DATE: 14/6

PCR - Promotors

PCR was performed to extract the promotors pTEF1, pPGK1, pTDH3, pCCW12 and pCIT2 from a *S. cerevisiae* genome given to us by Linnea. Genomic DNA was used as template, the following primers were used for each promotor

Promotor	**Primers**
1. pPGK1	28,29
2. pTEF1	30,31
3. pTDH3	32,33
4. pCCW12	34,35
5. pCIT2	36,37

The following volumes were used for the PCR

ddH2O	34 µl
PrimeStar buffer	10 µl
dNTP	1 µl
Template DNA	2 µl
Primer 1	1,25 µl
Primer 2	1,25 µl
PrimeStar polymerase	0,5 µl

The PCR was run according to the following protocol:

1. 98 °C - 1 min
2. 98 °C - 10s
3. 55 °C - 15s
4. 72 °C - 1 min
5. 72 °C - 4 min

with steps 2-4 being cycled through 32 times

Gel electrophoresis

The PCR products were run on a 1% agarose gel, at 90V for 30 min and the gel was post-stained. Expected lengths for the fragments were:

- pPGK1: 442bp
- pTEF1: 1014bp
- pTDH3: 706bp
- pCCW12: 1030bp
- pCIT2: 769bp

Note: the lengths above are wrong according to benchling
Corrects lengths, see below

- pPGK1: 984bp
- pTEF1: 412bp
- pTDH3: 676bp
- pCCW12: 739bp
- pCIT2: 1000bp

The results, shown below showed that pTEF1, pTDH3, pCCW12 and pCIT2 were successfully extracted, without byproducts

[INSERT GEL IMAGE]

PCR - pPGK1

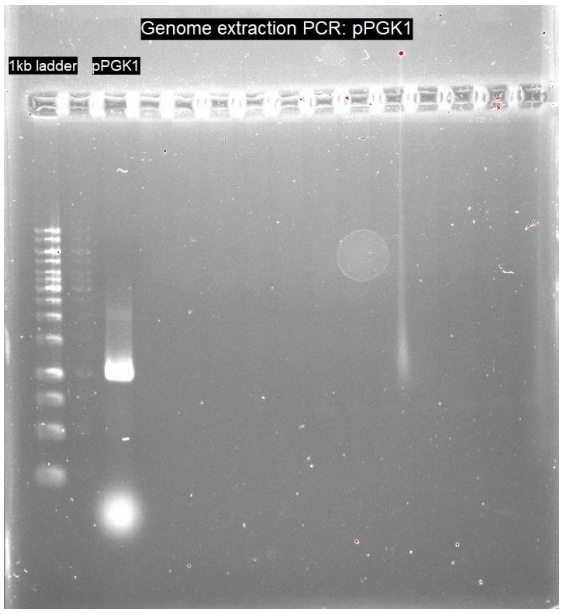
The PCR was remade for pPGK1, with the annealing step changed to touchdown, from 60-48 °C, and finally setting on 52 °C.

DATE: 17/6

WHAT WE DID:

Gel electrophoresis - pPGK1

The pPGK1 PCR product from the final PCR on the 14/6 was run on a gel, same settings as before. The results shown below indicated that pPGK1 had been successfully extracted from the yeast genome



PCR purification - Promotors

PCR purification was performed for all five PCR products for which the extraction had been successful, according to standard protocol (GeneJET PCR purification). The concentrations of the DNA solutions were measured on a nanodrop, with the following results:

- pPGK1: 22,6 ng/µl
- pTEF1: 145,7 ng/µl
- pTDH3: 114,2 ng/µl
- pCCW12: 104,8 ng/µl
- pCIT2: 90,5 ng/µl

Fusion PCR #1 - DP

Fusion PCR was performed in order to produce dual promoters, with the following combinations

Dual promotor	**Components**
DP1	pPGK1, pTEF1
DP2	pTDH3, pCIT2
DP3	pTEF1, pCCW12
DP4	pTEF1, pCIT2

For the first round of fusion PCR (without primers), the following volumes were used (differed between the different mixtures):

DP1	
ddH2O	13,04 µl
PrimeStar buffer	5 µl
dNTP	0,75 µl
pPGK1	2,21 µl
pTEF1	1,5 µl
PrimeStar polymerase	2,5 µl (1:10 dilution)

DP2	
ddH2O	8,05 µl
PrimeStar buffer	5 µl
dNTP	0,75 µl
pTDH3	3,0 µl
pCIT2	5,7 µl
PrimeStar polymerase	2,5 µl (1:10 dilution)

DP3	
ddH2O	11,55 µl
PrimeStar buffer	5 µl

dNTP	0,75 µl
pTEF1	1,5 µl
pCCW12	3,7 µl
PrimeStar polymerase	2,5 µl (1:10 dilution)

DP4	
ddH2O	9,55 µl
PrimeStar buffer	5 µl
dNTP	0,75 µl
pTEF1	1,5 µl
pCIT2	5,7 µl
PrimeStar polymerase	2,5 µl (1:10 dilution)

The PCR was run according to the following protocol:

1. 95 °C - 3 min
2. 98 °C - 10 s
3. 58 °C - 15 s
4. 72 °C - 1 min
5. 72 °C - 10 min

with steps 2-4 being cycled through 15 times

Fusion PCR #2 - DP

For the second round of fusion PCR, the following primers were used for each dual promotor:

Dual promotor	**Primers**
DP1	29, 31
DP2	33, 37
DP3	31, 35
DP4	31, 37

The following volumes were used:

(The DNA template is the product, from the “Fusion PCR #1, for each corresponding sample.)

ddH2O	33,5 µl
PrimeStar buffer (5X)	10 µl
dNTP (10mM)	1 µl
Primer (up)	1,5 µl
Primer (down)	1,5 µl
DNA template	2 µl
PrimeStar polymerase	0,5 µl

The PCR was run according to the following protocol:

1. 95 °C - 3 min
2. 98 °C - 10 s
3. 55 °C - 15 s
4. 72 °C - 1 min 40 s
5. 72 °C - 10 min

with steps 2-4 being cycled through 35 times

PCR amplification of genes

Gene labels and primers used for PCR

Genes	**Label (PCR tube)**	**Primer 1 (label on primer tube)**	**Primer 2**
bphA1	1	bphA1_Fwd (01)	bphA1_Rvs (02)
bphA2	2	bphA2_Fwd (03)	bphA2_Rvs (04)
bphA3	3	bphA3_Fwd (05)	bphA3_Rvs (06)
bphA4	4	bphA4_Fwd (07)	bphA4_Rvs (08)
bphB	B	bphB_Fwd (09)	bphB_Rvs (10)
bph	C	bphC_Fwd	bph_Rv

C		(11)	s (12)
bphD	D	bphD_Fwd (13)	bphD_Rvs (14)
bphK	K	bphK_Fwd (15)	bphK_Rvs (16)
pcbA5	5	pcbA5_Fwd (17)	pcbA5_Rvs (18)

WHAT WE DID:

We diluted the gene working stock to 1ng/µl.

We started our first PCR.

1. PCR 1, Genes(1-9) PrimeStar

A master mix was made out of H2O, dNTP and Buffer.

- 32 microL H2O
- 1.25 microL primer 1
- 1.25 micro-L primer 2
- 10 micro-L Buffer
- 4 micro-L dNTP
- 0.5 micro-L Enzyme
- 1 micro-L Template

PCR settings: PrimeStar PCR; Temp : Time (we have noted what we remember)
94oC,
52oC,
72oC : 1:30 (1min/kb)

Gel settings:

- 1 µl loading dye, 1 µl PCR solution, loaded the wells with 2 µl
- 15 wells
- 90 V, 30 min,
- post staining

Results: Fail. No results, only primer clouds.

Trouble shooting: We decided to do a Never Fail PCR instead.

2.1 Gene PCRs 2, Prime Star, Never Fail

This time we did both regular gene sequences and genes with tags.

Never Fail, and more amount of template, to ensure that it was not to little.
A master mix was made out of H2O, dNTP and Buffer.

- 23 and 28 µl H2O
- 1.25 µl primer 1
- 1.25 µl primer 2
- 10 µl Buffer
- 4 µl dNTP
- 0.5 µl Enzyme
- 10 and 5 µl Template

PCR settings: Never Fail; Temp : Time (we have noted what we remember)

[Unknown?]

Gel settings:

- 1 µl loading dye, 1 µl PCR solution, loaded the wells with 2 µl
- 15 wells
- 90 V, 30 min,
- post staining

Results: Fail. No results, only primer clouds.

DATE: 18/6

WHAT WE DID:

Gel electrophoresis - DP

1 µl of each fusion PCR product was run on a gel (1% Agarose, 90V, 30 min, GelRed post-staining) to examine results of fusion PCR. The expected fragments lengths were

DP1	1426 bp
DP2	1706 bp
DP3	1181 bp
DP4	1442 bp

The results showed clear bands at the expected lengths, in addition to other byproducts. The remaining PCR product was put on a gel, identical to the previous with the exception that the staining was done using GelGreen.

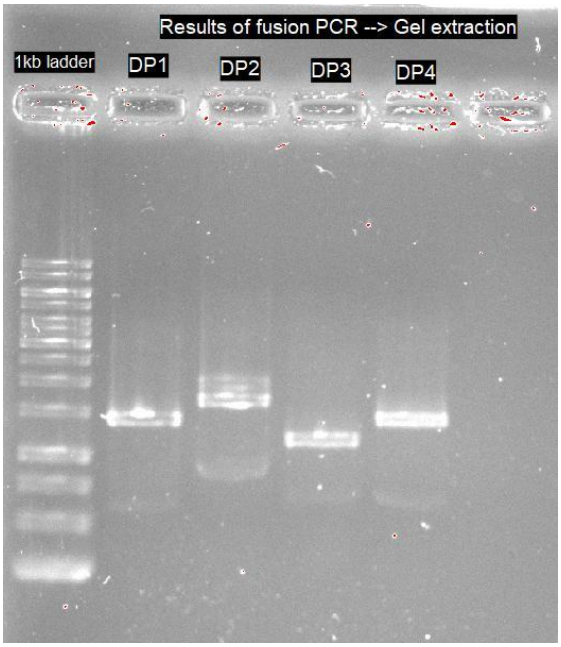
Gel purification - DP

The desired fragments were then cut out from the gel and purified according to standard protocol (GeneJet GelExtraction).
The concentrations were measured after purification to be the following:

DP1	34,4 ng/µl
DP2	27,2 ng/µl
DP3	22,0 ng/µl
DP4	16,0 ng/µl

Gel electrophoresis - DP

1 µl of each product, purified from the previous gel, was run on a gel (1% Agarose, 90V, 30 min, GelRed post-staining) to confirm a successful gel purification.



A successful purification was confirmed.

Amplification PCR - DP

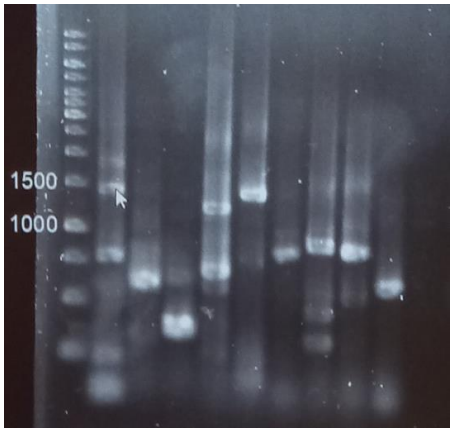
An additional PCR was performed on the purified double promotors in order to obtain higher concentrations (note that there is a higher risk of mutations). The primers, concentrations and PCR protocol used were the same as for the second round of fusion PCR, except that 1 µl of template DNA was used instead of 2 µl.

