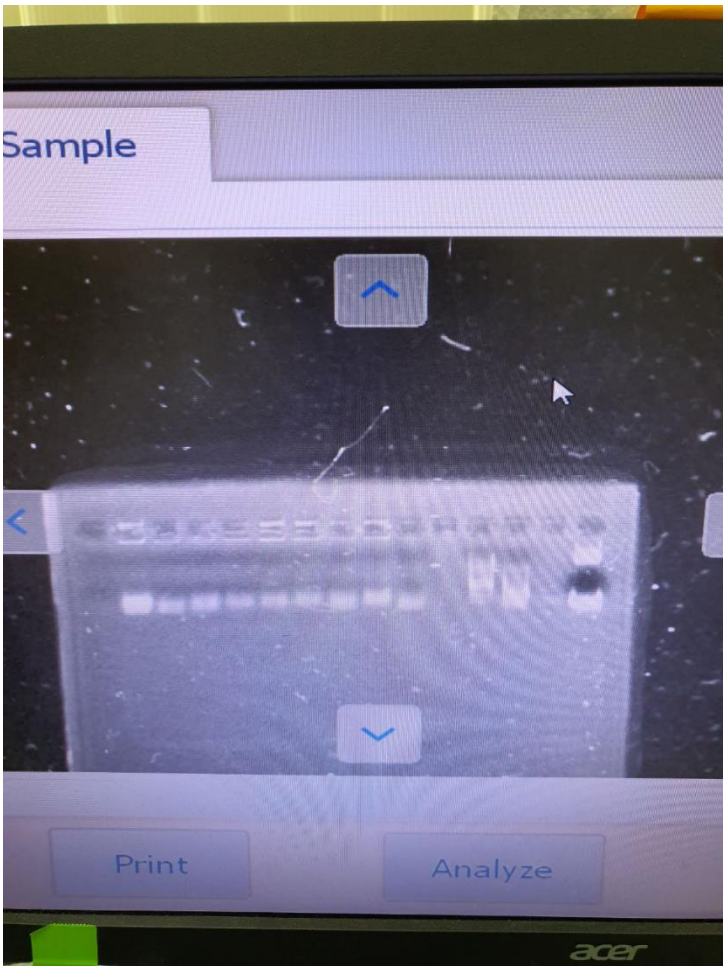
3/8

Sequencing results

Sequencing results for pCE_integrate colony 2a, 6a, 2b and 3b as well as pC120_mKO colonies 1, 6, 7 and 8 were received, all were positive. PCE_integrate colony 2a and pC120_mKO colony 7 were then transformed into Top10 E.coli as they contained the least number of sequencing errors

Colony PCR

Colony PCR was carried out on white colonies from the golden gate reaction to assemble 3xC120 using Lvl0vrf and C120_cyc_rvs



1st lane: Negative control with no colony, 2nd - 10th lane: 9 colonies from the patching

All lanes showed a positive band, and thus the PCR results from colony 1, 3, 4 and 8 were PCR-ed using 3xC120_fwd and rvs in a 50uL KOD reaction

1st -4th lane: Colonies 1, 3, 4 and 8

These bands were gel isolated and 10ul sent for sequencing, the rest was used for cloning 3xC120_mKO. pC120_mKO was amplified with Multi_bb fwd and rvs

1st lane: ladder, 2nd and 3rd lane: pC120_mKO amplified with multi_bb fwd and rvs

Backbone was gel isolated, and each 3xC120 repeat was gibson assembled with the backbone.

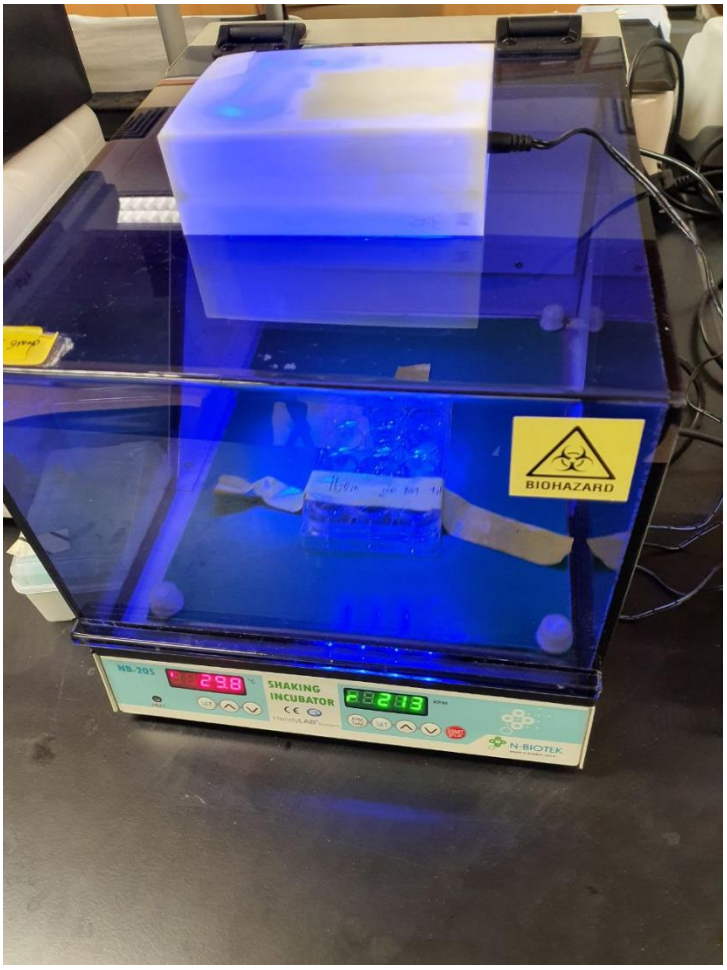
Fluorescence measurement

One 50ml culture of pC120_mKO was placed in a 30 degrees incubator shaking with the blue light shining on top

After 6 hours, the fluorescence was measured with 500 excitation and 560 emission, and compared against the culture in the dark

	OD	Average O	AU	Average AU	Normalized	
	1.192		14470			
	1.167		14814			
	1.184		14498			
	1.197		14310			
	1.184		14434			
Dark	1.179	1.183833	14565	14515.16667	12261.16	
	1.159		42516			
	1.19		42926			
	1.18		43203			
	1.179		43879			
	1.73		43101			Induction t
Light	1.173	1.2685	43320	43157.5	34022.47	2.774817

In general, an increase in fluorescence was observed. Absolute levels were quite high, but background leakiness levels were also high. The plate is thus an unsuitable reactor to culture, and future experiments should be use flasks.

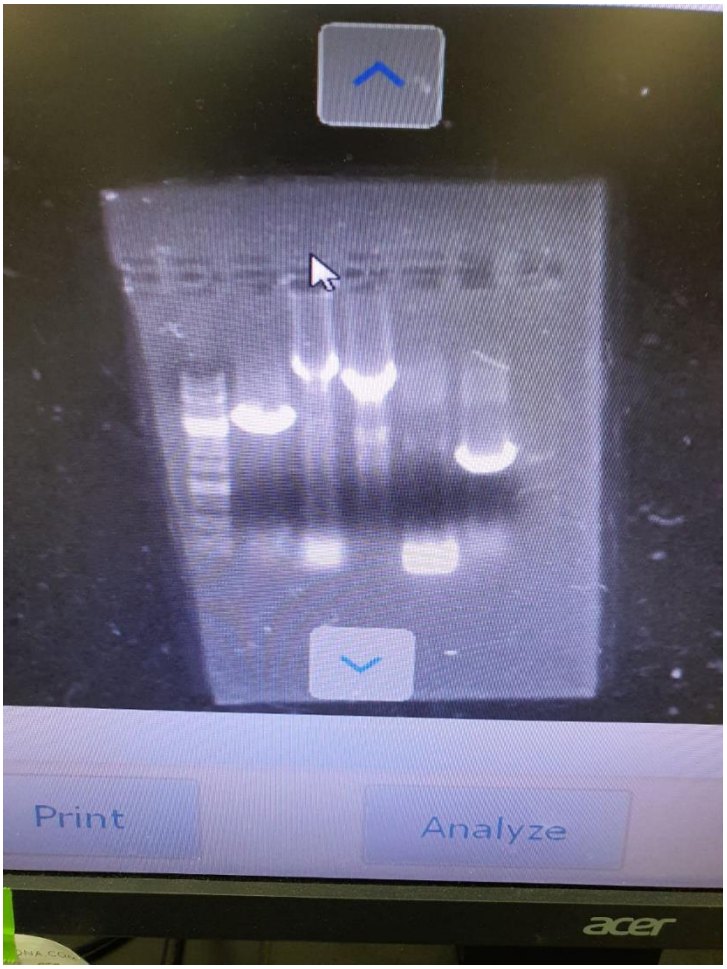


4/8

Gibson assembly

HBD2 gene was amplified from the vacciome plasmid using HDB_fwd and HDB rvs, with the backbone of pGMLfT-H amplified with mfa_his_bb_fwd/rvs.

C120_flo and C120_mKo were amplified with the primers C120_mko/flo_ins_fwd/rvs, and pGMLfT-H was amplified with pGLCM/F_bb_fwd/rvs primers as backbones to plasmids containing the blue light promoter without the EL222 gene



1st lane: Flo1 insert for pGLCF, 2nd lane: Backbone for pGLCF/M, 3rd lane: Backbone for pHBD, 4th lane: HBD insert, 5th lane: mKO insert for pGLCM

Since bands were all of the desired size, they were gel extracted and the purified fragments were run again in a gel electrophoresis.

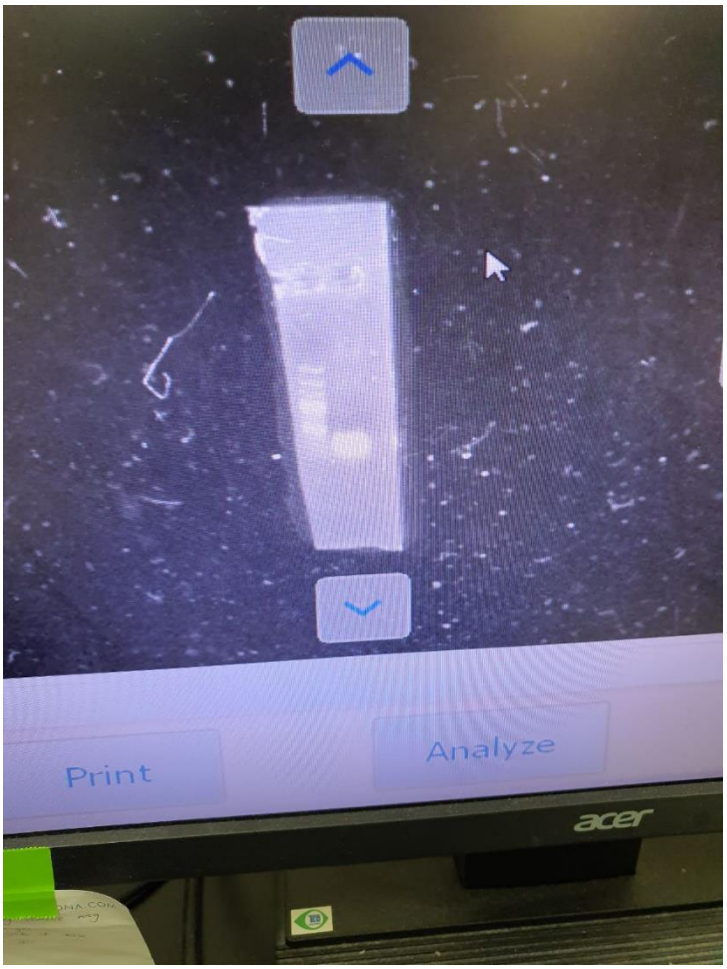
1st lane: Flo1 insert for pGLCF, 2nd lane: Backbone for pGLCF/M, 3rd lane: Backbone for pHBD, 4th lane: HBD insert, 5th lane: mKO insert for pGLCM

Gibson assembly for pGLCF, pGLCM and pHBD was carried out

3xC120

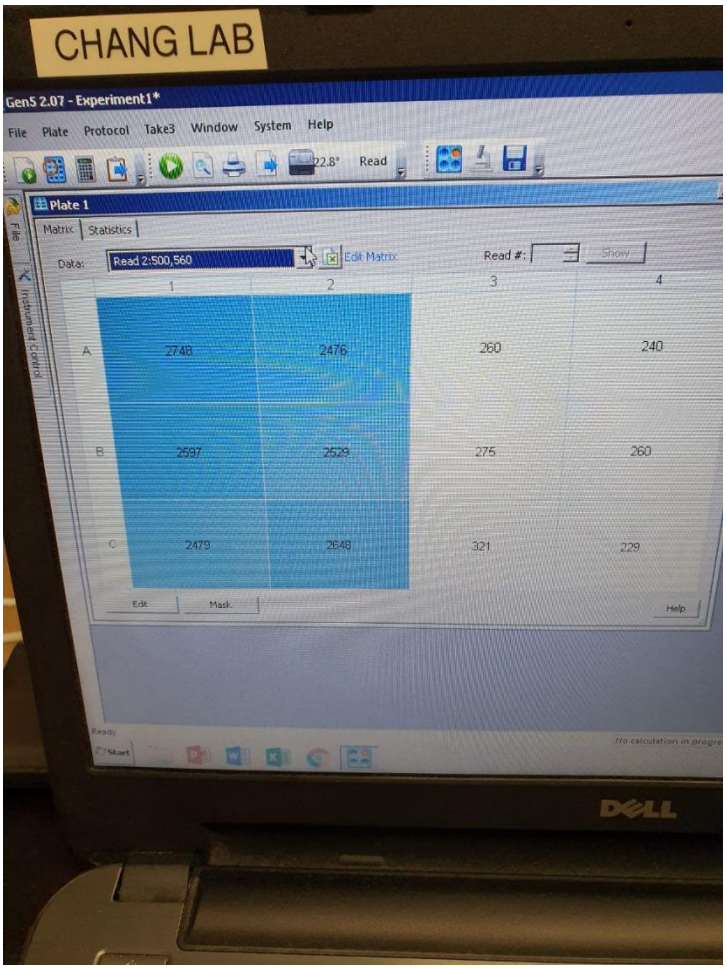
3xC120 fragment was amplified from colonies using Lvl0vrf and C120_cyc_rvs

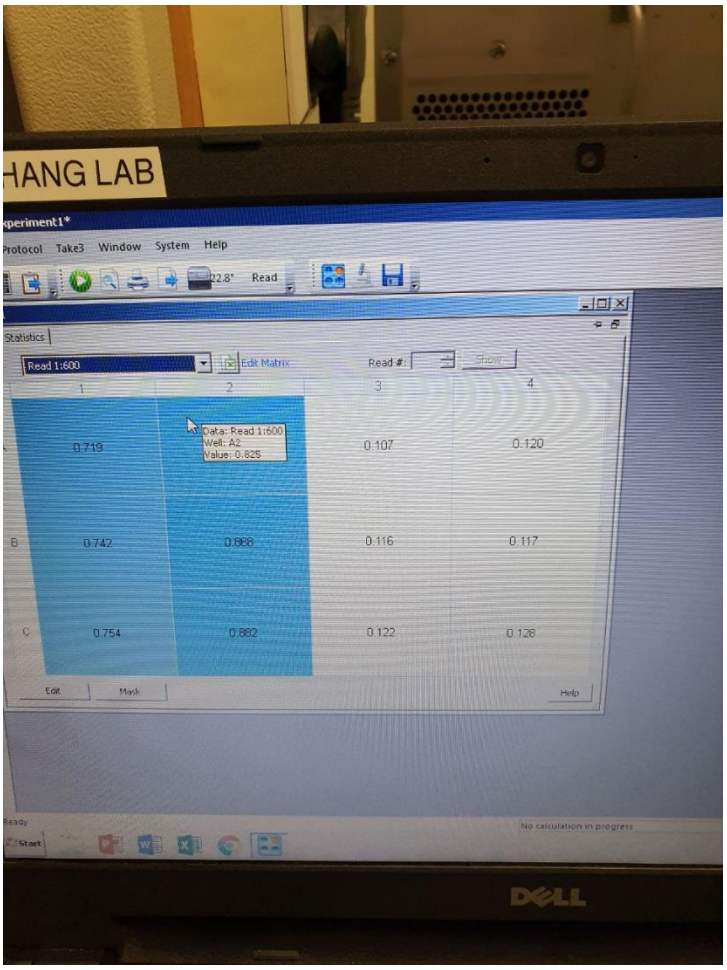
Fragment was gel isolated and sent for sequencing, and the remaining PCR reaction was scaled up to 50ul with Taq polymerase



Fluorescence

PC120_mko that was left in the incubator with blue light overnight was measured


CHANG LAB
GenS 2.07 - Experiment1*
File Plate Protocol Take3 Window System Help
Read
Plate 1
Matrix Statistics
Data: Read 2:500,560
Edit Matrix
Read #:
Show
1 2 3 4
A 2748 2476 260 240
B 2597 2529 275 260
C 2479 2648 321 229
Edit
Mask
Help
Ready
Start
DELL



Fluorescence seems to have disappeared overnight, and the induced culture had no higher fluorescence than the uninduced culture. This could be because of several reasons, the induction may have stopped due to the cells hitting stationary phase/carbon source depleted, and the EL222 degraded, or the promoter may have deactivated due to lack of oxygen as CYC1 is a aerobic activated promoter. Excessive levels of EL222 may also be toxic to the cell.

