Myr1_GFP

TORSDAG, 2018-07-19

Phusion PCR of MYR1 part 1 and 2 and GFP

Dilution of myrosinase constructs was done by adding 100 µL of TE to the ordered tubes, which gave us a concentration of 10 ng/µL. The tubes were incubated for 20 minutes at 50 °C.

The primers was also diluted and mixed in pairs, the dilution was done as usual i.e. 10 µL of each primer and 80 µL. The tubes were marked and put in out box in the fridge.

PCR

Phusion PCR was prepared for 50 µL according to the protocol for:

- MYR1_PART1
- MYR1_PART2
- GFP(MYR1)

We did not add the optional DMSO.

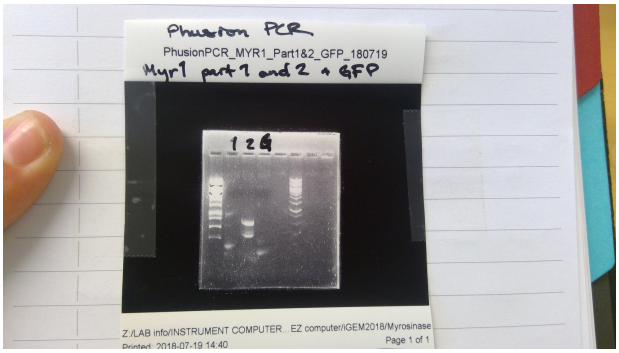
The settings for the PCR for the different construct was saved into the folder Myrosinase on the computer. The annealing temperature that was used was:

- MYR1_PART1: 63.3 °C
- MYR1_PART2: 66.5 °C
- GFP(MYR1): 60.4 °C

A gel was prepared.

The gel did not show such good results. Part 1 seems to be the right length but it is really weak. Part 2 has a strong band but it is positioned at around 600 bp which is too short, it's suppose to be around 1000 bp. GFP did not show any results at all.

180719_gel1.jpg



We are going to do the PCR tomorrow agoing, this time we'll ad DMSO and also use the lowest Tm for each primer pair and add 3°C for the annealing temperature. (It was Doris gave us a tip)

FREDAG, 2018-07-20

Phusion PCR for MYR1 part 1 and 2 and GFP

Today we redid the phusion Phusion PCR from yesterday to see if we could get better results. The things we changed from yesterday was to add DMSO in the preparation step of PCR and also to change the annealing temperature by looking at the lowest Tm for each primer pair, and to that add 3°C. The annealing temperatures was then:

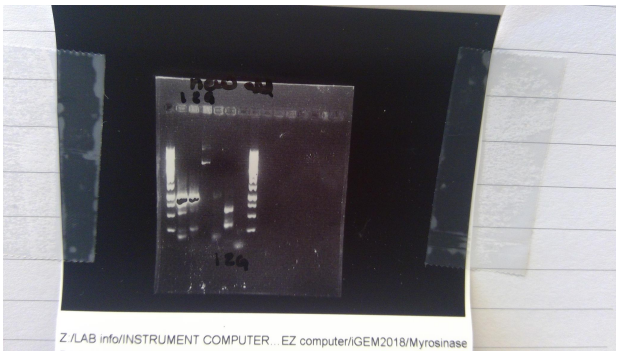
MYR1_PART1: 62.4 °C
MYR1_PART2: 66.8 °C
GFP(MYR1): 59.6 °C

Those were saved in the folder on the computer market as v2 (version 2).

Gel results:
The gel did show a mush better result for the MYR1 part 1 and 2 with sequences that seemed to be at very high concentration and at the right length. The GFP did only show the plasmid which concerned us.

PICTURE!!

180720_gel1.jpg



When talking to Angelo which had provided us with the GFP plasmid, he said that the plasmid was high concentrated (500 ng/µL) which is way to high. We decided to suspend it 1:100 to get a concentration of 5 ng/µL and try phusion PCR for the GFP again, this time with a gradient.

Phusion gradient PCR for GFP

8 PCR tubes where prepared according to the protocol for 50 µL. We added the DMSO.

Settings

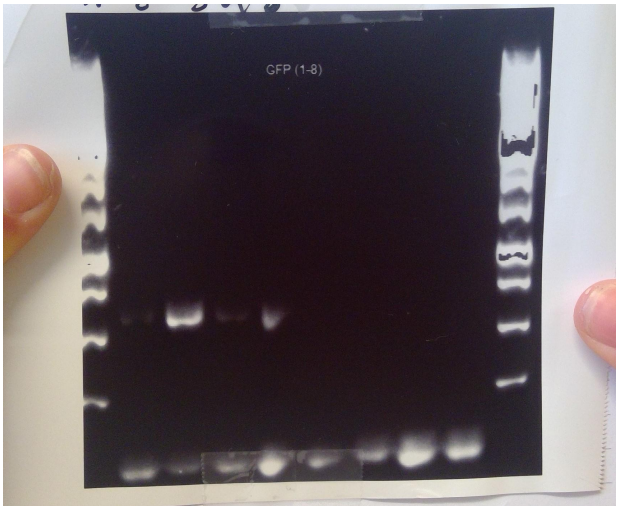
Table1

	A	B
1	Tube	Ta °C
2	1	65
3	2	64.5
4	3	63.3
5	4	61.4
6	5	59
7	6	57
8	7	55.7
9	8	55

The settings were saved in the folder "myrosinase" on the computer.