3. Gene PCR Never Fail, First Success! GPCR3

- 31 µl H2O
- 1.25 µl primer 1
- 1.25 µl primer 2
- 10 µl Buffer
- 1 µl dNTP
- 0.5 µl Enzyme
- 5 µl Template

PCR settings: Never Fail; Temp : Time
Fill in settings here!
Well-order:
bphA1
bphA2

bphA3
bphA4
pcbA5
bphB
bphC
bphD
bphK



4. PCR genes shorter annealing time, GPCR4

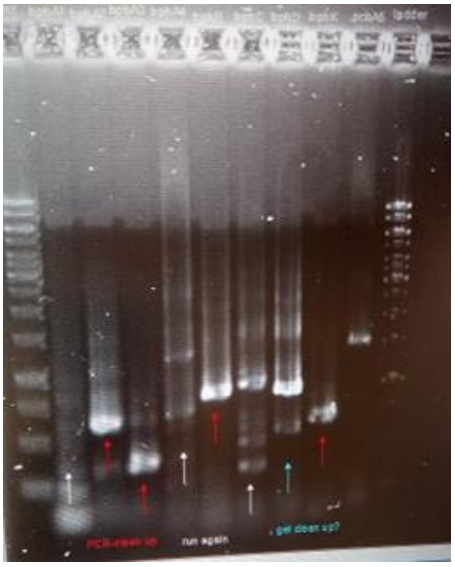
- 31 µl H2O
- 1.25 µl primer 1
- 1.25 µl primer 2
- 10 µl Buffer
- 1 µl dNTP
- 0.5 µl Enzyme
- 5 µl Template

PCR settings: Never Fail; Temp : Time

1. 95oC : 2:00
2. 95oC : 0:20
3. 60oC : 0:05
4. 72oC : 1:30
5. GOTO 2, 20 times
6. 95oC : 0:20
7. 53oC : 0:05
8. 72oC : 1:30
9. GOTO 6 20 times
10. 14oC forever

OBS! Different well-order

bphA1
bphA2
bphA3
bphA4
bphB
bphC
bphD
bphK
pcbA5



GPCR5 : Phusion polymerase: pbhA1, pbhA4, pbhA4, pbhD, pbhC, pcbA5

98 C: 30 sec
{
98C: 10 sec
70 C: 30 sec
72 C: 45 sec
}x 30 cycles
72 C: 5 min
12 C: forever

Did not work very well

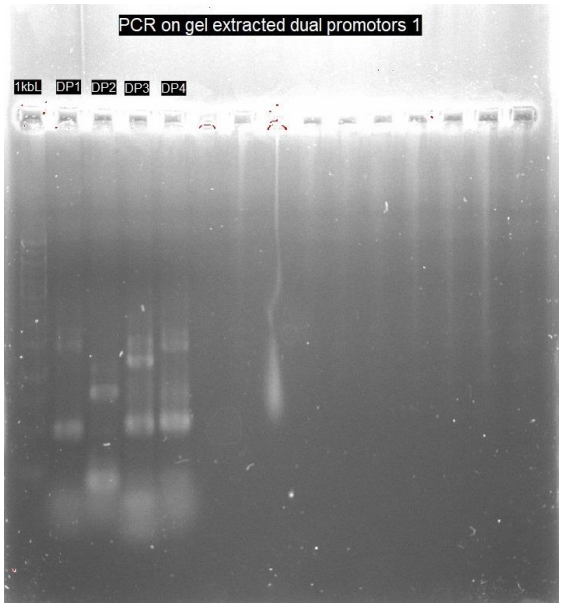
DATE: 19/6

WHAT WE DID:

Gel electrophoresis

1 µl of each PCR product (except for DP1, where 2 µl was accidentally used), was run on a gel (1% Agarose, 90V, 30 min, GelRed post-staining) to confirm a successful gel purification.

The expected length of the bands can be seen in the log for 18/6.



PCR troubleshooting

The gel results showed several, unwanted, bands. After analysis the following modifications were made to the PCR program:

- Increase annealing temperature by 3°C
- Decrease annealing time to 5 s.

Pipetting fail→PCR purification

During the pipetting, for the PCR, primers were accidentally transferred to the template DNA. Therefore the double promotor stocks were cleaned, using PCR purification.

Modified PCR - DP

A new, additional PCR was performed on the purified double promotors in order to obtain higher concentrations (note that there is a higher risk of mutations). The primers and concentrations used were the same as for the second round of fusion PCR, except that 1 µl of template DNA was used instead of 2 µl.

The PCR was run according to the following protocol:

1. 95°C - 3 min
2. 98°C - 10 s
3. 58°C - 5 s
4. 72°C - 1 min 40 s
5. 72°C - 10 min

with steps 2-4 being cycled through 35 times.

These are the same settings used as for the last amplification PCR, except for the annealing step, where the temperature was increased by 3°C to 58°C and the time was decreased to 5 s, from 15 s.

Gel electrophoresis

1 µl of each PCR product, was run on a gel (1% Agarose, 90V, 30 min, GelRed post-staining) to examine whether the modified PCR program produced the expected results.

[INSERT GEL RESULT - TILIA]

5. PCR of genes: bphA1, A4, C & D, & pcbA5

With this PCR we wanted to take the gene that did not show very nice bands on our last two PCRs, and try to get better bands for them. We did a PhusionPCR with some altered temperatures, because we

noticed that the annealing temp. was quite high for all primers of the genes.

We also noticed that the temp. difference between primer 1 and 2 was larger for these genes than for the ones that gave really nice bands previously.

PhusionPCR

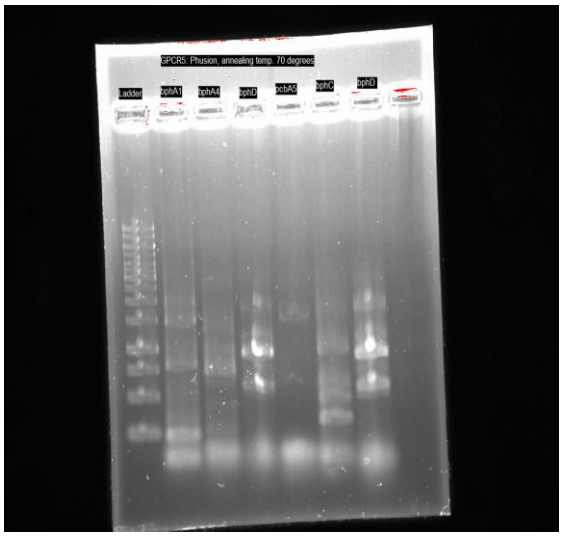
- 28.5 µl H2O
- 2.5 µl primer 1
- 2.5 µl primer 2
- 10 µl Buffer
- 1 µl dNTP
- 0.5 µl Enzyme
- 5 µl Template
- No DMSO!

Phusion PCR settings:

1. 98oC : 0:30
2. 98oC : 0:10
3. 70oC : 0:30
4. 72oC : 0:45
5. GOTO 2, 30 times
6. 72oC : 5:00
7. 12oC : forever

Well-order:
bphA1, bphA4, bphD, pbcA5, bphC, bphD

Results:



Bad, not useable

6. PCR of genes bphA1, A4, C, GPCR6

- 31 µl H2O
- 1.25 µl primer 1
- 1.25 µl primer 2
- 10 µl Buffer
- 1 µl dNTP
- 0.5 µl Enzyme
- 5 µl Template

PrimeStar PCR, Never Fail
Changes in yellow

1. 95oC : 2:00
2. 95oC : 0:20
3. 60oC : 0:09
4. 72oC : 1:30
5. GOTO 2, 20 times
6. 95oC : 0:20
7. 55oC : 0:09
8. 72oC : 1:30
9. GOTO 6 20 times
10. 14oC forever

Results: Bad I think?

7. PCR clean-up for genes bphA2, A3, B & K

PCR Purification protocol

- Add isopropanol to bphA3, because it is <500 bp.

Nanodrop results
bphA2: 170.2 ng/µl
bphA3: 246.2 ng/µl
bphB: 225.1 ng/µl
bphK: 218.7 ng/µl

Results: Coming 20/19

8. Gel extraction of bphD & pcbA5 from both GPCR3 & GPCR4

Well-order:
GPCR4 - bphD
GPCR3- bphD
GPCR4- pcbA5
GPCR3 - pcbA5

100 V, 30 min

Results:
Did not get bphD

Gel weight for pcbA5:
(There are two weights since we took two bands, coming from different PCRs)GPCR4 - pcbA5: 0.14315 g
GPCR3 - pcbA5: 0.15606 g

DATE: 20/6

WHAT WE DID:

Modified PCR - DP (61 °C)

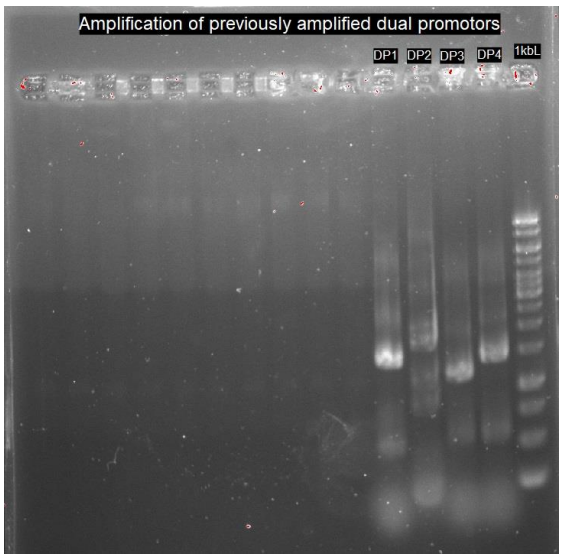
Volumes and procedure were the same as for the 58°C PCR, except that the temperature was raised by an additional 3°C, to 61°C

1. 95°C - 3 min
2. 98°C - 10 s
3. 61°C - 5 s
4. 72°C - 1 min 40 s
5. 72°C - 10 min

with steps 2-4 being cycled through 35 times.

Gel electrophoresis

1 µl of each PCR product (except for DP1, where 2 µl was accidentally used), was run on a gel (1% Agarose, 90V, 30 min, GelRed post-staining) to confirm a successful gel purification.



PCR purification x2 (fail)

A PCR purification was performed, in an attempt to remove the bands of smaller sizes. In the elution step the tubes were not switched to new, uncontaminated, tubes. Therefore another PCR cleanup had to be performed (of the product from the first purification), which may have resulted in lower yields overall.

Gel electrophoresis

1 µl of each PCR product (except for DP1, where 2 µl was accidentally used), was run on a gel (1% Agarose, 90V, 30 min, GelRed post-staining) to confirm a successful gel purification.

9. Gel Purification of pcbA5

Gel Purification Protocol

- 143.2 µl of Binding Buffer was added to.
- 156.1 µl of Binding Buffer was added to GPCR3 - pcbA5.
- We added elution buffer, first for GPCR4 - pcbA5, and then transferred the supernatant to GPCR3 - pcbA5, to elute that one.

Nanodrop results:

pcbA5: 67.6 ng/µl

10. PCR for genes bphA1, A4, C, D; GPCR7

Prime Star PCR

- 31 µl H2O
- 1.25 µl primer 1
- 1.25 µl primer 2
- 10 µl Buffer
- 0.8 µl dNTP
- 0.5 µl Enzyme
- 5 µl Template

Settings, NeverFail

1. 95oC : 2:00
2. 95oC : 0:20
3. 60oC : 0:10
4. 72oC : 1:30
5. GOTO 2, 20 times
6. 95oC : 0:20
7. 50oC : 0:10
8. 72oC : 1:30

9. GOTO 6 20 times
10. 14oC forever

Well order: A1, A4, C, D
90V, 35 min, Post Staining, gel Red

Results: Half Success!
Weak bands, but it looked like bphC and D had single bands. bphA1 and A4 did not look good. They seemed to lack bands (especially A4, see picture on Ellens account). It seemed like the staining was poor. Ladder was also weak. Thus, we did purification of C and D:

11. PCR purification for genes bphC, bphD

PCR purification protocol

- Assumed 49 µl PCR product in each sample. (Might have had 48 µl in one of them, unsure which) → Add 49 µl Binding Buffer

Nanodrop results
bphC: 132.6 ng/µl
bphD: 117.5 ng/µl

Next week:

Improve amplification for genes bphA1 and A4, or/and order primers for them.

