1.-M9 and X-Gal plates

THURSDAY the 6th of September, 2018

M9 Agar plates:

Plates where made by autoclaving M9 and agar and adding mgso4 3 ml from 100mm solution, biotin, 1ml of a 100x thiamine solution, estimated from the concentration written on the first PA media protocol. Final volume of 300 ml in cloramphenicol resistance. Two sets of plates were made, one with galactose and one with glucose. Ecnr2 was plated to assay growth in this type of plates.

X-gal; IPTG; carbimicine agar plates

FRIDAY the 7th of September, 2018

Plates were made and stored at the 4ºc degrees common room (intermintently exposed to light)
-100mm IPTG stock
-20mg/ml X-gal stock

5ul/ml of each of the reagents above were used (plus normal concentration of carbamicine 1000X).

FRIDAY the 19th of September, 2018

Plates were made and stored at the 4ºc degrees common room (intermintently exposed to light)

-100mm IPTG stock
-20% X-gal stock from Prof. Juana Diaz's Virology lab

5ul/ml of each of the reagents above were used (plus normal concentration of Carb 1000X).
-We got 2ml X-gal from Prof. Juana Diaz, all used for the plates in a 500ml batch of plates. Possible X-gal degradation due to its old "age" as been told.

FRIDAY the 5th of October, 2018

Fresh X-Gal IPTG LB plates were made using fresh reagents from Promega Corporation.

-100mM IPTG stock solution
-1mL 50mM X-gal solution.

5uL/mL IPTG was used for 500mL of media to pour onto plates.

2.- Generation of bla mutant

TUESDAY the 14th of August, 2018

Generation of Bla mutant

Conclusion:

Both **ECNR2** and **EcM2.1**. were used in order to estimate efficiency and prepare colonies for coselection. In many screening attempts (aprox 5-6 platings of 100 colonies) we did not find any recombinant that had lost Ampicillin resistance. We decided to switch to X-gal (blue-white screening) to improve screening speed.

MAGE protocol (Gallagher R, Li Z, Lewis A, Isaacs F. Rapid editing and evolution of bacterial genomes using libraries of synthetic DNA. Nat. Protoc. 2014;9(10):2301-2316.) was followed. 2µM oligo bla_mut was used.

Two cycles were performed. In the first cycle wrong electroporation program might have been applied. After first cycle a sample was plated and grown at 30˚, to see efficiency differences between first and second cycle.

WEDNESDAY the 15th of August, 2018

One more cycle was performed (3 cycles in total). Colonies resulting from the 2nd cycle where grown on LB+chloramphenicol plates. Colonies from the 1st cycle where simultaneously screened on LB+carbamicine and cloramphenicol plates. Striking was not performed correctly on the first plate so single colonies where difficult to pick. Colonies resulting from the 3rd cycle where plated.

THURSDAY the 16th of August, 2018

No ampicillin negative colonies where found on the screening of the first cycle. Cycle two colonies where plated for screening.

FRIDAY the 17th of August, 2018

Screening of 70 colonies from second cycle and 30 colonies from third cycle did not yield any *bla* mutants. Since heat shock in MAGE cycling was not performed in a shaking bath, this will be improved in the next cycle.

SATURDAY the 18th of August, 2018

Another MAGE cycle was performed. This time improvised 42ºC shaking water bath was used. Colonies where plated after 2h of growth.

SUNDAY the 19th of August, 2018

Colonies from the 4th MAGE cycle where picked for screening.
No bla negative colonies where found.

SUNDAY the 31th of August, 2018

Start of the cycles with carbamicine OFF oligos.

Strains used:

-ECNR2 (clor + carb resistant)

-ENC2 (carb resistant)

-LB+Carb plates were poured in order to assess efficiency for both strains after growth on respective first selection (following) plates;

-ECN2: LB plates

-ENCR2: LB+Clor plates

SUNDAY the 9th of September, 2018

Several cycles where performed with Amp off oligo but no recombinant colonies were found on LB+Carb plates.

3.-Generation of X-Gal recombinants.

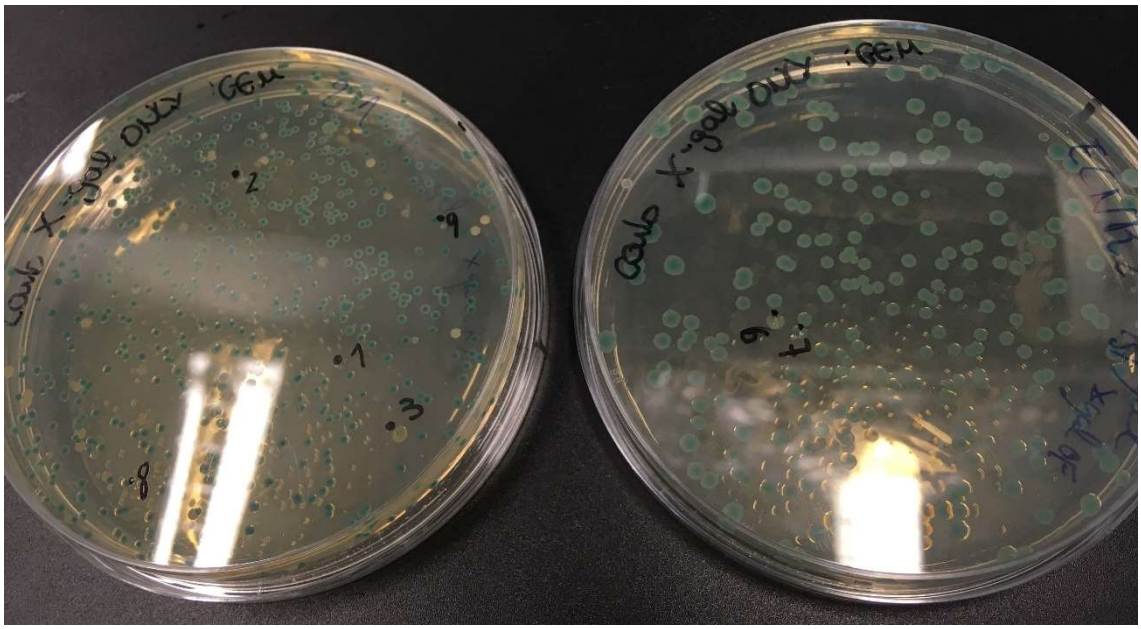
THURSDAY the 13th of September, 2018

Objective:

Estimate MAGE efficiency and set up controls for the MAGE experiment turning X-gal oligo off.

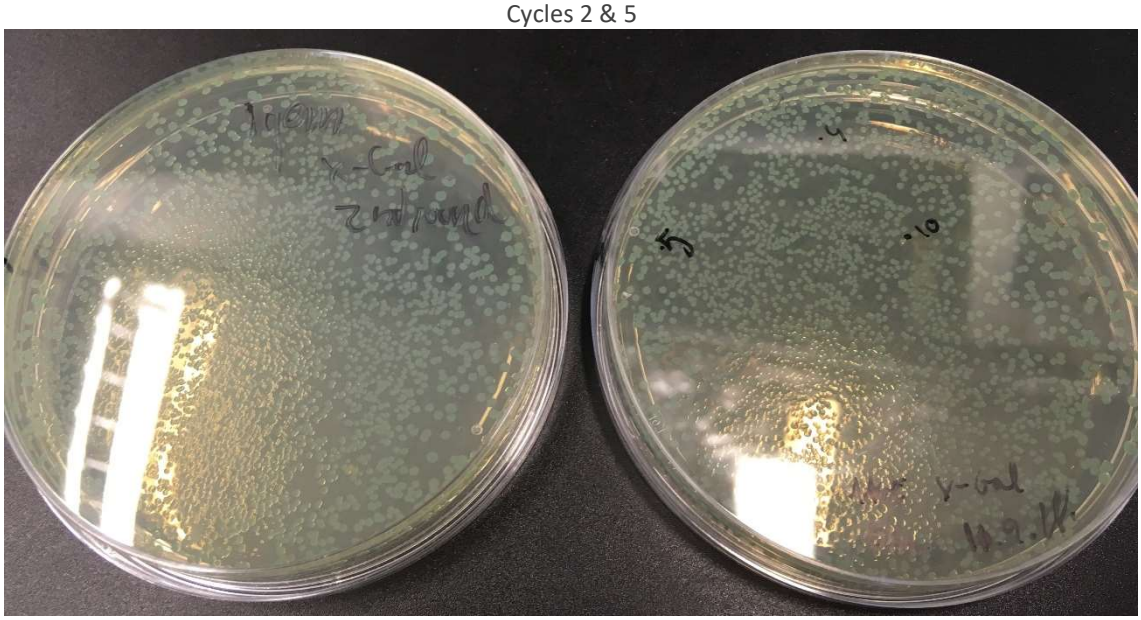
- After several MAGE cycles, colonies were plated:

Cycle 1



White colonies can be seen on the left plate, unfortunately, this colonies seem to be a contamination product.