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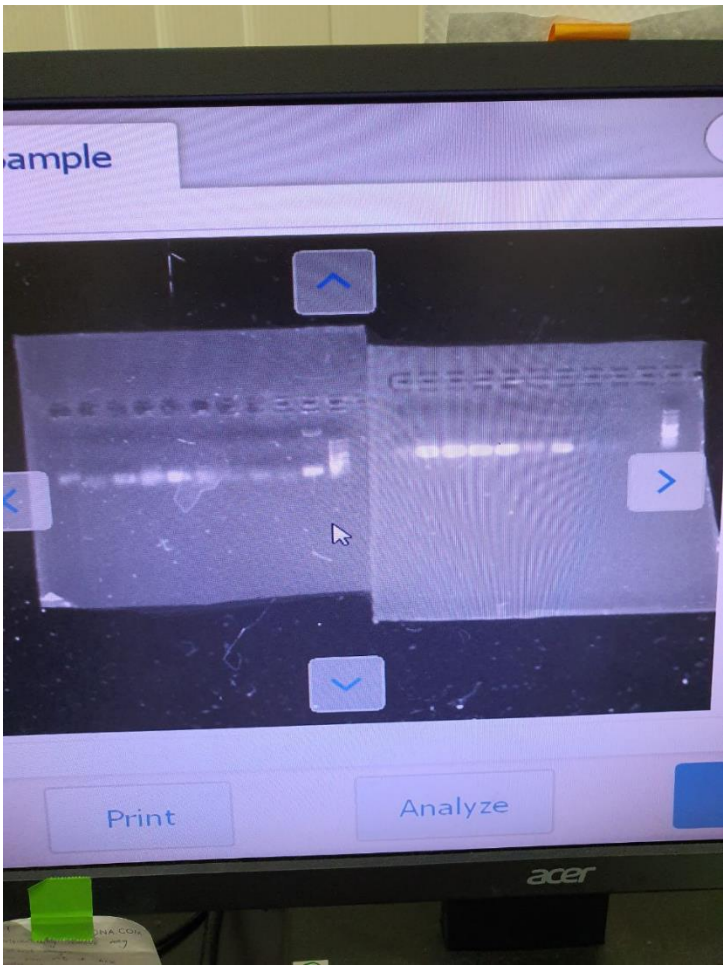
Colony PCR

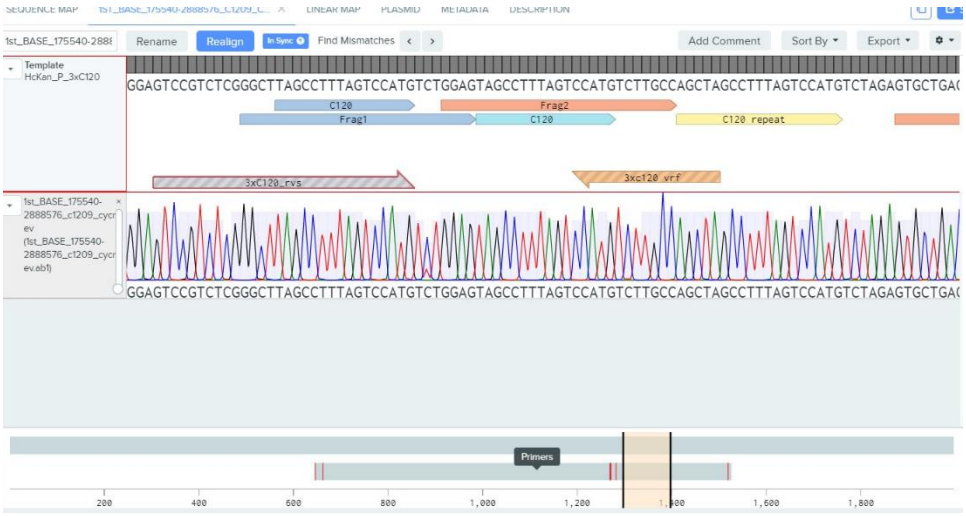
pHBD was verified by using HBD_fwd and HBD_rvs, whereas pGLCM and pGLCF were verified using C120_CYC fwd and rvs



Left gel, 1st -4th lane: colony 5 to 9 of pGLCF, 5th-9th lane: colony 5-10 of pGLCM, 10th lane: positive control using pC120_flo. Right gel. 1st-8th lane: colony 2, 3, 5-10 of pHBD, 9th lane: positive control of original vacciome plasmid

<u>3xC120 sequencing</u>

3xc120 sequencing was shown as positive



As such primers were ordered to facilitate cloning of 3xC120 into the 1xC120 sequencing, and extending the C120 repeats to 5, and replacing the current C120 repeat with the exact sequence from a paper.

NEW3xC120_fwd	gtgtcagcacTCTAGACATGGACTAAAGGCTAG	**HcKan_P_C120p**	Forward primer for 3xc120 fragment for gibson
NEW3xC120_rvs	taaatgttgtGCAGCAGATTACGCGCAG	**HcKan_P_C120p**	Reverse primer for 3xc120 fragment for gibson
NEWMulti_bb_fwd	aatctgctgcACAACATTTAACCTACATTCTTCC	Anything with 1xc120-cyc	Forward primer for 3xC120 backbone
NEWMulti_bb_rvs	catgtctagaGTGCTGACACTACAGGCATATATATATG	Anything with 1xc120-cyc	Reverse primer for 3xC120 backbone
NEW3xC120_vrf	AGTCCATGTCTGGAGTAG		
5xC120_fwd	aatggtctct**tgcc**GTCCGTCTCGGGCTTA	**HcKan_P_3xC120p**	Golden Gate for 5xc120 fragment
C121F	atggtctct**GGCT**TAGGTAGCCTTTAGTCCATG**GGAG**cgagaccta		
C121R	taggtctcg**CTCC**CATGGACTAAAGGCTACCTA**AGCC**aggagaccat		
C122F	atggtctct**GGAG**TAGGTAGCCTTTAGTCCATG**TGCC**cgagaccta		
C122R	taggtctcg**GGCA**CATGGACTAAAGGCTACCTA**CTCC**aggagaccat		
Circle_rectF	GGCTACCTAGCTACTAGTAGCGAAC	**pC120_mKO**	
Circle_rectR	GTAGCTAGGTAGCCTTTAGTCCATG	**pC120_mKO**	

Stocks

Glycerol stocks were made of E.coli transformed with pC120_flo, pCE_integrate, pHcKan_P_3xC120 and vacciome plasmid, and of By4741 transformed with pC120_flo, and strain BY474B(BY4741 with genome integrated El222)

BY474B was also spun down and resuspended in YPD

Blue light induction

1ml cultures of pC120_mKO and pC120_flo were kept in the dark as well in blue light, but no induction was observed, this may be because of a lack of oxygenation, carbon, or sufficient light. 100ml flasks of 25ml cultures of pC120_flo and pC120_mKO in the dark and light

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Fluoresence induction

Triplicates for the fluorescence of the pC120_mKO in the light and dark for overnight induction were taken

	OD	Average OD	AU	Average AU	Normalized	
	0.699		8033			
	0.869		1187			
Dark	0.807	0.791667	9934	6384.667	8064.842	
	1.129		46020			
	1.137		45375			
Light	1.113	1.126333	43878	45091	40033.44	4.963946

These were then split into 6 cultures in falcon tubes, and the remainder was kept in the 10 ml flask to be illuminated by blue light

1. Induced culture, continued to be induced
2. Induced culture, kept back in the dark
3. Induced culture, media swapped for fresh media, and continued to be induced
4. Induced culture, media swapped for fresh media, kept back in the dark
5. Uninduced culture, started induction
6. Uninduced culture, kept back in the dark

Measurements were taken every hour

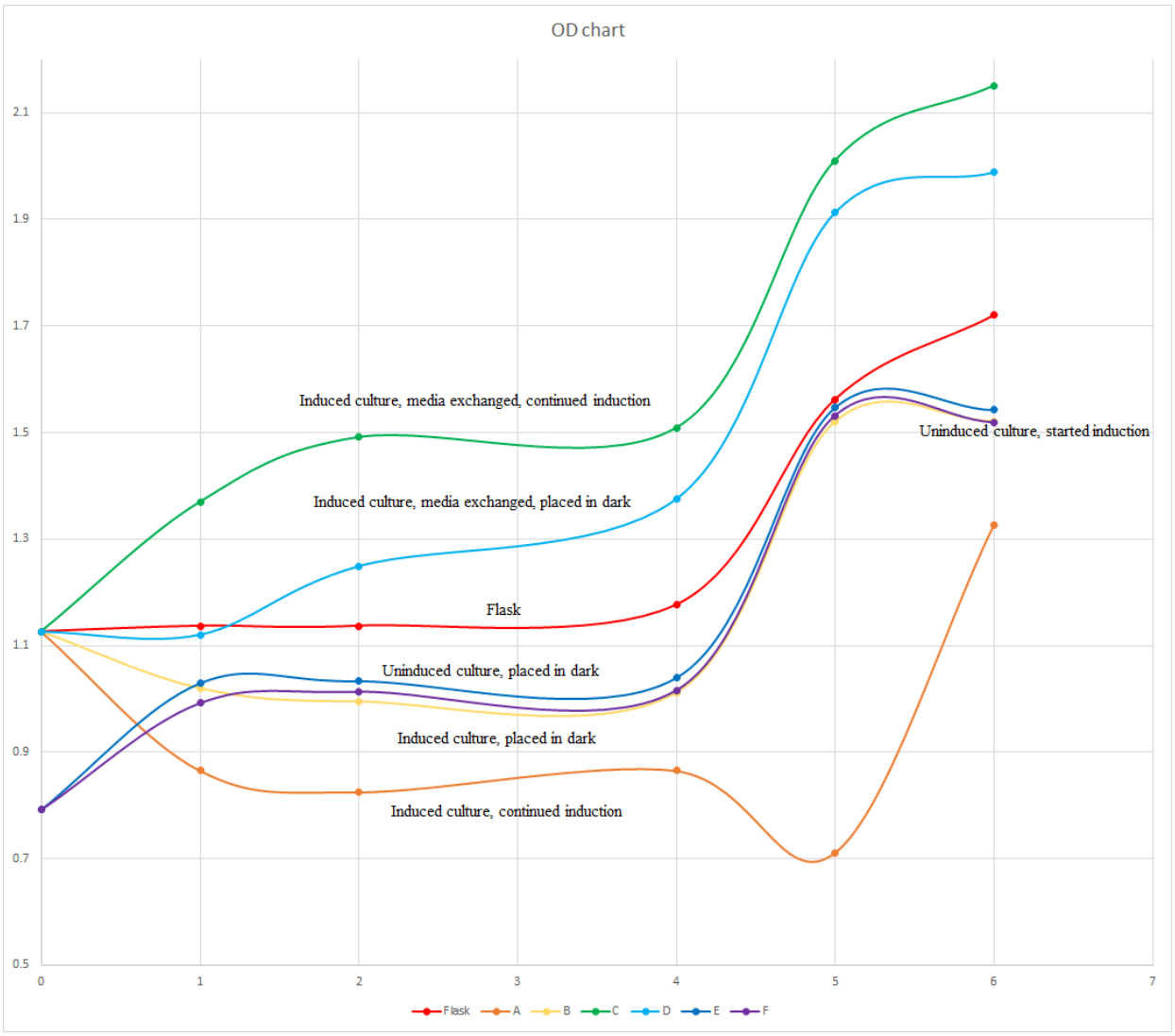
1030	OD	Average O	AU	Average AU	Normalized
	1.159		45040		
	1.142		43213		
Flask	1.109	1.136667	41639	43297.33	38091.5
	0.858		22197		
A	0.873	0.8655	22572	22384.5	25863.08
	1		34925		
B	1.018	1.02	35373	35149	34459.8
	1.366		64665		
C	1.373	1.3695	64279	64472	47077.04
	1		41756		
D	1.11	1.1205	40700	41228	36794.29
	1.009		13143		
E	1.05	1.0295	14207	13675	13283.15
	1.007		13928		
F	0.977	0.992	14178	14053	14166.33

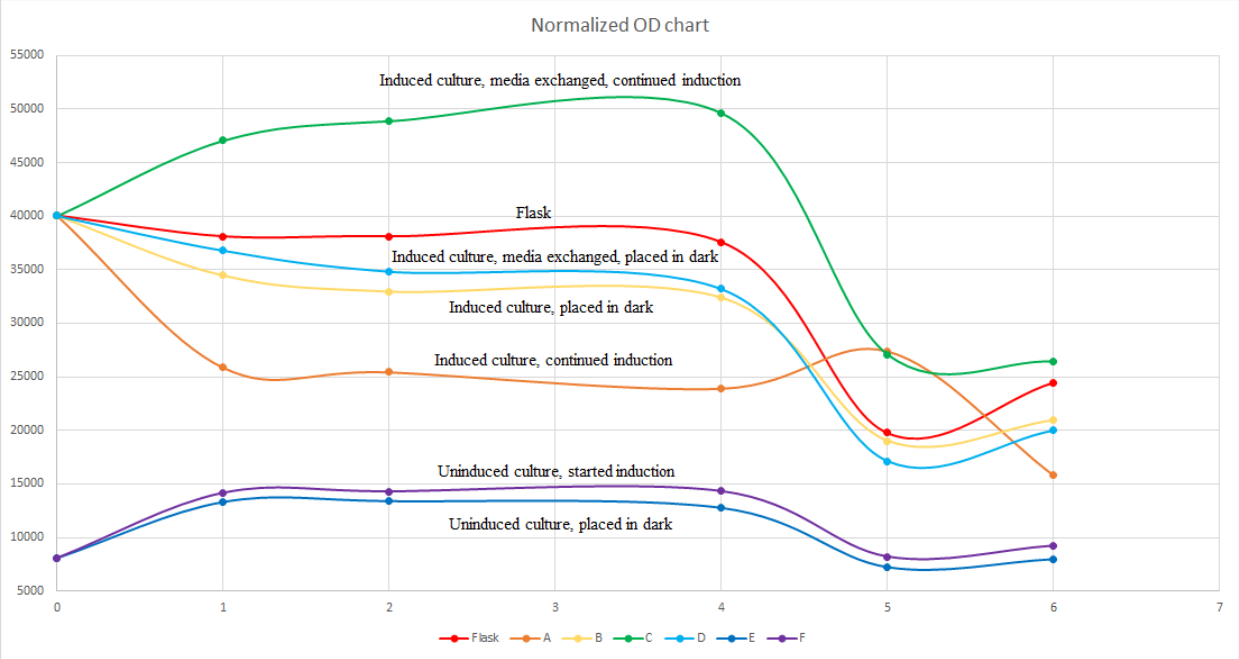
1130	OD	Average O	AU	Average AU	Normalized
Flask		0		0	#DIV/0!
	0.869		23010		
A	0.781	0.825	18960	20985	25436.36
	0.989		32334		
B	1.001	0.995	33257	32795.5	32960.3
	1.493		72884		
C	1.489	1.491	72979	72931.5	48914.49
	1.243		41890		
D	1.255	1.249	45116	43503	34830.26
	1.05		14359		
E	1.017	1.0335	13262	13810.5	13362.84
	1.016		14534		
F	1.011	1.0135	14453	14493.5	14300.44

1330	OD	Average O	AU	Average AU	Normalized
	1.198		44809		
	1.156		42966		
Flask	1.175	1.176333	44717	44164	37543.78
	0.858		20352		
A	0.872	0.865	21024	20688	23916.76
	0.992		32249		
B	1.031	1.0115	33290	32769.5	32396.94
	1.496		72370		
C	1.521	1.5085	77435	74902.5	49653.63
	1.362		44424		
D	1.388	1.375	46876	45650	33200
	1.041		13576		
E	1.038	1.0395	12898	13237	12734.01
	0.994		14234		
F	1.038	1.016	14854	14544	14314.96

1430	OD	Average O	AU	Average AU	Normalized
	1.492		26806		
	1.544		30341		
Flask	1.651	1.562333	35533	30893.33	19773.84
	1.291		19789		
A	0.1314	0.7112	19173	19481	27391.73
	1.529		28117		
B	1.512	1.5205	29674	28895.5	19003.95
	2.095		51788		
C	1.925	2.01	57143	54465.5	27097.26
	1.901		31883		
D	1.924	1.9125	33669	32776	17137.78
	1.559		11114		
E	1.535	1.547	11188	11151	7208.145
	1.537		12436		
F	1.526	1.5315	12611	12523.5	8177.277

1530	OD	Average O	AU	Average AU	Normalized
	1.716		42890		
	1.732		40776		
Flask	1.713	1.720333	42661	42109	24477.23
	1.327		20964		
A	1.324	1.3255	20942	20953	15807.62
	1.524		32395		
B	1.519	1.5215	31286	31840.5	20927.05
	2.2112		55490		
C	2.09	2.1506	58285	56887.5	26451.92
	1.991		40144		
D	1.986	1.9885	39461	39802.5	20016.34
	1.534		12787		
E	1.552	1.543	11786	12286.5	7962.735
	1.524		14120		
F	1.514	1.519	13881	14000.5	9216.919


OD chart
2.1
1.9
1.7
1.5
1.3
1.1
0.9
0.7
0.5
0
1
2
3
4
5
6
7
Induced culture, media exchanged, continued induction
Induced culture, media exchanged, placed in dark
Flask
Uninduced culture, placed in dark
Induced culture, placed in dark
Induced culture, continued induction
Uninduced culture, started induction
Flask
A
B
C
D
E
F



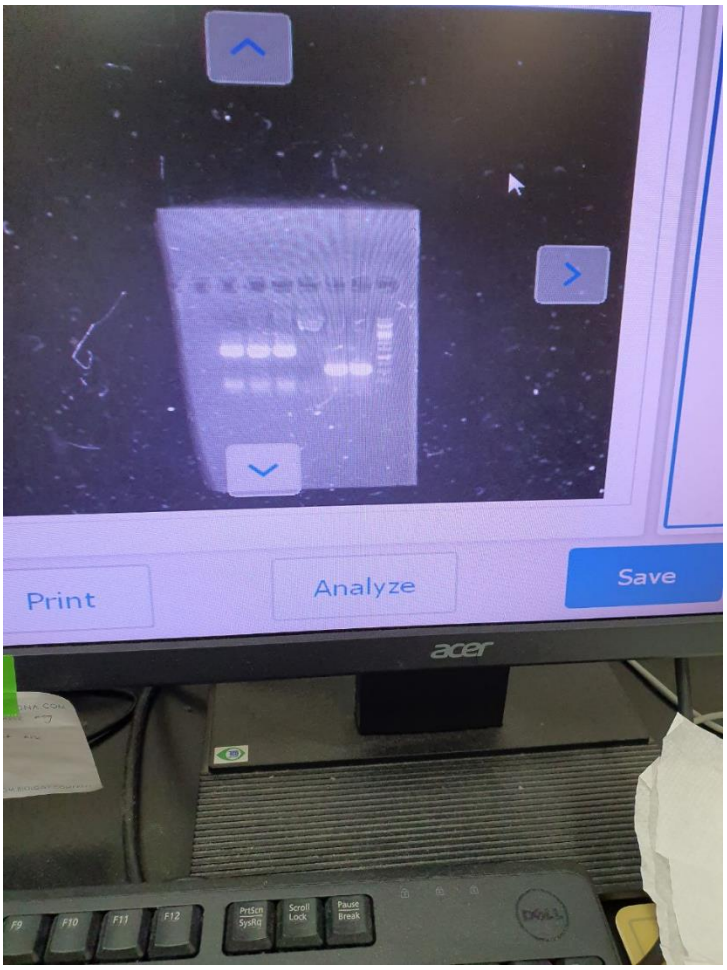
In general it seems that the OD rapidly enters a second exponential phase at 4 hours, and this causes a decrease in the normalized fluorescence. Future experiments should characterize variance in fluorescence over time.

