGEM protocol.

>Assembly of RFP and terminator
RFP (=Part A): 2047 bp
Termi (=Part B): 2044 bp
Backbone: pSB1K3 (KanR) 2096 bp

Ligation overnight at 4°C.

MARDI 18/07/2017

Follow up of the ligation: heat-kill at 80°C for 20 minutes.
Purification (Monarch kit), eluted in 10µL H2O: 9.5 ng/µL (280/280: 1.75).
Transformed in TOP10, plated out on LB agar + Kan and incubated overnight at 37°C.

CRISPR/Cas9: making theTyrA- auxotrophic strain
Cloning of sgRNA against TyrA in pTARGET (TetR) with Gibson assembly.

PCR (50 µL):

REV primer (10 µM)	2.5 µL
FWD primer (10 µM)	2.5 µL
5x buffer HF	10 µL
dNTPs (20 mM)	1 µL
DNA (30 ng/µL)	2 µL
Phusion Polymerase	0.5 µL
H2O	31.5 µL

Program: 98°C 5 minutes

98°C	30 sec	25 cycles
72°C	2 minutes	
72°C	7 minutes	
4°C	∞	

Purification (Monarch kit): ~180 ng/µL

3A assembly
> Assembly of pBAD and RBS
pBAD (=Part A): 959 bp

RBS (= Part B): 38 bp
Backbone: pSB1T3 (TetR) 2420 bp

Followed protocol, except for the ligation: overnight at 4°C.

MERCREDI 19/07/2017

3A assembly
Purification of pBAD-RBS, eluted with 10 µL H2O: 16.1 ng/µL (260/280: 1.91).
Transformed in TOP10 electrocompetent cells, incubated onvernight at 37°C in liquid LB + Tet.

Preculture of RFP-TERMI.

CRISPR/Cas9: making theTyrA- auxotrophic strain
Gibson assembly of pTARGET

NEB Gibson mastermix	10 µL
PCR product	1 µL
DpnI	0.5 µL
H2O	8.5 µL

Incubated for 1h at 50°C, purification (Monarch kit) and transformed in TOP10 commercial cells (NEB).

JEUDI 20/07/2017

Previous transformation of pBAD-RBS failed, redid in TOP10 and NC1000 (competent cells given by S.W.)

CRISPR/Cas9: making theTyrA- auxotrophic strain
Colony PCR for 6 clones. PCR identical as previous.

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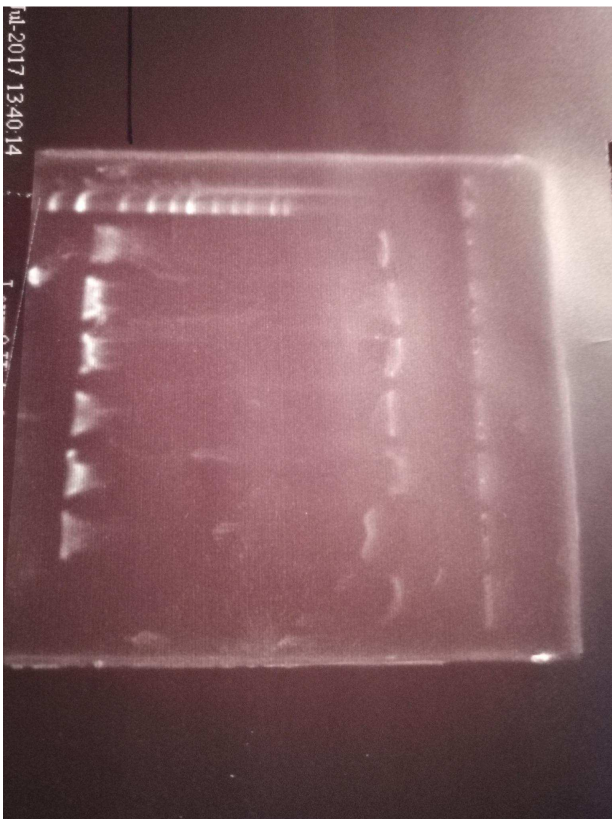

Purification (Monarch kit): 10 ng/µL
Send to sequencing, conserved on LB agar plates + Tet for the weekend at room temperature.

LUNDI 24/07/2017

Transformation failed: redid in TOP10.

