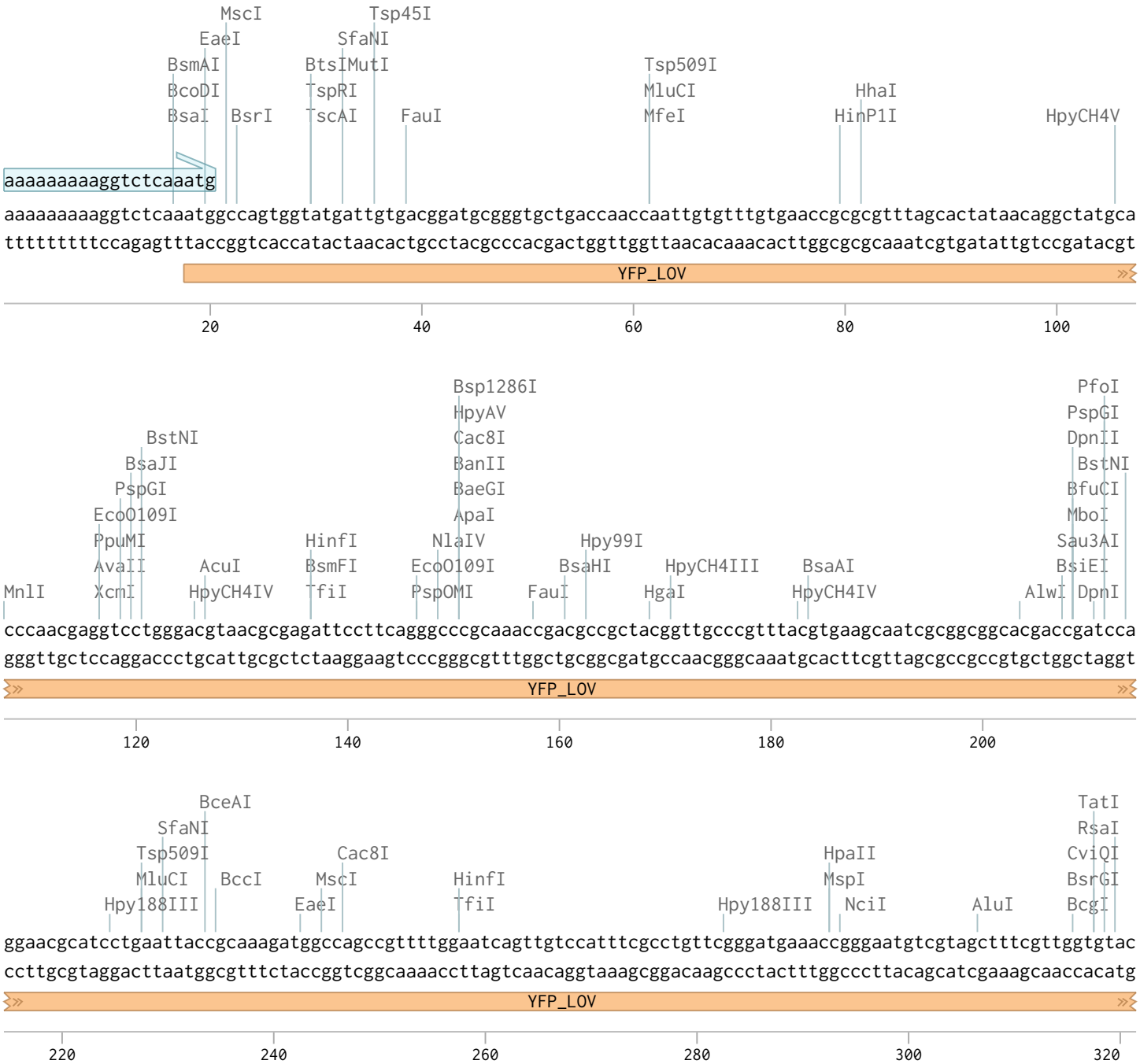
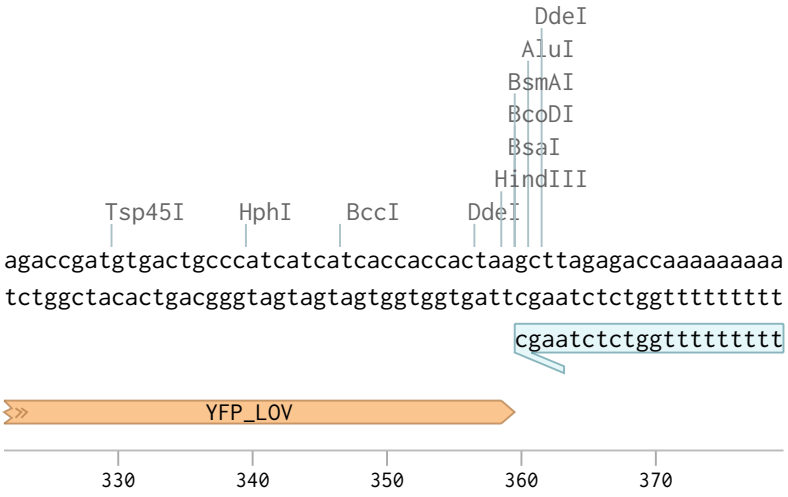
YFP (optimized) (379 bp)




DdeI
AluI
BsmAI
BcoDI
BsaI
HindIII
Tsp45I
HphI
BccI
DdeI
agaccgatgtgactgcccatcatcatcaccaccactaagcttagagaccaaaaaaaaa
tctggctacactgacgggtagtagtagtggtggtgattcgaatctctggtttttttt
cgaatctctggtttttttt
YFP_LOV
330
340
350
360
370

GG#1

WEDNESDAY, 7/22/2020

Done by Mathieu and Clémentine

Note : this manipulation was done in the same time with Inaccessible DNA Sequence

Note 2 : You need to extract and quantify with a nanodrop the plasmid before to begin the GG (here we used pBAD-Moclo serie 1 with a concentration of 77 ng/µL)

Material :

- IDT sequence insert ilvA_ccdB resuspended at 10 ng/µL (1 000 ng of sequence lyophylized = powder was given by IDT)
- thermocycleur
- H20 mQ
- 75 ng of plasmid pBAD-MOCLO
- kit Golden Gate NEB

Protocol for cloning golden gate for pBADMoclo:

Add in this order :

1. 15 µL of inserts with ratio 2:1 (insert:plasmid) (10 ng/µL) (H20 for control condition)
2. 2 µL of T4 DNA ligase buffer
3. 1 µL of plasmids pBADMoclo (75 ng/µL)
4. 1 µL of QSP H20
5. 1µL of NEB Golden Gate

NB: DONT FORGET TO PUT THE TUBES IN THE ICE AND DONT FORGET TO HAVE A CONTROL CONDITION

Table1

	A	B	C	D	E	F
1		plasmide de destination :	Insert with amplicon form (150 ng) :	T4 DNA ligase Buffer	NEB Golden Gate Enzyme mix	H2O
2	Volume (µL) for Golden Gate cloning	1 µL	15 µL (C = 10 ng/µL)	2	1-2	qsp 20 µL

We prepared 3 GG reactionnal tubes :

- pBAD-MOCLO + ilvA_ccdB (red point on the PCR tube)
- pBAD-MOCLO + stable_YFP (blue point)
- pBAD-MOCLO only (**black** point)

(Heat at 37°C for 1 min and then 16°C for 1min) for 30 cycles and 60°C for 5 min

After the GG cycle, we transformed bacteria DH5alpha.

Transformation of pBAD-MOCLO + optimized_stable_YFP GG#1

WEDNESDAY, 7/22/2020

Done by Clémentine, Inès and Mathieu

When the insertion of inserts in pBAD-MOCLO is done theorically

Matérials :

- 50 µL DHS alpha chemocompetentes
- LB pur
- plasmids :
  - pBAD-MOCLO serie 1+ ilvA_ccdB_GG (= insert 1, red point on the PCR tube)
  - pBAD-MOCLO serie 1+ optimized stable YFP (= insert 2, blue point on the PCR tube)
  - pBAD-MOCLO serie 1 + NOTHING **(= control, black point on the PCR tube)**
- plaque chauffante
- glace
- the kit "NEB Golden Gate Assembly kit (BSAI-HF v2))" from New England Biolabs

Protocols :

- DHS -> during 30 min
- 10 ng of plasmids in bacteria (= 2,7 µL), SHAKE, put tubes in the ice during 30 min
- Then, put it at 42°C during 1 min
- Put it in the ICE during 2 min
- Add 1 mL, of LB pur then put it at 37°C during 1 hour with shaking
- Spread bacteria on agar plates (LB + Amp + 0.02% glucose media)
  - 50 µL spread
  - 100 µL spread
  - 100 µL concentrated -> centrifuge 1 min at 5000 g, remove media and add 100 µL of media, dilute cells and spread it
- Put petri dishes in incubator at 37 °C all the night
- Observe :)

THURSDAY, 7/23/2020

Results : (observation of our transformants 23/07/2020)
Done by Mathieu

colonies are observable :
ccdb : a few (2/3 colonies) -> because the concentration of saccharose are lower (0.02 %), so the promoter of ccdb is active and the toxine are expressed.
YFP : colonies are observable
control : a few colonies are observable

Table2

	A	B	C	D
1	Plasmids	Number of colonies		
2		50 µL	100 µL	Concentrate
3	pBAD-MOCLO serie 1+ ilvA_ccdB_GG	2	0	2
4	pBAD-MOCLO serie 1+ optimized stable YFP	3	6	48
5	pBAD-MOCLO serie 1 without INSERT	2	0	2

N-B : the concentration of saccharose are lower (0.02 %), so the promoter of ilvA-ccdB is active and the toxine are expressed.
N-B bis : Yersterday, we added Amp when the media is very hot, so we heat again the media to degrade Amp and we added again Amp. So the concentration of Amp is maybe not optimized.

--> Today (23/07/2020), we prepare again the media LB + Amp + Agar + 0.2% glucose

Seeding of GG#1 : pBAD-MOCLO + optimized_stable_YFP

THURSDAY, 7/23/2020

Done by Clémentine and Mathieu

Note : The Amplification handling was done at the end of the day.
Note 2 : This handling was done in the same time of the Transformation pBAD-MOCLO + ilvA_ccdB
Note 3 : This handling was done in the same time with stable-YFP

Material :

- liquid media LB+ glucose 0,2% (=2g/L) + Amp 1 X (Ci= 1000 X)
- colonies containing pBAD-MOCLO + ilvA_ccdB
- toothpick
- 4 Falcon of 15 mL

Protocol :

Note : We did this handling in the same time that we prepared sampled of Template DNA for the PCR colony PCR#1 .
Note 2 : Colonies preleved are the SAME that we used in the PCR #1 samples.