Gel results:
Results from Phusion gradient PCR for GFP showed some fragments but it seems that they are too short. They are supposed to be around 800 bp which they clearly are not. It is only four wells that shows this fragment of which the well with 64.5 °C as annealing temperature showed the strongest band. I'm going to redo the Phusion PCR with this annealing temperature next week.

1807220_gel2.jpg



Yeast inoculation

We also started a yeast inoculation of the 11C strain to be able to extract the pFUS1 promoter from the genome.

We took a culture from a plate from our box in the frigde and resuspended it with 5 mL to inoculate overnight at 37 °C

Liquid cultures wehre put in fridge on next sunday to be ready on monday.

SÖNDAG, 2018-07-22

Colony PCR on Yeast for amplification of pFUS1

The OD was measured on the liquid that hade been ptu in the fridge the day before. There was one culture that had a much dense and darker color and therefore i namend the different cultures dark and light. The OD of the two different cultures was:

Dark: OD=1.389
Light: OD= 0.971

Preparation of Yeast before colony PCR
I prepared 7 tubes from each liquid culture, in tube 1-4 for each of them was diluted 0.5 µL of liquid culture into 50 µL of NaOH and in tube 5-7 was diluted 1 µL of liquid culture in 50 µL in 50 µL of NaOH.

The tubes where boiled in the PCR machine for 15 minutes at 98 °C. I saved the setting in the Myrosinase folder.

After boling the tubes were centrifuged for 1 minute aproximately to spin down cell content.

Phusion PCR

Phusion PCR was prepared according to protocol and as template 1 µL of the supernant from the tubes were used. The PCR settings were saved in the folder "Myrosinase" and the annealing temperature that was used was 63.1°C.

Gel results:

Gel order:

pFUS1 is 438 bp long so we want that sequence length

The PCR did obloiusly not work. We can see the primers for all

I'll redo the procedure tomorrow. this time by amplifying the pFUS1 promoter from the p28 construct to see if that works better. Then I can perform a positive control with the p28 primers to see that the PCR accturally worked.

MÅNDAG, 2018-07-23

Phusion gradient PCR of pFUS1 and GFP

First I prepared Phusion PCR according to the protocol, including DMSO, for 6 tubes for both GFP and pFUS1. One positive control containing the p28_GFP construct and the forward and reverse primers for this construct was used for the pFUS1 amplification.

Gradient PCR was performed as following

Table3

	A	B	C	D
1	Tube GFP	Ta °C	Tube pFUS1	Ta °C
2	1	69.5	1	68.1
3	2	69	2	67.6
4	3	67.9	3	66.5
5	4	65.9	4	64.5
6	5	63.5	5	62.1
7	6	61.5	6	60.1

The setting for the different PCR are saved in the folder on the compuder for the date of today.

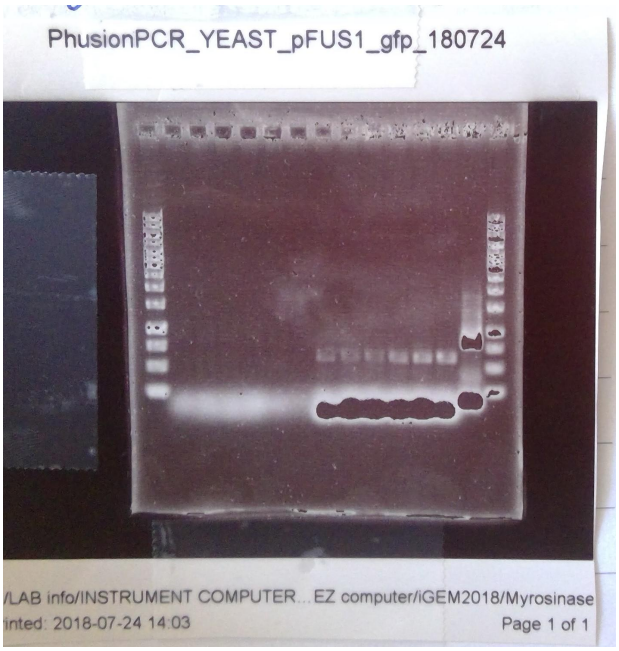
Preparation of post-stained gel:
The liquid was prepared in an old bx for pipet tips by adding 50 µL of gelred to 50 mL of MQ-water. The box was after that covered with alumina foile to protect it from light. The solution can be used for at least staining of three gels. The gel for the gel electrophoresis is prepared without any stain.

Gel results:

When I came to get the PCR I realized that it was turned of for the pFUS1 amplification. I want to remember that I did start alla the PCR reactions but I cannot be sure. One possibility is that someone else has turned off my reaction by accident or that I did not start it. I ran the gel anyways to see if something was there.

After the gel electrophoresis the gel was but in the post-staining box for 15 minutes. The box was shaked during those minutes.

