Week 26

Project: iGEM 2021
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Created at: 2021-06-28T15:23:16.949617+00:00

MONDAY, 28/6/2021

Purpose

Construction of gene fragments by PCR, gibson assembly/cloning and insertion into plasmids, followed by transformation and integration into yeast strain of first replacement FAS system part if time allows.

Methods

Group 1:

Ordered gene fragments were prepared by thawing them at room temperature and then protocol from IDT was followed which consisted of centrifuging at 3,1 kg in 1 minute, addition of 100 µL MQ water to each tube (Fragment 1-4 and antisense mrna) and then brief vortexing. The tubes were then incubated over 20 min at 50 °C, followed by centrifugation at 3 kg over 1 minute. Final concentration in each fragment mixture was 10 ng/µL.

Primer mixes containing both forward and reverse primers for a specific fragment were prepared for future PCR. Table 2 shows all the PCRs which are to be done, what the product will be, which plasmid/gene will be used as a template with, its corresponding primers and the expected PCR fragment lengths.

Primer mixes were prepared by pairing forward and reverse primers and diluting them to reach a concentration of 10 ng/µL. All primers that were used had a concentration of 1000 ng/µL. Table 2 shows the content of the primer mixes and to which PCR they correspond.

Table 1. PCRs, source plasmids, primers that will be used and expected lengths of the PCR fragments

	A	B	C	D	E	F
1	PCR nr	Fragment	Source Plasmid/Gene	Primer fw	Primer rev	PCR fragment length (bp)
2	1	Fragment 1	IDT fragment 1	fw_eFAS_frag/antisense	rv_eFAS/antisense	2900
3	2	Fragment 2	IDT fragment 2	fw_eFAS_frag/antisense	rv_eFAS/antisense	2800
4	3	Fragment 3	IDT fragment 3	fw_eFAS_frag/antisense	rv_eFAS/antisense	2800
5	4	Fragment 4	IDT fragment 4	fw_eFAS_frag/antisense	rv_eFAS/antisense	2900
6	5	Antisense mrna	IDT antisense	fw_eFAS_frag/antisense	rv_eFAS/antisense	1000
7	6	scCUP1p	pYTK031	fw_CUP1p_p413TEF	rv_CUP1p	326
8	7	miRFP670	pMC-3-miRFP670	fw_miRFP670_CUP1p	rv_miRFP670_p413TEF	988
9	8	tesBT	IDT tesBT	fw_CUP1p_tesBT	rv_TCYC1_tesBT	784
10	9	Andrea fragment 1	pYTK095-TetA - Andrea makes it	fw_tPGK1_p415TEF	p_TEF1p_rv	1665
11	10	TetON promotor TesA	pWS3033_-_pTet7_[2]	fw_LexOP_TEF1p	rv_LexOP_TesA	406
12	11	TesA	IDT TesA	fw_tesA_p415TEF tetON	rv_TesA_p415TEF TetON	647
13	12	TetON promotor BFP	pWS3033_-_pTet7_[2]	fw_LexOp_TEF1p	rv_LexOp_mTagBFP2	406
14	13	BFP	pmc-3-tagbfp2	fw_mTagBFP2	rv_mTagBFP2_tCYC1	728
15	14	Andrea fragment 2 GFP	pYTK095-Z3EV - Andrea makes it	p_p416-GFP_ADH1_fw	rv_pTEF1_highTm	2054
16	15	p416 - TEF	p416-GFP	p416GFP_backbone_fw	p416GFP_backbone_rev	5792
17	16	Estr promotor GFP	pWS2952_-_pZ3_(P1)_[2]	p_Estrp_fw	p_Estrp_rev	592
18	17	Estr promotor fatB	pWS2952_-_pZ3_(P1)_[2]	p_Estrp_fw	rw_EstradiolP_FatB	592
19	18	fatB	IDT fatB	fw_ScGAL_pFatB	rw_CYC1T_fatB	1280
20	19	Andrea fragment 2 FatB	pYTK095-Z3EV - Andrea makes it	p_p416_ADH1t_fw	rv_pTEF1_highTm	2054

Table 2. Primer mixes.

