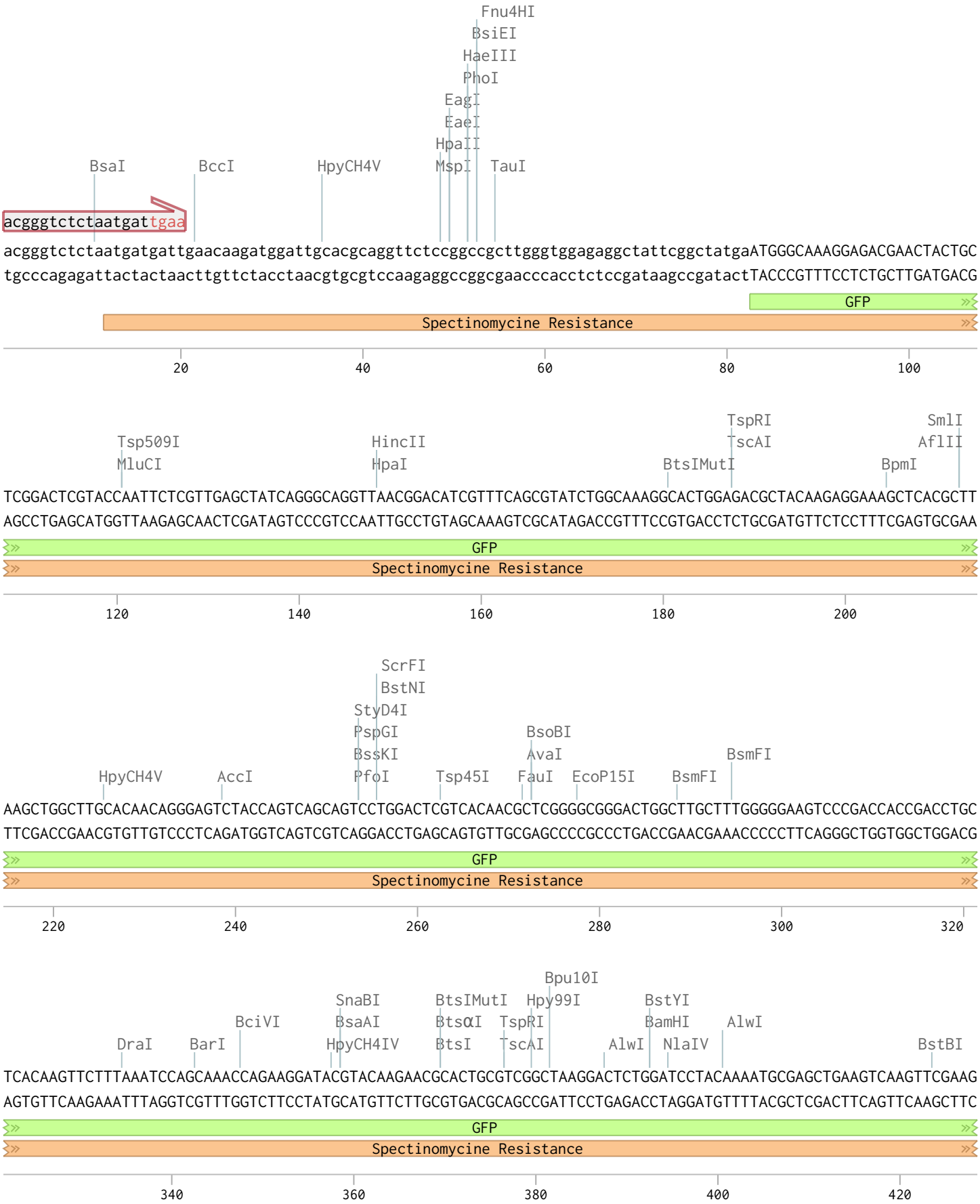
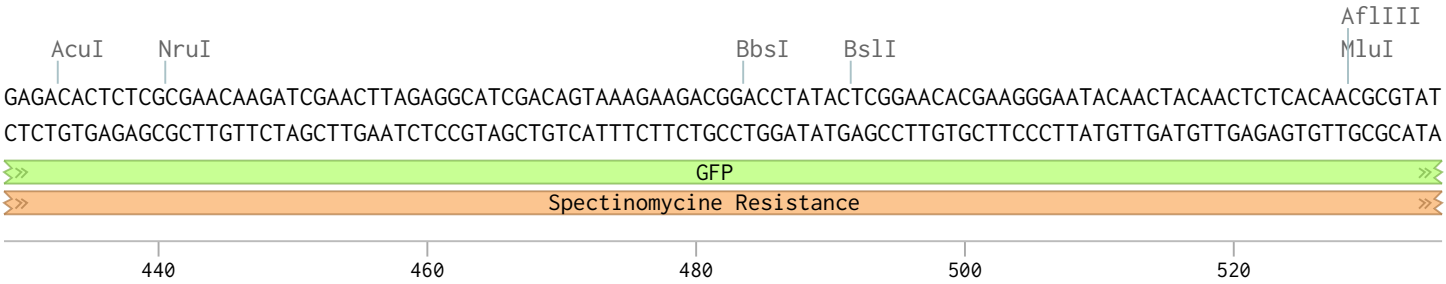
GFP_KanR (823 bp)

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AcuI
NruI
BbsI
BslI
MluI
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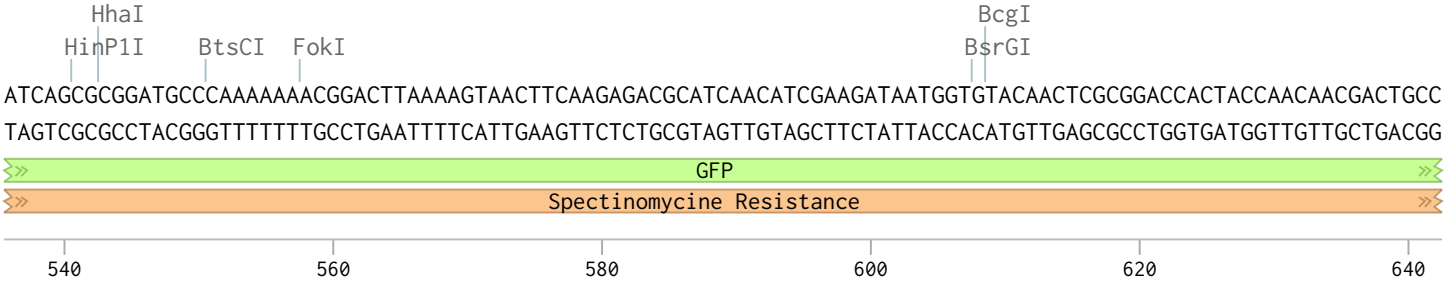
CTCTGTGAGAGCGCTTGTTCTAGCTTGAATCTCCGTAGCTGTCATTTCTTCTGCCTGGATATGAGCCTTGTGCTTCCCTTATGTTGATGTTGAGAGTGTTGCGCATA
GFP
Spectinomycine Resistance
440
460
480
500
520


HhaI
HinP1I
BtsCI
FokI
BcgI
BsrGI
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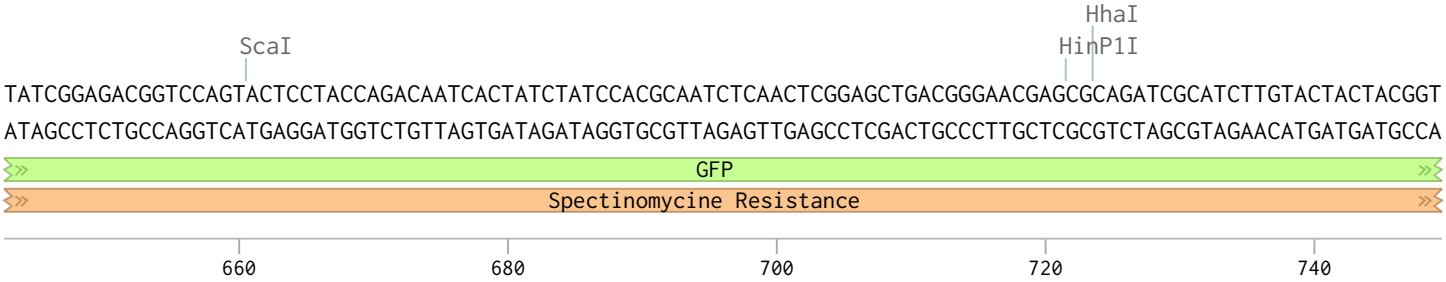
TAGTCGCGCCTACGGGTTTTTTTGCCTGAATTTTCATTGAAGTTCTCTGCGTAGTTGTAGCTTCTATTACCACATGTTGAGCGCCTGGTGATGGTTGTTGCTGACGG
GFP
Spectinomycine Resistance
540
560
580
600
620
640


HhaI
ScaI
HinP1I
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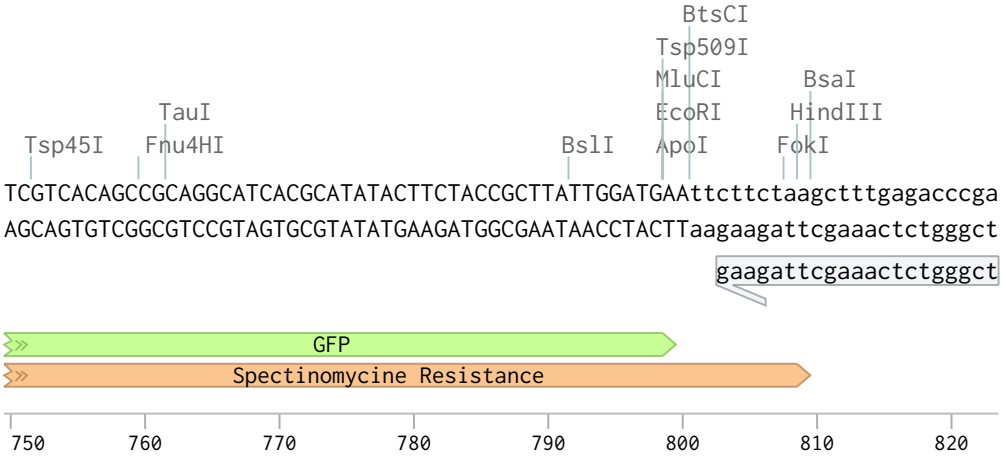
ATAGCCTCTGCCAGGTCATGAGGATGGTCTGTTAGTGATAGATAGGTGCGTTAGAGTTGAGCCTCGACTGCCCTTGCTCGCGTCTAGCGTAGAACATGATGATGCCA
GFP
Spectinomycine Resistance
660
680
700
720
740


BtsCI
Tsp509I
MluCI
BsaI
TauI
EcoRI
HindIII
Tsp45I
Fnu4HI
BslI
ApoI
FokI
TCGTCACAGCCGCAGGCATCACGCATATACTTCTACCGCTTATTGGATGAAttcttctaagctttgagacccga
AGCAGTGTCGGCGTCCGTAGTGCGTATATGAAGATGGCGAATAACCTACTTaagaagattcgaaactctgggct
gaagattcgaaactctgggct
GFP
Spectinomycine Resistance
750
760
770
780
790
800
810
820

Reception and resuspension

WEDNESDAY, 7/29/2020

IDT sequence has been received like 6 other sequences.
Resuspension finale à une concentration de 10ng/ul.

GG#3

WEDNESDAY, 7/29/2020

Done by Florent and Mathieu

Note 1 : 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)
Note 2 : **Note : this manipulation was done in the same time with kanR_GFP**

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 + knt_ccdB_gene272_BsaI
- pBAD-MOCLO + knt_GFP
- pBAD-MOCLO only

(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.

Table2

	A	B	C	D	E	F
1	GG#X	Plasmids	Number of colonies			Middles
2			50 µL	100 µL	Concentrate	
3	GG#3	pBad-MC_sfgfp_knt	0	0	0	LB + AMP + glucose
4		pBAD-MC_ccdB_knt	0	0	0	
5		control H2O (sans insert)	0	0	0	

Transformation 2 GG#3

THURSDAY, 7/30/2020

Seconde transformation based on GG#3 simultaneously to PCR #5 to determine if transformation is effective if PCR results are those expected (it's not the case)

Done by Florent.

We started by transforming chemocompetent DH5alpha.
Protocol of transformation Inaccessible Protocol

We than transformed the golden gate products :

- GG#3 reaction tube pBAD-MC knt-GFP
- GG#3 reaction tube pBAD-MC knt-ccdB
- GG#3 reaction tube pBAD digéré sans insert

Inaccessible Entry

PCR#5 from GG#3 and GG#4

THURSDAY, 7/30/2020

Done by Mathieu

We had no transformants with GG#3 and GG#4 so we did a PCR and an electrophoresis migration to verify if it's our Golden Gate or our transformation method that don't function.

Material:

- 2019GO-5-Seq-pBAD-F
- 2019GO-6-Seq-pBAD-R
- oligo EC632 (forward)
- oligo EC633 (reverse)
    - **-> 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
4	oligo EC632	pZE12	5' gccctttcgtcttcacctc		
5	oligo EC633	pZE12	tcaaaggcggtaatacggtt		

- results of GG#3 and GG#4
- PCR tube
- H2O milli Q
- Thermocycler
- toothpick
- ice

Protocol

1. 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)	Oligo Forward	2,5	0.5µM
5	4)	Oligo Reverse	2,5	1 X
6	5)	Nuclease free water	19 (20 for the negative control)	/

We prepare 7 tubes with the mix PCR

- GG#3 reaction tube pBAD-MC knt-GFP
- GG#3 reaction tube pBAD-MC knt-ccdB
- GG#3 reaction tube pBAD digéré sans insert
- GG#4 reaction tube pZE12 + Luc_consensus_Nter_BsaI
- GG#4 reaction tube Luc_GFP_gene 28
- GG#4 reaction tube Luc_knt_gene 36
- GG#4 reaction tube pZE12 + GFP_Luc1
- GG#4 reaction tube pZE12 + GFP_Luc2
- pBAD natif
- pZE12 natif
- pZE12 digéré par DPN1
- GG#4 reaction tube pZE12 digéré sans insert

PCR Program:

<u>Determination of the Temperature of elongation :</u>
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 - **60** s
5. Repeat from step 2 (5 times)
6. 98°C - 10 s
7. 52°C - 30 s
8. 72°C - **60** s
9. Repeat from step 6 (**20** times)
10. 72°C - 2 min
11. 4°C - Infinity

N-B : We changed the time for the temperature of elongation : in the protocol of PCR #1, the time was 3 min and in this protocol it's 1 min.
The products are analysed by electrophoresis

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

Electrophoresis of PCR#5 products

THURSDAY, 7/30/2020

Done by Mathieu

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 **25 minutes**.