CRISPR/Cas9: making theTyrA- auxotrophic strain
Sequencing results: clones 2 and 4 ok.
Clone 6 inoculated in liquid LB + Tet and incubated onvernight at 37°C.

MARDI 25/07/2017

Transformation failed again: re-assembly of pBAD-RBS. Followed protocol, except: RBS and pBAD concentration 2x that of the backbone, and ligation at 16°C overnight.

MERCREDI 26/07/2017

TyrA- strain plated on two M9 + photocaged tyrosine + Kan agar plates. Incubated for 6h outside, one in a cardboard box and the other in the light. Incubated further at 37°C overnight.

JEUDI 27/07/2017

Redid preculture of RFP-TERMI (previous tubes forgotten in the incubator).

Results for the photocaged tyrosine: bacteria grew on the one exposed to light

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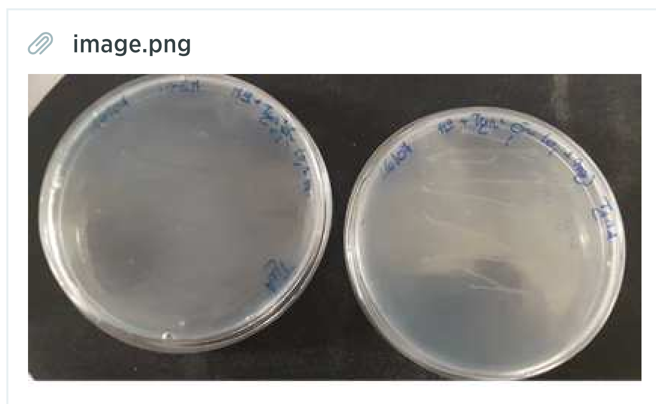

> Ideal concentration of tyrosine
Stock: 5 µg/mL

Table6

	A	B
1	Plate	Vol stock (mL)
2	1	1
3	2	0.75
4	3	0.5
5	4	0.3
6	5	0.1
7	6	0.05
8	7	0.2

Growth on every plate, but works the best with 0.2 mL of stock solution.

VENDREDI 28/07/2017

DNA extraction RFP-TERMI, eluted with 100 µL of elution buffer. Purification (Monarch kit).

Table7

	A	B	C
1		Conc (ng/µL)	260/280
2	Tube 1	40.3	1.92
3		46.2	1.77
4	Tube 2	39.2	1.81
5		43.6	1.90

LUNDI 31/07/2017

3A assembly
> Assembly of RFP-TERMI and pBAD-RBS
pBAD-RBS (= Part A): 3417 bp
RFP-TERMI (=Part B): 2931 bp
Backbone: pSB1C3 (CmR) 1070 bp

Followed protocol, but ligation overnight at 4°C.

MARDI 01/08/2017

Ligation complete plasmid
pBAD-RBS + RFP-TERMI = pTot1
T7-RBS + RFP-TERMI = pTot2

>Ligation of pTot1 (pSB1K3 backbone), transformed into TOP10 cells and plated out on agar LB (desalted for 20 minutes before transformation)
>Ligation of pTot2 (pSB1C3 backbone)

Controls: backbone without inserts

MERCREDI 02/08/2017

Transformation of pTot2 + control into TOP10 cells

pTot1: pick 5 colonies, preculture with 0.5% arabinose

JEUDI 03/08/2017

DNA extraction for pTot1 (Roche kit), eluted with 10 µL H_2O
NanoDrop: bad curves

Verificating ligation
Plasmid digestion with EcoR1-HF and PstI-HF (in CutSmart) : 4 µL DNA, 1 µL buffer, 0.5 µL enzymes
Agarose gel: no DNA

Precultures of pTot1 and pTot2 (4 each)

VENDREDI 04/08/2017

DNA extraction of pTot1 and 2

Table8

	A	B	C
1		Conc (ng/µL)	260/280
2	pTot1	9.2	2.1
3		6.2	2.44
4	pTot2	7	1.85
5		8	2.29
6		5.3	2.1

Plasmid digestion with EcoR1-HF and PstI-HF (in CutSmart) : 4 µL DNA, 1 µL buffer, 0.5 µL enzymes