	A	B	C	D	E	F	G
1	Primer mix name	For PCR	Primer fw	Primer fw (µL)	Primer rev	Primer rev (µL)	MQ water (µl)
2	pmix frag+antisense	1-5	fw_eFAS_frag/antisense	20	rv_eFAS/antisense	20	160
3	pmix scCUP	6	fw_CUP1p_p413TEF	10	rv_CUP1p	10	80
4	pmix miRFP670	7	fw_miRFP670_CUP1p	10	rv_miRFP670_p413TEF	10	80
5	pmix tesBT	8	fw_CUP1p_tesBT	10	rv_TCYC1_tesBT	10	80
6	pmix Andrea fragment 1	9	fw_tPGK1_p415TEF	10	p_TEF1p_rv	10	80
7	pmix TetON promotor TesA	10	fw_LexOP_TEF1p	10	rv_LexOP_TesA	10	80
8	pmix TesA	11	fw_tesA_p415TEF tetON	10	rv_TesA_p415TEF TetON	10	80
9	pmix TetON promotor BFP	12	fw_LexOp_TEF1p	10	rv_LexOp_mTagBFP2	10	80
10	pmix BFP	13	fw_mTagBFP2	10	rv_mTagBFP2_tCYC1	10	80
11	pmix Andrea fragment 2 GFP	14	p_p416-GFP_ADH1_fw	10	rv_pTEF1_highTm	10	80
12	pmix p416 - TEF	15	p416GFP_backbone_fw	10	p416GFP_backbone_rev	10	80
13	pmix Estr promotor GFP	16	p_Estrp_fw	10	p_Estrp_rev	10	80
14	pmix Estr promotor fatB	17	p_Estrp_fw	10	rw_EstradiolP_FatB	10	80
15	pmix FatB	18	fw_ScGAL_pFatB	10	rw_CYC1T_fatB	10	80
16	pmix Andrea fragment 2 FatB	19	p_p416_ADH1t_fw	10	rv_pTEF1_highTm	10	80
17							

3 different PCR batches were run, one with annealing temp at 60C, and two at 58C, totalling 10 batches: 1-5, 16 and 17 at 60C, 6, and 13 at 58C, and 15 at 58C over night.

The source plasmids were diluted with MQ water to have the same concentration as the IDT fragments at 10 ng/µL. The dilutions can be seen in table 3.

Table 3. Source plasmid dilutions

	A	B	C	D	E	F	G
1	PCR nr	Source plasmid	C source plasmid (ng/µl)	Source plasmid (µl)	MQ water (µl)		
2	16 - 17	pWS2952	38,7	1	2,87		
3	6	pYTK031	44	1	3,4		
4	13	pmc-3-tagbfp2	68	0,5	2,9		
5	15	p416-GFP	505	0,5	24,75		

For each PCR batch a master mix containing, phusion polymerase, phusion buffer, dNTPs and MQ water was made according to Phusion PCR protocol:
10 µL Phusion buffer/PCR tube
1 µL dNTPs/PCR tube
35 µL MQ water/PCR tube
0,5 µL Phusion polymerase/PCR tube

46,5 µL of the mastermix was added to each PCR tube, followed by
2,5 µL of respective primer mix and 1 µL of the source plasmids/gene fragments to each respective PCR tube. The tubes were then vortexed.

The tubes were put in the PCR machines with the following settings (at 40 seconds of extension time per 1000 base pairs):
PCR 1-5, 16 and 17 at 60C were set with an extension time at 120 second cycles and 40 cycles.

PCR 6, and 13 at 58C were set with an extension time of 40 seconds and 40 cycles.
PCR 15 at 58C over night was set with an extension time of 4 min and 40 seconds and 40 cycles.

Results

Ready primer mixes for all PCRs and diluted templates.

10 PCR reactions produced or running, to be checked the following the day.

Conclusions

We require more coffee.

TUESDAY, 29/6/2021

Purpose

Investigating the success of the 10 PCRs from yesterday.

Prepare construction of gene fragments into plasmids for gibson cloning, transformation and integration.