Since the staining was poor for GPCR7 (saved on Ellens account), consider doing a gel on A1 and A2 PCR products, and on C and D PCR cleanup products, with better staining, to confirm.

DATE: 1/7

WHAT WE DID:

Two-step PCR with higher temperature with DP (fail)

A two-step PCR with higher temperature were performed for the double-promotors (DP), in purpose to reach higher concentrations.
Solutions was prepared as followed:

DP1; DP2; DP3; DP4	
ddH2O	34,5µl
PrimeStar buffer	10 µl
dNTP	1 µl
Primer (29;33;31;31)	1,5 µl
Primer (31;37;35;37)	1,5 µl
Template	1 µl
PrimeStar polymerase	0,5µl
Total amount	50 µl

The PCR was run according to the following protocol:

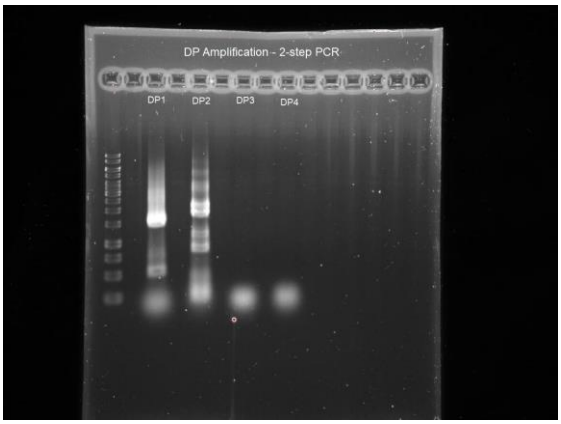
1. 95°C - 3 min
2. 98°C - 10 s
3. 68°C - 1min 40 s
4. 68°C - 10 min
5. 15°C - infinity

with steps 2-3 being cycled through 35 times.

Gel electrophoresis

The lengths can be seen under *date 18/6- Gel electrophoresis - DP.*
1 µl of DP1,DP2 and DP4 (2 µl for DP3) were mixed with 1µl loading dye and 2µl ladder.

The product was run on gel (1% Agarose, 90V, 30 min, GelRed post-staining).



No good bands, smeared for DP1 and DP2. Only one bigger band for DP3 and DP4 but not on the corrects position to benefit our result.

Go for Gibson with existing concentrations.

Amplification of tagged Genes (GTPCR1)

Genes bphA2, bphA3, bphB and bphK same as GPCR 4. pcbA5 and bphD were also included, without expectation for good results.

- 1 μl - dNTP
- 5 μl - template
- 10 μl - buffer
- 0.5 μl - primestar polymerase
- 1.25 μl - primer x2
- 31 μl H2O

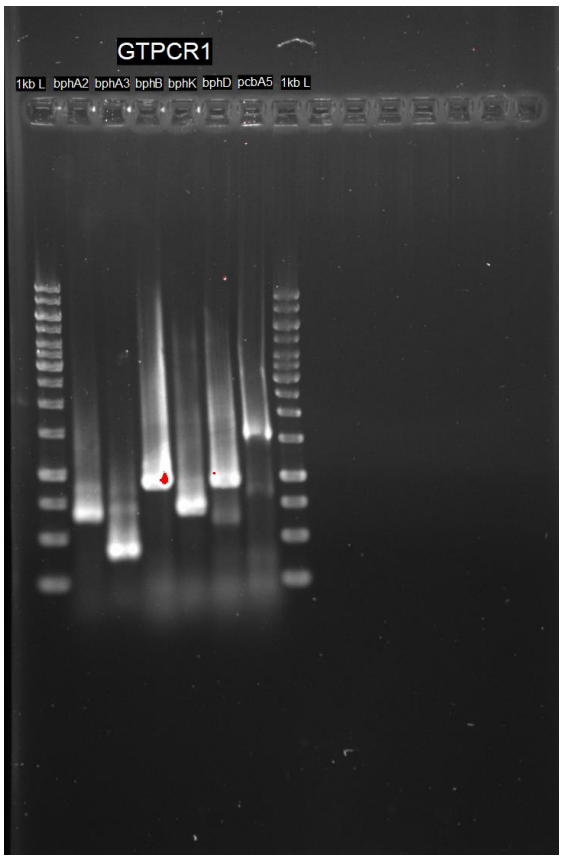
Never fail PCR protocol used

1. 95oC : 2:00
2. 95oC : 0:20
3. 60oC : 0:05
   - 0.6 per cycle
4. 72oC : 1:30
5. GOTO 2, 20 times
6. 95oC : 0:20
7. 53oC : 0:05
8. 72oC : 1:30
9. GOTO 6 20 times
10. 14oC forever

The PCR products were run on a short gel, for 15 min at 90V

[INSERT GEL PICTURE]

The results showed good results for bphA2, bphA3, bphB and bphK. Another longer gel was run for confirmation, and PCR cleanup was performed according to standard protocol. Note that isopropanol was added to bphA3 due to its length being merely 350bp.



The 2nd gel confirmed that the results for bphA2, bphA3, bphB, bphK were good enough for PCR purification. In addition, from these results it was decided that gel purificaiton would be performed for bphD and pcbA5.

Achieved concentrations from PCR purification of bphA2, bphA3, bphB, bphK:

Gene	**Concentration**
bphA2	105.1 ng/µl
bphA3	129.3 ng/µl
bphB	104.8 ng/µl
bphK	68.3 ng/µl

DATE: 2/7

Measuring concentrations of the amplified double-promotors. (Tim)

Measured concentration with nanodrop.

Amplified DP	Concentration in ng/µl
DP1*	158,6
DP2*	127,7
DP3*	130,7
DP4*	134,6

This concentrations was used in calculation for Gibson but the purified DP was used in the actual gibson assembly.

Pcf2903 Plasmid linearization using RE SFAA1

Linearization of the Pcf2403 was performed, using the restriction enzyme **SFAA1**, as a linear fragment is needed for Gibson assembly. The disgestion was made according to the standard protocol.

Gibson Assembly Plasmid 3 and Plasmid 4 (Erik and Ellen)

Note: Here a negative control was made, this is not needed for the future. Don't make a negative control (many plates is wasted for almost nothing).

Gibson assembly was performed according to standard protocol and positive mixtures were made for plasmid 3 and plasmid 4, according to the table below. Because of the small volumes required 5x-dilutions were made, for several of the promoters and genes and backbones.

Negative controls were also made, where the Gibson mix was substituted for water.

	P3	P4
Gibson master mix	10µl	10µl
Mq-water	0,9µl	5,79µl
DP	2,25µl *	0,56µl
Gene 1	1,0µl * (bhpB)	2,1µl * (bphD)
Gene 2	1,85µl * (bphC)	0,85µl * (bphK)
Backbone	4µl * (Pcf2903)	0.7µl (Pcfb3034)
Total volume:	20µl	20µl

*5x-diluted

The gibson mix for plasmid 3 was remade the next day, according to table below (according to protocol).

Gel extraction of bphD and pcbA5

The samples were loaded on gel, run for 30 min on 90V and post-stained with GelGreen. 5 µl green buffer was added to 49 µl of PCR product, and 24 µl of this mixture was added to each well (2 wells per gene)

Weight of gel fragments:

Gene	**Empty tube**	**Tube + Gel**	**Gel**
pcbA5 - tag	1099.6 mg	1231.2 mg	131.6 mg
bphD - tag	1103.1 mg	1235.7 mg	132.6 mg

Gel cleanup was performed according to standard protocol.

Achieved concentrations:

Gene	Concentration
pcbA5 - tag	13.4 ng/µl
bphD - tag	27.0 ng/µl

It was decided that the samples would be concentrated, however the concetration was overdone and all the water evaporated…
A volume of water was added to each sample such that the concetrations would become ~30 ng/µl (sufficient for Gibson).

Resulting solutions:

Gene	Volume	New conc.
pcbA5 - tag	20 µl	34.0 ng/µl
bphD - tag	40 µl	26.0 ng/µl

PCR of His-tagged bphC (GTPCR2)
Primers used: 11 and 24
PCR protocol (equal to GPCR7):
Prime Star PCR

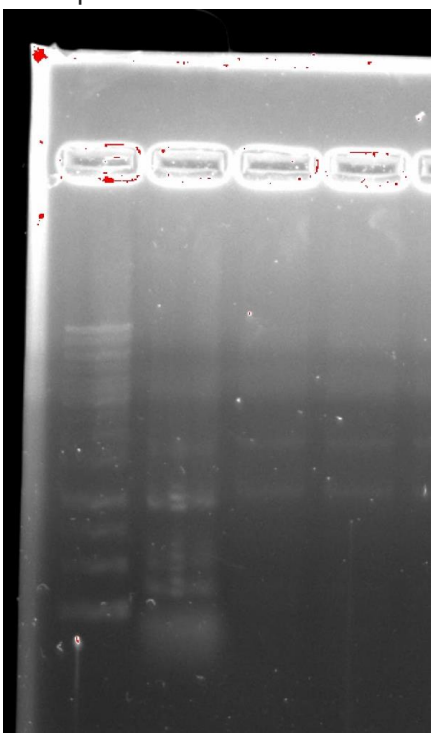
- 31 µl H2O
- 1.25 µl primer 1
- 1.25 µl primer 2
- 10 µl Buffer
- 0.8 µl dNTP
- 0.5 µl Enzyme
- 5 µl Template

Settings, NeverFail

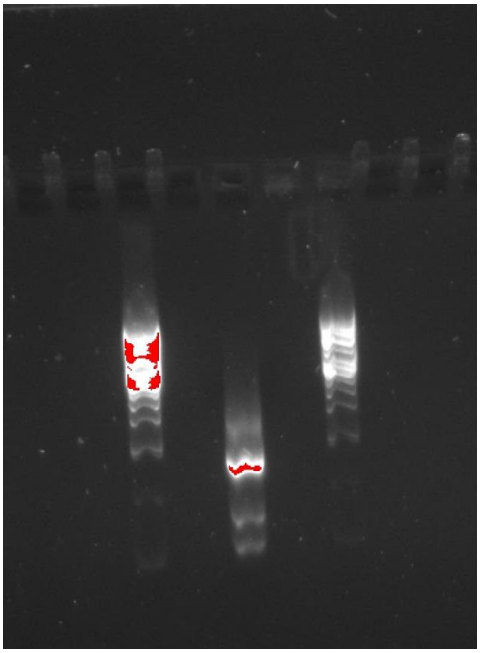
1. 95oC : 2:00
2. 95oC : 0:20
3. 60oC : 0:10
4. 72oC : 1:30
5. GOTO 2, 20 times
6. 95oC : 0:20
7. 50oC : 0:10
8. 72oC : 1:30
9. GOTO 6 20 times
10. 14oC forever

The PCR product was run on a gel, however the first attempt produced many bands that were probably the result of contaminated buffer. Another (prestained) gel aws therefore run (small gel, 140V, 15 min).

Attempt 1:



Attempt 2:



The resulting gel showed three bands, out of which one seemed to be the one we wanted. However it looked to lie around 750bp instead of ~1000bp, however it was till decided to do gel extraction of bphC.

Gel extraction of tagged bphC from GTPCR2

Weight of gel fragments:

Gene	Empty tube	Tube + Gel	Gel
bphC - tag	1102.1 mg	1269 mg	167 mg

Gel cleanup was performed according to standard protocol.

Achieved concentrations:

Gene	Concentration
bphC - tag	14.8 ng/µl

Need to re-do...

Plasmid MiniPrep: EasyClone vector pcfb2903

Culture volume prepared: 20 ml
Ampicillin added: 25 µl

Final concentration: 222.1 ng/µl
Final volume: 180 µl

Standard protocol was followed except for two points:

1. Elution buffer was heated before use
2. After the empty column was spun down, the column was heated for 5 min

DATE: 3/7

Gibson Assembly Plasmid 3

Note: Here a negative control was made, this is not needed for the future. Don't make a negative control (many plates is wasted for almost nothing).