Electrophoresis on :

We prepare 7 tubes with the mix PCR

- GG#3 reaction tube pBAD-MC knt-GFP
- GG#3 reaction tube pBAD-MC knt-ccdB
- GG#3 reaction tube pBAD digéré sans insert
- GG#4 reaction tube pZE12 + Luc_consensus_Nter_BsaI
- GG#4 reaction tube Luc_GFP_gene 28
- GG#4 reaction tube Luc_knt_gene 36
- GG#4 reaction tube pZE12 + GFP_Luc1
- GG#4 reaction tube pZE12 + GFP_Luc2
- pBAD natif
- pZE12 natif
- pZE12 digéré par DPN1
- GG#4 reaction tube pZE12 digéré sans insert

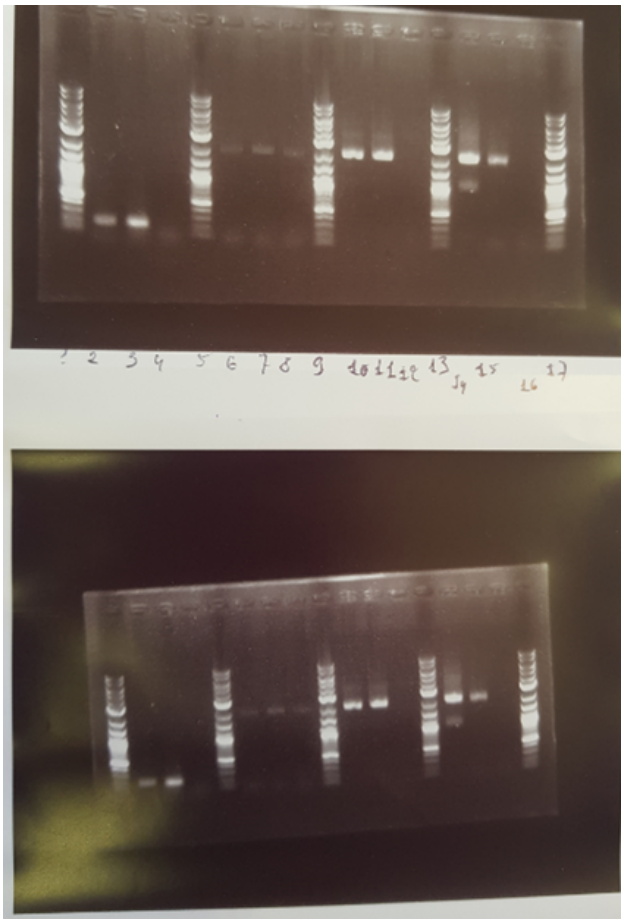
Depot map's + Results

1. Ladder
2. GG#3 reaction tube pBAD-MC knt-GFP
3. GG#3 reaction tube pBAD-MC knt-ccdB
4. GG#3 reaction tube pBAD digéré sans insert
5. Ladder
6. GG#4 reaction tube pZE12 + Luc_consensus_Nter_BsaI
7. GG#4 reaction tube Luc_GFP_gene 28
8. GG#4 reaction tube Luc_knt_gene 36
9. Ladder
10. GG#4 reaction tube pZE12 + GFP_Luc1
11. GG#4 reaction tube pZE12 + GFP_Luc2
12. pBAD natif
13. Ladder

14. pZE12 natif
15. pZE12 digéré par DPN1
16. GG#4 reaction tube pZE12 digéré sans insert
17. Ladder

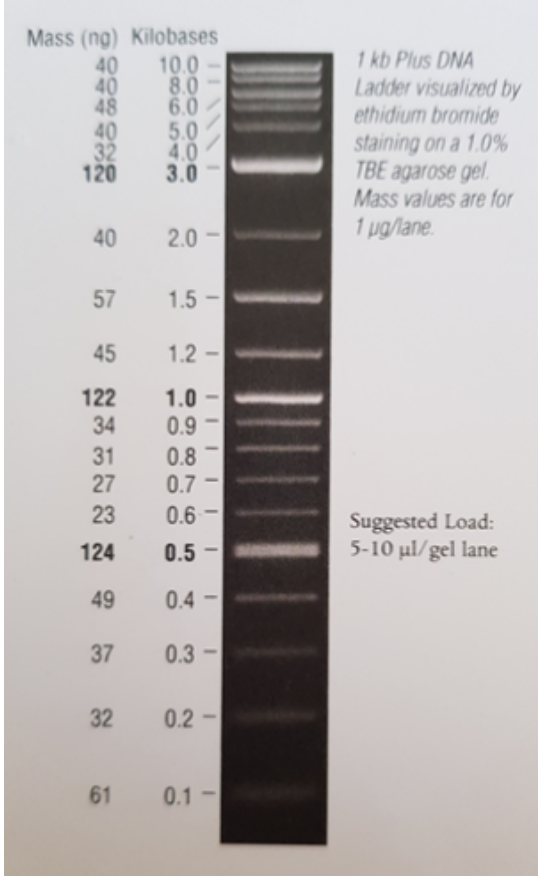
Results

image.png



Le Golden Gate n'a pas fonctionné, seuls les plasmides natifs semblent avoir été amplifés par PCR.

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

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)	/

Plan de dépot voir photos

PCR Program:

98° 10 sec

25 times

1. 55° 30 sec
2. 72° 1 min

78° min

Sub-cloning transformation GG#7

THURSDAY, 8/6/2020

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

Culture of colonies of sub-cloning GG#7 on selective middle + stocking + preparation tubes for PCR#9

FRIDAY, 8/7/2020

Done by Florent

Voir Inaccessible Entry

- Sub-clones knt-GFP were put on LB+AMP, LB +AMP+SPE 125uL et LB+AMP+SPE 250 uL.
- Sub-clones YFP were put on LB+AMP

PCR colony #10

MONDAY, 8/10/2020

PCR colony done with knt_GFP sub-clones from GG#7 grew on LB+amp+spe middle.

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 sub-clone knt_GFP GG#7 on box LB+amp (noir)
- colony sub-clone knt_GFP GG#7 on box LB+amp+spe (rouge)

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

PCR Program:

98° 10 sec

25 times
1. 55° 30 sec
2. 72° 1 min

78° min

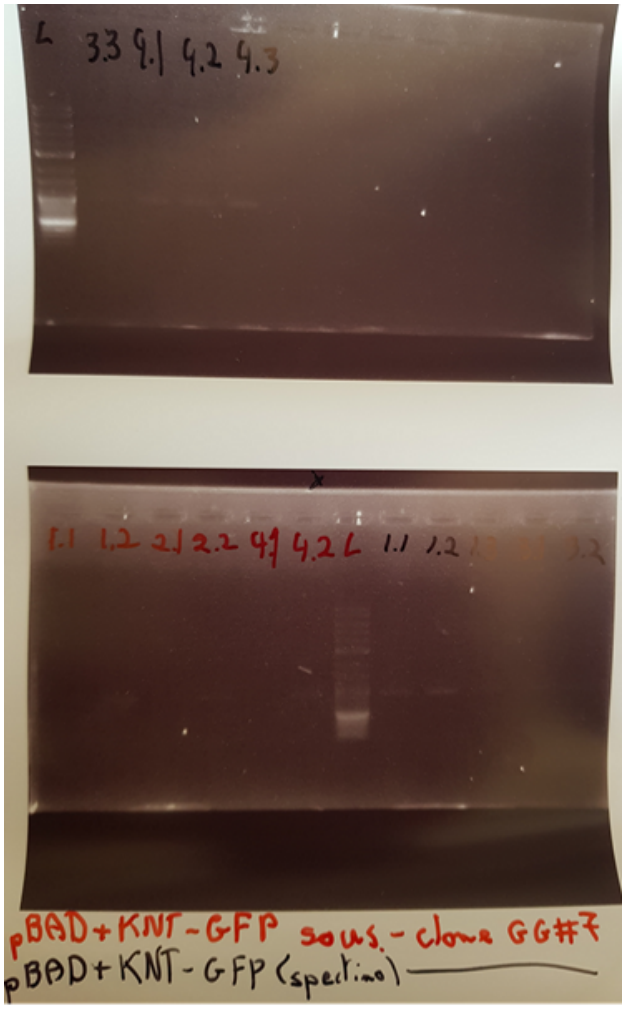
We use the kit " Q5 High-Fidelity 2 X Master MIX" from New England Biolabs.

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	

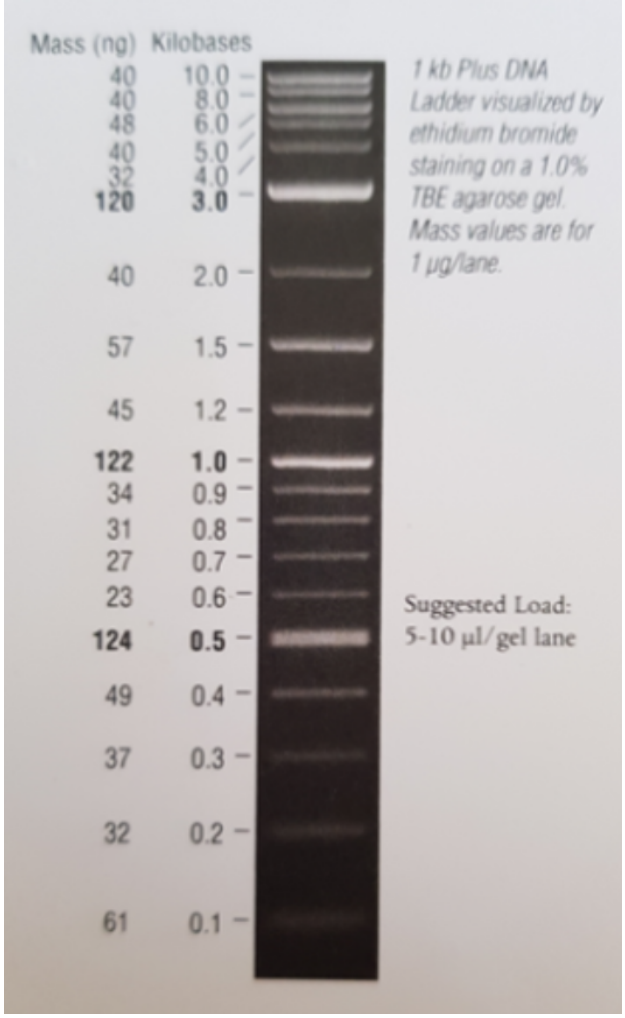
Plan de dépot voir photos

image.png



We can see a bande at the good length so it's possibly good we have an insert with the good length.

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

Electrophoresis PCR colony #10 from sub-clones knt-GFP from GG#7

MONDAY, 8/10/2020

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 :

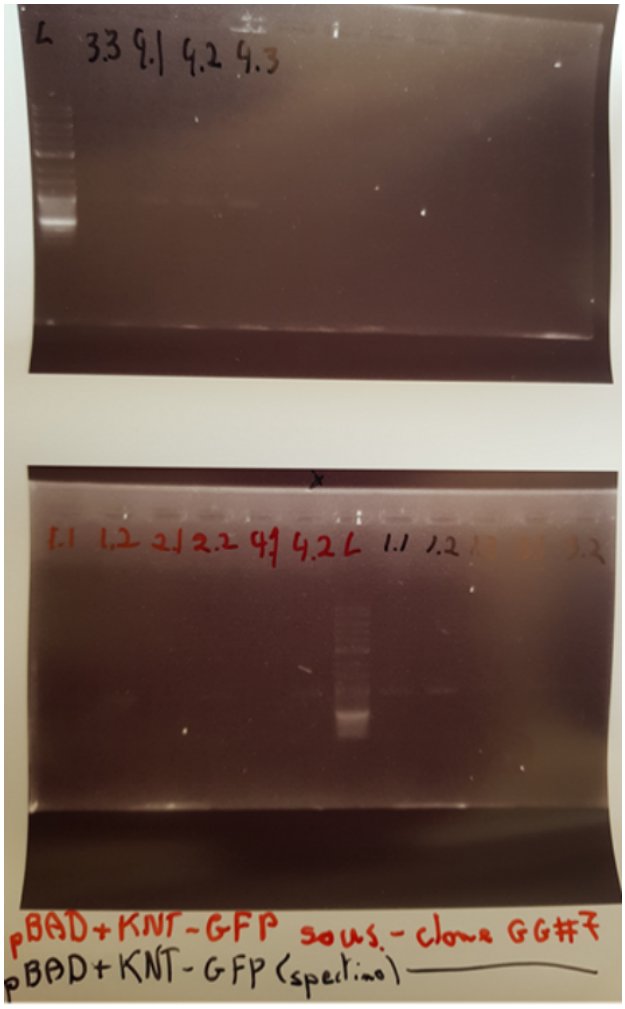
- colony sub-clone knt_GFP GG#7 sur boite LB+amp (black)
- colony sub-clone knt_GFP GG#7 sur boite LB+amp+spe (red)

Depot map's + Results

See photos

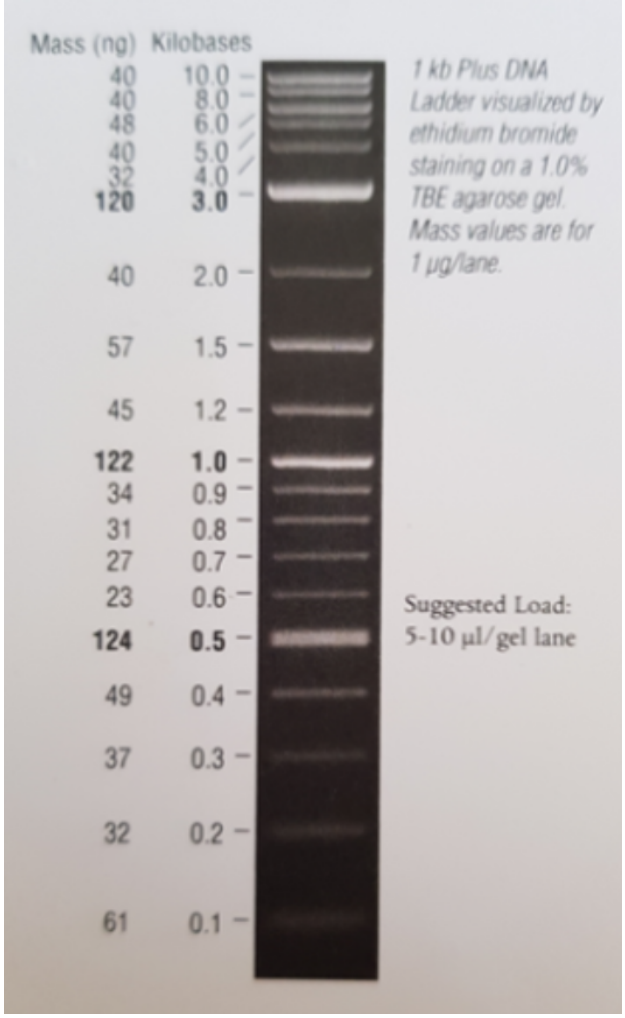
Results

image.png



The bands are at the good lenght (eviron 1kb) so it 's probably our insert but bands very weak, probably a TM (temperature melting) problem for the PCR. But there were no arabinose on the middle so finally it's not our insert.