Methods

Group 1:

Pre-stain gel electrophoresis for the 10 PCRs from yesterday were run on agarose.

Heated agarose (1%) was supplied with GelGreen via pouring into a plastic gel mould containing a comb for small wells, until half of the mold was full. The gel was left to set for 20 minutes, after which the comb was removed and the gel is transferred to a gel electroforesis box and covered with TAE buffer (1X). Drops of loading dye was pipetted on parafilm and 1 µL of the PCR fragments 1-5, 16, 17, 6, 13 and 15 is added to one drop each, followed by loading into the wells of the gel. 0,5 µL of 1 kb thermoscientific gel ruler was loaded into the wells flanking the samples, and the gel was run at 80V over 40 min. The gel was then imaged in and the image saved.

Two gels with bigger wells were created and gel purification was performed with post-staining in order to purify PCR products 1-5 and 15 due to the unwanted bands discovered in the analytical gel image for these PCR products. Agarose (1%) was poured in a gel mould containing a comb for big wells, until half of the mould was full. After setting for 20 minutes, the gels were then immersed in a post staining solution for about 40 minutes, composed of: 100 mL distilled water and 15 µL GelGreen, suitable for three gel post-stains. The gels were transferred to a gel electroforesis box and covered with TAE buffer (1X). 10 µL loading dye was added to the PCR tubes and 50 µL of the PCR product with the loading dye was then added into the big wells of the gels. 0,5 µL and 1 µL 1 kb gel ruler was then loaded into the two wells at the outer left edge of gel 1 and the outer right edge of gel 2. Gels were then run at 80 V over 40 min. The gels were imaged with UV light and the desired bands were cut and placed in tubes for future purification.

Group 2:

Three PCRs with annealing temp at 62 C were run: 7, 10 and 12.
Master Mix was prepared as previously described. The source plasmids were diluted to reach a concenration of approximately 10 ng/µL. Information about the dilutions can be found in table 4. Due to having a meeting, the Master mix was stored in freezer and then thawed for 30 minutes. Master mix, primer mix and template DNA is added in the same way as prevously desribed.

Table 4. Source plasmid dilutions

	A	B	C	D	E	F	G
1	PCR nr	Source plasmid	C source plasmid (ng/µL)	Source plasmid (µL)	MQ water (µL)		
2	7	pMC-3-miRFP670	51	0,5	2		
3	10, 12	pWS3033_-_pTet7_[2]	111,1	0,5	5		

A pre staining gel was prepared as previusly described, and loaded with sample 7, 10 and 12 and flanked with ladders, and run at 140 V for 20 min.

Results

All 10 group 1 PCRs were successfull, there were however unwanted PCR fragments in PCR product 1, 5 and 15, for which another gel was ran and a gel extraction was performed to harvest the wanted PCR fragments with the correct length.

Figure 1 shows the gel electrophoresis run for the PCR fragments 1, 2, 3, 4, 5, 16, 17, 6, 13 and 15 together with two ladders for reference.

Gel image 06-29_Genes, parts and backbone_PCR-1, 2,3,4,5,16,17,6,13,15.png

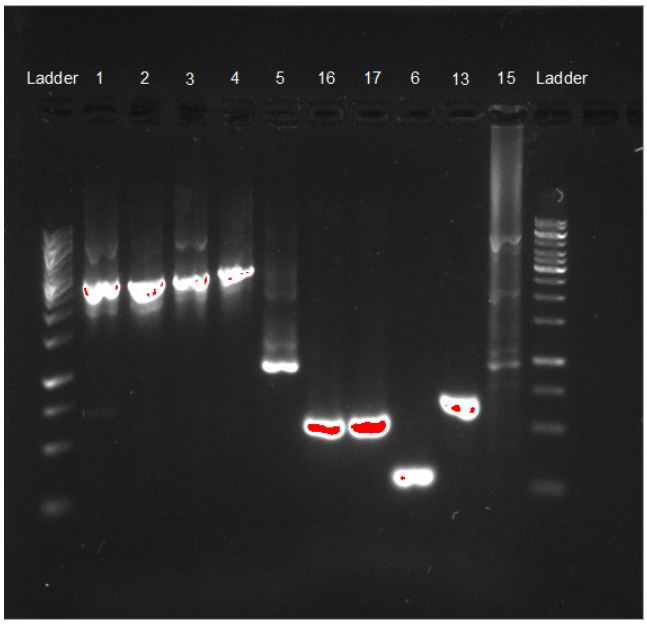

Figure 1: Gel electrophoresis of PCR products 1, 2, 3, 4, 5, 16, 17, 6, 13, 15.