1. Put 4 mL of media in each tube
2. With a toothpick, scrape the stripe of 1 cm of colony (this stripe was made with another toothpick used to prepare the template DNA PCR tube)
3. Put this toothpick in the Falcon
4. Incubate overnight at 37°C and shake tubes at 200 rpm

PCR colony #1 from GG#1

THURSDAY, 7/23/2020

Done by Clémentine and Mathieu

Previously manips on the file " Transformation of pBAD-MOCLO + insert Golden Gate

Samples came from the Golden GATE #1

Material:

- 2019GO-5-Seq-pBAD-F
- 2019GO-6-Seq-pBAD-R
  - **-> NB : 1 mutation between oligos and plasmid sequence for F and R.**

Table1

	A	B	C	D	E
1	Nom de l'oligo	Cible	Séquence (5'-3')	Tm (°C, IDT-oligo analyzer)	hairpin Tm (°C, IDT)
2	2019GO-5-Seq-pBAD-F	pBAD24	5'-TGGCTATGCCATAGCATTTTTATCC	55,6	50,0
3	2019GO-6-Seq-pBAD-R	pBAD24	5'- GGT CTG ATT TAA TCT GTA TCA GGC TG	55,3	38,7

- transformants pBAD-MOCLO serie 1+ ilvA_ccdB_GG (= insert 1, red point on the PCR tube)
- transformants pBAD-MOCLO serie 1+ optimized stable YFP (= insert 2, blue point on the PCR tube)
- Transformants pBAD-MOCLO serie 1 + NOTHING **(= control, black point on the PCR tube)**
- ligations tubes of this 3 conditions
- PCR tube
- H2O milli Q
- Thermocycler
- toothpick
- ice

Protocol

1. With a toothpick, stripe 1 colony on 1 cm; then, put it on an eppendorf containing 100 µL of H2O. Homogeneise the tube. You have the 'Template DNA' ready
2. Add composants in this order (cf below). Attention, all this tubes are in ice

We use the kit " Q5 High-Fidelity 2 X Master MIX" from New England Biolabs.

Table2

	A	B	C	D
1	Order of introduction	Components	Volume (µL) for 50 µL reaction	Final concentration
2	1)	Template DNA (pBAD-MOCLO +/- insert)	1 (0 for the negative control)	<1.000 ng
3	2)	Q5-High_Fidelity 2X Master Mix	25	0.5µM
4	3)	2019GO-5-Seq-pBAD-F	2,5	0.5µM
5	4)	2019GO-6-Seq-pBAD-R	2,5	1 X
6	5)	Nuclease free water	19 (20 for the negative control)	/

We prepare 9 tubes with the mix PCR :

- 1) transformant pBAD-MOCLO serie 1+ ilvA_ccdB_GG (= insert 1, 1 red point on the PCR tube) colonie 1
- 2) transformant pBAD-MOCLO serie 1+ ilvA_ccdB_GG (= insert 1, 2 red points on the PCR tube) colonie 2
- 3) transformant pBAD-MOCLO serie 1+ optimized stable YFP (= insert 2, 1 blue point on the PCR tube) clone 1
- 4) transformant pBAD-MOCLO serie 1+ optimized stable YFP (= insert 2, 2 blue points on the PCR tube) clone 2
- 5) 1 colonie transformant **boîte noire contrôle (1 black point + letter "c")**
- 6) all without pBAD-MOCLO **(= control, 1 black point + word "lig" on the PCR tube)**
- 7) ligation tube pBAD-MOCLO + ccdb (= insert 1, 1 red square + word "lig" on the PCR tube)
- 8) ligation tube pBAD-MOCLO + YFP (= insert 2, 1 blue square + word "lig" on the PCR tube)
- 9) tout sauf plasmide (negative control, **ensemble vide** on the tube)

PCR Program:

Determination of the Temperature of elongation :
size of pBAD-MOCLO : 45 110 pb
size of ilvA_ccdB : 1 582 pb
size of stable_YFP : 367 pb

4 510 + 1 582 = 6 092 pb
Processivité de la polymérase : 20-30 seconds / kb
6kb X 30 second = 180 seconds

1. 98°C - 2min
2. 98°C - 10 s
3. 52°C - 30 s
4. 72°C - 195 s
5. Repeat from step 2 (5 times)
6. 98°C - 10 s
7. 52°C - 30 s
8. 72°C - 195 s
9. Repeat from step 6 (25 times)
10. 72°C - 2 min
11. 8°C - Infinity

The products are analysed by electrophoresis

Preparation of electrophoresis gel (cf file "Electrophoresis pBAD- MOCLO")

Electrophoresis PCR colony #1 from GG#1

THURSDAY, 7/23/2020

Done by Clémentine, Mathieu and Inès

Protocol :

NB: Don't forget your gloves for the heat AND the Ethidium Bromide (BET) !!!

For the gel

- Weigh 4,8g of agarose (0,8% --> 0,8g pour 100 ml)
- Measure 600 ml of TAE buffer (0.5X) in a test tube
- Put the agarose and then add the buffert in a 1L (glass) bottle.
- Keep the gel boiling in the microwave
- Cast the gel and put the combs

For 1 well :

- on 1 PCR tube, put : 10µL of mix PCR + 2µL of blue charge. Then put 12µL of this mix in 1 well
- for the ladder : just put 5 µL of ladder

For the migration :

- Set the machine on 100 Volts during 30 minutes.

Electrophoresis on :

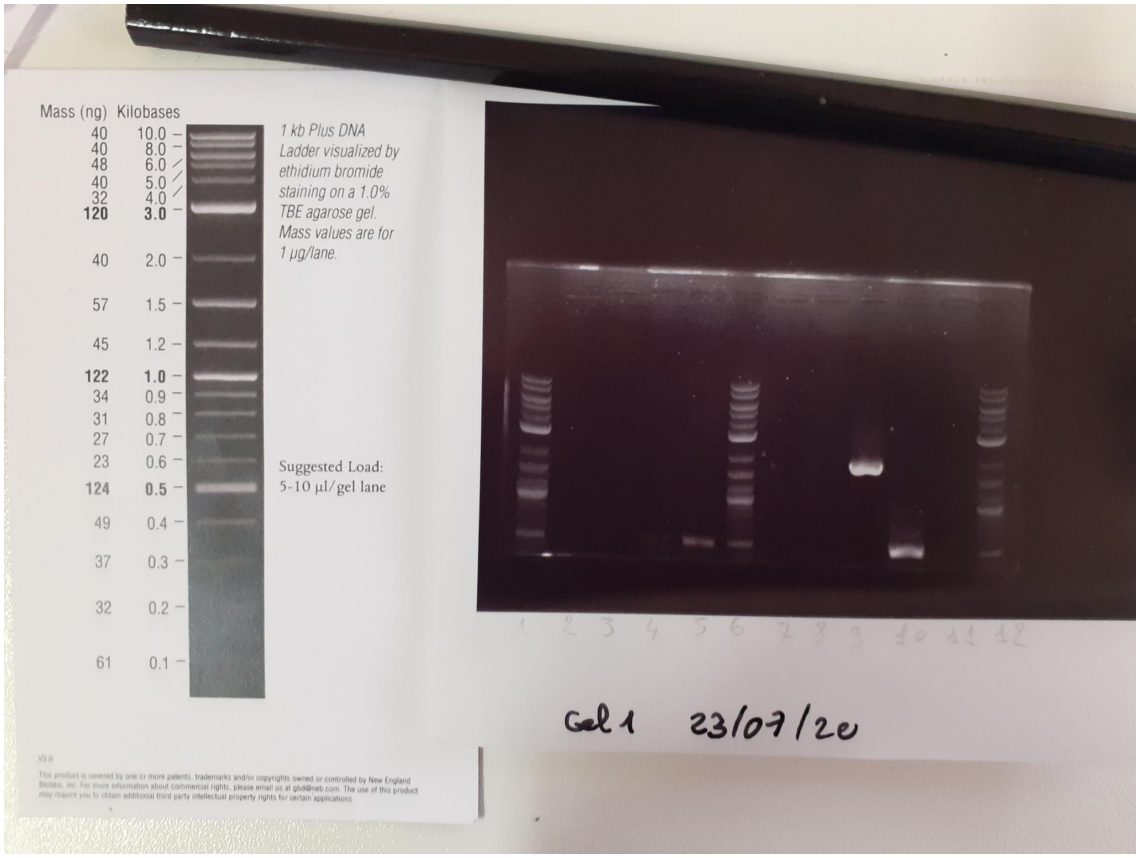
- 1) transformant pBAD-MOCLO serie 1+ ilvA_ccdB_GG#1 23/07/2020 (= insert 1, 1 red point on the PCR tube) colonie 1
- 2) transformant pBAD-MOCLO serie 1+ ilvA_ccdB_GG#1 23/07/2020 (= insert 1, 2 red points on the PCR tube) colonie 2
- 3) transformant pBAD-MOCLO serie 1+ optimized_stable_YFP_GG#1 23/07/2020 (= insert 2, 1 blue point on the PCR tube) clone 1
- 4) transformant pBAD-MOCLO serie 1+ optimized_stable_YFP_GG#1 23/07/2020 (= insert 2, 2 blue points on the PCR tube) clone 2
- 5) 1 colonie transformant **boîte noire contrôle 23/07/2020 (1 black point + letter "c")**
- 6) GG#1 reactionnal tube only pBAD-MOCLO 22/07/2020 **(= control, 1 black point + word "lig" on the PCR tube)**
- 7) GG#1 reactionnal tube pBAD-MOCLO + ilvA_ccdB 22/07/2020 (= insert 1, 1 red square + word "lig" on the PCR tube)
- 8) GG#1 reactionnal tube pBAD-MOCLO + stable_YFP 22/07/2020 (= insert 2, 1 blue square + word "lig" on the PCR tube)
- 9) H2O : all without plasmide (negative control, **ensemble vide** on the tube)

Depot map's + Results

Table1

	A	B	C	D	E	F	G	H	I	J	K	L	M	N
1		Puits n°	1	2	3	4	5	6	7	8	9	10	11	12
2	Tape (pb)	Condition	Ladder	pBAD-MOCLO serie 1+ ilvA_ccdB_GG (= insert 1, 1 red point on the PCR tube) clone 1	pBAD-MOCLO serie 1+ ilvA_ccdB_GG (= insert 1, 1 red point on the PCR tube) clone 2	pBAD-MOCLO serie 1+ optimized stable YFP (= insert 2, 1 blue point on the PCR tube) clone 1	pBAD-MOCLO serie 1+ optimized stable YFP (= insert 2, 1 blue point on the PCR tube) clone 2	Ladder	**Clone in control condition**	**GG#1 reactionnal tube, only pBAD-MOCLO**	GG#1 reactionnal tube, pBAD-MOCLO + ilvA_ccdB	GG#1 reactionnal tube, pBAD-MOCLO + stable_YFP	Negative Control (water and not DNA)	Ladder
3	1 500 - 1 600			nothing	nothing	nothing	nothing		nothing	nothing	1 tape, high intensity	nothing	nothing	
4	300			nothing	nothing	1 tape, low intensity	1 tape, medium intensity		nothing	nothing	nothing	1 tape, high intensity	nothing	

Electrophoresis gel - pBAD-MOCLO + YFP or ccdB.jpg



Note : Because it was late, 5 last minutes we select 130 Volt on this machine.