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

Electrophoresis PCR colony #10 from sous clones knt-GFP from GG#7 (2)

TUESDAY, 8/11/2020

Done by Florent

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 :

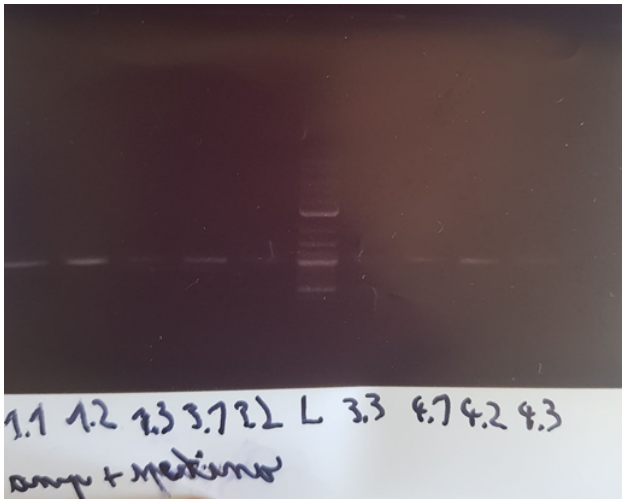
- colony sub-clone knt_GFP GG#7 sur boite LB+amp (black)
- colony sub-clone knt_GFP GG#7 sur boite LB+amp+spe (red)

Depot map's + Results

See photos

Results

image.png



The bands are at the good lenght (eviron 1kb) so it 's probably our insert but bands very weak, probably a TM (temperature melting) problem for the PCR. But there were no arabinose on the middle so finally it's not our insert.

image.png

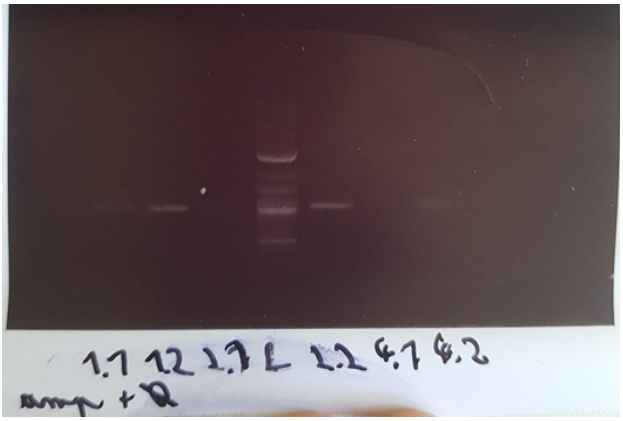
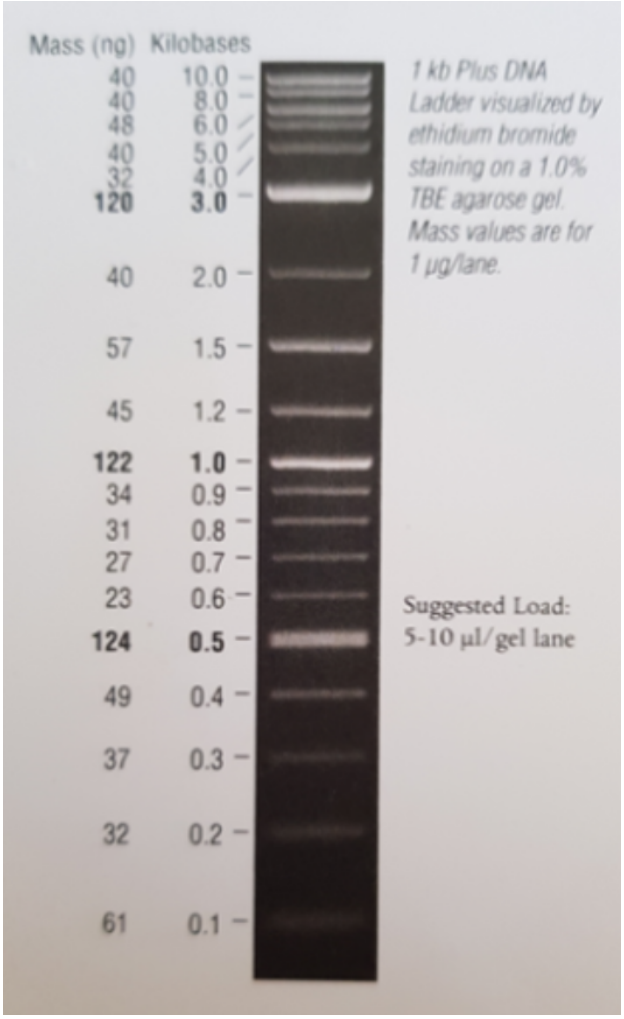


image.png



Observation of knt-GFP and YFP strains with UV table

TUESDAY, 8/11/2020

Done by Mathieu

knt-GFP GG#7 amp (1, 2, 3, 4) : no fluorescence
knt-GFP GG#7 sous clone amp + spectino (1, 4) : no fluorescence
YFP GG#7 (1, 2, 3, 4) : no fluorescence

Miniprep pBAD knt_GFP sub-clone GG#7

TUESDAY, 8/11/2020

Done by Baptiste

To have enought DNA to sequence it.

Material :

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

Protocol :

With 5 minustes of incubation and H2O for eluant.

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:

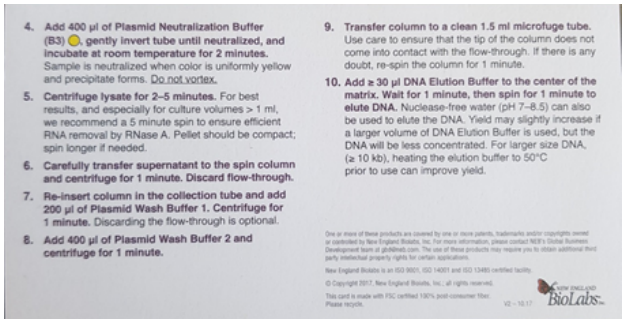
- Add 4 volumes of ethanol (≥ 95%) to one volume of Plasmid Wash Buffer 2.
- All centrifugation steps should be carried out at 16,000 x g (~13,000 RPM).
- If precipitate has formed in Lysis Buffer (B2), incubate at 30–37°C, inverting periodically to dissolve.
- Store Plasmid Neutralization Buffer (B3)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

continued on back →

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

Résultat :

Obtention of a pBAD-MC tube of concentration of 547 ng/uL

Measurement 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
- sub-clone pBAD-MC knt_GFP from GG#8 1 : 0.50 4
- sub-clone pBAD-MC knt_GFP from GG#8 2 : 0.48 5
- sub-clone pBAD-MC knt_GFP from GG#8 3 : 0.49 6
- sub-clone pBAD-MC YFP from GG#7 1 : 0.50 7
- sub-clone pBAD-MC YFP from GG#7 2 : 0.50 8
- sub-clone pBAD-MC YFP from GG#7 3 : 0.52 9

Culture by night of colonies knt-GFP and YFP for microscopique fluorescence observation and Clariostar observation + results observations

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 :

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

On middle liquid LB + chloro + Ara

- Witness which express GFP constituvely

Results :

Inaccessible Entry

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

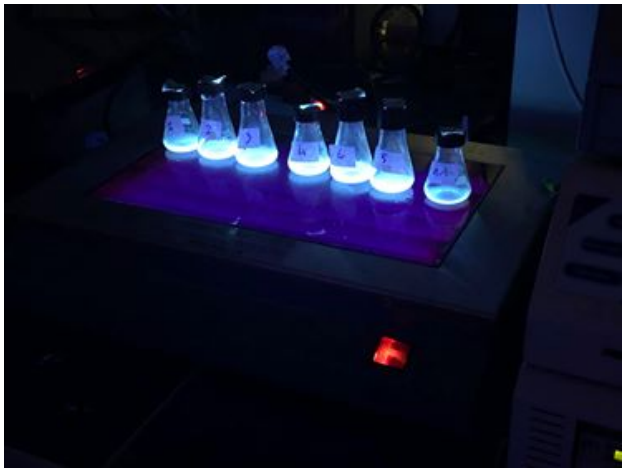
WEDNESDAY, 8/12/2020

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 10)
-Witness YFP (11)

Results

image.png

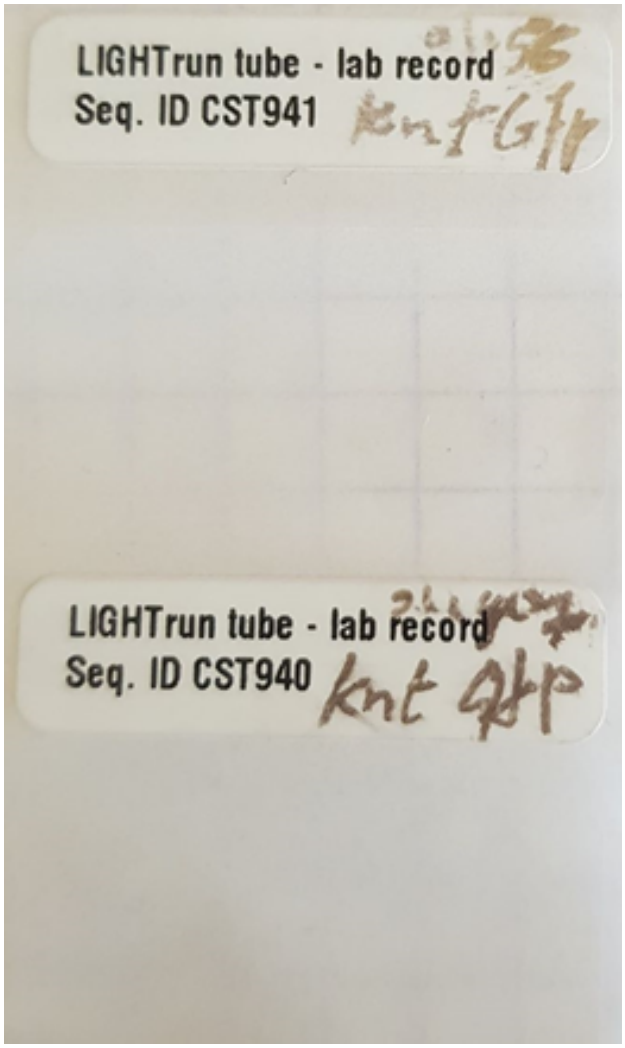


All the colonies seem to express fluorescence even negative witness

Sending DNA from miniprep colony knt-GFP 4 from GG#8 for sequencing

WEDNESDAY, 8/12/2020

image.png



Fluorescence microscope observation 1

WEDNESDAY, 8/12/2020

Culture of colonies knt-GFP on selective middle

THURSDAY, 8/13/2020

Objective :Culture of pBAD-MC_knt_GFP colonies on solid middles to show spectinomycine resistance.

Prtocol :

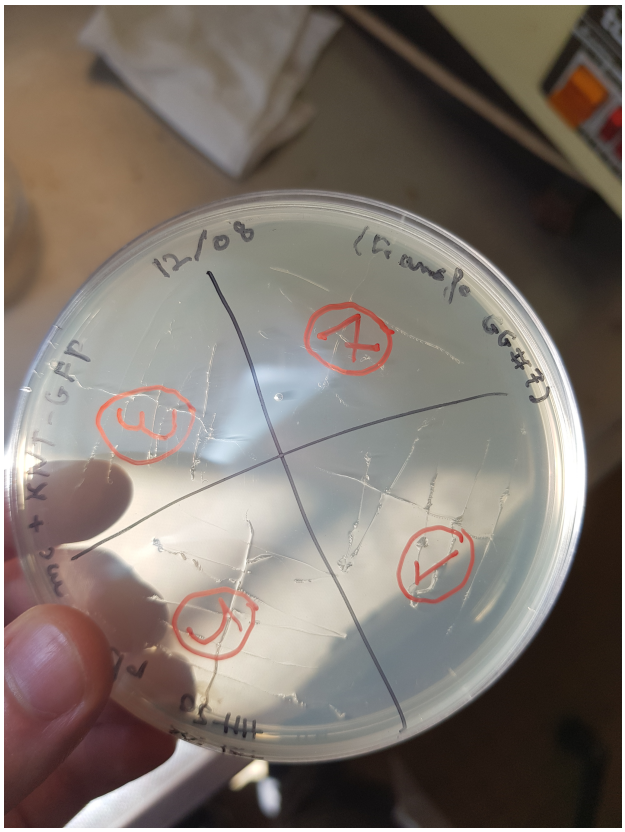
Culture of 4 colonies pBAD-MC_knt_GFP and pBAD-MC with no insert on three middles :

- LB + Amp + Ara + Spe (50 ng/mL)
- LB + Amp + Ara + Spe (100 ng/mL)
- LB + Amp + Ara