Flocculation

Flocculation induction initially seemed to be unsuccessful, but upon changing medium to YPD floculation could be observed after several hours of induction, although not as clearly as with the GAL promoter. A stronger promoter may be needed for flocculation, and induction should be done in YPD, I suspect that this may be because of the amount of calcium ions present in the medium

Plasmid sequencing

Plasmids for colonies 6 and 7 of pGLCM and colonies 8 and 9 of pGLCF were isolated and underwent PCR with pGLfT_seq1 and C120_cyc_rvs, and colonies 3 and 5 of pHBD were isolated and underwent PCR with Gal_seq and HBD_rvs



1st - 2nd lane: pGLCM, 3rd-4th lane: pGLCF, 5th -6th lane: pHBD

All 6 were sent for sequencing

Inoculation

BY474B in YPD was seeded into YNB-HIS medium

Transformation

Transformation for BY474B with pHBD, pGLCM and pGLCF were carried out

Golden gate reaction

Golden gate reaction was carried out with pHcKan_P, C1201, C1202 and amplifed fragment of 3xC120 from HcKan_P_3xC120. Mixture was run at 5 minute cycles of 37 and 18 degrees celcius, 60 times before being kept at 4 degrees

7/8

Transformation

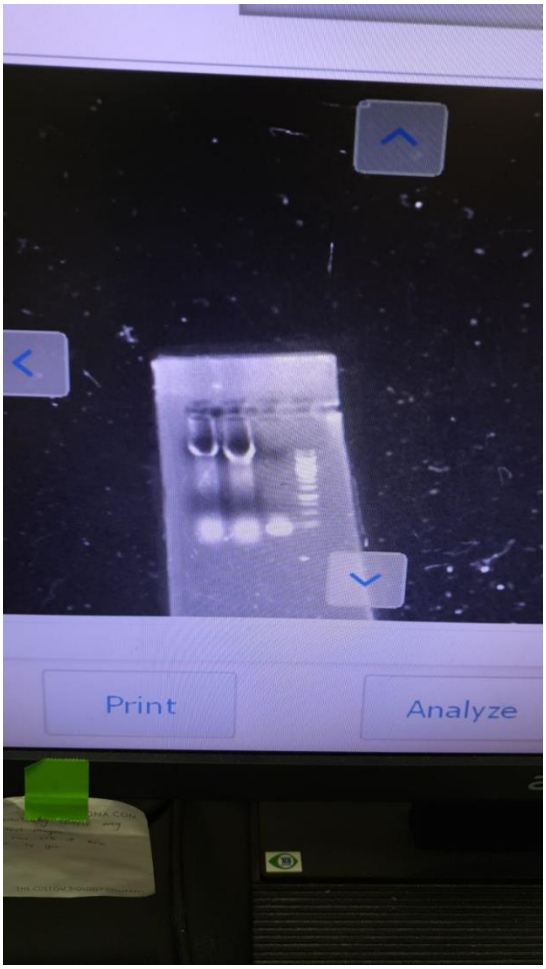
Transformation plates looked like a smear, and thus it was suspected that the HygB was non functional. Thus, recovering medium from the previous day was plated onto freshly made YPD-HygB

Golden gate tranformation

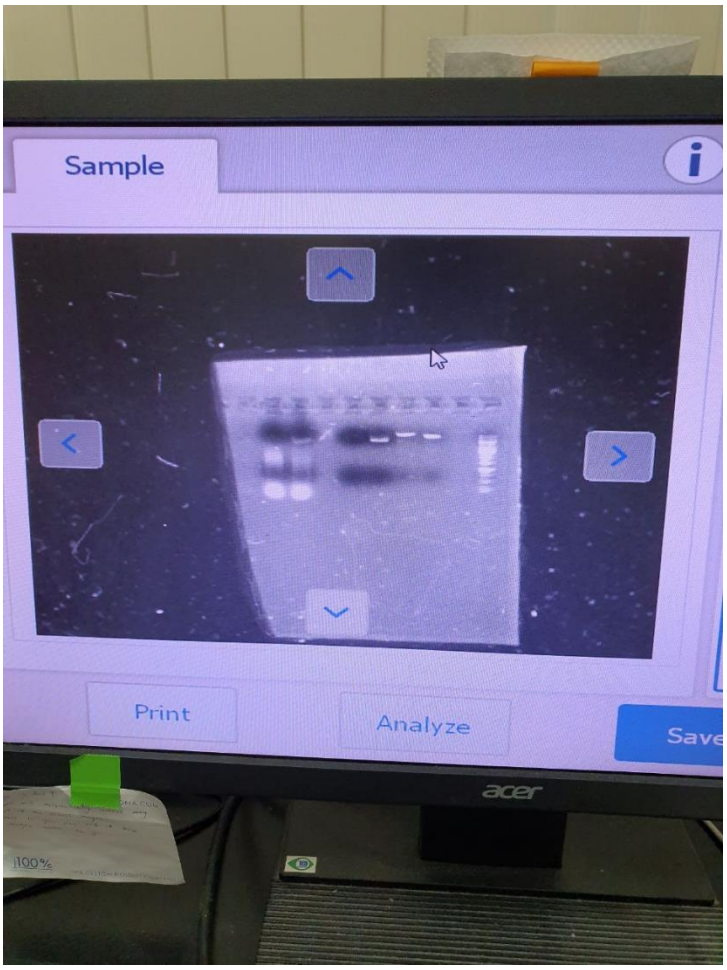
Golden gate mixture from overnight reaction was transformed into E.coli and plated on LB Kan

PCR for gibson assembly.

pGLCM and pC120_mko were amplified with NEWMulti_bb_rvs and fwd, but no band was seen with KOD. Reaction was then retried with Q5, but no band was seen either. HcKan_P_3xC120 was PCR-ed with NEW3xC120_fwd and rvs, and the band was gel isolated



1st lane: pGLCM amplified with NEWMulti_bb_rvs and fwd, 2nd lane: pC120_mKO amplified with NEWMulti_bb_rvs and fwd, 3rd lane: HcKan_P_3xC120 was PCR-ed with NEW3xC120_fwd and rvs



1st lane: pGLCM amplified with Q5, 2nd lane pC120_mKO amplified with Q5, 3rd lane pGLCM template diluted to 25uL, 4th lane: pC120_mKO template diluted to 25uL, 5th lane: pGLCM amplified with KOD and gel extracted, 6th lane: pC120_mKO amplified with KOD and gel extracted.

It seems evident that no band was being amplified for the backbone, and a large amount of primer dimers were present. Upon closer examination, the primer NEWMulti_bb_rvs had a palindromic sequence in the 3' end which may have been causing the dimers to appear and inhibiting amplification

Inoculation

Seed culture of BY474B in YNB-HIS grew, and was re-seeded into 50ml of YPD. 50ml culture of pC120_flo BY4741 in YNB-URA was started. Two 5ml cultures of pC120_flo BY4741 were media exchanged for YPD, one was left in the light and one was left in dark

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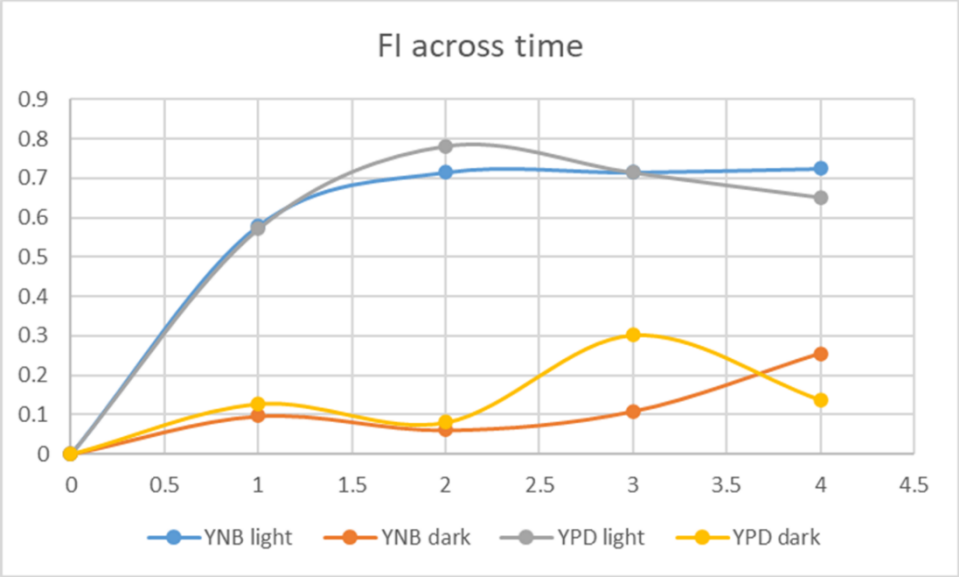
Flocculation testing

40ml culture of pC120_flo BY4741 was split into 4 tubes, and spun down. The media was discarded, and two tubes were filled with 5ml YNB, two tubes were filled with 5ml YPD. One of each was put in the dark, and the other pair was placed in the incubator with blue light induction. Every one hour, OD was

measured for each tube, and the culture left for 1 minute to settle. OD of the supernatant was measured again, and the flocculation index was taken as 1-(settled OD/initial OD).

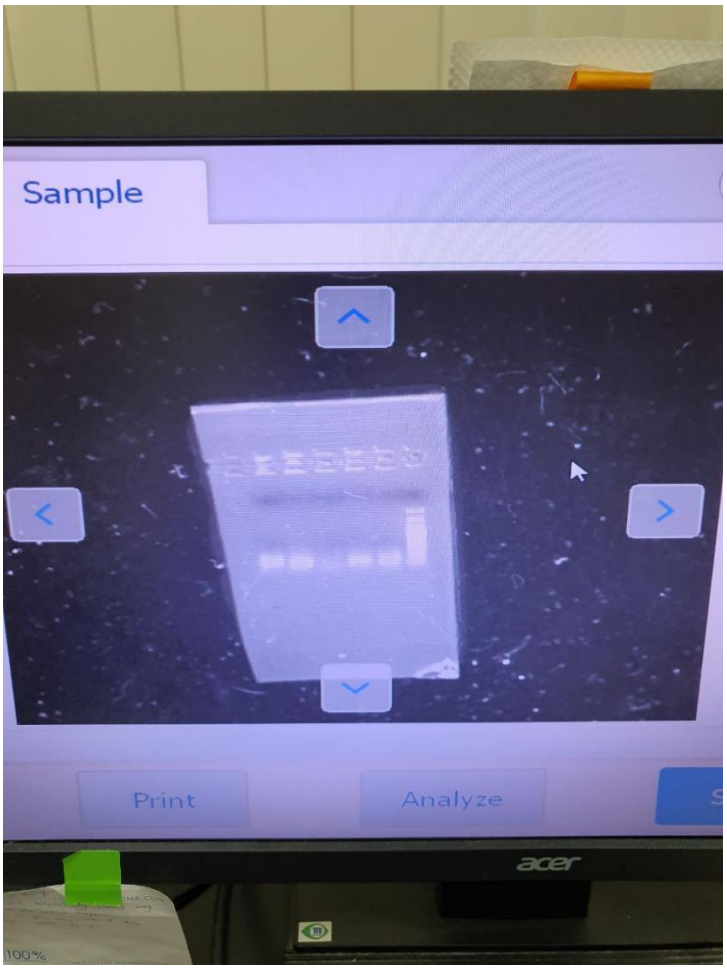
		YNB light	YNB dark	YPD light	YPD dark
1pm	Initial OD	4.5	3.7	3.6	5.4
	1m later	4.5	3.7	3.6	5.4
	FI	0	0	0	0
2pm	Initial OD	3.8	5.2	3.5	4.7
	1m later	1.6	4.7	1.5	4.1
	FI	0.578947	0.096154	0.571429	0.12766
3pm	Initial OD	4.9	6.6	5.5	6.2
	1m later	1.4	6.2	1.2	5.7
	FI	0.714286	0.060606	0.781818	0.080645
4pm	Initial OD	7	8.3	8.4	9.6
	1m later	2	7.4	2.4	6.7
	FI	0.714286	0.108434	0.714286	0.302083
5pm	Initial OD	10.5	9.8	11.2	12.3
	1m later	2.9	7.3	3.9	10.6
	FI	0.72381	0.255102	0.651786	0.138211



While flocculation was clearly faster for blue light induction, the non-induced batch showed non-negligible flocculation as well, once again confirming that the leakiness needs to be suppressed.

Colony PCR

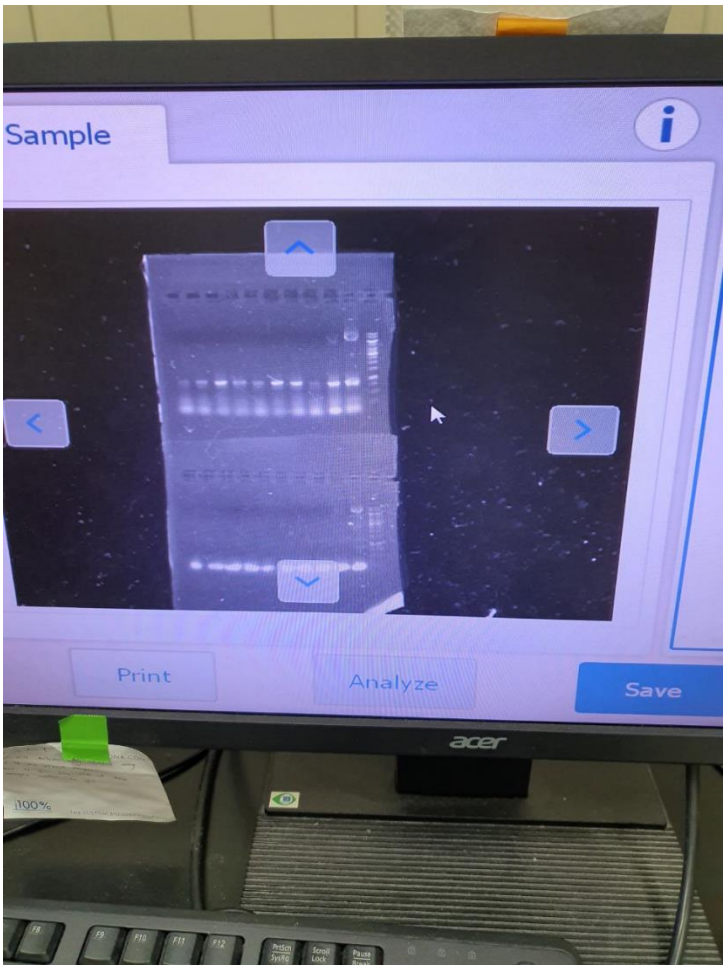
5 white colonies from the golden gate transformation were picked and underwent PCR with NEW3xC120_fwd and rvs



1st-5th lane: PCR reactions with NEW3xC120_fwd and rvs

Colonies 1 and 2 were inoculated in 5ml LB Kan200 and left at 30 degrees shaking

4 colonies from the transformation of BY474B with pGLCM and pGLCF were picked and boiled in NaOH 20mM for 20 minutes, and underwent PCR with HcKan_O_EL222_fwd and rvs to check for the EL222 integration in the genome, and C120_cyc_fwd and rvs to check for the episomal plasmid



Top gel, 1st-4th lane pGLCM amplified for EL222, 5th-8th lane: pGLCF amplified for EL222, 9th lane: pGLCM purified plasmid negative control amplified for EL222, 10th lane: pGLCF purified plasmid negative control amplified for EL222

Bottom gel, 1st-4th lane pGLCM amplified for C120_cyc, 5th-8th lane: pGLCF amplified for C120_cyc, 9th lane: pGLCM purified plasmid positive control amplified for C120_cyc, 10th lane: pGLCF purified plasmid positive control amplified for C120_cyc

While all colonies showed positive for EL222 and C120_cyc, the negative controls were also shown to give a band for EL222 for unknown reasons.

Inoculation

BY474B in YPD was seeded in 50ml YPD at 2%

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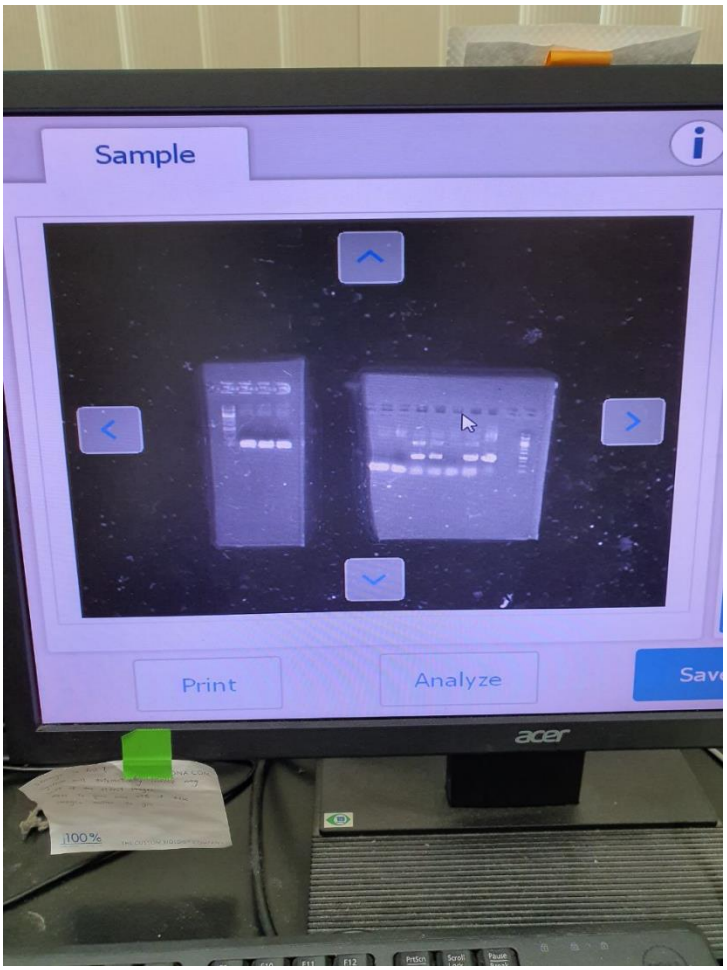
Colony PCR

1uL of BY474B liquid culture, 1uL of BY474B pGLCM, Colony 1 and 3from the patching of BY474B pGLCM were boiled in 20mM NaOH for 20 minutes

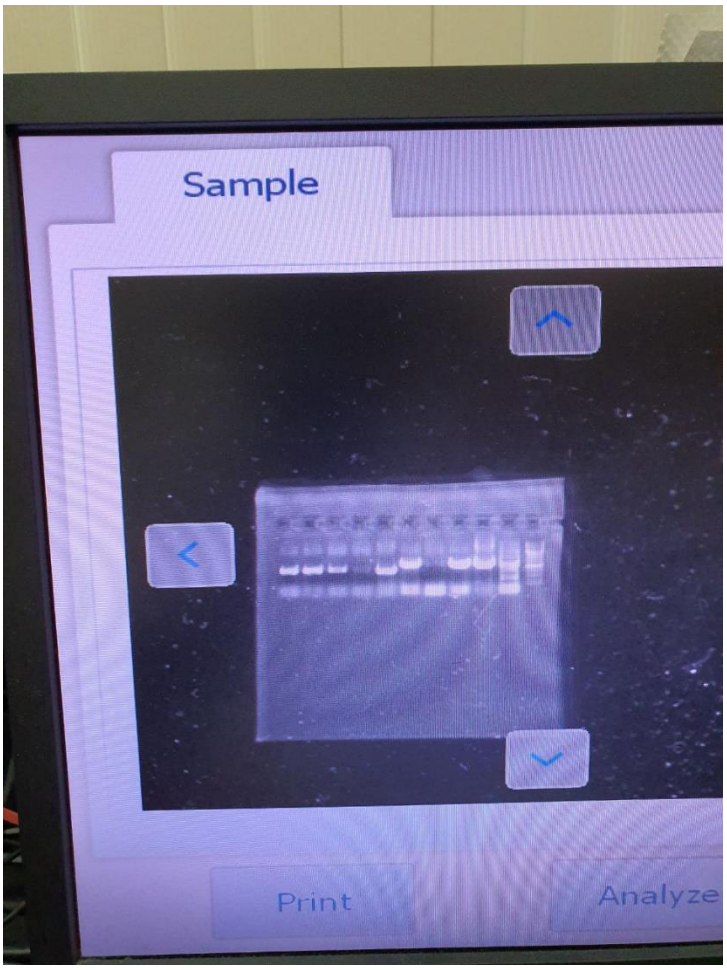
PCR was then carried out using primers to amplify EL222 and CYC_C120



1st lane: Col 1 CYC-c120 PCR, 2nd lane Col 3 CYC-C120_PCR, 3rd lane: Liquid culture CYC_C120 PCR, 4th lane: BY474B CYC_C120 PCR, 5th lane: positive control of pGLMC with CYC_C120, 6th lane: Col 1 EL222 PCR, 7nd lane: Col 3 EL222 PCR, 8rd lane: Liquid culture EL222 PCR, 9th lane: BY474B EL222, 10th Lane: pCE_integrate EL222 PCR.

