DATE: **28/8**

GC/MS trial run

Want to know if the protocol we found in literature will be able to detect PCB solutions, and at what concentrations. To that end, two samples consisting of PCB solution diluted with hexane were prepared.

Sample 1: 1:1000 dilution of the original PCB solution. Conc. = 0,2 µg/ml.
Was prepared by taking 1 µl of the original PCB solution and diluting it in 1 ml hexane

Sample 2: 1:1000 dilution of sample 1. Conc. = 0,0002 µg/ml.
Was prepared by taking 1 µl of sample 1 and diluting it in 1 ml hexane
(Note that the original intention was to make a 1:400 dilution, not 1:1000, however there was some stress involved which caused mistakes to be made)

Samples were loaded as follows on the GC:

1. Blank (hexane)
2. Sample 2
3. Sample 1

The GC was run on the following settings:

1. 70 °C for 2 min (initial oven temp.)
2. 150 °C (increased by 25 K/min)
3. 200 °C (increased by 3 K/min)
4. 280 °C (increased by 8 K/min)
5. Isocratic period: 10 min

Injection volume: 1 µl in splitless mode
Carrier gas: Helium, 18 psi, 1,9 ml/min
Constant pressure for injector at 250 °C
Detector temp.: 270 °C

The results were poor, even in the case of the blank, as the hexane was released over a far longer time than expected and blotted out any peaks we might have **detected otherwise. The people responsible for the GC/MS intend to investigate the origin of the problem as it does not seem like the problem is because of the program.**

DATE : 3/9

Responsible person: Moa

Western blot: Inoculation of cells for plasmid prep

6 different 50 ml falcon tubes were marked with accordingly,Wt. 5 ml YPD was added to each tube. One colony from each plate was added to according tube. The wildtype colony was taken from the plate labeled: *YPD+ G418 // CENPK // 3-11C + PCAb2312// 15 august 2019// for Dany from iGEM :) α*

The tagged plasmid plate used was labeled: X= number of plasmid *//Plasmid X tagged, restreak (Dany WT)// iGEM 2019// 27 aug 2019 //YPD//*

The colonies used for the tagged plasmids can be seen in the following table

Tagged plasmid	colony on plate
0	6
1	1
2	8
3	2
4	2

The falcon tubes was kept in saker in 30°C room.

DATE: 4/9

Dilution of the yeast strain w. tagged genes , in preparation for Western Blot (WB)

The cultures were diluted to an OD of 0.1 (see table above).

The tubes are marked iGEM, WB, P0-4 + wt.

The tubes where the cells were taken from have not been thrown away yet, and are stored together with the WB-cells in the 30 degree room.

DATE: 5/9

Responsible person: Moa and Emma

Western blot protein table

Plasmid	Gene	Amino acid	weight (kDa)	Tag
0	PCBA5	481	53,42	Flag
1	bphA1 (a)	460	51,6	His
1	bphA2(b)	187	22,02	Flag
2	bphA3(b)	107	11,54	Flag
2	bphA4(a)	413	44,03	His
3	bphB	275	28,73	Flag
3	bphC	303	33,21	His
4	bphD	286	31,45	His
4	bphK	203	22,4	Flag

Western blot: protein prep w Veronica

Veronicas protocol was followed with Veronicas guidance. Our own BSA stock was made and used in the qubit protein assay. The concentrations was measured with qubit protein assay, see in the table below. S1,S2,S3 was for calibration.

Sample	Measured	Concentration
S1	0,556	-
S2	2,58	-
S3	3,00	-
Wt	0,604	-
P0	1,32	-
P1	0,528	1,056 µg/µl
P2	0,602	1,204µg/µl
P3	0,582	1,164µg/µl
P4	0,994	1,988µg/µl

These concentrations are not good enough for western, which means new protein prep have to be made.

New OD measurement, preparation for new protein prep.

New dilutions of cells were made, see table below

Sample	OD	Vcells(ml)	Vypd (ml)
wt	0,353	0,170	5,83
P0	0,370	0,162	5,838
P1	0,340	0,176	5,824
P2	0,370	0,162	5,838
P3	0,363	0,165	5,835
P4	0,380	0,158	5,842

Note: even if all plasmids are annotated PX its the tagged plasmids were are using.