The gibson mix for plasmid 3 was remade, according to table below (according to protocol):

	Positive	Negative
Gibson master mix	10µl	-
Mq-water	0,9 µl	10,9 µl
DP3(5x-diluted)	2,25µl	2,25µl
bhpB (5x-diluted)	1,0µl	1,0µl
bhpC (5x-diluted)	1,85µl	1,85µl
Backbone (Pcf2903) linearized date: 2/7	4µl	4µl
Total volume:	20µl	20µl

Note: Gibson is expensive and 20 µl is not needed, a total volume of 10 µl works just fine. So for the future, use half as much of the components.

E.coli transformation

E.coli transformation was performed for both plasmid 3 and 4. The transformations steps was performed according to the new E.coli transformation protocol.

The plating was performed using glass beads and split into 10% and 90% for each transformation. The plates was incubated overnight (16h).

GPCR8

New primers arrived!
PCR was performed on the genes bphA1, bphA4 and pcbA5 (need new version with different promotor overhang)

The following primers were used:

Gene	Primers
bphA1	41, 39
bphA4	44, 39
pcbA5	45, 40

PCR protocol:

Prime Star PCR

- 31 µl H2O
- 1.25 µl primer 1
- 1.25 µl primer 2
- 10 µl Buffer
- 1 µl dNTP
- 0.5 µl Enzyme
- 5 µl Template

Settings

1. 95oC : 3:00
2. 98oC : 0:10
3. 55oC : 0:10
4. 72oC : 1:40
5. GOTO 2, 35 times
6. 72oC : 10:00
7. 15oC forever

DATE: 4/7

WHAT WE DID:

Colony PCR and single colony plate- DreamTaq

A PCR-master mix was performed, see below (protocol x4)

10x Dream taq buffer	20µl
dNTPs (10mM)	4µl
Primer 1 (fwd)	6µl
Primer 2 (rvs)	6µl
Template DNA	-
DreamTaq-polymerase	2µl
MQ-water	162µl
total volume	200µl

The master mix was gently mixed with the pipette after all components have been added.

Then 20 PCR-tubes was marked and 10µl of the master mix was added to the tubes. 10 colonies from each 90% plate were selected and marked with Plasmid 3:1-10 respectively Plasmid 4: 15-24.
The colonies was taken up by a pipette tip and gently spread to a new plate, the tip added to each PCR tube. Let the tip stay in the PCR tube and then shake the rack gently. When you take up the tip don't forget to press your finger against it to avoid solution to get stuck in the tip.

Primers for the colony PCR was given from Dharmik. The primers was prepared and now exists in a storing stock 1x10 dilution (100 µl from primer solution and 900µl autoclaved water (new) and working stock (100µl from storage). They are named 46 and 47 (his system 31 and 32).

The primers had an annealing temperature at 53 °C and the length 1,5 kb (+ added insert).

The total insert length for the plasmid were:

Plasmid 3	7,3 kb
Plasmid 4	7,4 kb

The PCR was run according to the following protocol:

1. 95°C - 3 min
2. 98°C - 30 s
3. 53°C - 30 s
4. 72°C - 9 min 50 s
5. 72°C - 15 min

with steps 2-4 being cycled through 29 times.

Note: Dream taq poly. takes a really long time and have a maximum capacity around 6kb and we have 10kb. In the future use Phusion (works for 10kb and takes shorter time).

Promotor- PCCW12 PCR1

For both PCR the following primes was used:

First PCR	Number	Melting temperature
Primer 1	38 short	62,5°C
Primer 2	35	66°C
Second PCR		
Primer 1	38 long	60,1°C
Primer 2	35	66°C

The following volumes were used for PCR 1. (only the first PCR were performed during this day)

ddH2O	34 µl
PrimeStar buffer	10 µl
dNTP	1 µl
Template DNA	2 µl
Primer 1	1,25 µl
Primer 2	1,25 µl
PrimeStar polymerase	0,5 µl
Total volume	50 µl

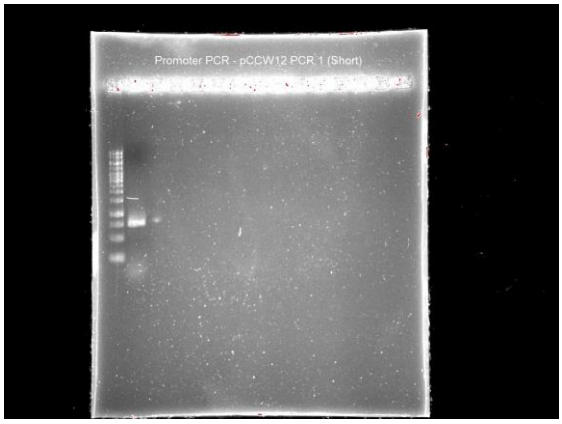
The PCR was run according to the following protocol:

1. 98°C - 1 min
2. 98 °C - 10s
3. 55 °C - 15s
4. 72 °C - 1 min
5. 72 °C - 4 min

with steps 2-4 being cycled through 32 times

Gel electrophoresis- PCCW12 PCR1

The PCR product was run on gel (1% Agarose, 90V, 30 min, GelRed post-staining).

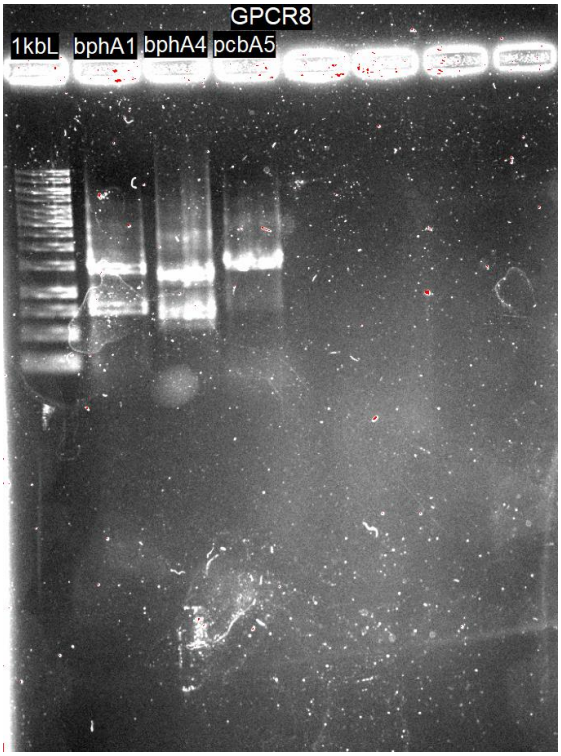


One band around 750 bp which means we have the right fragment.

PCR clean up and Nanodrop

A PCR clean up was performed on the rest of the PCR1 product, according to protocol. The concentration was checked to 112,2 ng/µl with nanodrop.

The products from GPCR8 were run on a gel (30 min, 90V)



Two clear bands were seen for A1 and A4 → re-do PCR with increased annealing temp. and decreased annealing time. Also do PCR cleanup for pcbA5 since results were good.

PCR cleanup

PCR cleanup of pcbA5 was performed according to standard protocol.
Achieved concentration:

Gene	Concentration
pcbA5	153.3 ng/µl

GPCR9

PCR protocol:

Prime Star PCR

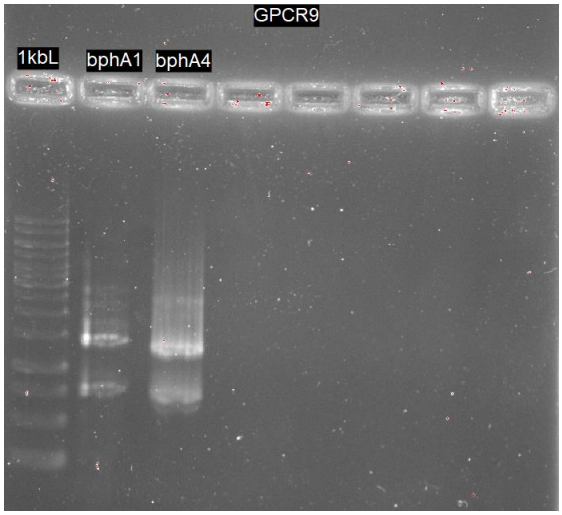
- 31 µl H2O

- 1.25 µl primer 1
- 1.25 µl primer 2
- 10 µl Buffer
- 1 µl dNTP
- 0.5 µl Enzyme
- 5 µl Template

Settings

1. 95oC : 3:00
2. 98oC : 0:10
3. 58oC : 0:05
4. 72oC : 1:40
5. GOTO 2, 35 times
6. 72oC : 10:00
7. 15oC forever

The products were run on a gel (30 min, 90V)



The results were pretty much the same as for GPCR9 → Combine the PCR products from GPCR8 and GPCR9 and do a gel cleanup!

Gel cleanup

A thicker gel as made, and 50 µl PCR product went into each well (two wells for each gene).

Weight of gel fragments:

Gene	Empty tube	Gel
bphA1	1104.2 mg	150.7 mg
bphA4	1099.9 mg	146.3 mg

Gel cleanup was performed according to standard protocol.

Achieved concentrations:

Gene	Concentration
bphA1	64.7 ng/µl
bphA4	53.8 ng/µl

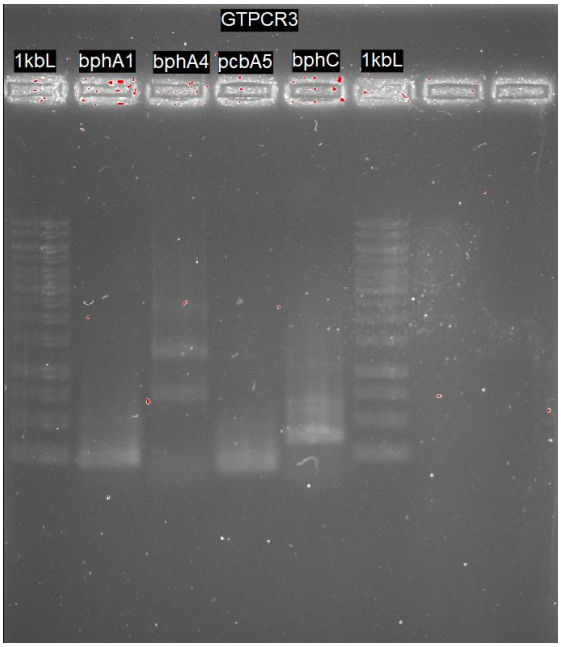
PCR of tagged bphC, bphA1, bphA4 and pcbA5 (GTPCR3)

The following primers were used:

Gene	Primers
bphC	11, 24
bphA1	19, 41
bphA4	22, 44
pcbA5	27, 45

Unknown protocol?

Products were run on a gel



No results from A1, A5 or C → Gel extract bphA4 - tag!

Plasmid miniprep: gRNAs and vectors

Miniprep followed as protocol, except for the changes noted earlier:

- Hot elution buffer used
- Tubes heated after empty tube spin-down

Achieved concentrations

Plasmid	**Concentration**
pcfb3042	265.8 ng/µl
pcfb3045	286.9 ng/µl
pcfb3035	173.5 ng/µl

GTPCR4

New PCR on tagged A1, A5 and C.

- 31.2 µl H2O
- 1.25 µl primer 1
- 1.25 µl primer 2
- 10 µl Buffer
- 0.8 µl dNTP
- 0.5 µl Enzyme
- 5 µl Template

PrimeStar PCR, Never Fail

1. 95oC : 2:00
2. 95oC : 0:20
3. 60oC : 0:09
4. 72oC : 1:30
5. GOTO 2, 20 times
6. 95oC : 0:20
7. 55oC : 0:09
8. 72oC : 1:30
9. GOTO 6 20 times
10. 14oC forever

No gel clean up was done for bphA4, because the bands were not good enough.