BY474B showed a positive band for CYC_C120 despite not being transformed with a blue light plasmid. BY474B transformed with pGLMC gave a negative band for EL222 despite the colony 3 that it originated from being positive for EL222

Another PCR was carried out with C120_CYC_fwd to mKO_rvs, and EL222_fwd to reccom vrf rvs



1st lane: Col 1 CYC-mKO PCR, 2nd lane Col 3 CYC-mKO _PCR, 3rd lane: Liquid culture CYC-mKO PCR, 4th lane: BY474B CYC-mKO PCR, 5th lane: positive control of pGLMC with CYC-mKO, 6th lane: Col 3 EL222 to reccom vrf PCR, 7th lane: Liquid culture EL222 to reccom vrf PCR, 8th lane: BY474B EL222 to reccom vrf, 9th Lane: pCE_integrate EL222 PCR, 10th lane pGLMC negative control

This time, BY474B did not show a lane for the mko to cyc promoter as expected, but the liquid culture also did not show EL222 was integrated into its genome. The negative control of pGLMC also shows many unspecific bands, which may mean that the plasmid stock in not pure.

As such, the plasmids pGLCM and pGLCF were re-transformed into E.coli for selection again

<u>Cloning pGL5CM/F</u>

The following primers were ordered to clone 5xC120 repeats

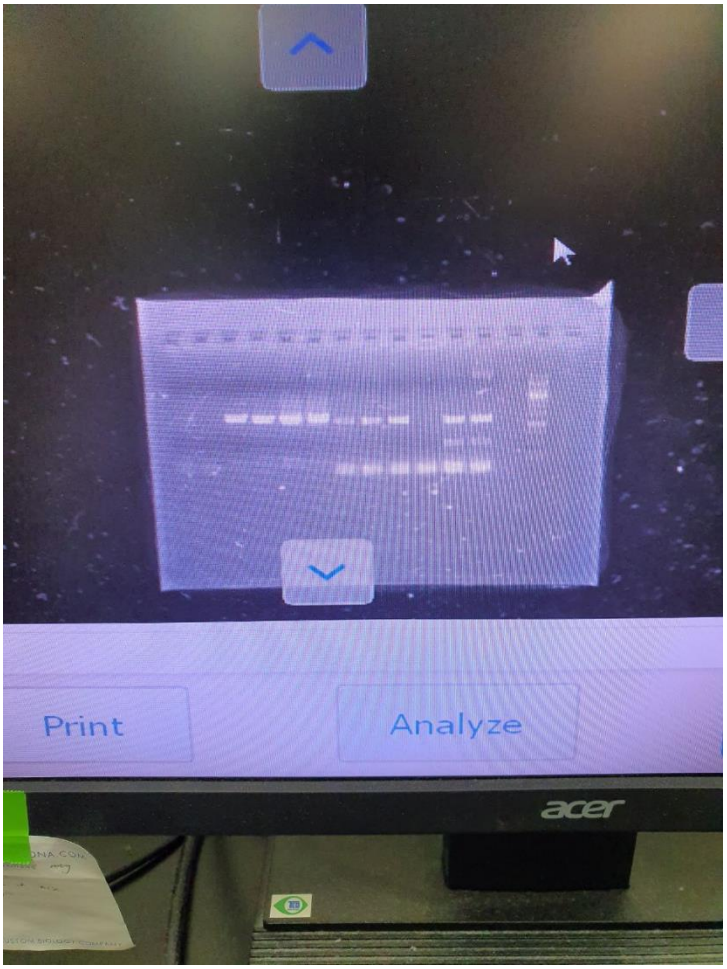
Name	Sequence	Template	Purpose
pGL5CM_bb_fwd	ggcctttcttGAAGCGGGTAAGCTGCCAC	pGLCM/F	Backbone for inserting 5xC120 repeats
pGL5CM_bb_rev	aa*attgtgagcggataacaatt*TATTCTTTCCTTATACATT	pGLCM/F	Backbone for inserting 5xC120

			repeats + lacO sequence
5c120ins_fwd	attgttatccgctcacaaTTTAGAGAAAAGAAGAAAACAAGAGTTTTATATACATACAG	HcKan_5xC120	Insert for 5xC120 +lacO
5c120ins_rev	tacccgcttcAAGAAAGGCCCACCCGTG	HcKan_5xC120	Insert for 5xC120

11/8

Colony PCR

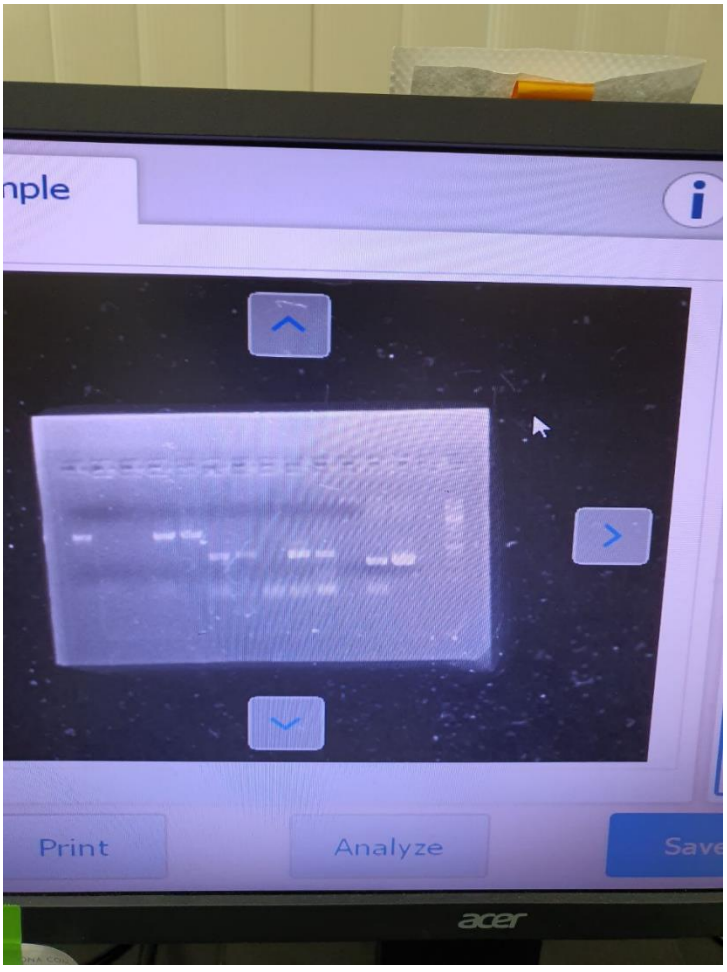
1uL of BY474B pGLCM and pGLCF liquid culture were boiled, 1 colony from each transformation was taken(pGLCM and pGLCF), and the original plasmid of each, were run with PCR for C120_cyc_rvs to pGLFT_seq1, and EL222rvs and fwd



1st lane: liquid culture of pGLCM with CYC-Lf PCR, 2nd lane: liquid culture of pGLCF with CYC-Lf PCR, 3rd lane: 1 colony of pGLCM transformants with CYC-Lf PCR, 4th lane: 1 colony of pGLCF transformants with CYC-Lf PCR, 5th lane: original pGLCM plasmid with CYC-Lf PCR, 6th lane: original

pGLCF plasmid with CYC-Lf PCR, 7th lane: liquid culture of pGLCM with EL222 PCR, 8th lane: liquid culture of pGLCF with EL222 PCR, 9th lane: 1 colony of pGLCM transformants with EL222 PCR, 10th lane: 1 colony of pGLCF transformants with EL222 PCR, 11th lane: original pGLCM plasmid with EL222 PCR, 12th lane: original pGLCF plasmid with EL222 PCR

Initially, it seems that the cultures of pGLCM and pGLCF did not carry the plasmid, despite the strain being BY474B. This correlated with the OD measurement of ~0.2 after overnight growth, which seemed to indicate that there was no growth. The transformed pGLCF carried the CYC-Lf fragment only, but it seems that the transformed pGLCM plasmid carried both the CYC-Lf fragmen as well as the EL222, thus more colonies needed to be screened. Once again, the original solution of both plasmids gave an unspecific EL222 band, as well as a faint band at around 500bp. Colony PCR was then carried out on 4 more colonies of pGLCM transformation, using HcKan_mKO_rvs and pGLFT_seq1, as well as checking for the same unspecific EL222 band. The lysed culture was also verified a second time using KOD PCR, and primers for amplifying mKO



1st -4th lane: colonies 1-4 amplified with HcKan_mKO_rvs and pGLFT_seq1, 5th lane positive control with sequenced pGLCM, 6th -9th lane: colonies 1-4 amplified with EL222 PCR, 10th lane: negative control with the sequenced pGLCM. 11th lane: culture amplified with mKO primers, 12th lane: positive control with pGLCM amplified with mKO primers.

The colonies showed no plasmid that had a positive mKO to Lf band, uet did not give a positive band for the EL222 as well. Thus I am inclined to believed that there is some unspecific binding of the primers. Colony 5 of pGLCM transformation and COlony 1 of pGLCF was inoculated in 5ml LB amp. The second PCR on the culture using mKO primers also yielded a band, combined with the growth of the culture to around 0.9 OD, the culture was spun down, and resuspended in 10ml of HygB YPD, and a positive control comparison of BY4741 with pC120_mKO was spun down and resuspended in 10ml of YNB-URA. Each culture was then split into two 5ml cultures and one was kept in the dark and the other was placed under blue light illumination overnight.

As a backup, transformation was repeated, and plated on HygB YPD, as well as YNB-URA to check for unspecific plasmids. The previous positive streak from the previous transformation was also reinoculated into 5ml of YPD HygB, and streaked on YPD HygB plate. BY474B was also streaked onto YPD HygB as a negative control.

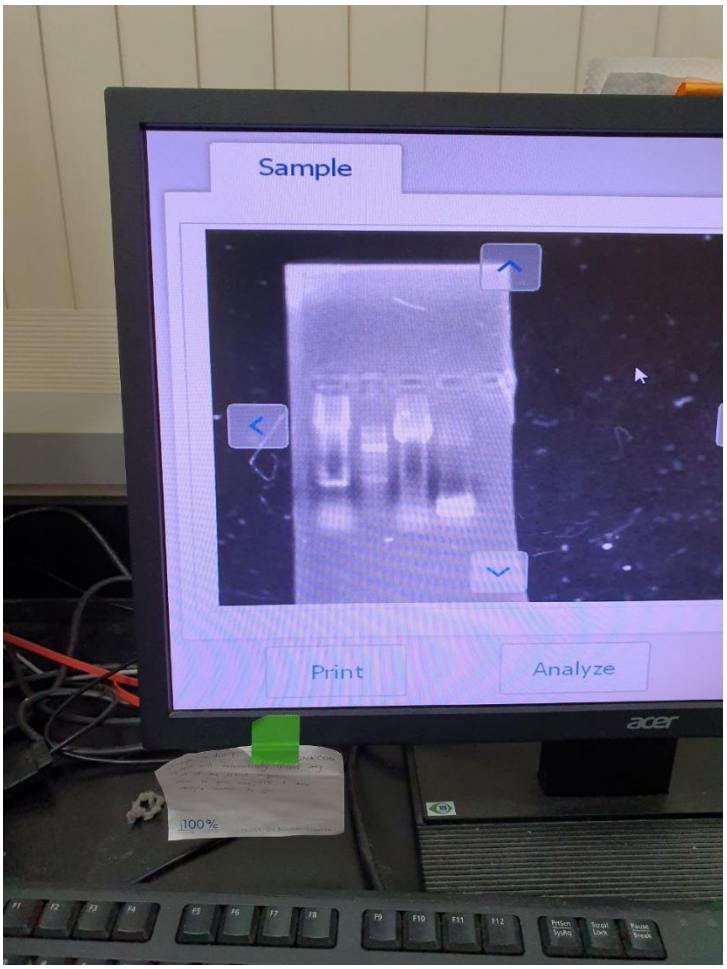
12/8

Sequencing

The newly inoculated pGLCM and pGLCF were plasmid isolated, and the old pGLCM and the new pGLCM were sent for sequencing with both pGLfT-Seq1 and EL222_fwd to see whether the plasmid was indeed correct, and what was giving the unspecific contaminating band.

PCR

5xC120_ins_fwd/rvs was used to amplify HcKan_5xC120, and pGL5CM_bb_fwd/rvs was used to amplify both the old pGLCM and pC120_mKO

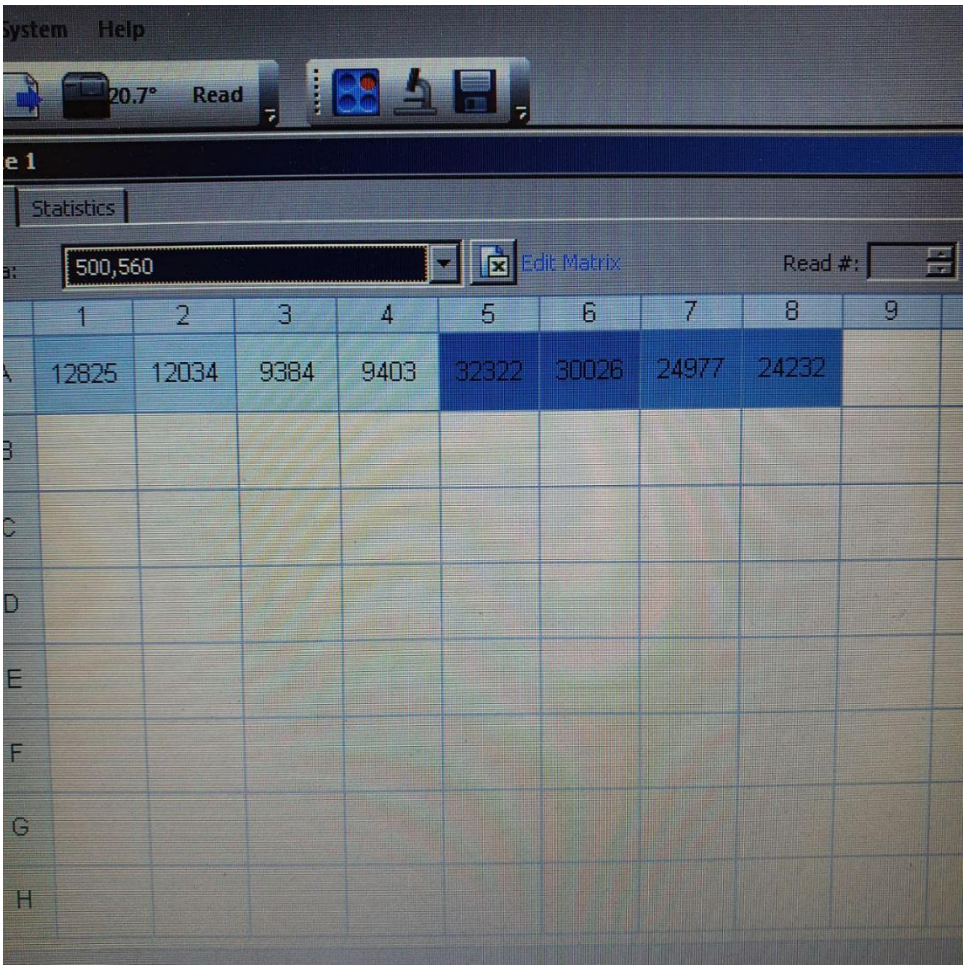


1st lane: pC120_mKO amplified with pGL5CM_bb_fwd/rvs, 2nd lane: ladder, 3rd lane: pGLCM amplified with pGL5CM_bb_fwd/rvs, 4th lane: HcKan_5xC120 amplified with 5xC120_ins_fwd/rvs

The 5xC120 insert was clear, but both the pGLCM and the C120_mko plasmids gave unclear bands, and in addition, the pGLCM plasmid gave an unspecific band around 1kb. As such, the higher segments were gel isolated, and the gel isolated segments as well as the original plasmids underwent 50 rounds of Q5 amplification again overnight.

Fluorescence testing

OD measurements today indicate that all colonies grew significantly, thus the overnight induction cultures of pC120_mKO and pGLCM were measured for their fluorescence. They did not differ significantly in their fluorescence.


