DATE: 8/7

Inoculation for miniprep:
Responsible person: Dharmik
Inoculation of Plasmids contained EColi in
15 ml ampicillin medium
pcfb2903
pcfb3035
pcfb3039
pcfb3042
pcfb3045

DATE: 9/7

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)

DATE: 10/7

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: 15/7

A decision was made to try a yeast transformation this week even though we have not yet confirmed the lengths of plasmid P0-P2. We might have time for a confirmation before transformation, but that we will see tomorrow.

Cell preparation - inoculation of E.coli

We took three colonies containing P1 respectively P2 and two colonies containing P0.

NAME SYSTEM:

First number represent plasmid number and second number represent colony number.

Name example -- 1.3

Is a sample of colony 3 containing plasmid 1 (P1)

Tubes:

0.5
0.7
1.1
1.3
1.5
2.1
2.3
2.5

These tubes were incubated at 37'C overnight for plasmid mini prep tomorrow.

Preparation for yeast transformation

From Dharmiks own stock we made a small amount of yeast culture that we can use for the transformation if it will come to that tomorrow.

DATE: 16/7

Yeast transformation

The idéa is to try this first transformation with the tree integration g-RNA plasmid.

STEP 1.

Measure OD600 of overnight culture
Blank: only media
Sample: 900µl + 100µl culture (9:1)

OD600: 0.230 *10 = 2.30

We want to start with an OD600 of 0.025

v1*c1 = v2*c2
1ml * 2.30 = v2 * 0.025 => v2= 92 ml

But 92 ml is a bit much so we changed the volume to 50 ml => v1 = 0.54 ml

Incubation 1 (~6h):
- 50 ml media
- 0.54 ml culture

A start conc of OD600=0.025 will after ~6h be in the exponential phase: between OD600: 0.2-0.8

Plasmid mini prep of E.coli containing plasmid P0-P2

1. Harvest cells by spin down
   We used the centrifuge in the "yeast room": 3000 rcf, 5 min
2. Resuspend -> mix!
3. Lysisbuffer -> mix!
4. Neutralisation buffer -> spin down
5. Transfer to spin column and follow the protocol

Nanodrop results:

Sample	ng/µl
0.5	19.8
0.7	39.2
1.1	102
1.3	39.9
1.5	57.9
2.1	40.9
2.3	31.8
2.5	41

Is the culture in exponential phase?

Dilution: 9:1
OD600: 0.101* 10 = 1.01 OK!

STEP 2. Transformation

Calculations:
Total cultural volume: 50 ml
OD600 ~1
That gives us 50 OD units

We want to do 4 transformations (N gRNA, N0, 3P and 2P)

5 OD units per transformation => We need 20 OD units of culture => We will take 20 ml of culture

This will later be divided into 4 eppendorf tubes.

DATE: 22/7

Colony PCR of YT1 (P2, P3, P4)

PCR fragment	Primers
Verification for insert site XI - 2, nr 1	Green Mastermix 1
Verification for insert site XII - 2, nr 1	Green Mastermix 2
Verification for insert site X - 3, nr 1	Green Mastermix 3
Verification for insert site XI - 2, nr 2	Green Mastermix 4
Verification for insert site XII - 2, nr 2	Green Mastermix 5
Verification for insert site X - 3, nr 2	Green Mastermix 6

Note: For information of what primers are in the Green Mastermixes, please refer easy clone marker free manual

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 - 5 min
2. 98 °C - 10s
3. 50 °C - 15s
4. 72 °C - 2min 30s
5. 72 °C - 10 min

with steps 2-4 being cycled through 25 times

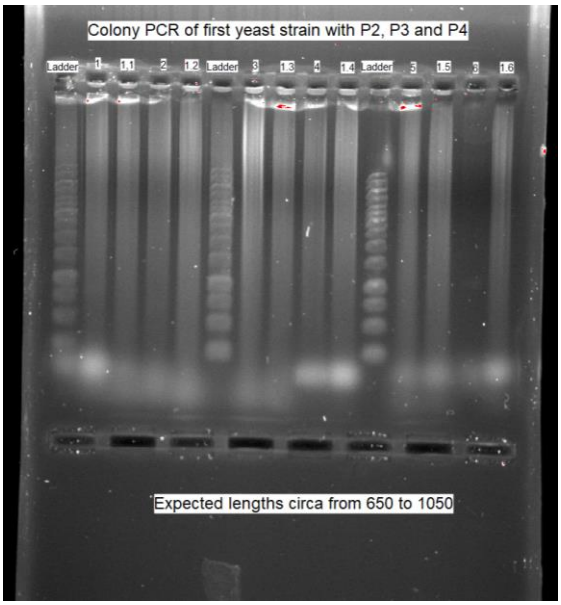
Gel electrophoresis

Expected lengths:

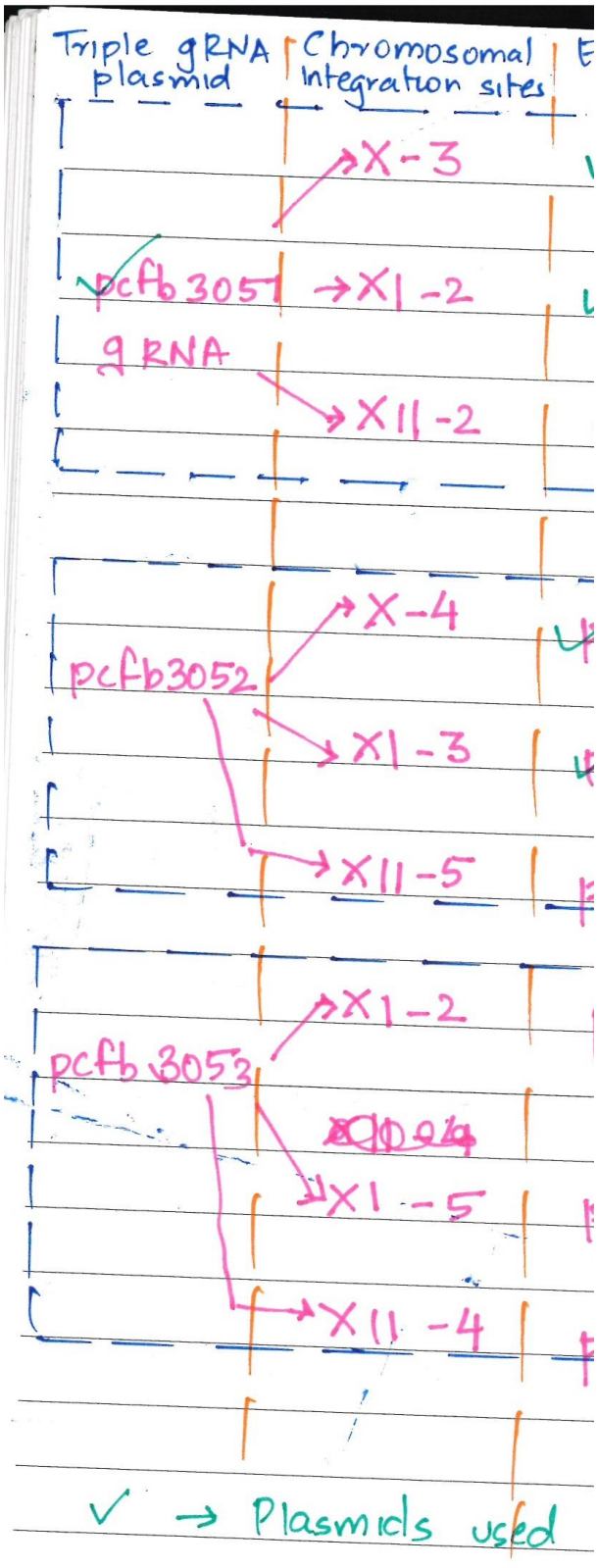
1	818 bp
2	666 bp
3	667 bp
4	963 bp
5	795 bp
6	1059 bp

Yeast overnight culture

Inoculated yeast from WT, colony 1 and colony 5 (colonies from YT1: P2, P3, P4) into 5 ml YDP. Let the cultures grow overnight (~17 h) at 30°C.



Conclusion: No visible bands, no result


Triple gRNA plasmid
Chromosomal integration sites
X-3
pcfb3051
X1-2
gRNA
X11-2
X-4
pcfb3052
X1-3
X11-5
X1-2
pcfb3053
X1-5
X11-4
✓ → Plasmids used

DATE: 23/7

Plasmid prep for PT0 and PT2

The protocol for plasmid miniprep was followed.
The first centrifugation was performed in falcon tubes. First after resuspension, lysis and neutralization buffer had been added the liquid it were transferred to eppendorf tubes.
Note: Transfer directly after the cells have been resuspened.
After the concentration was checked with nanodrop.

P0	216,3 ng/µl
P1	337,8 ng/µl

Replating and making <u>liquid culture of PT1,PT3 and PT4</u>

All colonies (1-8) for PT1,PT3 and PT4 were replated on new plates and kept in 37°C oven overnight.

Colony 3 and 6 for each plasmids were made into liquid culture in falcon tubes. 5ml of LB-media together with 5 µl ampicillin. The tubes were incubated in 37°C shaker overnight for plasmid prep.

Yeast transformation of P0, P1

It is most likely that LB medium was used instead of YPD, when staring the culture on 22/7. (deduced from colour of medium)
OD measurements:
Made 1:10 dilution of all samples, except for the blank.

Strain	**1:10**	**1:1**
WT	0.02	0.2
YT1	0.004	0.04
YT5	0.010	0.10

→ ODs too low for transformations
→ Plan: Inoculate cultures on wednesday, let incubate overnight and perform the work, that was planned for today, on thursday instead. Let plate incubate over weekend.

Colony PCR of YT1 (P2, P3, P4)

Colonies (from plate) checked: 1,2,5,6
Cells from each colony were resuspended in 15 µl 20mM NaOH and incubated at 96 °C for 15 min.

DreamTaq master mixes were provided by Dharmik (1-6) giving us the PCR samples 1.1-1.6, 2.1-2.6, 5.1-5.6 and 6.1-6.6, where the first number represents the strain and the second number represents the master mix/primers used.

The following volumes were used for the PCR:

Master mix	9 µl
DreamTaq Polymerase	0.5 µl
Cell DNA solution	1 µl

The PCR was run according to the following protocol:

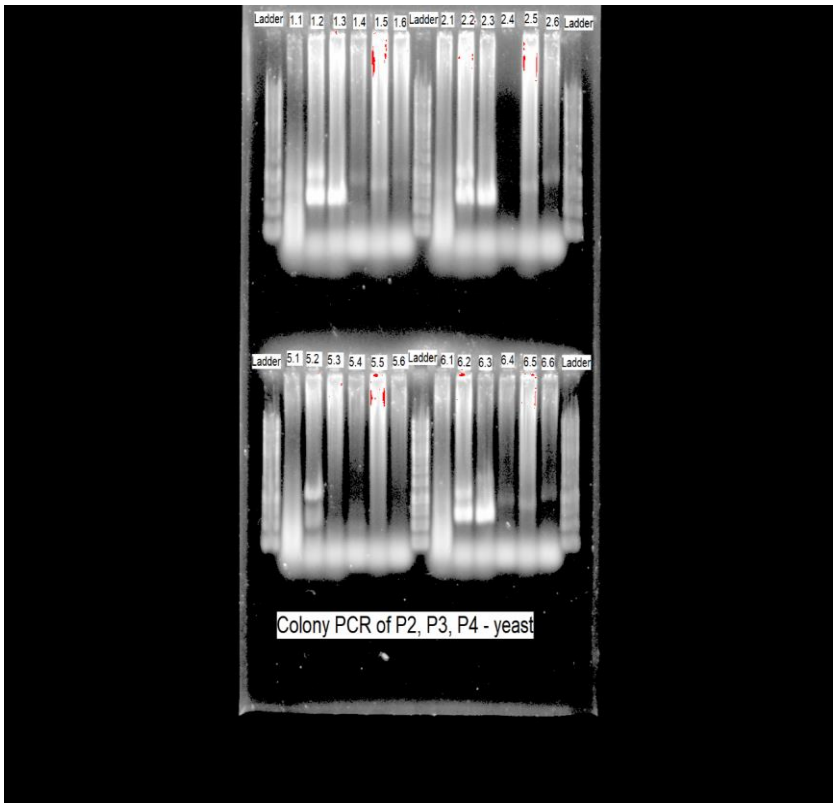
1. 98 °C - 5 min
2. 98 °C - 30s
3. 50 °C - 30s
4. 72 °C - 1 min 30s
5. 72 °C - 10 min

with steps 2-4 being cycled through 35 times

Gel electrophoresis

8 µl samples in wells. Ran for 30 min @90V.