Cycles 2 & 5



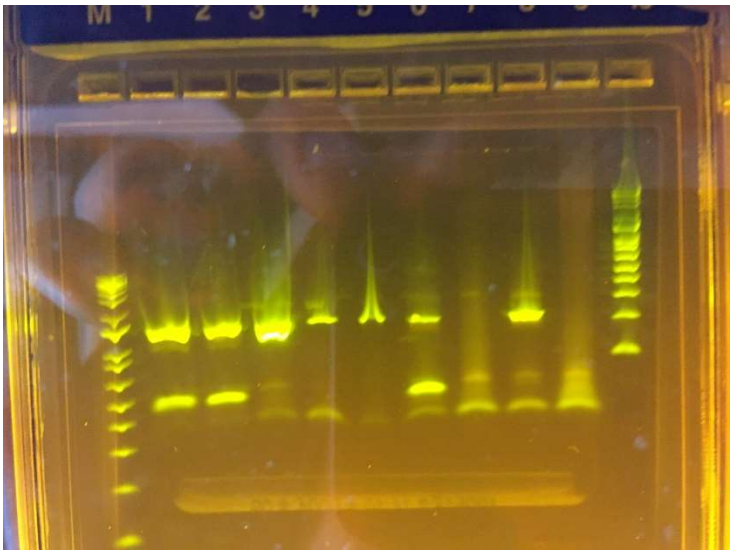
No white colonies where observed in cycled plates.

FRIDAY the 14th of September, 2018

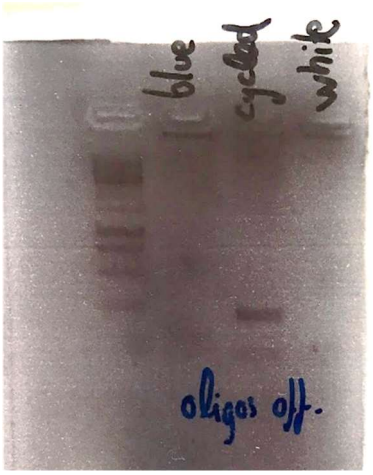
A cycle was performed in the 4ºC cold room as indicated in the protocol (Gallagher, 2014). Tcs where between 4.1 and 5.1, using both ECNR2 and ECM2.1 in Xgal and T7 combinations.
Shaking at 42 was performed manually in a 42ºC waterbath.

X-GAL PCR screening:

PCR with specific primers for X-Gal ON and X-Gal OFF was performed as screening in order to prove that recombination is taking place in cycled populations.
Several screening PCR reactions failed to retrieve results, we attribute this to the state of our NEB Taq polymerase, since other non-related PCRs also failed.

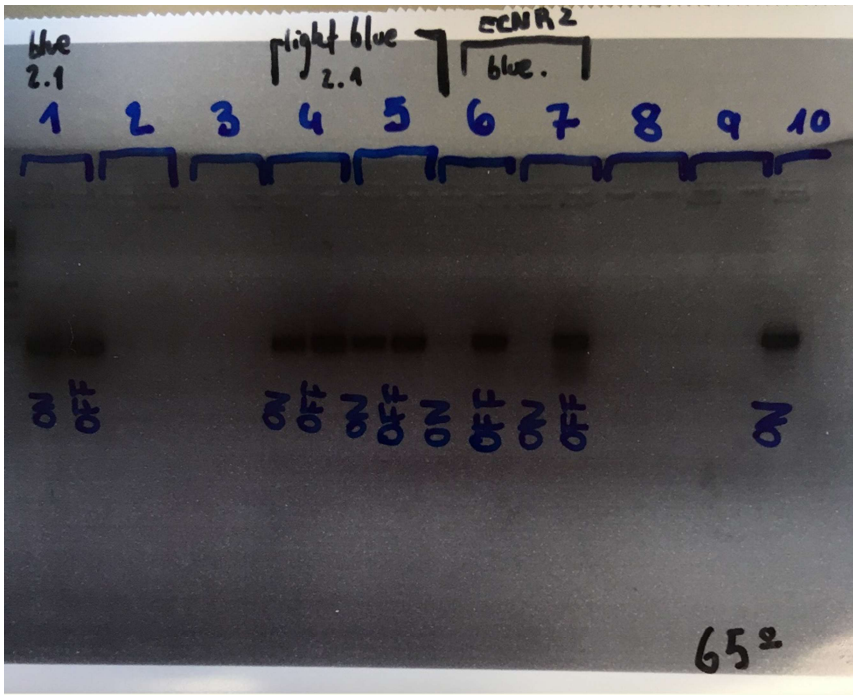


Kappa polymerase amplification (wells): WT (1), cycled1 (2), blue colony (3), white colony (4) white colony (4) (all ON) , WT (5), cycled1 (6), blue colony (7), white colony (OFF) (8).
For the X-gal ON screening, we can see that the first three lanes seem to contain more DNA than 4 & 5. This might be attributed to diminished amplification in mutants.



X-gal OFF colony PCR (326bp for *wt* allele)

SATURDAY the 15th of September, 2018



X-gal colony screening

1: blue colony; 2,3,8,9: white colonies from X-gal OFF plate. 4&5: ECM2.1 blue. 6&7. ECNR2 blue

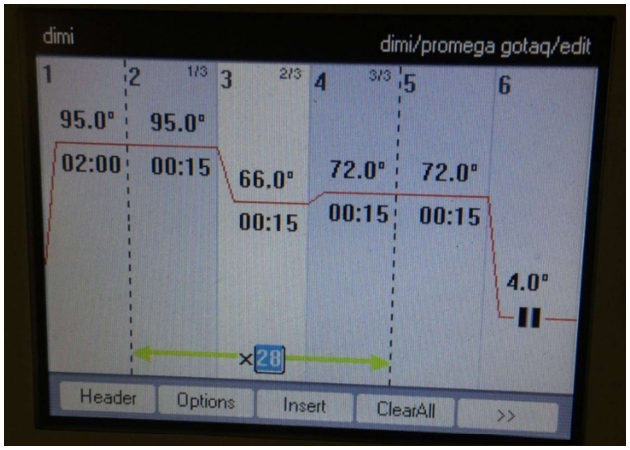
This gel suggests that X-gal OFF primer is not specific, and also that ECNR2 can't be amplified with X-gal ON primer. The only difference between two previous gels is extension time (from 15 to 30 seconds). All previous PCRs where used with promega GotaqHOTSTART

PCR optimization:

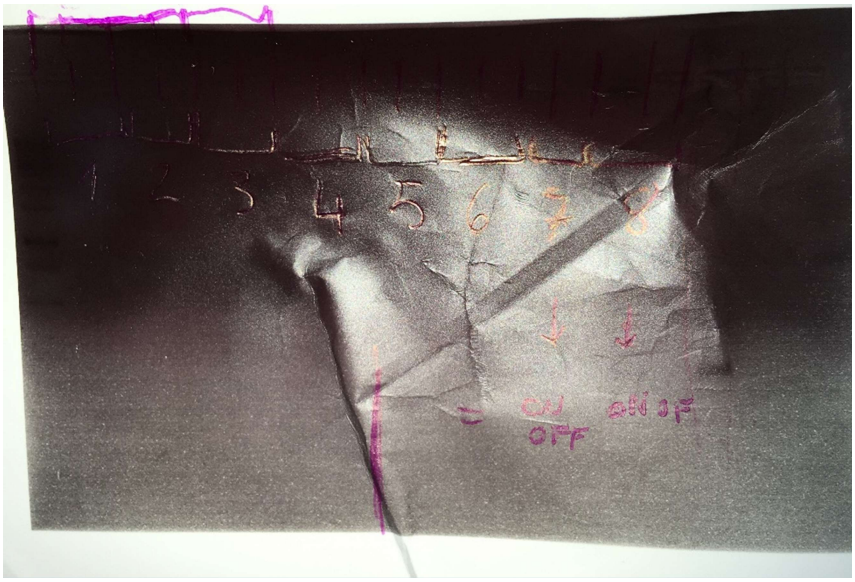
In order to determine primer specificity WT ECM2.1 and WT ECNR2 will be amplified with at 15 and 30 s.

SUNDAY the 16th of September, 2018

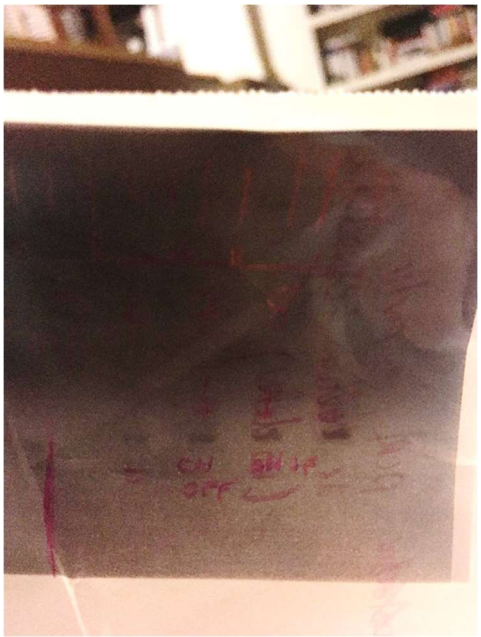
Since the PCR from the 15th of September provided unexpected amplification for blue (suspected ON) colonies, another reaction was performed using the following thermocycling conditions:



Which led to the following bands



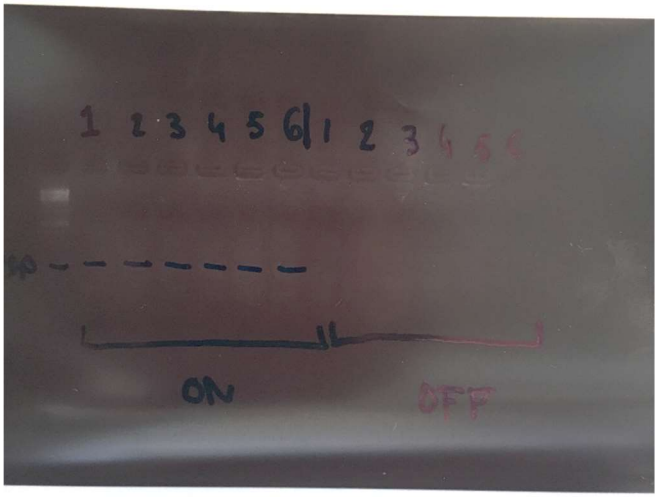
This photo should display 8 different template bacteria. 1,2,34 are replicates of a 1:100 dilution (10µL in 990µL water) and 1µL of template (ON and OFF) colonies 5, 6, 7 and 8 are from plates. Under these conditions, OFF oligo seems to gain specificity for screening, although we don't understand why colony 5 is not amplified.



Picture showing bands from colonies 6,7,8 (on can be seen, not OFF) (last band is not from colony 6,7,8).

MONDAY the 17th of September, 2018

Another Gel was set up under same conditions using non diluted culture as a template:



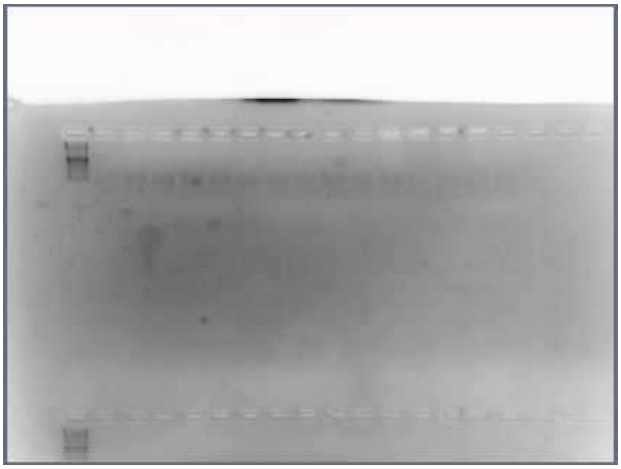
On amplicons work while OFF don't amplify. For all strains. Reactions from samples submitted to cycling don't show any band.

MONDAY the 17th of September, 2018

PCR screening

For each WT, old cycled and new cycled strains, eigh tubes: And optimize for temperature and extension temp 15 and 25 sec. (1:10 dilution) 30 cycles

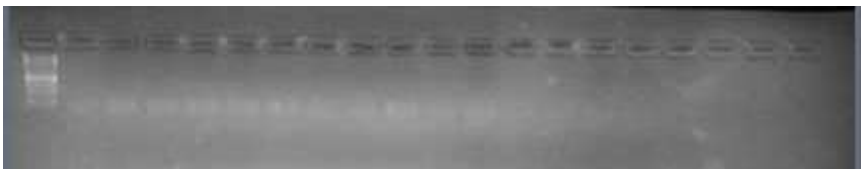
25µL (x25 reactions, 12,5 of 50µL) of X-gal OFF primer



ECM2 X-gal OFF cycled once. 15 sec extension (1-8)t gradient, and 30 sec extension (9-17)