Results: Bad

DATE: 5/7

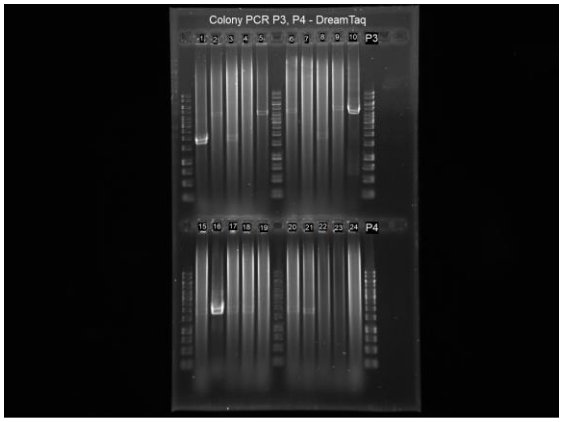
MEMBERS THIS DAY:

- Erik
- Moa
- Tim

WHAT WE DID:

Gel electrophoresis P3 and P4- Colony PCR Dream Taq

The colony PCR product(dream taq) was run on gel (1% Agarose, 120V, 30 min, GelRed post-staining).



No good bands on the right place, which confirms that Dream taq didn't work properly.

Colony PCR for plasmid 3 and 4 - Phusion

Colonies from plate, take samples from same colonies as the previous colony PCR (not the same plate).

A PCR master mix was prepared according to protocol:

MQ-water	134µl
5xHF buffer	40µl
dNTPs (10mM)	4µl
Primer 1 (46)	10µl
Primer 2: 47	10µl
Template DNA	--
Phusion Polymerase	2µl
total volume	200µl

The master mix was gently mixed with the pipette after all components have been added.

Then 20 PCR-tubes was marked and 10µl of the master mix was added to the tubes. Colonies from the plate was added with a pipette tip. Let the tip stay in the PCR tube and then shake the rack gently. When you take up the tip don't forget to press your finger against it to avoid solution to get stuck in the tip.

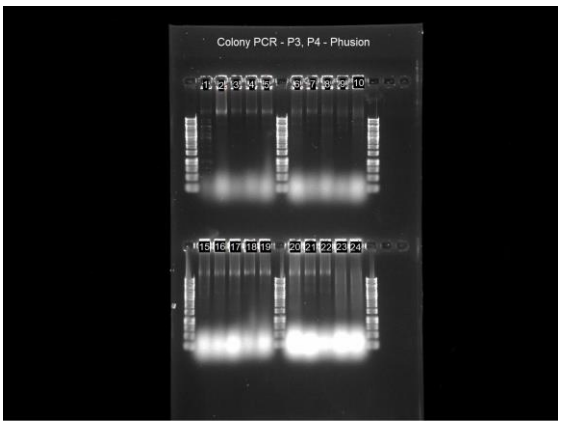
The PCR was run according to the following protocol:

1. 98°C - 3 min
2. 98°C - 10 s
3. 53°C - 30 s
4. 72°C - 4 min 30s
5. 72°C - 10 min

with steps 2-4 being cycled through 25 times.

Gel electrophoresis P3 and P4- Colony PCR Phusion

The colony PCR product(phusion) was run on gel (1% Agarose, 120V, 20 min, GelRed post-staining).



Good band for every colony except for 23 and 24.

Promotor- PCCW12 PCR2

The following volumes were used for PCR2.

ddH2O	27,9 µl
PrimeStar buffer (5x)	10 µl
dNTP master mix	4,1 µl
Template DNA	5 µl
Primer 1 (38 long)	1,25 µl
Primer 2 (35)	1,25 µl
PrimeStar polymerase	0,5 µl
Total volume	50 µl

The dNTP master mix was created by mistake. The master mix equals the amount of 4,1 µl master mix = 1µl dNTP (10mM)

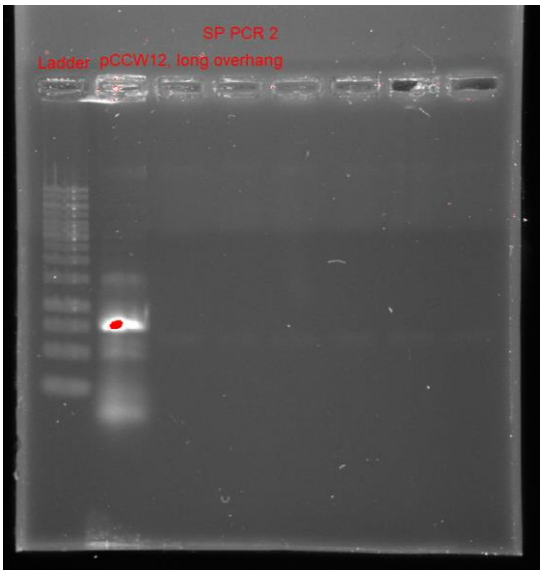
The PCR was run according to the following protocol:

1. 98°C - 1 min
2. 98 °C - 10s
3. 55 °C - 15s
4. 72 °C - 1 min
5. 72 °C - 4 min

with steps 2-4 being cycled through 32 times

Gel electrophoresis- pCCW12 PCR2

The PCR product was run on gel (1% Agarose, 90V, 30 min, GelRed post-staining).



Strong band at 750 bp but some extra bands. The strong band is cut out and now kept in a tube (freezer). **A gel clean up will be performed next week.**

For gel clean up:
Tube weight: 1009,0 mg
Tube+ gel: 1144,3 mg
Gel= 135,3mg

DATE: 8/7

GTPCR5

New PCR on tagged A1, A4, A5 and C.

- 31.2 µl H2O
- 1.25 µl primer 1
- 1.25 µl primer 2
- 10 µl Buffer
- 0.8 µl dNTP
- 0.5 µl Enzyme
- 5 µl Template

PrimeStar PCR, Never Fail

1. 95oC : 2:00
2. 95oC : 0:20
3. 60oC : 0:09
4. 72oC : 1:30
5. GOTO 2, 20 times
6. 95oC : 0:20
7. 55oC : 0:09
8. 72oC : 1:30
9. GOTO 6 20 times
10. 14oC forever

Gel Electrophoresis GTPCR5.1:

Ladder, A1, A4, A5, C

Results: No bands, not even on the ladder.

We ran a new gel, from the same PCR samples (same settings)

Gel purification, pCCW12

Gel was weighed again, because we couldn't find any weight from last week.
Gel weight: 0,1132 g → 113,2 µg.

Followed gel purification protocol.

- No optional steps were done.
- Binding buffer added: 113,2 µl.

Nanodrop: pCCW12: 33,3 ng/µl

Gel Electrophoresis GTPCR5. 2:

Ladder A1, A4 A5 , C

Results and Observations:
This time the ladder is much better! The bands for each of the tagged genes are the right lengths but the A1, A4 and C are weaker and double bands. Therefore, we decided to do PCR purification only on pcbA5_tag.

Nanodrop: pcbA5_tag: 156.6 ng/µl
Tomorrow: Gel purification on the rest?

Inoculation for miniprep:
Responsible person: Dharmik
Inoculation of Plasmids contained E.Coli in 15 ml ampicillin medium
pcfb2903
pcfb3035
pcfb3039

pcfb3042
pcfb3045

DATE: 9/7

Restriction digestion

Responsible people: Ellen and Dharmik
added 1 ug of vector backbone

	2904	3035	3039
backbone	4.41 ul	3.87ul	3.63ul
Enzyme +Buffer	2 + 2	2+2	2+2
Water	11.59	12.13	12.37
Total	20	20	20

Gibson Assembly:
Responsible people Ellen and Dharmik
Recipe for Gibson Used

1. *Gibson 3 aka Plasmid 2*
   D.P 4 - 3.3 ul
   BphA3 - 1 ul (25 x dilution)
   BphA4 - 0.77 ul
   pcfb3039 - 1 ul
2. *Gibson 4 aka Plasmid 1*
   DP 1 - 1.36 ul
   BphA1 - 0.72 ul
   BphA2 - 1 ul (10 x dilution)
   pcfb3035 - 1ul
3. *Gibson 5 aka Plasmid 0*
   pCCW12 - 1 ul (5 x dilution)
   pcbA5 - 0.35 ul
   pcfb2904 - 1 ul

Gel Clean up, GTPCR5
We decided to do a gel clean-up for bphA1, A4 and C.

Gel electrophoresis
Well order:
A1,A1, blank, A4, A4, blank, C, C

~30 µl in each well

48 µl PCR solution

12 µl Loading dye

Two wells per sample.

Gel extraction:

Gel weight:
bphA1: 0,23676 g
bphA4: 0,54378 g
bphC: 0,56723

Amount of Binding Buffer used:
bphA1: 237 µl
bphA4: 544 µl
bphC: 567 µl

Results Nanodrop:

bphA1: 29,4 ng/µl
bphA4: 24,1 ng/µl
bphC: 28,1 ng/µl

Good enough to use for Gibson!
Plasmid miniprep

Responsible person: Dharmik
Miniprep done as protocol except

1. Heated the elution buffer
2. Heated empty miniprep tubes before adding elution buffer to evaporate ethanol

pcfb2903 - 228.8 ng/ul
pcfb3035 - 275.6 ng/ul
pcfb3039 - 258.2ng/ul
pcfb3042
pcfb3045
(Must be almost 90 ul of these plasmids)

All tagged genes are now ready!

DATE: 10/7

Colony Restreak - Colonies from each Gibson plate were restreaked as a separate streak on new ampicillin plate
E. coli inoculation, plasmids 3 and 4

2 tubes.
In each tube:

- 5 ml LB media
- 5 µl Amp
- Cells from one colony on the plate

Plasmid 3: Colony 2 was used
Plasmid 4: Colony 16 was used.

OBS! There was a slight spill from the tube for plasmid 4 (without cells). We think it is no worries and that it wasn't a large volume.

DATE: 11/7

Colony PCR of Gibson

Primer(31 and 32) : annealing time - 5 min
annealing temperature: 53 C
Phusion polymerase needed

10 ul	buffer
1 ul	dNTP
1.5 ul	primer (x2)
0.5 ul	enzyme
35.5 ul	H2O

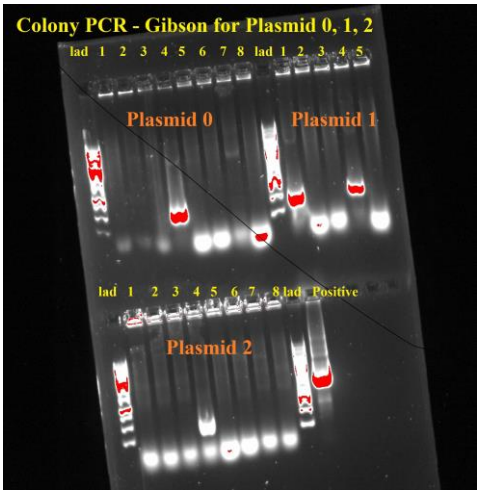
Positive control used - circular pcfb2903

PCR Cycle:

98 C - 10 min
98 C - 10 s
53 C - 30 s
72 C - 2 min 30 s
Goto 2 - 35x
72 C - 10 min
15 C - Forever

Expected Band size -
plasmid 0 - 3.65 kb
plasmid 1 - 4.65 kb
plasmid 2 - 4.85 kb
positive control - 1.5 kb

Gel run - 5 ul F.D green added to colony PCR samples. 140 V, 20 min



Freezing stock - E.Coli plasmid 3 and plasmid 4

800 µl culture + 200 µl 80 % glycerol

Plasmid miniprep of Plasmid 3 and Plasmid 4

Plasmid 3:132.7 ng/µl
Plasmid 4: 91.5 ng/µl

Date 12/7

Colony PCR:- P0, P1, P2

4 colonies per plasmid

Mastermix

5 µl Dreamtaq buffer
2 µl dNTP
1.25 µL primer 1
1.25 µl primer 2
0.5 µl Dreamtaq polymerase
42 µl water