DATE: 6/9

Responsible person: Moa,Emma

New protein prep for western blot

New OD measurement of cell culture was made and calculated with following equation V=1.5/OD , see table below:

Sample	OD	Volume added to eppendorf tube
wt	2,97	505 µl
Pr0	3,41	440µl
Pr1	3,61	416µl
Pr2	3,28	457µl
Pr3	3,33	450µl
Pr4	3,73	402µl

Protein preparation

The protocol for protein prep was followed and the concentration was measured with qubit protein assay, see table below:

Sample	Measured	actual Concentration (measuredx 2)
Pr0	3,72	7,44 µg/µl
Pr1	2,04	4,08 µg/µl
Pr2	0,584	1,168 µg/µl
Pr3	1,74	3,48 µg/µl
Pr4	0,802	1,604 µg/µl
wt	1,43	2,86 µg/µl

60µl of supernatant was added with 15 µl LB(4x SDS-PAGE loading buffer), 7,5 1M DTT.

For western blot want we to have 20 µg of proteins, which means a concentration of 20 µg/10 µl in the tubes.

The dilutions made for western blot next week can be seen in table below.

Sample	Concentration	Sample volume (µl)	Mq water (µl)	Final conc:
Pr0	7,44 µg/µl	16,13	43,87	2µg/µl
Pr1	4,08 µg/µl	29,41	10,59	2µg/µl
Pr2	1,168 µg/µl	**60**	-	1,688 µg/µl
Pr3	3,48 µg/µl	34,48	5,52	2µg/µl
Pr4	1,604 µg/µl	**60**	-	1,604 µg/µl
wt	2,86 µg/µl	41,96	18,04	2 µg/µl

To each tube 7,5 µl DTT and 15 µl Loading buffer was added.

DATE: 9/9

Western blot no1:
The protocol for westernblot was followed besides the following notes:
120V until the samples are in the gel
185V 30 min.

DATE: 10/9

Western blot no1- flag

The gel was taken out of the 4°C room and the antibody was removed.
The membrane was washed 6 times (5min) with TBST.
HRP: 1,5ml+1,5ml in falcon-tube.
Note. Its light sensitive and should be kept in drawer when the solution was added.
The solution was added to the membrane (kept from light) and was added over the lanes (smear out gently) and put the foil back on. Keep still for 5 minutes and afterwards the solution was poured out in the sink. Take the membrane and put it in the plastic.

[insert western picture]

The picture was high exposed and thereby hard to tell if we have our proteins or not. We may have number 3 but should confirm with another western.

Suggestion for the future: rerun the high exposed samples again on one new gel for both flag- and his-tag.
Run sample 2 and 3 on one gel and the other ones on another gel. From this you should see if something has happened to number 2.

DATE: 11/9

Responsible person: Dharmik, Emma

Western Blot no2- Flag and His tag

The samples were boiled 98°C for 10 min and then centrifuged 14rpm, 15 min.
The electrophoresis module was assembled.
Rinsed wells
Filled wells with samples (10µl/sample, 3µl/ladder, 3µl/blank).

	2	3	L	L	0	1	4	wt		2:Flag
	wt	0	1	L	2	3	4			1:His

Run at 120V until samples are in the gel and not in wells anymore, press stop.
Run at 185V,30 min
Rinsed gels (packed in) and electrophoresis setup w dest. water
Image-> stain free tray/select:protein stain free gel
No need for gel activation of gel, we used Linneas.

Protein transfer

Assembled gel together with transfer kit from Linea, in transfer cassette. Don't use transfer buffer , since we have Linneas kit.
Run machine for A and B (mini gel) for 7 min.
Put in box with TBST before imaging.
Imaged-> select/protein/ stain free blot.

Probing with antibody

Blocked with blocking solution on "rocking" plate, overnight in 4 °C room.

Antibody working solution.

5 µl antibodies and 5 ml western blocker solution.
The following solutions were prepared.
1*AB-anti his +HRP
1* Flag
2*AB- anti mouse (Flag).

DATE: 12/9

Responsible person: Dharmik, Emma

Antibody incubation

Incubated with primary antibodies for 1h.
Note: Don't waste the AB, put them back.
Washed with TBST 4 times x 5min.
Incubated His tag with HRP for 5 min, ready for image.
Incubated Flag tagged protein 1 h with 2* anti-mouse AB for Flag.
Washed Flag with TBST 6 times x 5min.
Cut the membrane to separate 2 and 3 from each other.
Incubates Flag tag with HRP for 5 min, ready for image.