THURSDAY the 20th of September, 2018

MAGE X-gal OFF1



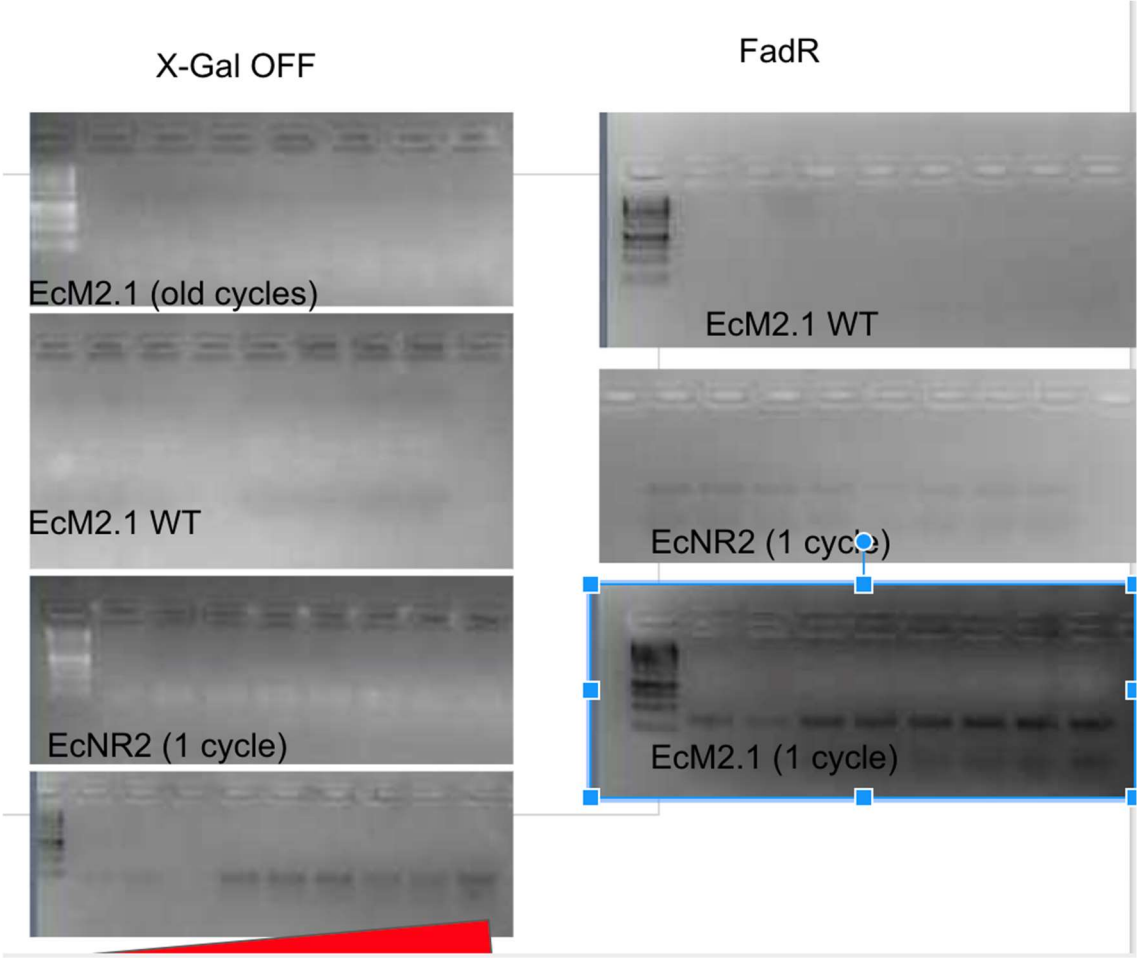
1-8 Xgal OFF ECM2.1 cycled once. T° gradient

MAGE X-gal OFF2

1-9 T gradient of ECNR2 cycled one Xgall OFF primers. (9-17)

MAGE X-gal F3

old ECNR2 cells cicles

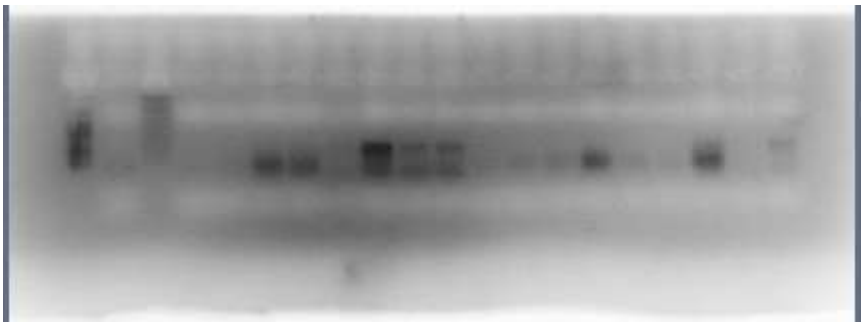


It seems that fadR recombination also works!

WEDNESDAY the 3rd of October, 2018

PCR for X-gal OFF screening.

Reagent	2mM MgCl2		
H20	12.5	275	
Buffer	4	88	
MgCl2	2.4	52.8	
dNTP	0.4	8.8	
pol	0.1	2.2	
Primer Mix	0.4	8.8	217.8
template DNA (1:20 culture dil)	1	11	
total	20.8	446.6	



1st, 2nd, 3rd & 4th cycle cells were plated onto X-Gal+IPTG+Carb LB Plates in order to assess eficiency of recombination.

FRIDAY the 12th of October, 2018

Recombination screening method was changed for a more efficient one (see WIKI page).

4.- Introduction of T7 prom& FADR KO.

SATURDAY the 15th of September, 2018

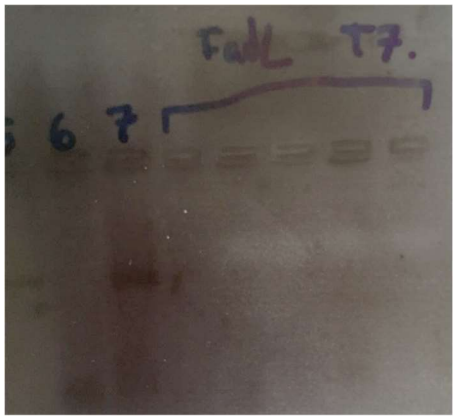
Cycling:

Up to three cycles where performed with a mixed oligo pool including all T7 promoters and KO of fadR.

WT screening PCR:

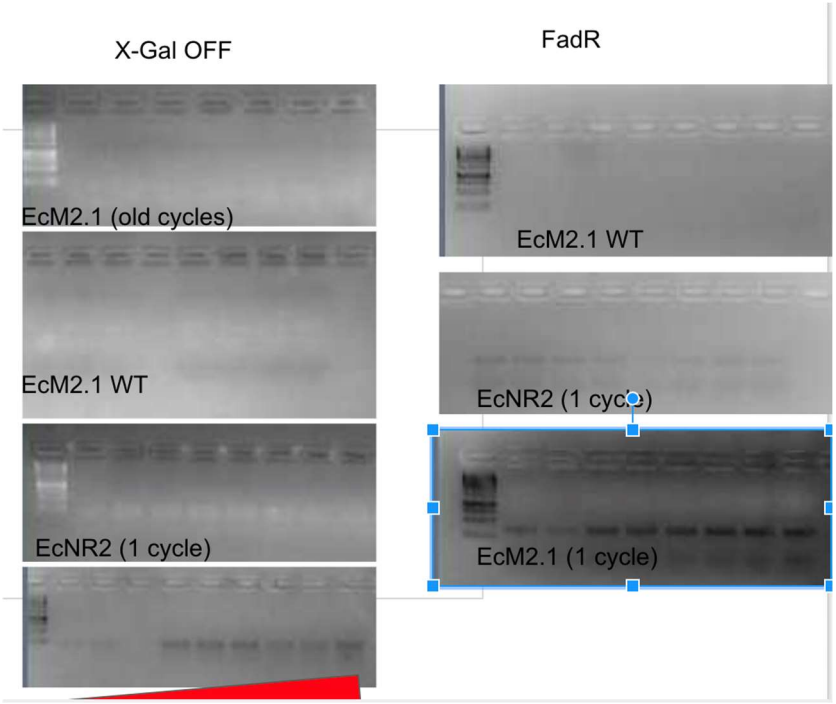
First attempt of multiplex PCR failed, resulting in NO bands. SIngle amplifications where performed with each region:

T7 *fadR* KO screening PCR:



FadL T7 screening PCRS at 4 different temperatures. No results.

