Lab Journal - May

Project: iDEC 2024

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DIMARTS, 30/4/2024

In lab: Gabriel, Philip, Pau

Prepping plasmids from EcoFlex kit

5 ml liquid cultures started from EcoFlex kit for the following plasmids (37 ^{o}C):

Liquid cultures from EcoFlex kit

	A	B	C
1	**Plasmid**	**Well**	**Resistance**
2	pTU1-A-RFP	A6	Amp
3	pTU1-B-RFP	A7	Amp
4	pTU1-C-RFP	A8	Amp
5	pTU1-D-RFP	A9	Amp
6	pBP	B2	Chlor
7	pTU2-A-RFP	B9	Chlor
8	pBP-J23108	C7	Chlor
9	pBP-SJM901	C9	Chlor
10	@SJM910	D3	Chlor
11	pBP_BBa_B0034	D11	Chlor
12	pBP-L3S2P21	F6	Chlor

DIMECRES, 1/5/2024

Prepping plasmids from EcoFlex kit Qiagen Miniprep

Miniprep from EcoFlex kit

	A	B	C
1	**Plasmid**	**Concentration (ng/ul)**	**Notes**
2	pTU1-A-RFP	230	
3	pTU1-B-RFP	257	
4	pTU1-C-RFP	251	
5	pTU1-D-lacZ	236	
6	pBP	203	
7	pTU2-A-RFP	229	
8	pBP-J23108	191	
9	pBP-SJM901	194	
10	@SJM910	189	A260/230 0.86
11	pBP_BBa_B0034	225	
12	pBP-L3S2P21	193	

Plates poured

20 LB-Carb

20 LB-Chlor

In cold room

Resuspension of Twist genes

Twist genes were resuspended to a concentration of 10 ng/uL according to the yield found on spec sheet

Resuspension of Twist genes

	A	B	C	D
1	**Fragment**	**Well**	**Yield (ng)**	**Resuspension Volume (uL)**
2	RNAP N-Term	A1	1331	133.1
3	SmR	A2	1072	107.2
4	UBCH5A	B1	1331	133.1
5	Ubiquitin	B2	1174	117.4
6	EGLN3	C1	1191	119.1
7	KanR	C2	1514	151.4
8	EGLN1	D1	2181	218.1
9	a-synuclein	E1	1072	107.2
10	KLF10	F1	2337	233.7
11	Combined_2	G1	1027	102.7
12	CymR	H1	1344	134.4

Restriction digest Restriction digest

The following reactions were set up for NdeI and SphI digestion:
A1: RNAP N-Term
B1: UBCH5A
B2: Ubiquitin
C1: EGLN3
D1: EGLN1
E1: a-syn
F1: KLF10
pBP

A 20 ul system was set up for the inserts from the Twist order, containing roughly 200 ng DNA, with 0.5 ul of each enzyme

A 50 ul system was set up for pBP containing 1 ug of DNA with 1 ul of each enzyme

DIJOUS, 2/5/2024

in the Lab: Noemie

Clean Up

Restriction digests were cleaded up using the NucleoSpin Gel and PCR Clean-up kit (Protocol: NucleoSpin Gel and PCR Clean-up)
according to protocol, eluted in 20 ul Elution Buffer

Yields were rather low

PCR cleanup results

	A	B	C	D	E	F	G
1	Fragment	Label	Yield (ng/uL)	Length (bp)	Yield (fmol/uL)		
2	RNAP N Term	A1	4.4	582	12.3		
3	UBCH5A	B1	5	487	16.7		
4	Ubiquitin	B2	3.5	274	20.7		
5	EGLN3	C1	3.7	762	7.9		
6	EGLN1	D1	4.5	1323	5.5		
7	a-synuclein	E1	5.4	465	18.85		
8	KLF10	F1	5.4	1485	5.9		
9	pBP	pBP	5.5	2091	4.3		
10							

Ligation Reaction

The following ligation reactions were setup for Level 0 construction:
RNAP N-Term (pES0003)
UBCH5A (pES0007)
Ubiquitin (pES0004)
EGLN3 (pES0013)
EGLN1 (pES0014)
a-syn (pES0015)
KLF10 (pES0016)

Molar ratio of 1:3 vector to insert was used
Total reaction volume: 20 uL
Incubation 2 h at RT

LIgation Protocol

	A	B
1	Component	20 uL Reaction (ul)
2	10x T4 DNA Ligase Buffer	2 uL
3	Vector	20 fmol
4	Insert	60 fmol
5	Water	to 20 uL
6	T4 DNA Ligase	1 uL

Transformation

Transformation of all the ligation reactions above, following the High efficiency Protocol: Transformation
plate in LB + Chlor & incubate at 37 °C ON

PCR

Dilute all primers to 10 uM
Total # Reactions: 5
Master Mix: 30 uL 5X Reaction Buffer; 3 uL 10 mM dNTPs, 94.6 uL Water, 1.5 uL Polymerase, 6 ul Template -> distribute 22.5 uL to each Tube
add 1.25 ul of each 10 uM Primer

PCR for Twist_Combined_2

	A	B	C	D	E	F	G
1	Descriptiion	Label	Template	Primer 1	Primer 2	Ta	
2	Dummy TU1-B	P 1	Twist_combined_2	o028	o029	59	
3	Dummy TU1-D	P 2	Twist_combined_2	o030	o031	59	
4	Linker 3	P3	Twist_combined_2	o024	o025	59	
5	Linker 4	P 4	Twist_combined_2	o026	o027	61	
6	Ps70 + RBS	P 6	Twist_combined_2	o013	o014	59	
7							
8							

Cycling conditions
98 C 30 s

30 x
98 C 10 s
60 C 30 s
72 C 20s

72 C 2 min

Hold 4 C

oligo annealing (Procedure from IDT)
Mix fwd and rv oligo in equal molar amounts
Primer concentration: 100 uM, Total reaction volume: 100 uL

Heat at 94 °C for 2 minutes, turn off heat block, leave them in heat block until cooled back down to room temperature

Oligo annealing

	A	B	C	D
1	**Name**	**Label**	**Oligo 1**	**Oligo 2**
2	T7 Promotor CGG	A1 CGG	o005	o006
3	T7 Promotor GAC	A2 GAC	o007	o008
4	Linker 1	A3 L1	o020	o021
5	Linker 2	A4 L2	o022	o023

DIVENDRES, 3/5/2024

in Lab: Noemie

PCR Purification and Restriction Digest

Purification of PCR reaction P1 - P5 from twist_combined, according to NucleoSpin Gel and PCR Clean-up

Dummy TU-1 B and Dummy TU1-D stored, purified.

for P3-P5: set up 20 ul digestion reactions with SphI and NdeI, according to Restriction digest
Incubation: 1h at at 37C, 20 min at 65C

For pBP: set up 50 ul digestion reactions with SphI and NdeI, according to Restriction digest
Incubation: 1h at at 37C, 20 min at 65C

Restriction digests were cleaded up using the NucleoSpin Gel and PCR Clean-up kit (Protocol: NucleoSpin Gel and PCR Clean-up)
according to protocol, eluted in 20 ul Elution Buffer

Ligation Reaction
The following ligation reactions were setup for Level 0 construction:

from oligo annealing reactions A1 - A4 (diluted reaction 1:10)
T7 CGG (pES0017)
T7 GAC (pES0018)
Linker 1 (pES0020)
Linker 2 (pES0021)

from PCR + digest + clean up
Linker 3 (pES0002)
Linker 4 (pES0022)
Ps70 + RBS (pES0005)

Molar ratio of about 1:3 vector to insert was used
Total reaction volume: 20 uL
Incubation 1 h at RT

LIgation Protocol1

	A	B
1	Component	20 uL Reaction (ul)
2	10x T4 DNA Ligase Buffer	2 uL
3	Vector	ca. 20 fmol
4	Insert	ca. 60 fmol
5	Water	to 20 uL
6	T4 DNA Ligase	1 uL

Transformation

Transformation of all the ligation reactions above, following the High efficiency Protocol: Transformation
plate in LB + Chlor plates & incuabated at 37 °C ON

Retransformation of Jin216

- cut small piece out of the paper & dissolved it in water

- Transformed & directly inoculated a liquid culture 5 mL LB + Carb (no outgrowth)

Analysis Transformants from 02.05
no colonies for pES0013, pES0014, pES0016
for pES0003, pES0004, pES0007, pES0015 -> picked 2-3 colonies each -> inoculated 4 mL LB + Chlor liquid cultures
shake ON

DISSABTE, 4/5/2024

In lab: Gabriel

Miniprep of liquid cultures 3/5 Qiagen Miniprep
pJin216 did not grow

Miniprep of cultures from 3/5

	A	B	C
1	**Plasmid**	**Concentration**	**Notes**
2	pES003a	171	
3	pES003b	178	
4	pES003c	164	
5	pES004a	164	
6	pES004b	168	
7	pES004c	176	
8	pES007a	168	
9	pES007b	133	
10	pES015a	168	
11	pES015b	170	

Restriciton digest of failed products Restriction digest

Restriction digest of failed transformations

	A	B	C
1	**DNA**	**Well**	**Amount**
2	EGLN3	C1	400 ng
3	EGLN1	D1	400 ng
4	KLF10	F1	400 ng
5	pBP	-	1 ug

IMPORTANT:
reaction tube 1 has both enzymes, 2, 3, and 4 have NdeI, but NO SphI (ran out smfh), can try to rerun digest

DIUMENGE, 5/5/2024

In lab: Gabriel

New chloramphenicol stock
Stock concentration: 25 mg/ml
50 ml falcon tube put in freezer in main lab (same drawer as twist genes)

Liquid cultures for transformations from 03/05
Colonies on plates show different morphologies. Does not seem to be plate contamination, as unused half of plate showed no growth at all. Either inconsequential or cell stock contamination.
5 ml liquid cultures (18) set up for:

- pES002 (3)
- pES005 (3)
- pES017 (3)
- pES018 (3)
- pES020 (2)
- pES021 (1): only weird morphology colony grew
- pES022 (3)

Plate containing pES020 and pES021 put back in incubator to see if growth continues
Other plates in the fridge

Digest from 04/05 moved to fridge under bench

DILLUNS, 6/5/2024

in Lab: Noemie

Miniprep of liquid cultures 5/5 Qiagen Miniprep

- Miniprep failed for pES002a, pES017c, pES018c

Sequencing

all Plasmids prepped on 6.5. and 4.5.

Sequencing of plasmids from 6/5 and 4/5

	A	B	C	D	E
1	**Plasmid**	**Tube Nr**	**Primer**	**Results (7.5.)**	**Comment**
2	pES002 b	1	o017	ok	4 silent mutations
3	pES002 c	2	o017	no insert	
4	pES003a	3	o017	no insert	
5	pES003b	4	o017	no insert	
6	pES003c	5	o017	no insert	
7	pES004a	6	o017	no insert	
8	pES004b	7	o017	no insert	
9	pES004c	8	o017	no insert	
10	pES005 a	9	o017	ok	
11	pES005 b	10	o017	ok	
12	pES005 c	11	o017	ok	
13	pES007a	12	o017	no insert	
14	pES007b	13	o017	no insert	
15	pES015a	14	o017	no insert	
16	pES015b	15	o017	no insert	
17	pES017 a	16	o017	ok	
18	pES017 b	17	o017	ok	
19	pES018 a	18	o017	ok	
20	pES018 b	19	o017	ok	
21	pES020 a	20	o017	wrong insert	has PT7 GAC insert (pES0018)
22	pES020 b	21	o017	no insert	
23	pES021 a	22	o017	no insert	
24	pES022 a	23	o017	ok	
25	pES022 b	24	o017	wrong insert	has Ps70 + RBS insert (pES0005)
26	pES022 c	25	o017	ok	

Transformation of pES020 and pES021

only contamination grew, needs to be repeated

Check for Contaminations on Chlor plates and cells

- Plate empty: empty plate
- Plate DH5a: 50 uL untransformed DH5a stock

Put plates in 37C incubator ON

Finish Digests from 04/05

- added SphI (0.5 uL per reaction) to reactions 2-4, incubated at 37C for 15 min, 65C for 20 min
- stored back in the fridge

DIMECRES, 8/5/2024

in Lab: Noemie

Sequencing Results

see Table 9 (on 6.05)

pooled correct plasmids & diluted them to 100 ng/uL

Contamination Check

no growth observed on the two plates: source of contamination are not plates or competent cells

Hypothesis: spatula made from pasteur pipette that I used for streaking was not sterile

Resuspension of Twist Clonal genes

resuspended to a concentration of 100 ng/uL, stored at -20 C

DIJOUS, 9/5/2024

in Lab: Gabriel

Attempt 2 of twist genes into pBP

Restriciton digest Restriction digest

Restriciton digest of genes using NdeI and SphI, 1 uL of each enzyme used

NdeI-SphI restriction digest of twist genes 9/5/24

	A	B	C	D	E
1	**Part**	**Well**	**Amount (ng)**	**Reaction size**	**Product size (for GE)**
2	RNAP-Nterm	A1	300	30	
3	UBCH5A	B1	300	30	
4	Ubiquitin	B2	300	30	
5	EGLN3	C1	300	30	
6	EGLN1	D1	300	30	
7	a-syn	E1	300	30	
8	KLF10	F1	300	30	
9	HsUba	Tube	800	50	3220
10	TuUba	Tube	800	50	3199
11	RNAP-Cterm1	Tube	800	50	2158
12	RNAP-Cterm2	Tube	800	50	2158

Additionally, 4 x pBP (1 ug DNA, 50 ul) reactions were set up to be gel extracted

Gel extraction of pBP NucleoSpin Gel and PCR Clean-up

Twist clonal gene (30 ul each) and all pBP reactions (total 200 ul) were run on a gel until pBP bands no longer match those of the undigested plasmid and then gel extraction was performed.