180724_gel1.jpg



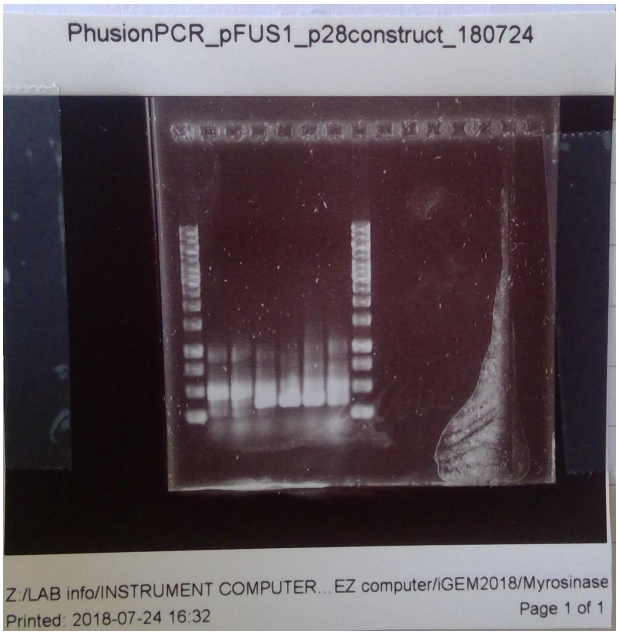
The Gel showed band for the GFP but nothing for the pFUS1 amplification. This indicates that the PCR did not run. I wil try to run it with the same tubes once again and if that does not work I'll redo the whole PCR for the pFUS1.

The Gel order for the second try was:

Table5

	A	B	C	D	E	F	G
1		pFUS1					
2	Ladder	1	2	3	4	5	6

180724_gel2.jpg



It looks like it worked good either way because the pFUS1 construct should be around 450 bp including the primers. I'm going to redo the amplification with 1 tube using the annealing temperature 64.5 °C

Realization!!!

We just realized that the reverse primers below was ordered in the wrong direction. This explaines why the GFP looked too short in the previous amplifications. The Part2_Myr1 and the GFP amplification for Myr1 needs to be redone the other ones have not started yet.

Table6

	A	B
1	Rv_p28	gacataactaattacatgactcagtcatcgggtttaag
2	Rv_GFP(Myr1)	atgtaagcgtgacataactaattacatgacCTATTTGTATAGTTCATCCATGCC
3	Rv_Par2Myr1(GFP)	GAGAACCTCCCCCGCCtcaTGCGTCCTCGAATTTCTTGTTC

TISDAG, 2018-07-24

Amplification of gRNA_Bar1-construct

Phusion PCR was prepared for one tube. The primers that was used was borrowed from Alex and it was a mix with the primer concentration 10 ng/µL.
The protocol for the phusion PCR was followed, including DMSO and that I took 3 µL of primer mix instead of 2.5µL.

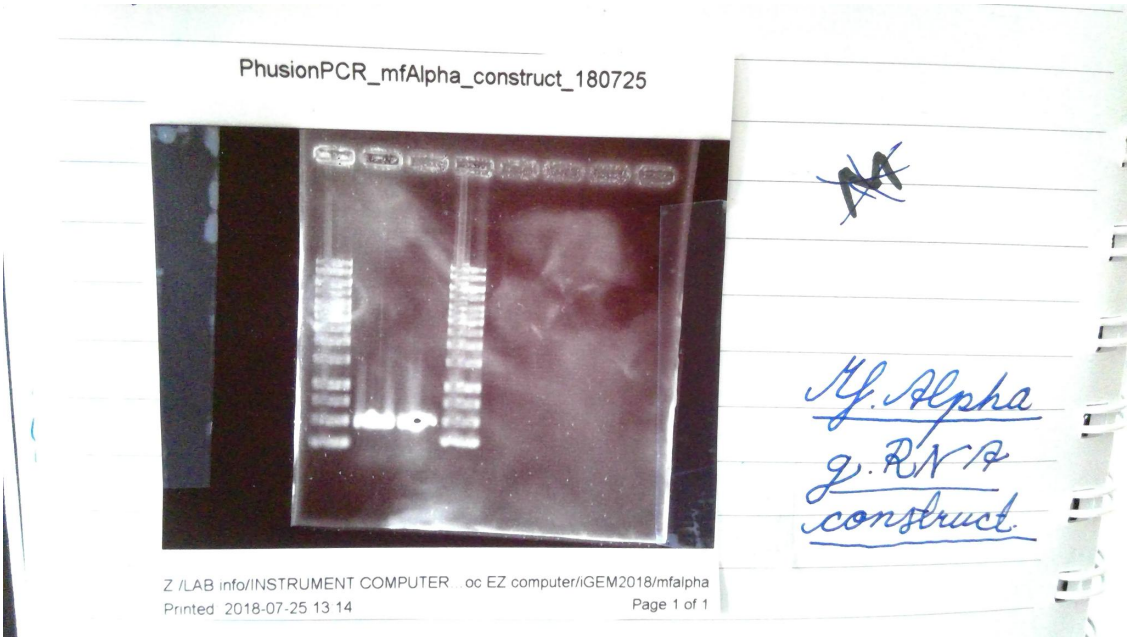
The gRNA-construct is suppose to be arround 455-500bp

PCR settings is stored in the folder mfAlpha and the annealing temperature that were used was 55 °C.

Gel results:
A post-stained gel was prepared as usual, 20 mL of agarose gel.
The gel was run for 40 minutes at 89V.