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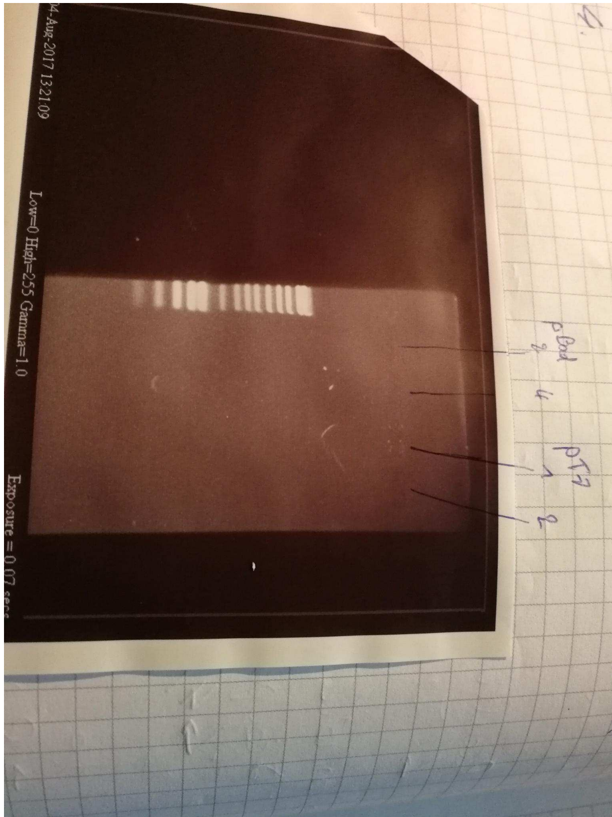

No DNA.

Concentration of samples with Monarch kit, new agarose gel: no DNA

Control
plates CM30 lig-/lig+
plates CM30 + drying (either with laminar flux (FL) or 1h at 60°C) + TOP10 control cells
Left on the bench for the weekend

LUNDI 07/08/2017

CM30 lig-/lig+ : both grew
CM30 FL and 60°C : both grew despite antibiotic

Strain contamination ? Bad Cm stock ?

>Precultures of RFP-TERMI and pBAD-RBS
> M9 agar without tyrosine preparation
> Several plates with differents concentrations of tyrosine (0.5-2.5 mL tyrosine per plate, 250 mg/L stock): 2 plates each with TyrA- cells

MARDI 08/08/2017

Growth in all plates: 5 mg/L of tyrosine enough
Redo test (0-0.4 mL tyrosine per plate, 250 mg/L stock)

Preculture of TyrA- (KanR) and TOP10 (SmR, from S.W.)

MERCREDI 09/08/2017

Making TyrA- and TOP10 electrocompetent cells
Inoculated 250 mL of LB with 5 mL preculture + antibiotic, incubated at 37°C for3h30
Centrifugated at 4000 RPM for 10 minutes, 4°C. Resuspended in 250 mL chilled glycerol 10% (v/v).
Redid washing with 3x with half volume glycerol each time.
Resuspended in 1.5 mL glycerol, aliquoted in 50 µL portion, stored at -80°C.

Control plates:
> TyrA- plated out on M9 + KAN30, M9 + CM30, and M9 alone (half stock, half competent cells)
> TOP10 competent cells plated out on LB: KAN30, CM30, Sm + Tet, and Sm

CRISPR/Cas9
Retransformed BL21 with pCas, plated out on agar LB (half transformed, half not)

JEUDI 10/08/2017

Results
>TyrA- : no growth on M9 alone or Cm (normal), two colonies on Kan
> BL21: no resistance to Kan or Cm
> TOP10 resist only to Sm

PCR amplification for Cas9 and RFP
>Agarose gel: correct profile for Cas9 (tubes 100 and 10) and RFP (tubes 1 & 4)

Assembly
Ligation RFP + TERMI (backbone Tet) as per iGEM protocol (control: no ligase)
Transformed into TOP10, plated out on agar LB + Sm and Tet at 37°C

>Ligation of RFP into plasmid 33 (pBAD-containing plasmid from S.W.)
Primers: n°331 and 332 from database
Amplification of RFP and iGEM backbone Kan with PCR

PCR for RFP (50 µL):

REV primer (10 µM)	2.5 µL
FWD primer (10 µM)	2.5 µL
5x buffer HF	10 µL
dNTPs (20 mM)	2 µL
DNA (30 ng/µL)	2 µL
Phusion Polymerase	0.5 µL
H_2O	30.5 µL