Gel extraction was performed as normal on the products of the clonal gene digests

Concentrations were seen as followed:

Gel extraction results 9/5/24

	A	B	C
1	**Part**	**Concentration**	**Comment**
2	HsUba	16.3	
3	TuUba	18.9	
4	RNAP-C1	12.7	
5	RNAP-C2	13.3	
6	pBP1	3.1	
7	pBP2	2.6	
8	pBP3	2.2	
9	pBP4	2.4	
10	pBP average	2.575	

results for pBP were very poor, and there was evry little plasmid left over, so 4 overnight liquid cultures were prepared from the EcoFlex kit

DIVENDRES, 10/5/2024

In lab: Gabriel

Miniprep of overnight cultures from 9/5

4 overnight cultures for pBP were miniprepped:

Miniprep of liquid cultures from 9/5

	A	B	C
1	**Name**	**Concentration**	**Comment**
2	pBP1	190	
3	pBP2	204	
4	pBP3	241	
5	pBP4	267	

Restriciton digest Restriction digest

4 x 50 ul reactions were set up containing 1 ug pBP DNA and 1 ul of each enzyme. Left to incubate over the day

DILLUNS, 13/5/2024

in Lab: Philip

Prepared LB-medium (2x1L), **LB-Agar** (2x1L,1x2L)

DIMARTS, 14/5/2024

in Lab: Noemie, Philip

Poored 45 LB-agar Chloramphenicol plates, 20 LB-agar carbenicillin

Attempt 3 to clone twist fragments

Restriction Digest

Restriction digest

Master Mix: for 11x20 uL Reactions (Inserts) and 4x50 uL Reactions (pBP)

10x Cutsmart Buffer: 45 uL

SphI: 9 uL

NdeI: 9 uL

For 50 uL Reactions: added 7 uL to each Tube + 5000 ng (25 uL) + 18 uL Water

For 20 uL Reactions: added 3 uL to each Tube + 170 ng (17 uL)

Incubation: 1 h at 37 C

NdeI-SphI digest of twist genes

	A	B	C	D	E	F	G	H	I
1	**Part**	**Location**	**Tube Label**	**Conc after clean up (ng/uL)**	**Size (after Digest)**	**uL Insert to add to Ligation Reaction (60 fmol)**	Water to add		
2	RNAP-Nterm	A1	3	13	537	1.5	3.5		
3	UBCH5A	B1	7	26	441	1	4		
4	Ubiquitin	B2	4	15	262	1	4		
5	EGLN3	C1	13	12	717	2.3	2.7		
6	EGLN1	D1	14	12	1278	4	1		
7	a-syn	E1	15	8	420	2	3		
8	KLF10	F1	16	9	1440	5	0		
9	HsUba	Tube	1	82	3220	1.5	3.5		
10	TuUba	Tube	6	91	3156	1.3	3.7		
11	RNAP-Cterm1	Tube	8	65	2158	1.2	3.8		
12	RNAP-Cterm2	Tube	9	53	2158	1.5	3.5		
13	pBP (3x)	Tube	BP	29	2091	1 (for 20 fmol)			

Took Sample of pBP for Gel electrophoresis before continuing

Clean up Digest Reaction: NucleoSpin Gel and PCR Clean-up

incubate column with elution buffer in heat block at 70C for 5 min before elution (elute in 15 uL)

A260/A230 values were all too low (most were < 1)

concentrations were ok (all > 8 ng/uL, for pBP around 30 ng/uL)

Ligation

Master Mix for 11 Reactions (20 uL each) -> 15 uL per tube

10x Ligase Buffer: 24 uL

Vector: 12 uL

Water: 132 uL

T4 DNA Ligase: 12 uL

1h at RT

Took Sample for Gel Electrophoresis after clean up (5 uL + 1 uL Loading Dye)

MoClo Reaction: Level 1 Assemblies

Test direct assembly from twist genes to Level 1 - only for clonal genes, bcs here we have larger amounts: HsUba1 (pES1002), TuUba (pES1003)

Diluted all Plasmids to 100 ng/uL first

Vector: pTU1-B-RFP

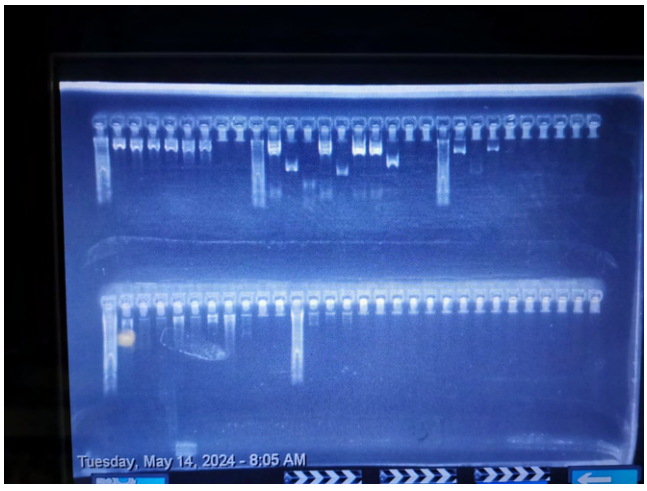
Inserts: pBP-SJM901, pBP_BBa_B0034, pBP-L3S2P21, respective E1 Insert

Ran 15 cycles of assembly protocol

EcoFlex Assembly

Gel electrophoresis

Gel 14/05



Gel loading scheme of samples

	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P	Q	R	S	T
1	ladder	pBP 1 (digested)	pBP 2 (digested)	pBP 3 (digested)	pBP 1 (digested, after clean-up)	pBP 2 (digested, after clean-up)	BP3 (digested, after clean-up)	ladder	pES0001 (digested, clean-up)	pES0003 (digested, clean-up)	pES0004 (digested, clean-up)	pES0006 (digested, clean-up)	pES0007 (digested, clean-up)	pES0008 (digested, clean-up)	pES0009 (digested, clean-up)	pES0013 (digested, clean-up)	ladder	pES0014 (digested, clean-up)	pES0015 (digested, clean-up)	pES0016 (digested, clean-up)
2	ladder	pES0001 (ligated)	pES0003 (ligated)	pES0004 (ligated)	pES0006 (ligated)	pES0007 (ligated)	pES0008 (ligated)	pES0009 (ligated)	pES0013 (ligated)	ladder	pES0014 (ligated)	pES0015 (ligated)	pES0016 (ligated)							

Transformation: Transformation

- 5 uL of Ligation Reaction to cells
- 5 uL of MoClo reaction

- Transformation of pJin216 (2 Reactions)
- 1 h Recovery
- Plated on plates with +Chlor/+Carb

DIMECRES, 15/5/2024

in Lab: Noemie, Philip

Analysis Transformation 14/05
All plates show colonies, again some colonies show different/weird morphologies (large colonies with fuzzy ends)
Picked 3 colonies per plasmid: Ecoli NightSeq and inocculated overnight culture (in approprate antibiotics)

Inocculation of pBP from cyrostock: 3 ON cultures

oligo annealing (Procedure from IDT)
Mix fwd and rv oligo in equal molar amounts
Primer concentration: 100 uM, Total reaction volume: 20 uL
Linker 1 (L1): o020, o021
Linker 2 (L1): o022, o023

Heat at 94 °C for 2 minutes, turn off heat block, leave them in heat block until cooled back down to room temperature -> stored in fridge ON

PCR PCR: Q5 Polymerase
added 2 uL of respective template
Cycling conditions: according to protocol (35 cycles), Ta: 61C, Extension time: 45 s

	A	B	C	D	E	F	G
1	Descriptiion	Label	Template	Primer 1	Primer 2	Ta	Comment
2	PCymR RBS	C1	pSBO sGFP	o011	o012	61	
3	PCymR RBS	C2	pSBO CymRAM 4	o011	o012	61	
4	luxAB	L	pJin216	o015	o016	61	length: 2.2 kb

DIJOUS, 16/5/2024

in Lab: Philip

Preparation:

PCR cleanup:
NucleoSpin Gel and PCR Clean-up

DNA concentrations after PCR cleanup

	A	B	C
1	Part	Conc (ng/ul)	Comment
2	PCymR RBS 1	60.2	low 260/230
3	PCymR RBS 2	63.2	low 260/230
4	LuxAB	112	

Digest of PCR products and pBP:

3x20 uL Reactions (Inserts) and 1x50 uL Reactions (pBP)

Restriction digest

PCR cleanup:

NucleoSpin Gel and PCR Clean-up

digested DNA concentrations after PCR cleanup

	A	B	C
1	Part	Conc (ng/ul)	Comment
2	PCymR RBS 1	3.7	low 260/230
3	PCymR RBS 2	8.5	low 260/230
4	LuxAB	7.0	low 260/230
5	pBP	5.3	low 260/230

Ligation:

T4 Ligation

Ligation

	A	B	C	D
1	DNA fragment	mass (ng) needed for 60 fmol (insert) / 20 fmol (backbone)	volume needed for calculated mols (ul)	water added
2	C1	5.84	1.6	7.6
3	C2	5.84	0.7	11.5
4	LuxAB	79.6	11.4	0.6
5	L1	- (1:20 in ligation mix)	2	15
6	L2	- (1:20 in ligation mix)	2	15
7	pBP	25.61	4.8	

Transformation:

Transformation

Used 10 ul of competent bacteria (recommendation of Kim)

Analysis of Sequencing Results

Sequencing Results Ecoli NightSeq

	A	B	C	D	E	F
1	**Plasmid**	**Replicate**	**Well**	**Primer**	**Results**	**Comment**
2	pES001	A	A1	o017	no insert	
3		B	B1	o017	no insert	
4		C	C1	o017	no insert	
5	pES003	A	D1	o017	no insert	
6		B	E1	o017	no insert	
7		C	F1	o017	no insert	
8	**pES004**	A	G1	o017	no insert	
9		B	H1	o017	**ok**	
10		C	A2	o017	no insert	
11	**pES006**	A	E2	o017	**ok**	
12		B	F2	o017	no insert	
13		C	G2	o017	no insert	
14	**pES007**	A	H2	o017	no insert	
15		B	A3	o017	sequencing too short	
16		C	B3	o017	ok	has a stop codon at the end (which is not there in the benchling plasmid)
17	**pES008**	A	C3	o017	no insert	
18		B	D3	o017	ok	
19		C	E3	o017	no insert	
20	**pES009**	A	B2	o017	ok	
21		B	C2	o017	no insert	
22		C	D2	o017	sequencing failed	
23	pES0013	A	F3	o017	no insert	
24		B	G3	o017	no insert	
25		C	H3	o017	no insert	
26	**pES0014**	A	A4	o017	no insert	
27		B	B4	o017	ok	
28		C	C4	o017	no insert	