***Primers used: P0 (40, 39), P1 (31,39), P2 (33,37)

For P0

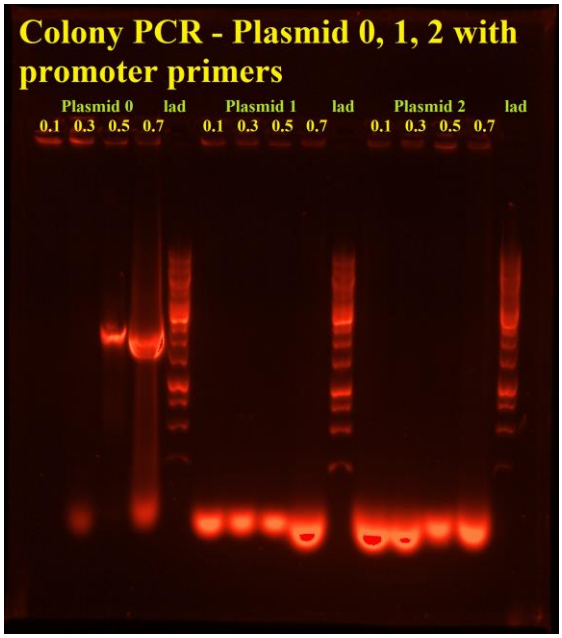
95 C: 3 min
{
95 C: 10 sec
53 C: 30 sec
72 C: 1 min 30 sec
}x26 cycles
72 C: 5 min
12 C: forever

For P1 and P2

95 C: 3 min
{
95 C: 10 sec
57 C: 30 sec
72 C: 1 min
}x26 cycles
72 C: 5 min
12 C: forever

DATE: 14/7

Gel Run of Colony PCR of Plasmid 0, 1, 2
by Tim and Tilia
Prestaining, 96 V, 40 min

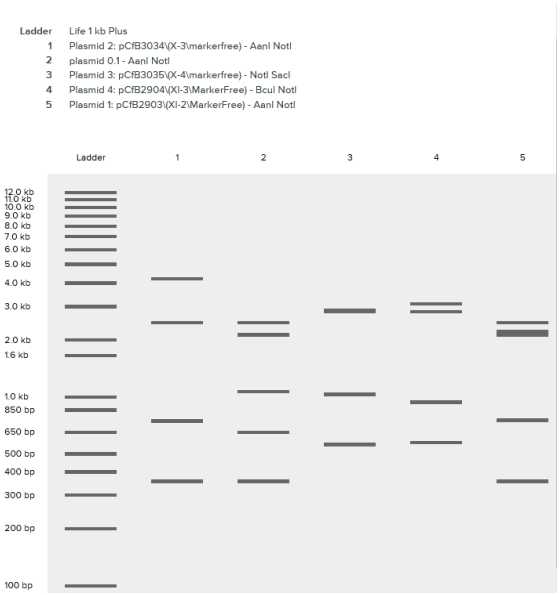


DATE: 15/7

WHAT WE DID:

Plasmid verification: P3 and P4

For plasmid verification the plasmids are digested with restriction enzymes.



For plasmid 3 and 4, Fastdigest NotI is used for both and SacL for plasmid 3 and BcuI for plasmid 4.

Fastdiegset restriction enzymes protocol was followed and the following mixture was prepared. The components were mixed in order.

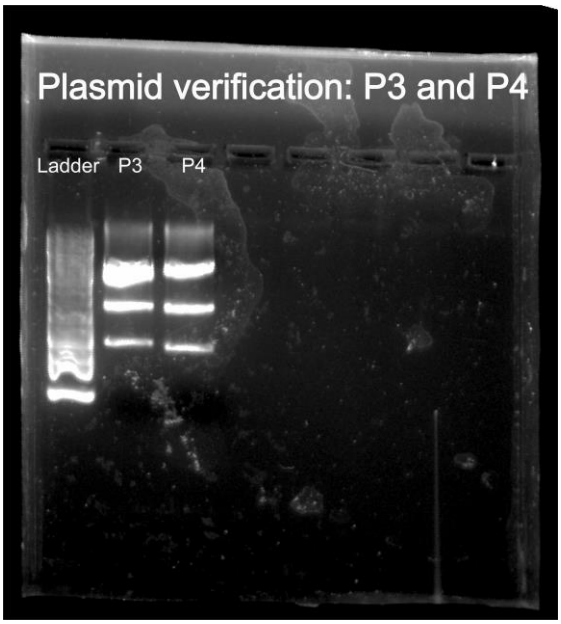
	Plasmid 3	Plasmid 4
water	14µl	14µl
10X fast diegest buffer	2µl	2µl
DNA	2µl (plasmid3)	2µl (plasmid 4)
Enzyme 1	1µl (NotI)	1µl (NotI)
Enzyme 2	1µl (SacI)	1µl (BcuI)
Total amount	20µl	20µl

The tubes were mixed gently and incubated at 37 °C in heating block for 5 min.

Gel electrophoresis- Plasmid verification P3 and P4

The mixture was run on gel (1% Agarose, 90V, 30 min, GelRed pre-staining (according to SOP 0,5 µl)).

20µl plasmid was mixed with 3µl loading dye (note: only 20µl was loaded in total) and 4µl DNA 1kb ladder was used.



Good bands for both plasmids, compared to benchling. The verification worked and we have both plasmid 3 and 4.

Gibson assembly for tagged genes

The volumes for gibson assembly is calculated with *Gibson Calculation sheet-pmol* that can be found on in *Lab schedules and notes* folder. Change the concentration of backbone and genes to the concentration of interest.

Backbone used for the different plasmids

Plasmid	Name	Concentration ng/µl
P0	pcfb 2904	50
P1	pcfb 3035	50
P2	pcfb 3039	50
P3	pcfb 2403	222,1
P4	pcfb 3034	134

‘

The recipe for gibsson assembly tagged genes for the different plasmid was as follows.

Plasmid 0	
Backbone (Pcf2904) linearized date:	1µl
SP	0,62µl
pcbA5	1,24µl
Mq-water	2,14 µl
Gibson master mix	5µl
Total volume:	10µl

Plasmid 1	
Backbone (Pcfb3035) linearized date:	1µl
DP1	1,15µl
bphA1	1,53µl
bphA2 (diluted x3)	0,51µl
Mq-water	0,81 µl
Gibson master mix	5µl
Total volume:	10µl

Plasmid 2	
Backbone (Pcfb3039) linearized date:	1µl
DP2	1,74µl
bphA3 (diluted x6)	0,52µl
bphA4	1,50µl
Mq-water	0,24 µl
Gibson master mix	5µl
Total volume:	10µl

Note: the amount of MQ-water was hard to pipette the right amount of obvious reasons. The added amount can thereby not be guaranteed.

Plasmid 3	
Backbone (Pcfb2403) linearized date:	1µl
DP3	2,55µl
bphB	0,5µl
bphC	1,69µl
Mq-water	-
Gibson master mix	5µl
Total volume:	10µl

The calculated amount of bphB is 0,41 µl, but 0,5 µl was added because the small amount is hard to pipette.

Plasmid 4	
Backbone (Pcfb3034)	1µl

linearized date:	
DP4	1,6µl
bphD	0,50µl
bphK (diluted x3)	0,54µl
Mq-water	1,36 µl
Gibson master mix	5µl
Total volume:	10µl

The tubes were incubated in the manual PCR at 50°C for 1h (lid/cove temperature 50°C).

The tubes were kept in freezer overnight, transformation the next day.

Tubes are marked with P0 GA-P4 GA.

DATE: 16/7

WHAT WE DID:

Verification digestion: P0,P1,P2

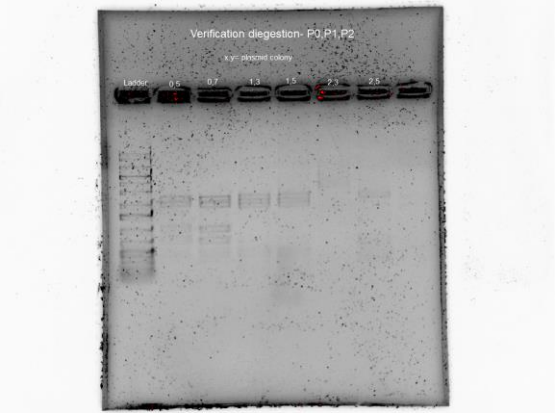
A mastermix was made according to the table below.

Mq water	108 µl
10X fast diegest buffer	18 µl
Fast diegest enzyme: AanI	9 µl
Fast diegest enzyme: NotI	9 µl
Total	144 µl

16 µl of mastermix was added to each tube together with 4 µl of DNA.

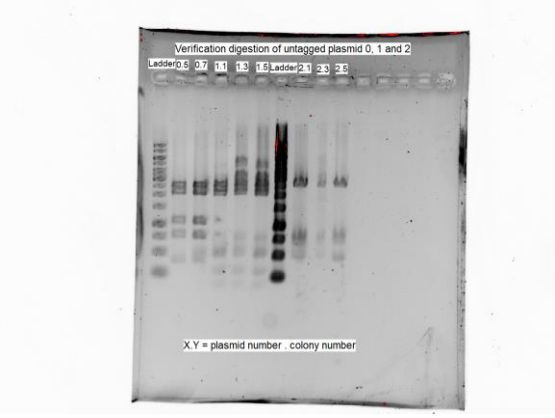
Gel electrophoresis- Verification diegestion: P0,P1,P2

12,5 µl of digest solution (mastermix+DNA) were mixed with 2,5 µl loading dye and 3µl ladder(1kb) were used. 12,5 µl of mixture(diegest solution+LD) was loaded on the gel, except for 0.5 when 15µl was used on the gel. 2.3 may have been contaminated by a small amount of 2.1, the LD drop got a little fused together.
The product was run on gel (1% Agarose, 90V, 30 min, GelRed pre-staining).



No good bands.

The samples was re-run on a new gel (1% Agarose, 110V, 20 min, GelRed pre-staining). In total 3µl solution was added for each sample to the gel. The soultion was made of 1µl loading dye, 2µl ladder (1µl 1kbplus ladder mixed with 1µl LD), 2µl plasmid
Note: Gel electrophorersis wasn't remade for 1.1 and 2.1.



Not so clear bands, can't draw any conclusion.

E.coli transformation: Gibson assembled tagged genes

For E.coli transformation protocol was followed. The bacteria plated on LB-Amp plates with glass beads. Two plates was made, 10% and 90%. For

plasmid 0 and 1, the supernatant was poured out after centrifuging and the pellet was resuspended in water, when making the 90%. For plasmid 2,3,4 some of the supernatant was poured out and the pellet was resuspended in the rest. For plating glass beads was used.

The plates was incubated at 37°C overnight.

DATE: 17/7

WHAT WE DID:

E.coli transformation: Gibson assembled tagged genes

All plates had colonies, see table below.

Note: the values are probably underestimated.

	10%plate	90% plate
Plasmid0	<10	~20
Plasmid1	~20	~300
Plasmid2	~10	~50
Plasmid3	>100	>500
Plasmid4	~20	~50

8 colonies was taken from the original plates and plated on a new LB-Amp plate. The pipette tips (used to pick up colonies) was left in a PCR-tube with 6,7 µl MQ-water. The tubes are kept in 4°C room overnight, for colony PCR with new primers.

For plasmid 0 and 2 the colonies were taken from the 90%-plate and for plasmid 1,3 and 4 from the 10%-plate.