N-B : The time that we have choosen (30 min) are too long because tapes had migrated too far. -> Next time we will choosen a time of 25 min.

N-B bis : We forget 1 control : the condition only plasmid

Conlusion / Discussion

Globally, on the gel, 2 tapes of 2 differents sizes are observable (1,5-2 kb and 0,3 kb). The tape of 1,5 kb can matched with the ccdb insert's which have a size of 1582 pb. The tape of 0,35 kb can matched with the YFP insert's which have a size of 367 pb. This tapes are visible in ligation tube after Golden Gate and colonies owning pBAD-MOCLO + YFP.
The insertion of sequences of interest in the plasmid pBAD-MOCLO has worked (cf tube ligation). However, only the transformation of YFP functionned. ccdB transformation hasn't worked, maybe, because the concentration of glucose was too low in the media LB + agar + Amp (0,02 % in place of 0?2%). Then, the toxine ccdB was produced in a little quantity but sufficient to kill bacteria (hence the very low number of colony in petri dishes) . The concentration of Amp, maybe higher than the normal, can be rule out because bacteria + plasmid + YFP had grown.

GG#6

MONDAY, 8/3/2020

Done by Godeffroy

GG with PCR cleaned of pZA31 et pBAD-MC

Material :

- IDT sequence inserts resuspended at 10 ng/µL (1 000 ng of sequence lyophylized = powder was given by IDT)
- thermocycleur
- H20 mQ
- 75 ng of plasmid pBAD-MOCLO (same sample used in GG#1)
- kit Golden Gate NEB

Protocol for cloning golden gate for pBADMoclo:

Add in this order :

1. 15 µL of inserts with ratio 1:1 (insert:plasmid) (10 ng/µL) (H20 for control condition)
2. 2 ,5 µL of T4 DNA ligase buffer
3. 75 ng de plasmide
4. 1 ,5µL of QSP H20
5. 1µL of NEB Golden Gate
6. 0,5 uL of T4 DNA ligase
7. 1uL DpnI pour pZA31

NB: DONT FORGET TO PUT THE TUBES IN THE ICE AND DONT FORGET TO HAVE A CONTROL CONDITION

Table1

	A	B	C	D	E	F	G	H
1		plasmide de destination :	Insert with amplicon form (150 ng) :	T4 DNA ligase Buffer	NEB Golden Gate Enzyme mix	T4 DNA ligase	H2O	DpnI pour pZA31
2	Volume (µL) for Golden Gate cloning	75ng	15 µL (C = 10 ng/µL)	2,5 uL	1uL	0,5 uL	qsp 20 µL	1 uL

We prepared 5 GG reactionnal tubes :

- pBAD-MC + YFP stable
- pZA31 cleaned + luc consensus
- pBAD-MC + knt-ccdB
- pZA31 cleaned digested without insert
- pBAD-MC digested without insert

(Heat at 37°C for 1 min and then 16°C for 1min) for 30 cycles and 60°C for 5 min

After the GG cycle, we transformed bacteria DH5alpha.

Inaccessible Entry

Table2

	A	B	C	D	E	F
1	GG#X	Plasmids	Number of colonies			Middles
2			50 µL	100 µL	Concentrate	
3	GG#6	pBAD-MC_YFP stable	0	0	0	LB + AMP + glucose
4		pZA31 cleared_Luc consensus	0	0	0	LB + CM
5		pBAD-MC_knt_ccdB	0	0	0	LB + AMP + glucose
6		pZA31 cleared_digested_alone	0	0	0	LB + CM
7		pBAD-MC_digested_alone	0	0	0	LB + AMP + glucose
8		pBAD-MC_alone	25	45	>200	LB + AMP + glucose
9		pZA31_alone			200	LB + CM

GG#7

WEDNESDAY, 8/5/2020

Done by Goddefroy

New protocol !!!!!!!!!

Material :

- IDT sequenc inserts resuspended at 10 ng/µL (1 000 ng of sequence lyophylized = powder was given by IDT)
- thermocycleur
- H20 mQ
- plasmids (pBAD-Mc or pZ)
- kit Golden Gate NEB

Protocol modifications :

- T4 DNA ligase : 1000U become 500U
- Nb of cycles is variable (15min 37°C, 30 cycles, 45 cycles, OV (overnight)
- insert : 100 ng become 150 ng
- BsaI : 30U become 15U

Protocol for cloning golden gate for pBADMoclo:

Add in this order :

1. 15 µL of inserts with ratio 1:1 (insert:plasmid) (10 ng/µL) (H20 for control condition)
2. 2 ,5 µL of T4 DNA ligase buffer
3. 4uL of plasmid
4. 0,25 uL of T4 DNA ligase
5. BsaI-HF-V2 (20U/uL) : 0,75uL
6. H2O MQ : 3uL

NB: DONT FORGET TO PUT THE TUBES IN THE ICE AND DONT FORGET TO HAVE A CONTROL CONDITION

Table1

	A	B	C	D	E	F	G	H
1		plasmide de destination :	Insert with amplicon form (150 ng) :	T4 DNA ligase Buffer	BsaI-HF-V2 (20U/uL)	T4 DNA ligase	H2O	
2	Volume (µL) for Golden Gate cloning	75ng	15 µL (C = 10 ng/µL)	2,5 uL	0,75uL	0.25uL	3 uL	

We prepared 10 GG reactionnal tubes :

- pBAD-MC + YFP stable 15min 37°C
- pBAD-MC + YFP stable 30 cycles
- pBAD-MC + knt_GFP 15min 37°C
- pBAD-MC + knt_GFP 30 cycles
- pBAD-MC + knt_GFP45 cycles
- pBAD-MC + knt_GFP OV
- pBAD-MC digested without insert 15min 37°C
- pBAD-MC digested without insert 30 cycles
- pBAD-MC digested without insert 45 cycles
- pBAD-MC digested without insert OV

After the GG cycle, we transformed bacteria DH5alpha for 15min and 30 cycles conditions, no for the others.

Inaccessible Entry

Table2

	A	B	C	D	E	F
1	GG#X	Plasmids	Number of colonies			Middles
2			50 µL	100 µL	Concentrate	
3	GG#7	pBAD-MC_YFP stable 15'	3	20	98	LB + AMP + glucose
4		pBAD-MC_YFP stable 1h30	0	5	38	
5		pBad-MC_sfgfp_knt 15'	3	22	80	
6		pBad-MC_sfgfp_knt 1h30	3	8	37	
7						
8						
9		pBad-MC digested alone 15'	4	5	88	
10		pBad-MC digested alone 1h30	0	0	11	
11						
12						
13						

PCR colony #7 from GG#7

WEDNESDAY, 8/5/2020

Done by Godeffroy

Material:

- 2019GO-5-Seq-pBAD-F
- 2019GO-6-Seq-pBAD-R

Table1

	A	B	C	D	E
1	Nom de l'oligo	Cible	Séquence (5'-3')	Tm (°C, IDT-oligo analyzer)	hairpin Tm (°C, IDT)
2	2019GO-5-Seq-pBAD-F	pBAD24	5'-TGGCTATGCCATAGCATTTTTATCC	55,6	50,0
3	2019GO-6-Seq-pBAD-R	pBAD24	5'- GGT CTG ATT TAA TCT GTA TCA GGC TG	55,3	38,7

- colony GG#7 knt_GFP 15′
- colony GG#7 knt_GFP 1h
- colony GG#7 YFP 154
- colony GG#7 YFP 15′
- colony GG#7 H2O
- colony GG#7 native colony

Protocol

1. With a toothpick, stripe 1 colony on 1 cm; then, put it on an eppendorf containing 100 µL of H2O. Homogeneise the tube. You have the 'Template DNA' ready. NOTE : in PCR#1 and 2, I pipette 1µL of this 100µL H2O + plasmid as DNA Template. I was wrong You need to pipette 19+1 µL H2O + plasmid.
2. Add composants in this order (cf below). Attention, all this tubes are in ice

We use the kit " Q5 High-Fidelity 2 X Master MIX" from New England Biolabs.

Table2

	A	B	C	D
1	Order of introduction	Components	Volume (µL) for 50 µL reaction	Final concentration
2	1)	Template DNA (pBAD-MOCLO +/- insert)	1 (0 for the negative control)	<1.000 ng
3	2)	Q5-High_Fidelity 2X Master Mix	25	0.5µM
4	3)	2019GO-5-Seq-pBAD-F	2,5	0.5µM
5	4)	2019GO-6-Seq-pBAD-R	2,5	1 X
6	5)	Nuclease free water	19 (20 for the negative control)	/

PCR Program:

98° 10 sec

25 times

1. 55° 30 sec
2. 72° 1 min

78° min

Preparation of electrophoresis gel (cf file "Electrophoresis pBAD- MOCLO")

Electrphoresis PCR colony#7 from GG#7

WEDNESDAY, 8/5/2020

Done by Godeffroy

Protocol :

For the gel

- Weigh 4,8g of agarose (0,8% --> 0,8g pour 100 ml)
- Measure 600 ml of TAE buffer (0.5X) in a test tube
- Put the agarose and then add the buffert in a 1L (glass) bottle.
- Keep the gel boiling in the microwave
- Cast the gel and put the combs

For 1 well :

- on 1 PCR tube, put : 10µL of mix PCR + 2µL of blue charge. Then put 12µL of this mix in 1 well
- for the ladder : just put 5 µL of ladder

For the migration :

- Set the machine on 100 Volts during **25 minutes**.