Colony 1 seemed to be the most promising, with 5 out of 6 expected bands (see picture).



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 flacon 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

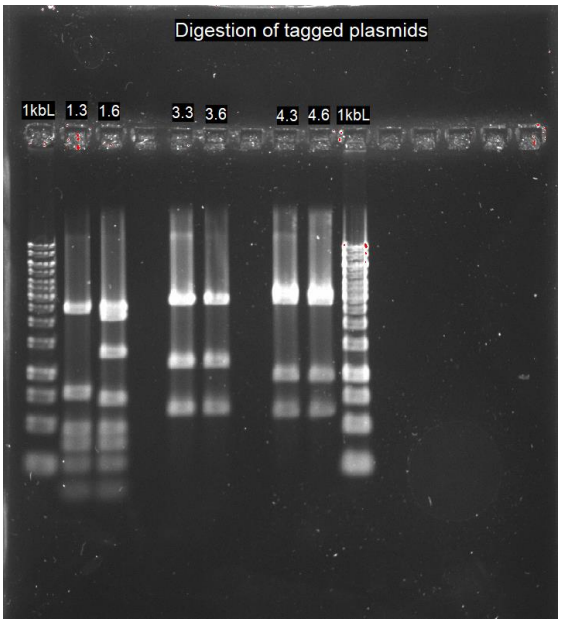
The enzymes used can be seen below:

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 enyme.

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.

Inoculation of YT1.1 and WT for yeast transformation (P1)

Inoculated from culture 1, on plate from YT1 (--> YT1.1), which showed a positive result for the tranformation of P2,P3,P4 (see gel from 22/7).
Inoculated wild type Δ80 (WT)
Both were inoculated into 50mL falcon tubes, containing 20 mL YPD.
Put in 30°C overnight.

DATE: 25/7

Linear fragment of P1 for yeast transformation

Protocol was followed.

15 µl of water, 2µl 10x Fas digest buffer, 2 µl plasmid (up to 1µg of DNA) and 1µl of NotI fast digest enzyme were mixed and incubated in heating for 30 min at 37°C. Then thermal inactivation at 85°C for 25min (shouldn't be for so long time, we forgot).

Incubation in liquid culture of plasmids P0,P1,P2,P3,P4 for plasmid prep.

The concentration of plasmid were quite low (see table below) so a new plasmid prep was performed.

Plasmid (colony)	Concentration (ng/µl)	Plasmid (colony)	Concentration (ng/µl)
P0 (0.5)	19,8	PT0 (0.5)	216,3
P1(1.1)	102	PT1 (1.6)	141,6
P2 (2.5)	41	PT2 (2.5)	337,8
P3 (3.2)	132,7	PT3 (3.3)	121,0
P4 (4.16)	91,5	PT4 (4.3)	82,3

For plasmid 1 and 2 we just added more LB and amp to the culture left from making freezer stock. For the other plasmids colonies were picked from plate.

Plasmid	added LB (ml)/ added ampicillin	colony from?
	(µl)	
P0	5/5	plate
P1	3,75/3,75	solution for freezing stock
P2	2,75/2,75	solution for freezing stock
P3	5/5	plate
P4	5/5	plate

The tubes were incubated in the 37°C shaker overnight.

Yeast transformation (P1)

OD measurement:

OD of overnight cultures:

Strain	**1:10**	**1:1**
WT(Δ80)	0.216	2.16
YT1	0.211	2.11

Planning:

Because all constructs needed for the transformation, only one positive transformation was planned: P1 into the YT1(P2,P3,P4). Two negative controls were also planned.

Diluted culture YT1 to OD≅0.1

Since a final volume of 30 mL is needed (10 mL per sample if final OD=0.5, to get a total of 5OD-units)

TRAFO master mix:

Made TRAFO MM according to:

PEG	720 µl
LiAc	270 µl

OD measurement (14.00-ish):

YT1.1: OD=0.012

Concentration much lower than expected and too low for transformations. It was concluded that the likely cause was not

mixing the culture tubes, before diluting for the second incubation (done in the morning, see above).

OD measurement (17.00-ish):
YT1.1: 0.074 (1:10-dil)

5OD-units → 5/0.74=6.76 mL≅7 mL

Preparation of solution:
Transferred 3*7=21 mL (1 pos., 2 neg. and 7mL each) to new falcon tube and spun down.
Resuspended in milliQ.
Spun down and 1mL LiAc (1M) was added. (0.5 mL per 5OD-units * 3 tubes, but some left over liquid from previous step was still in the tube and therefore the full 1.5 mL were not added).

Divided into separate tubes and 330 µl of the TRAFO MM was added each tubes.
Added 2 µl of gRNA (368 ng/µl)
Added 10 µl of the digested plasmid (500 ng in total).
Diluted all, with milliQ, to 20 µl V_{tot}.

Tube markings:

N_O	Water
N_G	No gRNA
P_1	Positive

Incubation:
Incubated for 30 min @30°C

Heat shock:
Heated in heating block for 15 min @42°C

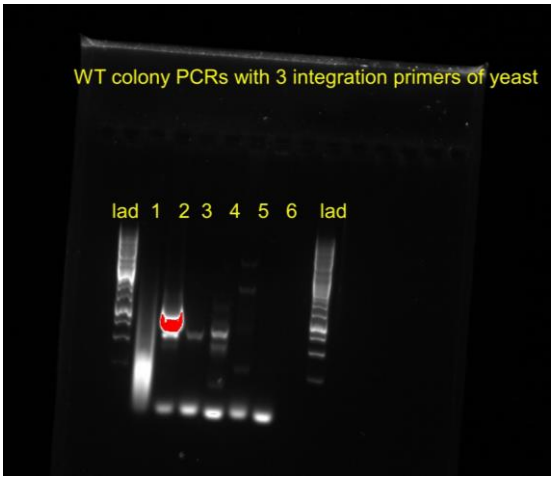
Final incubation:
The tubes were centrifuged and the supernatant discarded.
The pellets were resuspended in 150 µl YPD.
Incubated for 1h 45 min at the bench (the lab was quite hot because of the weather).

DATE: 26/7

WHAT WE DID:

Plasmid prep-P0,P1,P2,P3,P4

The protocol for plasmid miniprep was followed. For P0.5 350µl of neatulization buffer was used, for the rest only 300 µl was used. When transferring from eppendorf tubes to spin columns, there was a lot of supernatant left. It was realised after washing that this could be added to increase concentration. This was done, which also meant that the columns was washed twice. The concentrations was measured using nanodrop.



Plasmid (colony)	Concentration (ng/µl)
P0 (0.5)	275,8
P1 (1.1)	251,0
P2 (2.5)	161,7
P3 (3.2)	353,1
P4 (4.16)	232,5

Negative control yeast colony PCR

WT strain used against green mastermix
1 - 6: Expect no bands
Positive control: From dharmik

Recipe
9 µl Mastermix,
0.5 µl Dream taq polymerase
1 µl sample
PCR didn't help. Reason: Used old buffer for gel run

95 C - 5 min
{
95 C - 0.5 min
50 C - 30 sec
72 C - 2 min
}x35 cycles
72 C - 10 min

DATE: 29/7

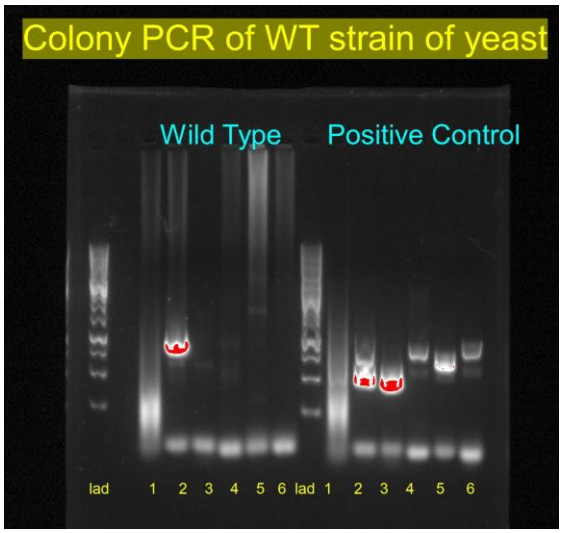
Colony PCR for wild type yeast

1W,.....,6W = 6 wild type sachharomyces
1P,.....,6P = 6 positive control from dharmk

PCR sample composition:
Mastermix - 9 µl
Dream Taq- 0.5 µl
DNA - 1 µl

Mastermix composition:
Primers (both): 1.5 µl each
Dream Taq green buffer: 5 µl
Water: 40 µl
dNTP (10µM): 1 µl

PCR cycle - 35 X
98 C : 5 min
95 C : 30 sec
50 C : 30 sec
72 C : 2 min
72 C : 5 min
15 C : Forever



Inoculation of E.Coli with pcfb3041, pcfb3044 and pcfb3048

Inoculation of E.Coli with pcfb3041, pcfb3048, pcfb3044. Liquid culture with 5 ml LB + 5 µl ampicillin. Kept in shaker incubator at 11.15 am

Note: Accidentally cracked the bottom of pcfb3044 plate. Should be okay. Tapped red at the crack area!

Preparations for yeast transformations

Aim was to perform 10 transformations: Single transformations into WT yeast for P0, P1, P3, P4, PT0, PT1, PT2, PT3 and PT4, as well as transformation of P1 into the strain already containing P1-P4.

6 ml WT yeast culture (in YPD) was inoculated and incubated for later use. Due to uncertainties about whether last weeks transformation needed to be redone or not, 5 ml of YT2 yeast culture (containing P1, P2, P3 and P4) was inoculated, and so was 5 ml of YT1 (containing P2, P3 and P4).

Digestions of gene plasmids
All gene plasmids should be digested prior to transformation to achieve higher efficiency. The aim was to acquire a 20 ul solution that in total contained 2 ug of DNA, note that this was not possible for PT3 or PT4 due to low DNA concentrations, and therefore these solutions would contain 1 ug instead. Also note that a digestion was made for P2 even though it won't be used for tomorrows experiment, but will be used later.