Both bands shown in the picture are taken from the same PCR tube.

180725_gel1.jpg



The gel shows that the gRNA_BAR1 construct seems to have been amplified successfully.

ONSDAG, 2018-07-25

Phusion PCR pFUS1

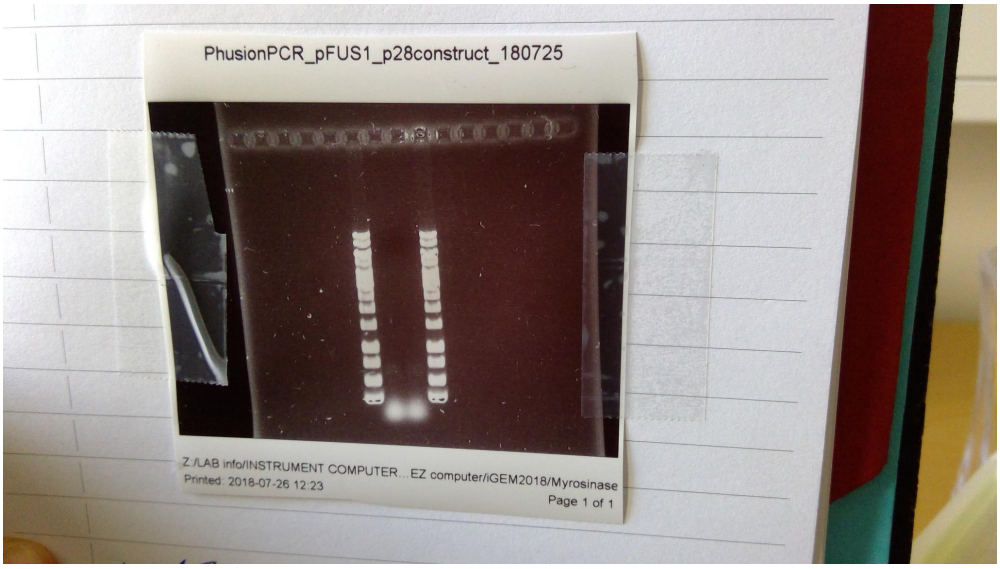
Phusion PCR was prepared according to the protocol, includin DMSO and also by adding 3 µL of primer mix.

PCR was run according to protocol and the setting are saved in the folder with todays date. The annealing temperature was set to 64.5 °C.

Gel Results:
A post stained gel was prepared.

Both wells that where loaded where loaded from the same sample.

180726_gel1.jpg



The gel showed nothing, so I'll redo a gradient PCR tomorrow.

MÅNDAG, 2018-07-30

Phusion PCR Part2_Myr1, GFP(Myr1) ; Gradient PCR for pFUS1(Myr1)

The annealing temperatures for the constructs were:

Part2_Myr1: 66.5 °C
GFP(Myr1): 59.6 °C

Temperatures for gradient PCR for pFUS1(Myr1)

Table7

	A	B	C	D	E	F	G	H
1	Tube	1	2	3	4	5	6	7
2	Temp. °C	68.1	67.6	66.5	64.5	62.1	60.1	58.8

All the settings for the gradient PCR were saved in the folder.

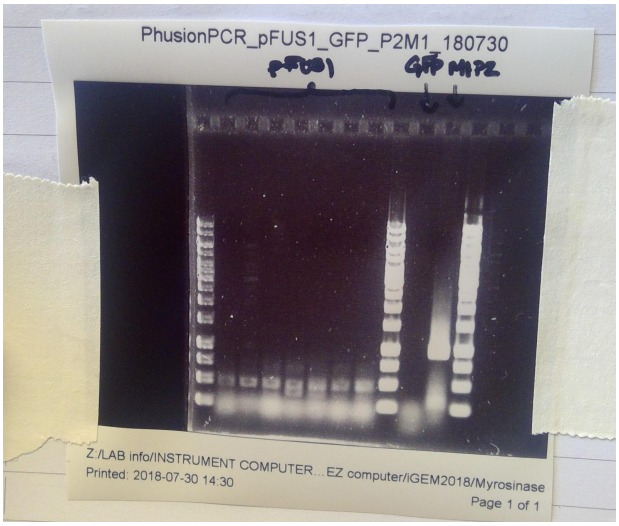
Gel order:

pFUS1(Myr1)

Ladder 1 2 3 4 5 6 7 Ladder GFP P2M1

Gel Results:

180730_gel1.jpg



pFUS1 and part2_Myr1 seems to have worked. We will redo the GFP with a gradient.

ONSDAG, 2018-08-01

Plasmid PCR KanMX Cas9 plasmid

We got the plasmid from Alex and we did a Phusion PCr of the plasmid with a extension time of 6.5 minutes. This made the PCR take 4 hours to do. The annealing temperature was set to 60 °C and 2 ng of implant was used for the PCR

The plasmid had the stock concentration 166.6 ng/µL so we diluted it 100 times to 1.66 ng/µL.