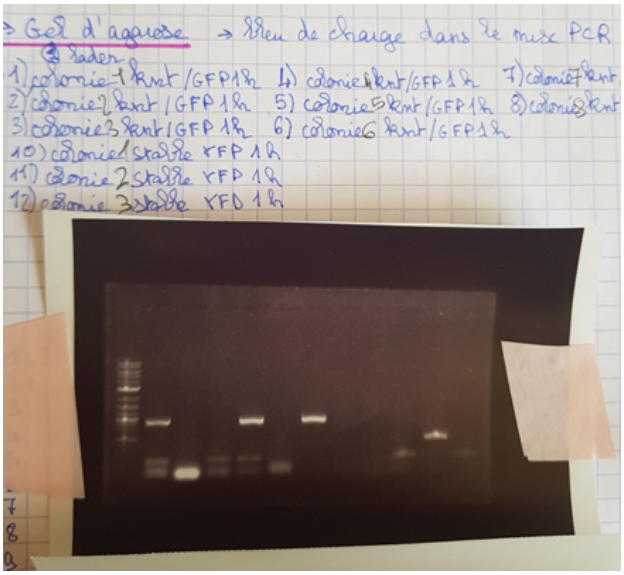
Electrophoresis on :

- colony GG#7 knt_GFP 15′
- colony GG#7 knt_GFP 1h
- colony GG#7 YFP 154
- colony GG#7 YFP 15′
- colony GG#7 H2O
- colony GG#7 native colony
- pBAD-MC native
- digestion BSAI mix PCR6 pZA31
- digestion BSAI + H2O
- digestion BSAI pZA31luc
- digestion BSAI + H2O

Depot map's + Results

Results

image.png



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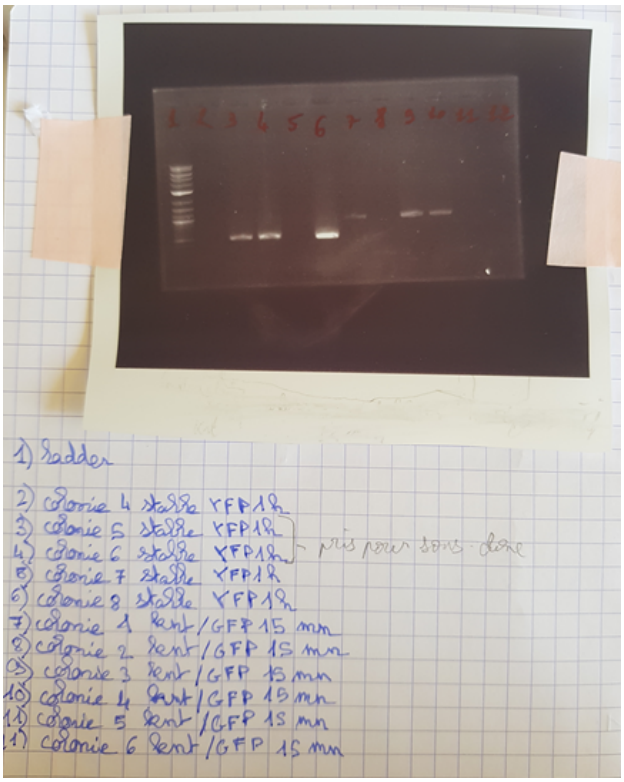

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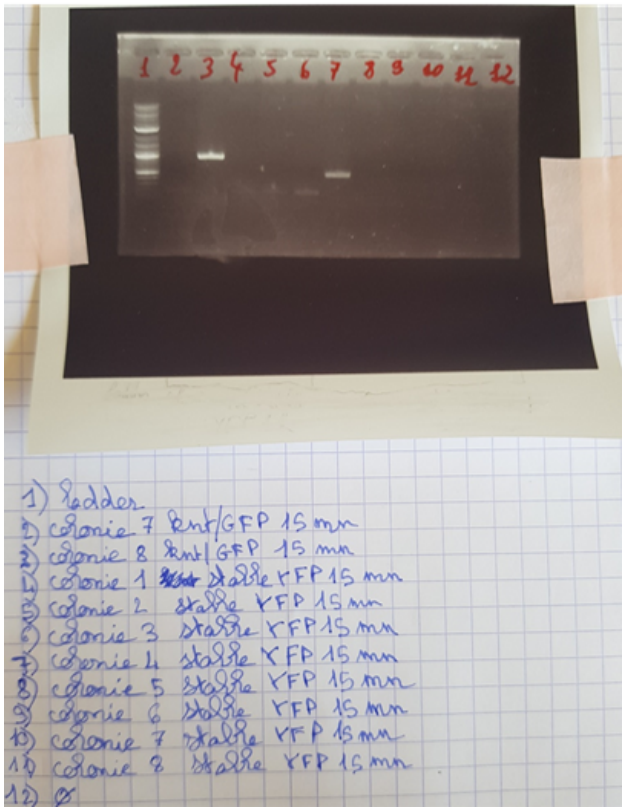
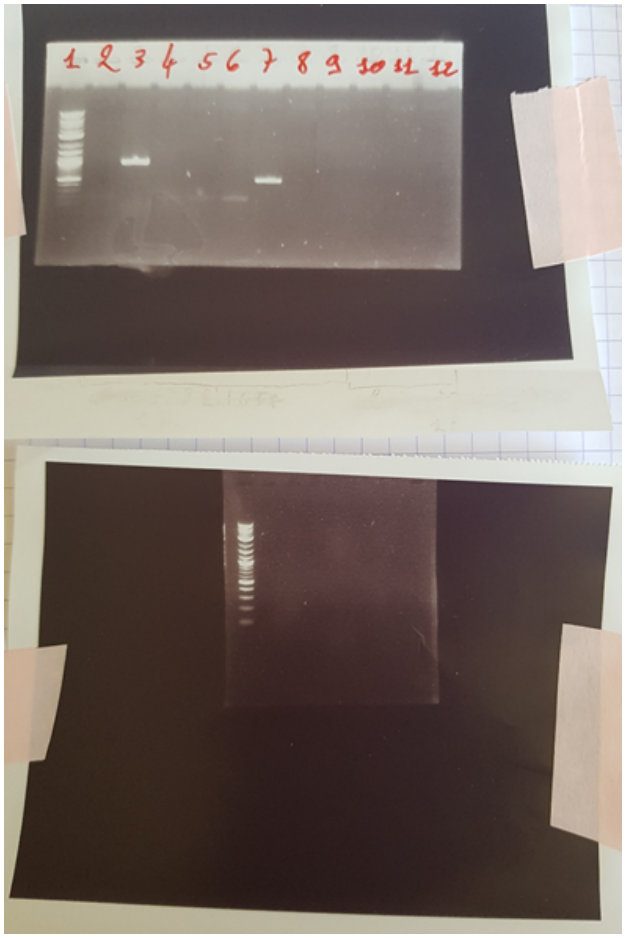

1 2 3 4 5 6 7 8 9 10 11 12
1) ladder
2) colonie 7 kmt/GFP 15 mm
3) colonie 8 kmt/GFP 15 mm
4) colonie 1 stalle YFP 15 mm
5) colonie 2 stalle YFP 15 mm
6) colonie 3 stalle YFP 15 mm
7) colonie 4 stalle YFP 15 mm
8) colonie 5 stalle YFP 15 mm
9) colonie 6 stalle YFP 15 mm
10) colonie 7 stalle YFP 15 mm
11) colonie 8 stalle YFP 15 mm
12) Ø

image.png



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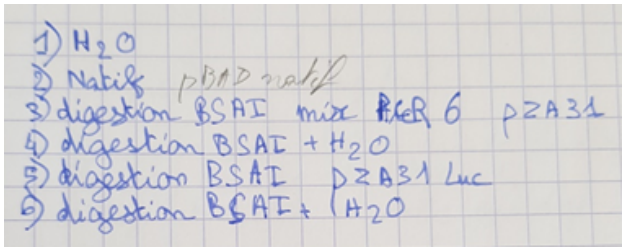
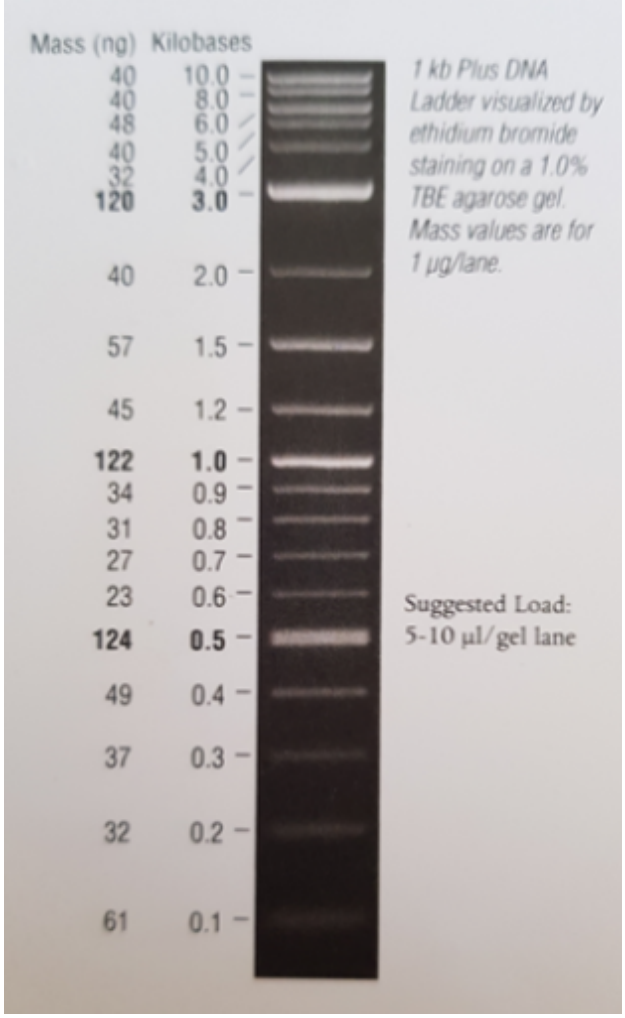


image.png


Mass (ng) Kilobases
40 10.0
40 8.0
48 6.0
40 5.0
32 4.0
120 3.0
40 2.0
57 1.5
45 1.2
122 1.0
34 0.9
31 0.8
27 0.7
23 0.6
124 0.5
49 0.4
37 0.3
32 0.2
61 0.1
1 kb Plus DNA Ladder visualized by ethidium bromide staining on a 1.0% TBE agarose gel. Mass values are for 1 µg/lane.
Suggested Load: 5-10 µl/gel lane

Sub-cloning transformation GG#7

THURSDAY, 8/6/2020

Stries with transformants from Inaccessible Entry (transformants YFP and knt_GFP) on LB + Amp middle to obtain isolated transformants wich will be used for selection, sequencing, caracterization and stocking.