Volumes calculated based on DNA concentrations:

	DNA	RE	Buffer	dH2O
P0	8 ul	2 ul	2 ul	8 ul
P1	10 ul	2 ul	2 ul	6 ul

P2	13 ul	2 ul	2 ul	3 ul
P3	6 ul	2 ul	2 ul	10 ul
P4	9 ul	2 ul	2 ul	7 ul
PT0	10 ul	2 ul	2 ul	6 u
PT1	15 ul	2 ul	2 ul	1 ul
PT2	6 ul	2 ul	2 ul	10 ul
PT3	9 ul	1 ul	2 ul	8 ul
PT4	13 ul	1 ul	2 ul	4 ul

The solutions were incubated for 5 min at 37oC

It was decided that we will carry on transforming the strain from last week.

Tubes were prepared for tomorrow's transformation, each containing 250 ul PEG and 10 ul ssDNA. The tubes were labelled in the following way:

- P0-P4: Single plasmid transformations
- PT0-PT4: Single tagged plasmid transformations
- P0- - P4-: Negative controls (with gRNA) for WT
- WT-: Negative control (no DNA) for WT
- YT2: Transformation of P0 into YT2 (strain from last week)
- YT-: Negative control (no DNA) for YT

YT- + gRNA: Negative control (with gRNA) for YT

DATE: 30/7

Plasmid miniprep for pcfb3041, pcfb3044, and pcfb3044.

Cultures from previous day were minipreped. Followed protocol the same way as given except the elution buffer was heated before use and the columns were heated after ethanol wash of columns.

The concentrations after the miniprep were -

pcfb3041 - 458 ng/µl
pcfb3044 - 514 ng/µl
pcfb3048 - 510 ng/µl

Yeast transformations

OD measurements were performed on incubated WT and YT2. The following ODs were measured:

- WT: 4
- YT2: 3.54

1:10 dilutions were made (100 ul of yeast culture in 900 ul YPD), and the WT was transferred to a 100 ml culture while the YT was transferred to a 20 ml culture. Both yeast cultures were then incubated.

To the 18 tubes that were prepared the day before, the following was added:

Tube	Dig. plasmid	gRNA plasmid	MQ-water
P0	10 ul	2.60 ul	7.4 ul
P1	10 ul	2.72 ul	7.28 ul
P3	10 ul	1.95 ul	8.05 ul
P4	10 ul	2.18 ul	7.82 ul
PT0	10 ul	2.60 ul	7.40 ul
PT1	10 ul	2.72 ul	7.28 ul
PT2	10 ul	1.95 ul	8.04 ul
PT3	10 ul	2.18 ul	8.05 ul
PT4	10 ul	2.18 ull	7.82 ul
P0-	-	2.60 ul	17.40 ul
P1-	-	2.72 ul	17.28 ul
P2-	-	1.96 ul	18.04 ul
P3-	-	1.95 ul	18.05 ul
P4-	-	2.18 ul	17.82 ul
WT-	-	-	20 ul
YT2	10 ul	2.60 ul	7.4 ul
YT-	-	-	20 ul
YT- + gRNA	-	2.60 ul	17.4 ul

After ~3 hours, OD was measured again, on 1:10 dilutions of the cultures:

- WT: OD600 = 0.033 → 0.33
- YT2: OD600 = 0.024 → 0.24

Since OD was not high enough, the cultures were kept in 37oC for an additional 2 hours, and OD was then measured again:

- WT: OD600 = 0.079 → 0.79
- YT2: OD600 = 0.076 → 0.76

In order to make the transformations, we need:

- 75 OD units of WT = 94.93 ml → Use to 50 ml tubes with 47.46 ml in each
- 15 OD units of YT = 19.74 ml in one 50 ml tube

Tubes were centrifuged for 5 min (3000g), the supernatant was removed. 10 ml autoclaved water was added to each tube, which was then centrifuged for 5 min (3000g) once again.

18.75 ml LiAc was added to each WT tube, while 7.5 ml LiAc was added to the YT tube. The tubes were vortexed, and then centrifuged for 5 min (3000g).

The cell pellets were then resuspended in LiAc:

- WT tubes: 675 ul
- YT tube: 270 ml

Falcon tubes were vortexed to help resuspend.
90 ul from the falcon tubes were added to the eppendorf tubes containing TRAFO mix, as follows:

- YT → YT2, YT-, YT- + gRNA
- WT → P0, P1, P3, P4, PT0, PT1, PT2, PT3, PT4, P0-, P1-, P2-, P3-, P4-

Tubes were vortexed for 1 min, and then kept for 30 min in 30oC shaker.
To heatshock the cells the tubes were put in a 42oC heatblock for 15 min, after which they were put on ice.
The tubes were then centrifuged for 2 min (3000g), and the supernatant was removed. 100 ul water was added to the cell pellet, which was resuspended by pipetting. The tubes were centrifuged for 2 min (3000g), supernatant was removed, 200 ul YPD was added to each tube and the cells were again resuspended by pipetting. The tubes were then incubated in 30oC shaker for 1.5h.

Plating on YPD+nourseo

- Divided negative controls into groups of 3+3+2 on plates
- The rest were divided into groups of two on the plates
- There was likely a mistake made where P4 and P3 were accidentally swapped, meaning that the markings on the plate are incorrect.
- Some plates might have been contaminated by their neighbours (spill over of culture)

DATE: 31/7

Primer ----> Mastermixes

Primers ordered, arrived and we created stocks for the primers using autoclaved water. *These stocks were directly used to make the following mastermixes*

Primers - 45, 46, 128, 129, 130, 131 (as named earlier by Dharmik)

Mastermix composition

Primer 1	2 µl
Primer 2	2 µl
Dream Taq green Buffer	50 µl
dNTP	10 µl
Water	416 µl
Total	**480 µl**

Plasmid 1 - Mastermix 7 (128, 45)
Mastermix 8 (129, 46)
Plasmid 0 - Mastermix 9 (130, 45)
Mastermix 10 (131, 46)

PRIME STAR

DATE: 1/8

Some strange things were noticed about the plates from the yeast transformation from the 30/7. WT yeast was growing on selection plate → Might be something wrong with the plates. Also, YT2 strain might not have lost the plasmid after the previous transformation.

Need to check these things → Started cultures of WT and YT2 from previous plates. WT will be plated on selection plate, if it grows something is wrong with the plates. YT2 will be plated on YPD plate to ensure loss of plasmid.

Yeast colony PCR - YT 2 transformation

Discussion about the experiment
Problem: 30 July yeast screen to grow everywhere in all plates
Experiment aim: Regardless of growth everywhere, let's see if YT-2 transformation succeeded. Hypothetical outcomes and experiment:

Yeast colony PCR

Yeast YT - 2 - Mastermix 7,8 (for integration of plasmid 1); Mastermix 9,10 (for plasmid 0 integrated); Mastermix 1,2 (Positive control).
WT yeast - Mastermix 1,2, 7,8,9,10.
Negative control

Didn't work, need big changes

Mastermix - 9 µl
Template - 1 µl
Dream Taq - 0.5 µl

98 C - 5 min
{
95 C - 30 sec
50 C - 30 sec
72 C - 2 min
}x35 cycles
72 C - 5 min
15 C - forever

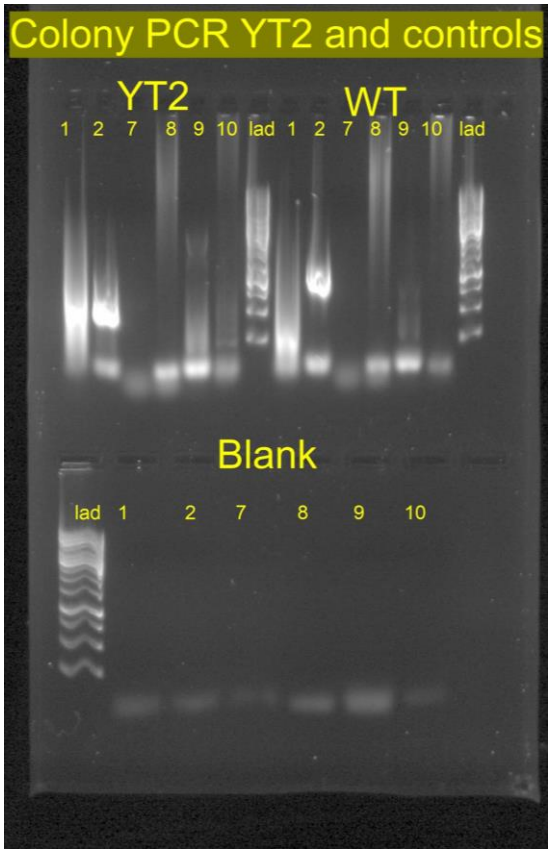
DATE: 2/8

Plated 100 ul each of the cultures made the previous day

- WT on YPD+nourseo
- YT2 on YPD

Gel run of colony PCR

140 V, 20 min, pre-stain
Result - no integration both during YT2 and YT3



DATE: 5/8

PCR to obtain single gRNA vectors (without Amp resistance) and URA marker , PRIMESTAR

Templates	primers
pcfb3041	156, 157
pcfb3042	156, 157
pcfb3044	156, 157
pcfb3045	156, 157
pcfb3048	156, 157
template for URA	158, 159

PCR mixture for every template:
dNTP: 2 µl
buffer: 10 µl
primer 1: 1 µl
primer 2: 1 µl
template: 1 µl
enzyme: 1 µl
H2O: 31 µl

PCR protocol for the pcfb templates(1-5)
98 C : 1 min
98 C : 0:10 sec
55.8 C : 0:15 sec
72 C : 5:30 min
35 X
72 C : 4 min
15 C : Forever
(this takes >4h)

PCR protocol for the URA templates(6)
98 C : 1 min
98 C : 0:10 sec
55.0 C : 0:15 sec
72 C : 1:10 min
35 X
72 C : 4 min
15 C : Forever

Gel Electrophoresis, URA

For URA template:
85V, 40 min

Results: Bad. Only primer clouds.

Gel Electrophoresis, gRNA vectors

90V, 35min

Results: Bands for 41, 45, 48 got cut out. No bands for 42, 44.

A) Checked for plates from previous week

Colonies on both plates - this means that our WT strain has resistance to nouveseo and we can not use it as a selection marker.

YT2 plate had colonies, hopefully have lost plasmid. But because of weird PCR last week. YT2 and YT3 needs to be redone with new marker -> Primers that changes backbone to UREA marker. TEAM AQUA will start PCR with primers to change selection marker and then continue with new YT2.

B) We made working stocks of twist DNA

From twist

From twist:			
Name	[ng]	Conc. we want	Add MQ
BbhR2	1000 ng	10 ng/µl	100
BBhR1	998 ng	10 ng/µl	98,8

pTDH3	1000	10 ng/µl	
pCIT2	1000	10 ng/µl	
pPGK1	1000	10 ng/µl	

WORKING STOCK
10 µl from storage with 90 µl MQ -> 1 ng/µl
The working stocks are stored in genebox
The storage tubes in primer storage box
The promoter tubes in paper box, I think...

Overnight culture of WT yeast cells
An overnight culture of Wt yeast cells from the stored plate was prepared. The culture was kept in 30C shaker overnight for PCB measurement tomorrow.

DATE: 6/8

New try!

PCR to obtain single gRNA vectors (without Amp resistance) and URA marker, Phusion

Since we got bands for 3 of the templates on the last primestar PCR, we tried it again. Oliver said then we should get bands for all of them. This time, we also did NeverFail.

PCR mixture for every template:
dNTP: 1 µl
buffer: 10 µl
primer 1: 1.5 µl
primer 2: 1.5 µl
template: 1 µl
enzyme: 0.5 µl
H2O: 31 µl

PCR protocol
??