29	pES0015	A	D4	o017	no insert	
30		B	E4	o017	no insert	
31		C	F4	o017	no insert	
32	pES0016	A	G4	o017	no insert	
33		B	H4	o017	no insert	
34		C	H5	o017	sequencing failed	wrong sequencing primer used
35	pES1002	A	B5	o017	sequencing failed	
36		B	C5	o017	????	sequenced something, does not align
37		C	D5	o017	sequencing failed	
38	**pES1003**	A	E5	o017	sequencing failed	
39		B	F5	o017	assembly ok	has promoter sjm910 instead of sjm901
40		C	G5	o017	sequencing failed	
41	**pJin216**	A	A5	AmpStop	ok?	wrong sequencing primer used
42		B	A6	AmpStop	ok	
43		C	B6	AmpStop	ok	

Miniprep of succesfully cloned plasmids and pJin216, pBP retransformed cultures (fill up pJin216/pBP stock):

Qiagen Miniprep

Plasmid concentrations post Miniprep

	A	B	C
1	Plasmid	Conc. (ng/ul)	Comment
2	pES0004	62	
3	pES0006	163	
4	pES0007	92	
5	pES0008	135	
6	pES0009	135.5	
7	pES0014	79	
8	pES1003	215	
9	pJin216 A	60	
10	pJin216 B	60	
11	pJin216 C	37.5	
12	pBP 1	60	
13	pBP 2	88	
14	pBP 3	57	

DIVENDRES, 17/5/2024

In Lab: Noemie, Philip

PCR of Luciferase in pJin216 (from1 16.5.24) to mutate SphI/BsmBI restriction sites and incorporate NdeI/SphI restriction sites at ends of Luciferase. + DpnI digest + Transformation (no recovery, only pJin216C as sequencing data was the best)
PCR twist fragments + clean up

PCR: Q5 Polymerase

PCR of pJIN216 and Twist fragments (for missing Lvl 0 plasmids), 50ul reactions, …

	A	B	C	D	E
1	Sample	Tm (°C)	Primers	Extension time (s)	amount of DNA (ng)
2	pJin216 A	56	0001/0002	120	1
3	pJin216 B	56	0001/0002	120	1
4	pJin216 C	56	0001/0002	120	1
5	EGLN3	59	0032/0033	30	1
6	a-syn	59	0032/0033	30	1
7	KLF10	59	0032/0033	30	1
8	RNAP N-term	59	0032/0033	30	1

NucleoSpin Gel and PCR Clean-up

Level 1 Assemblies EcoFlex Assembly

11 Reactions Total

Run Assembly protocol over night

Level 1 Assemblies 17/05

	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O
1	**Name**	**Description**	**TU Type**	**Resistance**	**Backbone**	**Insert Part 1**	**Insert Part 2**	**Insert Part 3**	**Insert Part 4**	**Insert Part 5**					
2	pES1001	NtermRNAP_L3_Ub	A	Amp	pTU1-A-RFP	pES0005	pcr amplified twist 3	pES0002	pES0004	pBP-L3S2P21					
3	pES1002	HsUba1_E1	B	Amp	pTU1-B-RFP	pBP-SJM901	pBP_BBa_B0034	water	twist clonal HsUba	pBP-L3S2P21					
4	pES1004	UBCH5A E2	C	Amp	pTU1-C-RFP	pBP-SJM901	pBP_BBa_B0034	water	pES0007	pBP-L3S2P21					
5	pES1007	EGLN3_L3_CtermRNAP (CGG)	A	Amp	pTU1-A-RFP	pES0005	pcr amplified twist 13	pES0002	pES0008	pBP-L3S2P21					
6	pES1008	EGLN3_L3_CtermRNAP (GAC)	A	Amp	pTU1-A-RFP	pES0005	pcr amplified twist 13	pES0002	pES0009	pBP-L3S2P21					
7	pES1011	EGLN1_L3_CtermRNAP (CGG)	A	Amp	pTU1-A-RFP	pES0005	pES0014	pES0002	pES0008	pBP-L3S2P21					
8	pES1012	EGLN1_L3_CtermRNAP (GAC)	A	Amp	pTU1-A-RFP	pES0005	pES0014	pES0002	pES0009	pBP-L3S2P21					
9	pES1013	asyn_L3_CtermRNAP (CGG)	A	Amp	pTU1-A-RFP	pES0005	pcr amplified twist 15	pES0002	pES0008	pBP-L3S2P21					
10	pES1014	asyn_L3_CtermRNAP (GAC)	A	Amp	pTU1-A-RFP	pES0005	pcr amplified twist 15	pES0002	pES0009	pBP-L3S2P21					
11	pES1015	KLF10_L3_CtermRNAP (CGG)	A	Amp	pTU1-A-RFP	pES0005	pcr amplified twist 16	pES0002	pES0008	pBP-L3S2P21					
12	pES1016	KLF10_L3_CtermRNAP (GAC)	A	Amp	pTU1-A-RFP	pES0005	pcr amplified twist 16	pES0002	pES0009	pBP-L3S2P21					

DISSABTE, 18/5/2024

In Lab: Philip

No colonies on Plated pJin216 (SphI mutated)

-> Running PCR again with subsequent ligation and NdeI digest,

PCR: Q5 Polymerase

PCR (SphI restriction site mutation), 50 ul, 25 cycles

	A	B	C	D	E
1	sample	DNA (ng)	Primers	Tm (°C)	Extension time (s)
2	pJin216 A	5	0001,0002	57	120
3	pJin216 B	5	0001,0002	57	120
4	pJin216 C	5	0001,0002	57	120

NucleoSpin Gel and PCR Clean-up

Concentrations PCR reactions (pJin216 A,B,C)

	A	B	C
1	Sample	Conc (ng/ul)	Comment
2	pJin216 A	217	
3	pJin216 B	164.8	low 230/260
4	pJin216 C	126.2	

DpnI digest

Restriction digest

50 ul reaction, 14 ul DNA (pJin216 A/B/C, everything), 1 ul DpnI, 25 min

20 min 80°C heat inactivate

NucleoSpin Gel and PCR Clean-up

Concentrations DpnI digest reactions (pJin216 A,…

	A	B	C
1	Sample	Conc (ng/ul)	Comment
2	pJin216 A	153.4	
3	pJin216 B	146.3	low 230/260
4	pJin216 C	73.8	

Phoshporylation (T4PKN) + Ligation

T4 Ligation T4 PKN phsophorylation: (https://www.neb.com/en/protocols/0001/01/01/non-radioactive-phosphorylation-with-t4-pnk-or-pnk3-phosphatase-minus)
Added 1 ul T4PKN to reaction before adding T4 Ligase, can work in same raction buffer, see:
https://www.neb.com/en/faqs/2011/08/13/can-i-use-t4-polynucleotide-kinase-and-t4-dna-ligase-in-the-same-reaction-buffer

After 20 min at 37°C, add T4 Ligase, incubate at RT for 20 mins

Heat inactivate at 65°C, 20 min

Transformation: 1ul Ligation product in 10 ul competent bacteria (outgrowth 30 min)
Transformation

DIUMENGE, 19/5/2024

In Lab: Philip

View colonies (6) observed on pJin216 C plate, no colonies on pJin216 A/B plate.
Process seems to be inefficient.

Colonies will be picked, direct **PCR performed to mutate BsmBI** site (primer: 0003,0004)

PCR: Q5 Polymerase

PCR (BsmBI mutation), 50ul reaction

	A	B	C	D	E
1	colony	primers	Annealing temp (°C)	Extension time (min)	Initial denaturation (min)
2	1	0003/0004	57	2	10
3	2	0003/0005	57	2	10
4	3	0003/0006	57	2	10
5	4	0003/0007	57	2	10
6	5	0003/0008	57	2	10
7	6	0003/0009	57	2	10

DpnI digest: Add 6ul Cutsmart to PCR product, 1 ul DpnI, incubate 37°C, 1h

PCR celanup:
NucleoSpin Gel and PCR Clean-up

Concentrations of PCR and DpnI digested plasmi…

	A	B	C
1	Sample	Concentration ng/ul	comments
2	1	22.4	low 230/260
3	2	9.3	low 230/260
4	3	14.7	low 230/260
5	4	23.5	low 230/260
6	5	9.0	low 230/260
7	6	27.3	low 230/260

Phosphorylation with PNK
Add 2ul T4 ligase buffer to extracted DNA, add 1 ul T4 PNK, fill up to 20ul with water. Incubate at 37°C for 30 min
Add 1ul T4 ligase, incubate at RT for 1h

Restriction digest of Twist frament PCR products (EGLN3, a-syn,KLF-10, RNAP N-term fragments)
Restriction digest

NucleoSpin Gel and PCR Clean-up

Concentrations of digest frgaments/pBP:

	A	B	C
1	Sample	Concentration ng/ul	comments
2	Fragment 0003	51.7	low 230/260
3	Fragment 0013	21.9	low 230/260
4	Fragment 0015	50.2	low 230/260
5	Fragment 0016	63.5	low 230/260
6	pBP	28.9	low 230/260

Ligation:

T4 Ligation Vector: Insert ratio: 1:10 (20 fmol vector, 200 fmol insert)

Ligation

	A	B	C
1	reaction	Insert (ul)	vector (ul)
2	Fragment 0003 + pBP	1.4	0.9
3	Fragment 0013 + pBP	4.2	0.9
4	Fragment 0015 + pBP	1.1	0.9
5	Fragment 0016 + pBP	2.9	0.9

Transformation of pBP0003, pBP0013, pBP0015, pBP0016 and pJin216 colony PCR-digested-ligated products

Transformation 1 ul DNA added to 10 ul com bacteria

Plated 50 ul on respective LB-agar plates (Lvl1 + lux on carb, Lvl0 on chlor)

DILLUNS, 20/5/2024

Observing plates:

Colonies on Lux 3/5 plate

PCR on 5 Lux3 (colony 3) colonies and 1 Lux5 (colony 5) colony (Addition of NedI and SphI restrcition sites)

PCR: Q5 Polymerase

PCR on Lux colonies (SphI, BsmBI mutant)

	A	B	C	D
1	Sample	T annealing (°C)	Extension time (min)	
2	Lux3.1	71	1	
3	Lux3.2	71	1	
4	Lux3.3	71	1	
5	Lux3.4	71	1	
6	Lux3.5	71	1	
7	Lux 5.1	71	1	

Digest of Lux PCR prodcuts with DpnI and subsequently NdeI and SphI

Restriction digest

Digest of KanR, CymR, and pTU2-A

PCR purification

NucleoSpin Gel and PCR Clean-up

Concentrations after PCR cleanup

	A	B	C
1	**Sample**	**concentration**	**Comment**
2	pTU2-A	17.6	
3	pTU2-A	28.3	
4	pTU2-A	28.9	
5	KanR	10.3	
6	CymR	14.7	
7	Lux PCR3.1	13.0	
8	Lux PCR3.2	11.0	
9	Lux PCR3.3	32.2	
10	Lux PCR3.4	14.6	
11	Lux PCR3.5	12.0	
12	Lux PCR5.1	13.2	

Ligation T4 Ligation

20 ul reactions for the replacement of resistance on pTU2-A-RFP and the insertion of lux into pBP

pbp: 26 ng (13+1)
lux: 132 ng

pTU2: 28.15 ng (3+6+1)
CymR: 44.54 ng
KanR: 58.27 ng

1-6 lux

7,8 pTU2

Transformation Transformation

25 ul cells used for each transformation

1 hr recovery

cells spun down and all plated

Send Ecoli NightSeq to microsynth for all constructs assembled in the past week (order see mail)

Inocculate pre-culture wit hthe same colonies

DIMARTS, 21/5/2024

Sequencing analysis: updated cloning checklist

nightseq 21.05.xlsx

- most assemblies could be confirmed
- luciferase didn't work: either we order the sequence or we try again (checked the sequences, looks all right)
- pES0015 didn't work (asyn)
- pES0016, pES1016, pES1015 all didn't work (thay all contain KLF10) I double checked the sequence and it looked fine, don't know what the issue is here
- the strategy for assembling pES0020 and pES0021 doesn't seem to work (we tried it twice now)
- pES0019: only partial prooter was inserted (marked with ?) -> reason: Primer that we used to amplify the sequence has 2 binding sites