Culture of colonies from GG#7 sub-clones on selective middle + mise en réserve + tubes for PCR#9 preparation

FRIDAY, 8/7/2020

Done by Florent

See Inaccessible Entry

- Sub-clones knt-GFP has been cultivated LB+AMP, LB +AMP+SPE 125uL et LB+AMP+SPE 250 uL.
- Sub-clones YFP has been cultivated LB+AMP

PCR colony #11

TUESDAY, 8/11/2020

Done by Godefroy

WE used the same reactionnal mix as Inaccessible Entry

Material:

- 2019GO-5-Seq-pBAD-F
- 2019GO-6-Seq-pBAD-R

Table1

	A	B	C	D	E
1	Nom de l'oligo	Cible	Séquence (5'-3')	Tm (°C, IDT-oligo analyzer)	hairpin Tm (°C, IDT)
2	2019GO-5-Seq-pBAD-F	pBAD24	5'-TGGCTATGCCATAGCATTTTTATCC	55,6	50,0
3	2019GO-6-Seq-pBAD-R	pBAD24	5'- GGT CTG ATT TAA TCT GTA TCA GGC TG	55,3	38,7

- sub-clones pBAD_knt-ccdB 1 to 8 from GG#8
- sub-clones pBAD_ilva-ccdB 1 to 8 from GG#8
- sub-clones pBAD_YFP 1 to 8 from box 5 from GG#7

Protocol

1. With a toothpick, stripe 1 colony on 1 cm; then, put it on an eppendorf containing 100 µL of H2O. Homogeneise the tube. You have the 'Template DNA' ready. NOTE : in PCR#1 and 2, I pipette 1µL of this 100µL H2O + plasmid as DNA Template. I was wrong You need to pipette 19+1 µL H2O + plasmid.
2. Add composants in this order (cf below). Attention, all this tubes are in ice

We use the kit " Q5 High-Fidelity 2 X Master MIX" from New England Biolabs.

Table2

	A	B	C	D
1	Order of introduction	Components	Volume (µL) for 50 µL reaction	Final concentration
2	1)	Template DNA (pBAD-MOCLO +/- insert)	1 (0 for the negative control)	<1.000 ng
3	2)	Dream taq Buffer	0,5	0.5µM
4	3)	2019GO-5-Seq-pBAD-F	0,5	0.5µM
5	4)	2019GO-6-Seq-pBAD-R	0,5	1 X
6	5)	Nuclease free water	19 (20 for the negative control)	/
7	6)	Dream taq polymerase	0,125	

Miniprep pBAD+ YFP from colony from sous-clone 5 from GG#7

TUESDAY, 8/11/2020

Done by Godeffroy

To know our YFP concentration for sequencing.

Material :

- Monarch^{R} Plasmid Miniprep Kit (#T10105 lot: 10040564)

Protocol :

image.png

Monarch
Plasmid Miniprep Kit Protocol Card

NEB #T1010

For a detailed protocol or to download the full manual, visit www.neb.com/T1010

BEFORE YOU BEGIN:

- Add 4 volumes of ethanol (≥ 95%) to one volume of Plasmid Wash Buffer 2.
- All centrifugation steps should be carried out at 16,000 x g (~13,000 RPM).
- If precipitate has formed in Lysis Buffer (B2), incubate at 30–37°C, inverting periodically to dissolve.
- Store Plasmid Neutralization Buffer (B3) at 4°C after opening, as it contains RNase A.

PROTOCOL STEPS:

1. **Pellet 1–5 ml (not to exceed 15 OD units) bacterial culture by centrifugation for 30 seconds. Discard supernatant.** 1.5 ml of culture is sufficient for most applications. Ensure cultures are not overgrown (12-16 hours is ideal).
2. **Resuspend pellet in 200 µl Plasmid Resuspension Buffer (B1)**. Vortex or pipet to ensure cells are completely resuspended. There should be no visible clumps.
3. **Add 200 µl Plasmid Lysis Buffer (B2), gently invert tube 5–6 times, and incubate at room temperature for 1 minute.** Color should change to dark pink, and solution will become transparent and viscous. Do not vortex.

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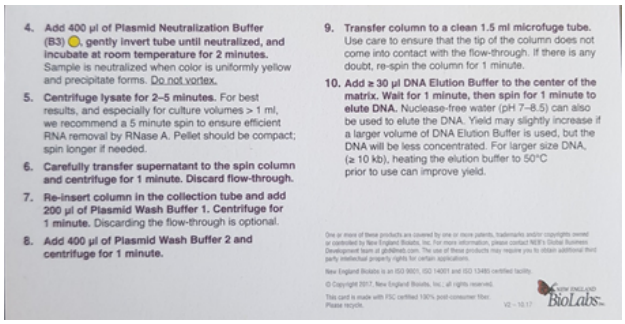
image.png



4. **Add 400 µl of Plasmid Neutralization Buffer (B3), gently invert tube until neutralized, and incubate at room temperature for 2 minutes.** Sample is neutralized when color is uniformly yellow and precipitate forms. Do not vortex.
5. **Centrifuge lysate for 2–5 minutes.** For best results, and especially for culture volumes > 1 ml, we recommend a 5 minute spin to ensure efficient RNA removal by RNase A. Pellet should be compact; spin longer if needed.
6. **Carefully transfer supernatant to the spin column and centrifuge for 1 minute. Discard flow-through.**
7. **Re-insert column in the collection tube and add 200 µl of Plasmid Wash Buffer 1. Centrifuge for 1 minute.** Discarding the flow-through is optional.
8. **Add 400 µl of Plasmid Wash Buffer 2 and centrifuge for 1 minute.**
9. **Transfer column to a clean 1.5 ml microfuge tube.** Use care to ensure that the tip of the column does not come into contact with the flow-through. If there is any doubt, re-spin the column for 1 minute.
10. **Add ≥ 30 µl DNA Elution Buffer to the center of the matrix. Wait for 1 minute, then spin for 1 minute to elute DNA.** Nuclease-free water (pH 7–8.5) can also be used to elute the DNA. Yield may slightly increase if a larger volume of DNA Elution Buffer is used, but the DNA will be less concentrated. For larger size DNA, (≥ 10 kb), heating the elution buffer to 50°C prior to use can improve yield.

V2 – 10.17

Résultat :

Obtention of a pBAD-MC tube of concentration of 18,858 ng/uL

PCR colony#11,5 YFP

WEDNESDAY, 8/12/2020

Done by Godefroy

WE used the same reactionnal mix as Inaccessible Entry

Material:

- 2019GO-5-Seq-pBAD-F
- 2019GO-6-Seq-pBAD-R

Table1

	A	B	C	D	E
1	Nom de l'oligo	Cible	Séquence (5'-3')	Tm (°C, IDT-oligo analyzer)	hairpin Tm (°C, IDT)
2	2019GO-5-Seq-pBAD-F	pBAD24	5'-TGGCTATGCCATAGCATTTTTATCC	55,6	50,0
3	2019GO-6-Seq-pBAD-R	pBAD24	5'- GGT CTG ATT TAA TCT GTA TCA GGC TG	55,3	38,7

- sub-clones pBAD_YFP 1 to 4 from box 5 from GG#7

Protocol

1. With a toothpick, stripe 1 colony on 1 cm; then, put it on an eppendorf containing 100 µL of H2O. Homogeneise the tube. You have the 'Template DNA' ready. NOTE : in PCR#1 and 2, I pipette 1µL of this 100µL H2O + plasmid as DNA Template. I was wrong You need to pipette 19+1 µL H2O + plasmid.
2. Add composants in this order (cf below). Attention, all this tubes are in ice

We use the kit " Q5 High-Fidelity 2 X Master MIX" from New England Biolabs.

Table2

	A	B	C	D
1	Order of introduction	Components	Volume (µL) for 50 µL reaction	Final concentration
2	1)	Template DNA (pBAD-MOCLO +/- insert)	5 (0 for the negative control)	<1.000 ng
3	2)	Dream taq Buffer	5	0.5µM
4	3)	2019GO-5-Seq-pBAD-F	1	0.5µM
5	4)	2019GO-6-Seq-pBAD-R	1	1 X
6	5)	Nuclease free water	19 (20 for the negative control)	/
7	6)	Dream taq polymerase	0,250	
8	7)	dNTP	1	

Electrpohoresis PCR colony#11,5

WEDNESDAY, 8/12/2020

Done by Godefroy

Protocol :

For the gel

- Weigh 4,8g of agarose (0,8% --> 0,8g pour 100 ml)
- Measure 600 ml of TAE buffer (0.5X) in a test tube
- Put the agarose and then add the buffert in a 1L (glass) bottle.
- Keep the gel boiling in the microwave
- Cast the gel and put the combs

For 1 well :

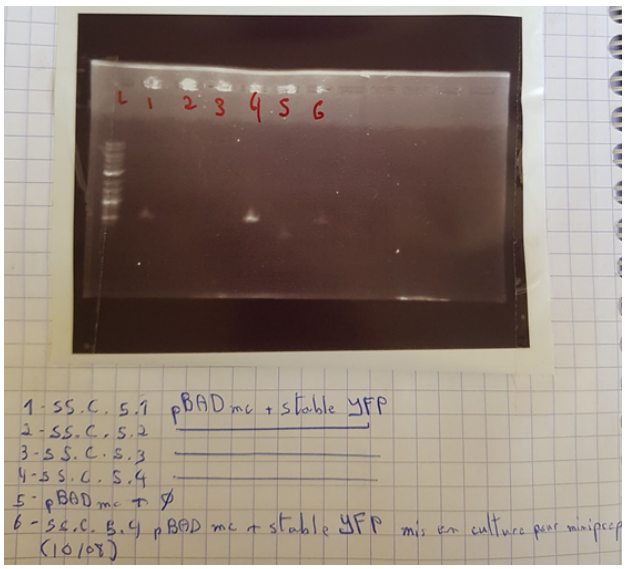
- on 1 PCR tube, put : 10µL of mix PCR + 2µL of blue charge. Then put 12µL of this mix in 1 well
- for the ladder : just put 5 µL of ladder

For the migration :

- Set the machine on 100 Volts during **25 minutes**.