Phusion Gradient PCR for GFP(Myr1)

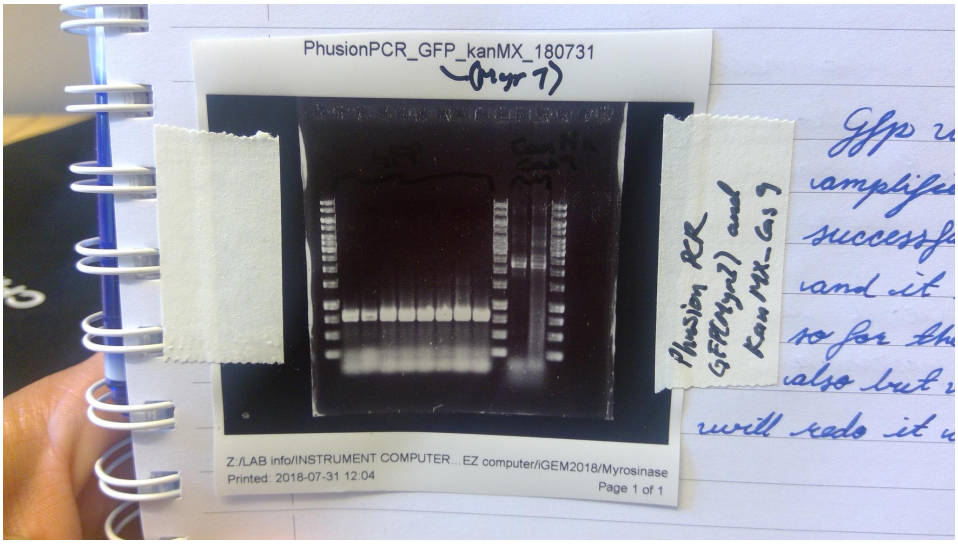
Was done according to the old settings in the folder.

Gel results

Gel order:

	GFP							kanMXCas9		
Ladder	*1*	*2*	*3*	*4*	*5*	*6*	*7*	*Ladder*	*1*	*2*	*Ladder*

180731_gel1.jpg



GFP was amplified successfully, but the kanMXCas9 looks wrong. We looked at the log for the PCR machine and realized that the rund was canceled due to an error in the middle of the run

Phusion PCR of mfα part 1 and part 2

TORSDAG, 2018-08-02

Purifications

Purification of:

- mfα part 1 and 2
- Myr1 part 1 and 2
- pFUS1(Myr1)
- GFP(Myr1)

For the GFP we took the fragment from well 1 in the gel picture on 180731.
For pFUS1 we took the fragments from well 7 in the gel picture on 180730

Purification was done according to purification protocol for all the samples.

Concentrations:

Table8

	A	B	C
1	sample	ng/µL	260/280
2	P1M1	97.7	1.77
3	P2M1	71.6	1.68
4	GFP(Myr1)	35.8	1.85
5	mfα part1	56.4	1.88
6	mfα part2	24.1	1.89
7	pFUS1	12.2	1.73

Inoculation of cas9 plasmid containing gRNA for deletion of BAR1

We prepared 4 overnight cultures of 5mL LB media and 5 µL amp.

FREDAG, 2018-08-03

Miniprep E.coli with kanMX cas9 gRNA

The inoculation tubes were centrifuged at 4500 rpm for 5 minutes

Miniprep was performed according to the protocol

Concentrations:

Table9

	A	B	C
1	tube	ng/µL	260/280
2	1	399.9	1.88
3	2	495.4	1.88
4	3	375.5	1.87
5	4	457.2	1.87

Digenstion of kanMX gRNA

Fast digest protocol was followed. We used 2 different restriction enzymes => only 14 µL of MQ water

180803_gel1.jpg

It looks like we transformed the right plasmid in three out of four samples. The first and the second well from the left look okay but probably not all of the plasmids where digested correctly, explained by the double band at the bottom.

MÅNDAG, 2018-08-06

Yeast transformation

Andrea out a 5 mL culture for us to use, OD~2.8. It was dilute 10 times using 3mL culture and 27 mL of YPD to an OD~0.28. This culture was grown for 4h in 30°C.

after thid the transormation was carried out according to the protocol. However there was a slight hold up when adding the DNA to the yeast. I did not have the exact measurments for the concentrations but it should be fine.

FREDAG, 2018-08-10

Phusion PCR

Phusion pcr was performed for p28 (withouth GFP) , myr1 part 1 and part 2 (With GFP) and mfα part 1 and part 2

The annealing tempertures was:

Table10

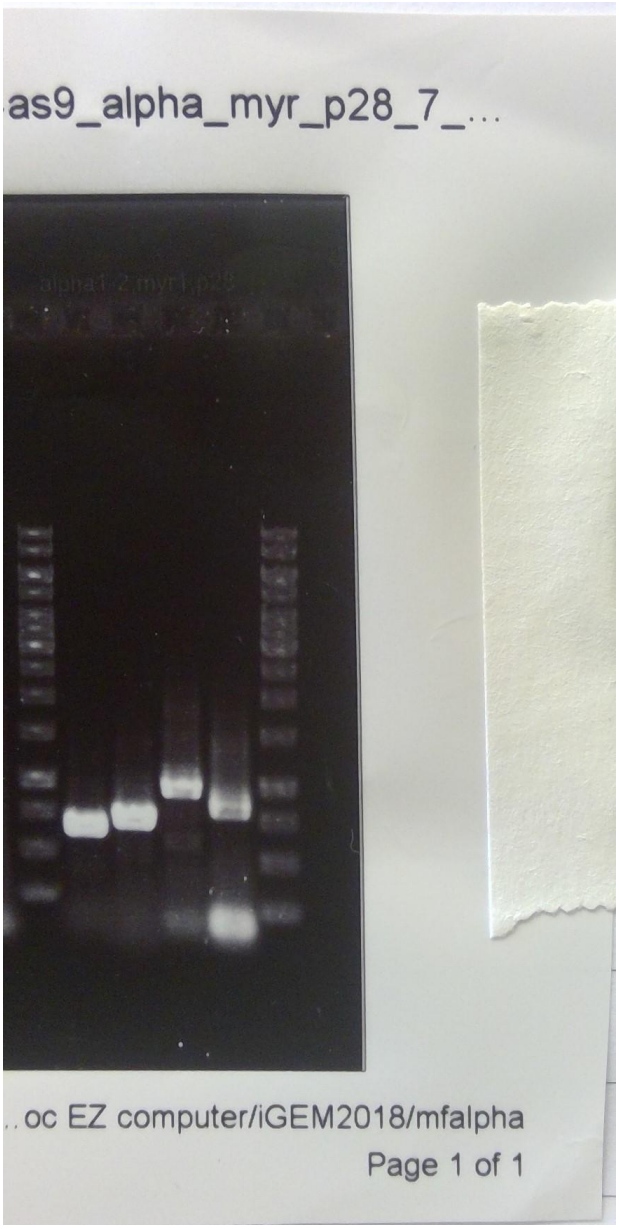
	A	B
1	construct	temp. °C
2	p28	56.9
3	mfα part 1	61.3
4	mfα part 2	62.8
5	Myr1 part 1	63.1
6	Myr1 part 2	67.2

Gel results:

Gel order:

Ladder alpha part 1 alpha part 2 myr1part 1 p28 Ladder

180807_gel1.jpg



Seems like all the constructs were amplified correctly. myr1 part 2 was lost because of errors in the settings of the pcr machine so Axel will redo this amplification tomorrow.

LÖRDAG, 2018-08-11

Preparation of colony PCR

Yesterday Axel did a transformation of kanMX plasmid with cas9 and gRNA for deletion of BAR1 together with mfα construct into p413TEF strain of yeast. Those were cultured on YPD + MX plates.
29 colonies were streaked and 28 colonies were prepared for the colony PCR today.
The colonies were prepared by lysing the yeast. A sample of each culture was extracted by the handle tip of a spreader and diluted in 5 µL of NaOH in pcr tubes. Those were incubated at 98 °C for 15 minutes.
After incubation the tubes were centrifuged in a mini centrifuge for 1 minute.

DreamTaq colony PCR

Colony PCR was performed according to protocol.
A mastermix was prepared of all the components exept the tamplate according to following volumes:

Table11

	A	B
1	Compound	volume µL
2	MQ water	1572
3	DreamTaq 10x buffer	160
4	10X dNTP	3.2
5	primer mix	160
6	DreamTaq polymerase	32

49 µL of the master mix were added to 28 pcr tubes and 1 µL of the supernatant from each culture was added to each tube for the colony PCR.

Colony PCR was performed with an annealing temperature of 52.

Results

Results were good! We had bands on 5, 8 and 10 on the "black" plate. On the green plate we had bands on ...

Yeast plate was put in fridge. Resteraking of positive cultures on YPD plate on monday.

MÅNDAG, 2018-08-13

Restreaked the positive colonies 5, 8 and 10 from green and black plate respectively.

TISDAG, 2018-08-14

PCR of Myr1_P2, Myr1_P2(GFP), pFUS(Myr1)
Annealing temperatures (Phusion)
pFUS-> 63.7 degrees celsius
Myr1_P2->67.2
Myr1_P2(GFP)->66.5

PCR was a success. For all of the constructs.
Since pFUS<500bp isopropanol was added to the purification column durring the first steps of purification.

ONSDAG, 2018-08-15

PCR of pFUS(GFP), GFP(pFus)

Digestion ofp413TEF
Gibson of:

- pFUS(GFP)-GFP(pFUS)
- pFUS(Myr)-M1P1-MyrP2(No GFP)
- pFUS(Myr)-M1P1.MyrP2(GFP)
- p28(no GFP)

Transformation of all of the above.
PCR
pFUS(GFP) primers:

- Fw_PFUS(Myr1)
- Rv_PFUS(GFP)

GFP(pFUS) primers:

- Fw_GFP(pFUS)
- Rv_GFP(pFUS)

Used a gradient PCR for more robust process.
Good results on the gel
doing a pooled gel extraction to achive a higher final concentration.