System Help
20.7°
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e 1
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12825 12034 9384 9403 32322 30026 24977 24232
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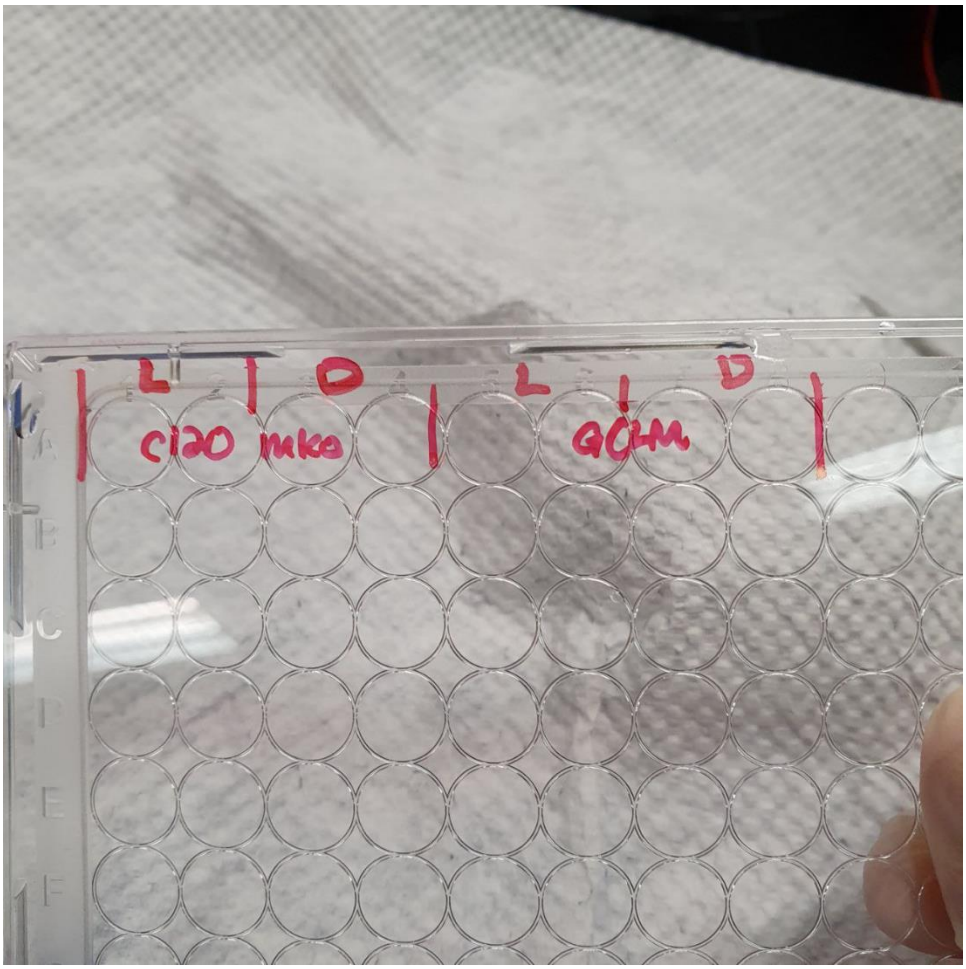
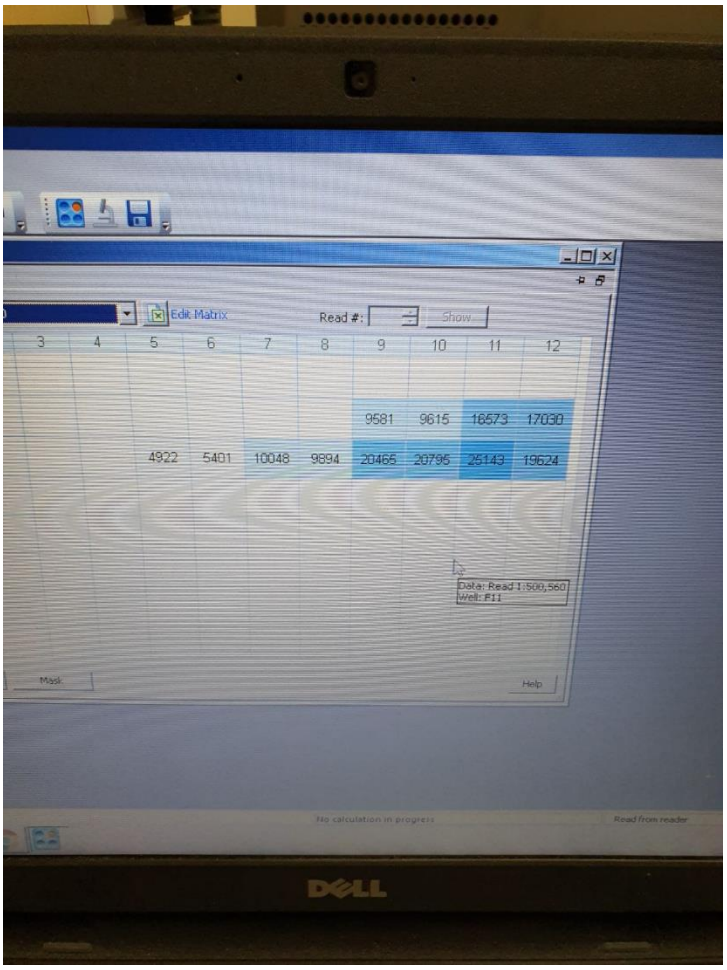
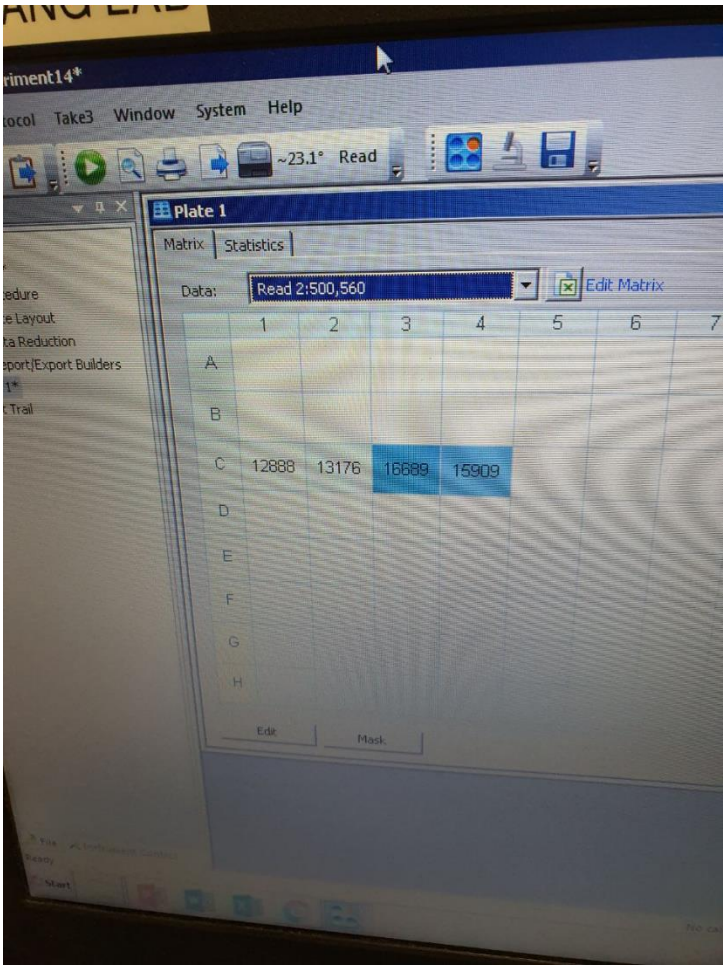


As such, the backup culture was induced as well, and the fluorescence was measured after 2 hours. The induction showed a lower fluorescence than the non-induced culture.



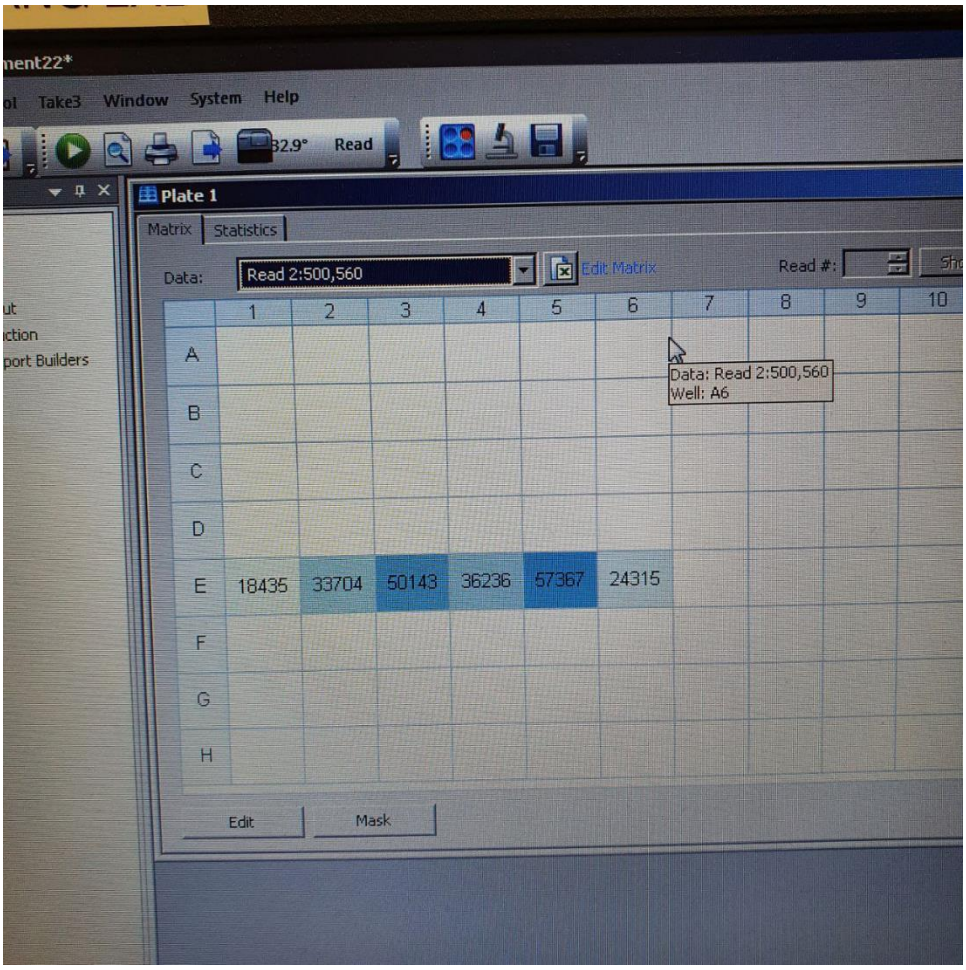
B9/10: induced backup culture, B11/12: uninduced backup culture, C5/6, induced pC120_mKO, C7/8:uninduced pC120_mKO, C9/10: induced pGLCM, C11/12, uninduced pGLCM.

The light was swapped for a stronger one, and the fluoresence was measured again after an hour, but the trend did not change



C1/2: induced backup culture, C3/4 uninduced backup culture.

500uL of each culture was spun down, washed with water and measured to prevent interference from the medium



E1: pC120_mKO induced, E2: pC120_mKO uninduced, E3: pGLCM induced, E4 uninduced, E5: backup culture induced, E6: backup culture uninduced.

The pGLCM for this measurement showed a more expected trend, and thus YPD medium may be a conflating factor for mKO.

Due to the inconsistent results, a more controlled experiment will be carried out. PC120_mKO and BY474B pGLCM were inoculated in 50ml of their selective medium from glycerol stocks, and will be induced for 6 hours, with measurements of the washed solutions taken every hour. This is a late stationary phase induction.

Tomorrow, a seed culture will be inoculated and immediately induced(exponential phase induction), and a seed culture will be inoculated and induced after 4 hours(stationary phase induction)

13/8

Sequencing results

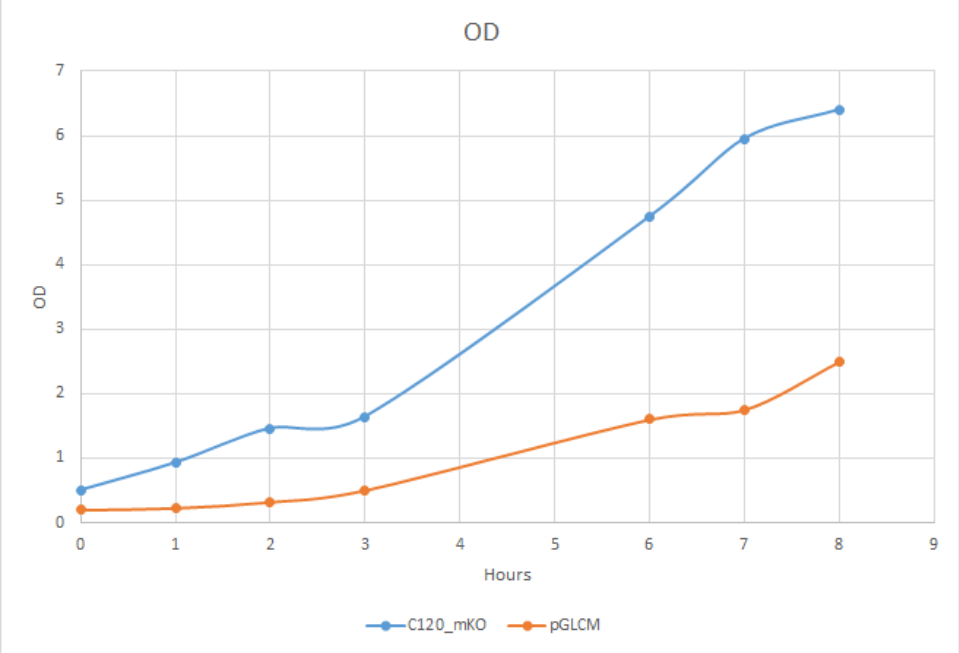
Both the old and the new pGLCM showed a positive assembly with pGLfT_seq1 primer, and no EL222 was detected with the HcKan_EL222_rvs primer

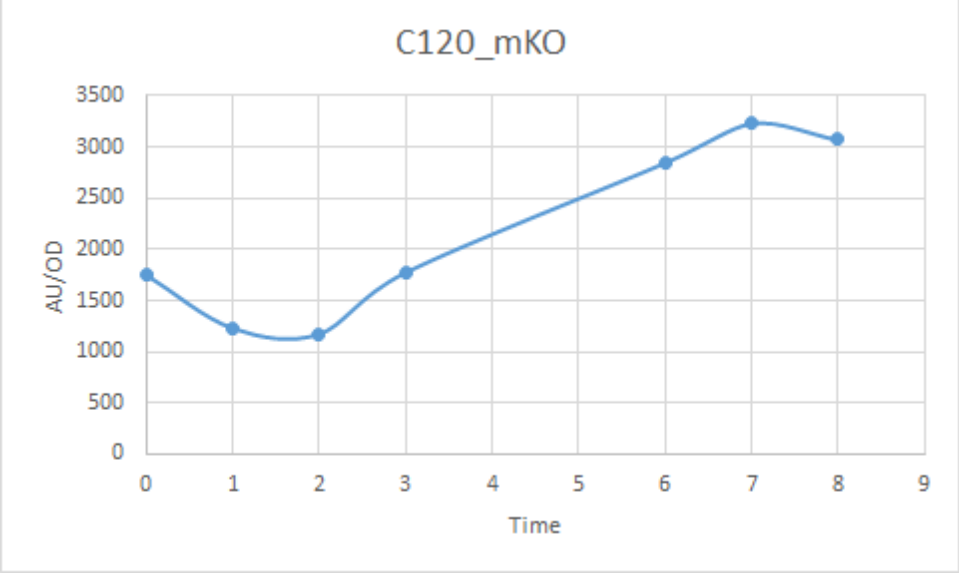
PCR

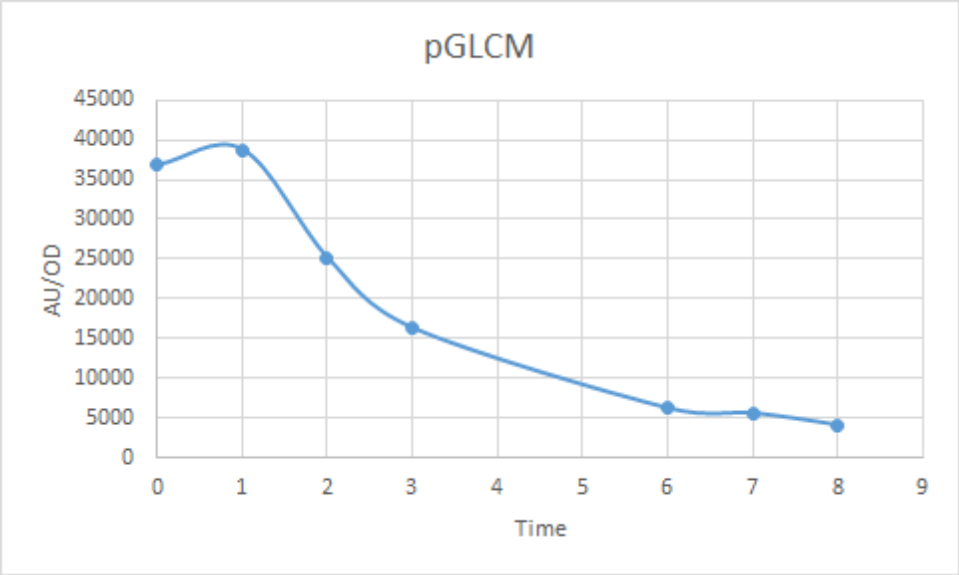
PCR was carried out on pGLCM and pC120_mKO with pGL5CM_bb_rvs/fwd, but no band was observed. PCR of pGLCM using pGL5CM_bb_rvs and HcKan_O_mKO_rvs as well as pGL5CM_bb_fwd and pGLfT_seq 1 was succesful. PCR of pC120_mKO using pGL5CM_bb_rvs and HcKan_O_mKO_rvs as well as pGL5CM_bb_fwd and HcKan_O_EL222_rvs was succesful. This indicates that either primer binds correctly, and it is the interaction between these two primers that may be an issue