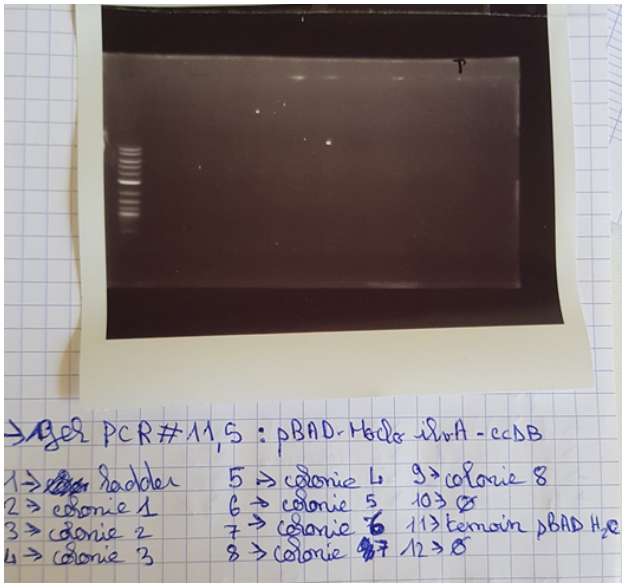
Results and plan :

image.png



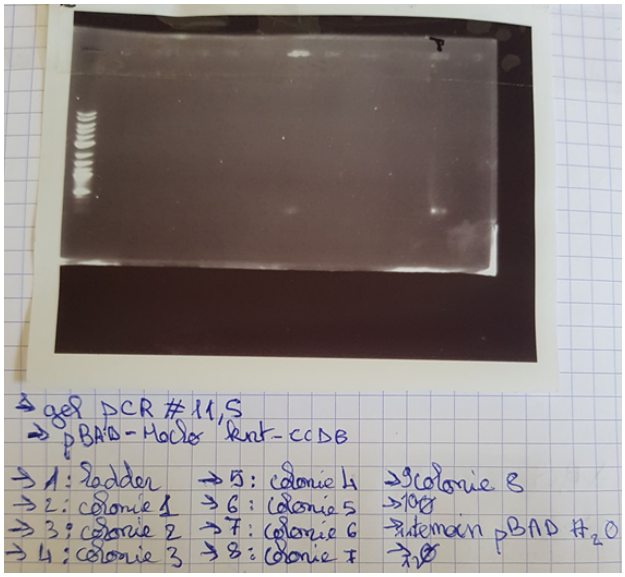
All the colonies tested seems to have an insert.

image.png



Seems that no colony has insert.

image.png



Seems that no colony has insert.

Miniprep sub-clone 5.4 pBAD-MC YFP GG#7

WEDNESDAY, 8/12/2020

Done by Godeffroy

To know our YFP concentration for sequencing. (the same as Inaccessible Entry to have more DNA)

Material :

- Monarch^{R} Plasmid Miniprep Kit (#T10105 lot: 10040564)

Protocol :

image.png

Monarch
Plasmid Miniprep Kit Protocol Card

NEB #T1010

For a detailed protocol or to download the full manual, visit www.neb.com/T1010

BEFORE YOU BEGIN:

- Add 4 volumes of ethanol (≥ 95%) to one volume of Plasmid Wash Buffer 2.
- All centrifugation steps should be carried out at 16,000 x g (~13,000 RPM).
- If precipitate has formed in Lysis Buffer (B2), incubate at 30–37°C, inverting periodically to dissolve.
- Store Plasmid Neutralization Buffer (B3) at 4°C after opening, as it contains RNase A.

PROTOCOL STEPS:

1. **Pellet 1–5 ml (not to exceed 15 OD units) bacterial culture by centrifugation for 30 seconds. Discard supernatant.** 1.5 ml of culture is sufficient for most applications. Ensure cultures are not overgrown (12-16 hours is ideal).
2. **Resuspend pellet in 200 µl Plasmid Resuspension Buffer (B1)**. Vortex or pipet to ensure cells are completely resuspended. There should be no visible clumps.
3. **Add 200 µl Plasmid Lysis Buffer (B2)**, **gently invert tube 5–6 times, and incubate at room temperature for 1 minute.** Color should change to dark pink, and solution will become transparent and viscous. Do not vortex.

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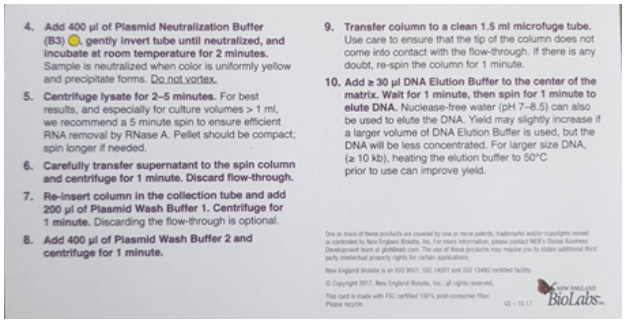
image.png



4. **Add 400 µl of Plasmid Neutralization Buffer (B3)**, **gently invert tube until neutralized, and incubate at room temperature for 2 minutes.** Sample is neutralized when color is uniformly yellow and precipitate forms. Do not vortex.
5. **Centrifuge lysate for 2–5 minutes.** For best results, and especially for culture volumes > 1 ml, we recommend a 5 minute spin to ensure efficient RNA removal by RNase A. Pellet should be compact; spin longer if needed.
6. **Carefully transfer supernatant to the spin column and centrifuge for 1 minute. Discard flow-through.**
7. **Re-insert column in the collection tube and add 200 µl of Plasmid Wash Buffer 1. Centrifuge for 1 minute.** Discarding the flow-through is optional.
8. **Add 400 µl of Plasmid Wash Buffer 2 and centrifuge for 1 minute.**
9. **Transfer column to a clean 1.5 ml microfuge tube.** Use care to ensure that the tip of the column does not come into contact with the flow-through. If there is any doubt, re-spin the column for 1 minute.
10. **Add ≥ 30 µl DNA Elution Buffer to the center of the matrix. Wait for 1 minute, then spin for 1 minute to elute DNA.** Nuclease-free water (pH 7–8.5) can also be used to elute the DNA. Yield may slightly increase if a larger volume of DNA Elution Buffer is used, but the DNA will be less concentrated. For larger size DNA, (≥ 10 kb), heating the elution buffer to 50°C prior to use can improve yield.

V2 – 10.17

NEW ENGLAND BioLabs

Result :

Obtention of a pBAD-MC tube of concentration of 48.057 ng/uL

Mesurement of DO of sous-clones from GG#7 and GG#8 grew in liquid middle by night

WEDNESDAY, 8/12/2020

Done by Baptiste

Mesurement of DO of sous-clones from GG#7 and GG#8 grew in liquid middle by night before observe them with microscope to mesure their fluorescence.

Results :

- pBAD-MC H2O 1 : 0.91 1
- pBAD-MC H2O 2 : 0.85 2
- pBAD-MC H2O 3 : 0.75 3
- sous-clone pBAD-MC knt_GFP from GG#8 1 : 0.50 4
- sous-clone pBAD-MC knt_GFP from GG#8 2 : 0.48 5
- sous-clone pBAD-MC knt_GFP from GG#8 3 : 0.49 6
- sous-clone pBAD-MC YFP from GG#7 1 : 0.50 7
- sous-clone pBAD-MC YFP from GG#7 2 : 0.50 8
- sous-clone pBAD-MC YFP from GG#7 3 : 0.52 9

Mesurement of DO of sous-clones from GG#7 and GG#8 grew in liquid middle by night

WEDNESDAY, 8/12/2020

Done by Baptiste

Mesurement of DO of sous-clones from GG#7 and GG#8 grew in liquid middle by night before observe them with microscope to mesure their fluorescence.

Results :

- pBAD-MC H2O 1 : 0.91 1
- pBAD-MC H2O 2 : 0.85 2
- pBAD-MC H2O 3 : 0.75 3
- sous-clone pBAD-MC knt_GFP from GG#8 1 : 0.50 4
- sous-clone pBAD-MC knt_GFP from GG#8 2 : 0.48 5
- sous-clone pBAD-MC knt_GFP from GG#8 3 : 0.49 6
- sous-clone pBAD-MC YFP from GG#7 1 : 0.50 7
- sous-clone pBAD-MC YFP from GG#7 2 : 0.50 8
- sous-clone pBAD-MC YFP from GG#7 3 : 0.52 9

Culture by night of colonies knt-GFP and YFP for microscopy fluorescence observation and Clariostar observation

WEDNESDAY, 8/12/2020

Done by Baptiste

Initial OD of our culture was 0,1.

On middle liquid LB + AMP + Ara
-pBAD-MC H2O :

- col 1 : 549 uL
- col 2 : 588 uL
- col 3 : 666 uL

-pBAD-MC knt_GFP :

- col 4 : 1mL
- col 5 : 1041 uL
- col 6 : 1020 uL

-pBAD-MC YFP (5.4) :

- col 7 : 1mL
- col 8 : 1041 uL
- col 9 : 961 uL

On middle liquid LB + chloro + Ara

- Witness which express GFP constituvely (11)

Cultures of knt-GFP and YFP for fluorescence observation on UV table in LB

WEDNESDAY, 8/12/2020

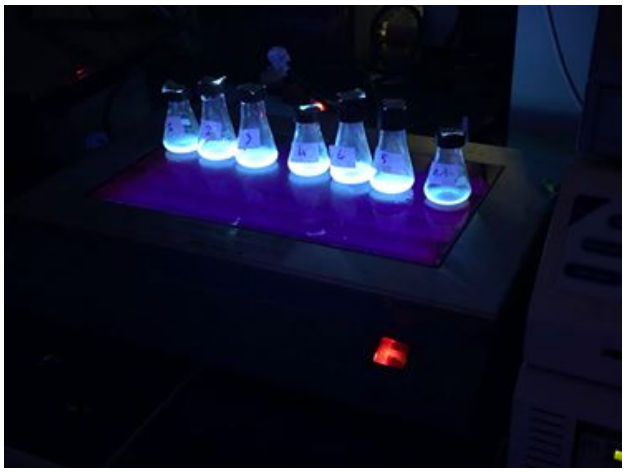
Incubation overnight

-pBAD-MC H2O (col 1, 2, 3)
-pBAD-MC knt_GFP (col 4, 5, 6)
-pBAD-MC YFP (col 7, 8, 9)
-Witness which express GFP constitutively (11)
-Witness YFP (10)

Results :

There were fluorescence of negative control so the results cannot be interpreted.