Program: 98°C 30 sec

98°C	5 sec	35 cycles
59°C	15 sec	
72°C	30 sec	
72°C	7 minutes	
4°C	∞	

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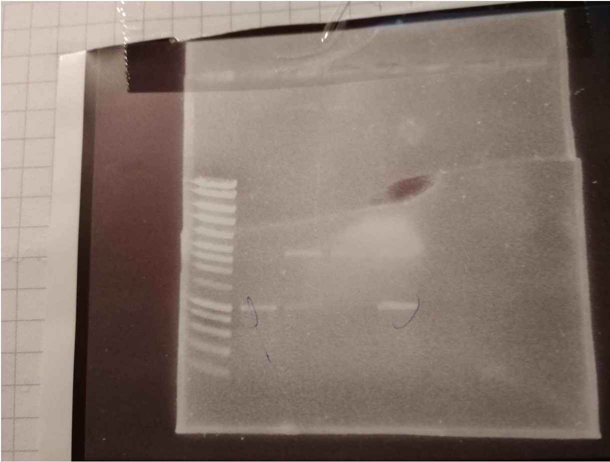

PCR for backbone: same (annealing temp: 71°C, primers n°317 and 320)

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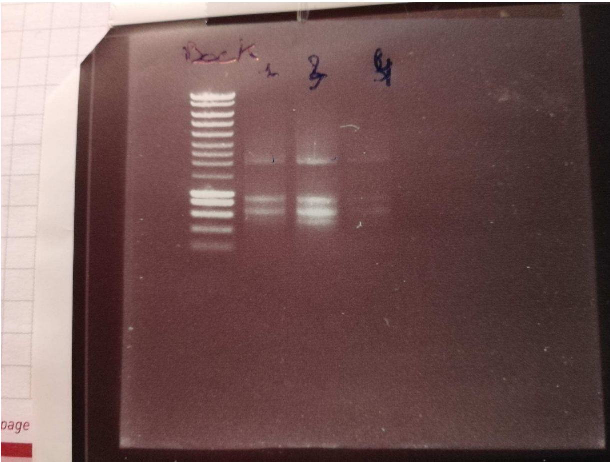

PCR of pCas (primers: n°1 and 52), GoTaq polymerase

Program: 98°C 30 sec

98°C	5 sec	25 cycles
52°C	15 sec	
72°C	45 sec	
72°C	7 minutes	
4°C	∞	

Results

>pCas: no DNA

>backbone: tubes 1, 3 & 4 ok

>RFP: tubes 1 &4 ok

VENDREDI 11/08/2017

Purification for ok tubes

Table9

	A	B	C
1		Conc (ng/µL)	260/280
2	RFP 1	21.6	1.72
3	RFP 2	18.3	1.82
4	BB 1	23.9	1.94
5	BB 3	86.7	1.82
6	BB 4	89.7	1.70

Ligation of RFP into plasmid 33 by restriction-ligation
>Enzymes: XbaI, EcoRI & NheI

Agarose gel:

Table18

	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P
1	Lane	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
2	Sample	pBAD	RBS	RFP	TERMI	Control Backbone	pBAD+RBS	RFP+TERMI	Control plasmid 33	RFP 1	RFP 2	10(Cas)-1	10(Cas)-2	100(Cas)-1	100(Cas)-2	Ladder

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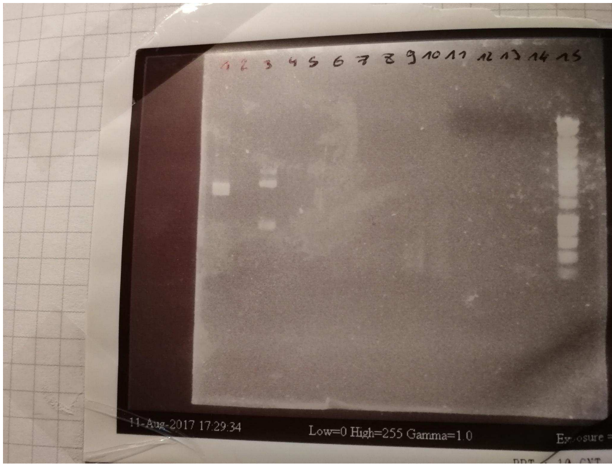

LUNDI 14/08/2017

Preculture Bl21-pCas.