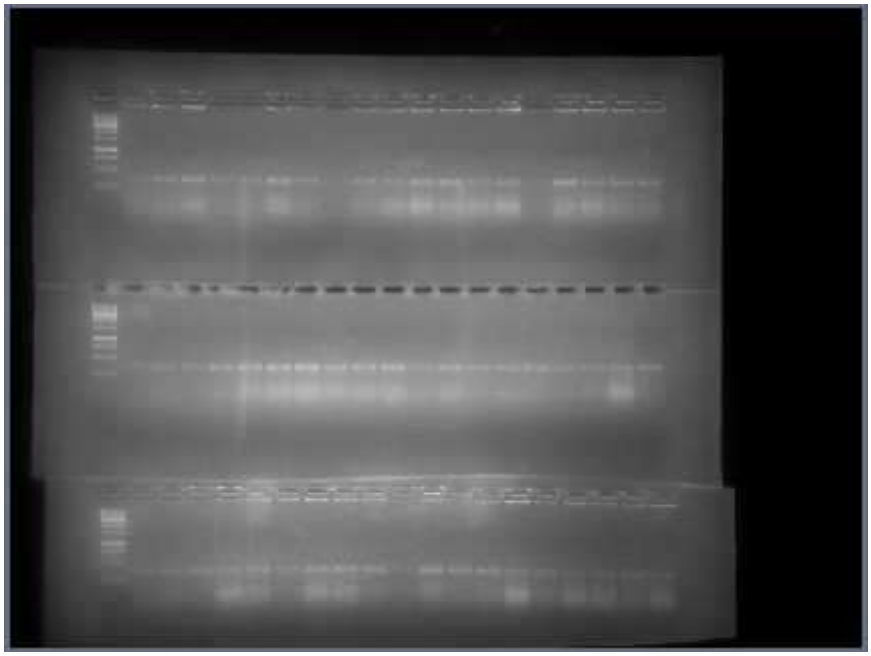
THURSDAY the 20th of September, 2018

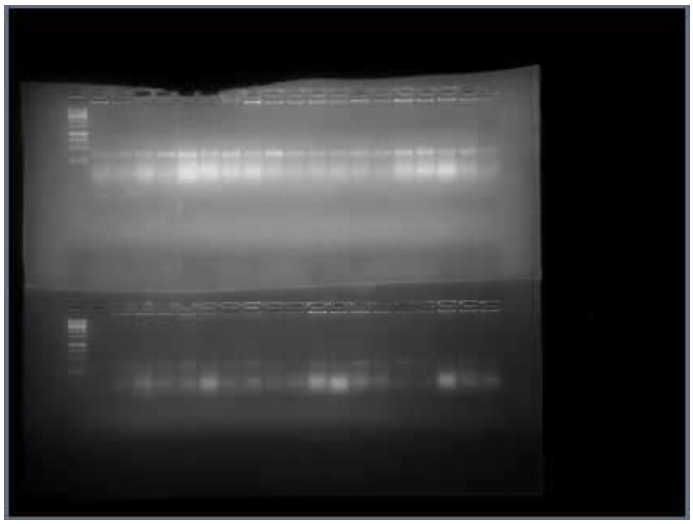


FadR possible recombinants observed after one cycle, plated afterwards

SATURDAY the 22nd of September, 2018

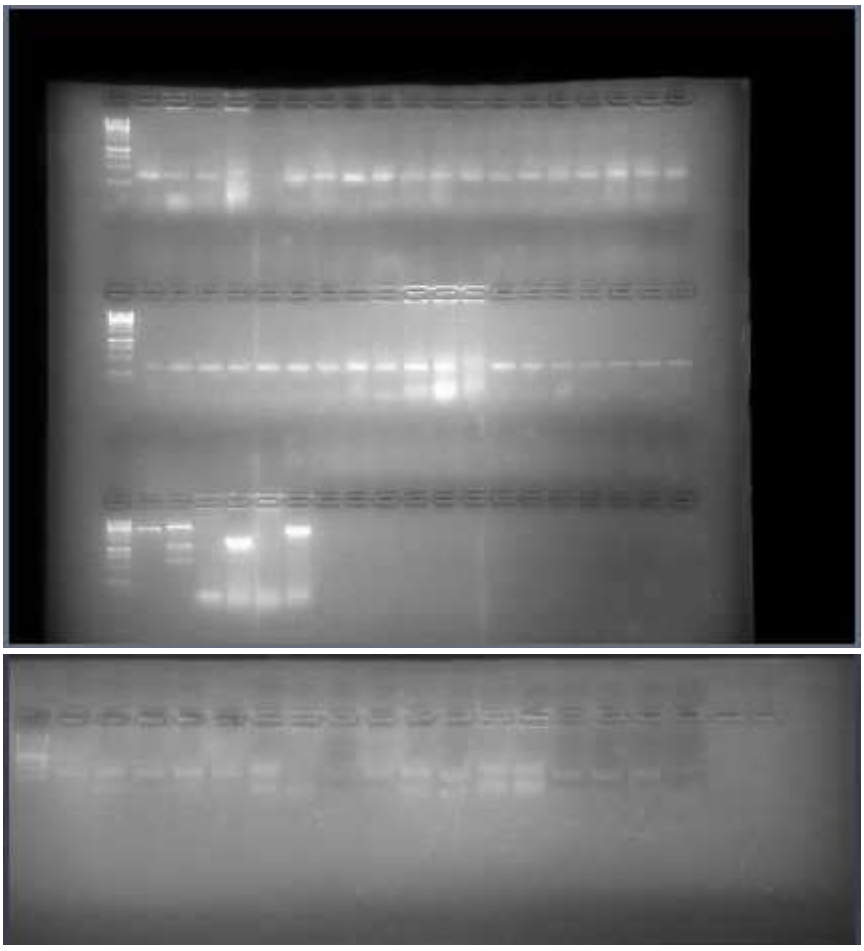
Performed parallel colony PCR and restreak for the plated colonies to screen for FadR KO recombinants with fadR KO & WT primers.





FadR KO screening PCRs

Multiple bands could be observed, possibly as a result of streaking multiple single colonies into each reaction.



WEDNESDAY the 26TH of September, 2018

PCR fadR KO primer was used for this screening PCR.

REAGENT	1mM MgCl2	2mM MgCl2	16 reactions		
H20	13.3	12.5	425.6	400	
Buffer	4	4	128	128	
MgCl2	0.8	1.6	25.6	51.2	

dNTP	0.4	0.4	12.8	12.8	
pol	0.1	0.1	3.2	3.2	297.6
Primer Mix	0.4	0.4	12.8	12.8	6.4
template DNA (1:20 culture dil)	1	1	32	32	152
total	20	20	640	640	

Conditions		
95	5'	
95	30"	x 25 cycles
65-72	30"	
72	30"	
72	5'	

Gel was empty!!!!

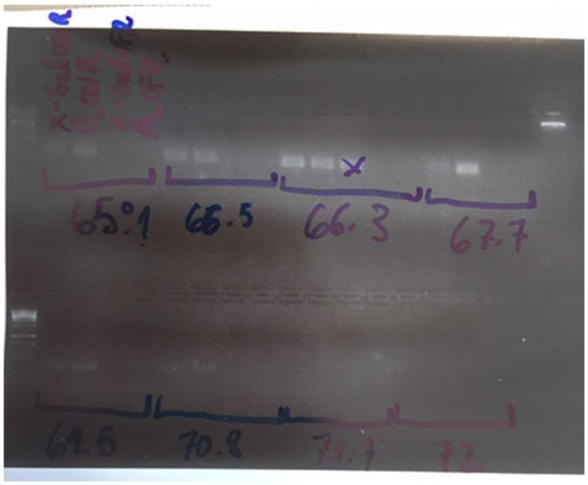
FRIDAY the 28th of September, 2018

We set up a new DNA polymerase from Promega (GoTaq G2 Hot Start). Performed again with also fadR ON colonies as a control.

Reagent	1mM MgCl2	2mM MgCl2	16 reactions		
H20	13.3	12.5	425.6	400	
Buffer	4	4	128	128	
MgCl2	0.8	1.6	25.6	51.2	
dNTP	0.4	0.4	12.8	12.8	
pol	0.1	0.1	3.2	3.2	148.8
Primer Mix	0.4	0.4	12.8	12.8	6.4
template DNA (1:20 culture dil)	1	1	32	32	77.6
total	20	20	640	640	

PCR was performed for primers targetting Xgal ON/OFF strains and fadR WT/KO (due to an error).