Gel Electrophoresis
2 gels, 24 wells
2 wells per PCR product, 25µl per well
90V, 35 min

Transferred 5,55 µl of FD green buffer dye to each PCR product.

Post stained with gelGreen.

Results: Bad. No bands at all.

→ New try

PCR to obtain single gRNA vectors (without Amp resistance) and URA marker, PRIMESTAR, NeverFail

dNTP: 1 µl
buffer: 10 µl
primer 1: 1.5 µl
primer 2: 1.5 µl
template: 10 µl
enzyme: 0.5 µl
H2O: 25 µl

PCR protocol, URA-marker

98 C : 1 min
98 C : 0:10 sec
55 C : 0:15 sec
72 C : 1:30 min

98 C : 0:10 sec
52 C : 0:15 sec
72 C : 1:30 min

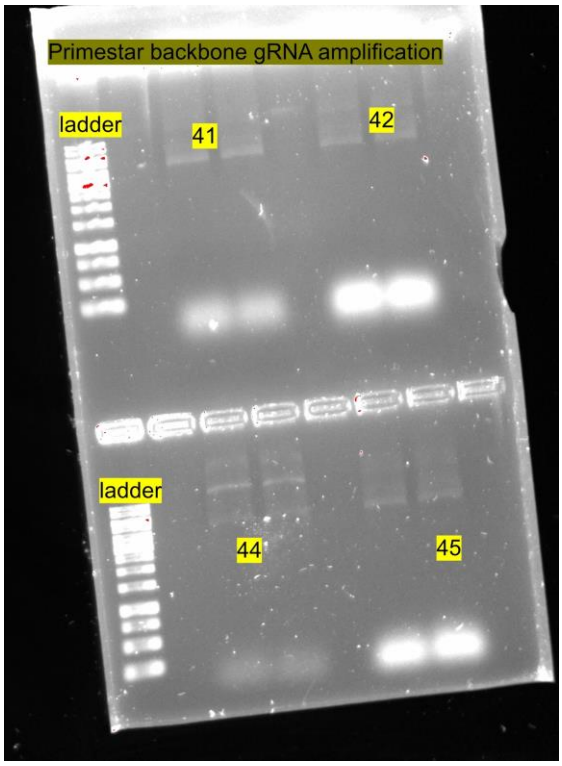
PCR protocol, gRNA vectors
98 C : 0:20 sec
60 C : 0:09 sec
72 C : 5:30 min

98 C : 0:10 sec
55 C : 0:09 sec
72 C : 5:30 min

DATE: 7/8

Gel run of previous PCR.

URA marker amplification worked but gRNA amplification seems to be failed. This was gel green, when loaded with all of the PCR product. But then the same gel was checked in the gel machine. There it seems to show a faint band. Which means the PCR actually works. Means there is hope!



→ New try

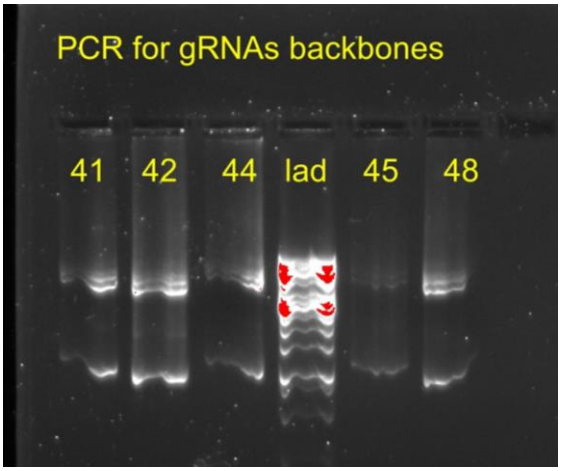
PCR amplification for gRNA vectors

PCR tried with a lower temperature for annealing.

98 C : 1 min
{
98 C : 10 sec
54 C : 15 sec
72 C : 5 min 30 sec
}35 x
72 C : 4 min
15 C : Forever

1 µl was loaded onto the gel in order to have a temporary confirmation so that we can do a gel purification. Was found out that there are 2 bands, but 5 kb bands really stands out. Oliver said that even if we try to PCR purify it and there is an impurity of 1kb in gibson, it will not help E.Coli to survive because amp gene and ORI together are more than 1 kb.

Anyways, so, the decision was to PCR purify the gRNA backbone fragments



Gel purification of URA

*Note: Gel purification of gels from earlier PCR attempt didn't go very well. pcfb3041, pcfb3042 and pcfb3045 had concentration below 5 ng/*µl. *So, dumped those samples*

Gel weight - 0.65 gm of gel
Binding buffer added - >700 µl

Final concentration - 50 µl of 89.2 ng/µl

PCR purification of gRNA backbones

Binding buffer added - 200 µl

pcfb3041 - 125 ng/µl
pcfb3042 - 111.1 ng/µl
pcfb3044 - 104 ng/µl
pcfb3045 - 55.1 ng/µl
pcfb3048 - 101.8 ng/µl

Gibson Assembly

10 µl gibson mix was added to 10 µl of DNA + water

basal molar amount set - 0.125 pmol (1 µl of URA marker fragment)

Gibson mix

	Gibson mix	Water	Ura	gRNA
G41	10 µl	5.4	1	3.6
G42	10	5	1	4
G44	10	4.7	1	4.3
G45	10	0.8	1	8.2
G48	10	4.6	1	4.4
N41	-	5.4	1	3.6

E.Coli transformation of Gibson Mixes

6 µl Gibson mix to 50 µl of competent cells

Note: Forgot to do the Dpn1 digestion. It is going to cost us probably.

DATE: 8/8

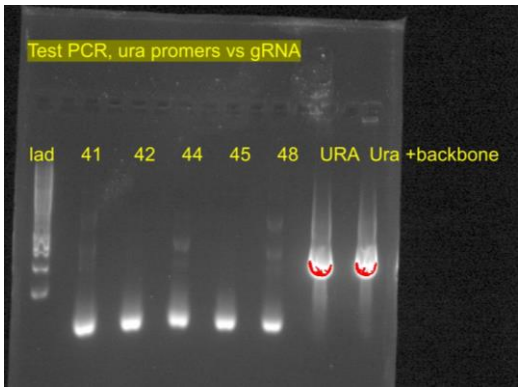
Restreak of Gibson colonies

8 colonies from each plate had been transferred to new LB amp plates

Test PCR for colony PCR of gibson colonies

all old gRNA plasmids, URA marker and URA plasmid were subjected to Dream Taq PCR amplification using primers for URA marker.

95 C : 3 min
{
95 C : 30 sec
55 C : 30 sec
72 C : 1 min 30 sec
}35 x
72 C : 10 min
14 C : Forever



Result: Primers work for colony PCR !!

DATE: 9/8

Colony PCR for Colonies from gibson

Mastermix for colony PCR

Dream Taq Green buffer	90 µl
dNTP 10 mM	9 µl
Primer 1	13.5 µl
Primer 2	13.5 µl
Water	315 µl

10 µl Mastermix + DNA polymerase were added to each well.
40 colonies (5 gRNAs x 8 colonies per plate) x 2 Primer pairs for verification. So 80 colony PCRS

For gRNA

95 C : 3 min
{
95 C : 30 sec
55 C : 30 sec
72 C : 5 min 30 sec
}20 x
72 C : 10 min
14 C : Forever

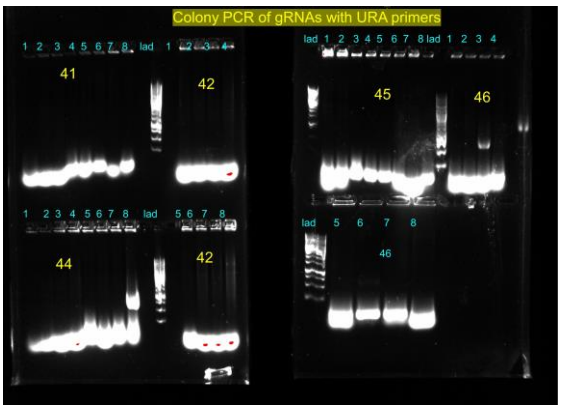
For URA primers

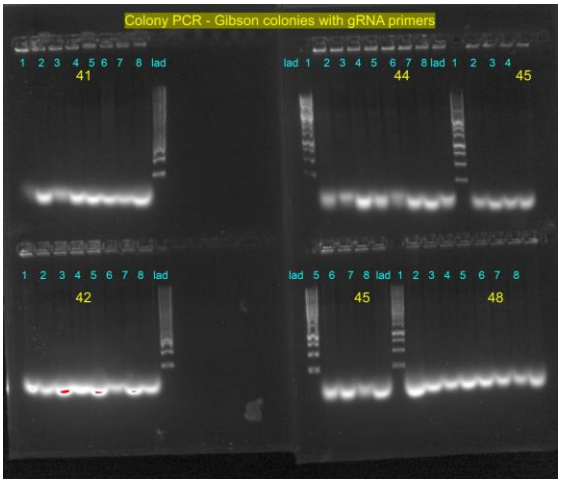
95 C : 3 min
{
95 C : 30 sec
55 C : 30 sec
72 C : 1 min 30 sec
}35 x
72 C : 10 min
14 C : Forever

Gel: URA, pre stain

120V, 20 min
No bands, maybe one super weak one at 1kb. Hard to say.

Gel: gRNA, pre stain
120V, 20 min
No bands.





What will we do?
Either gibson didn't, the transformation didn't work or the colony PCR didn't work. We will do a PCR on the gibson products to see if gibson worked.

PCR on gibson product of URA+gRNA vectors, Dream Taq

Here we do PCR with the URA primers and the gRNA primers on all 5 vectors. That is 10 PCR tubes, 5 per primer pair.

dNTP	1 µl

buffer DreamTaq	10 µl
primer 1	1..5 µl
primer 2	1.5 µl
enzyme	0.5 µl
template	1 µl
water	34.5 µl

- Primers used for the 5 URA PCRs: 158, 159
- Primers used for the 5 gRNA PCRs: 156, 157

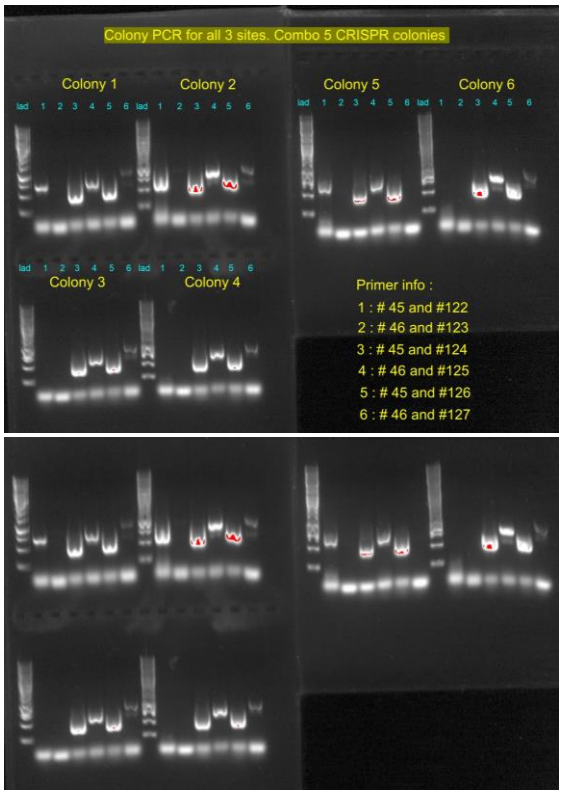
Gels will be checked on monday. They are pre stained!