MARDI 15/08/2017

Making BL21-pCas electrocompetent cells

Follow CRISPR/Cas9 protocol (see "Day 2")

PCR (step 11): protocol with Taq polymerase (primers n°336 and 337)

Program: 98°C 30 sec

98°C	5 sec	30 cycles
48°C	15 sec	
72°C	1min20 sec (1300 bp)	
72°C	7 minutes	
4°C	∞	

Precultures of 33RFP (Sm + 0.2% arabinose) and pBAD-RBS-TERMI

MERCREDI 16/08/2017

PCR of inserts

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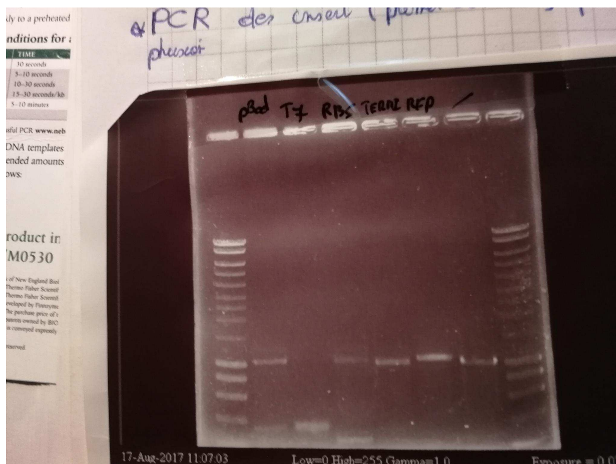

Precultures of pBAD, RBS and TERMI

JEUDI 17/08/2017

DNA extraction for pBAD, RBS and TERMI

Table10

	A	B	C
1		Conc (ng/µL)	260/280
2	pBAD	28.7	1.79
3	RBS	67.9	1.85
4	TERMI	44.7	1.92

Agarose gel with inserts + extracted plasmids

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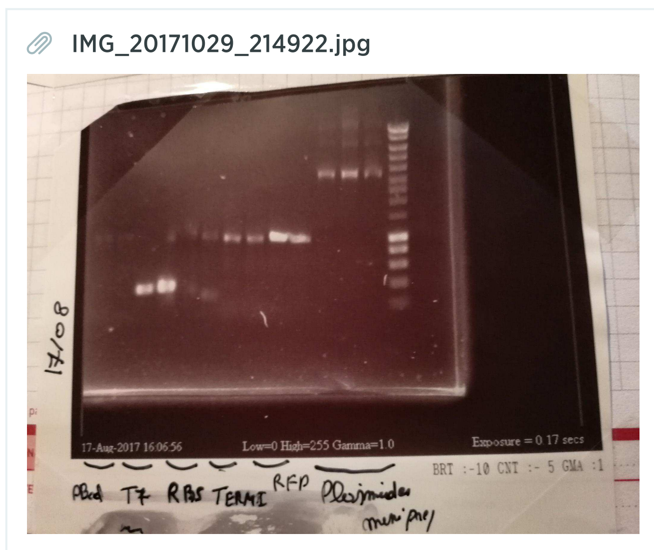

Purification of samples (Monarch kit), assembly as per 3A protocol of pBAD+RBS and RFP+TERMI (both into AmpR backbone)
Except: digestion for 1h at 37°C.

VENDREDI 18/08/2017

Purification (Monarch kit) after digestion

Table11

	A	B	C
1		Conc (ng/µL)	260/280
2	pBAD	53.1	1.6
3	RBS	13.2	2.43
4	TERMI	39.8	1.9
5	RFP	85.6	1.91
6	BB	29.6	1.79

Ligation for 1h at room temperature: pBAD+RBS, RFP+TERMI, control (only backbone, no insert).
Purification (Monarch kit), transformation into TOP10 electrocompetent cells (given by S.W.), recovered for 45 minutes at 37°C then plated out on agar LB + Amp

Mesure of TOP10 competence: transformation of DNA (100 ng/µL) into TOP10, recovered in SOC at 37°C, then plated out.
All plates left on bench for the weekend.