DIJOUS, 23/5/2024

In Lab: Philip
Setup ON cultures of successful assemblies

DIVENDRES, 24/5/2024

In Lab: Philip
Miniprep of successful assemblies
Qiagen Miniprep

DISSABTE, 25/5/2024

In Lab: Gabriel

ON culture p15A ori plasmids
5 ml cultures from bacterial stabs

qPCR of TadA pace
Cp: around 15 (values in spreadsheet)
Flow rate increased to 2 v/h

Media change for PACE
1 L LB added
Antibiotic concentrations decreased to relieve stress on bacteria (400 ul of stock for carb, spec, chlor. 200 ul for kan)

DIUMENGE, 26/5/2024

In Lab: Gabriel

Miniprep of p15A ori plasmids Qiagen Miniprep

Concentrations

- p15A ori pTU2-A-RFP: 181 ng/ul
- p15A ori pTU2a: 241 ng/ul

qPCR of TadA PACE

Mean Cp of 18.9

Arabinose additione

arabinose usage seems to be going faster than intended

Media change for PACE

1 L of LB kan-spec-carb-chlor + glucose added

DILLUNS, 27/5/2024

in Lab: Noemie and Philip

Luciferase: Q5 Mutagenesis

- Primer 3, 4; Annealing Temp: 57; Elongation Time 3 min PCR: Q5 Polymerase
- Dpni Digest (2h at 37C), in PCR Buffer
- Clean up NucleoSpin Gel and PCR Clean-up
- PNK (30 min at 37 C) in T4 Ligase Buffer
- Split reaction in to 2: 60 fmol Ligation and remaining Reaction (ca. 10x more)
- Ligation: 1 h at RT T4 Ligation
- Split Reaction again: one Part Tranformed into competent cells Transformation , second part stored in the fridge ON (will be BsmBI digested before transformation)

Resistance exchange p15A oir plasmids:

- Digest with BaeGi and SacI (2h at 37C; BaeGI onls has 25% efficiency in rCutSmart)
- Clean up NucleoSpin Gel and PCR Clean-up -> mixed up the two backbones at some point, need to veryfiy which one is which with sequencing!
- Ligation: 15 min at RT T4 Ligation
- Tranformation & plated on LB Agar + Spec plates after 30 min recovery

TadA PACE:

- qPCR: mean Cp of 22.5 -> no stringency adjustment
- Preparation of 5L Bottle of LB

Troubleshooting Cloning:

- Luciferase: Retry Q5 Mutagensis & if this doesn't work, ask Lukas for his
- L1/L2: designed for direct insrt on level 1 -> Level 0 plasmids are not possible!

- PCymR: new Primers designed & ordered

Level 0 Assemlby of asyn and KLF10:

- PCR o ftwist fragments (Primer 32/33, Annealing at 59C, Elongation for 45 s) PCR: Q5 Polymerase
- Clean up NucleoSpin Gel and PCR Clean-up
- Set up Digest for entire PCR Reactions and pBP: Buffer and Water is already added to the Tubes, but we ran out of SphI (is reordered) -> stored in Temp Box at 4 C

DIMARTS, 28/5/2024

in Lab: Philip, Noemie

Luciferase

- Change of strategy: use Luciferase from pLS-194g730 - no need to romve BsmBI site (protein sequence is the same)
- 2 PCR Reactions: to get 1) Luciferase and 2) gIII transcriptionally linked to Luciferase
- Protocol: Ta 61C, Elongation for 2 min, 35 cycles
- clean up of PCR Reaction & stored in fridge ON NucleoSpin Gel and PCR Clean-up

Table1

	A	B	C	D	E
1	PCR Reaction	Template	fwd Primer	rev Primer	
2	giii-lux	pLS-194g730	o009	o016	
3	lux	pLS-194g730	o015	o016	

PCymR EcoFlex Assembly

- Assembly of lvl 1 construct with the following components: pTU1-A-RFP, pCymRC, PPa_B0034, pES0005, L3S2P21
- Level 1 Assembly, 15 Cycles
- Transformation

TadA PACE:

- Media exhcange
- qPCR: Mean Cp 21.73
- sent Phage Sample for NightSeq (Primer: oLS670)
- Arabinose is empty again - decided to leave it like it is until Sasha fixes it

SP plasmids:

- Golden gate assembly of SP plasmids
- Insert: SIAH1/2 Backbone: SP231.1
- SapI used

Liquid overnight cultures:

- 2 colonies per plate from Resistant exhcange 27/05
- Pick more promoters, RBS, Terminators from EcoFLex Cryostock: SJM911, J23100, pET-RBS, TL1, TL12, TL4

DIMECRES, 29/5/2024

in Lab: Jakob, Noemie

Miniprep of p15A resistance exchange plasmids and EcoFlex Promoter plasmids Qiagen Miniprep

Concentrations

- pBP - TL1: 243 ng/µl
- pBP - TL4: 324 g/µl
- pBP - TL12: 264 ng/µl
- pBP - SJM911: 258 ng/µl
- pBP - pET - RBS: 170 ng/µl
- pBP - J23100: 369 ng/µl

- p15A TU2 1a - 160 ng/µl
- p15A TU2 1b - 173 ng/µl
- p15A TU2 2a - 132 ng/µl
- p15A TU2 2b - 108 ng/µl

Concentrations adjusted to 100 ng/µl

Level 1 Assemblies of various constructs EcoFlex Assembly

- Luciferase and giii-lux from PCR products from 05/28
- Various split RNAP Linker combination lvl1 construct
- Made 6 Master Mixes (see colours)
- Ran assembly for 15 cycles, then transformed (quick protocol with no recovery) Transformation

Level 1 Construct Assembly 05/28

	A	B	C	D	E	F	G	H	I	J	K	L
1	pES1001	NtermRNAP_L3_Ub	A	Amp	pTU1-A-RFP	pES0005	pES0003	pES0002	pES0004	pBP-L3S2P21		
2	pES1017	NtermRNAP_L1_Ub	A	Amp	pTU1-A-RFP	pES0005	pES0003	Linker 1	pES0004	pBP-L3S2P21		
3	pES1015	KLF10_L3_CtermRNAP (CGG)	A	Amp	pTU1-A-RFP	pES0005	KLF10 PCR from fragment	pES0002	pES0008	pBP-L3S2P21		
4	pES1016	KLF10_L3_CtermRNAP (GAC)	A	Amp	pTU1-A-RFP	pES0005	KLF10 PCR from fragment	pES0002	pES0009	pBP-L3S2P21		
5	pES1018	p70_EGLN3_L2_CtermRNAP (CGG)	A	Amp	pTU1-A-RFP	pES0005	pES0013	Linker 2	pES0008	pBP-L3S2P21		
6	pES1019	p70_EGLN1_L2_CtermRNAP (CGG)	A	Amp	pTU1-A-RFP	pES0005	pES0014	Linker 2	pES0008	pBP-L3S2P21		
7	pES1020	p70_asyn_L2_CtermRNAP(CGG)	A	Amp	pTU1-A-RFP	pES0005	asyn PCR from fragment	Linker 2	pES0008	pBP-L3S2P21		
8	pES1021	p70_KLF10_L2_CtermRNAP (CGG)	A	Amp	pTU1-A-RFP	pES0005	KLF10 PCR from fragment	Linker 2	pES0008	pBP-L3S2P21		
9	pES1022	p70_EGLN3_L4_CtermRNAP (CGG)	A	Amp	pTU1-A-RFP	pES0005	pES0013	pES0022	pES0008	pBP-L3S2P21		
10	pES1023	p70_EGLN1_L4_CtermRNAP (CGG)	A	Amp	pTU1-A-RFP	pES0005	pES0014	pES0022	pES0008	pBP-L3S2P21		
11	pES1024	p70_asyn_L4_CtermRNAP(CGG)	A	Amp	pTU1-A-RFP	pES0005	asyn PCR from fragment	pES0022	pES0008	pBP-L3S2P21		
12	pES1025	p70_KLF10_L4_CtermRNAP (CGG)	A	Amp	pTU1-A-RFP	pES0005	KLF10 PCR from fragment	pES0022	pES0008	pBP-L3S2P21		
13	pES1009	Luciferase (CGG)	B	Amp	pTU1-B-RFP	pES0017	pBP_BBa_B0034	-	lux PCR	pBP-L3S2P21		
14	pES1010	Luciferase (GAC)	B	Amp	pTU1-B-RFP	pES0018	pBP_BBa_B0034	-	luxPCR	pBP-L3S2P21		
15	pES1028	Luciferase (CGG) D Part	D	Amp	pTU1-D-RFP	pES0017	pBP_BBa_B0034	-	lux PCR	pBP-L3S2P21		
16	pES1026	gIII-lux (D Part)	D	Amp	pTU1-D-RFP	pES0017	pBP_BBa_B0034	-	gIII lux PCR	pBP-L3S2P21		
17	pES1027	gIII-lux (B part)	B	Amp	pTU1-B-RFP	pES0017	pBP_BBa_B0034	-	gIII lux PCR	pBP-L3S2P21		

TadA PACE :

- NightSeq from 28/05 failed (probably concentration was too low) -> try again when Cp is < 15
- (B4-6) lagoon Cp: 18.31, 14.98, 26.64
- (C4-5) chemostat Mean Cp: 18.48, 21.08, 22.08 -> probably phage contamination of chemostat => reset PACE tomorrow

Liquid overnight cultures + NightSeq:

- SIAH1 SP, SIAH2 SP, pCym Lvl1

DIJOUS, 30/5/2024

in Lab: Philip, Jakob

TadA PACE:

- Phage titer qPCR:
  Phage titer dropped to 2.96E7 -> turbidostat showed biofilm formation and signs of phage contamination
- completely new setup of tubridostat and lagoon. New tubing installed.

- Phage were reinocculated: 0.3 mL (25.5), 0.4 mL (26.5), 0.2 mL (29.5) -> total phage titer: 2.374E8 pfu/mL, 6.1 mL of LB (with antibiotics added)
- PCB died while setting up. PCB will be replaced and Controller calibrated tomorrow. Bacteria and Phage vial on 4°C
- Prepared 3x2L LB

Miniprep of SIAH plasmids and pCym lvl1 Qiagen Miniprep

Concentrations

- SIAH1 - SP cl1: 308 ng/µl
- SIAH1 - SP cl2: 378 ng/µl
- SIAH2 - SP cl1: 151 ng/µl
- SIAH2 - SP cl2: 161 ng/µl
- pCym lvl1 cl1: 182 ng/µl
- pCym lvl1 cl2: 505 ng/µl

Concentrations adjusted to 100 ng/µl

Sequencing

Seqeuncing of TU2 backbones from 28.05.24

E. coli night seq of transformations from 29.5.24

Liquid overnight cultures

of transformations from 29.05.24

Transformation

pES1010, pES1016, pES1020, pES1028

DIVENDRES, 31/5/2024

in Lab: Philip, Jakob

PCR of pSBO cymR:

50 ul reaction

primers: o012, o037v2

Elongation time: 20s

Annealing temp: 60°C

PCR: Q5 Polymerase

NucleoSpin Gel and PCR Clean-up

Concentration: 140.7

Sequencing Analysis

- lvl1 plasmids
  - all asyn and KLF10 assemblies are wrong, some others as well
- Resistance Exchange
  - SmR is sucessfully inserted?