NB : Arabinose (Ara) is necessary because MC is an inducible vector

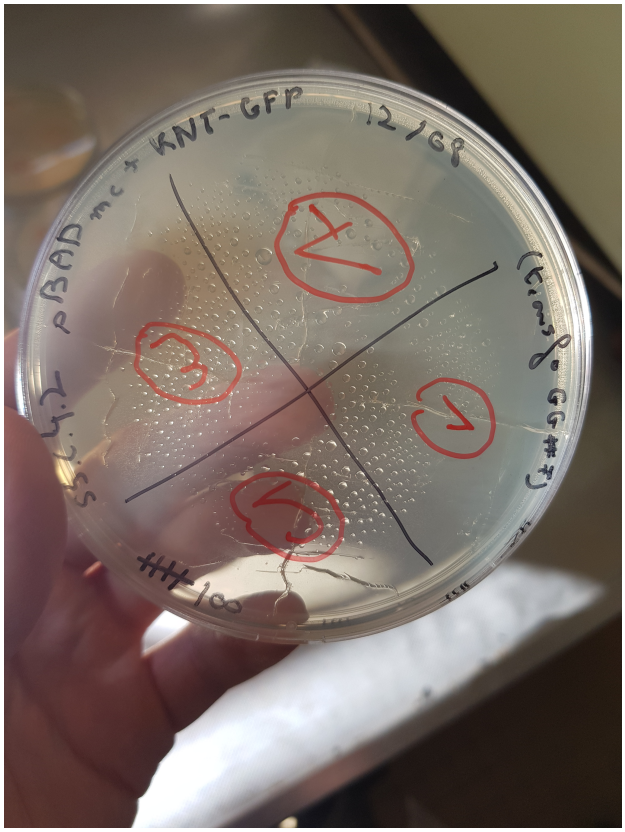
Results :

image.png



No colonie grew on the middles with spectinomycine so their is no resistance given by our entangled gene.

image.png



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

THURSDAY, 8/13/2020

Same protocol and same colonies as Inaccessible Entry

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 :

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

On middle liquid LB + chloro + Ara

- col 11 : Witness which express GFP constituvely
- col 10 : Witness which express YFP constutively (not stable YFP)

FRIDAY, 8/14/2020

Results :
1 : 0,34
2 : 0,35
3 : 0,34
4 : 0,34
5 : 0,33
6 : 0,37
7 : 0,24
8 : 0,27
9 : 0,28
10 : 0,09
11 : 0,45

NB : the cultures have been diluted by factor 10 because DO was superior to 1,5

Inaccessible Entry
Reuslts microscope

Culture of knt-GFP colonies one spectinomycin gradient

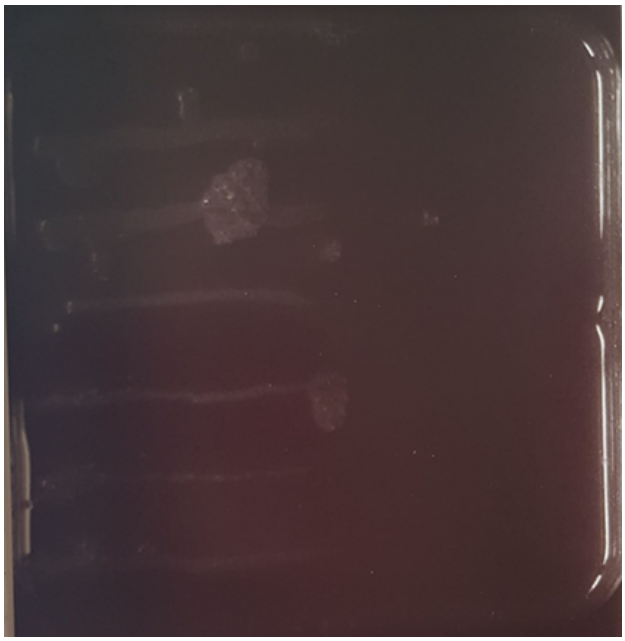
THURSDAY, 8/13/2020

Middle = LB + Arabinose + amp + spe gradient

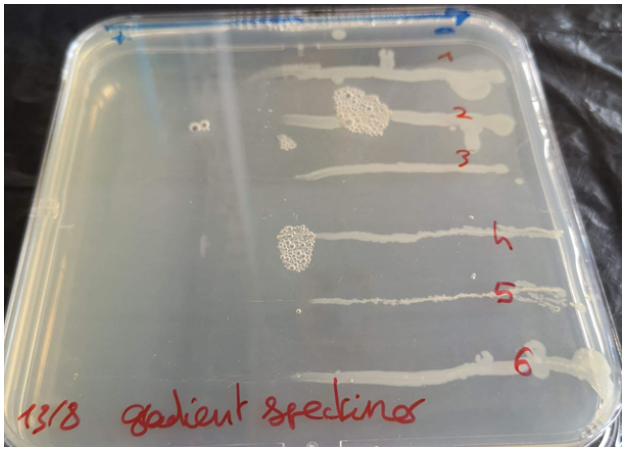
Résults

Transformants and witnesses seem to present no resistance to spectinomycine.

Gel 1 UV

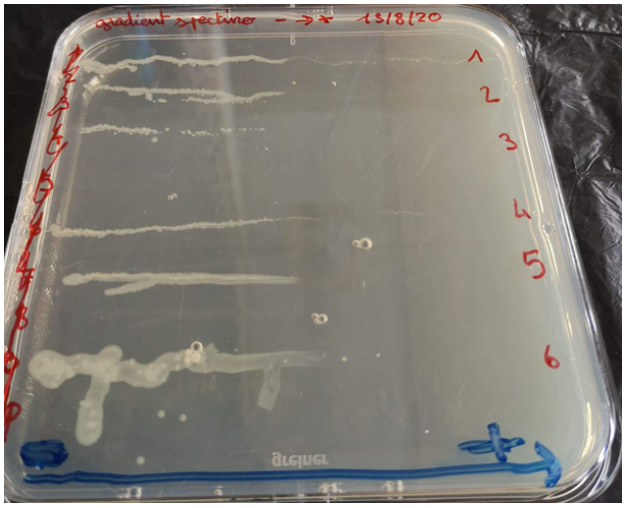


Gel 1



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.

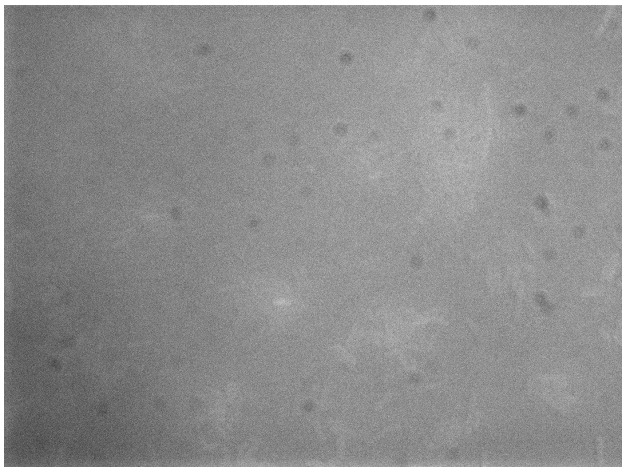
Fluorescent microscope observation 2

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.

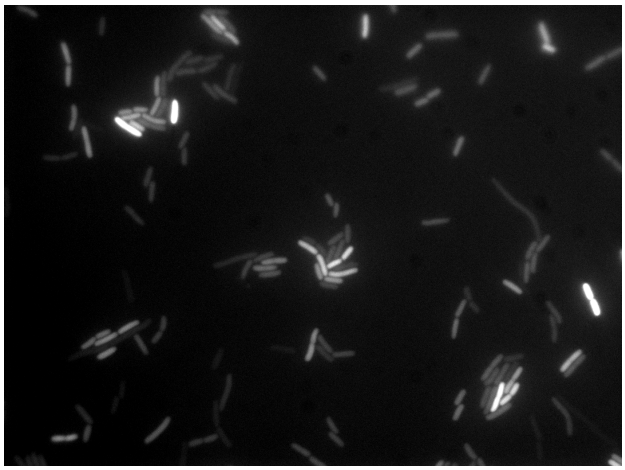
Negative witness :

DH5alpha + pBAD-Moclo cl2 image2_w2GFP.jpg



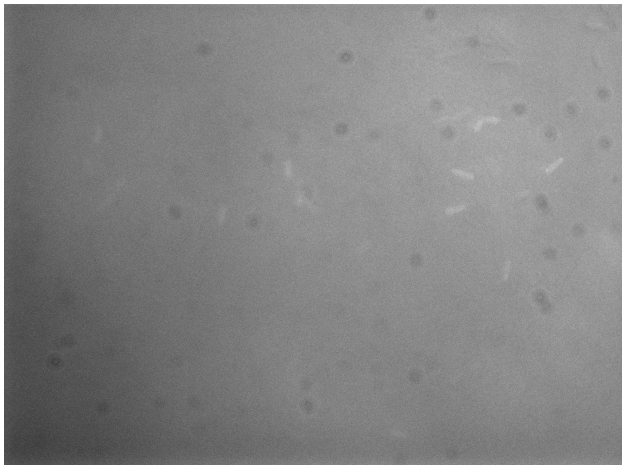
Positive witness :

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.

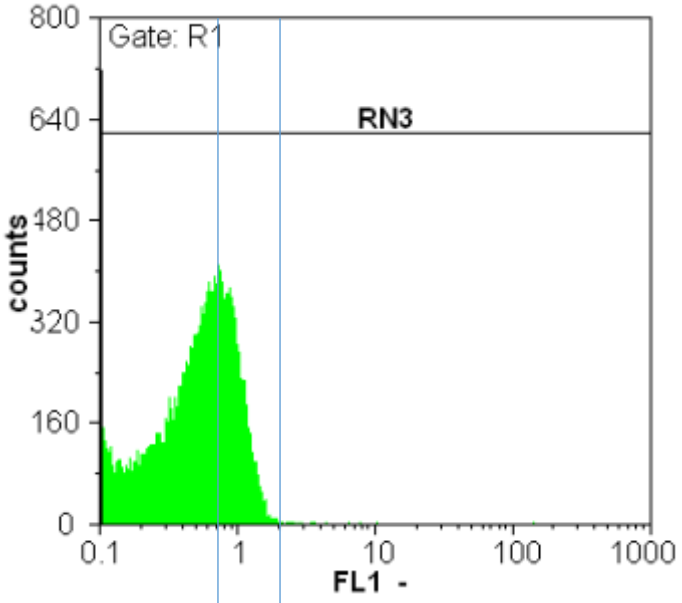
Results :

Résultats cytométrie.pptx

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

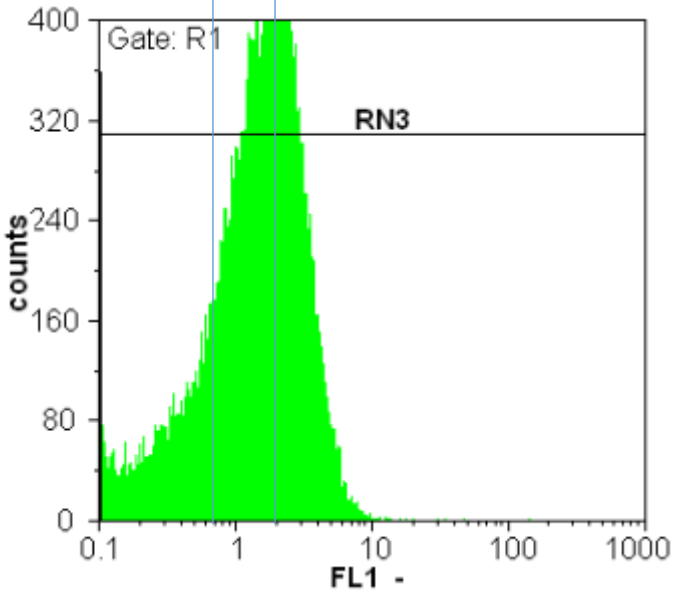
Negative control
(empty vector)

Mean 0.63



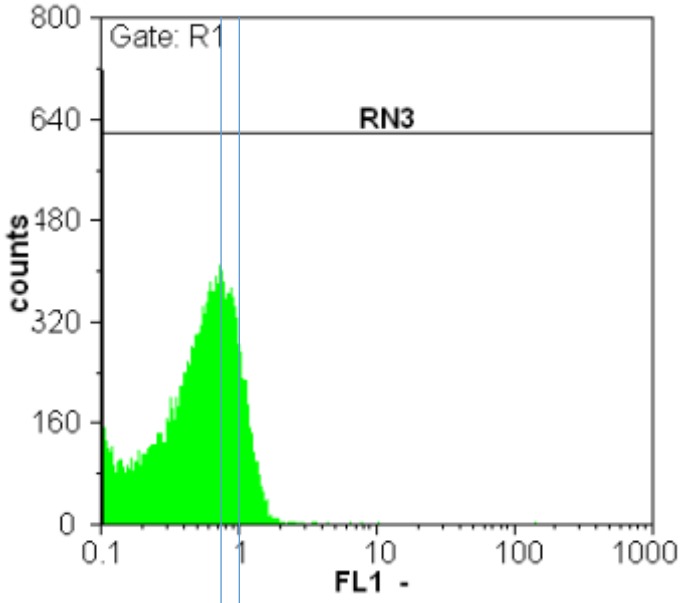
Stable YFP - LOV

Mean 1.83



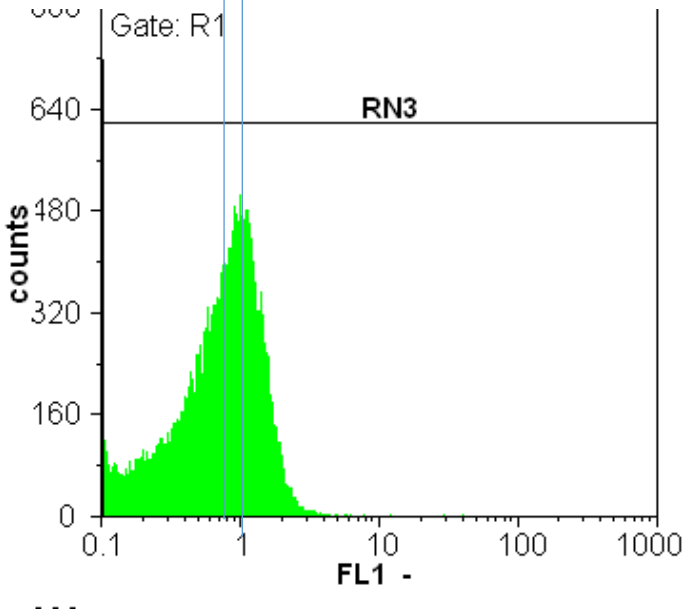
Negative control
(empty vector)