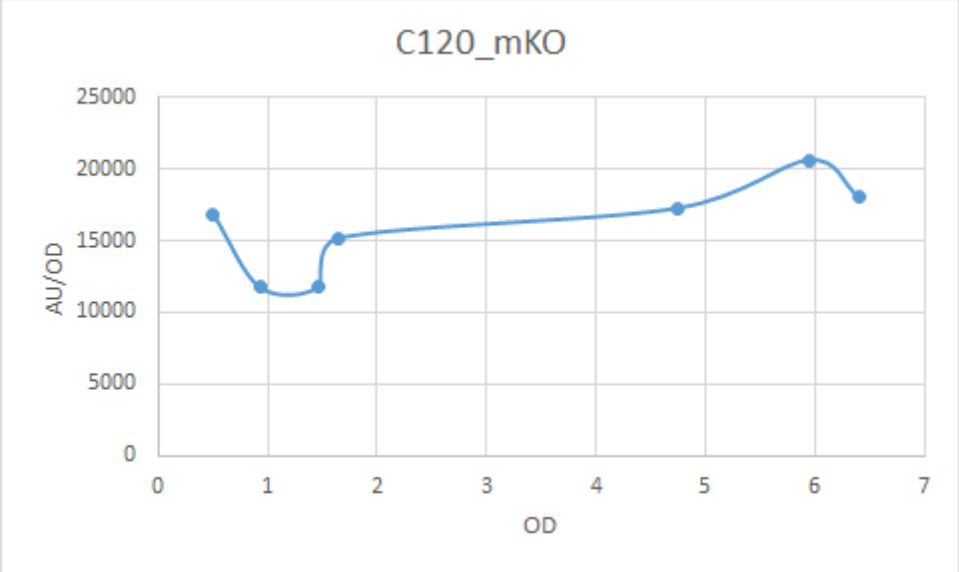
Fluorescence measurements

The cultures of pGLCM and pC120_mKO had their OD measured, and were immediately placed in blue light induction. Every hour, their OD and fluorescence was measured









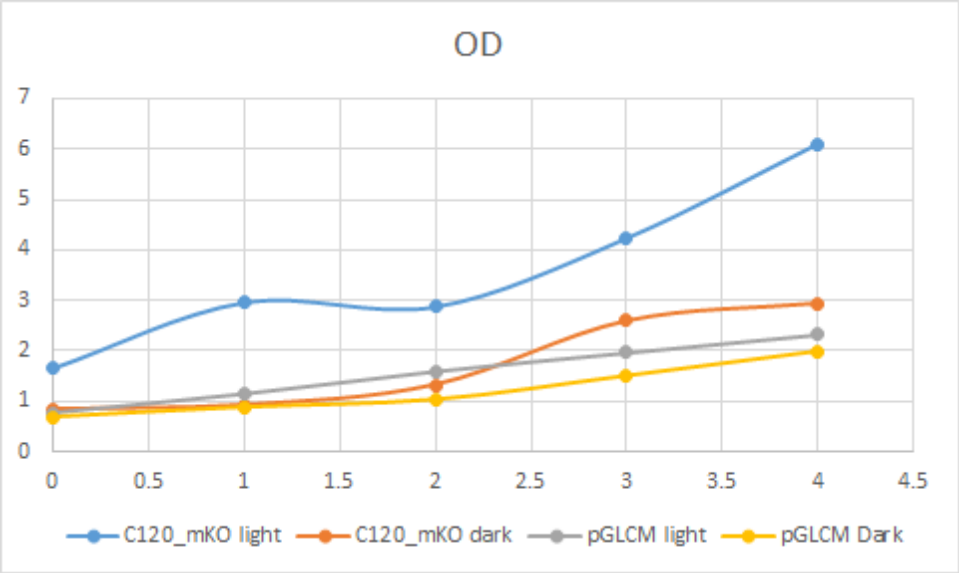
Results for C120_mKO indicate that the idea time to induce is around OD 0.5-1, and results should be visible for abour 4 hours, but results were only valid for C120_mKO. It is suspected that the YPD medium is interfering with the fluorescence measurements

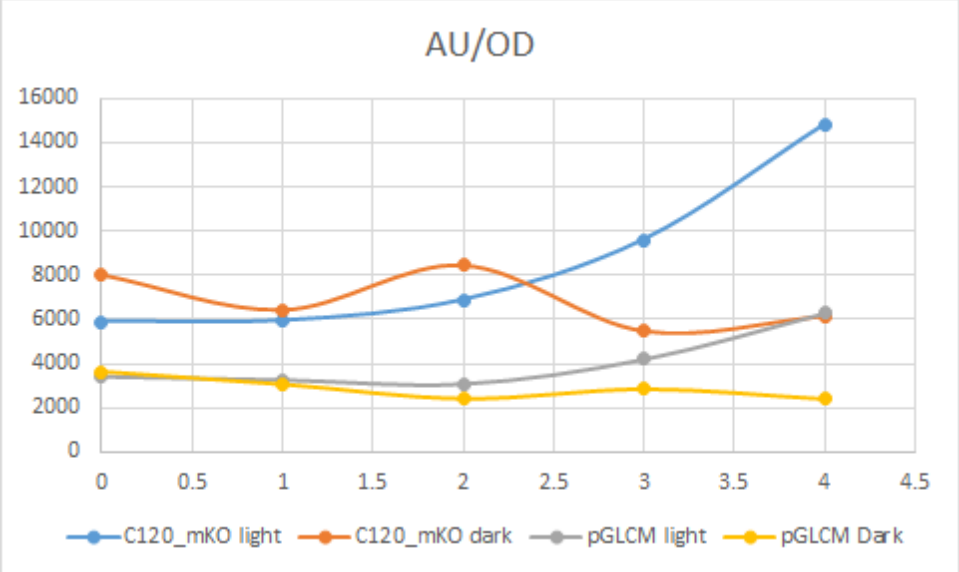
BY4741 was transformed with pGmFaHBD2 plasmid and inoculated into both pH buffered YPGR and regular YPGR. Induction was carried out over the weekend.

14/8

Fluorescence measurement

Cultures of B474B pGLCM in the dark and light, as well as cultures of BY4741 pC120_mko in the dark and light were induced with blue light, and their OD measured every hour. 300uL was aliquoted out, washed with water twice, and fluoresence was measured with ex:500 and em:560

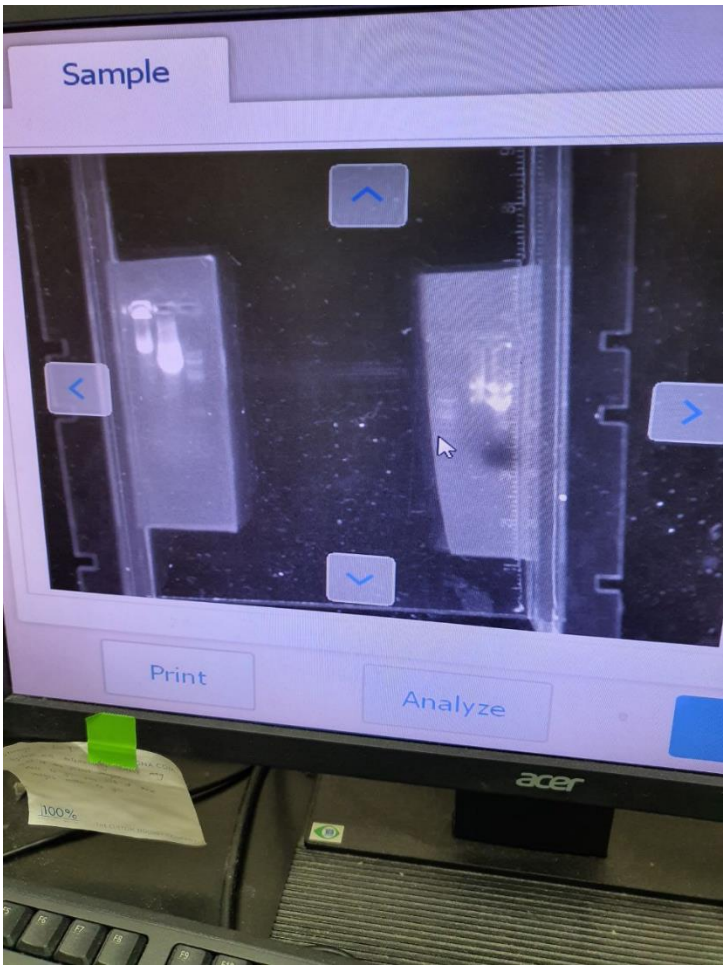




It seems that pGLCM is less leaky than the pC120_mKO, and the ideal OD to induce the culture at is about 3, within 4 hours a 2.5x fold was observed for pC120_mKO.

PCR

PCR was reattempted on pGLCM with pGL5CM_bb_rvs/fwd, with a step down of –1 degree per cycle from 65 for 10 cycles, and a 1:40 long extension. Two bands were observed



16/8

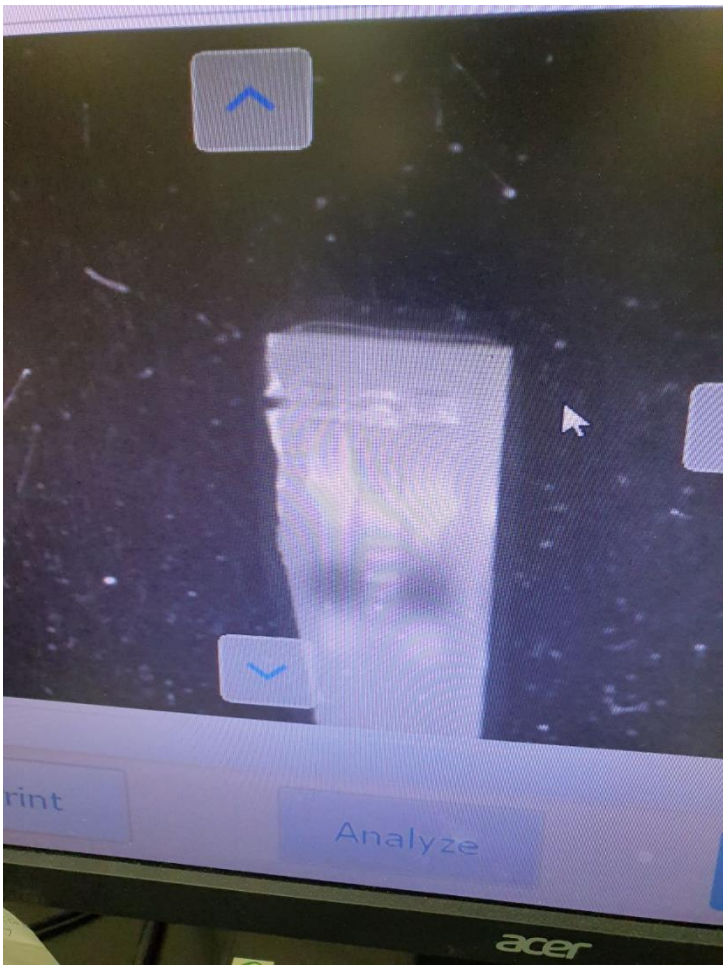
Fluorescence measurement

Cultures of B474B pGLCM in the dark and light, as well as cultures of BY4741 pC120_mko in the dark and light from saturday as well as friday were measure for their fluorescence and OD. Light went out half way, but pC120_mKO and pGLCM increased by a significant amount. Another important factor to consider is the decay of the induction after blue light is turned off, and the possible cross talk with red light

Gibson assembly

Gel extracts for PCR of pGL5CM underwent a second round of PCR, and backbones and inserts for PH_integrate were PCR-ed

1st lane: pure pGLCM plasmid, 2nd lane: ladder, 3rd lane: PCR amplification of pGL5CM backbone. Lower band was isolated, as higher band seems to be the original pGLCM plasmid.



1st lane: Integrative backbone from pGAU, 2nd lane: ladder, 3rd lane: PCB28 pathway

Gibson assembly was carried out for both pGL5CM and PH_integrate

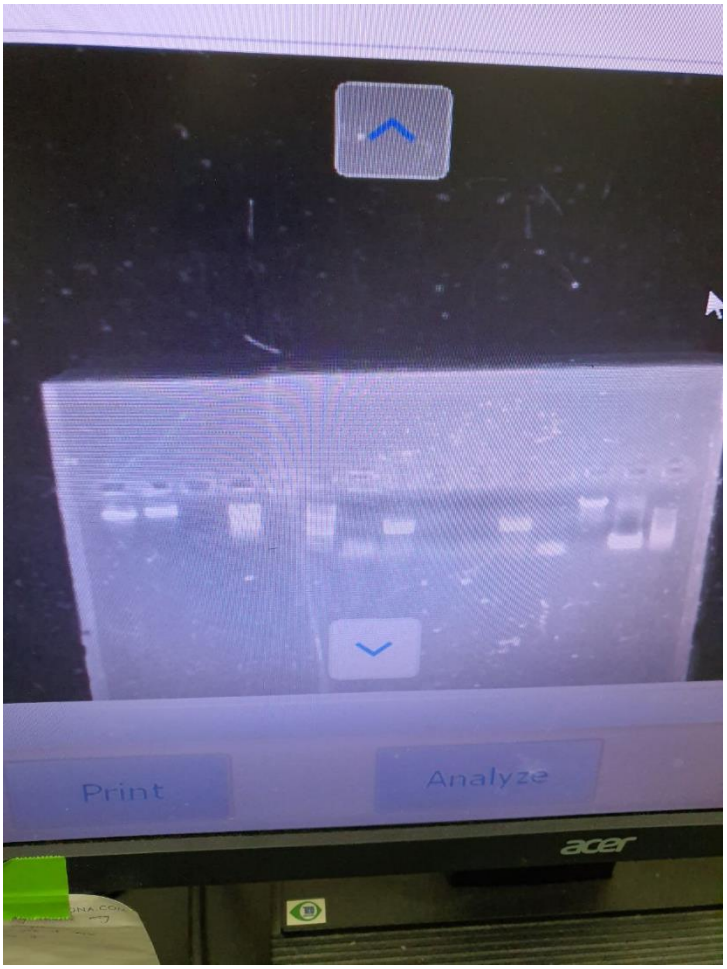
48 hr cultures were spun down for protein precipitation. YPGR culture showed significant growth in cell biomass over the weekend. However buffered YPGR culture failed to grow significantly (due to optimal pH for beta-defensin action against yeasts?). Buffered YPGR was left to incubate for another 24 hours. Spun down YPGR supernatant was precipitated by 20% TCA (w/v) for 2 hours. Protein precipitates were washed 3x in acetone and boiled in sample loading buffer. Sample was run in SDS-PAGE. Half of gel was used for Coomassie blue staining, and the other half for electro-transfer. Electro-transfer encountered issues with full transfer even after regular 30 min cycle + 7 min turbo transfer (unsure if it is due to transfer buffer or simply additional nitrocellulose membrane added in part for sandwich transfer method).

17/8

PCR

No colonies were observed for the assembly of pGL5CM, so the PCR fragments of pGL5CM_bb and insert were run on the gel. No band was visible for the insert, thus PCR was redone, and gibson assembly

was carried out. Colony PCR for 5 colonies for the PH_integrate assembly were verified with PH_integrate_vrf_fwd/vrf. Fragments for PGKrepress were also run

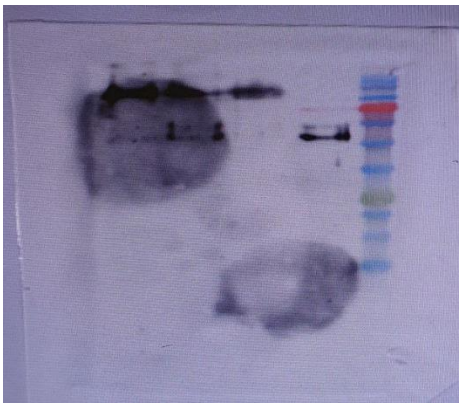


1st / 2nd lane: backbone for pGL5CM, 3rd lane: insert for pGL5CM, 7th-11th lane: colonies for PH_integrate, 12th lane: insert for pGKrepress, 13th lane: backbone for pGKrepress, 14th lane: re-PCR-ed insert for pGL5CM

Gibson assembly was reattempted for pGL5CM, and pGKrepress, Colony 2 and 5 of PH_integrate was inoculated in LB amp, as well as pRL_GI_Del1.

18/8

TCA Precipitation (20%) was performed on buffered YPGR (pH 7.0) and precipitate was on 15% SDS-PAGE. Western blot was performed on the transferred proteins. Western blot yielded bands at wrong sizes and many unspecific bindings. The nitrocellulose membrane was stripped in mild stripping buffer and re-blocked and re-stained.



Left – Right: 1/3 diluted precipitate, ½ dilution, neat dilution, +ve control, ladder

Patches of TMB substrate were left on the membrane (note to drop substrate out of membrane surface and spread with sterile spreader across membrane next time). Positive control seems to have degraded somewhat as the size of the positive control band has shifted down about 10 kDa. (verify again on next western blot.)

3 cultures were started:

BY4741 transformed with pHBD2 – 2x 50 ml cultures were made (48 hr extraction and 72 hr extraction).

BY4741 transformed with pHBD2 – 3x Hygromycin B was added.

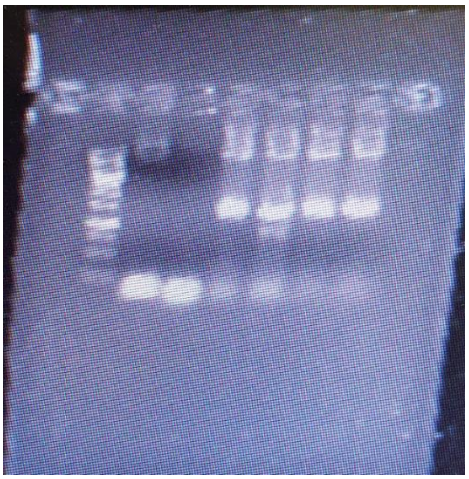
Negative control – empty BY4741 was used.

19/8

PCR isolation of the PH_inte fragment was performed from the assembled plasmid stock and colonies on selection plate to verify the integrity of the assembly.

Gel image of fragment isolation via PCR. (Ladder, Col2, 5, 6, 7). Amplification yielded positive results for both the isolated plasmid stock and colony from selection plate. at 10000 bp mark.

PCR verifications for HBD2 plasmid in BY4741 and PH_Inte plasmid was performed. HBD2 fragments yielded bands at about 100 bp and PH_inte plasmid fragments at 10000 bp.



Gel image for HBD2 fragment and PH_inte fragment verification. (Ladder, pHBD2, HBD2 col PCR, PH_inte col2, 5, 6, 7).

Stripped membrane was reblotted, and substrate reapplied to the membrane for imaging. Reblot yielded poor results that were inconclusive.

Left – Right: 1/3 diluted precipitate, ½ dilution, neat dilution, +ve control, ladder

BY4741 was transformed with PCR product from 1st PCR verification reaction of colony 2 and plated onto YNB -Ura agar.