Prepare gel extraction for tomorrow:

- Add 1.5 microlitres GelGreen to 1% agarose gel
- 120 microlitres pFUS sample + 20 microlitres LD in tripple well
- 100 microlitres MyrP2 sample - 20 microlitres LD in double well
- 100 microlitres MyrP2(GFP) + 20 microlitres LD in double well

TORSDAG, 2018-08-16

Ran gel prepared as stated above
Gel weight: pFUS(GFP) 250 mg
Myr(GFP) 280 mg
Myr 320 mg
Purified the collected dna
Concentrations:

- pFUS(GFP) -> 18.9
- MyrP2(GFP)-> 24.8
- MyrP2(No GFP) ->42.2

Pretty high 260/280->2.3

Did not get rid of all bands :(
Bothe pFUS and MYrP2 still got bands

Digested p413TEF.
Gibson of the constructs:
Myr-GFP

- pFUS(Myr) -> 63ng/microlitre 3microlitres
- M1P1 -> 17.7 ng/microlitres 8 microlitres
- MyrP2(GFP) -> 24.8 ng/microlitres 7 microlitres
- GFP(Myr) -> 35.8 ng/ml 6 microlitres

Myr

- pFUS(myr) -> 63 ng/microlitre 3 microlitres
- M1P1 -> 17.7 ng/microlitre 8 microlitres
- MyrP2 -> 42.2 ng/microlitres 5 microlitres

pFUS-GFP

- pFUS(GFP) -> 18.9 ng/microlitre 7 microlitres
- GFP(pFUS) -> 67.4 ng/microlitre 3 microlitres

p28

- p28 -> 97.7 ng/microlitres

E.coli transformations done according to protocol

FREDAG, 2018-08-17

Colony PCR x55
Digested backbone with wrong enzyme yesterday, will redo gibson.
Digest p413TEF with Xho1 and Sac1 30 min 37 degrees
Then same gibson as yesterday.
Will redo pcr for GFP(pFUS) and GFP(Myr)

MÅNDAG, 2018-08-20

PCR

- GFP(Myr)
- GFP(pFUS)

used p413TEF-GFP As template.

ONSDAG, 2018-08-22

- Going to sequence the yeast to check for deletion of BAR1 and insertion of mf-alpha into the genome.
- Transform Myrosinase with pFUS and with GFP into e.coli
- transform pFUS+GFP into e.coli

All the construcs in plasmid p413TEF. Transformation of e.coli done in accordance with protocol. MyrGFPx2, pFUS+GFP and Myr

TORSDAG, 2018-08-23

Going to check the insert in the yeast
Protocol:

- 15 microlitres NAOH mixed with one colony
- 15 min at 98 degrees
- quickly vortex
- centrifuge 15k g for 3 min
- use 1 microlitres for PCR

Ran the gel but got no bands :(

FREDAG, 2018-08-24

Check insert in e.coli for the Myr-gfp, Myr and pFUS-gfp. Redoing check of the insert in yeast.
Running 28x colony PCR to check insert. After gradient PCR, G5.2 nr one at highest temp, G10.1 nr 8 at highest temp, G8,2 nr 1 at the highest temp and B10 nr 8 at the highest temp.

Realized that mfalpha might be 1600bp and not 800 which means mistakes were made. So the isolation of DNA from yeast is going to be redone and the pcr need to be rerun with a longer extension time of about one minute.

SÖNDAG, 2018-08-26

- Ran gel for pfus-gfp
- Prepped ON-cultures for pfus/gfp/myr
- Prepp ON-Culture for yeast mfalpha
- Check lenght of insert in yeast again
- Prepped new LB+AMP, 1 ml to 1 microlitre

MÅNDAG, 2018-08-27

Starting preparation for transformation of yeast. Added 50 ml to ON-cultures and waited 6 hours.
Miniprepped , according to protocol, 17 ON cultures of e.coli.

	Sample	Concentration ng/microlitre	260/280
1	1	58.1	1.89
2	2	321	1.89
3	3	315.5	1.89
4	4	326	1.88
5	5	112.2	1.88
6	6	310	1.88
7	7	298.4	1.88
8	8	250	1.88
9	9	62.2	1.93
10	10	287.4	1.88
11	11	297.2	1.88
12	12	209	1.88
13	13	313.6	1.88
14	14	290.3	1.88
15	R1	9.4	1.99
16	R2	20	2.01
17	R3	32	1.99
18			

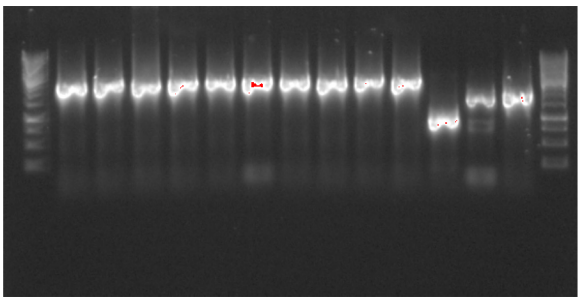
Run PCR for pfus1+GFP to verify insert, only run on them with bands aka: 2, 3, 4, 6, 7, 8, 10, 11, 12, 13, 14..
11x PCR mix.
Ran gel. Might have had to high concentration of plasmids.

TISDAG, 2018-08-28

Cut with Nco1 and Sac1 should get a band with 1.4 k b. Miniprepp 14 e.coli cultures with pfus-myr
gonna cut em and run on gel 65.5 celsius for pcr for sequencing of mfalpha 1.6k if good. 2.3k if bad.
Run PCR, same as yesterday, but with diluted plasmids(1:100).

Nco1 was out, so had to used pcr with colony pcr primers. 1-10 was successfull.
Doing transforation with the succsessfull ones. Doing one for myrosinase and one for gfp.
Gonna pick nr 5 for the myr and nr 2 for the gfp trnasformation.