Mean 0.63



Knt-GFP

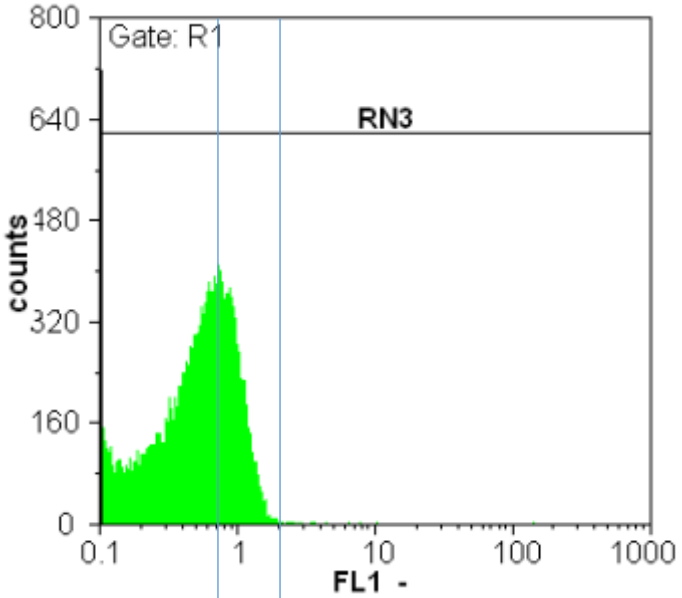
Mean 0.87



Remercier Elodie Hudik et Sophie Dupré de l'équipe d'Oliver Nüsse de l'Institut de Chimie Physique de l'Université Paris-Saclay

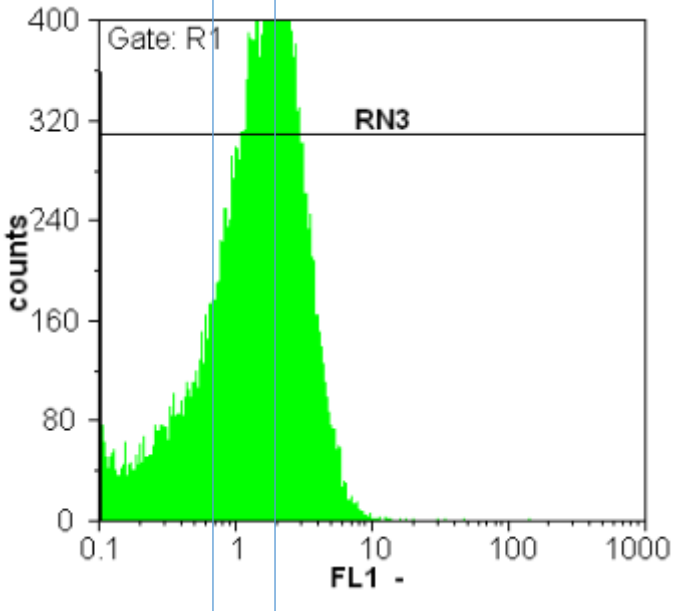
Negative control
(empty vector)

Mean 0.63



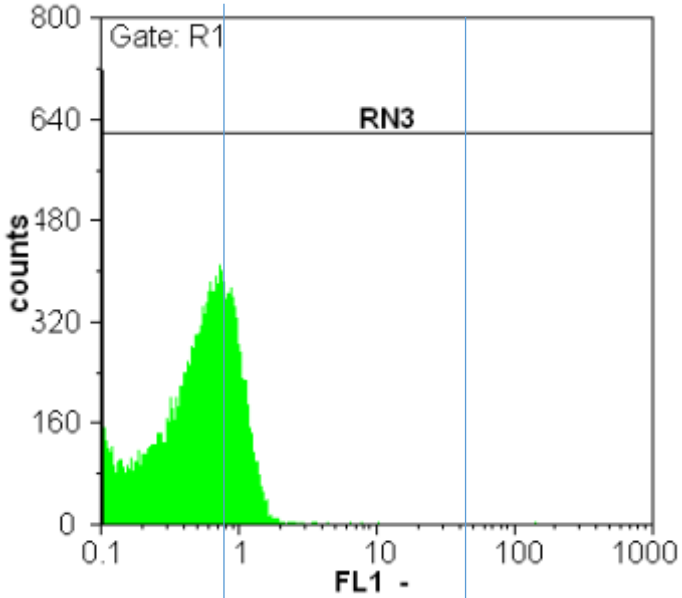
Stable YFP - LOV

Mean 1.83



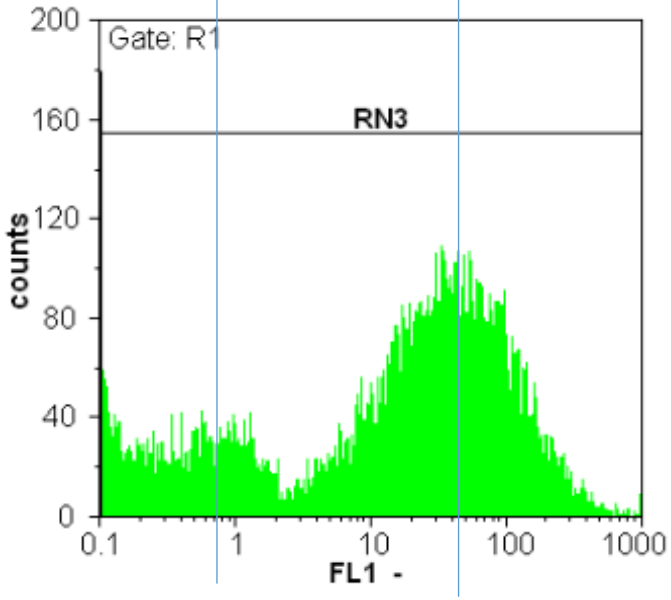
Negative control
(empty vector)

Mean 0.63



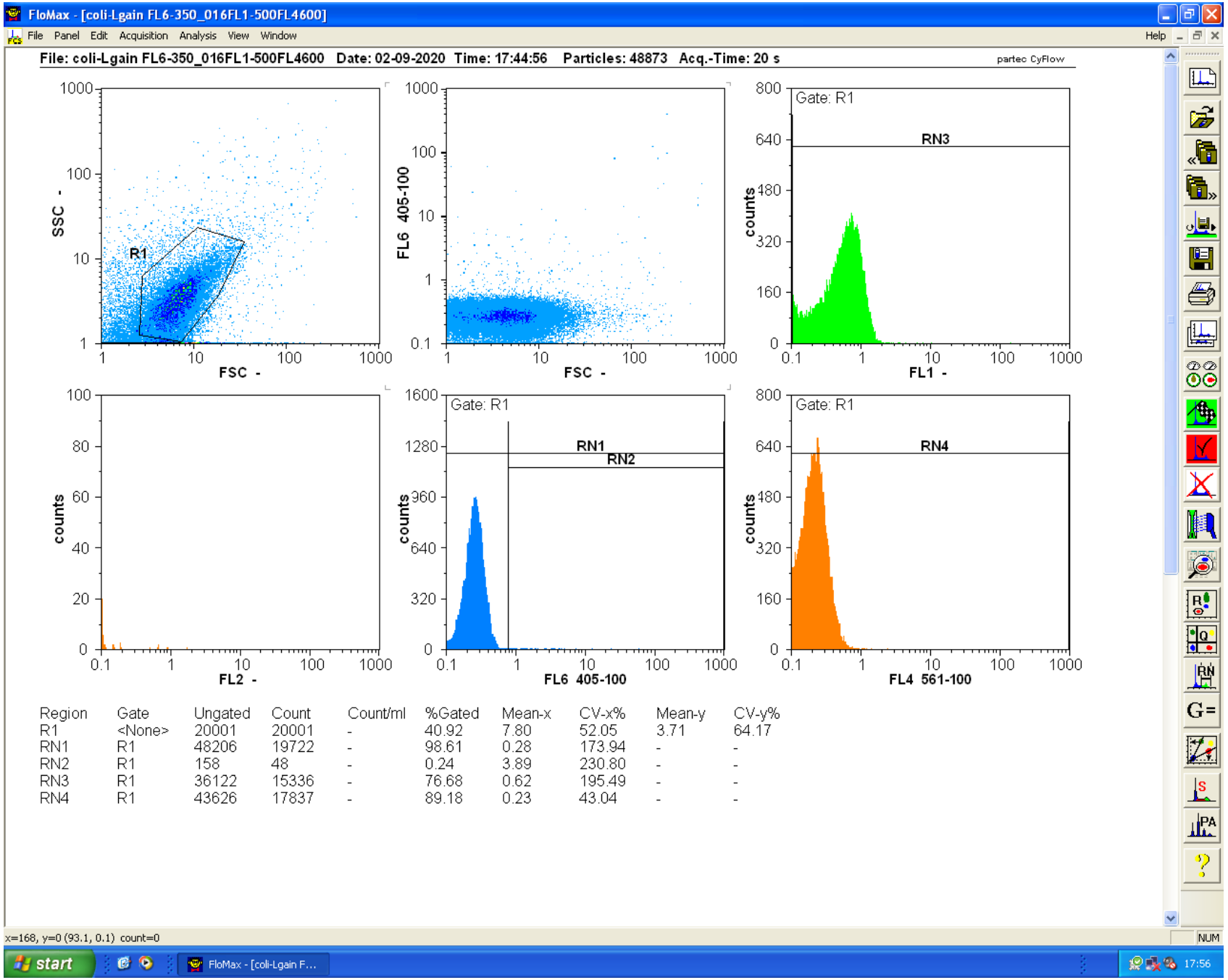
Positive control
YFP (pTH3)

Mean 50.54



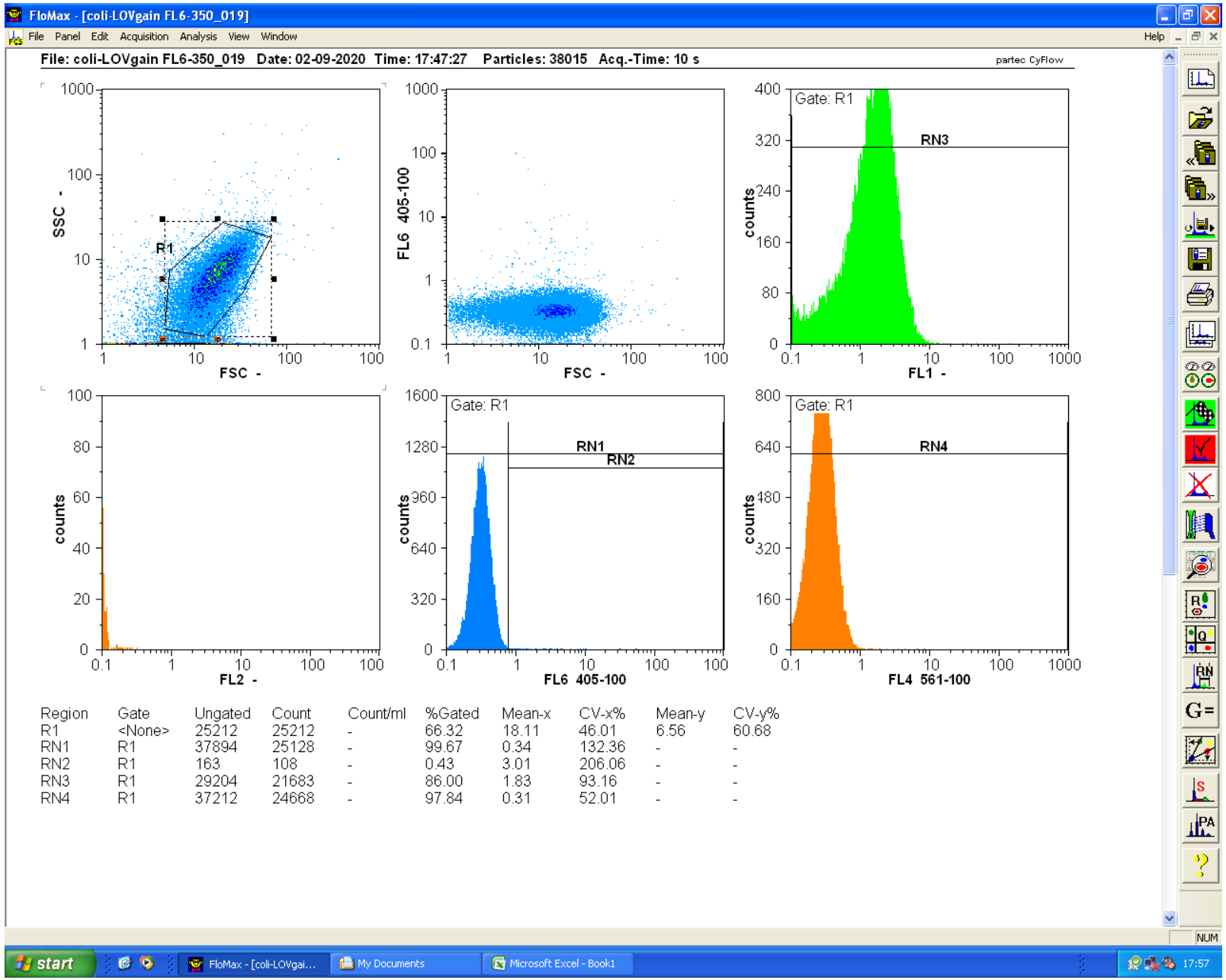
Remercier Elodie Hudik et Sophie Dupré de l'équipe d'Oliver Nüsse de l'Institut de Chimie Physique de l'Université Paris-Saclay