DATE: 12/8

MEMBERS THIS WEEK:

- David
- Dharmik
- Emma

Products from the previous PCR were run on gel to verify Gibson results



Results were undesirable. g-samples were all very smeared while nothing was shown for the u-samples (not even primer clouds). Likely no primers had been included in the PCR tubes, or some other mistake had been made.
→ Repeat colony PCR while simultaneously attempting to re-do the Gibson assembly (with DpnI digest this time).

Colony PCR
Made two master mixes of 500 ul each, MM1 containing URA primers (158 & 159) and MM2 containing gRNA primers (156 & 157).

dNTP	10 µl
buffer DreamTaq	100 µl
primer 1	15 µl
primer 2	15 µl
water	350 µl

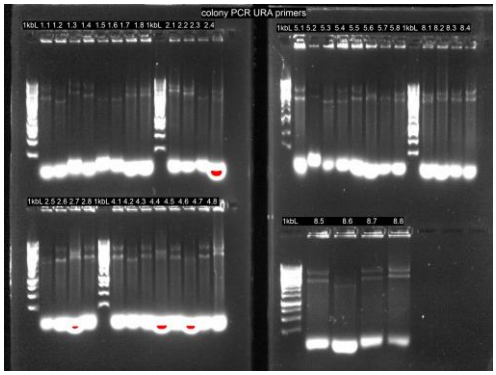
1 ul DreamTaq polymerase was added to 81 PCR tubes (8 colonies for each of the 5 plasmids, for both of the different primer mixes), 10 ul of Master Mix was added to the tubes and lastly cells were picked from plates with pipette tips.
The PCR was run on the following settings:
For gRNA primers

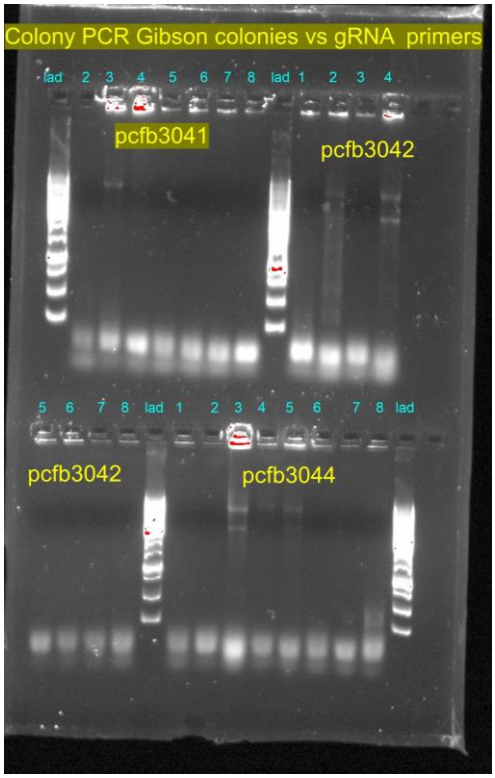
98 C : 10 min
{
95 C : 30 sec
55 C : 30 sec
72 C : 5 min 30 sec
}20 x
72 C : 10 min
14 C : Forever

For URA primers

98 C : 10 min
{
95 C : 30 sec
55 C : 30 sec
72 C : 1 min 30 sec
}35 x
72 C : 10 min
14 C : Forever

Results were run on gel (pre-stain, 140V 20 min)





→ None of the correct bands appeared! Re-do Gibson.

DpnI digestion

Want to digest remaining plasmids to avoid false positives. Digest 1 ug of each DNA type (gRNA vectors & URA marker), in a volume of 17 ul (according to Gibson protocol). Dilutions was made according to the table below. Note that due to the low concentration it was not possible to get a volume of 17 ul containing 1 ug of pcfb3045. For this reason, it was decided to make the solution contain 900 ng instead.

DNA	Conc. [ng/ul]	Vol DNA [ul]	Vol water [ul]
URA	89.2	11.21	5.79
3041	125	8	9
3042	111	9	8
3044	104	9.62	7.38
3045	55.1	16.33	0.67
3048	101.8	9.82	7.18

DpnI was added to the solutions, which were incubated at 37 C for 15 min. The enzyme was then heat inactivated at 82 C for 5 min.

Gibson assembly

Mix

- 1.5 ul URA solution (1.35 ul in the case of pcfb3045 to compensate for lower amount of this DNA)
- 7.5 ul gRNA plasmid backbone
- 1 ul H2O (1.15 ul in the case of pcfb3045)
- 10 ul Gibson mix
  → Incubate at 50 C for 15 min

A negative control, with water added instead of Gibson mix (pcfb4044 backbone was used), was also made.

Transform Gibson product into E.coli

Each Gibson product (+ negative control) was transformed into E. coli cells.

Transformation was carried out mostly according to standard protocol. The big difference was that only 100 ul LB media was added after the heat shock, and then after incubation transfering all of the media in the tube to a single plate (no 10% and 90% plates). The reason for this was simply that due to the low amount of DNA used for Gibson assembly only few

successful transformations were expected.

Colony PCR (again)
At supervisors suggestion colony PCR was attempted again to optimize the settings (since nothing worked previously). A smaller colony PCR was therefore made, with only gRNA primers (156 & 157) for only one of the plasmids (pcfb3041) as well as a positive control (pcfb4044 w/o the URA marker).
The following was added to each tube:

dNTP	1 ul
DreamTaq buffer	10 ul
Primer 1	1.5 ul
Primer 2	1.5 ul
H2O	35 ul
DreamTaq polymerase	1 ul

It was decided to try touchdown PCR at the following settings

- 98 C 10:00

- 95 C 0:30
- 52 C 0:30 (decrement by -0.6 each cycle)
- 72 C 5:30
  ---20x-----

- 95 C 0:30
- 55 C 0:30
- 72 C 5:30
- ---20x-----

Restriction Verification
As a contingency in case the colony PCR fails again cultures from five colonies from each gRNA plasmid were inoculated so that restriction digestion verification of the Gibson assembly can be attempted tomorrow.

DATE: 14/8

Plasmid miniprep
Miniprep was done on the cultures inoculated the day before, according to standard protocol. pcbf3051 (triple integration which has a URA marker) was also included for use as positive control.

The following concentrations were achieved

DNA	Concentation [ng/ul]
41.1	297.9
41.2	262
41.3	299.6
41.4	378.5
42.1	243.0
42.2	378.5
42.3	283.2
42.4	418.8
44.1	369.9
44.2	221.5
44.3	298.5
44.4	340.5
45.1	312.3
45.2	201.0
45.3	377.1
45.4	252.3
48.1	212.5
48.2	366.1
48.3	245.6
48.4	327.4
3051	151.7

Restriction digestion
For digestion, want 1 ug of DNA in a volume of 10 ul. pcfb3042 was included as negative control. Dilutions were therefore made as follows:

DNA	Vol DNA [ul]	Vol water [ul]
41.1	3.36	6.64
41.2	3.82	6.18
41.3	3.34	6.66
41.4	2.64	7.36
42.1	4.12	5.88
42.2	2.64	7.36
42.3	3.53	6.47
42.4	2.39	7.61
44.1	2.70	7.3
44.2	4.51	5.49
44.3	3.35	6.65
44.4	2.94	7.06
45.1	3.20	6.8
45.2	4.98	5.02
45.3	2.65	7.35
45.4	3.96	6.04
48.1	4.71	5.29
48.2	2.73	7.27
48.3	4.07	5.93
48.4	3.05	6.95
pos control	6.59	3.41
neg control	2.71	7.29

To each sample was then added:

- 1 ul NheI
- 1 ul Eco32I
- 2 ul FD Green buffer
- 8 ul water

from a master mix

Samples were incubated at 37 C for 15 min, then enzymes were heat inactivated at 65 C for 5 min. Afterwards samples were run on a gel at 90V, 30 min

[INSERT GEL PICTURE]

Sample 41.2 and 48.3 seem to be correct, the rest are not.

Preparation for yeast transformation

Acquired new colony from lab, this one needs G418 to keep Cas9 plasmid. Inoculated 5 ml culture in 50 ml tube, and added 20 ul G418, such that the concentration in the media was 4 ul/ml.

Inoculation of E.coli for tomorrow's miniprep

Strains inoculated: pcfb 3042, 3044, 3045, corresponding to plasmids 1, 3 and 0 respectively.

LB 5 ml
Amp 5 µl
1 colony per tube
2 tubes per strain

DATE: **15/8**

Inoculation of WT for transformation tomorrow.

- ~50 ml YPD
- A bunch of cells
- Put on shaker

RE digestion mix for P0 *called POD*

8µl P0 (2µg)
8µl H2O
2µl enzyme (Not1)
2µl FD Buffer

Inoculation of E.coli for tomorrow's miniprep:

Strains that we are inoculation. They are corresponding to integration of plasmid 0,1 and 3

Pcfb 3045
pcfb 3042
pcfb 3041

5ml LB + 5µl AMP
1 colony for each tube

DATE: **16/8**

Yeast transformation
OD of overnight culture from previous day was determined to be 0.45. All of the media was transferred to a shake flask along with 95 ml YPD and 400 ul G418.

TRAFO mixes were made for the 12 transformations that would be made (10 single inserts and 2 controls) by mixing 240 ul PEG, 10 ul ssDNA and DNA and water volumes which can be found in the table below

Tube	Dig. plasmid	gRNA plasmid	MQ-water
P0	10 ul	2.60 ul	7.4 ul
P1	10 ul	2.72 ul	7.28 ul
P2	10 ul	1.95 ul	8.05 ul
P3	10 ul	1.95 ul	8.05 ul
P4	10 ul	2.18 ul	7.82 ul
PT0	10 ul	2.60 ul	7.40 ul
PT1	10 ul	2.72 ul	7.28 ul
PT2	10 ul	1.95 ul	8.04 ul
PT3	10 ul	2.18 ul	8.05 ul
PT4	10 ul	2.18 ull	7.82 ul
P2 -	-	1.95 ul	18.05 ul
WT	-	-	20 ul

The OD was checked after 5 hours, and determined to be 0.1. It was then checked again after an additional 3 hours, and determined to be around 0.15. Since the culture was growing so slow it was decided that the transformation would be carried out during the next day (saturday 17/8).

Miniprep
Colonies 42.7, 42.8, 44.7, 44.8, 45.7 & 45.8 from the second Gibson attempt were miniprepped with the intention of verifying if the Gibson was successful. No deviations from protocol were made.