Temp	Time	
95	5'	
95	30"	x 25 cycles
65-72	30"	
72	30"	
72	5'	

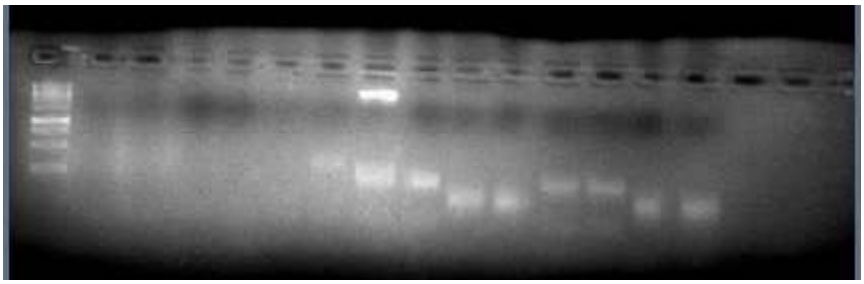


SATURDAY the 29th of September, 2018

PCR will be performed using 45 second periods and 6mM mgcl2

REAGENT	2mM MgCl2		
H20	12.5	106.25	
Buffer	4	34	
MgCl2	2.4	20.4	
dNTP	0.4	3.4	
pol	0.1	0.85	
Primer Mix	0.4	3.4	84.15
template DNA (1:20 culture dil)	1	8.5	
total	20.8	176.8	

We performed 25, 27, 28 and 29 cycles. Received no results



SUNDAY the 30th of September, 2018

In order to keep optimizing PCR conditions, the following will be performed:

PCR with kappa:

both X-gal ON and x gal OFF and fadR ON and fadR OFF primers will be used from twice cycled tubes in order to test.

PCR with promega:

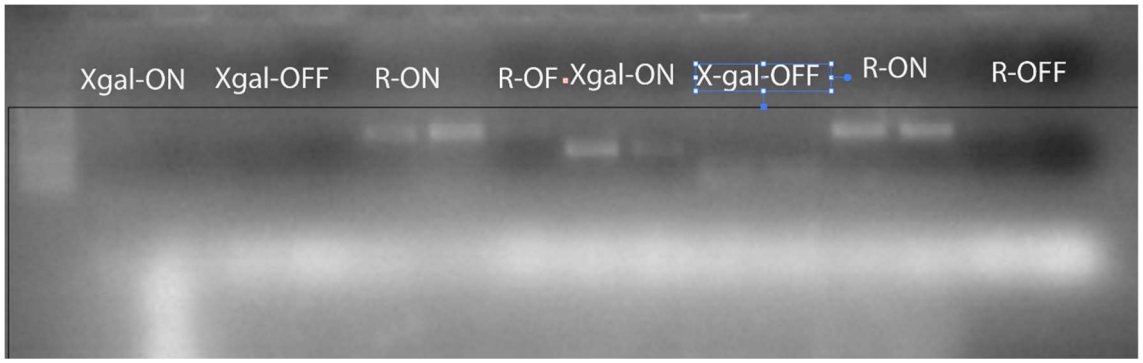
Reagent	2mM MgCl2		
H20	12.5	212.5	
Buffer	4	68	

MgCl2	2.4	40.8	
dNTP	0.4	6.8	
pol	0.1	1.7	
Primer Mix	0.4	6.8	168.3
template DNA (1:20 culture dil)	1	8.5	
total	20.8	345.1	

Pcr with gotaq and 30 cycles will be performed pippeting diluted culture in each case to see how it affects. OFF, WT and R.

TUESDAY the 2nd of October, 2018

No amplicons resulted with PCR kappa amplification.
PCR kappa amplification, 1:10 diluted culture of Xgal and R cycled for each case.



THURSDAY the 4th of October, 2018

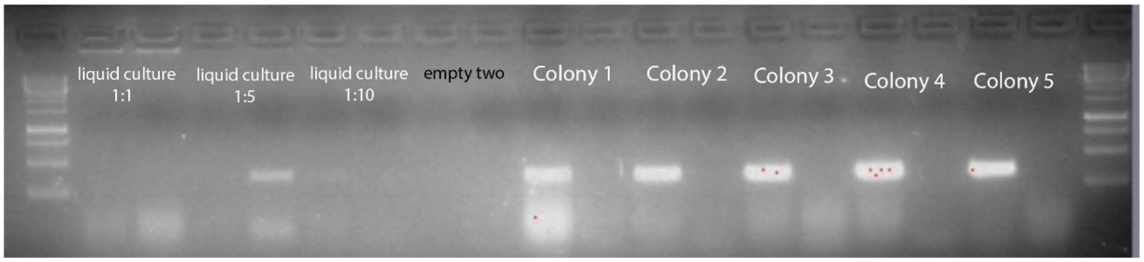
fadR ON, fadR OFF:

1:1 culture, 1:5 culture, 1:10 culture, six colonies.

Reagents	2mM MgCl2		
H20	12.5	275	
Buffer	4	88	
MgCl2	1.6	35.2	
dNTP	0.4	8.8	
pol	0.1	2.2	204.6
Primer Mix	0.4	8.8	
template DNA (1:20 culture dil)	1	22	
total	20	440	

Temp	time	
95	5'	
95	30"	x 25 cycles
65-72	30"	
72	30"	
72	5'	

Gel.
Each condition corresponds to ON, OFF:



It seems that liquid culture is not amplifiable any more.

THURSDAY the 7th of October, 2018

PCR with liquid cultures from new cycles will be amplified.

Reagent	2mM MgCl2		
H20	12.5	56.25	
Buffer	4	18	
MgCl2	1.6	7.2	
dNTP	0.4	1.8	
pol	0.1	0.45	41.85
Primer Mix	0.4	1.8	
template DNA (1:20 culture dil)	1	4.5	
total	20	90	

Temp	time	
95	5'	
95	30"	x 25 cycles
65-72	30"	
72	30"	
72	5'	

Screening colony PCR (1)			
Reagent	2mM MgCl2		
H20	12.5	1250	
Buffer	4	400	
MgCl2	1.6	160	
dNTP	0.4	40	
pol	0.1	10	930
Primer Mix	0.4	40	
template DNA (1:20 culture dil)	1	100	
total	20	2000	

WEDNESDAY the 10th of October, 2018

PCR screening for FadR KOs was performed on 20 single colonies in order to search for FadR KO 4th cycle recombinants. Two conditions were used, KO recombinant primers and WT screening primers. 5uL MilliQ were used to spread the colony on each PCR reaction tube, deducted from the mix.

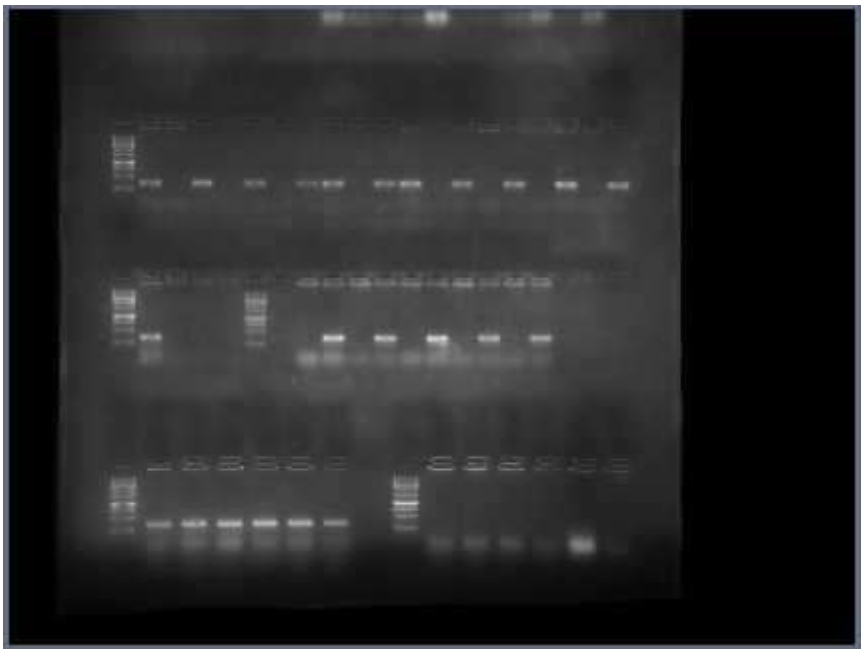
Screening colony PCR (1)		
Reagent	2mM MgCl2	
H20	1.75	38.5
Buffer	2	44
MgCl2	0.8	17.6
dNTP	0.2	4.4
pol	0.05	1.1
Primer Mix	0.2	4.4
total	10	220