Témoin *E. coli* vecteur vide
+ arabinose



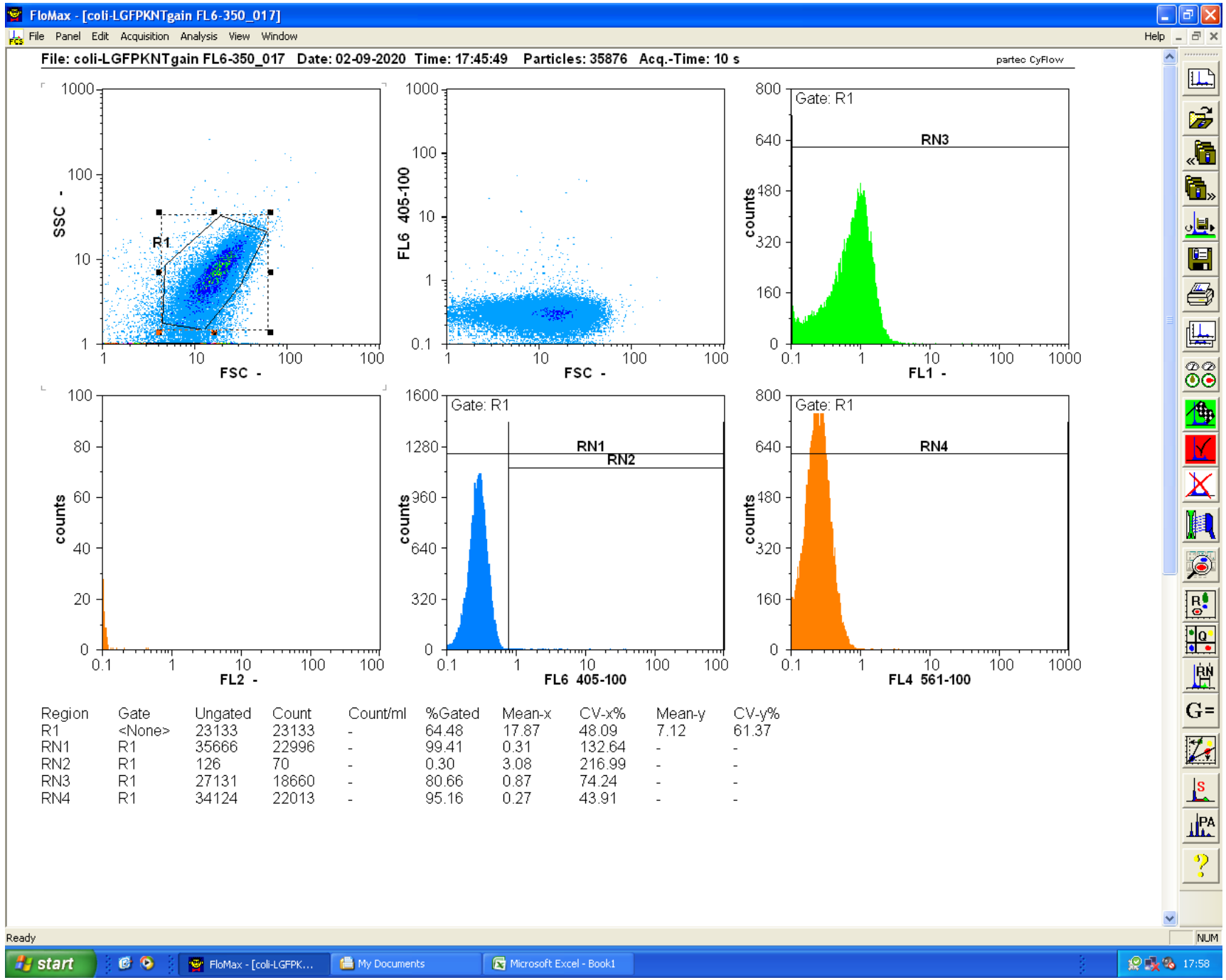
Region	Gate	Ungated	Count	Count/ml	%Gated	Mean-x	CV-x%	Mean-y	CV-y%
R1	<None>	20001	20001	-	40.92	7.80	52.05	3.71	64.17
RN1	R1	48206	19722	-	98.61	0.28	173.94	-	-
RN2	R1	158	48	-	0.24	3.89	230.80	-	-
RN3	R1	36122	15336	-	76.68	0.62	195.49	-	-
RN4	R1	43626	17837	-	89.18	0.23	43.04	-	-

Témoin *E. coli* YFPstable-LOV
+ arabinose



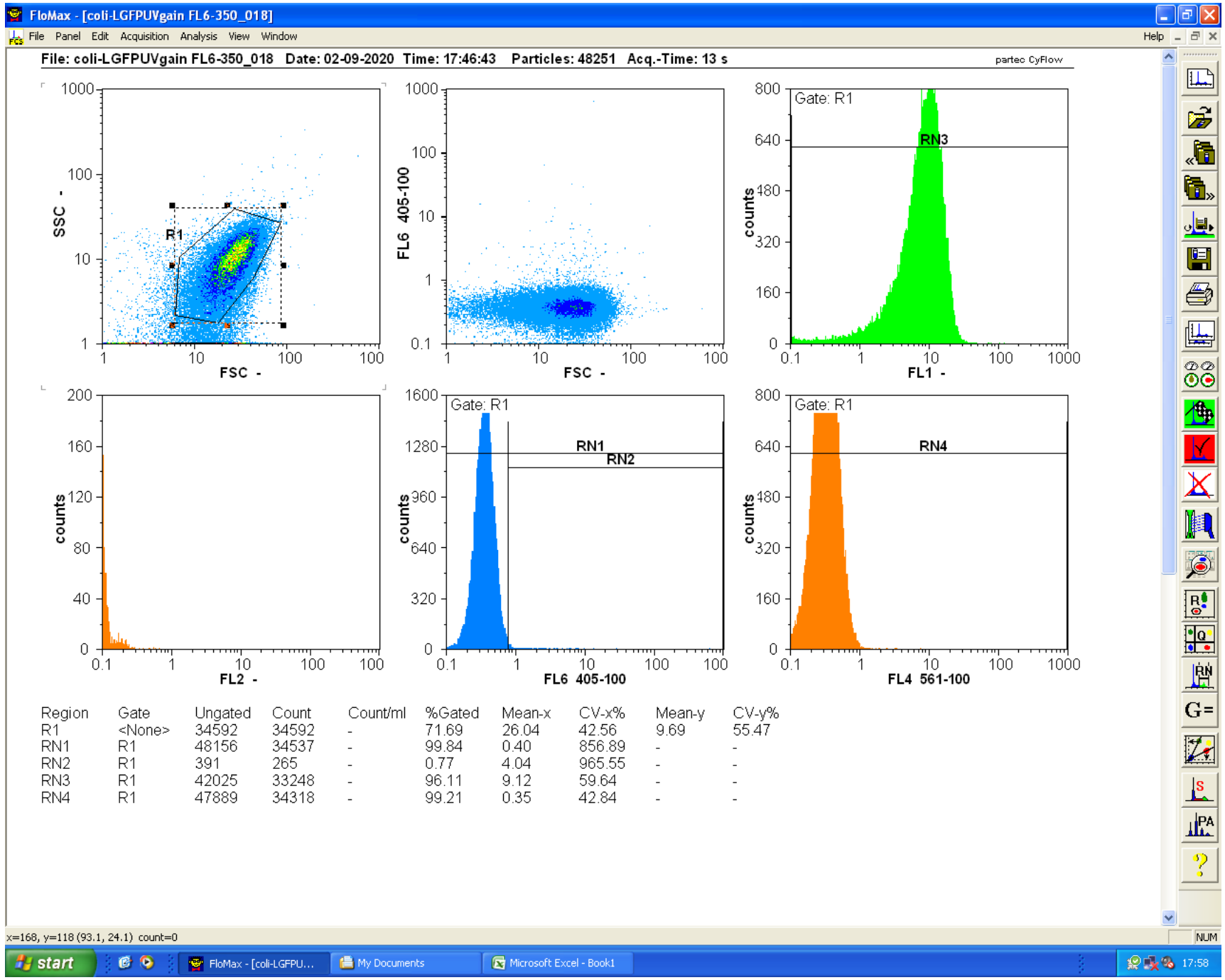
Region	Gate	Ungated	Count	Count/ml	%Gated	Mean-x	CV-x%	Mean-y	CV-y%
R1	<None>	25212	25212	-	66.32	18.11	46.01	6.56	60.68
RN1	R1	37894	25128	-	99.67	0.34	132.36	-	-
RN2	R1	163	108	-	0.43	3.01	206.06	-	-
RN3	R1	29204	21683	-	86.00	1.83	93.16	-	-
RN4	R1	37212	24668	-	97.84	0.31	52.01	-	-

Témoin *E. coli* knt-GFP
+ arabinose



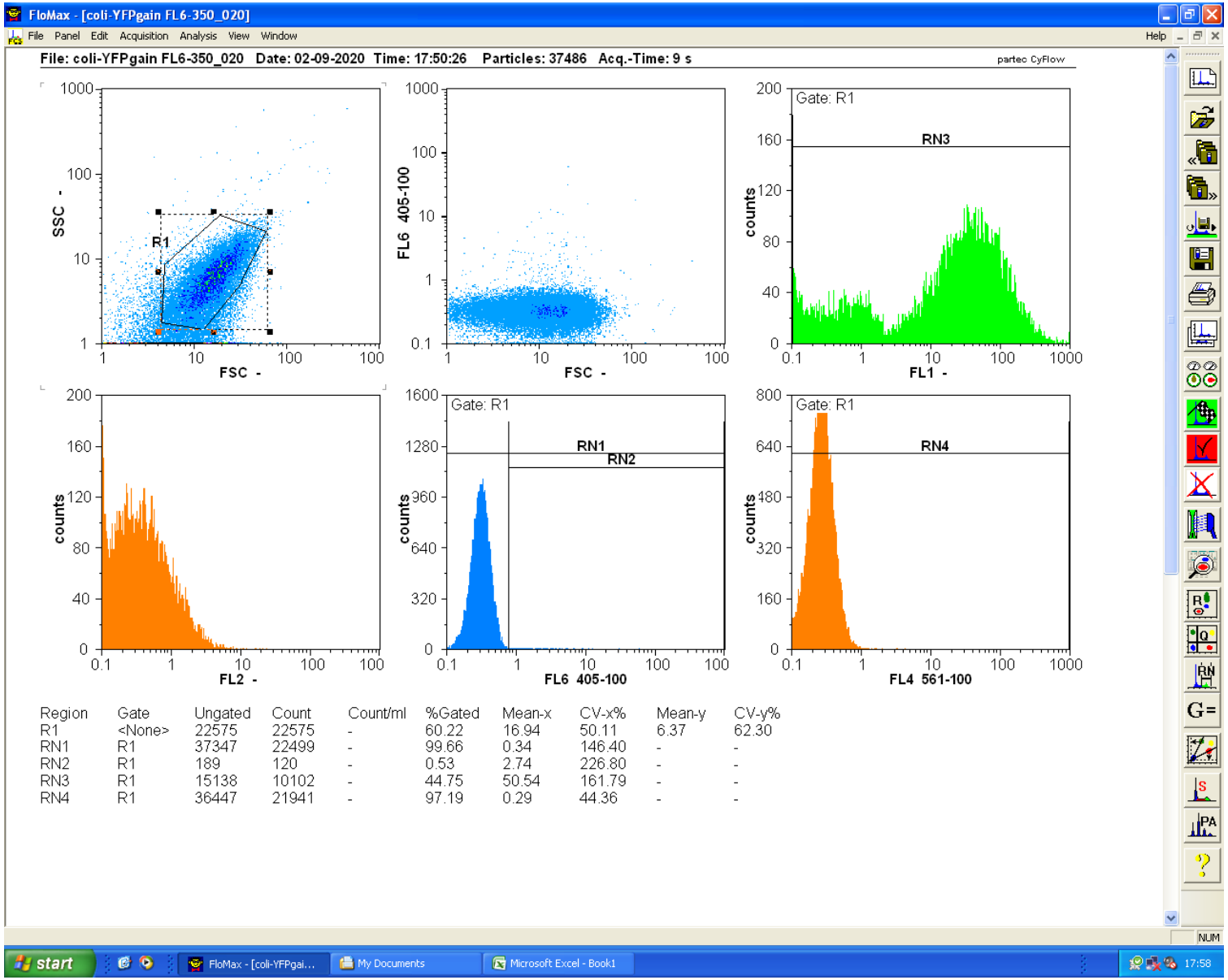
Region	Gate	Ungated	Count	Count/ml	%Gated	Mean-x	CV-x%	Mean-y	CV-y%
R1	<None>	23133	23133	-	64.48	17.87	48.09	7.12	61.37
RN1	R1	35666	22996	-	99.41	0.31	132.64	-	-
RN2	R1	126	70	-	0.30	3.08	216.99	-	-
RN3	R1	27131	18660	-	80.66	0.87	74.24	-	-
RN4	R1	34124	22013	-	95.16	0.27	43.91	-	-

Témoin *E. coli* GFP-uv
+ arabinose



Region	Gate	Ungated	Count	Count/ml	%Gated	Mean-x	CV-x%	Mean-y	CV-y%
R1	<None>	34592	34592	-	71.69	26.04	42.56	9.69	55.47
RN1	R1	48156	34537	-	99.84	0.40	856.89	-	-
RN2	R1	391	265	-	0.77	4.04	965.55	-	-
RN3	R1	42025	33248	-	96.11	9.12	59.64	-	-
RN4	R1	47889	34318	-	99.21	0.35	42.84	-	-

Témoin *E. coli* pTH3 (YFP)
+ arabinose



Region	Gate	Ungated	Count	Count/ml	%Gated	Mean-x	CV-x%	Mean-y	CV-y%
R1	<None>	22575	22575	-	60.22	16.94	50.11	6.37	62.30
RN1	R1	37347	22499	-	99.66	0.34	146.40	-	-
RN2	R1	189	120	-	0.53	2.74	226.80	-	-
RN3	R1	15138	10102	-	44.75	50.54	161.79	-	-
RN4	R1	36447	21941	-	97.19	0.29	44.36	-	-

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.