Verification diegestion: P0,P1,P2 (again)

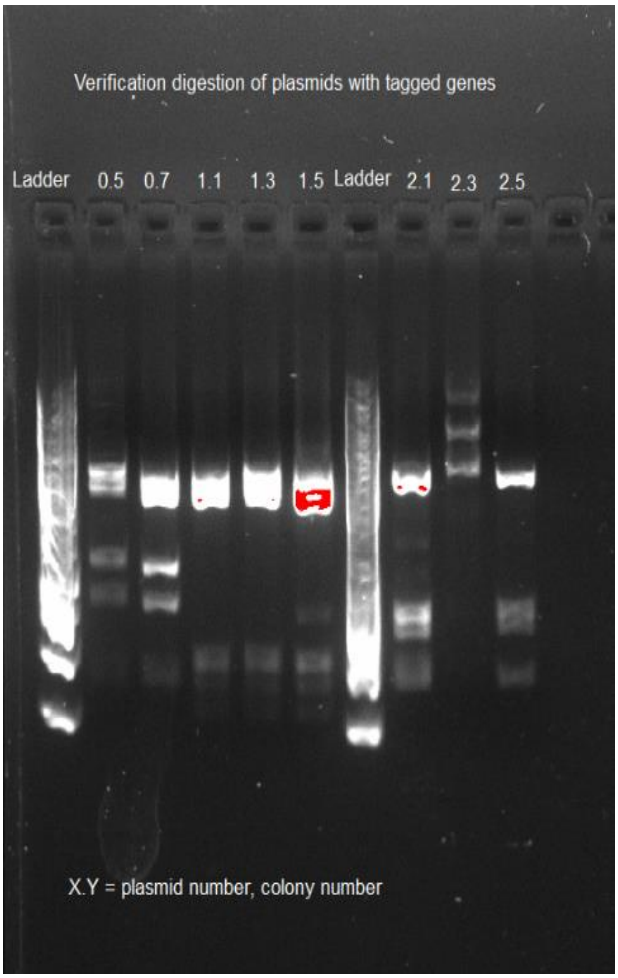
A mastermix was made according to the table below.

Mq water	108 µl
10X fast diegest buffer	18 µl
Fast diegest enzyme: AanI	9 µl
Fast diegest	9 µl
enzyme: NotI	
Total	144 µl

16 µl of mastermix was added to each tube together with 4 µl of DNA.

Gel electrophoresis- Verification digestion: P0,P1,P2

10 µl of diegest solution (mastermix+DNA) were mixed with 2 µl loading dye and 3µl ladder(1 µl 1kb plus mixed with 2 µl LD) were used. 10 µl of mixture(digest solution+LD) was loaded on the gel. The product was run on gel (1% Agarose, 90V, 30 min, GelRed pre-staining).



DATE: 19/7

WHAT WE DID:
Took out the plates from the 37°C (which we forgot yesterday).
Sequencing primers arrived and is in a store (100pmol/µl) and working stock (10pmol/µl).

Colony PCR- tagged plasmids (genes) P0-P4

The PCR tubes is taken from 4 °C room. There is already 6,7 MQ-water in the tubes which made the mastermix different.
The different plasmids had different primers seen, below. Primer 2 is a sequencing primer.

Plasmid	Primer 1	Primer 2
0	40	0.2
1	40	1.3
2	40	2.3 / 4.3
3	40	3.4
4	40	2.3 / 4.3

The mastermix is seen below and was made for every plasmid with the according primers.

Mq water	55,8 µl
5x HF buffer	36 µl
dNTPs	3,6 µl
Primer 1	11,25 µl
Primer 2	11,25 µl
Phusion poly	1,8 µl
Total	119,7

13,3 µl of master mix into every tube.
Mistake was made and the mastermix for plasmid 2 was put into plasmid 1 tubes. The mastermix for plasmid 1 was put in the tubes for plasmid 2. Another 1,25 µl of the right/ now missing primer was added to the according tubes and hopefully it will work anyways.

The PCR program for colony PCR is below.

1. 98 °C - 5 min
2. 98 °C - 10 s
3. 53 °C - 30s
4. 72 °C - 30 s
5. 72 ° C - 10 min
6. 4 ° C - Forever

step 2-4 was repeated 25 times

The PCR tubes are kept in frezeer.

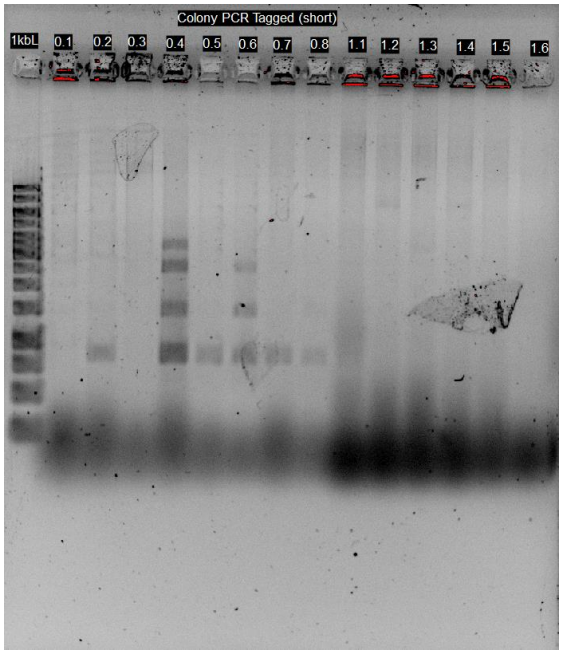
DATE: 22/7

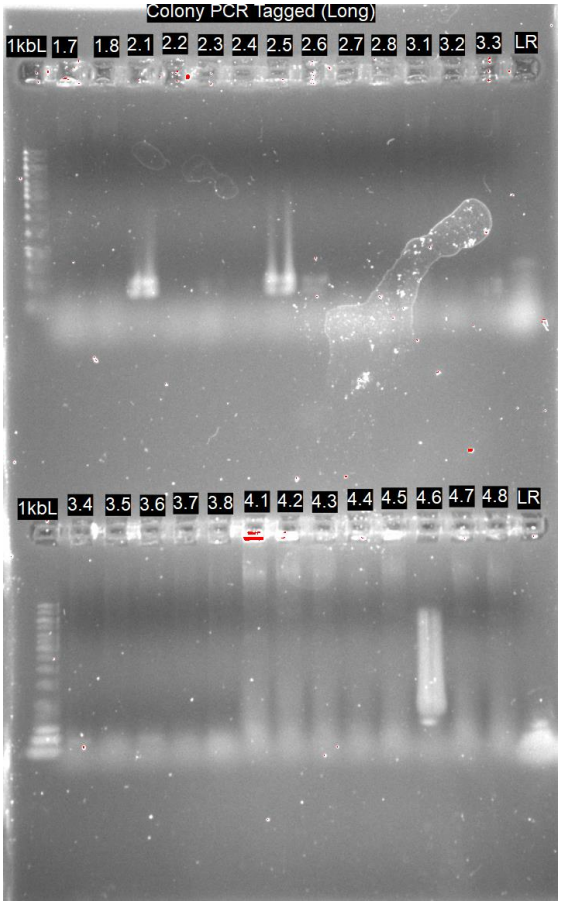
WHAT WE DID:

Gel-electrophoresis Tagged plasmids

The PCR product from friday week 5 were run on gel.
15µl sample was mixed with 3µl loading dye and a total of 17µl was loaded on the gel, for 0.1-0.8 and 1.1-1.6.

For 1.7-1.8, 2.1-2.8,3.1-3.8,4.1-4.8 only 5 µl of sample were mixed with 3 µl loading dye and a total volume of 7µl were loaded on the gel. For both gels 3 µl 1 kb ladder was used and for one gel the Low-range ladder was also used (3µl).
The product was run on gel (1% Agarose, 90V, 30 min, GelRed post-staining).





Colony 0.2,0.5,0.7,0.8,2.1,2.5 looks fine and is now verified. For the rest we can't say anything, re-do.

The expected length of tagged plasmids can be seen below:

TaggedPlasmid (PT)	Length (bp)
PT0	809
PT1	862
PT2	467
PT3	434
PT4	755

Colony PCR 2 tagged plasmids- PT1,PT3,PT4

The primers used for PCR can be seen below, primer 2 is sequencing primers.

Plasmid	Primer 1	Primer 2
1	40	1.3
3	40	3.4
4	40	2.3 / 4.3

The mastermix is seen below and was made for every plasmid with the according primers.

Mq water	58,0 µl
5x HF buffer	18 µl
dNTPs	1,8 µl
Primer 1	5,6 µl
Primer 2	5,6 µl
Phusion poly	0,9 µl
Total	90µl

Colonies was taken from the plate stored in 4 °C room.

10 µl of master mix into every tube. 3.1 had a little less MM compared to the others. For plasmid 1, toothpicks were used for picking colonies and a lot of liquid disappeared. Some extra MM was added in 1.1. For the rest, pipette tips was used when picking colonies.

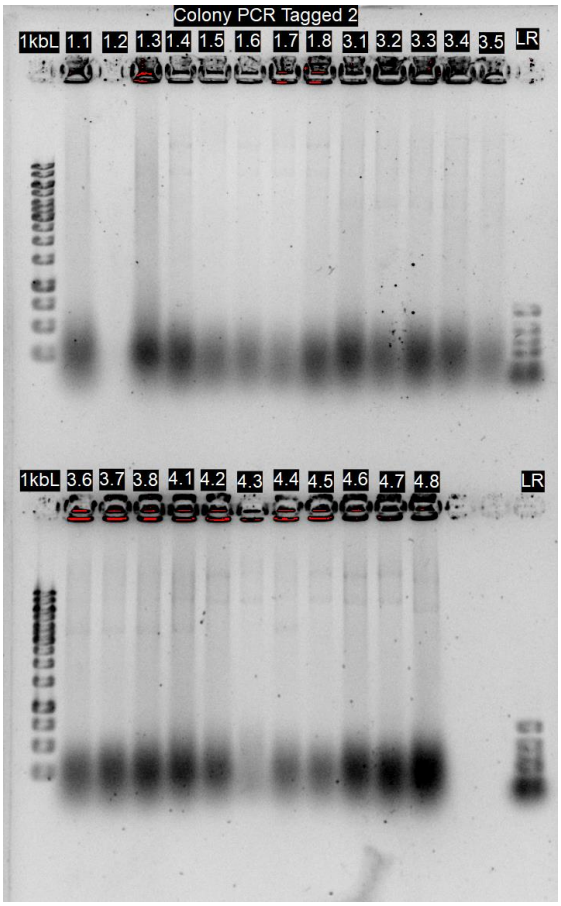
The PCR program for colony PCR is below.

1. 98 °C - 5 min
2. 98 °C - 10 s
3. 53 °C - 30s
4. 72 °C - 30 s
5. 72 ° C - 10 min
6. 4 ° C - Forever

step 2-4 was repeated 25 times.

Gel-electrophoresis ColonyPCR verification PT1,PT3,PT4

XX µl sample was mixed with 3µl 10X FD green buffer and a total of xxµl was loaded on the gel.
The product was run on gel (1% Agarose, 90V, 30 min, GelRed post-staining).



None of them were good. Try to verify by using diegestion instead.

Inoculation of P0 and P2- preparation of freezer stock

5ml of LB-media was mixed with 5 µl of ampicillin in cultivation tube (white cap). For plasmid 0 colony 5 was taken from the plate (marked red on plate) and for plasmid 2 colony 1 was used. Each colonies was added in their tube of media and incubated in 37 °C shaker for 3h. After 3h OD was checked, 1ml of solution against 1 ml LB media (used as blank).
When the OD is between 0.3-0.5 it's good for freezer stock.
The final concentration of glycerol was made to 20%, which means 0,25 ml of

80% glycerol was added to the freezer tubes together with 0,75ml cell culture.

The freezer stock tubes was labeled according to below:
iGEM
e.coli
Plasmid X, tagged
22/7-19
OD600=
The OD was checked just before storage and was for OD600 (P0)= 0,369 and OD600 (P2)= 0,317. The tubes are now stored in -80°C freezer lower iGEM box.

2,25 ml of LB media together with 2,5 µl of ampicillin was added to the rest of cell culture (again total volume of 5 ml). The solution was transferred to 50ml falcon tube (yellow cap, in order to give e.coli enough oxygen) and was incubated in 37°C shaker for plasmid prep tomorrow.