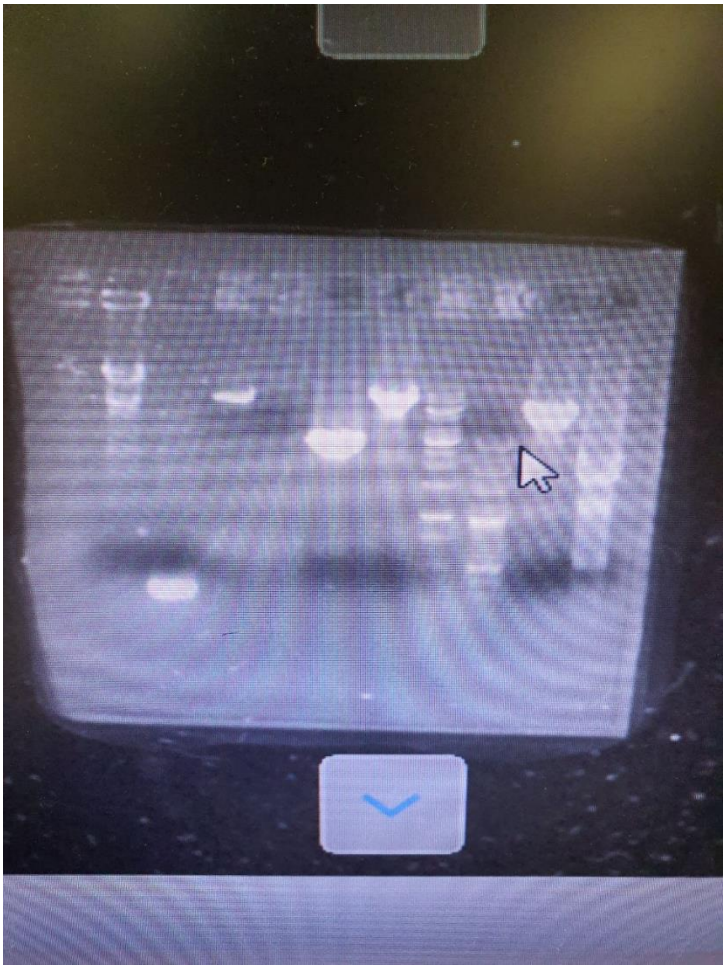
20/8

BY4741B was transformed with pGmFaGFP plasmid and spread onto YPD + hygromycin plate. HBD2 culture was spun down after 48 hr mark - supernatant and cell pellets were frozen at –80 degrees for later analysis.

23/8

PCR

Backbone for pGL5CM, insert for pGL5CM, as well as the backbone of pGL5CM spilt into two using hLF_rev and pGHLf_T_fwd, backbone and insert of PP_integrate was amplified. Backbone and insert of pRepress was run in the gel as well

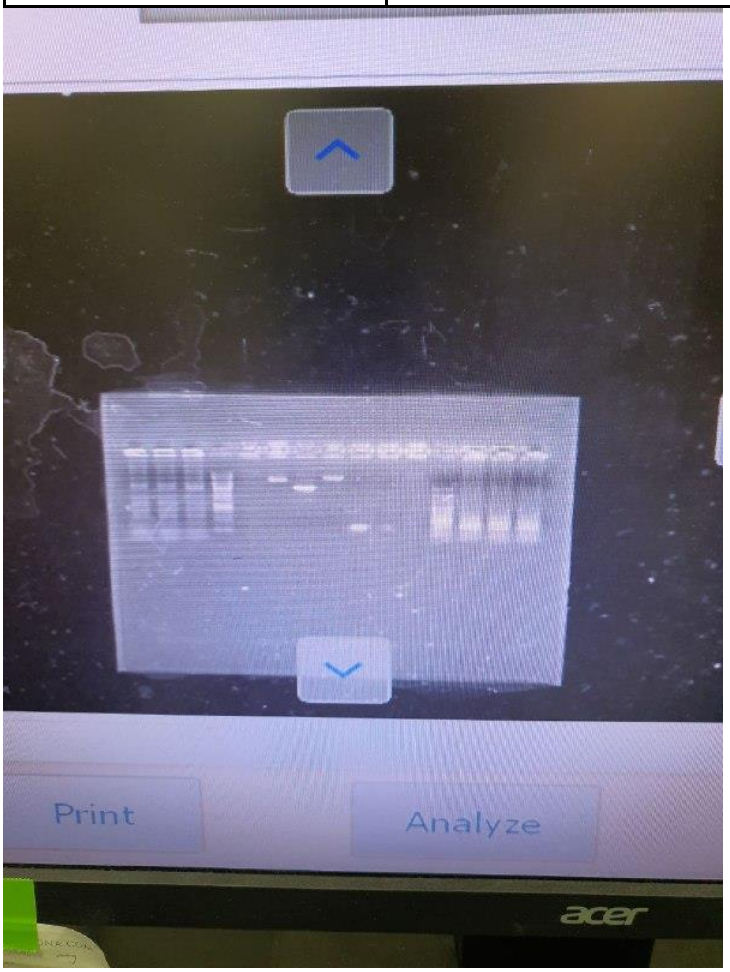


1st lane: backbone for pGL5CM, 2nd lane: insert for pGL5CM, 3rd lane: backbone for pRepress, 4th lane: insert for pRepress, 5th lane: fragment 1 of pGL5CM up until hLF_rev, 6th lane: fragment 2 of pGL5CM from pGhLF-T_fwd onwards, 7th and 8th lane: ladder, 9th lane: bacbone for PP_integrate, 10th lane: insert for PP_integrate.

Backbone for pGL5CM did not seem to yield a significant band, while the two fragment amplifications gave accurate sizes. Thus these two bands were excised along with the insert for pGL5CM, and used for a 3 part gibson assembly. While the PP_integrate backbone displayed the correct band size, the insert showed bands around 1kb. Thus the following primers were ordered to carry out a 3 fragment insert for PP_integrate, as it may be possible that long amplifications are not efficient.

PP_mid_fwd	gttcctatcggtacagccat
PP_mid_rvs	atggctgtaccgataggaac

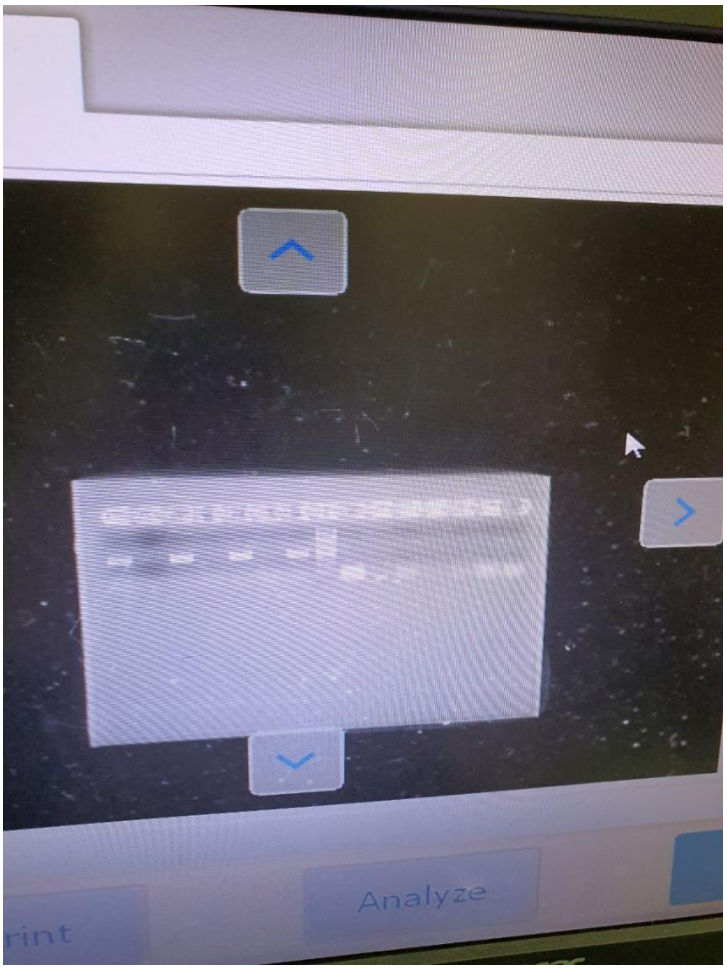
NEWGL5CM_bb_fwd	ggcctttcttGAAGCGGGTAAGCTG
NEWGL5CM_bb_rvs	aattgtgagcggataacaattTATTCTTTCCTTATACATTAGGACC



6th lane: Gel isolated PP_integrate backbone, 7th lane: Gel isolated Fragment 1 for pGL5CM backbone, 8th lane: Gel isolated Fragment 2 for pGL5CM backbone, 9th lane: Gel isolated insert for pGL5CM

Gibson assembly was thus reattempted using a new set of hifi mix for both pGL5CM and pREPRESS

After transformation, 7 colonies underwent PCR from each assembly. PGL5CM was verified with NEW3xC120_vrf and HcKan_O_mKO_rvs, and pRepress was verified with c120repress_ins_fwd/rvs



1st - 7th lane: verification of pGL5CM, 9th-15th lane: verification for pRepress

Colonies 5 and 7 for pGL5CM were inoculated and colonies 1 and 7 for pRepress were inoculated in LB amp

Protein work:

Cultures were spun down and supernatant was separated from cell pellets. Spun down culture supernatants were concentrated in 10 kDa columns to a final volume of 1.5 ml. During spin process culture media was replaced with Tris-HCL buffer (pH 7).

Cell pellets were resuspended in Tris-HCL buffer and sonicated at 5 second bursts with 3 second rest intervals for 3 times. The lysate was centrifuged and the supernatant was concentrated as well.

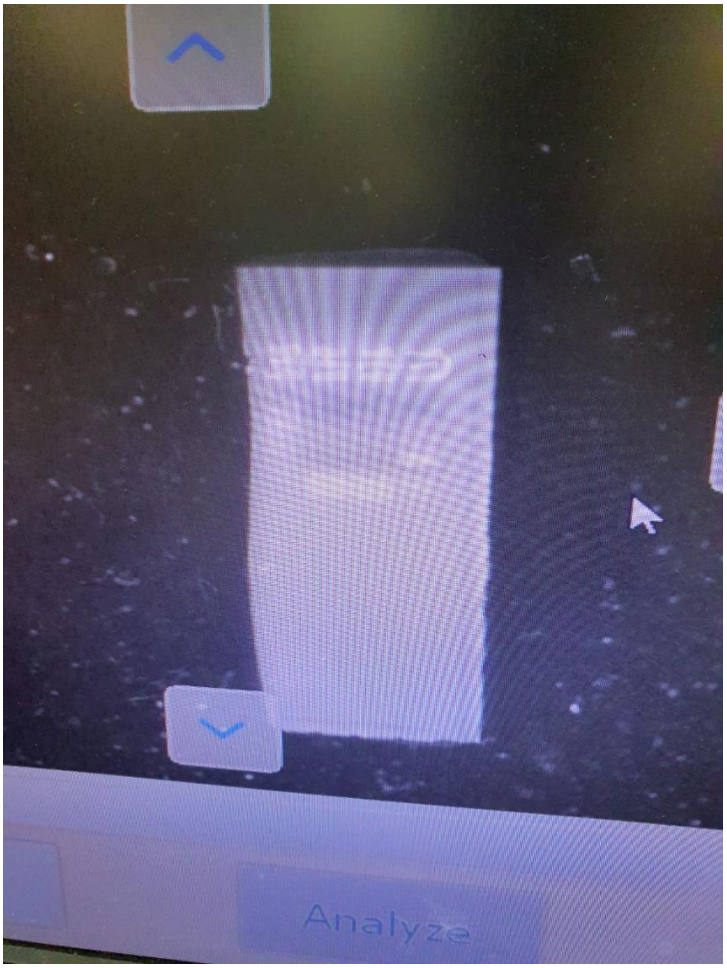
24/8

Protein concentrate was His-purified using Ni-NTA spin column, washed twice with wash buffer and eluted in elution buffer. Elutant was further concentrated in 10 kDa spin column and loaded into sample loading buffer.

24/8

PCR

Only colony 7 of pGL5CM grew, while both colonies of pRepress grew. All 3 were plasmid isolated and underwent the same PCR verification



1st lane and 2nd lane: pRepress verification, 3rd lane: pGL5CM verification

pRepress was sent for sequencing with c120repress_ins rvs and pGL5CM was sent for sequencing with HcKan_O_mKO_rvs

Transformation

BY474B was transformed with pGL5CM, pTurquoise, pRepress and pmFaGFP

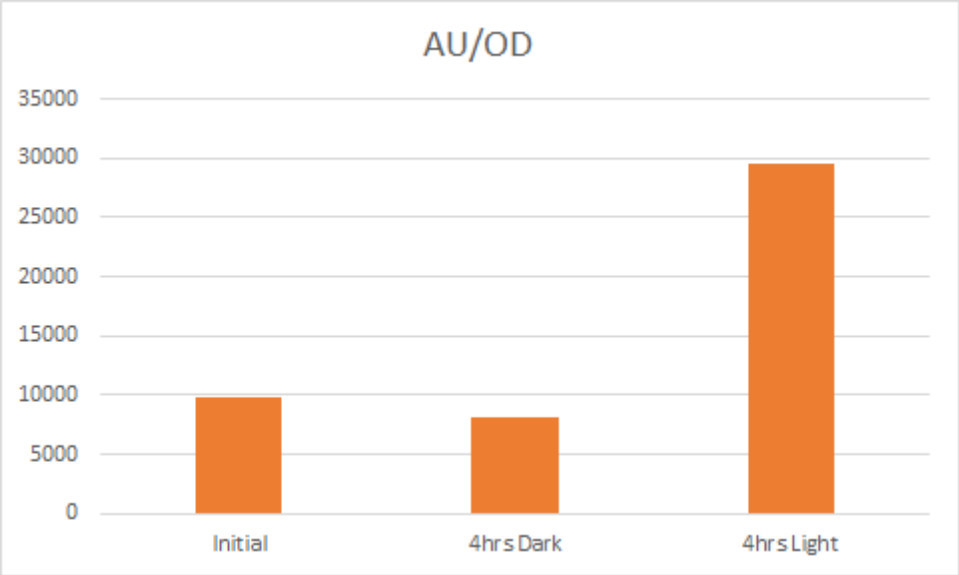
3/9

Blue light induction

Overnight culture of pGL3CM was measured to have an OD of 15, it was then diluted into 50ml of YPD to an OD of 1.2. 50ml was then split into 25ml cultures in two 250ml flasks, with one placed in blue light and 30 degrees shaking, while the other was placed into a dark incubator wrapped in foil for 4 hours.

At the end of 4 hours, both were measured to be an OD of 2.1, and their OD and fluorescence at ex515 and em560 was measured

	Water blank			Initial culture			4 hours dark			4 hours light		
OD	0.037	0.036	0.038	0.091	0.089	0.092	0.181	0.177	0.185	0.147	0.144	0.146
AU	90	88	107	1053	1065	1248	1512	1414	1490	4439	4160	4281
Averages	0.037	95	2567.568	0.090667	1122	12375	0.181	1472	8132.597	0.145667	4293.333	29473.68
Normalized						9807.432			8132.506			29473.59



PH integrate

When liquid culture of PH_integrate was verified with PH_vrf_fwd/rvs, no bands were observed other than the positive control, and thus transformation needs to be reattempted.

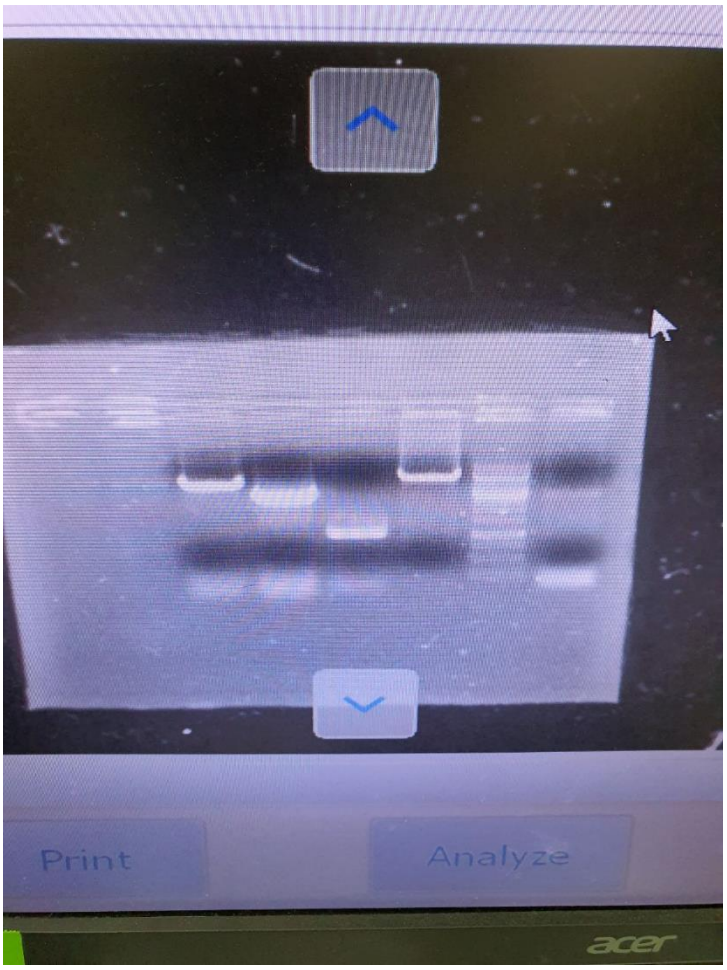
8/9

50 mL HBD2 culture + neg control was spun down and cell pellet was separated from the supernatant. Media Supernatant was split into 2 (1 for His purification, 1 for concentration + His purification). Cell pellet was washed with PBS 3x. Washed cell pellet was sonicated on ice for 1 minute in 30 sec pulses + 30 sec rest. Cell lysate and media supernatant were both purified via His spin column and eluted into 300 uL elution buffer.