THURSDAY the 11th of October, 2018

REAGENT	2mM MgCl2		
H20	3.25	325	
Buffer	2	200	
MgCl2	0.8	80	
dNTP	0.2	20	
pol	0.05	5	315
Primer Mix	0.2	20	
template DNA (1:20 culture dil)	0	0	
total	6.5	650	

FRIDAY the 12th of October, 2018

FadR KO screening



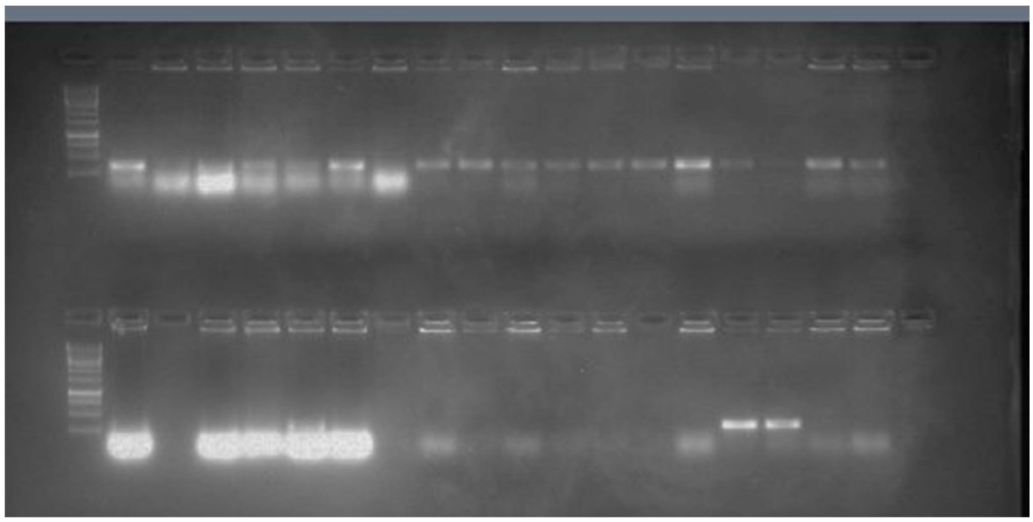
Reagent	2mM MgCl2		
H20	2.75	275	
Buffer	2	200	
MgCl2	0.8	80	
dNTP	0.2	20	
pol	0.05	5	290
Primer Mix	0.2	10	
template DNA (1:20 culture dil)	0	0	
total	6	600	

SATURDAY the 13th of October, 2018

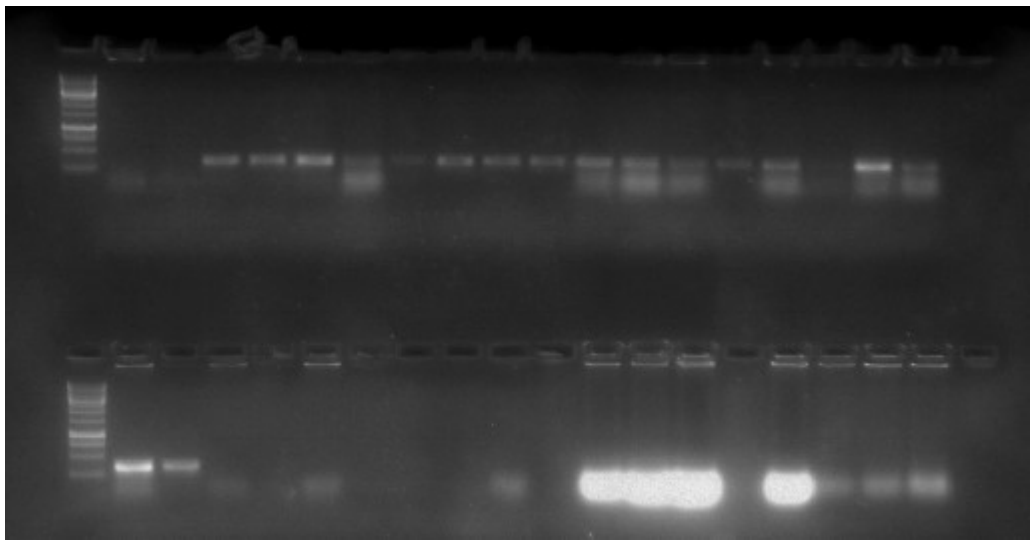
A

It seems we have achieved fadR OFF allele amplification

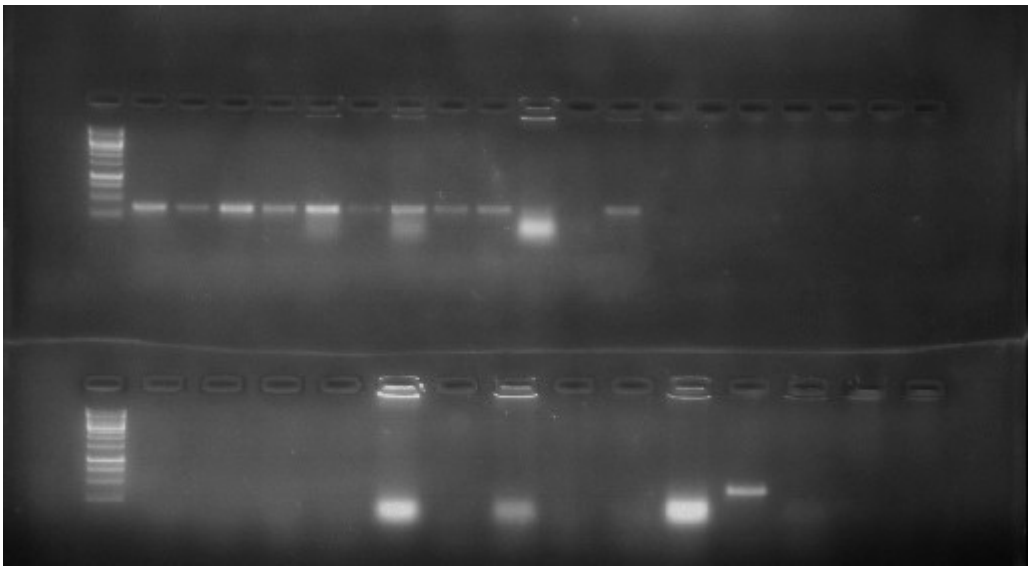
Screening from **biosensor** transformed colonies yielded FadR KO recombinants (see more in the WIKI page).



The first row shows colony PCR using WT plasmids as negative controls to screen for recombinant strains. The second row of the gel has been generated using KO primers for the PCR reaction. As it can be seen, two colonies yielded positive for FadR KO.



As it can be seen, two colonies tested positive for FadR KO.



One more colony yielded positive results for FadR KO.

SUNDAY the 14th of October, 2018

Colony PCR to screen for T7 promoter insertion upstream of fad genes from liquid cycled (6th cycle) culture yielded no amplicons.