- can't see from sequencing which is p15A-TU2-a SmR and which is p15A-TU2-A SmR --> Sequencing again with primer 017
- pTU2_2a (KanR+CymR) is finished

Liquid overnight cultures

pES1010, pES1016, pES1020, pES1028

Sequencing

Seqeuncing of TU2 backbones from 28.05.24 with primer 017

CymR PCR product, primer 037

Miniprep of lvl1 plasmids Qiagen Miniprep

Concentrations

- pES1001 cl. B: 252 ng/µl
- pES1009 cl. C: 146 ng/µl
- pES1018 cl. B: 378 ng/µl
- pES1019 cl. B: 348 ng/µl
- pES1022 cl. A: 277 ng/µl
- pES1023 cl. B: 436 ng/µl
- pES1026 cl A: 133 ng/µl
- pES1026 cl C: 139 ng/µl --> kept
- pES1027 cl A: 292 ng/µl --> kept
- pES1027 cl B: 159 ng/µl
- pES1027 cl C: 241 ng/µl

Concentrations adjusted to 100 ng/µl

TadA PACE:

Restarted with new reactor

Lab Journal - June

Project: iDEC 2024
Author: Gabriel Cervera
Entry Created On: 30 Apr 2024 08:37:30 UTC
Entry Last Modified: 03 Oct 2024 12:50:31 UTC
Export Generated On: 08 Oct 2024 09:23:21 UTC

DISSABTE, 1/6/2024

in Lab: Gabriel, Jakob

Sequencing Analysis

- p15a TU2 2a SmR = p15a TU2 A SmR
- p15a TU2 1a SmR = p15a TU2 a SmR

Miniprep of lvl1 plasmids

Qiagen Miniprep

Concentrations

- pES1017: 273 ng/µl
- pES1022 cl. B: 285 ng/µl
- pES1010 cl. A: 232 ng/µl
- pES1010 cl. B: 201 ng/µl
- pES1020 cl. A: 436 ng/µl
- pES1020 cl. B: 376 ng/µl
- pES1020 cl C: 463 ng/µl
- pES1028 cl A: 282 ng/µl
- pES1028 cl B: 342 ng/µl
- pES1028 cl C: 290 ng/µl

Concentrations adjusted to 100 ng/µl

PCR of g3-negative

50 ul reaction
primers: o009, o010
Elongation time: 20s
Annealing temp: 60°C

PCR: Q5 Polymerase

NucleoSpin Gel and PCR Clean-up

Level 1 Construct Assembly - missing lvl1 + negative promoter + CymR + Lvl 2 constructs

	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O
1	pES1010	Luciferase (GAC)	B	Amp	pTU1-B-RFP	pES0018	pBP_BBa_B0034	-	luxPCR	pBP-L3S2P21					
2	pES1018	p70_EGLN3_L2_CtermRNAP (CGG)	A	Amp	pTU1-A-RFP	pES0005	pES0013	Linker 2	pES0008	pBP-L3S2P21					
3	pES1019	p70_EGLN1_L2_CtermRNAP (CGG)	A	Amp	pTU1-A-RFP	pES0005	pES0014	Linker 2	pES0008	pBP-L3S2P21					
4	pES1020	p70_asyn_L2_CtermRNAP(CGG)	A	Amp	pTU1-A-RFP	pES0005	asyn PCR from fragment	Linker 2	pES0008	pBP-L3S2P21					
5	pES1024	p70_asyn_L4_CtermRNAP(CGG)	A	Amp	pTU1-A-RFP	pES0005	asyn PCR from fragment	pES0022	pES0008	pBP-L3S2P21					
6	pES1028	Luciferase (CGG) D Part	D	Amp	pTU1-D-RFP	pES0017	pBP_BBa_B0034	-	lux PCR	pBP-L3S2P21					
7	pES1033	p70_EGLN3_L4_CtermRNAP (GAC)	A	Amp	pTU1-A-RFP	pES0005	pES0013	pES0022	pES0009	pBP-L3S2P21					
8	pES1047	CymR_EGLN3_L4_CtermRNAP(CGG)	A	Amp	pTU1-A-RFP	CymR + RBS	pES0013	pES0022	pES0008	pBP-L3S2P21					
9	pES1055	CymR_EGLN3_L4_CtermRNAP(GAC)	A	Amp	pTU1-A-RFP	CymR + RBS	pES0013	pES0022	pES0009	pBP-L3S2P21					
10	pES1059	CymR_EGLN3_L3_CtermRNAP (CGG)	A	Amp	pTU1-A-RFP	CymR + RBS	pES0013	pES0002	pES0008	pBP-L3S2P21					
11	pES1060	CymR_EGLN3_L3_CtermRNAP (GAC)	A	Amp	pTU1-A-RFP	CymR + RBS	pES0013	pES0002	pES0009	pBP-L3S2P21					
12	pES1034	p70_EGLN1_L4_CtermRNAP (GAC)	A	Amp	pTU1-A-RFP	pES0005	pES0014	pES0022	pES0009	pBP-L3S2P21					
13	pES1037	CymR_EGLN1_L3_CtermRNAP (CGG)	A	Amp	pTU1-A-RFP	CymR + RBS	pES0014	pES0002	pES0008	pBP-L3S2P21					
14	pES1038	CymR_EGLN1_L3_CtermRNAP (GAC)	A	Amp	pTU1-A-RFP	CymR + RBS	pES0014	pES0002	pES0009	pBP-L3S2P21					
15	pES1048	CymR_EGLN1_L4_CtermRNAP(CGG)	A	Amp	pTU1-A-RFP	CymR + RBS	pES0014	pES0022	pES0008	pBP-L3S2P21					
16	pES1056	CymR_EGLN1_L4_CtermRNAP(GAC)	A	Amp	pTU1-A-RFP	CymR + RBS	pES0014	pES0022	pES0009	pBP-L3S2P21					
17	pES2003	AP2pos	SmR	pTU2-a p15a SmR	pES1007	pTU1-B Dummy									
18	pES2005	AP2pos (luciferase)	SmR	pTU2-a p15a SmR	pES1007	pES1009									
19	pES2007	AP1 (HsUba_1) (for luciferase)	KanR (+CymRam)	pTU2-A-RFP	pES1001	pES1002	pES1004	pTU1-D Dummy							

DIUMENGE, 2/6/2024

in Lab: Philip, Jakob

Miniprep of lvl1 plasmids Qiagen Miniprep

Concentrations

- SIAH2 SP cl 3: 241 ng/µl
- SIAH2 SP cl 4: 288 ng/µl
- SIAH2 SP cl 5: 311 ng/µl

Concentrations adjusted to 100 ng/µl

--> Samples prepared for sequencing

PCR of gIII-lux and Luciferase:

primer: o009, o016 (gIII-lux)

primer: o015, o016 (luciferase)

template: pLS194g730

annealing temp: 60°C

elongation time 1:30 min

Oligo annealing - Linker 2

50 µL o022 and 50 µL o023 combined

Primer concentration: 100 µM, Total reaction volume: 100 uL

Heat at 94 °C for 2 minutes, turn off heat block, leave them in heat block until cooled back down to room temperature

Concentration: 1069 ng/µL --> adjusted to 100 ng/µL

Lvl 1 assembly - Linker 2

	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O
1	pES1018	p70_EGLN3_L2_CtermRNAP (CGG)	A	Amp	pTU1-A-RFP	pES0005	pES0013	Linker 2	pES0008	pBP-L3S2P21					
2	pES1029	p70_EGLN3_L2_CtermRNAP (GAC)	A	Amp	pTU1-A-RFP	pES0005	pES0013	Linker 2	pES0009	pBP-L3S2P21					
3	pES1043	CymR_EGLN3_L2_CtermRNAP(CGG)	A	Amp	pTU1-A-RFP	CymR + RBS	pES0013	Linker 2	pES0008	pBP-L3S2P21					
4	pES1051	CymR_EGLN3_L2_CtermRNAP(GAC)	A	Amp	pTU1-A-RFP	CymR + RBS	pES0013	Linker 2	pES0009	pBP-L3S2P21					
5	pES1019	p70_EGLN1_L2_CtermRNAP (CGG)	A	Amp	pTU1-A-RFP	pES0005	pES0014	Linker 2	pES0008	pBP-L3S2P21					
6	pES1030	p70_EGLN1_L2_CtermRNAP (GAC)	A	Amp	pTU1-A-RFP	pES0005	pES0014	Linker 2	pES0009	pBP-L3S2P21					
7	pES1044	CymR_EGLN1_L2_CtermRNAP(CGG)	A	Amp	pTU1-A-RFP	CymR + RBS	pES0014	Linker 2	pES0008	pBP-L3S2P21					
8	pES1052	CymR_EGLN1_L2_CtermRNAP(GAC)	A	Amp	pTU1-A-RFP	CymR + RBS	pES0014	Linker 2	pES0009	pBP-L3S2P21					

Gel extraction

50 µL from PCR run on gel, cut and purrified

NucleoSpin Gel and PCR Clean-up --> 20 µL

Concentration:

upper band: 32 ng/µL

upper band: 38 ng/µL

--> prepared for sequencing

Transformation

pES1010, pES1020, pES1024, pES1028, pES1033, pES1034, pES1037, pES1038, pES1047, pES1048, pES1055, pES1056, pES1059, pES1060

pES2003, pES2005, pES2007

DILLUNS, 3/6/2024

in Lab: Noemie, Jakob

Transformation

pES1018, pES1019, pES1029, pES1030, pES1043, pES1044, pES1051, pES1052

Sequencing

pES1010, pES1024, pES1028, pES1033, pES1034, pES1037, pES1038, pES1047, pES1048, pES1055, pES1056, pES1059, pES1060
pES2003, pES2005, pES2007
(for pES2003 and pES2005 the plates were overgrown - for A and B of each a tiny colony was picked, for C many colonies were added, new plates were then made to obtain single cultures)
Twist orders of: SIAH2, KLF10, ASYN
! the plate containing the twist fragment was not properly sealed. There was frozen liquid on top of the plate and some wells (including asyn) were empty. PCR product of the amplified sequence was sent for sequencing instead.

Preparation for Autoclaving
3x 0.5L LB-Agar
PACE tubing and small parts

Preparation PACE How to PACE

- Prepare Tubing, Media, Adaptors etc. for autoclaving
- Inocculated some media with bacteria & grew for approx. 8 h

Phage Production Day 1: GG Phage Production

- donor Plasmid: SP-SIAH 1 (I used plasmid prep 1)

DIMARTS, 4/6/2024

In Lab: Philip, Noemie

Spec - plates
overgrew again - maybe antibiotic is no good

PCR cleanup gIII-lux, lux PCR from 2.6.24
Concentrations:
gIII-lux: 141.3
lux: 177.2

Level 0 Assemblies:

- lux, gIII-lux, asyn, KLF10, PcymR
- digest with Sph1 and Nde1
- PCR Clean up
- Ligation: 1 h at RT -> stored in fridge ON

Sequencing Analysis

- SIAH2 twist: Probably twist messed up the synthesis, aligment shows DNA mixture
- KLF10 & Asyn: both sequences are correct, but the got switched up at some point in the lab (either when being sent for sequencing of before that).
- gIII-neg: sequencing failed - either concetration was too low (needs to be 40 ng/uL), or PCR failed
- SIAH2 SP: one of the 3 tested colonies has the entire SIAH2, but has a Ser -> Arg mutation at position 46
- pES1010: A ok, B has A75S mutation

- pES1024: A, B ok; C sequencing broke off after 300 bp
- pES1028: A, B, C ok
- pES1033: A, B wrong, C mostly ok but has a Linker 3 instead of Linker 4.
- pES1034: looks ok, but sequencing doesn't cover linker reagion, might have the same issues as pES1033
- pES1037: sequencing failed
- pES1038: plasmid mix, very low quality
- pES1047: wrong promoter (has p70 instead of CymR;), Linker 3 instead of Linker 4
- pES1048: there was no file for this sequence
- pES1055: wrong promoter (p70 instead of CymR), contains Linker 3 instead of Linker 4
- pES1056: Sequencing failed
- pES1059: wrong promoter region: has p70, not cymR
- pES1060: A has p70 promoter and not CymR; B, C have HsUbai instead of EGLN1
- pES2003: sequencing failed
- pES2005: C might be ok (partially covered), A, B are wrong
- pES2007: A mixture; B and C first TU looks ok, but second TU is Dummy TU1 -B (wrong Dummy was used - should have been Dummy TU1-D)

-> Probably there is Linker 3 in pES0022 instead of Linker 4 -> probably got messed up during Miniprepping, bcs sequence was confirmed with sequenceing
-> even though PCR product of pCymR seemed to be correct, the assemblies did not contain the correct fragment
-> there are overall many wrong sequences (probably pipetted wrong or tubes with the wrong label)