The following concentrations were achieved:

DNA	**Concentation [ng/ul]**
42.7	267.8
42.8	254.6
44.7	351.7
44.8	320.6
45.7	315.4
45.8	249.4

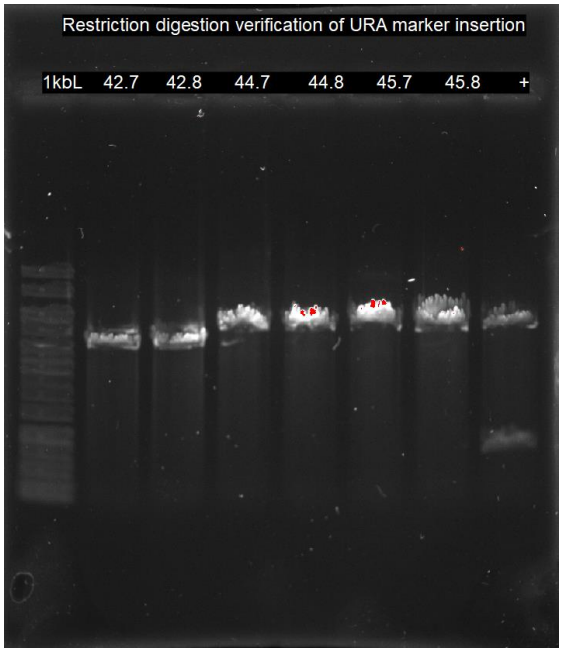
Restriction digestion
pcfb3051 was used as positive control

To each tube was added:
- 1 ul NheI
- 1 ul Eco32I
- 2 ul FD green buffer

as well as (for each tube):

DNA	**Vol DNA [ul]**	**Vol water [ul]**
42.7	3.78	12.27
42.8	3.93	12.07
44.7	2.84	13.16
44.8	3.12	12.88
45.7	3.17	12.83
45.8	4.00	12.00
pcfb3051	6.59	9.41

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



All colonies showed negative results → Gibson was unsucessful.

DATE: **19/8**

Fusion PCR of gRNA backbone and URA marker

Want to construct gRNA plasmids with URA markers instead of nourseothricin marker, corresponding to the integration sites of plasmid 0 and 1 to complement triple integration.

Strategy: gRNA backbone (pcfb3042, pcfb3045) + URA → equimolar ratio!

Run 15 cycles, add primers (156, 158), run another 25 cycles. Due to not having any pcfb3045 left we will test with just pcfb3042 and see if it works.

Step 1

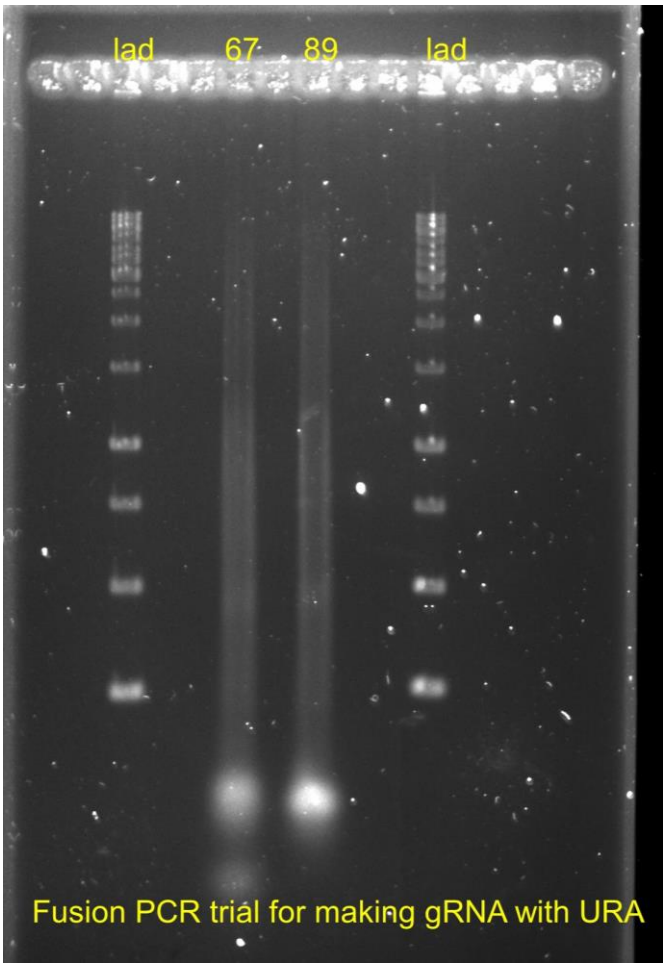
Primestar buffer	10 µl
dNTPs (10mM)	1 µl
pcfb3042	5 µl
URA	1 µl
Enzyme	0.5 µl
Water	29 µl

95 °C	3:00
95 °C	0:10
53 °C	0:15
72 °C	5:30
GOTO 2	x 15
72 °C	10:00
12 °C	forever..

Step 2 (add to previous PCR mixture)

Enzyme	0.5 µl
Primer 156/157	1.5 µl
Primer 158/159	1.5 µl

95 °C	3:00
98 °C	0:10
53 °C	0:15
72 °C	6:30
GOTO 2	x 35
72 °C	10:00
12 °C	forever..



DATE: **21/8**

Amplification of gRNA

01 pcfb 3042
00 pcfb 3045

Primer numbers: 157,156 for both

Follow the recipe from 6th Aug
First try: No bands

2nd try:
PCR protocol

98'C 3.0 min
98'C 10 s
54'C 15 s
72'C 5.30 min
GOTO x 35
72'C 10.0 min
15'C forever

Worked!

DPN1 digestion
Add 1µl enzyme to PCR reaction mix. Incubate for 30-60 min (37'C). Heat inactivate 80'C 5 min. TIP: use the PCR mashine for heat inactivation - we made a program for this in the iGEM folde

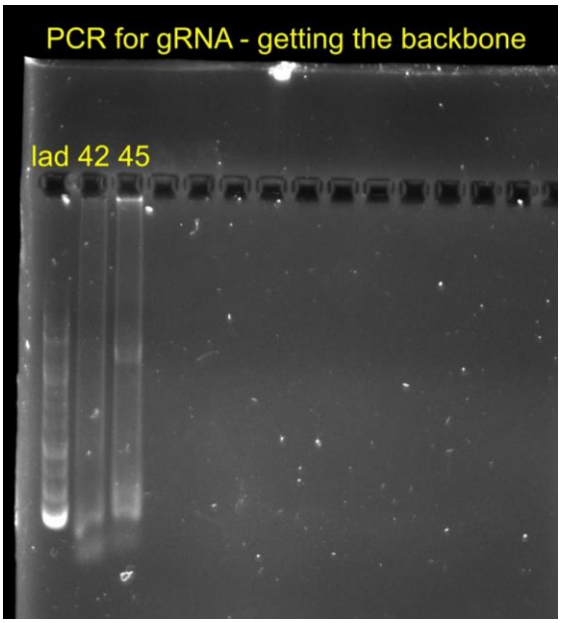
Fusion PCR

#1
Enzyme 0,5 µl
gRNA and URA marker in equal molar amount
This time:
1 µl of gRNA ⇔ 4µl URA
Buffer 10 µl
dNTP 1 µl
H20 up to 50 µl
NO PRIMERS
Run PrimeStrar protocol with annealing temp: 53'C and elongation time 5,5 min
x15

#2
ADD PRIMERS
Primer numer: 159 and 157

Run PrimeStrar protocol with annealing temp: 53'C and elongation time 6,5 min
x35

Results:
It did not work



DATE: **22/8**

Plasmid miniprep
Done according to protocol
Accidentally added elution buffer twice to PT2 tube, so that tube became 100

Nanodrop conc.

Plasmid	Conc. [ng/µl]
P0	160.3
P1	193.3
P2	152
P3	230.5
P4	140
PT0	130
PT1	188
PT2	81.8 (163.6)
PT3	198.4
PT4	146.6

Fast digestion:

Max of 17µl plasmid DNA or max 1µg.
Lowest conc. PT2: 81.8 ng/µl
17 µl => 81.8 * 17 = 1390.6 ng = 1.396 µg > 1 µg

Highest conc. P3: 230.5 ng/µl
17*230.5 = 3.918 µg > 1 µg

Total volume of 20 µl
Max conc. 1µg/20µl = 50 µg/µl

Fusion PCR :

Enzyme	0.5 µl
gRNA	1 µl
URA	4 µl
Buffer	10 µl
dNTPs	1 µl
dH2O	36.5 µl

98 C : 3 min
{
98 C : 10 sec
53 C : 15 sec
72 C : 5 min 30 sec
}15 x
72 C : 10 min
14 C : Forever

After this step, add primers 157 and 159,

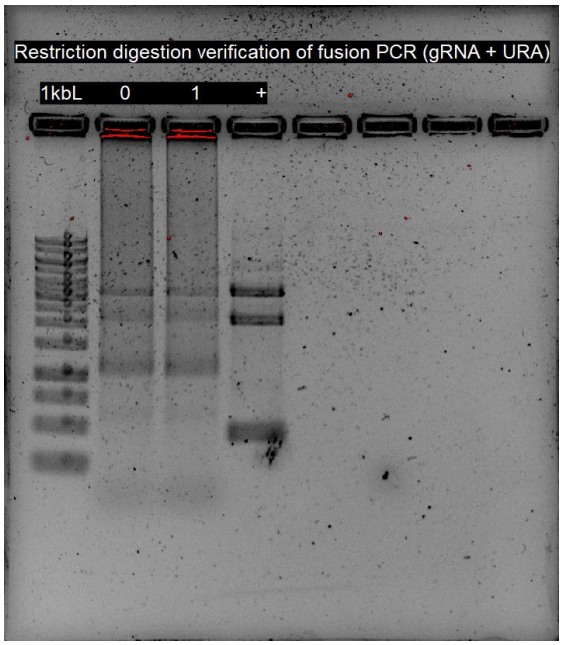
98 C : 3 min
{
95 C : 10 sec
55 C : 15 sec
72 C : 6 min 30 sec
}35 x
72 C : 10 min
14 C : Forever

DATE: **23/8**

Test digest of colony PCR product

Xba1 digestion was performed on the products from the previous day. Since concentration was unknown. The following was added to each PCR tube
Positive control - pcfb3051

Xba1	1 µl
MQ water	13 µl
DNA	3 µl
FD green buffer	2 µl



Restriction digestion: Repair fragment isolation from plasmid

P1	47 µl
P2	39 µl
P3	50 µl
P4	32.6
P0	40 µl+ 8 µl from old stock
PT0	40 µl
PT1	22.4 µl (old stock)
PT2	38 µl
PT3	50 µl + 10 µl (old stock)
PT4	42 µl

7.5 µl enzyme + 7.5 µl buffer + 60 µl DNA + water
Restriction digestion set for 7.5 µg of DNA

Yeast Transformation

Make trafo mix - 12 tubes
240 µl - PEG
10 µl - ssDNA
20 µl - DNA + water

gRNA vol added

sample	**gRNA**	**gene**
P0	2.6	18
P1	2.72	18
P2	1.95	18
P3	2.18	18
P4	2.18	18
PT0	2.6	18
PT1	2.7	18
PT2	1.95	18
PT3	2.18	18
PT4	2.18	18
WT	0	0
WT + gRNA	2.72	0

Note : due to low concentrations after digestion, 18 µl of digest was added from each tube

Culture O.D - 2.25

DATE: **26/8**

E.Coli transformation - Plasmid 0, Plasmid 1, pcfb3042 (with URA) and pcfb3045 (with URA)

E.Coli was transformed with
{1} Plasmid 1 colony 1
(2) Plasmid 0 colony 5
(3) gRNA for plasmid 0
(4) gRNA for plasmid 1

plasmid 1.1 and plasmid 0.5 are supposed to be liquid culture for miniprep and plasmid 3045 and plasmid 3042 - plated for colony PCR

Note: By mistake plasmid 1.1 was frown in liquid culture than on plate

Restreak of Backup transformations

Transformation worked!! - So all cells were plated on YPD plates
Why YPD? - plans for second transformation for the untagged plates has been cancelled for now. So, no need for cas plasmid and gRNA plasmid in the cells. To both remove plasmids and facilitate faster growth of cells, we used . YPD

DATE: **27/8**

E.Coli Transformation 2

Transformation from yesterday didn't work. Plasmid in liquid culture had shown some growth. But the rest had no growth whatsoever. So, repeat

Plasmid 0.5 worked,
miniprep - 238.7 ng/μl

Date: 28/8

Miniprep of colony PCR - 1.1

Concentration of 1.1 plasmid - 108 ng/μl

DATE: **29/8**

Colony PCR of non-tagged(u) and tagged strains.