LUNDI 21/08/2017

TOP10 competence
Dilution 10^-0 : 54 CFU
Dilution 10^-1 : 0 CFU

Ligation verification
Colony PCR (16 colonies of both RFP-TERMI and pBAD-RBS) + positive control (RFP alone) + negative control (no DNA)

PCR (10 µL):

REV primer (10 µM)	0.2 µL
FWD primer (10 µM)	0.2 µL
Green flexi buffer	2 µL
dNTPs (20 mM)	0.4 µL
MgCl2	0.6 µL
Taq polymerase	0.05 µL
H2O	6.55 µL

(Annealing temp: 49°)

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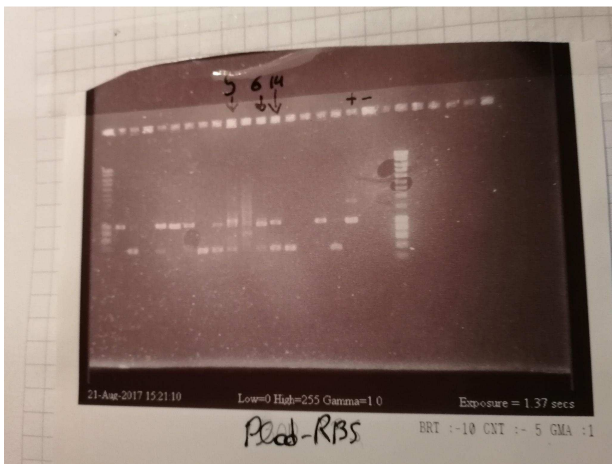

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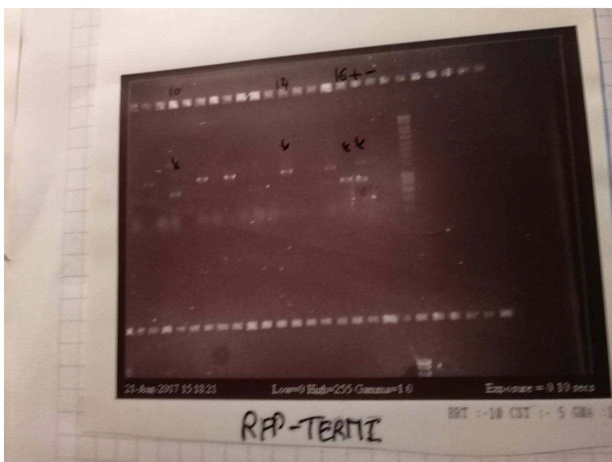

MARDI 22/08/2017

PCR amplification of RFP pBAD-RBS colonies 5-6-14 and RFP-TERMI colonies 10-14-16
Purification (Monarch kit)

Table12

	A	B	C	D
1			Conc (ng/µL)	260/280
2	pBAD-RBS	5	246.5	1.85
3		6	316.5	1.85
4		15	135	1.8
5	RFP-TERMI	10	370.9	1.88
6		14	224	1.84
7		16	301.1	1.86

Sent to sequencing.

JEUDI 24/08/2017

Sequencing: bad results.

MARDI 29/08/2017

PCR: amplification of RFP plasmid from iGEM well N11 (plate 3, 2016) = piFinal

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Purification (Monarch kit), eluted with 20 µL H2O

Table13

	A	B	C
1		Conc (ng/µL)	260/280
2	Tube 1	123	ok
3	Tube 2	128	ok

JEUDI 31/08/2017

Test competence for BL21 and TyrA-
> Tranformed DNA 100 ng/µL, plated out on agar LB + Cm (dilution: 10^-0, 10^-1, 10^-2 and 10^-3)

VENDREDI 01/09/2017

Results for competence test (CFU)

Table14

	A	B	C	D	E
1		10^-0	10^-1	10^-2	10^-3
2	BL21	Uniform cell layer	71	3	1
3	TyrA-	20	1	0	0