Miniprep of correct Assemblies (marked in green)

Send for Sequencing:

- Transformants from 03.06 sent for NightSeq (no growth for: 1030, only red colonies for 1052, only one colony for 1019, 1044)
- Also: Economy Run from pES0002, pES0022, to ensure that the tubes that we are storing are labeled correcty

Phage production Day 2

- Transformation according to Phage Production

TadA PACE

set up chemostat & let it run ON for bacteria to reach appropriate OD

DIMECRES, 5/6/2024

in Lab: Philip, Noemie

Plated 5 spec plates (check whether spec antibiotic is not good)

Transformation of Ligation reaction from 4.6.24, pES0002 (little left)

Transformation

negative (untransformed) plated on new and old spec plates

Sequencing analysis:

- Sequence results from pES0002 and pES0022 look good. Tubes seem to be labelled correctly
- pES1018: both have the wrong linker (3 instead of 2)
- pES1019: sequencing failed
- pES1029: both have the wrong linker (3 instead of 2) (alings with pES1007, which we already have)
- pES1043: wrong promoter, wrong linker (3 statt 2) (alings with pES1007, which we already have)
- pES1044: all components are not what they should be (align perfectly with pES1001)
- pES1051: wrong promoter, wrong linker (3 statt 2)

TadA PACE:

- inocculate Lagoon: mixture from different phage samples from previous evolution -> together the have Cp=25
  150 uL from 25.5.
  100 uL from 28.5.
  3 uL from 22.5.
- restarted PACE at 11:20
- reactor broke down in the afternon, restarted again an 8 pm
- Took waste sample shortly after lagoon was connected (mean cP: 27.23) could be contamination, though samples was not directly taken from turbidostat. Samples will be taken directly from wasteline of turbidostat in the next view days

Phage production Day 3

- first Plaque Assay Phage Production
- sent phages to sequencing (NightSeq)

Golden Gate Assemblies: only pES2008 and pES2009 were assembled due to lack of pCymR (cloned into lvl0 at the moment)

EcoFlex Assembly

BsmBI enzyme, high efficiency (35 cycle) protocoll (components listed in table bellow)

GG Assemblies (lvl 1 and lvl 2)

Lvl2 assemblies

	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O
1	pES2008	AP1 (for plaque assay)	KanR (+CymRam)	pTU2-A-RFP KanR CymRAM	pES1001	pES1002	pES1004	pES1026	-						
2	pES2009	AP1 (for plaque assay)	KanR (+CymRam)	pTU2-A-RFP KanR CymRAM	pES1001	pES1003	pES1004	pES1026	-						

DIJOUS, 6/6/2024

in Lab: Philip, Jakob

Transformation of lvl2 assemblies from 05.06.24 and pES0002

Transformation

lvl2 assmelbies plated, pES0002 set up as ON culture

Sequencing

- SIAH2 SP clone 4 again sent for sequencing
- pES0002, pES0012, pES0015, pES0016, pES0019, pES0027, pES2005

Liquid overnight cultures

- pES0002 (from old miniprep-stock)
- picked cultures of pES0002, pES0012, pES0015, pES0016, pES0019, pES0027, pES2005

Phage production: Determine phage titer

- SIAH1 phages: 4.75 *10^7 pfu/mL
- pos. ctrl: 8.25 *10^8 pfu/mL
- Sequencing: SIAH1 is ok, not sure about which backbone -> ask Lukas next week

Setup of User interface for PACE with Graziano

DIVENDRES, 7/6/2024

In Lab: Jakob, Philip

TadA Pace

- PACE UI protocol written
- Flow rate increased to 2 v/h
- P3c culture innoculated in new turbidostat, put in fridge after 6 hours

Phage production

- S2208 ON inocculated by Lukas, Tube in fridge, might need to grow a bit more
- 3 phage plaques picked --> liquiid ON culture

Miniprep of lvl0 and lvl2 plasmids Qiagen Miniprep

Concentrations
pES0002: 172 ng/µL
pES0002 reserve 1: 178 ng/µL
pES0002 reserve 2: 167 ng/µL
pES0015: 180 ng/µL
pES0019: 255 ng/µL
pES2005: 239 ng/µL
Concentrations adjusted to 100 ng/µl

Sequencing and ON cultures

pES2008 --> many cultures were red
only red cultures for pES2009 --> retransformed

Sequencing analysis

Sequencing worked this time on full Siah2 gene (inculding N-term). Mutation in S46 (AGC) to AGA (would be Arg). This was allready observed in previous sequencing run

Table3

	A	B	C
1	pES0002 A	ok	
2	pES0002 B	ok	
3	pES0002 C	ok	
4	pES0012 A	wrong	sequencing started very late (primer?)
5	pES0012 B	wrong	
6	pES0012 C	wrong	
7	pES0015 A	wrong	
8	pES0015 B	wrong	
9	pES0015 C	wrong	
10	pES0016 A	wrong	
11	pES0016 B	ok	is actually pES0015 (asyn)
12	pES0016 C	ok	is actually pES0015 (asyn)
13	pES0019 A	ok	insert before promoter
14	pES0019 B	wrong	
15	pES0019 C	nothing	
16	pES0027 A	wrong	
17	pES0027 B	wrong	
18	pES0027 C	wrong	
19	pES2005 A	ok	linker has silent mutations
20	pES2005 B	ok	linker has silent mutations
21	pES2005 C	ok	linker has silent mutations

--> KLF10 and asyn have been swapped a long time ago

old KLF10 plasmids were checked again --> pES1021 from 29.05. was actually correct pES1020 --> plate put into incubator for picking

Lvl 1 assemblies

	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O
1	pES1018	p70_EGLN3_L2_CtermRNAP (CGG)	A	Amp	pTU1-A-RFP	pES0005	pES0013	Linker 2	pES0008	pBP-L3S2P21					
2	pES1019	p70_EGLN1_L2_CtermRNAP (CGG)	A	Amp	pTU1-A-RFP	pES0005	pES0014	Linker 2	pES0008	pBP-L3S2P21					
3	pES1043	CymR_EGLN3_L2_CtermRNAP(CGG)	A	Amp	pTU1-A-RFP	pCymR PCR	pES0013	Linker 2	pES0008	pBP-L3S2P21					
4	pES1044	CymR_EGLN1_L2_CtermRNAP(CGG)	A	Amp	pTU1-A-RFP	pCymR PCR	pES0014	Linker 2	pES0008	pBP-L3S2P21					
5	pES1047	CymR_EGLN3_L4_CtermRNAP(CGG)	A	Amp	pTU1-A-RFP	pCymR PCR	pES0013	pES0022	pES0008	pBP-L3S2P21					
6	pES1048	CymR_EGLN1_L4_CtermRNAP(CGG)	A	Amp	pTU1-A-RFP	pCymR PCR	pES0014	pES0022	pES0008	pBP-L3S2P21					
7	pES1061	P70_NtermRNAP_L1_Ub	A	Amp	pTU1-A-RFP	pES0005	pES0003	pES0020	pES0004	pBP-L3S2P21					
8	pES1062	PCym_NtermRNAP_L1_Ub	A	Amp	pTU1-A-RFP	pCymR PCR	pES0003	pES0020	pES0004	pBP-L3S2P21					
9	pES1063	pCymR_NtermRNAP_L3_Ub	A	Amp	pTU1-A-RFP	pCymR PCR	pES0003	pES0002	pES0004	pBP-L3S2P21					

DISSABTE, 8/6/2024

In lab: Jakob, Pau, Noemie

TadA Pace

qPCR - Average Cp: 19.97, 7.5*10^8 PFU

- Reminder: primers are diluted 1:5, account for that when preparing master mix

Turbidostat Cp: 25.91 --> constant

Liquid overnight cultures

pES1020, pES2009

Plaque Assay

1 plate Noemie, low S2208 concentration -> let grow S2208 some more & retry

2 plates, each with all 3 clones (picked on 07.06.) and positive control

Miniprep of pES2008 cl. B Qiagen Miniprep --> stored in lvl 2 box, sequence yet to be checked

Concentration - pES2008: 369 ng/µL

Concentrations adjusted to 100 ng/µl

Transformation of lvl1 assemblies from 07.06.24

Transformation

pES1018, pES1019, pES1043, pES1044, pES1047, pES1048, pES1061, pES1062, pES1063

DIUMENGE, 9/6/2024

- [x] Put P3c turbidostat back in incubator
- [x] miniprep pES1020 and pES2009, send for sequencing
- [x] Double-check Jakobs Primer Design (fix them) and order primers (in Jakob Test folder) --> Gabriel
- [x] pick lvl1 assemblies (ON cultures and sequencing - or leave open until monday, since they won't be sequenced before then anyways) --> postponed to Monday

- [x] Keep PACE alive + qPCR, swap turbidostat out for P3c culture
- [x] Change fresh medium PACE

In lab: Gabriel, Pau

TedA PACE

qPCR - Mean Cp: 25.09 - $2.16 \cdot 10^7$ pfu/mL
Host strain changed for P3C culture (new sterile needles used).
Fresh medium bottle changed. Previous bottle is left next to it to fill the current bottle when half empty.

Miniprep of pES1020 an pES2009 Qiagen Miniprep

3 colonies of each miniprepped and sent for sequencing
Concentrations (ng/ul)
pES1020a: 140
pES1020b: 263
pES1020c: 399
pES2009a: 227
pES2009b: 201
pES2009c: 345

Lvl1 assembly check

Plates with > 3 white colonies: pES1018, pES1043, pES1047
Plates with 1-2 white colonies, no white colonies, or colonies too small to tell: pES1019, pES1044, pES1048, pES1061, pES1062, pES1063

We decide to leave all plates in the incubator ON and tomorrow do picking + sequencing in multi-well plate (n = 27).

Protocol update suggestion Transformation

When using few competent cells (e.g., 20 uL) or transforming problematic plasmids, add 200-500 uL of LB after heat-shock and centrifuge before plating to make sure you plate all cells. This will increase the probability of some transformed cell with the right plasmid.

Plaque assay plates

Stored in 4ºC.

DILLUNS, 10/6/2024

- [x] Analyse sequencing results of pES2008 N
- [x] Look at plaque assay plates to determine phage titer + Full plasmid seq for phages
- [x] Pick lvl1 assemblies + sequencing
- [x] Keep PACE alive + qPCR
- [x] Change waste bottles PACE
- [x] Primer design SIAH2
- [x] PCRs 11, 12, 27 + clean u

GG 2008

In lab: Noemie, Pau

Idea: do not erase to-do list, insted leave it to see what was done and maybe also what was not.

TedA PACE

qPCR --> Mean Cp: 26.05 - $1.11 \cdot 10^7$ pfu/mL
Chemostat at Cp 26.4.
Waste bottles changed for new clean ones.
Reduced FLow rate to 1 v/h
Arabinose flow is veery low (close to none)

Plaque Assay SIAH1 clones 1-3

S2208 lawn was not dense enough to count plaques -> needs to be repeated to get exact titer
estimated phage titer:

- pos. ctrl (SIAH1 from 6.6): few colonies at -3 -> approx. 2*10^6 pfu/mL
- clone 1: around 20-50? single plaques at -6 -> approx: 5*10^9 pfu/mL
- clone 2: around 20-50? single plaques at -6 -> approx: 5*10^9 pfu/mL
- clone 3: around 20? single plaques at -7 -> approx: 5*10^10 pfu/mL

Lvl1 assembly check

Plates have big colonies and small colonies around them. --> probably satellite colonies.

- only red colonies for: pES1062

Colonies picked for sequencing and incubated in 96w plate 37ºC ON.

- Picked colonies: pES1018a-c, pES1019a-b, pES1043a-c, pES1044a-c, pES1047a-c, pES1048a-c, pES1061a-b, pES1063a-c, pES2008a
- pES1062 was not picked nor sent to sequencing

Order placed in Microsynth and plate deposited. We send additional 200 uL of 1:10 o017 primer (miliQ diluted)
Plates with colonies deposited in 4ºC.