Image His tag

The following setup was used:
Multichannel/Channel 1: Chem Hi sensitivity(or R) Channel 2: Colorimetric
Run protocol.
As result: all bands are on the wrong place for all stains.

Image Flag tag

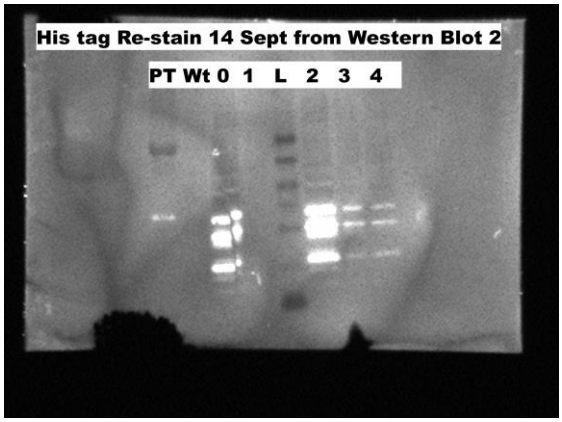
Same setup as for His tag.
As result: couldn't see any bands at all, super long time for image.

DATE: 15/9

Responsible person: Tim

Western blot imaging.

Both pieces of the membrane with FLAG-tagged proteins and the membrane with the His-tagged ones were incubated with antibodies, washed and incubated with HRP again. No results.



Date: 17/9

Inoculation of Yeast cells for western blot protein extraction
PT(0-4) was inoculated in YPD for western blot protein extraction.

Protein sample from Veronica
A positive control for western blot were received from Veronica (2ml in total). 0,5 ml of the positive control is stored in iGEM box -20°C freezer and the rest is stored in -80°C freezer (proteins should be stored in the -80°C freezer if possible, can be kept there for 3 months.

Date: 18/9

Protein prep for western blot.
The standard protocol was followed with the following remarks.
Boiled samples for 10 min at 95°C.
Centrifuged samples 15 min, 14krpm
Assembled gel electrophoresis module
Rinsed the wells
Loaded wells

All samples were loaded to their gel, according to the following scheme. PC=positive control.
4µl of SDS(s) was used, 3µl ladder(L) and a total of 10µl of samples.

s	s	s	s	wt	0	1	L	2	3	4	Pc	s	s	s*	**H**
			pc	wt	0	1	4	L	L	2	3	PC			**F**

H=Histag
F=Flag tag
*Added 0,5µl of ladder to get polarity in the membrane.

Gel run Western blot
120V until blue dye is inside the gel
185V, 30 min
Run extra time until the blue dye is out of the gel. (Image looked good).
Transfer to blot
The Blot for Flag-tag looked good but the blot for His-tag looked dirty.

Incubate both His and Flag overnight.

DATE: 19/9

Emma

Protein prep for WB3

The OD was measured for the incubated cells, see following table

Cells	OD
wt	3,42
0	3,75
1	3,67
2	4,16
3	3,76
4	4,39

The according volume of cells were transferred to a eppendorf tube, see table below:

Cells	Transferred volume (µl)
wt	438
0	400
1	409
2	361
3	400
4	342

The Protein prep protocol from Lucy was followed.
Centrifuge the cells, remove supernatant and then flash freeze in liquid nitrogen.
Add TCA to each tube and centrifuge again. After add acetone. Mix the solution, centrifuge again and remove supernatant.
Add resuspension solution (75µl), boil the solution (around 96 °C for 10 min). Let the tubes cool down, spin the tubes for 1 min until the cell pellet debrises. Transfer 60µl to a fresh tube and measure the protein concentration.
The concentrations of protein obtained from the Qubit Protein Assay was as follows:

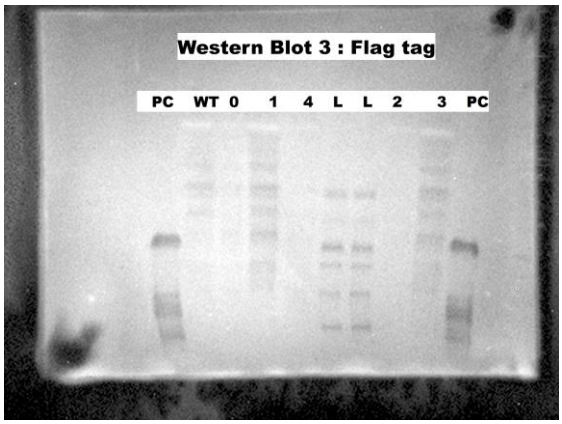
sample:	Conc. (µg/µl)
wt	3.34
0	3.61
1	3.71
2	3.38
3	3.41
4	3.51
S1	177
S2	37413
S3	61483