Figure 2 shows the 2 larger welled post staining gels for purification. 1, 2 and 3 had clear band and were easy to cut out, 4 and 5 had pretty clear bands. 15 was hard to cut out due to smear.

image.png

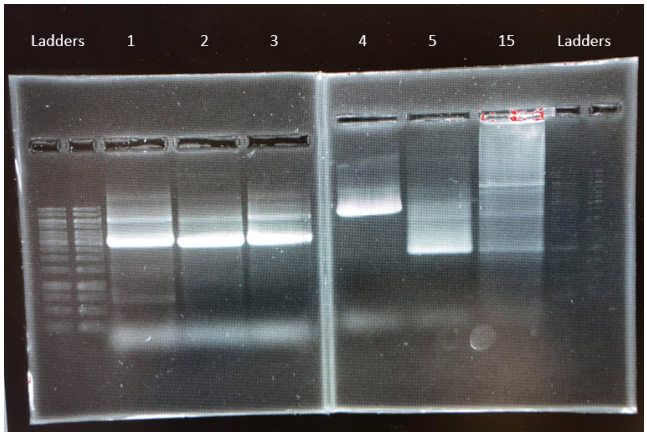

Figure 2: Gel electrophoresis for purification of PCR products 1, 2, 3, 4, 5, 15.

Figure 3 shows the gel electrophoresis run for the PCR fragments 7, 10 and 12 together with two ladders for reference.

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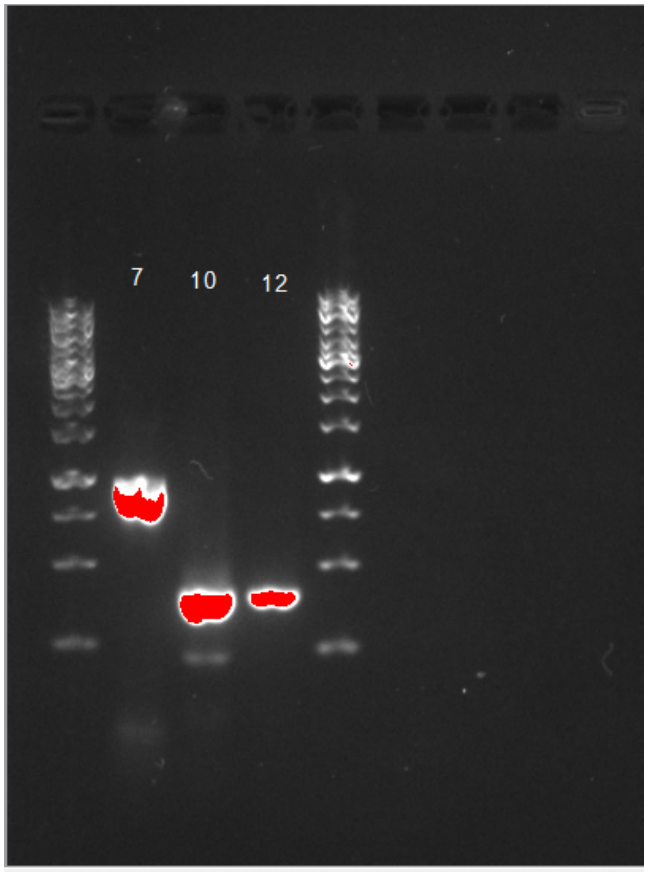

Figure 3: Gel electrophoresis of PCR products 7, 10, 12.

Conclusions

PCR 1-6, 13, 16 and 17 seems to have be successful.

PCR 15 might need to be redone.