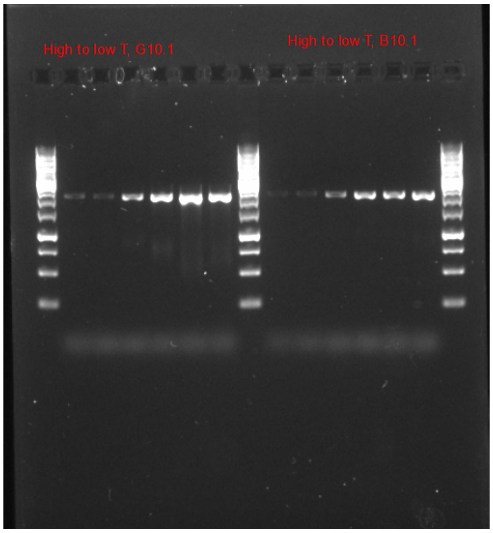
pFUS_Myr_plasmidPCR_280818.tif



ONSDAG, 2018-08-29

The pcr for mfalpha was a faliure only bands at 2.3 kb (Bar1).
Redoing it today hoping for better stuff. Also messed up yesterday and spread about 80% of the cells on the wrong plate. His instead of Ura plates. But the remaining cells were put on the right plate.

mfalpha from genome G10 and B10 16h42m28s 18-10-14.png



TORSDAG, 2018-08-30

Supervisor said "Run dreamTaq 55 degrees" so that was done.
Only got the wrong bands anyway.
Gonna re run som colony pcr to check the insert ones more, Also picking eight colonies of the transfomred yeast to check flourescens.

FREDAG, 2018-08-31

Mixing 20 microMolar in each tube.
M(alphapheromone)=1682 g/mol
1 mg/l is $5.94*10^{-5}$ mol/l -> 594 micromol/l =c1
V2=100 microlitres
c2=20 microMolar
V1=3.37 microlitre.

After 2 h incubation with pheromone the yeast was checked underflouroscenc microsope. GFP expresion in both posetive and negative control. Quite possible that the TEF promoter has not been replaced with the pFUS1.

MÅNDAG, 2018-09-03

Checking cancer toxin efficacy. Sinigrin M=397 -> concentration of sulforaphane greater than 20 microMolar had negative effects on cancer cell growth. Final Volume of abour 200 microlitres. Going to aim for a concentration that is four times higher, want to have a clear impact on the cacer cells. Need $0,4 * 10^{-3}$ mol -> 0.0004*397=0.158 grams. Going to make 4 ml of final culture. Spun down 50 ml culture and separated it into two tubes with 2 ml each. Only the one with added substrate should affect the cancer. Added 66 microlitres of pheromone solution to both the cultures. Will filter it throug a syringe and then add it to the cancer cells. 6x positiv and 6x contol.

Re checking the pfus1 with/without alphapheromone. Checked the control first.
They all had GFP expression once again, SAD DAY.

Decided to do 4 tubes of 2 ml each for the yeast with the myr construct. Added 66 microlitres of pheromone to them and our supervisor will filter them tomorrow and add the sinigrin and finally put it into the cancer cell medium.

LÖRDAG, 2018-10-06

Biobrick preparation of Myrosinase from same plasmid that was used to transform the yeast and was sent for sequencing, nr 5. Anneal at 72 degrees according to calculator, very high according to me. Should have checked how much overlap it would have to to the biobrick plasmid and how much to the original construct. But what ever will just do gradient anyway. From 70 degrees down to 62 Removed so that only the 20 relevant basepairs were calculated and got a more reasonable temperature of 53 degrees. Since its unsure how much overlap our construct has and i don't have accesss to a computer im just gonna run a gradient from 54 up to 64 and see if it works. made a 8x pcr mixture and ran it. Only got bands in 6, 7 and 8. (the lower temperature range) Nr 8 did not really have the alotted 50 microlitres so i will continue only with 7/6. Purified the PCR product according to protocol.
Nanodrop: 6 -> 56.7 ng/microlitre 1.92
7 -> 47.5 ng/microlitres 1.94

Gibson time baby :^)
Plasmid is pSB13c

- Total volume:20 microlitres
- Gibsonmix: 10 microlitres
- Fragments 3:1 for the insert to plasmid

Max ammount is about 0.5 pmols 50ng of 500bp -> 0.15 pmols
Plasmid is about 3k bp -> 50ng -> 0.025 pmols
inser 2k bp -> 50ng -> 0.0375 pmols
4 microlitres of plasmid -> 0.025pmols and 2 microlitres of insert -> 50 ng -> 0.075.
total of 0.1 pmols well within the allowed interval.
Ran the gibson and preformed transformation of ecoli according to protocol with negative control.

SÖNDAG, 2018-10-07

Got some colonies on the plate and zero on the control.
Picked out six and cultured them in LB+Chloramphenicol in 2ml over night

MÅNDAG, 2018-10-08

Miniprepped the cultures.
Got pretty low concentrations.
Cut with Nco1, if the insert is right should get two bands at 1.8kb and one at 2.3 kb but for a miss insert only one band.
Ran a gel and got two bands on all of them, very cool.

MyrBiobrick all is right boiiii 2018-10-09_00h04m17s.png