PCR: gIII-neg PCR: Q5 Polymerase

- Template: p231.1 (should be pDB016?)
- Primers: 009, 010
- Conditions: Ta 61C and elongation for 30 s
- Purified reaction

Sequencing Results

- pES2008: A matches pES1002, B matches pES1001 -> failed

Repeat GG for pES2008

- changed assembly protocol, becasue apperently BsmBI-v2 is active at higher temperatures (NEBridge Golden Gate Assembly Kit (BsmBI-v2) | NEB)
- (42°C, 1 min → 16°C,1 min) x 30 → 60°C, 5 min

- Transformed and plated after 1 h recovery

DIMARTS, 11/6/2024

In lab: Noemie, Jakob

- [x] Keep PACE alive + qPCR (incl culture from glycerol stock to check for phage contamination in the glycerol stock)
- [x] Sequencing Analyse WE
- [x] Inoculate lvl1 assemblies based on sequencing results --> miniprep on wednesday
- [x] buy stickers for tubes to make labeling more clear (especially for ecoflex plasmids)
- [x] Agarose Gel: Assembly GG lvl 2 (run PCR first, extension 40-50s/kb) and PCR products
- [x] send pTU2-A-RFP KanR CymR for sequencing -> check overhangs of RE
- [x] retransformation of 2008 on new Kan-plates

TadA PACE
qPCR --> Mean Cp: 19.58
Chemostat --> Mean Cp: 19.61
--> probably added too much primer, disregard results
innoculated new P3c chemostat, also innoculated P3a-g to check for phage contamination in glycerol stock

Sequencing Analysis

- pES1020: a, c -> correct, b has one mutation
- pES2009: does not look ok, seems to be missing 2nd insert, aligns perfectly to 1001 => check resistance of plate
- pES2008: sequencing failed
- SIAH1 phage clones: clone 2 and 3 are correct, clone 1 is wrong
- pES1018: wrong linker
- pES1019: possibly wrong linker? not sequenced far enough
- pES1043: a and c wrong linker, promoter wrong (b completely wrong)
- pES1044: a and c promoter correct, linker possibly wrong (b completely wrong)
- pES1047: a and b correct (c wrong linker and promoter)
- pES1048: c correct (b failed, a has mutations, can't see linker)
- pES1061: b is correct but linker has many silent mutations (a completely wrong)
- pES1063: a and b are correct but have insert before promoter (c failed)

--> pBP-L3S2P21, pES0008, pES0013 or pTU1-A-RFP might contain Linker 3 (pES0002) and Ps70 (pES0005), sent one of each for sequencing and performed PCR with o024 and o025 (Linker 3 primers)

PCR PCR: Q5 Polymerase

- Templates: pBP-L3S2P21, pES0008, pES0013 or pTU1-A-RFP
- Primers: o024, o025
- Conditions: Ta 72C and elongation for 60 s

ON cultures
1019a+b, 1044a+c, 1047a+b, 1048c, 1061b, 1063a
S2208

pES2008 assembly --> no cultures

- retransformation of ligation reaction with new Kan plate to make sure plate is not the issue

Lvl 2 troubleshooting:

- PCR amplification of ligation reactions 2008 and 2009 with primers 019 and 016 to amplify lvl2 inserts to check of there are correct assemblies and transformation efficiency is the issue => run PCR reaction on gel (see below)
- sequencing of Tu2-A-RFP KanR CymRam to chekc for correct GG cloning sites (rule it out as a reason why lvl 2 assemblies are not working)
- sequencing of 2005 with primer 018 to check if both insert are there

Agarose Gelelectrophoresis

- 1% Agarose Gel, ran at 120 V for 60 min
- PCR products of lvl 2 insert amplification
- PCR products of gII neg, gIII-lux, lux, asyn, KLF10
- Marker: GeneRuler 1 kb
- Loading scheme:
  M2 - - - - - - - - M4 (gIIIneg) - (gIIIlux) - (lux) - (aSyn) - (KLF10) - (2008) - (2009) - (2008 10.06) - M2

IMG_4949.jpg

DIMECRES, 12/6/2024

In lab: Noemie, Jakob

- [x] sequencing analysis 2005 reverse and lvl2 backbone
- [x] qPCR of PACE samples, glycerol stocks, and LB
- [x] Make 2x2L LB for PACE and 1x0.5L LB-Agar
- [x] E-Mail Twist about SIAH2
- [x] make glycerol stock of S2208 -> stored at -80, in a blue box where also the ecoflec kit is
- [x] SIAH2 SDM PCR + KLD + Transformation
- [x] Box mitnäh

- [x] run gel to check for linker3 contamination
- [x] Miniprep Lvl1 assemblies
- [x] repeat plaque assay for SIAH1 clones 2-3
- [x] decide what to do with KLF10 -> reorder? try to find in the twist plate? use an old PCR product? --> might not be super important at the moment, as we have many more promising substrates

PCR PCR: Q5 Polymerase

- Templates (10 ng): pES0002
- Primers: o024, o025
- Conditions: Ta 72C and elongation for 60 s

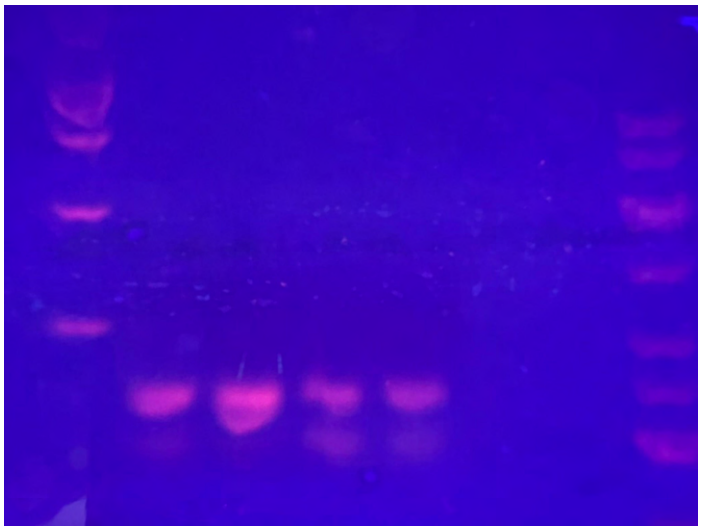
Sequenzing analysis

- pES2005 reverse - sequencing failed
- pBP-L3S2P21, pES0008, pES0013 all look fine, does not seem to be plasmid mixes
- pTU1-A-RFP - sequencing failed

Agarose Gelelectrophoresis

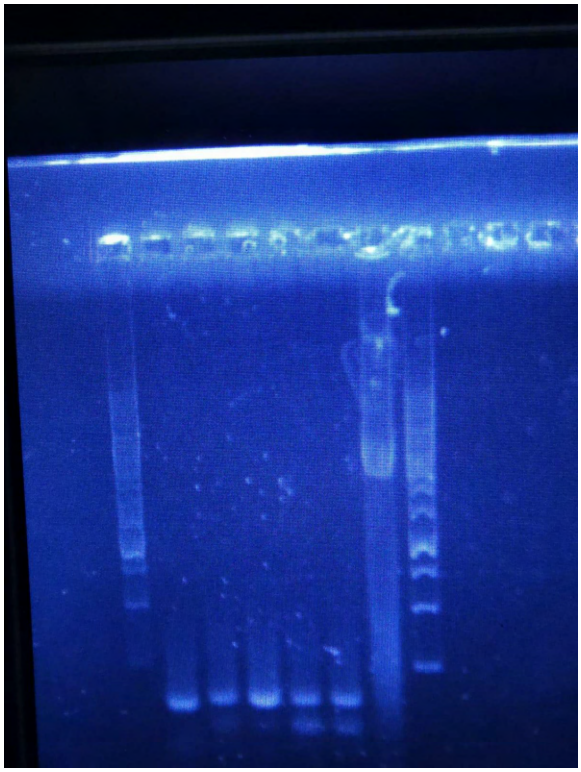
- 1% Agarose Gel, ran at 120 V for 60 min
- pBP-L3S2P21, pES0008, pES0013 or pTU1-A-RFP - linker 3 amplification
- Marker: GeneRuler 1 kb
- Loading scheme:
  M - pES0008 - pES0013 - pTU1-A-RFP - pBP-L3S2P21

5B359E6A-33AB-4851-B7C7-A1338A2CEE41_1_105_c.jpeg



- Repeated with pES0002 as positiv control (rest same as above)
  Loading scheme: M, pES0002, pES0008, pES0013, pTU1-A-RFP, pBP-L3S2P2, SIAH2SP-SDM

WhatsApp Bild 2024-06-12 um 16.30.44_5ffabf48.jpg



TadA PACE

qPCR --> Mean Cp: 25.53

Chemostat --> Mean Cp: 26.01

P3a-h, LB --> Cp ~26

--> rerun by Sasha --> all Cp ~27.7

Miniprep of lvl1 plasmids Qiagen Miniprep

pES1019a: 513 ng/µL

pES1019b: 556 ng/µL

pES1044a: 171 ng/µL

pES1044c: 348 ng/µL

pES1047a: 311 ng/µL

pES1047b: 606 ng/µL

pES1048c: 173 ng/µL

pES1061b: 310 ng/µL

pES1063a: 206 ng/µL

Concentrations adjusted to 100 ng/µl

SIAH2-SP SDM

1. Q5 Mutagenesis PCR: Q5 Polymerase

    - Templates (10 ng) : SIAH2-SP cl. 4
    - with GC-Enhnacer (bcs Primer bind in a very GC rich region)
    - Primers: o039, o040
    - Conditions: Ta 72C and elongation for 2 min
    - PCR seemed to have worked (see gel image above)
2. KLD Treatment: 1 uL PCR reaction, 5 uL KLD Buffer, 1 uL KLD Enzyme Mix, 3 uL Water, icubated at RT for 5 min

Plaque Assay

2 plates, positive control + SIAH1 clone 2 and 3

1 plate of P3a-h + positive control

Transformation Transformation

- SIAH2-SP SDM, pES2008 Ligation reaction from 10.06
- used 50 uL of competent cell + 5 uL Ligation/KLD reaction
- Recovery: 1 h in LB
- plated 1/10 and 9/10 on seperat plates

DIJOUS, 13/6/2024

- [x] determine phage titer siah1 clones 2&3
- [x] linker 3 PCR with water and with 1019, 1044, 1048
- [x] Test kanamycin plates
- [x] look at new 2008 plate and asses what to do next
    - [x] try lvl2 assemblies with new protocol
- [x] check SIAH2SP SDM plate -> pick colonies

In lab: Philip, Jakob

Plaque Assay Analysis

- P3a-h are all clean
- cl.2 - 26 plaques in -7 --> 9*10^10 pfu/mL
- cl. 3 - 29 plaques in -7 --> 10^11 pfu/mL

Sequencing

3 colonies from SIAH2-SP SDM plate picked and sent for Night-Seq

Kanamycin Plate Test

3 old Kan plates and 3 new Kan Plates

one with pES2008 (positive control), one with untransformed cells (negative control) and one with SIAH2-SP SDM (resistance check)

Linker 3 Troubleshooting

- Templates (10 ng): MiliQ H20 (new and old), Ultra Pure H2O, pES1019, pES1044, pES1048

- Primers: o024, o025
- Conditions: Ta 70°C and elongation for 1 min
- Loading Scheme: M, pES1019, pES1044, pES1048, MiliQ old, Ultra Pure H20, MiliQ new