Table over which green mastermix to use for verification of genome insertion.

Plasmid	Mastermix
P0	9 and 10
P1	7 and 8
P2	2 and 5
P3	1 and 4
P4	3 and 6

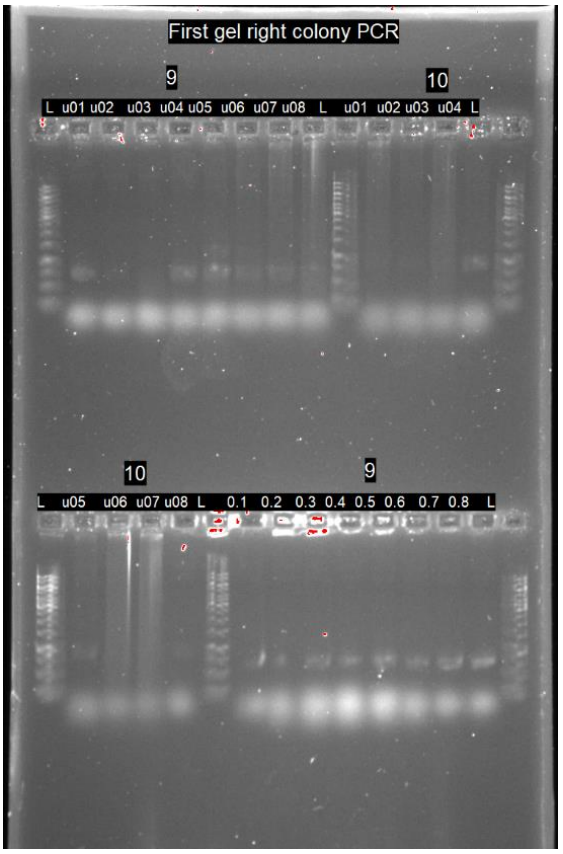
Eight colonies for each transformation were tested with both mastermixes corresponding to the specific integration site.

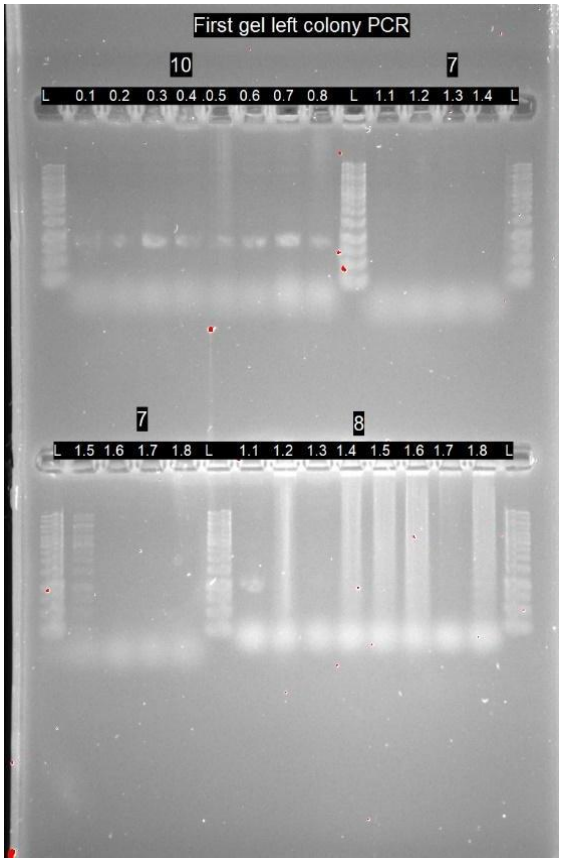
The genome was extracted through 96 degree celsius incubation for 15 minutes in 0.02 M NaOH.
Recipe:

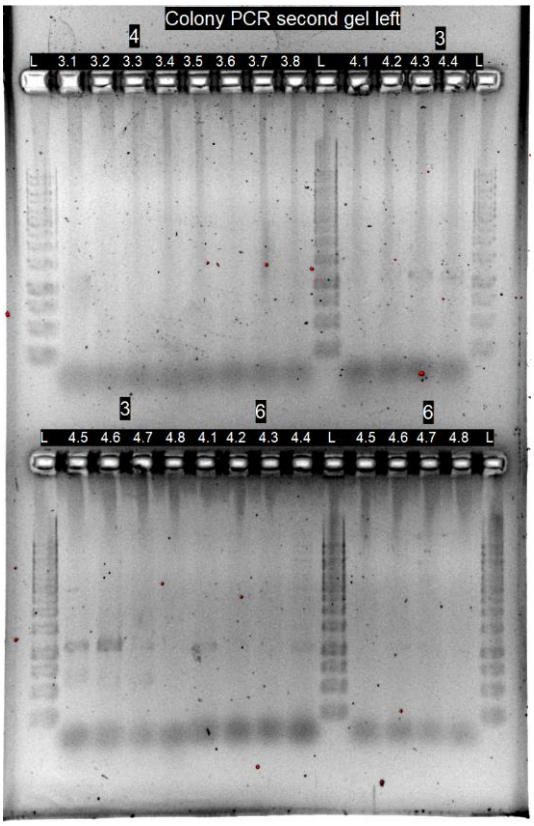
Component	Volume
Mastermix	9 µl
DreamTaq	0.5 µl
Template	0.5 µl

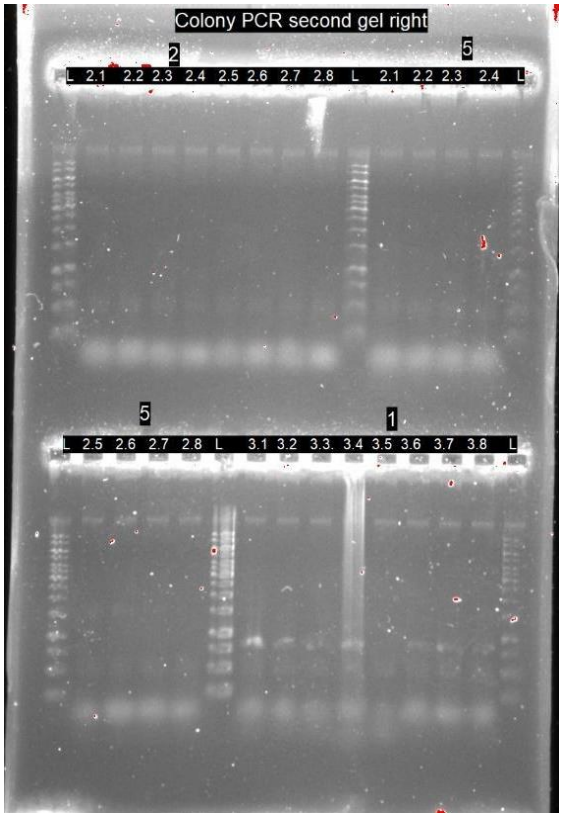
PCR protocol

95 °C	3:00
98 °C	0:30
55 °C	0:30
72 °C	1:30
GOTO 2	x 35
72 °C	10:00
14 °C	forever..




First gel left colony PCR
10
7
L 0.1 0.2 0.3 0.4 0.5 0.6 0.7 0.8 L 1.1 1.2 1.3 1.4 L
7
8
L 1.5 1.6 1.7 1.8 L 1.1 1.2 1.3 1.4 1.5 1.6 1.7 1.8 L


Colony PCR second gel left
4
3
L 3.1 3.2 3.3 3.4 3.5 3.6 3.7 3.8 L 4.1 4.2 4.3 4.4 L
3
6
6
L 4.5 4.6 4.7 4.8 4.1 4.2 4.3 4.4 L 4.5 4.6 4.7 4.8 L


Colony PCR second gel right
2
5
L 2.1 2.2 2.3 2.4 2.5 2.6 2.7 2.8 L 2.1 2.2 2.3 2.4 L
5
1
L 2.5 2.6 2.7 2.8 L 3.1 3.2 3.3. 3.4 3.5 3.6 3.7 3.8 L

DATE: **2/9**

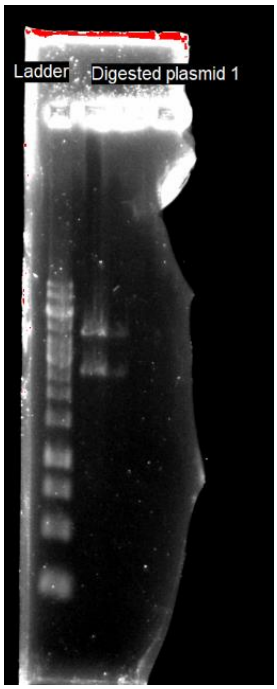
Preparation for transformation

Plasmid 1 was digested using NotI

Recipe:

Component	Volume
MQ water	15 µl
Green buffer	2 µl
Template	2 µl
FastDigest NotI	1 µl

Digestion was performed according to protocol and the enzyme was heat inactivated.



The bands on the gel were at the expected lengths around 3kb and 5kb, the digestion seem to have been successful.

With the digested plasmid a trafo mix was done.

Recipe:

-240 µl PEG 50% w/w

- 36 µl LiAC 1 M
- 20 µl ssDNA
- 40 µl MQ water to get as much as possible
- 18 µl P1, linear
- 4 µl gRNA

DATE : **3/9**

Transformation of plasmid 1 into YT1

The OD of the overnight culture was 0.246 with a 1 to 10 dilution, meaning the OD in the culture was 2.46.
The desired OD of the new culture was 0.1, meaning 0.8 ml of the overnight culture were added to 20 ml of YPD in a sterile culture flask. (C1V1=C2V2)

When the new culture had the OD of 0.326, 9.21 ml were extracted for the transformation and 2 negative controls. The protocol was followed from there on and incubated in 30 degree Celsius.

Colony PCR

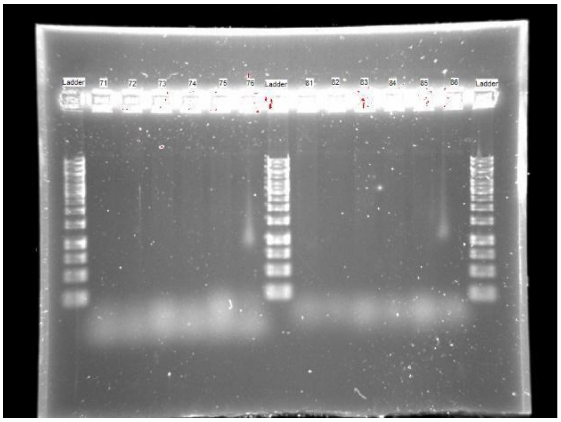
6 colonies were replated and colony PCR was performed. During this, a mistake was made which lowered the amount of cells in the PCR and on the plate. The cells were added to NaOH and incubated in 96 degrees Celsius for 15 minutes to extract the DNA.
Mastermix 7 and 8 used for verification.
The recipe for the PCR:

Component	Volume
Mastermix	9 µl
Template	2 µl
DreamTaq polymerase	0.5 µl

PCR protocol

95 °C	3:00
98 °C	0:30
55 °C	0:30
72 °C	1:30
GOTO 2	x 35
72 °C	10:00
14 °C	forever..