Top10 E. coli was inoculated in LB overnight

Gibson assembly

pGL3CM was PCR-ed with the primers 3C120_mko fwd/rvs and pRL_GI_yeGEP was PCR-ed with the primers RL-URA_fwd/rvs, LacI gene fragment was PCR-ed with lac_fwd and lac_rvs, pTurq was amplified with lac_bb_fwd/rvs



1st lane: pGL3CM fragment, 2nd lane RL_GI_yeGFP fragment, 3rd lane: LacI fragment, 4th lane: Backbone for pConLac

pGL3CM fragment and pRL_GI_yeGFP fragment were gibson assemblied, and LacI fragment and pConLac fragment were gibson assemblied

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His purified cell lysate + media for both negative and HBD2 culture were tested for antimicrobial activity using Agar well diffusion assay. Top10 culture was diluted with sterile H2O to an OD600 value of 0.5 and 150 uL of diluted culture was spread on LB plates. 50 uL of both His-purified HBD and –ve control cell lysate (cell lysate contained more HBD2) were aliquoted into wells. Negative control

Screening

pRL3CM were screened by amplifying the GFP gene, pConLac was screened by amplifying the lacI gene

10/9

Transformation

BY474B strain with pGL3CM already transformed, was transformed with pConLac, and plated on YNB-URA-AS HygB, and BY474B strain was transformed with pRL3CM and plated on YNB-URA

14/9

Lac testing

BY474B strain with pGL3CM and ConLac, was seeded at OD 1.2 and left to grow for 24 hours in light, dark and light+IPTG, and the fluoresence measured afterwards. Ligth and dark were both close to baseline, but light+IPTG showed around a 4x induction fold

18/9

Transformation

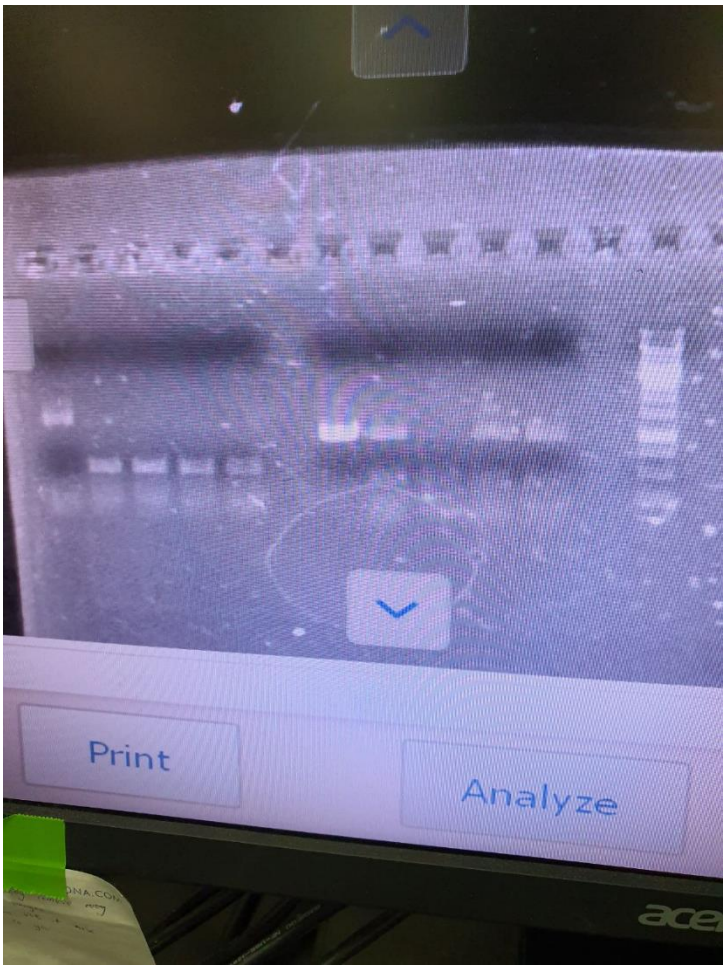
BY474C strain was transformed with replacement fragment to substitute URA marker in PH_integrate fragment with a primer sequence to integrate the red light system, and selected for with FOA plates

21/9

Transformation

BY4741 strain transformed with replacement fragment that showed positive PCR was transformed with the plasmid pRL_CT_GI containing the red light system and selected for with YNB-Leu. 5 colonies of pRepress2 were selected and inoculated in LB ampicillin

PCR of the replacement fragment

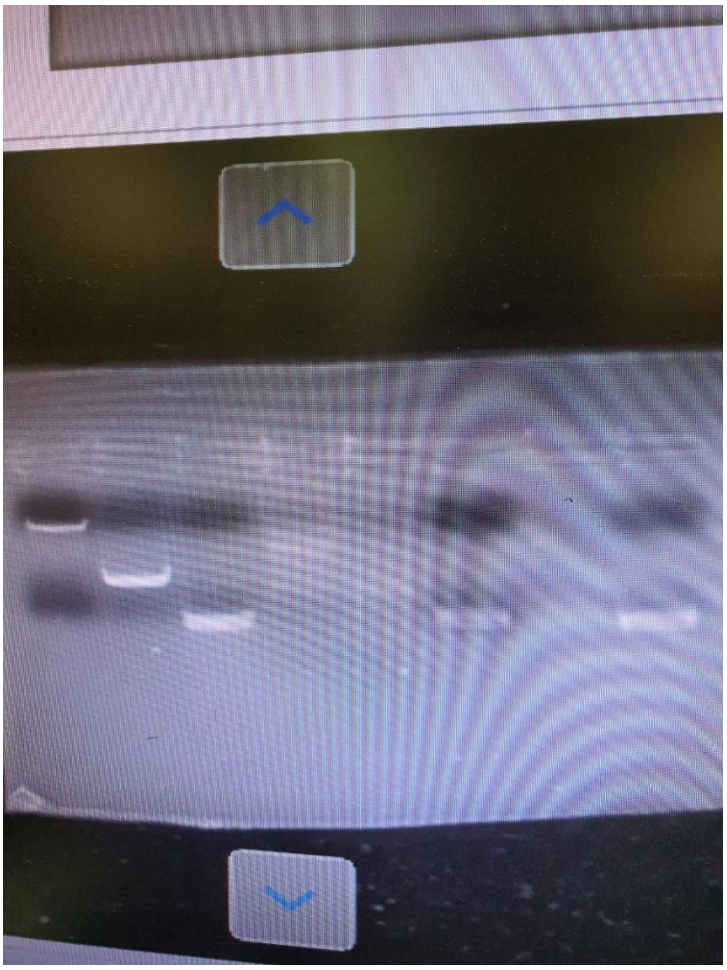


Lane 1: PH_integrate plasmid PCR-ed with PH_vrf_fwd and PH_vrf_rvs. Lane 2-5: Colony PCR of colonies with PH_vrf_fwd and PH_vrf_rvs. Lane 7: Replacement fragment amplified with Replace_fwd and Replace_rvs. Lane 8-12: Colony PCR of colonies with Replace_fwd and Replace_rvs.

While the replacement fragment seems to be present, the original PH_integrated plasmid looks like it may have been truncated as the PCR-fragment is only 500bp long

22/9

PCR and gibson assembly

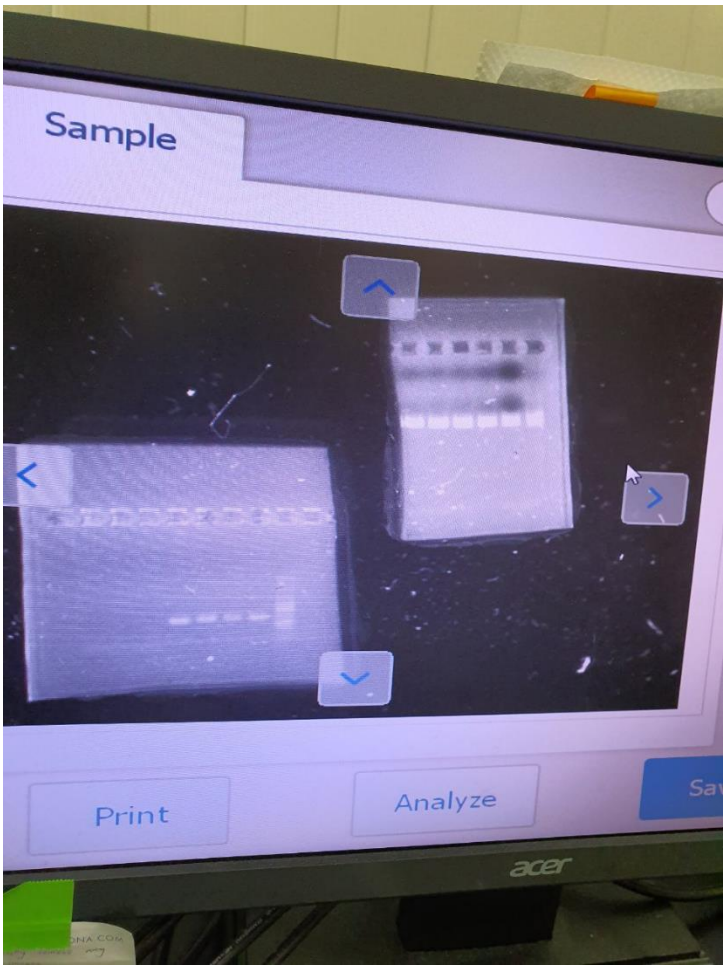


Lane 1: Backbone for driver plasmid, amplified from pCE_integrate with drive_bb_fwd and driver_bb_rvs. Lane 2: EL222 fragment amplified from pCE_integrate with EL222_ins_fwd and EL222_ins_rvs. Lane 3: C120 promoter amplified from pGL3CM with c120_ins_fwd and c120_ins_rvs. Lane 6: Nuclease A with NLS, amplified with nls_nuc_fwd and nuc_rvs. Lane 8: Nuclease A amplified with nuc_fwd and nuc_rvs.

Driver plasmid was then assembled with the backbone, EL222 plasmid and C120 promoter, and pGNLSnucA and pGNucA were assembled with their respective fragments

23/9

PCR verification



Left 4 lanes: PCR for nuclease fragment in pNLSnucA transformants. Right 4 lanes: PCR for C120 promoter in pDriverintegrate transformants. One colony of each was picked and inoculated in LB amp.

23/9

Plasmid isolation

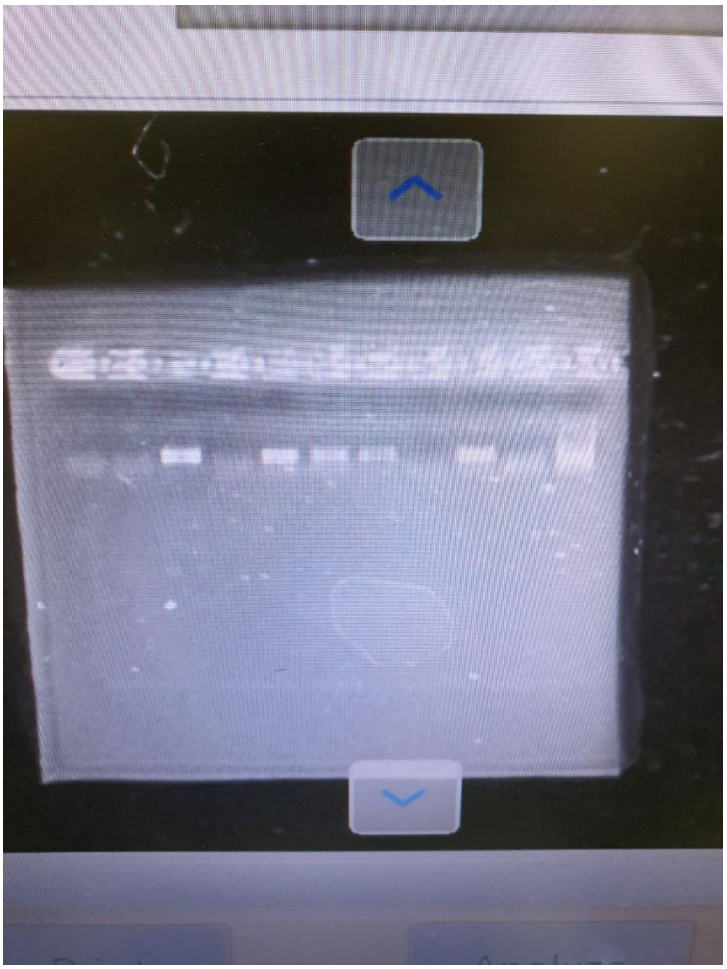
Plasmids for pNLSnucA and pDriverintegrate were isolated, and pDriverintegrate was digested with Eco31RI to release the integrative cassette. Backbone and insert for pDriverConlac and backbone and insert for PHI_integrate

Lane 1 and 2: Digested pDriverintegrate. Lane 3: Backbone for pDriverConlac amplified with pLac_bb_fwd and pLac_bb_rvs. Lane 4: Constitutive lacI fragment amplified with dlac_ins_fwd and dlac_ins_rvs. Lane 5: PHI backbone amplfied with PHI_bb_fwd and PHI_bb_rvs. Lane 6: Replacement fragment amplified with PHI_ins_fwd and PHI_ins_rvs

The digested pDriverintegrate were excised and gel isolated, and transformed into BY4741. pDriverConlac backbone showed a smear and a 1kb band, whereas a 6kb band was expected. It is possible that the dual EL222 sequences are interfering with the PCR. The rest of the bands were as expected, thus PHI_integrate was gibson assembled. pNucA was also assembled with Gibson assembly.

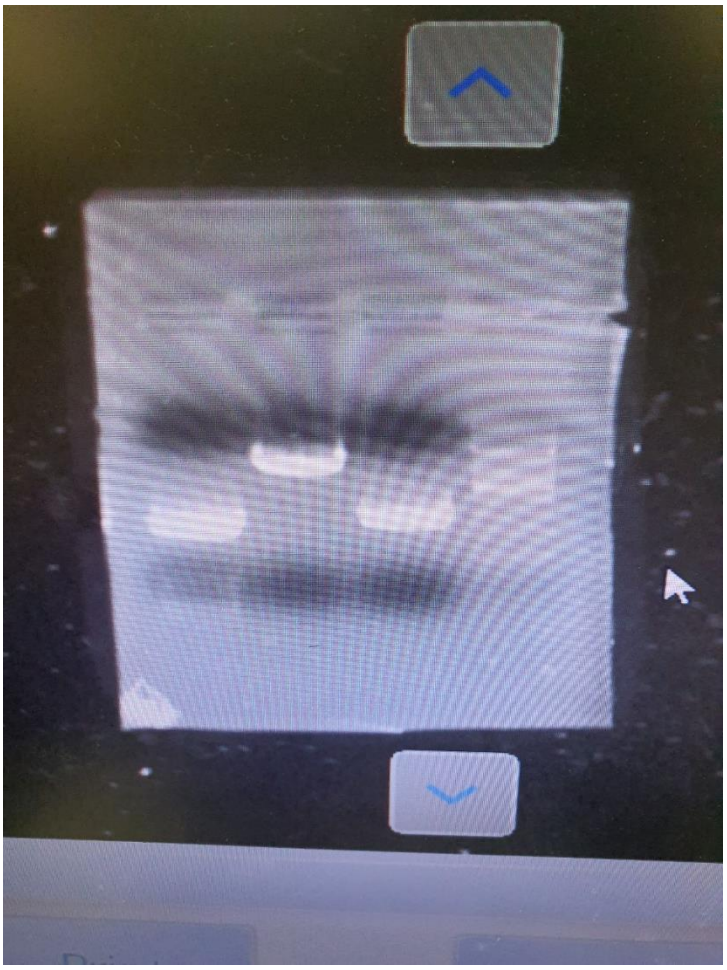
24/9

PCR verification



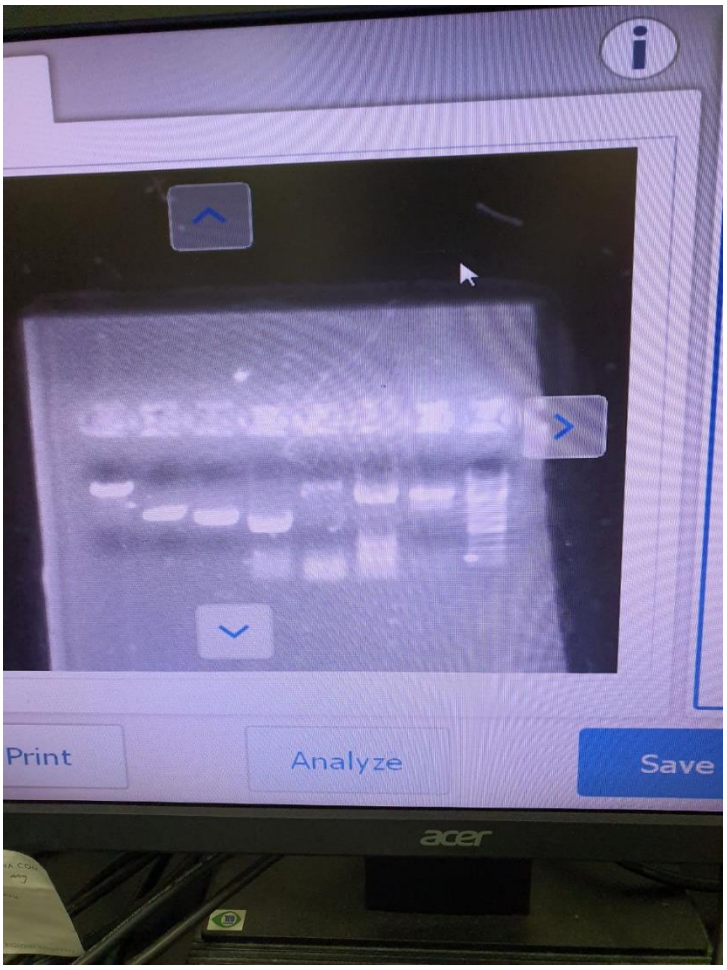
Lane 1-5: Nuclease fragment colony PCR-ed from pGNucA assembly transformants amplified with nucA_fwd and nucA_rvs, lane 6-10: PHI_integrate assembly transformants amplified with PHI_ins_fwd and PHI_ins_rvs. Positive colonies were inoculated in LB amp.

Overlap PCR



To overcome the issue with the EL222 sequences interfering with the PCR for the assembly of pDriverConLac, overlap PCR was used to join EL222 and C120 promoter, while remaining fragments were obtained from the original pCE_integrate, such that the pDriver_integrate plasmid did not need to be used as a template for PCR.

These fragments were then combine with the constitutive lac fragment to form pDriverConLac.



Lane 5: PCR of PH_integrate pure plasmid, Lane 6: PCR of isolated genome of pBY474R with the same primers, Lane 7: PCR of pRL_CI_GT with verifying plasmids, Lane 8: PCR of isolated genome of BY474R with the same plasmids

It seems that while the pRL_CL_GT with the key transcription factors are present in the genome, there is issue visualizing the PH_integrate plasmid, which is unlikely as that plasmid was neccesarry to integrate the pRL_CL_GT. Thus a more thourough genome isolation protocol may be needed.

BY474R was transformed with pRL3CM

BY474B was transformed with pRepress2

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Transformation

BY4741 was transformed with an BsaI digested pDriver to produce BY474D, as well as pGNucA

27/9

Transformation verification

BY474R transformed with pRL3CM colonies were placed under blue light, and GFP fluoresence was clearly visible, indicating that the red light promoter was active but may be leaky

Blue light repression testing

BY474B transformed with pRepress 2 was cultured in YNB-URA in either blue light or darkness for 6 and 24 hours, but no difference was observed in either, indicating that this blue light repression system did not work

Transformation

BY474D culture was transformed with pRL3CM

Nuclease testing

BY4741 transformed with pGNucA were cultured in YPD HygB overnight, and the next day they were inoculated at OD1.5 into either YPD HygB or YPGR HygB, and left to culture

28/9

Transformation verification

BY474R transformed with pRL3CM colonies were placed under blue light, and GFP fluoresence was clearly visible, indicating that the red light promoter was active but may be leaky

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