PCR for fragments 7, 10 and 12 were successful, with 10 needing gel purification to remove unwanted side product.

WEDNESDAY, 30/6/2021

Purpose

Purify gel to get DNA fragments for Gibson cloning.

Methods

Group 1:

GeneJet Gel extraction for samples: 1-5 and 15. The purification protocol is followed.

The tubes with cut out gel were weighed to know how much buffer to use. The tube weighs about 1 g. Weighs are found in table 5.

Table 5: Weights of gel.

	A	B
1	Gel DNA	Weight (mg) no tube
2	1	444,3
3	2	489,0
4	3	597,1
5	4	655,0
6	5	553,6
7	15	302,1

1:1 (volume: weight) binding buffer was added to the gels. They were then incubated at 60C until dissolved (about 10 minutes).
For the PCR products 1:1 volume of binding buffer. Sample 6 is under 500bp long, and therfore 1 gel volume of 100% isopropanol.

From now, the GeneJet PCR purification protocol can be followed for the gel solutions.
For both gels and PCR products, up to 800 µL of the solutions was added to PCR columns which are then centrifuged at max for 1 min. The flow through is discarded and the columns are added with 700 µL of wash buffer and centrifuged again at 1 min and the flow through is discarded. The empty columns are then centrifuged again for 1 min to completely remove wash buffer. The GeneJET purification column is transferred to a clean 1.5 mL microcentrifuge tube and 50 µL of Elution Buffer is added. The tube is centrifuged 1 min. The purification columns are discareded. The concentration of DNA is measured using NanoDrop by placing 1 µL of sample. The concentrations can be seen in table 6. The purified DNA is stored in freezer.

Group 2:

A gel purification for PCR fragment 10 was performed as previously described according to the GeneJet gel extraction protocol, with 1 µL and 2 µL ladder respectively, followed by post-staining and gel cutting. The GeneJet PCR purification protocols were followed for PCR product 7,10 and 12, with 10 having additional gel purification steps. Concentration of purified PCR products were measured using Nanodrop and the purified DNA was put in the freezer.

PCR for fragment 8 and 11 was performed at 58C, with 30 second elongation, followed by gel electrophoresis at 140V for 20 min. Fragment 11 was successful, purified according to protocol, measured using nanodrop and stored in freezer.

Results

Table 6: Concentrations of purified samples.

	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O
1	Sample	1	2	3	4	5	6	13	15	16	17	7	10	12	11
2	ng/µL	38,5	51,7	45,1	37,9	14,7	83,4	69,4	3,2	48,0	69,3	180,2	118	197,2	78,4

Figure 4 shows the

[Insert picture of the post staining gel for sample 10]

Figure 4:

Figure 5 shows the gel electrophoresis run for the PCR fragments 8 and 11 together with two ladders for reference.