The PCR was run on a 1% gel, for 28 minutes on 90V



There’s an indication that colony 6 might have been successfully transformed but the results are otherwise inconclusive.

DATE : **4/9**

Freezer Stock for yeast with tagged genes - 20% glycerol, Total volume: 1ml

800 µl yeast culture
200 µl glycerol

OD of the yeast cultures is noted below.

Their caps are also labeled wt, 0, 1, 2, 3, 4 with blue marker

OD (strains are numbered after the plasmids where the new genes are from)
wt: 4,56
0: 4.59
1: 4,64
2: 4,94
3: 4,38
4: 4,34

DATE: 6/9

Colony PCR of YT2-transformation from 3/9

4 colonies growing, no colonies on any negative plate.

All the 4 colonies as well as colony 6 (only one that grew on the new plate) from previous transformation were tested.

Genome extraction using NaOH and 96 degree incubation was done.

Mastermix 7 and 8 used for verification.

The recipe for the PCR:

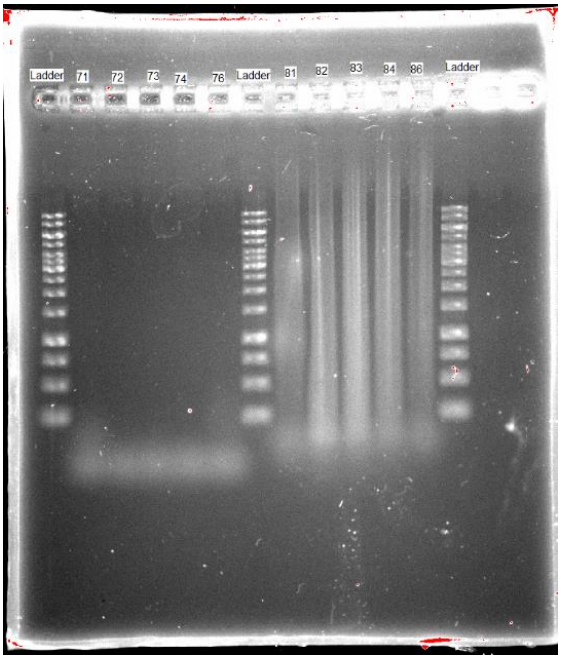
Component	Volume
Mastermix	9 µl
Template	2 µl
DreamTaq polymerase	0.5 µl

Note! For colony 6 0.5 µl template-mix was used since a larger cell mass was used.

PCR protocol

95 °C	3:00
98 °C	0:30
55 °C	0:30
72 °C	1:30
GOTO 2	x 35
72 °C	10:00
14 °C	forever..

The PCR was run on a 1% gel, for 30 minutes on 90V



XY = mastermix, colony.

For example: 71 = mastermix 7, colony 1

No results of use at this point, seem to be some nonspecific binding by the primers in mastermix 8.

DATE: 7/9

Preparation for colony PCR

Colonies 1 to 4 (from transformation 3/9) all grow on the plate they were restreaked on during the genome extraction.

Colony 3 of the plasmid 0-transformation (27/8) inoculated in 10 ml YPD.

DATE: **10/9**

Colony PCR of YT2 (3/9 transformation)

- Genome extraction

A LiAC/SDS-buffer was prepared. Received SDS-buffer with an assumed concentration of 1%, resulting in the final buffer having the concentration of 0.8% SDS, 0.2 M LiAC instead of 1% SDS, as it was in the protocol.
The cells were incubated in the lysis buffer for 10 min in 75 degrees. 50µl was used since the incubation was performed in a PCR machine and that was the max volume.
Mixture was transferred to eppendorf tubes and centrifuged for 3 min at 15 000 g.

YT2 colony	**Concentration genomic DNA (ng/µl)**
Colony 1	8,1
Colony 2	5,8
Colony 3	5,9
Colony 4	3,3
Colony 6	1,2

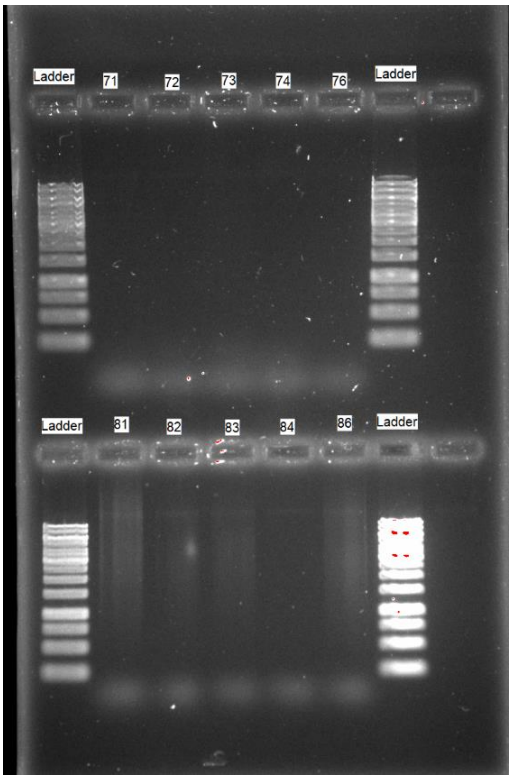
Very low concentrations, try anyway.

Mastermixes 7 and 8 are used to confirm integration of P1.

The PCR-recipe differs since it's adopted after the low concentrations:

Component	**Amount for colony 1-3 (µl)**	**Amount for colony 4 and 6 (µl)**
Mastermix	12	16
Template	4	8
DreamTaq polymerase	0.5	0.5
Final volume	16.5	24.5

Standard colony PCR program used
1% gel, 90V, 30 min



XY=mastermix nr, colony nr
No positive results, net attempt will be with a more proper genome extraction

DATE: 12/9

Preparation for sequencing of inserts in yeast strains

Inoculated tagged single integration strains, YT1 and P0-strain in 3 ml YP each.

Strain	**Colony**
Tagged P0 (TP0)	**6**
TP1	**1**
TP2	**8**
TP3	**2**
TP4	**2**
P0	**6**

Freezer stock of YT1 was used

DATE: 13/9

Preparation for sequencing

The cells were spun down for 5 minutes, 5525g. Supernatant was removed and the “Crude genome prep”-protocol was followed.
Double amount of lysis buffer was used since the amount of cells was way higher than in the protocol.
200 µl of cell/lysis buffer-mix was transferred to eppendorf tubes.

Final DNA concentrations

Strain	**Concentration (ng/µl)**
YT1	1870
P0	4260
TP0	3960
TP1	3860
TP2	3820
TP3	2890
TP4	2550

DATE: 14/9

Responsible person: Erik

Inoculation of YT1+ P1 for colony PCR

Inoculated culture 1-4 from plate, in 4x5ml YPD. Put in 30°C room over night.

DATE: 15/9

Genome prep:

Used 1 ml of overnight cultures from culture 1-4 on plate (Team Magma book for more details)
Followed protocol with 3X volumes (since 1 ml overnight was used instead of 100 μl)
Spin down @3000g, 3 min
Resuspend in 300 μl 0.2 LiAc, 1% SDS
Heated for 10 min @ 75 C
Added 900 μl 100% ethanol
Spin @ 13000 rpm for 3 min
Added 450 μl 70% ethanol
Spin @ 13000 rpm for 3 min
Dried on heating block @ 50 C
150 μl Elution buffer added
Spin @ 13000 g for 1 min
130 μl of each mixture (1-4) added to separate eppendorf tubes

Nanodrop concentrations

Sample	Concentrations
1	263.7 ng/μl
2	245.7 ng/μl
3	375.5 ng/μl
4	182 ng/μl

Colony PCR

9 μl Mastermix (7 & 8), 0.5 μl Dream taq polymerase, 0.5 μl template

98 C : 3 min
{
98 C : 30 sec
53 C : 30 sec
72 C : 1.5 min
} 35 x
72 C : 5 min
15 C : forever

Preparation for sequencing of inserts in yeast strains
All solutions with genomic DNA were diluted to 100 ng/µl (calculated with C1V1=C2V2)

A PCR to extract the inserts was performed.
Recipe:

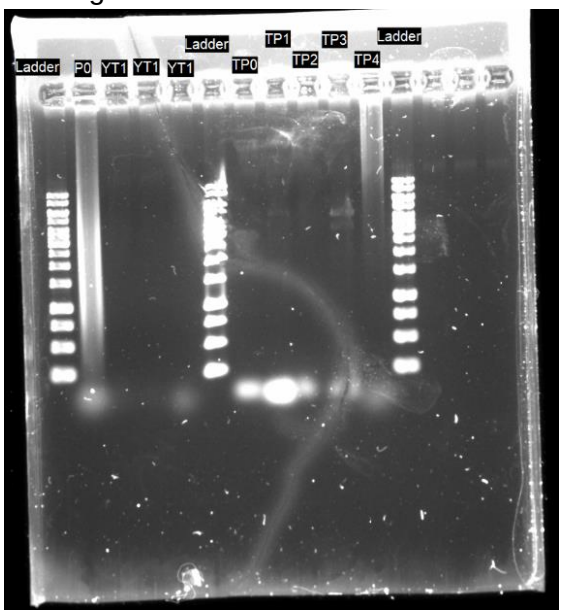
Component	**Amount (µl)**
MQ-water	35
Primestar buffer	10
Primer 39	1.25
Primer 40	1.25
dNTP	1
Template	1
Primestar polymerase	1 (by accident, meant to be 0.5)

Standard primestar PCR protocol with elongation time of 1.5 minutes

DATE: 16/9

Gel for sequencing of TP0-4, YT1 and P0

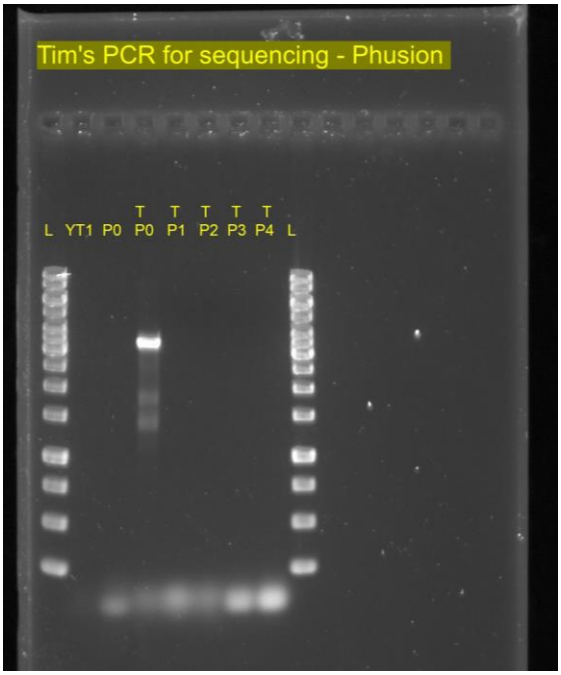
1% gel, 90V, 30 min. It broke during post-staining but is still readable



Possibly a band of the right size for TP3 but it would have to be a gel clean up.

DATE: **19/9**

PCR for sequencing of TP0-4, YT1 and P0



Component	**Volume (µl)**
MQ-water	35
HF-buffer	10
Primer 39 and 40	1,25 each
Template	1
dNTP	1
Phusion polymerase	0.5

PCR program

Temperatur (Celsuis)	**Time (min.sec)**
98	10.0
98	0.10
53	0.10
72	1.30
72	5.0
15	Forever