Absorbance piFinal

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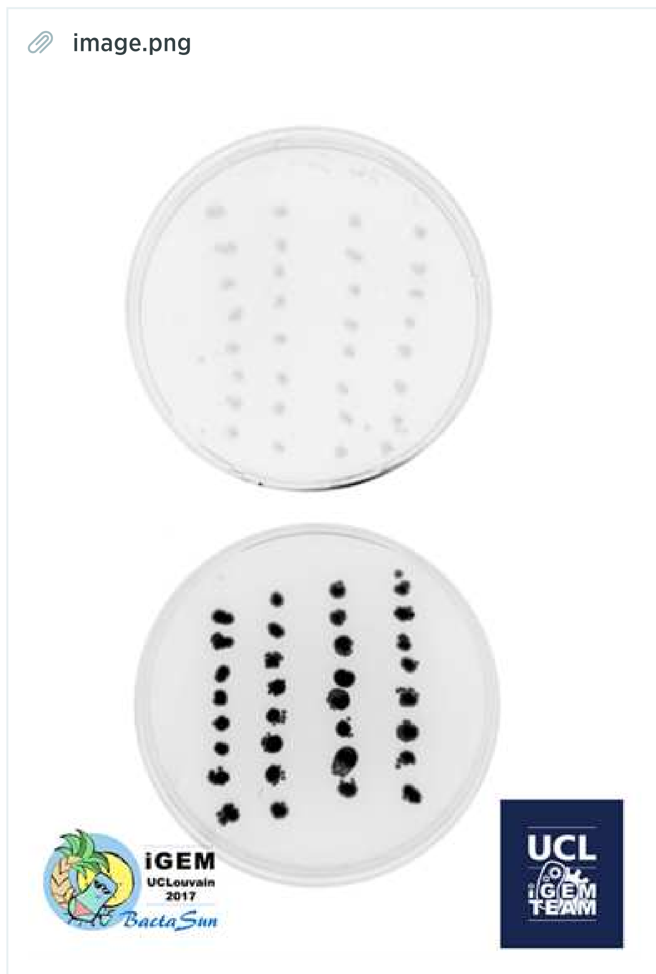

First: medium without arabinose. Second: medium + ara 0.1%

>Preparation of M9 + Tyr (0.1 mg/mL) with different concentration of arabinose

LUNDI 04/09/2017

Plated out TOP10 and TyrA- with piFinal on agar + 0.05% to 1 % arabinose

DNA extraction of piFinal in TOP10 (Roche kit). Tubes 2.14, 3.13, 1.16 and 3.15 (= most fluorescent) and purification (Monarch kit)

Table15

	A	B
1		Conc (ng/µL)
2	1.16	83.3
3	2.14	45.6
4	3.13	77.3
5	3.15	103.4

Stored at -20°C.

MARDI 05/09/2017

Preculture of TyrA- + piFinal (CmR)

MERCREDI 06/09/2017

Plates with different concentration of tyrosine (M9)
Tyr concentration: 0.1, 0.3, 0.5, 0.7 and 1%
Arabinose and Cm
TurA- + piFinal plated out and incubated overnight at 37°C.

RFP induction
Diluted preculture to OD600 ~ 0.1 and added 0.1% glucose. Incubated at 37°C, 125 RPM for 1h40
Centrifugated and resuspended in liquid LB + Cm, added 0.1% arabinose.
10 mL taken every hour, conserved on ice.

Table16

	A	B
1	Time after induction	OD600
2	1h50	0.432
3	2h50	0.546
4	3h50	0.543
5	4h27	0.580

>Make photocaged tyrosine (ONB-Tyr) stock: 0.5 mg/mL
>Make 3 plates with M9 + CM + 0.04 mg ONB-Tyr

Preculture of TyrA- + piFinal

JEUDI 07/09/2017

Redid RFP induction test.

Table17

	A	B
1	Time after induction	OD600
2	1h	0.793
3	2h	0.897

VENDREDI 08/09/2017

4 plates incubated at 37°C for >48h
>TyrA- + piFinal + CM30 + Ara + Tyr
>Concentration Ara: 0 (control), 0.2, 0.6, 0.8%

LUNDI 11/09/2017

Results:
>0% Ara: white colonies, some pink -> probable contamination
> other plates: all colonies are pink

Precultures of TyrA-/piFinal with different concentration of arabinose + CM30 + Tyr (0.1 mg/mL)
> 0, 0.1, 0.3, 0.5, 0.7, 1 & 2%
Incubated at 37°C, checked after 24h and 48h.

MARDI 12/09/2017

Preculture TyrA-/piFinal + Tyr

MERCREDI 13/09/2017

Overexpression of RFP
Pellet preculture
Resuspended in 50 mL LB + CM30, incubated until reached OD600 ~0.4-0.5
Thermal shock: put in ice for 10 minuted (agitated every 2 min)
Added 0.05% ara then agitated at 30°C for 4h. Mesured OD.
Mesured OD600 after 24h and 48h.