iGEM2020 Cytometry data

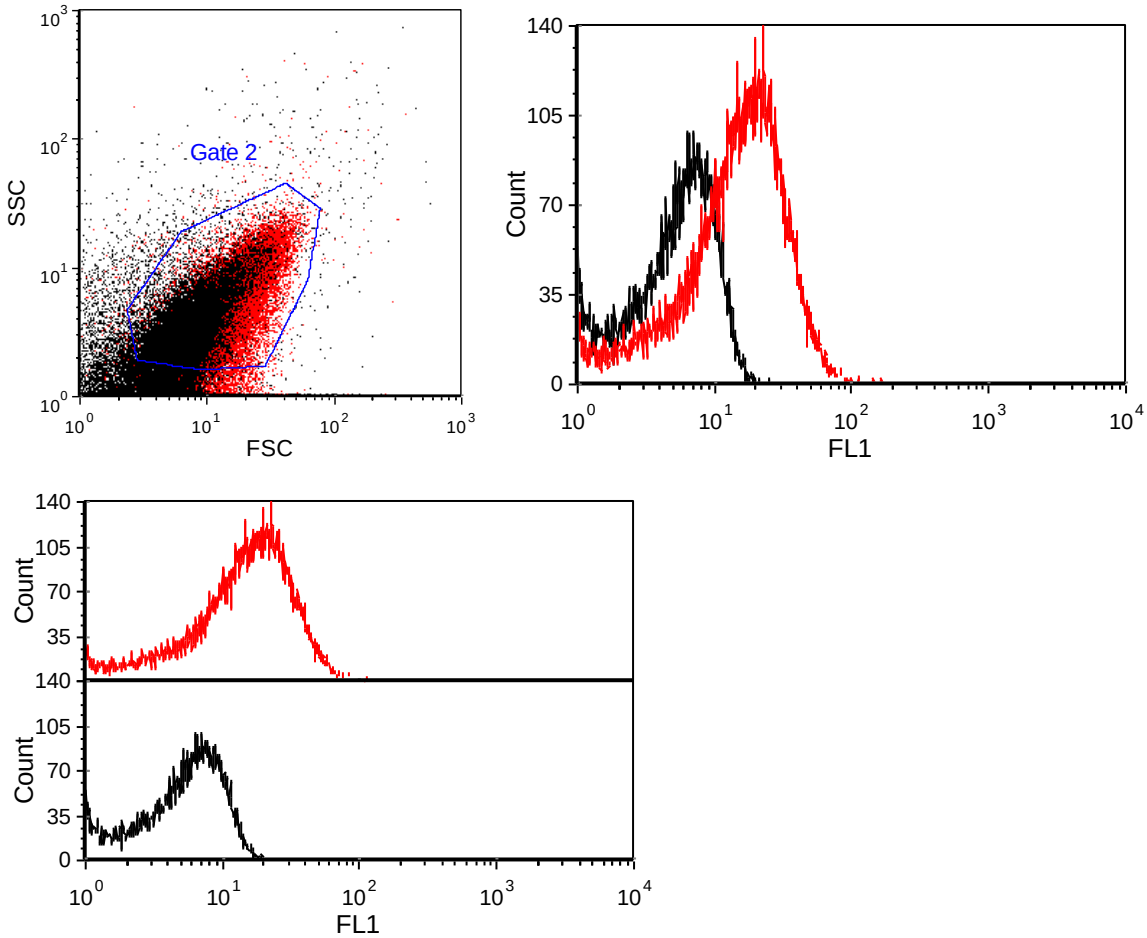
Fluorescence detection:

Laser blue (excitation): 488 nm (20 mW)

Emission: 536 nm (/40 nm)

First experiment (Partec CyFlow ML)

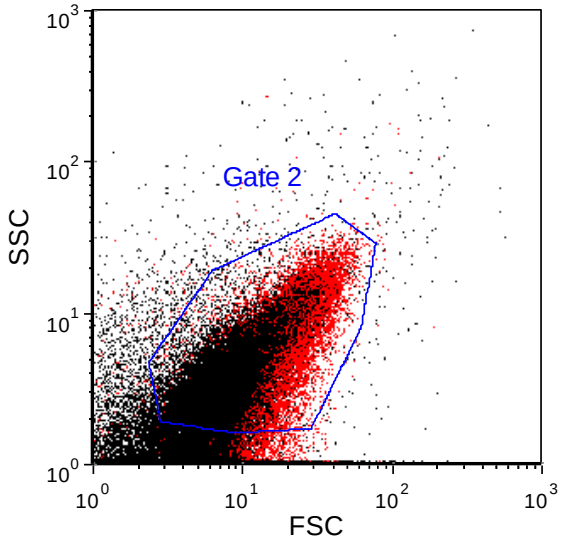
StableYFP(LOV)-versus negative control

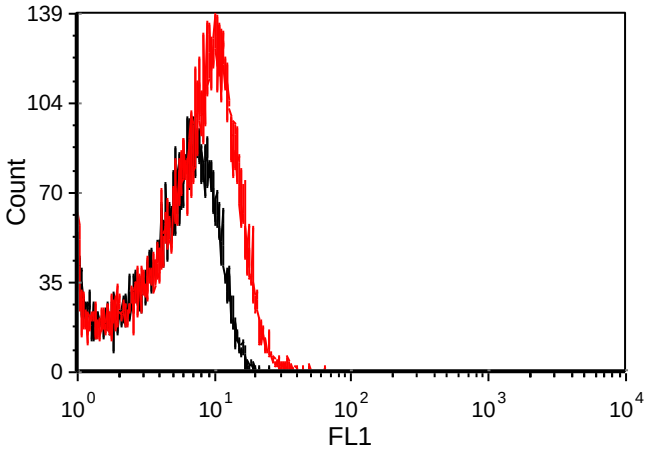


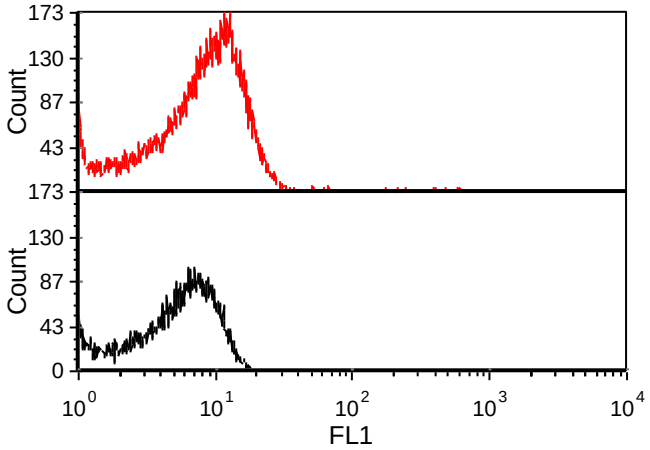
Green fluorescence (arbitrary units)

Filename	# of Events	Median	Geometric Mean
Negative control	856154	46	0
YFPstable(LOV)	99147865536	111	0

kntGFP-versus negative control



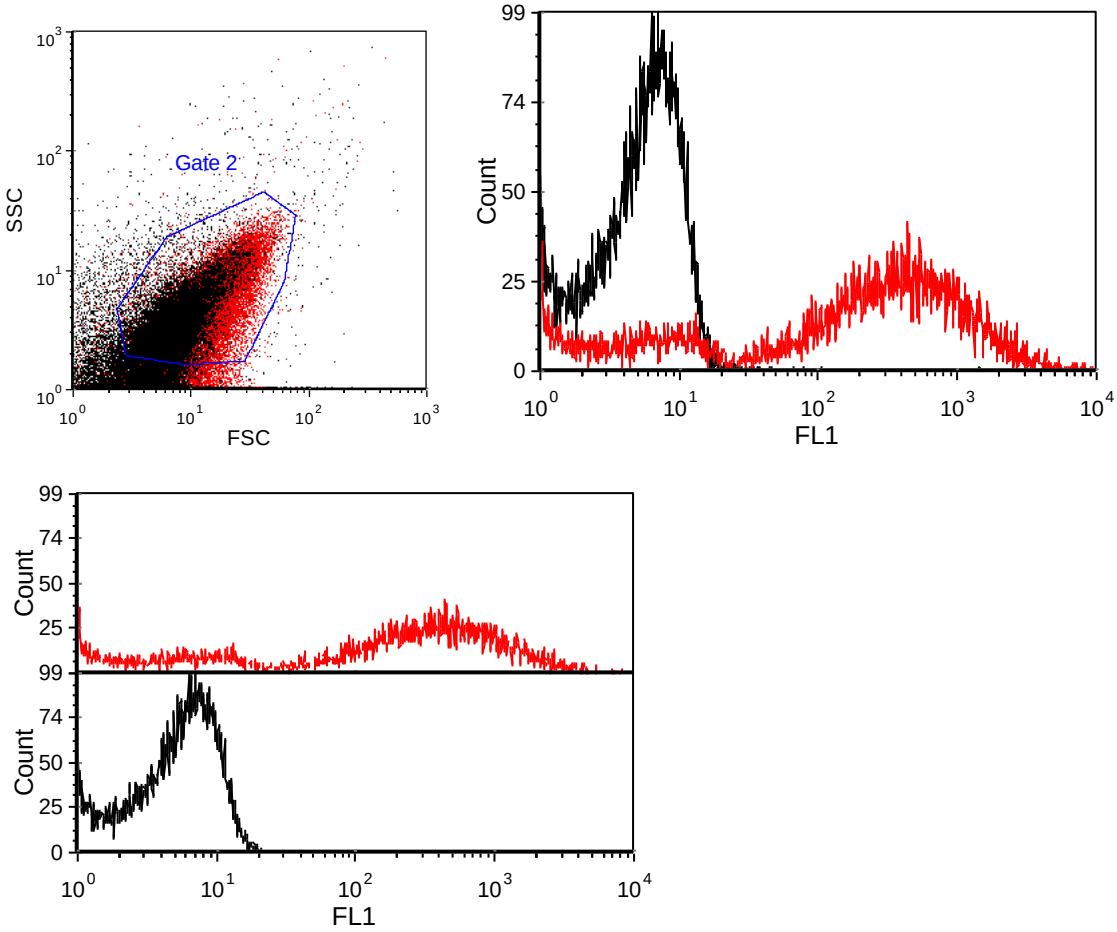




Green fluorescence (arbitrary units)

Filename	Median	Geometric Mean
Negative control	46	0
coli-LGFPKNTbisgain FL6-350_021.FCS	41	0

Positive YFP control condition-versus negative control

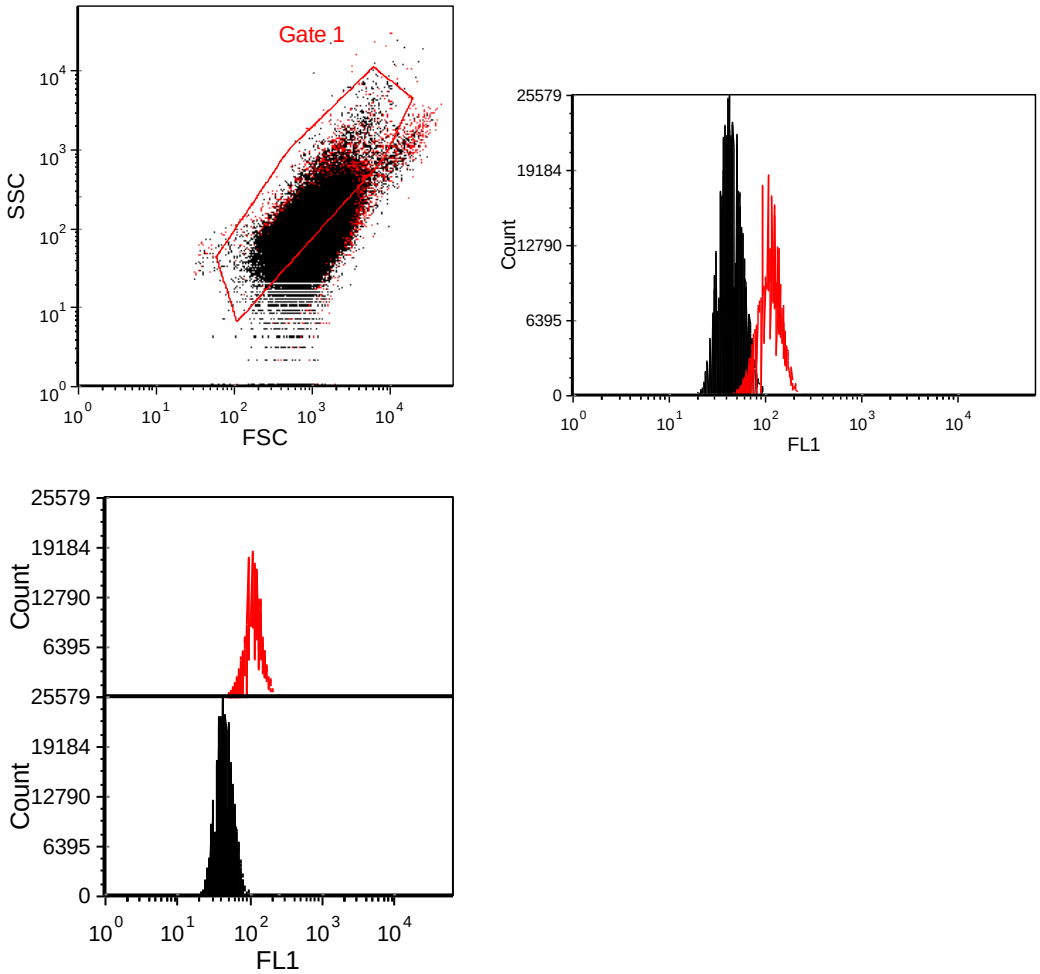


Green fluorescence (arbitrary units)

Filename	Median	Geometric Mean
Negative control	46	0
YFP positive control	611	4.94

Second experiment (performed with PARTEC CUBE1 cytometer)

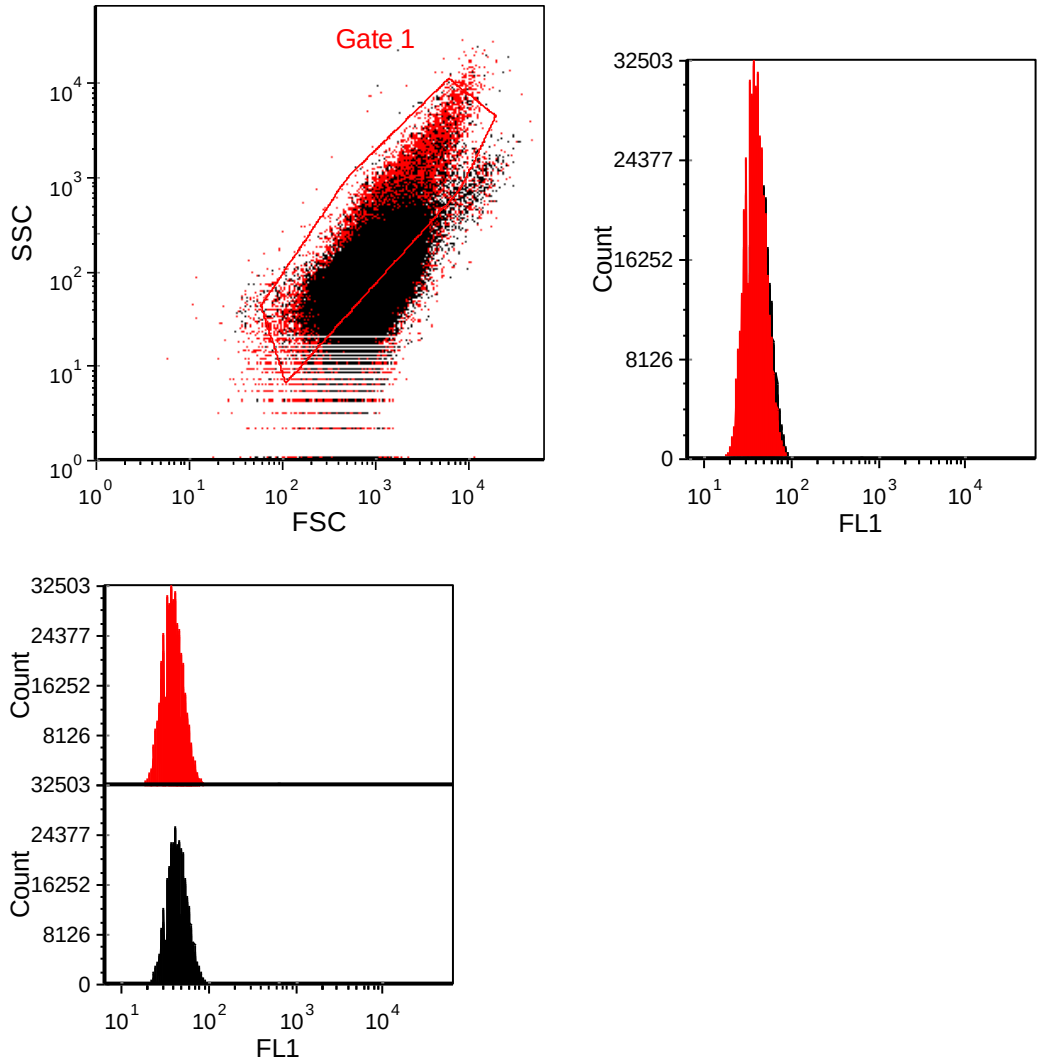
StableYFP(LOV)-versus negative control



Green fluorescence (arbitrary units)