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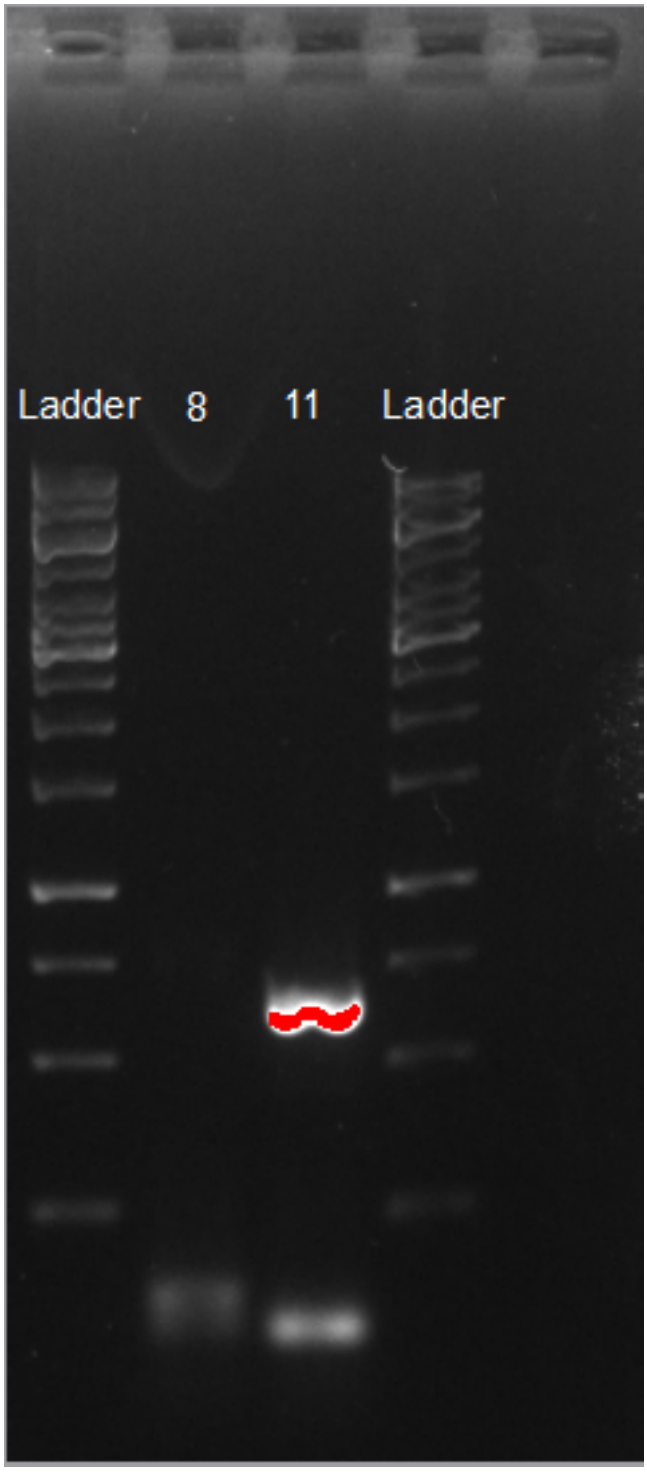

Figure 5: Gel electrophoresis of PCR products 8 and 11.

Conclusions

Sample 15 probably needs to be redone, we will try gibson cloning but if it does not work we will try with a gradient PCR.

Fragment 8 needs to be rerun due to unknown error, hopefully the error in PCR was a fluke and product will be obtained in a second attempt. Fragments 7, 11 and 12 gave satisfactory yields.

THURSDAY, 1/7/2021

Purpose

Digestion of plasmids: pCfB-(3040,2909,3035,3038,3039) using restriction enzyme. Planned to do gibson assembly as well, but did not have time. PCR on fragment 8 and digestion of plasmids TEF 413 and TEF 415 for future gibson assembly.

Methods

Group 1

Digested plasmids using restriction enzyme SacI, according to gibson protocol. Pipett was not changed between application to 3040 and 2909, and thus 2909 was remade. However SacI restriction enzyme might also be contaminated, and so we may have to redo some plasmid digestions, though our lab supervisor suggested that we check it by using restriction enzymes specific to induvidual plasmids and checking the resulting gel electrophoresis to determine if there are other uncleaved plasmids present.

The digested plasmids were put into agarose gel, electrophoresis performed over 40 mins at 80 V, the gel cut and put into tubes. Plasmid dna was extracted according to gel purification protocols.

Group 2

Repeated PCR for fragment 8 was run at 58 C and 30 s elongation, followed by analysis using gel electrophoresis. To our suprise, a strong band was now found and the mixture then purified using PCR purification protocols as previously described. Measurement with nanodrop revealed 103,7 ng/µL.

Digestion of plasmids p413TEF and p415TEF were performed following protocol as follows:

Table3

	A	B	C	D	E
1	Plasmid	H2O (µL)	Buffer (µL)	DNA (µL)	Res.Enzyme (µL)
2	p413TEF	13,2	2	3,8	1
3	p415TEF	14,2	2	2,8	1

The digestion mixes were incubated at 38 C for 20 minutes.

Results

Purified plasmid-DNA stored in freezer.

Purified fragment 8 yielded 50 µL of 103,7 ng/µL DNA and was stored in freezer.