Mesure: take 5 mL, pellet then resuspend in 1 mL PBS buffer. Lysed with FastPrep then conserved on ice. Fluorescence mesured in 96-well (excitation 589 nm, emission 607 nm). No difference compared to negative control.

JEUDI 14/09/2017

>TyrA- plated out on M9+ONB-Tyr+Ara, different UV exposure times (BioRad instrument)
2 plates : 0, 2, 4, 6 min and 0, 5, 10, 15 min
Plate "BactaSun" M9 + Tyr (stock 25x diluted)

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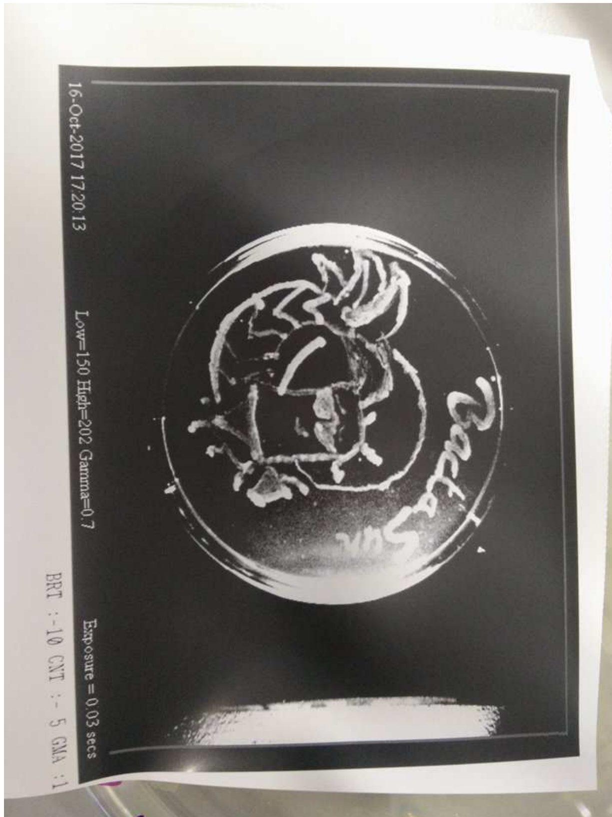

LUNDI 25/09/2017

UV lamp borrowed from Prof. Elias
Exposure of TyrA-/piFinal : different times and intensities
Times: 0, 10 sec, 1 min, 5 min, 10 min

JEUDI 28/09/2017

Nothing grew.
Redid test with exposure times: 0, 10 sec, 1 min, 5 min, 10 min and intensities 0, 1, 2, 3 UV.
Plated out before and after exposure.
Plates: M9 + ONB-Tyr + CM30 + Ara 0.1%
Negative controls: M9 + CM30 + Ara 0.1%, plated out after exposure.
Positive controls: M9 + Tyr + CM30 + Ara 0.1%, 3 UV, plated out before and after exposure, and same with 1 UV.

MARDI 03/10/2017

Results analysis
Less and less colonies on positive control plates after greater exposure time. Colonies are pink.
Nothing grew on negative control.
Nothing grew on plates with ONB-Tyr
>> the tyrosine was not decaged and cells resist UV exposure

VENDREDI 27/10/2017

Mesure RFP fluorescence (see protocol)
>Different times of exposure to UV light (0, 12, 60, 300 minutes)
>Toxicity test for ONB-Tyr: add 50-50% Tyr-ONBTyr

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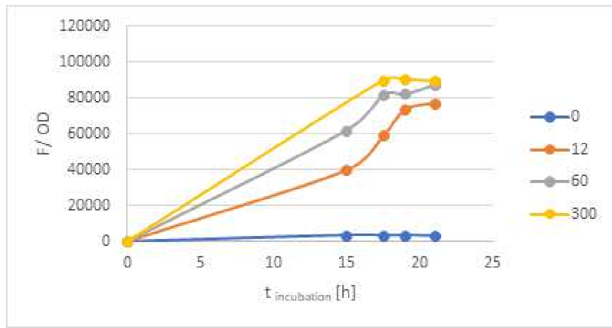

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