Filename	Median	Geometric Mean
Negative control	4.49	3.55
StableYFP(LOV)	13.49	9.51

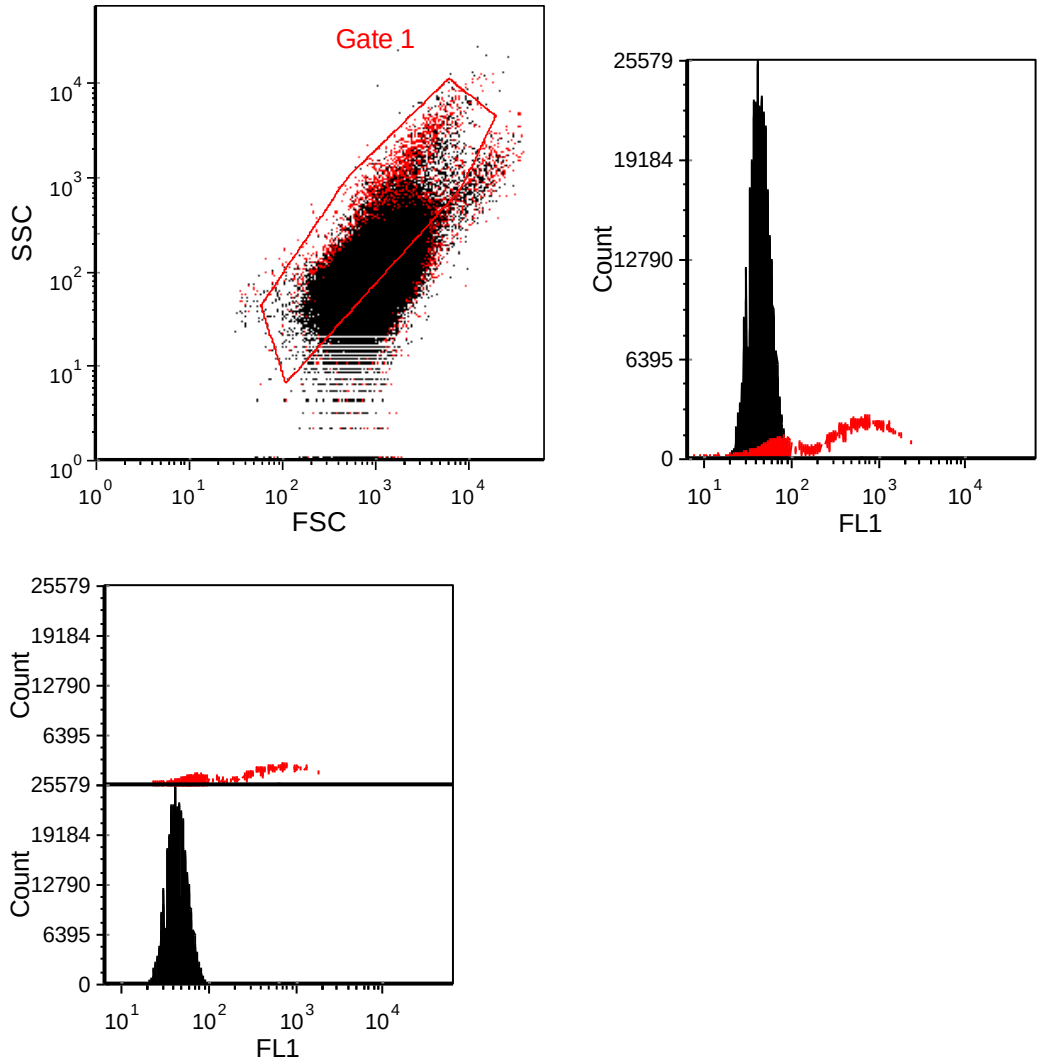
kntGFP-versus negative control



Green fluorescence (arbitrary units)

Filename	Median	Geometric Mean
Negative control	4.49	3.55
Knt-GFP	6.63	4.94

Positive YFP control condition-versus negative control



Green fluorescence (arbitrary units)

Filename	Median	Geometric Mean
Negative control	4.49	3.55
YFP positive control	101	8.70

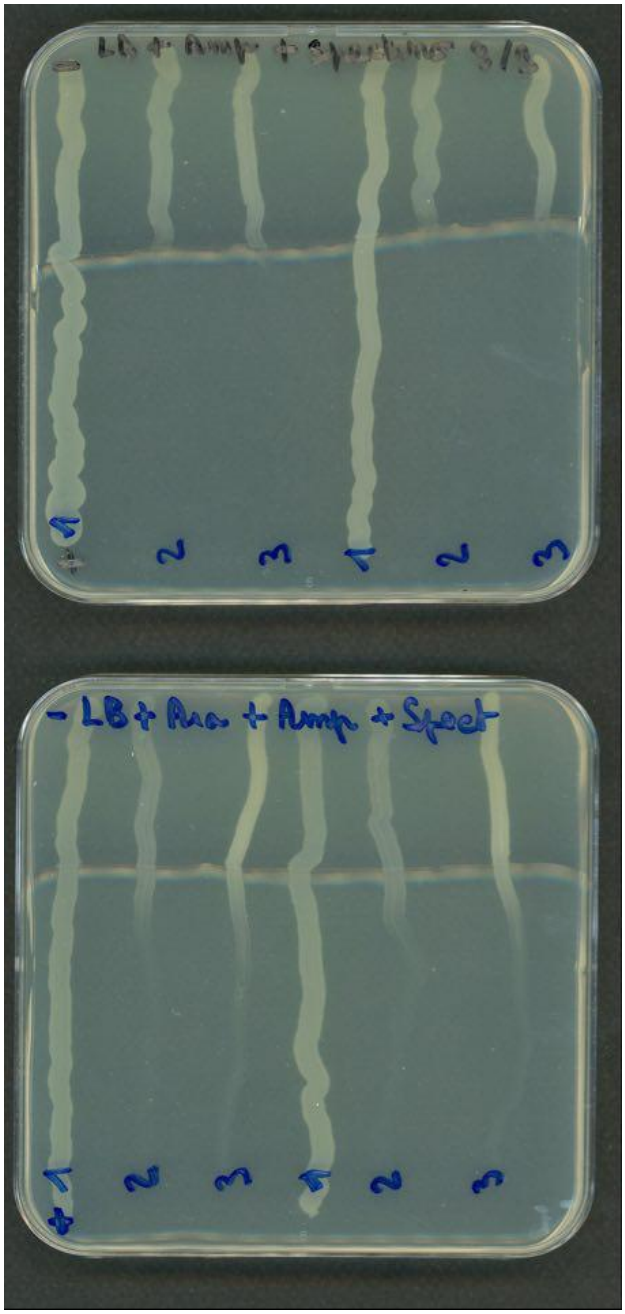
Culture of YFP and knt-GFP transformants on middles with different antibiotics concentration gradients

THURSDAY, 9/3/2020

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

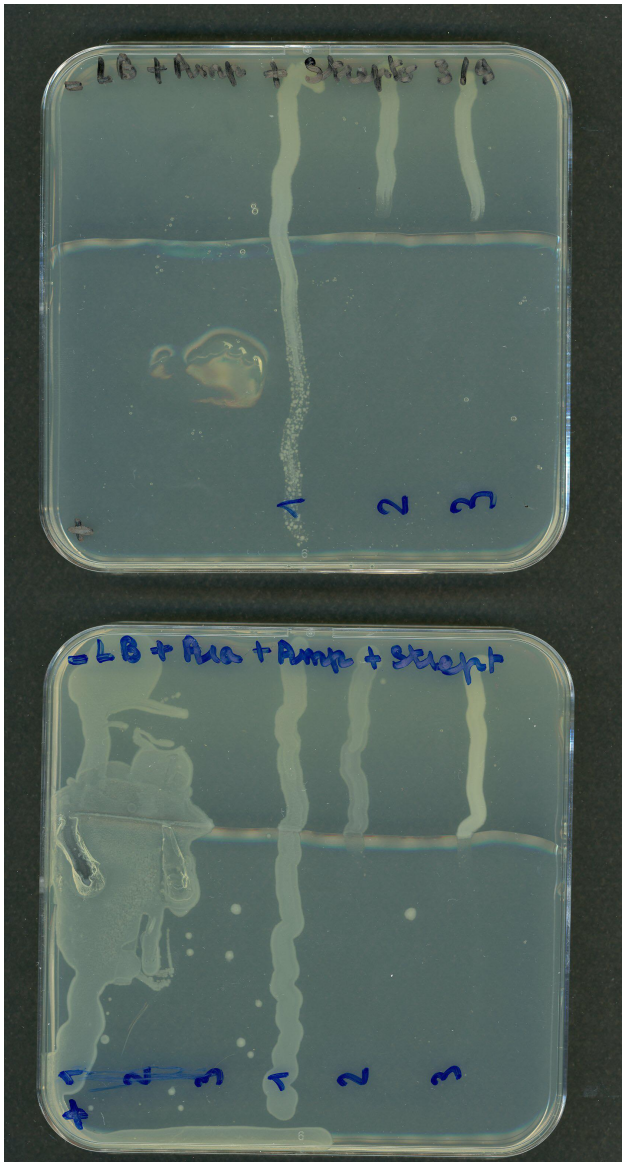
1 : positive control DH5alpha (plasmid with SpecR)
2 : DH5alpha (pBAD24-MoClo-knt-GFP)
3 : negative control DH5alpha (pBAD24-MoClo)

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 knt-GFP designed and 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 99,87% for forward and reverse primer.

For the sequencig with the forward primer it seems that there is a single mutation of type substitution at the nucleotide 63 of our gene where a C has been replaced by a A. However, the sequencing shows that at this position (753) sequencing's quality isn't optimal, so there is a possibilty there is in fact no mutation.

For the sequencig with the reverse primer it seems that there is a single mutation of type substitution at the nucleotide 708 of our gene where a G has been replaced by a T.

In conclusion because two diferent mutation have been detected with the two primers, we can suppose there is no mutation of our gene knt-GFP.

Screen captures :

Forward sequences :

Blast :

image.png

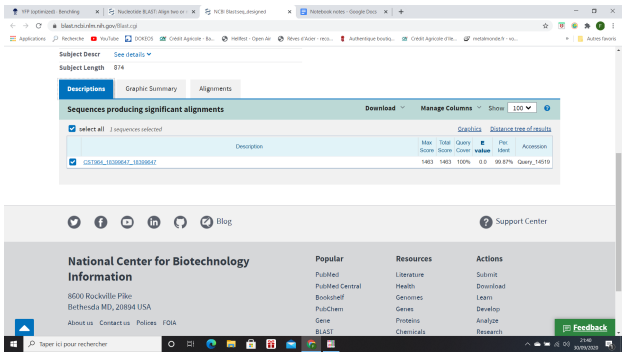
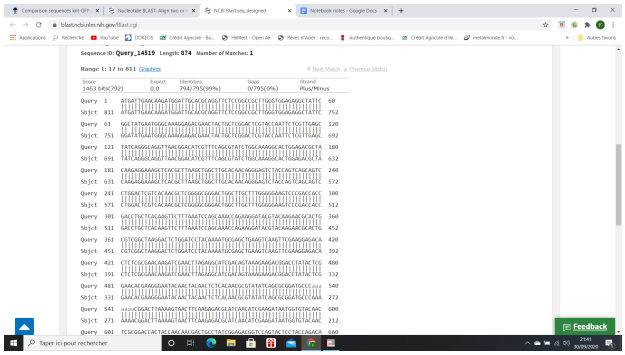


image.png



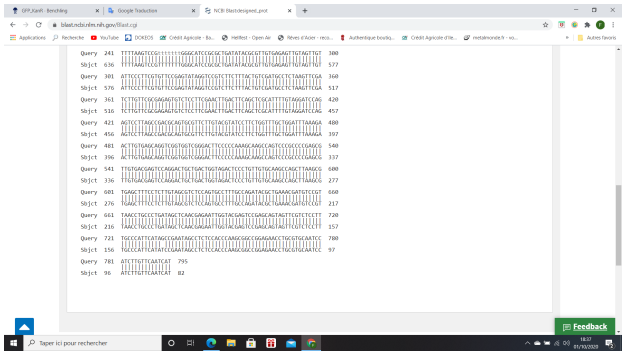
Sequencing :

image.png

<u>Reverse sequences :</u>

Blast :

image.png



Sequencing :

image.png


CST964_18399647_18399647.pdf
CST965_18399654_18399654.pdf
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Fixe en cours de synchronisation
1 sur 3
Lire à haute voix
Dessiner
Mettre en surbrillance
Effacer
Sequence: 18399654
Samples: 13888
Bases: 1167
Average spacing: 12.0
Average quality: 19.20, 20.00, 20.100
Quality: 0 - 9
10 - 19
20 - 29
>= 30
Page 1/3
25.03.2022
Taper ici pour rechercher
18:20
01/10/2022