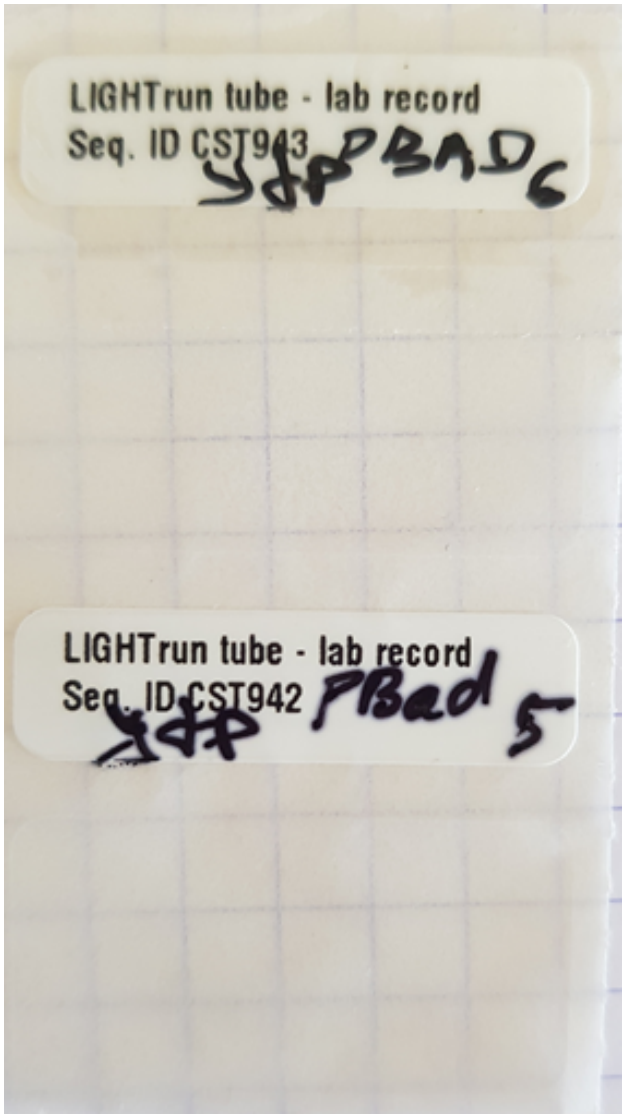
image.png



Sending sequencing DNA from miniprep colony YFP from GG#7

WEDNESDAY, 8/12/2020

image.png



Fluorescence microscope observation 1

WEDNESDAY, 8/12/2020

Culture of knt-GFP colonies one spectinomycine gradient

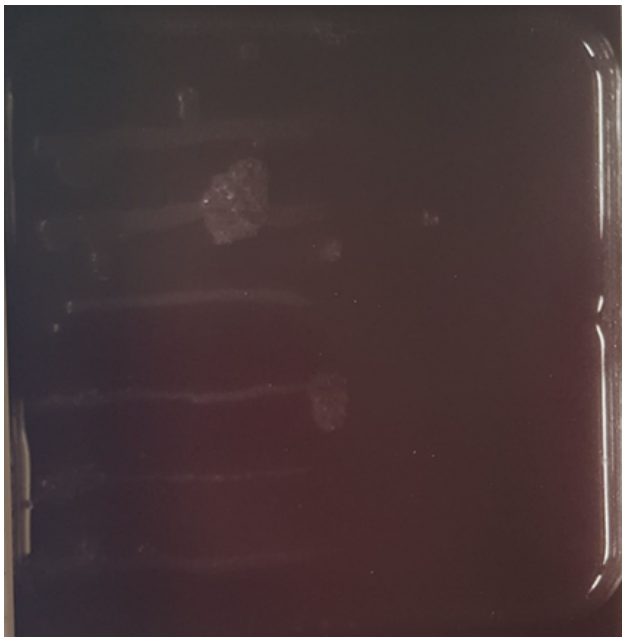
THURSDAY, 8/13/2020

Middle = LB + Arabinose + amp + spe gradient

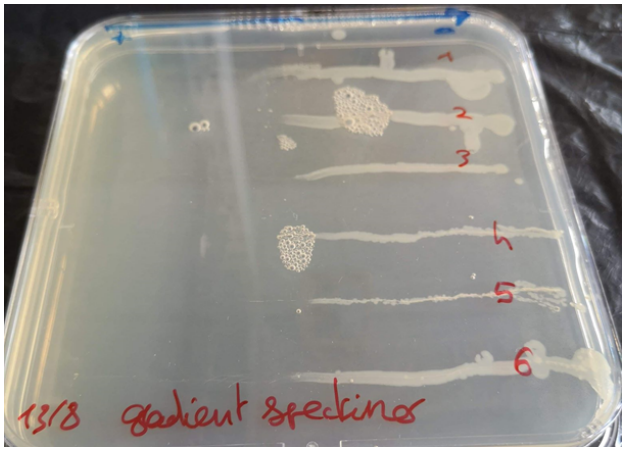
Résults

Transformants and witnesses seem to present no resistance to spectinomycine.

Gel 1 UV

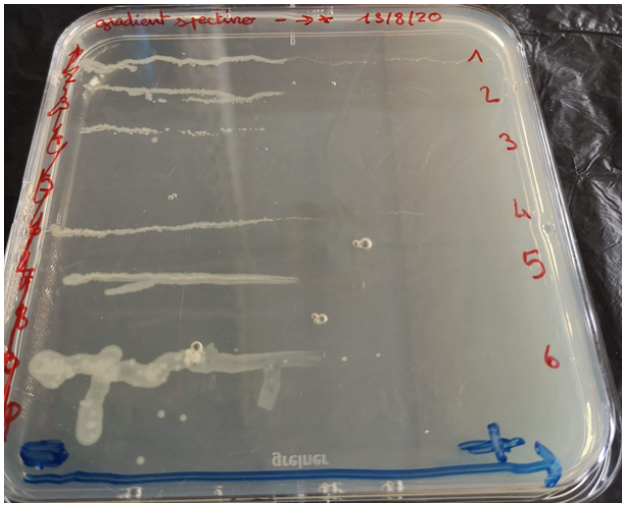


Gel 1



Gel 2 UV

Gel 2



Clariostar knt-GFP and YFP

FRIDAY, 8/14/2020

Goal

Use Clariostar to mesure fluorescence of our transformed bacteria.

Plate plan :

Table1

	A	B	C	D	E	F	G	H
1	blanc 1	blanc 2	1	2	3	4	5	6
2	7	8	9	10	11			

blanc 1 : LB liquid + Amp + Arabinose
blanc 2 : LB liquid + Cmp + Arabinose
Numbers 1 to 9 refer to colonies of Inaccessible Entry
10 : YFP witness
11 : GFP witness

NB : our YFP witnesses do not express the same YFP as our transformed colonies wich express our stable YFP.

Results

Clariostar_YFP_14082020_3_valeurs.xlsx

Clariostar_OD600_14082020_3_valeurs.xlsx

Clariostar_GFP_14082020_3_valeurs.xlsx

The results seem to show that their is no fluorescence of our bacterias due to our biobricks knt-GFP and YFP.

Protocol preparation for fluorescent microscope observation

FRIDAY, 8/14/2020

1. OD mesurment and respusention of bacterias for OD = 1
2. centrifugation
3. resuspension in 100uIL of MQ water of bacterias
4. 10 uL of solution on LB bubble on lamina
5. observation

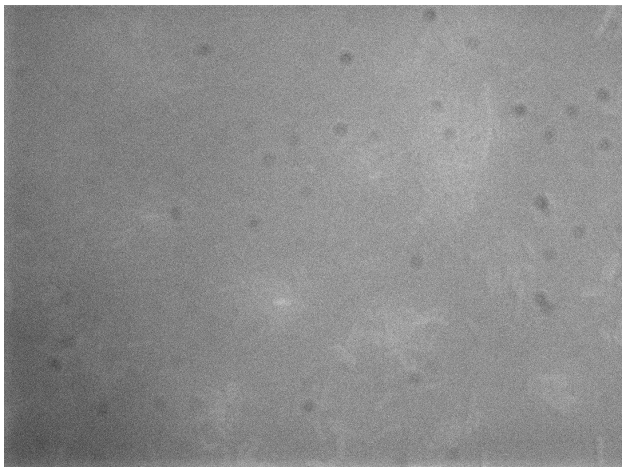
Fluorescent microscope observation 2

FRIDAY, 8/14/2020

It seems that there is was no clear fluorescence of our bacteria expressing knt-GFP compared to the negative and positive controls.

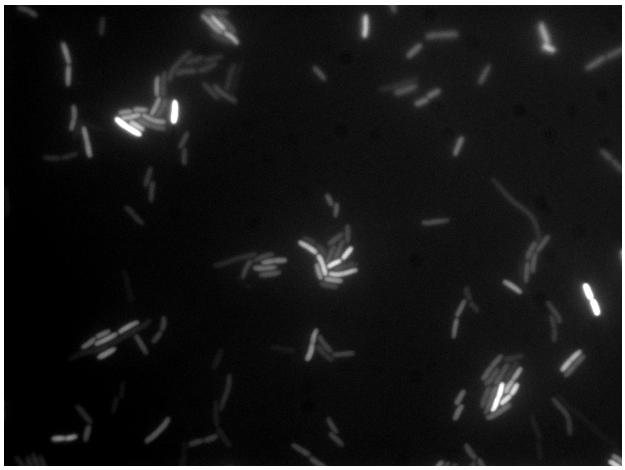
Negative witness :

DH5alpha + pBAD-Moclo cl2 image2_w2GFP.jpg



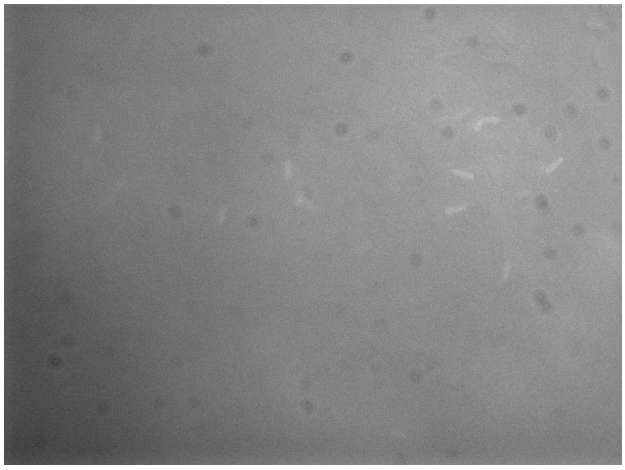
Positive witness :

Témoin positif GFP Philippe image2_w2GFP.png



Knt-GFP :

DH5alpha + pBAD-KntGFP cl1 image3_w2GFP.png



Cytometry YFP and knt-GFP

WEDNESDAY, 9/2/2020

Cytometry test to determine if our transformants of stable YFP and knt-GFP express fluorescence.

Results :

Résults cytométy.pptx

We observe a clear fluorescence due to YFP but results for knt-GFP seem not clear, another cytrometry experiment is required.

Cytometry YFP and knt-GFP 2

THURSDAY, 9/3/2020

The results of the first cytometry were not sufficient to determine if knt-GFP permitt fluorescence so we did a second cytometry test.

Results :

iGEM-Cytometry 2_data_analyses.docx

It seems that transformants of stable YFP express fluorescence compared to negative control but less fluorescence than positive controle.
There is no clear proof fluorescence due to knt-GFP.