DATE: 23/7

WHAT WE DID:

Colony PCR 3 tagged plasmids- PT1,PT3,PT4

The primers used for PCR can be seen below, primer 2 is sequencing primers.

Plasmid	Primer 1	Primer 2
1	40	1.3
3	40	3.4
4	40	2.3 / 4.3

The mastermix is seen below and was made for every plasmid with the according primers.

Colonies was picked with pipette tips from plate stored in 4°C room. The pipette tips was put in PCR tubes containing 6,4 µl of water

5x HF buffer	18 µl
dNTPs	2 µl
Primer 1	5,5 µl
Primer 2	5,5 µl
Phusion poly	1 µl
Total	90µl

3,6 µl of master mix into every tube. (in order to have the enzyme last)
The PCR program for colony PCR is below.

1. 98 °C - 10 min
2. 98 °C - 10 s
3. 60 °C - 30s
4. 72 °C - 30 s
5. 72 ° C - 10 min
6. 15 ° C - Forever

step 2-4 was repeated 25 times.

DATE: 24/7

WHAT WE DID:

Plasmid prep for PT1,PT3,PT4

The protocol for plasmid miniprep were follower and the first centrifugation step was in falcon tubes. After the cells had been resuspenden the liquid were transferred to eppendorf tubes.
The concentration for plasmid:

Plasmid	Concentration
1.3	74,4 ng/µl
1.6	141,6 ng/µl
3.3	121,0 ng/µl
3.6	64,0 ng/µl
4.3	82,3 ng/µl
4.6	78,0 ng/µl

Verification digestion 1- PT1,PT3,PT4

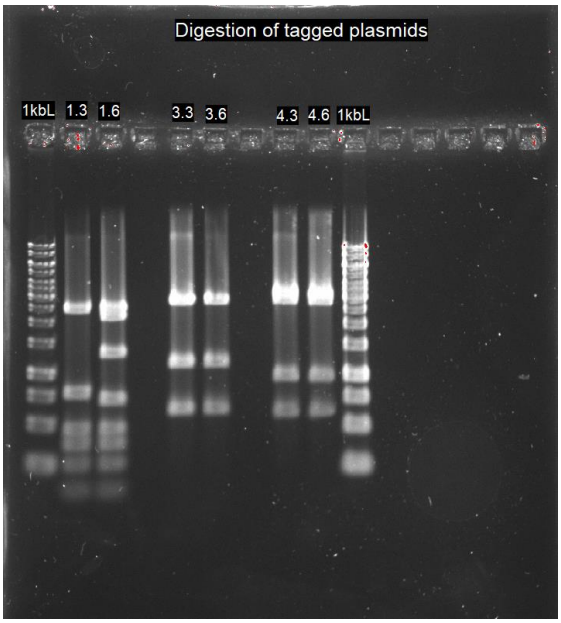
The enzymes used can be seen below:

PT1	AanI	NotI
PT3	SacI	NotI
PT4	BcuI	NotI

84 µl of water was mixed with 14µl 10xFD Green buffer. 14µl of mix was added to each tube together with 1 µl of each enzyme and 4 µl of plasmids. Each tube was incubated for 15 min in 37°C.

Gel electrophoresis- Verification digestion 1- PT1,PT3,PT4

7 µl of samples was loaded on the gel with 4µl of 1kb ladder. The product was run on gel (1% Agarose, 90V, 30 min, GelRed post-staining).



Gel showed nice result for plasmid 3 and 4. Colony 3.3 and 4.3 from plates were transferred to liquid culture (5ml LB+ 5µl Amp) to make freezer stock. Plasmid 1 showed strange results (too many bands), need to do new verification.

After 3h 30min the OD measurement was still too low for both PT3 and PT4. Redo the incubation tomorrow again with new culture (wanted to to home).

Verification digestion 2- PT1

A new verification digestion mix with a new enzyme.

water	12µl
10x FD green buffer	2µl
NotI	1µl
NheI	1µl
DNA	4µl

total	12µl

The tube was incubated for 15 min in 37°C.

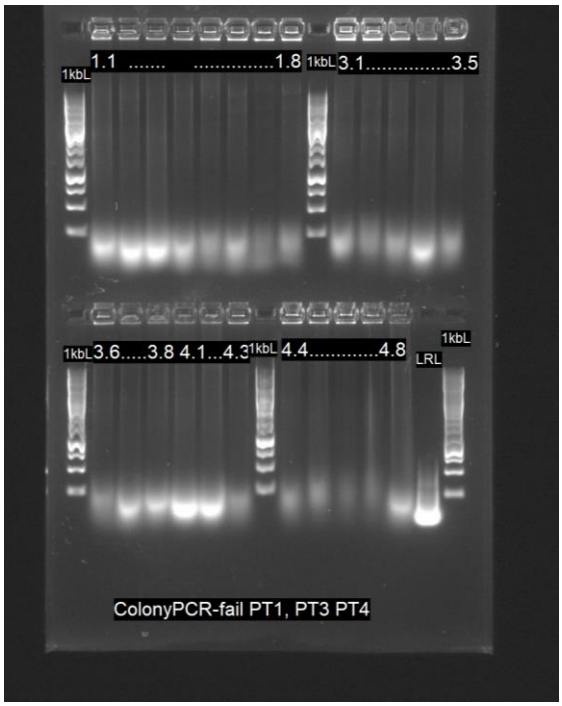
Gel electrophoresis- Verification digestion 1- PT1,PT3,PT4

7 µl of samples was loaded on the gel with 4µl of 1kb ladder. The product was run on gel (1% Agarose, 90V, 30 min, GelRed post-staining).

Something happened to the first gel and no bands could be seen. A new gel was made and was run under the same conditions, (1% Agarose, 90V, 30 min, GelRed post-staining) (new TAE buffer). 1.3 showed the wrong bands but 1.6 was correct. We have all tagged plasmids.

Gel-electrophoresis ColonyPCR 3 verification- PT1,PT3,PT4

2 µl of green buffer was used in each tube.
3 µl of 1kb ladder was loaded in the gel and total 4 µl of sample on the gel.
The product was run on gel (1% Agarose, 90V, 30 min, GelRed pre-staining).



DATE: 25/7

WHAT WE DID:

Freezer stocks of E.coli- untagged and tagged

We already have freezer stock for P3, P4 (team Magma) and for PT0, PT (team aqua).
In the table below the colony used for each freezer stock can be shown.

Plasmid	colony	Tagged plasmid	colony
P0	0.5	PT0	0.5
P1	1.1	PT1	1.6
P2	2.5	PT2	2.5
P3	3.2	PT3	3.3
P4	4.16	PT4	4.3

Colonies for plasmid P0,P1,P2 were picked from the plate marked: *//Restreak // Plasmid X - Gibson 5// iGEM 2019// 10 july 2019//*

Colonies for plasmid P0,P1,P2 were picked from the plate marked: *LB-amp e.coli, iGEM plasmid X, tagged 23/7-19*

Each colony were picked with plastic pipette tips and put in a falcon tube with 5ml of LB-media together with 5 µl of ampicillin. The tubes were incubated in 37°C shaker for > 3h.

After some time OD was measured, see in table below below.

Plasmid	time in incubator	OD600 when made into freezer stock
PT4 (4.3)	~4h	0,545
P1 (1.1)	4,5h	0,295
P2 (2.5)	5,5h	0,278
P0 (0.5)	5h	0,341
PT1 (1.6)	4,5h	0,430
PT 3 (3.3)	~4h	0,395

For OD measurement of P0 only 0,8 ml was used (0,8ml blank as well) for the rest 1ml solution was used (1ml blank).

For freezerstock 0,25 ml 80% glycerol was added to 0,75ml culture in freezing stock tube and then in lower iGEM box in -80°C freezer. iGEM 2019 freezing stock E.coli have a red dot on the cap (easier to find).

The freezer stock tubes was labeled according to below:
iGEM
e.coli
Plasmid X, tagged
25/7-19
OD600=

DATE: 24/9

Sequencing samples for verification

Not possible to do same day since not enough DNA

DATE: 25/9

Inoculation for miniprep (for sequencing)

Transformed and inoculated (10 ml + 10 μl ampicillin) @ 8.30 pm
PT0, PT1, PT2, PT3, PT4

DATE: 26/9

Plasmid miniprep for tagged plasmids

PT0	297.1 ng/μl
PT1	367.9 ng/μl
PT2	334.9 ng/μl
PT3	244.2 ng/μl
PT4	392.6 ng/μl

All with excellent purity

Date: 27/9

P0 strain C1 experiment

Yesterday PO strain was inoculated in 20 ml YPD
Results: Looks good
O.D @ 10.30 AM: 0.144 X 10 = 1.44
Dilution to O.D 0.1 - 5 times measurement.
10 ml culture in each. 100ml * 0.1 = V1 * 1.44 -> V1 = 6.94 ml + 93 µl YPD

Incubate cells for 24 hours

Plasmid 0 gene fragment isolation using easy clone primers from genome

PT0 strain, grown overnight (10 ml) was centrifuged (2500 rpm, 5 min); pellet washed with 10 ml water and again centrifuged; pellet resuspended in 1 ml. NaOH 0.02 M and 100 µl of tis was used for genome prep.

100 ml of PTO cells (confirmed colony)
Boiled - 99C, 25 min
Centrifuged - 15 min
Supernatant used for PCR template

PCR phusion - Primers 131 and 130 were used

Mastermix - for 10 samples
5X HF buffer - 100 µl
Primer 131 - 15 µl
Primer 130 - 15 µl
dNTP 10 mM - 10 µl
Water - 345 µl

48.5 µl Mastermix + 1 µl DNA template + 0.5 µl Polymerase - PCR for 8 samples

98 C - 3 min
{
95 C - 10 sec
52 C to 60 C - 10 sec
72 C - 2.5 min
} x 35 cycles
72 C - 5 min
15 C - forever

Gradient PCR to see what works

DATE: 29/9

Gel Run of PCR of plasmid 0 cells (yeast)

Aim of experiment; To see if primers from Easy clone system work for amplification of cassette from genome.
Pro tip from Veronica -> Phusion polymerase annealing temperature is +8C from Dream Taq
Gel run; Prestain (1.5 ml in 22.5 ml gel). 1% Agarose. 140 V, 20 min, FD green buffer loading dye. Sample - 5 ml; ladder - 2 ml

DATE: 1/10

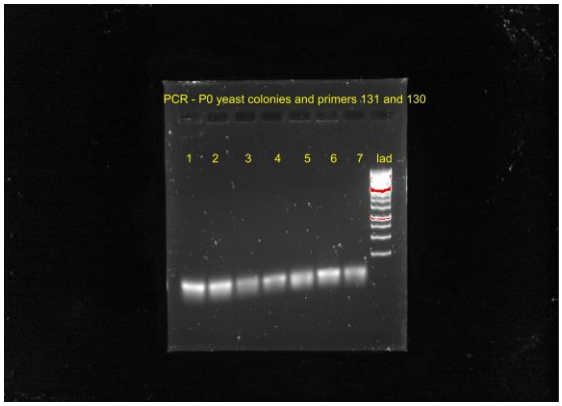
Colony PCR of P0 plasmid (Genome gene extraction)

Yeast DNA extraction - using NaOH (done earlier). Same mastermix, same protocol as 28 September colony PCR

- Gel: 140 V, 25 min, prestain (long gel, 2 µl Gel Red), (5 µl + 1 µl FD green buffer) and 2 µl ladder

Gel extraction of Tim's product

- Post stain, gel green, 2% agarose gel
- Gel Run went pretty well. But could not get PCR product through column. Bad product purity



DATE: 7/10

As a precautionary measure a gel cleanup is performed. ~40 µl of each sample loaded on a pre-stained 1% agarose gel. Run 20 min, 25 min, 125 V

TP0 1 - 9.4 ng/µl
TP 2: 8.8 ng/µl