The samples were prepared for a con. of about 3.5 µg/µl, 15 µl SDS and 7,5µl Dtt was added to each tube and the tubes were kept in the freezer.
We found out we should always make them as 2 µg/µl, so the samples were later diluted with 45 µl of Western loading buffer.

Imaging of Western Blot 3: 2*AB-staining

After we realized we had the incorrect secondary FLAG antibody, we made a solution of the correct one (anti-mouse).
Washed with TBST 5min X 4 times
Incubated with correct antibody (2* AB anti-mouse,Flag).
Washed TBST, 5 min x 6 times
Incubated with HRP
Imaged.

We saw only the ladder and the positive control, nothing else.



DATE: 23/9

Responsible person: Emma

Western blot from protein prep 3
Western blot 5

Western blot was performed according to protocol.

Remarks:

Gel electrophoresis
Samples were boiled 10 minutes
Samples were centrifuged 15 min at 14000 rpm, in RT.

Samples were loaded in the gel as follows

His
wt, 1, 2, Ladder, 3, 4, Positive Control

Flag
wt, 0, 1, 2, Ladder, 3, 4, Positive Control

The gel was run first at 120 V for 2 minutes, then at 185 V for 30 minutes.

Protein transfer
The blot meant for his antibodies got dry. Therefore it was rinsed with ethanol before it was put in TBST. This seemed to help and it looked good when imaged.

The blots were placed in blocker solution in 4 ^{o}C for 3 hours.

Antibody incubation
The incubation of the blots in blocker solution was continued in room temperature, for one hour.

Results: No wanted bands were visible

DATE: 24/9

Western Blot - Flag Tag (9 Sept) restain

- Kept in Western Blocker overnight in 4 C
- Kept in HRP for 5 min
- Imaging: Exposure time was forever

DATE: 27/9

Emma

Protein Prep 4

Proteins were extracted from tagged strains 0-4. 3 different colonies were taken from strain 0 - these samples were named A, B and C. The B-colony is the one that has been used for all other protein preps as well.

8:00: OD was measured. Thereafter the cultures were diluted to OD 0.1.

strain	OD
wt	0.2
A	1.52
B	2.2
C	2.5
1	2.03
2	1.23
3	1.3
4	1.3

11:30: OD was measured again, for some of the strains.

strain	OD
wt	0.3
A	0.3
3	0.3

13:15

strain	OD
wt	0.3
A	0.48

We wanted to wait until 14:15, but there were no LAF benches available so the cells were taken out of the 37 degree room and the next measurements were taken at 15:00.

strain	OD
wt	0.7
A	0.74
B	1.05
C	1.04
1	1.17
2	1.83
3	1.89
4	1.3

Enough volume of the samples for all of them to have OD 5 were transferred to fresh inoculation tubes.

The samples were centrifuged to collect the cells in the bottom of the tubes. Most of the supernatant was removed. The cells were resuspended in the rest of the supernatant and the samples were transferred into 2 mL eppies.

Samples 2 and 3 were accidentally transferred into the same tube, a new tube could be made of sample 2 with the same OD, but not for sample 3. Therefore we took what was left of sample 3 anyways, but also kept the 2+3 sample. Sample 3 did frome here on have less OD than the rest of the samples.

The samples were treated according to Protein prep protocol, with the following adjustments:

Remarks:
Since we had more OD than the protocol recommends, there some adjustments were made:

- 600 uL of acetone was added to the samples.
- The samples were washed with acetone 3 times.

Qubit Protein Assay Results

There was a problem reading the standards, so the concentrations measured might not be correct.

strain	Conc. (ug/uL) = Ci
wt	3.88
A	2.84
B	3.12
C	2.28
1	2.68
2	2.84
3	2.20
2+3	2.23
4	3.97

The samples were diluted to a concentration of 2ug protein/uL, by transferring the corresponding volumes into fresh tubes. MQ water was added to a total volume of 60 uL.