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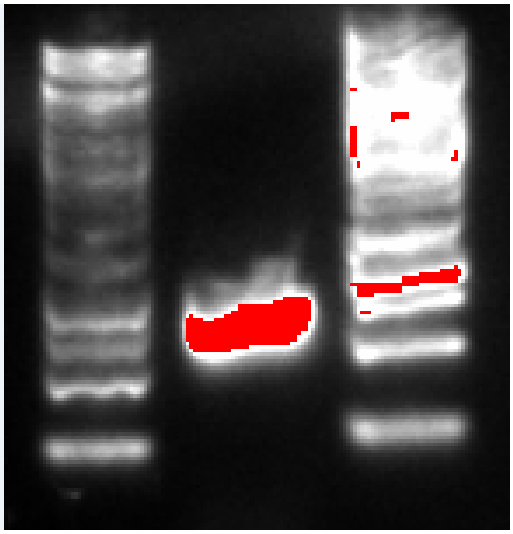
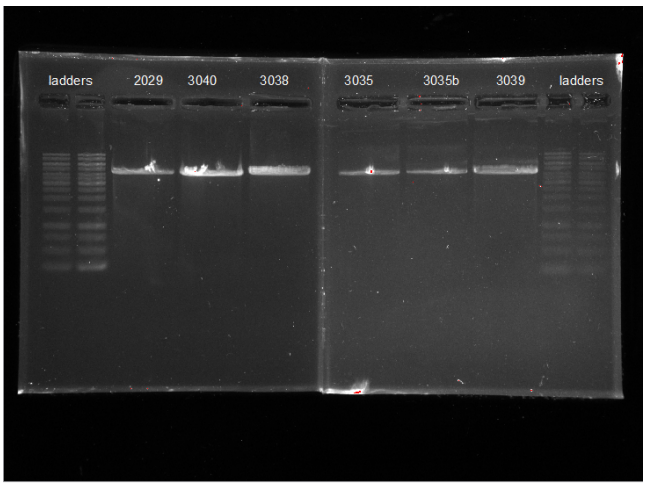


Figure 6: Result of second PCR on fragment 8.

7-1-21, plasmid digestions of 2029,3040,3038,3035,3039.PNG



Conclusions

Crosscontamination of plasmids may require digesting more from stock once more enzyme has been obtained. Fragments are otherwise to be used in gibson assembly.

The cause for the failure of PCR of fragment 8 yesterday is theorised to be accidential non-addition of template or primers to the PCR mixture.

FRIDAY, 2/7/2021

Purpose

Perform gibson assembly for integration plasmids and transform into E.coli for overnight cultivation. Gel purification of the digested TEF 413 and TEF 415 plasmids.

PCR of TetA and Estradiole induction fragments 9, 14 and 19. Gel electrophoresis check from PCR result of fragments 9, 14 and 19.

Methods

Group 1

Fragment 15 was dehydrated and resuspended into 10 µL mQ water in order to concentrate it. Concentrations of plasmid DNA from yesterdays digestion were measured using nanodrop:

Table1

	A	B
1	Plasmid	Conc ng/µL
2	3040	32,5
3	2909	14,5
4	3035	24,0
5	3038	40,9
6	3039	33,2

Using the prepared plasmids and fragments 1 through 5, gibson assembly was performed as follows:
To each mix was added 5 µL gibson master mix. Final vol for all samples was 10 µL.

Table2

	A	B	C	D
1	Plasmid + fragment	Vol plasmid (µL)	Vol fragment (µL)	Vol H2O (µL)
2	3040 + 2	0,8	1,51	2,69
3	2909 + 3	1,7	1,63	1,67
4	3035 + 5	1,05	3,95	N/A
5	3038 + 1	0,6	1,97	2,43
6	3039 + 4	0,8	2,1	2,1

The mixes were incubated at 50 C over 60 mins.