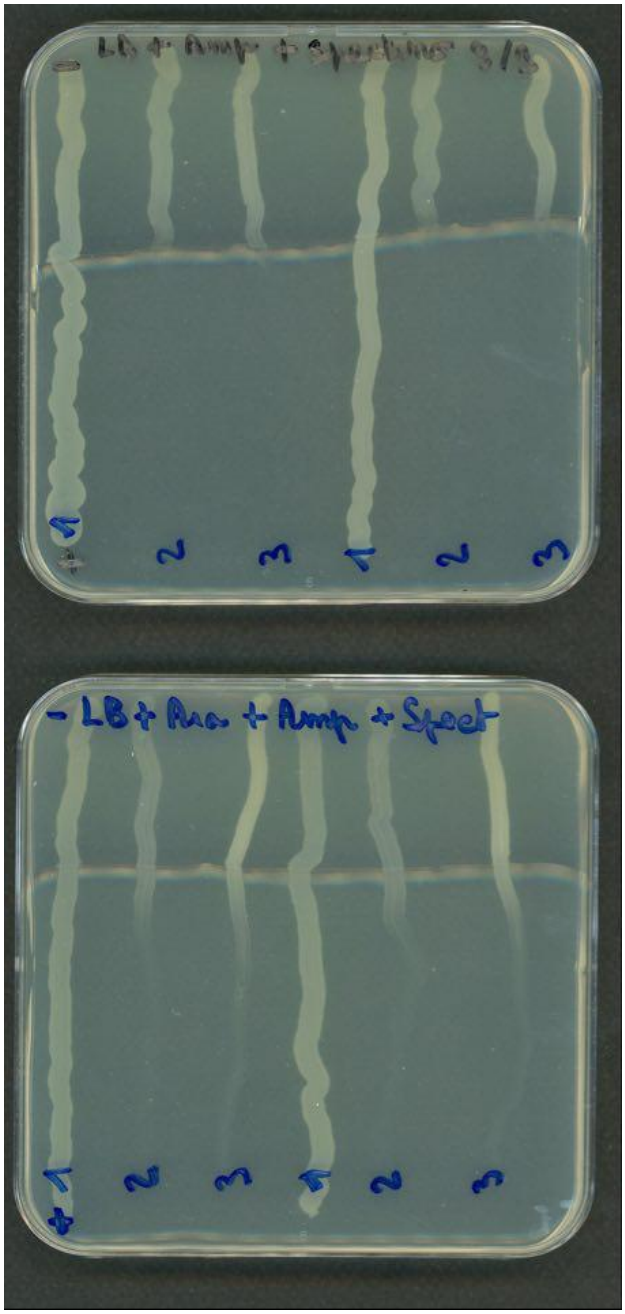
Culture of YFP and knt-GFP transformants on middles with different antibiotics concentration gradients

THURSDAY, 9/3/2020

Composition of media is written on the photos. We tried the following aminoglycoside antibiotics: spectinomycin, streptomycin and kanamycin.

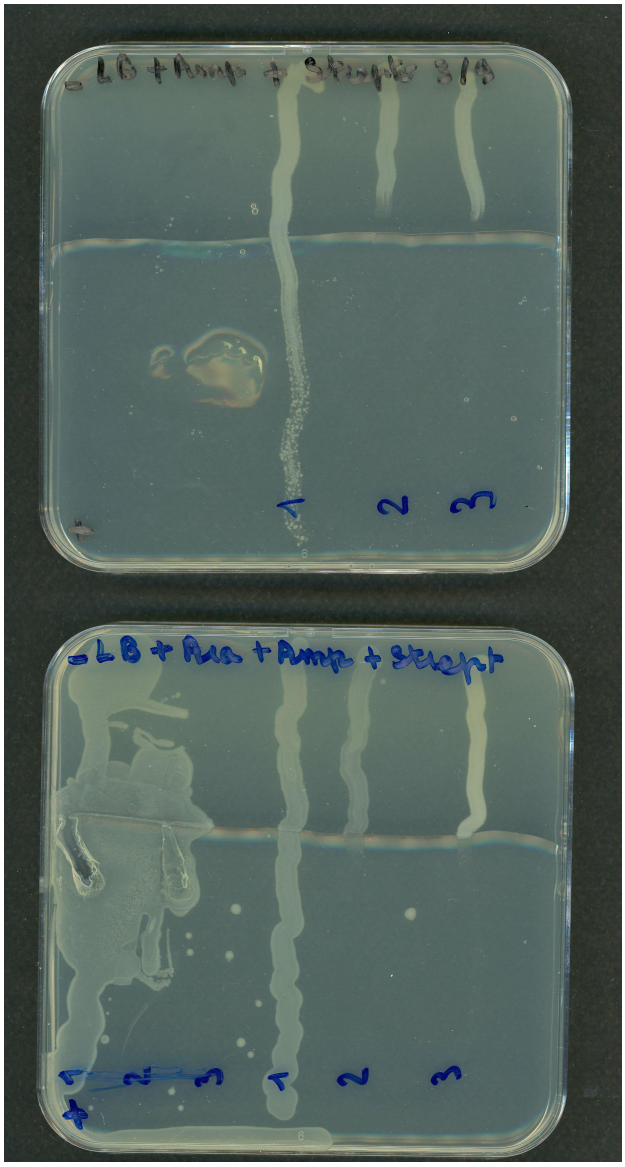
1 : positive control DH5alpha (plasmid with SpecR)
2 : DH5alpha (pBAD24-MoClo-knt-GFP)
3 : negative control DH5alpha (pBAD24-MoClo)

118774506_3482563185140124_1457991375884648640_n.jpg



No difference between 2 and 3 for spectinomycin resistance

118780229_3562645253754703_4969674500013421743_n.jpg



No difference between 2 and 3 for streptomycin resistance

118771223_254389875635796_7414920641372441991_n.jpg

No difference between 2 and 3 for kanamycin resistance

It seems that knt-GFP did not confer resistance against aminoglycoside antibiotics to our bacteria.

Comparison sequences YFP designed and YFP sequenced

WEDNESDAY, 9/30/2020

We send DNA inserted in our transformants bacterias Stable-YFP to IDT for sequencing with the amorces : 5′-TGGCTATGCCATAGCATTTTTATCC (forward)

5′- GGT CTG ATT TAA TCT GTA TCA GGC TG (riverse)

When we received the results of the sequencing we compared it to the sequence we designed with CAMEOS and Benchling with BLAST.

The result is that the query cover is 100% with an E-value of 0,00 and an identity percentage of 100% so the sequences are strictly identical.

Screen captures :

Forward sequences :

image.png

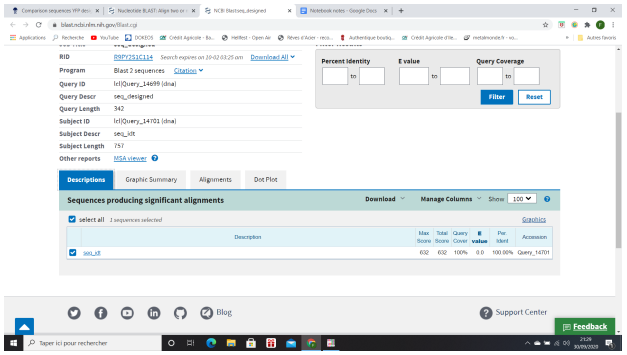
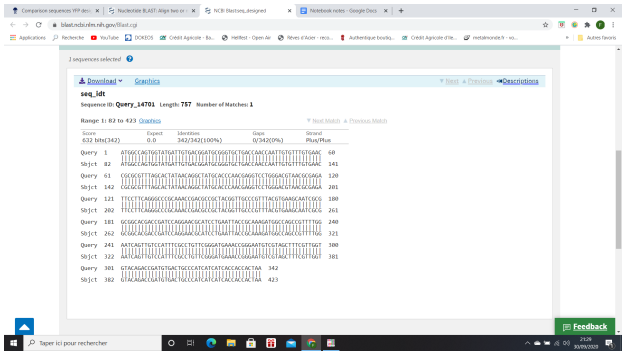
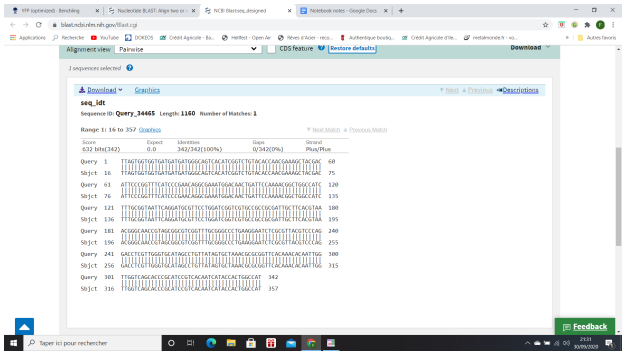


image.png



Reverse sequences :

image.png


Alignment view
Pairwise
CDS feature
Restore defaults
Download
1 sequences selected
Download
Graphics
Next
Previous
Descriptions
seq_idt
Sequence ID: Query_34465 Length: 1160 Number of Matches: 1
Range: 1: 16 to 357 Graphics
Next Match
Previous Match
Score
Expect
Identities
Gaps
Strand
632 bits(342)
0.0
342/342(100%)
0/342(0%)
Plus/Plus
Query 1 TTAGTGGTAGTGATGATGAGGCAGTCACATCGGTCTGTACACCAACGAAAGCTACGAC 60
Sbjct 16 TTAGTGGTAGTGATGATGAGGCAGTCACATCGGTCTGTACACCAACGAAAGCTACGAC 75
Query 61 ATTCCCGGCTTTCATCCCGAACAGGCGAATGGACAACTGATTCCAAAACGGCTGGCCATC 120
Sbjct 76 ATTCCCGGCTTTCATCCCGAACAGGCGAATGGACAACTGATTCCAAAACGGCTGGCCATC 135
Query 121 TTTGCGGTAATTCAGGATGCGTTCCTGGATCGGTCGTGCCGCCGCGATTGCTTCACGTAA 180
Sbjct 136 TTTGCGGTAATTCAGGATGCGTTCCTGGATCGGTCGTGCCGCCGCGATTGCTTCACGTAA 195
Query 181 ACGGGCAACCGTAGCGGCCTCGGTTTGCGGGCCCTGAAGGAATCTCGCGTTACGTCCCAG 240
Sbjct 196 ACGGGCAACCGTAGCGGCCTCGGTTTGCGGGCCCTGAAGGAATCTCGCGTTACGTCCCAG 255
Query 241 GACCTCGTGGCGCATGGCTCTCTTATGCGCTAAACGCGCGCTTCACAACACGATTCG 300
Sbjct 256 GACCTCGTGGCGCATGGCTCTCTTATGCGCTAAACGCGCGCTTCACAACACGATTCG 315
Query 301 TTGGTCAGCACCCGCATCCGTCACAATCATACCACTGGCCAT 342
Sbjct 316 TTGGTCAGCACCCGCATCCGTCACAATCATACCACTGGCCAT 357
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