	2mM MgCl2	
H20	6.75	13.5
Buffer	2	4
MgCl2	0.8	1.6
dNTP	0.2	0.4
pol	0.05	0.1
Primer Mix	0.2	0.4
total	10	20

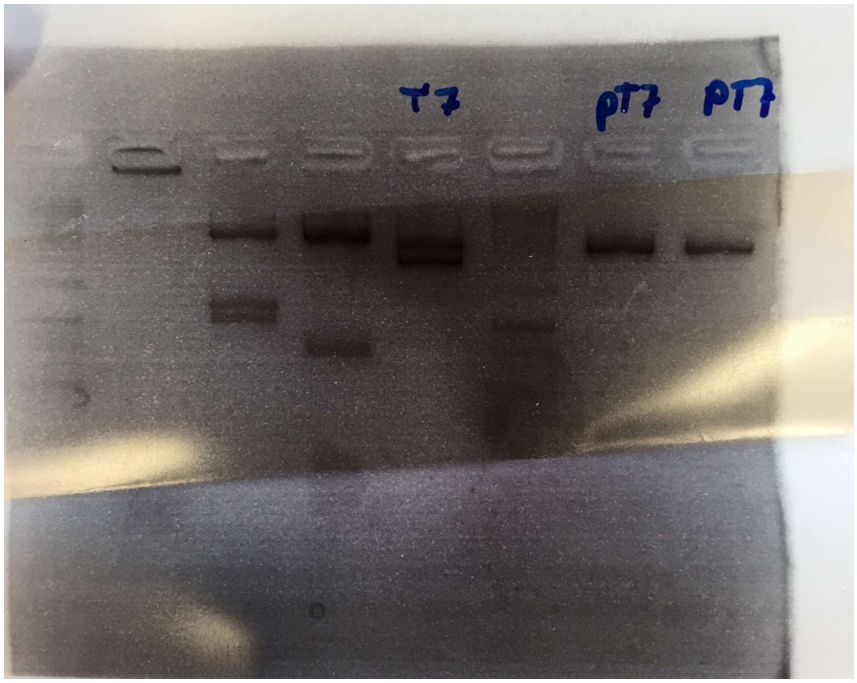
PCR of *fadR* cultures:

Reagent	2mM MgCl2		
H20	6.75	108	
Buffer	2	32	
MgCl2	0.8	12.8	
dNTP	0.2	3.2	
pol	0.05	0.8	78.4
Primer Mix	0.2	3.2	
total	10	160	

5.- pTET-T7 construct

SATURDAY the 1st of September, 2018

Carla performed a cloning by SP and XP digestion. Two colonies where digested and sequenced but there were no inserts:



More colonies will be screened.

SATURDAY the 15th of September, 2018

Guillem Performed a 3A assembly (details):
t14+ptet+34 -ES- (pK), T7 polymerase -XP-(pC) and pA backbone -EP- (D4 in the lab spreadsheet) were assembled;
-Two colonies grew after the assembly on the verge of the plate, not sure if contamination.

WEDNESDAY the 19th of September, 2018

Guillem Performed a 3A assembly:
t14+ptet+34 -ES- (pK), T7 polymerase -XP-(pC) and pA backbone -EP- (D4 in the lab spreadsheet) were assembled;

SATURDAY the 22th of September, 2018

Digestion

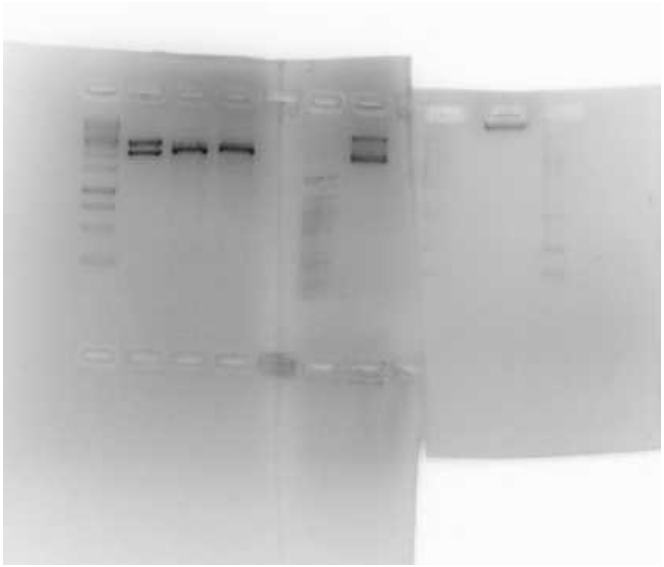
Digestion pTET 34	
Buff megamix	2
DNA	5.6
H20	11.4
E	0.5
s	0.5

Digestion T7	
Buff	2
DNA	1.7
H20	15.3
X	0.5
P	0.5

Digestion linearized backbones	
Buff	2
DNA	10
H20	7
E	0.5
P	0.5

WEDNESDAY the 24th of September, 2018

T7 an bacbones were cut from gel and frozen. pTET had no insert,



THURSDAY the 25th of September, 2018

Jordi digested pTET again and it had no insert.

TUESDAY the 28th of September, 2018

We asked PhD Student and iGEM instructor Eva Gonzalez for promoter part. Will be digested again. Part was not assembled corrrectly. Enzyme E was not working propperly eather or no insert.

Buff magamix	2
DNA	5.6
H20	11.4
E	0.5
s	0.5

SATURDAY the 3rd of October, 2018

t14ptetRBS32 will be digested again

t14pTET Digestion; E-S	
Buff magamix	2
DNA	5.5555555556
H20	11.4444444444
E	0.5
s	0.5

t14pTET digestion; S-P	
Buff magamix	2
DNA	5.5555555556
H20	11.4444444444
E	0.5
s	0.5

80 min 37 and 10 min 80 digestion was done in a thermoblock.

Ligation	
T7	10
Backbone	5
Buff	2
H20	2
T4	1

Was digested for 1h.

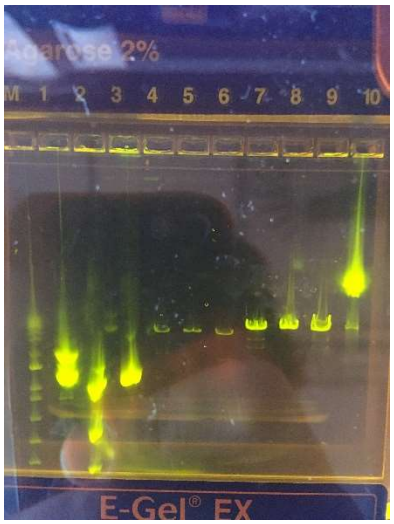
reagent	2mM MgCl2		
H20	12.7	127	
Buffer	4	40	
MgCl2	2.4	24	
dNTP	0.4	4	
pol	0.1	1	
Primer Mix	0.4	4	100
template DNA (1:20 culture dil)	0	0	
total	20	200	

SATURDAY the 9th of October, 2018

Last cloning attempt

ptet T14			
buff	2	2	2
DNA	7.5	7.5	7.5
E	0.4		0.4
S		0.4	0.4
h20	10.1	10.1	9.7

T7 backbone			
buff	2	2	2
DNA	1.6	1.7	1.8
E	0.4		0.4
X		0.4	0.4
h20	16	15.9	15.4



4,5,6 is ptet digested with E , s and ES (faint band at 300bp could be seen in ES digestion. 7,8,9 T7 digested E, X and EX

T7	1	1
PTET	12	0
Buff	2	2
H20	4	16
T4	1	1

ligation aprox 1h.

reagent	2mM MgCl2		
H20	12.7	25.4	
Buffer	4	8	
MgCl2	2.4	4.8	
dNTP	0.4	0.8	
pol	0.1	0.2	
Primer Mix	0.4	0.8	20
template DNA (1:20 culture dil)	0	0	
total	20		