The finished plasmids were then transformed into E.coli for growth as described by protocol: Chemically competent E.coli cells were thawed on ice for 30 minutes, after which 5 tubes with E.coli were supplied with each with 2 µL of one of the finished plasmids. Following this, the cells were heat shocked at 42 C for 90 s in a water bath, and were subsequently placed back on ice briefly and each was loaded with 1000 µL of agar growth media, and finally followed by incubation at 37 C for 60 min.
After incubation, 10 agar plates with ampicillin were withdrawn and to 5 of these, 100 µL solution from each tube was applied. The tubes were then centrifuged, supernatant discarded and the pellet resuspended in 100 µL mQ water, after which the full 100 µL volume was applied to a new set of plates, creating one 10% cellular concentration plate and one 90% cellular concentration plate for each gibson-constructed plasmid. These were then incubated overnight at 37 C, and placed into a fridge for storage.

Group 2

Agarose gel was poured without stain and 15 thick wells. Three wells were filled with p415TEF due to a slight excess of dye and two wells with p413TEF due to small well size. The gel was subsequently run at 80 V for 40 minutes. PCR for fragments 9, 14 and 19 was prepared as previously described, and fragment 9 run at 58 C with 70 second elongation, while 14 and 19 were run at 59 C with 90 seconds of elongation.

Following completion of PCR run, an analytical agarose gel with gel green prestaining according to protocol. PCR products of fragments 9, 14 and 19 were run and analysed, revealing only a short band below 250 bp for all mixtures, indicating failure of each PCR reaction.

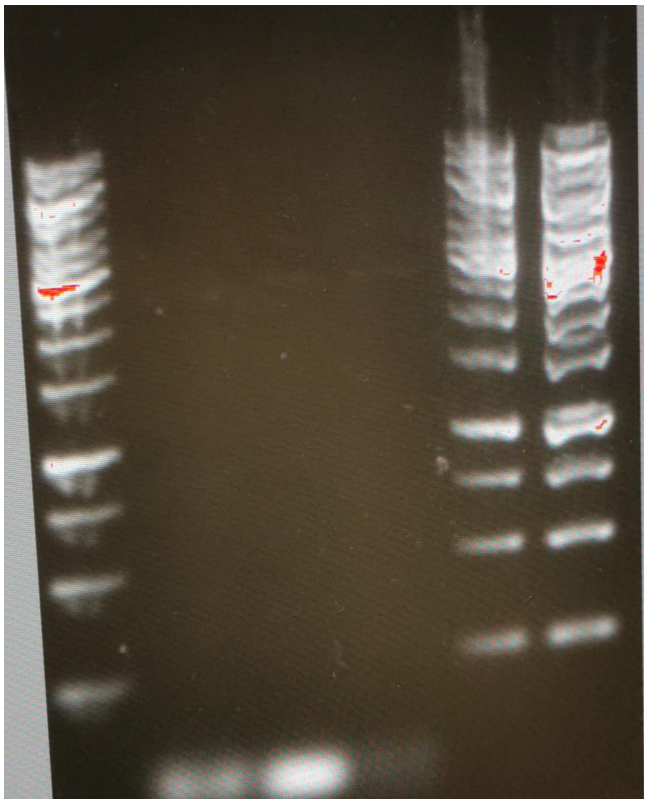
From the purification gel were the two sections containing 413 and 415 cut, followed by gel purification as established by protocol. p413TEF yielded 35 µL of 20,8 ng/µL and p415TEF yielded 35 µL of 2,3 ng/µL.

Results

Integration plasmids were assembled from digested fragments. Contamination may have occured, but the constructed plasmids were none the less transformed into E.coli and plated to be grown overnight, followed by cold storage.

Digested induction system plasmids p413TEF and p415TEF were successfully digested and purified using gel purification. PCR for fragments 9, 14 and 19 failed.

202107020_PCRGelCheck_9&14&19.jpg



Conclusions

Work will resume next week by preparation for and eventual transformation of the assembled integration plasmids into yeast, starting with 3040 + 2, the likely contaminant. All integration plasmids will be checked for possible contamination by 3040 + 2, but 3040 + 2 itself should be useful for transformation regardless.

p413 and p415 have been stored awaiting induction system plasmid assembly in the future. Fragments 9, 14 and 19 will be subjected to PCR again, in the hopes that the error was human error somewhere. Should this fail again, an annealing gradient will likely be the next course of action.