Volume of sample to transfer was calculated according to:

Vi=60 uL * 2 ug/uL / Ci

Samples were stored in freezer.

OBS! These samples were not boiled after adding SDS and DTT. This will have to be done before western blot.

DATE: 28/9

Emma

Preparation of Positive Control Working Stock

- 30 uL positive control stock
- 2 uL 1M DTT
- 8 uL 4X Western Loading buffer a.k.a SDS Loading Buffer

Western blot of samples from protein prep 3

Western blot protocol was followed.

Remarks:

Gels were loaded as follows:

His:
wt, wt, 1, 1, L, PC, 2, 2, 3, 3

The samples to the right had 20 uL in each well, whilst the left samples had 10 uL.

FLAG:
wt, wt, 0, 0, L, 1, 1, PC, 2, 2

The samples to the right had 20 uL in each well, whilst the left samples had 10 uL.

After the proteins were transferred to blots, the blots were washed in TBST, then put in western blocker overnight.

DATE: 29/9

Emma

Western blot 8 -Gel electrophoresis and protein transfer of samples from protein prep 4

Western blot protocol was followed.

Remarks:
Samples were boiled 5 min, then centrifuged for 2 min, at 16 rpm, RT.

Samples + PC were then put on heating block at 95 ºC, then centrifuged 1 min, 14 rpm, RT.

Gels were loaded as follows:

His:
wt, 1, 2, PC, L, 3, 2+3, 4

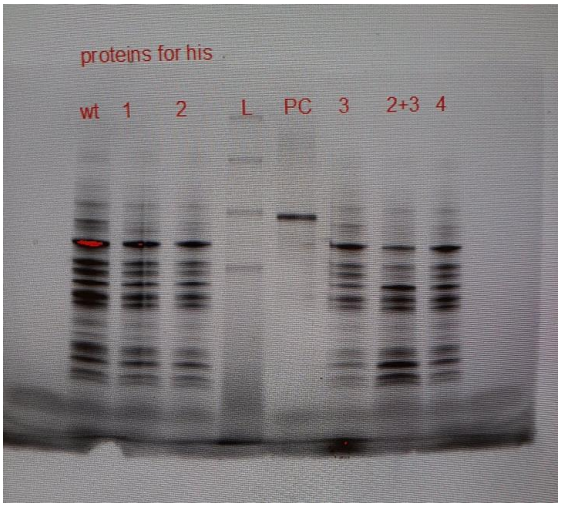
OBS! It looked like a bit of sample 3 floated into the well with PC. The loading of the samples didn't go very well in general, but probably good enough.

FLAG:
A1, A2, wt, L, B1, B2, PC, C1, C2

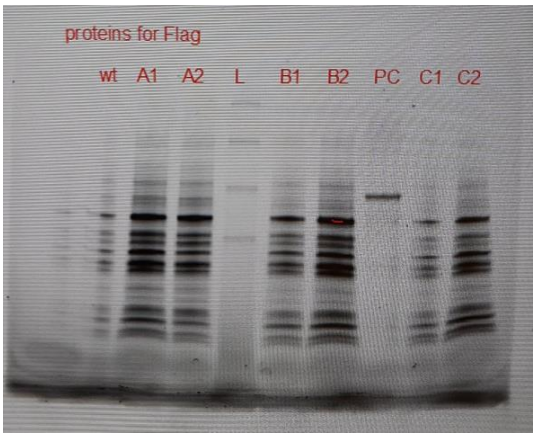
The samples to the right had 20 uL in each well, whilst the left samples had 10 uL.

OBS! Especially the C-samples didn't go into the wells well. Looked like a bit of wt sunk over into the empty well to its left.

Results
Images:



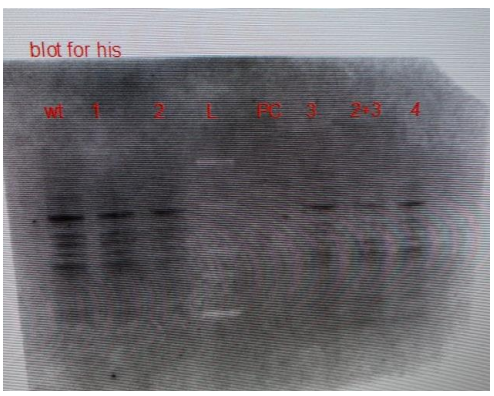
Gel for His



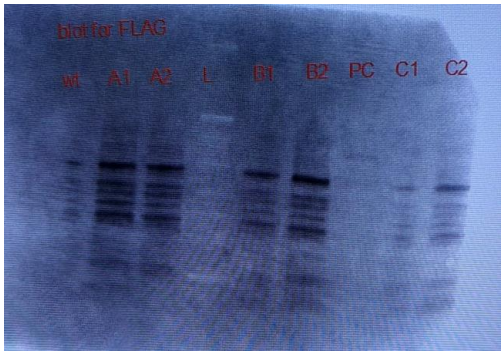
Gel for FLAG

Protein Transfer results
Went well enough. The wt proteins in the FLAG blot seemed to have been transferred enough for us to see something with the antibodies, if they bind to the wt proteins.

Images:
Blot for His



Blot for FLAG



The blots were put in western blocker overnight.

Antibody staining for Western Blot from yesterday

Primary Antibody (Flag tag) - 1:1000 in Western antibody 5 µl in 5 ml western blocker

Note! - We had no cells with GFP (been under UV) So we had to transform some more cells with GFP

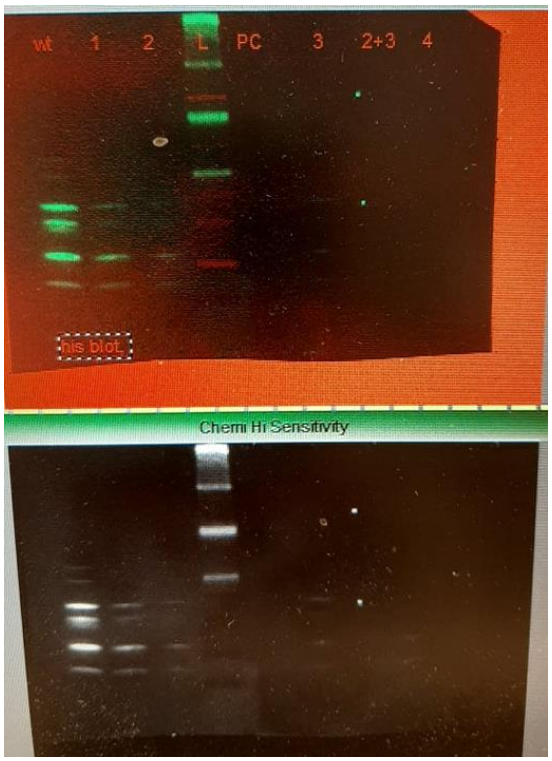
DATE: 30/9

Emma

Western Blot 8 - Antibody incubation

Results: The images shows no wanted bands. Same bands in all strains including wt, and on both blots.

His blot

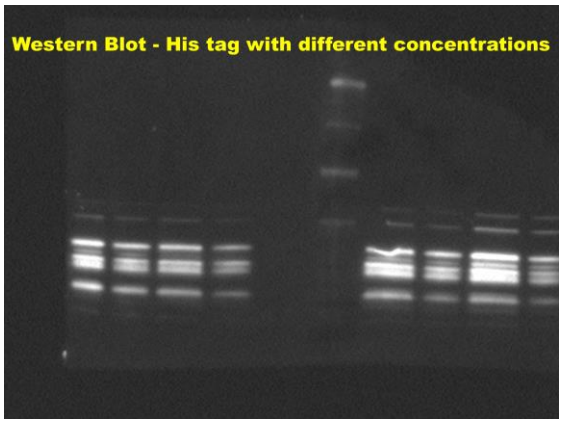


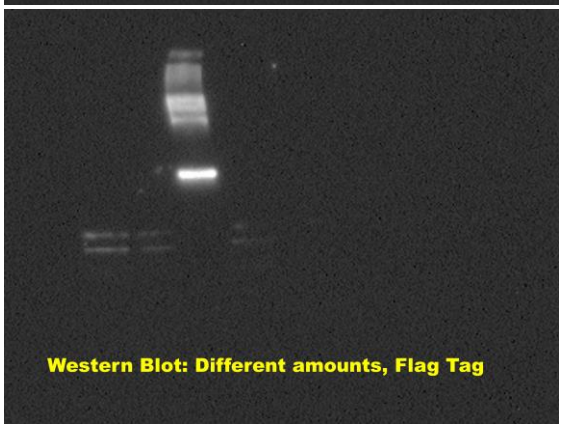
Flag blot

Date : 1/10

Western Blot staining for Emma

Went well, but blot did not show appropriate bands





DATE: 2/10

Emma

Western Blot 9 - Gel electrophoresis and protein transfer of PP4 (protein prep 4), FLAG only, strains 1-4

Since we have already done a western onstrain 0, but never on the other strains, with flag-tag, this western was done to look at the rest of the strains as well.

The Western Blot protocol was followed

Remarks

Gel Electrophoresis

Since the samples were not boiled after the protein prep, they were boiled now, for 5 minutes, before centrifuging them for 2 minutes.

The electrophoresis setup was assembled whilst the samples were heated again (this time with PC), at 95°C, for 10 min, whereafter they were centrifuged for 1 min.

The gel was loaded:
wt, 1, PC, L, 2, 3, 2+3, 4

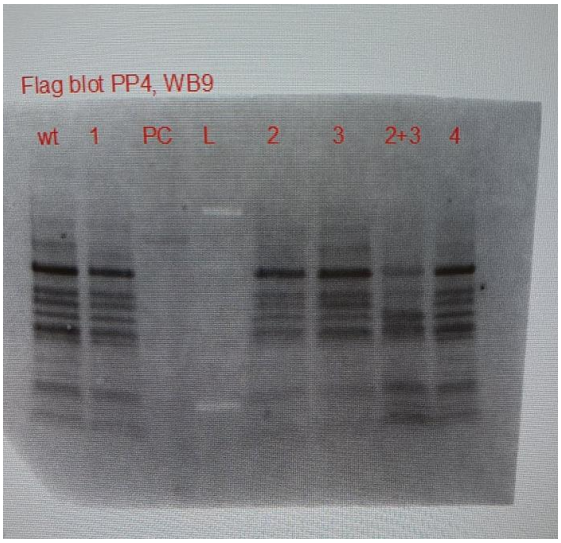
The gel was run at 120 V for 3 minutes, until the samples were inside the gel. Thereafter they were run at 185V for 33 minutes, to make sure the proteins were well separated at the bottom of the gel.

The gel looked good when imaging it.

Protein Transfer

Results: The proteins were transferred nicely to the blot. The blot got a bit dry but seemed to recover from washing with ethanol.

FLAG blot after protein transfer



OBS!!
The blot is usually cut on the top right corner, to enable us to know which way to image it. Today it was accidentally cut on the left bottom corner instead.

DATE: 5/10

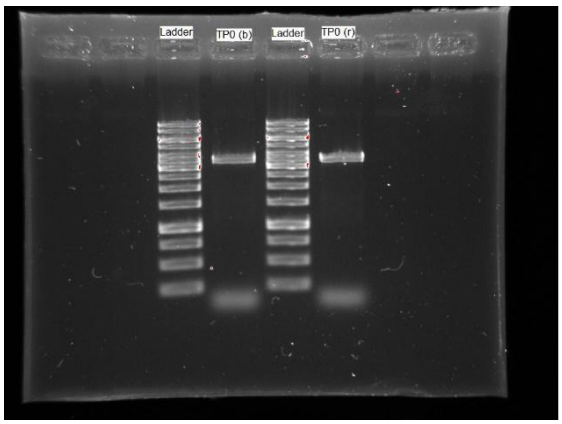
Genome prep for sequencing: Forgot to incubate will do later. So prep can be performed at a later date.
PCR for extraction insert from TP0 genome

35 µl M2 water
10 µl HF-Buffer
1.25 µl Primer 39
1.25 µl Primer 40
1 µl Template 100 ng/µl
1 µl dNTP
0.5 µL Phusion polymerase

There are gels that can be used for verification on bench

98C - 10 min
{
98C - 10 sec
53 C - 10 sec
72 C - 1 min 30 sec
}x32 cycles
72 C - 5 min
15 C forever

Inoculated TP1-TP4 (Yeast strains) in YPD
Intended to do YT1 and P0 as well but could not find the freezer stock



DATE: 6/10

Loaded gels from yesterday's sample.
B = Black mark on top
R = red mark on top
2µl sample, 1 µl L.D, loaded 2 µl mixture.
Loaded 2 µl ladder
1% agarose gel, 90 V, 30 min
Good results for both!

Western Blot restaining
According to the protocol
Spilled ~1ml of antibody 1 for Flag tag