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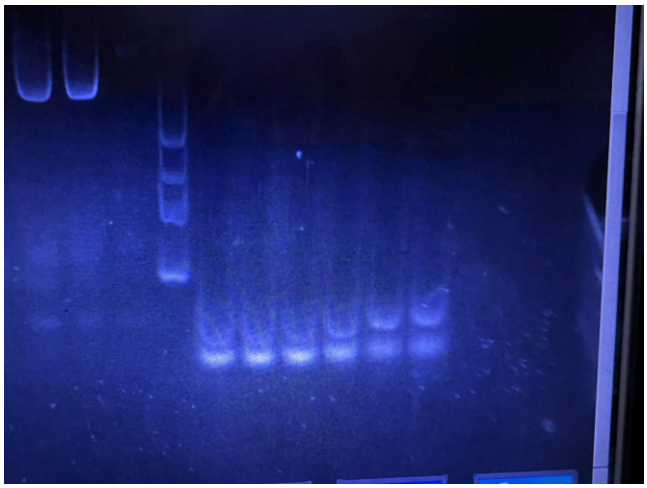
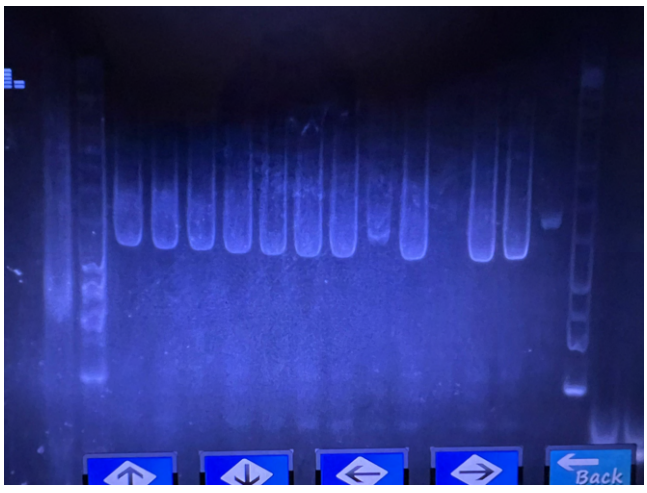


Colony PCR pES2008

13 colonies from pES2008 picked and ran PCR

- Primers: o018, o019
- 25 cycles
- Conditions: Ta 62°C and elongation for 5 min
- Loading Scheme: M, colonies 1-13

A11E3A9D-6868-4AAF-8597-FE4CC53D3575_1_102_o.jpeg



Level 2 assembly

EcoFlex Assembly --> transformed

Table2

	A	B	C	D	E	F	G	H
1	pES2003	AP2pos	SmR	pTU2-a p15a SmR	pES1007	pTU1-B Dummy		
2	pES2009	AP1 (for plaque assay)	KanR (+CymRam)	pTU2-A-RFP KanR CymRAM	pES1001	pES1003	pES1004	pES1026
3	pES2010	AP1 (for plaque assay), inducible	KanR (+CymRam)	pTU2-A-RFP KanR CymRAM	pES1063	pES1002	pES1004	pES1026
4	pES2011	AP1 (for plaque assay), inducible	KanR (+CymRam)	pTU2-A-RFP KanR CymRAM	pES1063	pES1003	pES1004	pES1026

DIVENDRES, 14/6/2024

- [x] check sequencing results of SIAH2 SP SDM
  - [x] If correct --> miniprep, assemble with SplitC - Split D and transform
- [x] Check Kanamycin plate test
- [x] colony PCR of lvl 2 assemblies (+ one from empty backbone)
- [x] run everything on gels
- [x] find out where the fuck linker3 is in
- [x] qPCR dilution curve for positive control

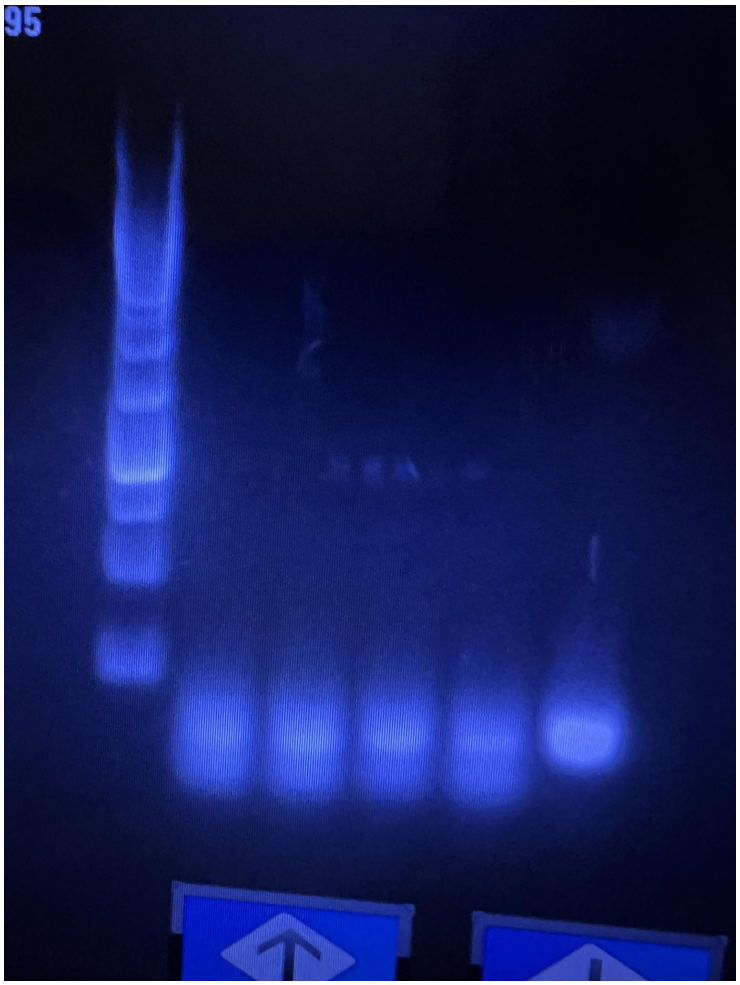
In Lab: Philip Jakob

Kanamycin plates are alright
All water was alright
Cleaned Bench and Pipettes with DNA Away

Linker 3 Troubleshooting

- MiliQ H20, Ultra Pure H2O,pES1001
- Primers: o024, o025 --> old and new each
- Conditions: Ta 70°C and elongation for 1 min
- Loading Scheme: M - old primers/MiliQ - new primers/MiliQ - old primers/new ultra pure - new primers/ultra pure - pES1001 (ultra pure)

92EF9045-A924-4959-B6A3-28783D28AD82.jpg



Analysis, water negative controls reveal only primers in gel

pES1001, only 024 binds, thats probably why only one primer band, upper very faint band at around 3 kb which is expected with single primer annealing and 1 min elongation

Colony PCR pES2003, pES2008, pES2009, pES2010, pES2011

20 colonies picked and ran PCR (1-5 pES2003, 6-7 pES2008, 8-12 pES2009, 13-16 pES2011, 17-20 pES2010)

- 21 = pTU2-A-RFP control
- Primers: o018, o019
- 35 cycles
- Conditions: Ta 62°C and elongation for 5 min
- Loading Scheme: M, colonies 1-21

PCR: Q5 Polymerase

Gelelectrophoresis analysis

IMG_6062.jpg

- pES2003 insert expected size at 3kb, which is the case in gel

- pES2008 inser expected size 8650, 2 could be something (runs too low though)
- pES2009 insert expected size 8600, 2 and 3 look good
- pES2011 1 could be something
- pES2010 2 and 3 could be something

picking:
pES2003 (1,2), pES2008 (7), pES2009 (9,10), pES2011 (13), pES2010 (18,19)
sending for sequencing

Sequencing Analysis

- pTU2-A-RFP looks good, has one insertion and one bacterial terminator missing

PCR positive control (linker 3)
template: 1001
Primer: 018,024
anealing temp: 62°C
Extension time: 30s
PCR: Q5 Polymerase --> see gel of colony PCR (left of ladder)

- also for pBP-L3S2P21, pES0008, pES0013 or pTU1-A-RFP, pES0002 (positive control)
- from 12.06. pES1019 cl. A and B, pES1044 cl. A and C, pES1048
- Loading Scheme: pES0002, pES0008, pES0013, pTU1-A-RFP, pBP-L3SP21, pES1019 A, B, pES1044 A, C, pES1048

BB688DD5-0DC7-4314-8B6E-61F988660E0A.heic

--> still nothing makes sense!

Sequencing results Siah2 SP
A and B look good
A and B were miniprepped
Qiagen Miniprep

Phage assembly - Day 1
Siah2 SP A was used as Donor plasmid
Phage Production

DISSABTE, 15/6/2024

TO do:

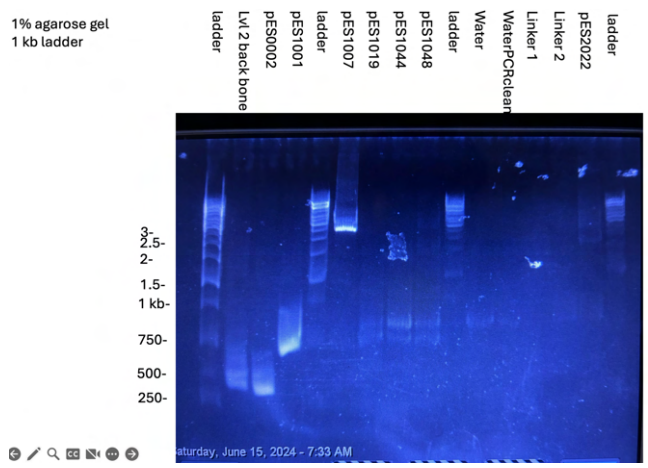
- [ ] qPCR dilution series with replicates
- [ ] Phage production
- [ ] Prepare PACE tubing for autoclaving - restart PACE on monday
- [ ] colony PCR of lvl1s that keep being wrong
- [ ] Make s2208 competent cells (someday next week))

In Lab: Philip

PCR troubleshooting PCR: Q5 Polymerase

PCR of pES0002, pEs1001, pES1007, pES1019, pES1044, pES1048, pES0022,Linker1, Linker 2 (primer 024,018) check whether bands implicate contamination
LVL 2 backbone (019,018) as followup from yesterday
Annealing temp: 60, 61, 62 (ask philip for specifics)
Elongation time: 1.5 min, 35 cycles
loaded on gel, run for 55 min

image.png



Analysis:
LVL 2 backbone expected amplified fragment: 1331 bp, most likely forgot to add DNA template, seen band is probably contamination (not sure)
Positive controls:
pES0002 expected fragment: 331bp -> correct
pES1001 expected fragment: 529 bp -> correct
pES1007 expected fragment: 2413 bp -> correct
Samples contaminated?
pES1019 expected fragment: X -> same bands seen as in negativ control -> probably nothing
pES1044 expected fragment: X -> same bands seen as in negativ control -> probably nothing
pES1048 expected fragment: X -> same bands seen as in negativ control -> probably nothing
Negative controls
Water: two faint bands allready observed in the past view days -> probably nothing
Water PCR cleanup: bands a bit less intense
Linker1: nothing (not contaminated)
Linker2: nothing (not contaminated)
pES2022: very faint, could be single stranded amplification products because primer 018 is alligning
Comments:
Ladders seem to have different intensities, same amount was loaded, could ba that left side of the gel is imaged with higher intensity, observed this allready in previous gels

Tested samples should not be contaminated with linker 3 , positive controls worked well, will be used to test for contamitions or succesful assemblies in the future

DIUMENGE, 16/6/2024

- [ ] Phage production
- [ ] qPCR dilution series (triplicates)

In Lab: Philip, Gabriel

Phage production day 2

Phage Production

S2208 cells precultured for plating tomorrow
Transformation of phage plasmid assembly

qPCR quality control

Ask Sasha for formula for CP

DILLUNS, 17/6/2024

In Lab: Noemie, Philip

- [x] Sequencing results -> either prep or start new assembly
- [x] 2007 is 2008 in sequencing data

Sequencing Analysis

- pES2003: 1 and 2 both look ok, but sequencing only partially covers first TU (we can not differentiate btw lvl 1 and 2 backbone, as we have had issues with this previously)
- pES2007: 8 is ok, though the promoter of HsUba1 is not the one in the bechling assembly (SJM910)
- pES2009: 9, 10 both look ok (sequencing coverage to beginning of second TU)
- pES2010: 18, 19 both look ok (sequencing coverage to beginning of second TU)
- pES2011: 13 looks ok (sequencing coverage to beginning of second TU)

Miniprep of correct assemblies Qiagen Miniprep

Send for sequencing:

- Siah2 phage pool nightseq
- full plasmid seq of SIAH1 clone 2, plasmids 2008, 2009, 2010, 2011,

Phage production day 3

Phage Production

plaque assay: positive control is siah1 cl.2

Comptetent Cells:

Set up ON culture of S2208

DIMARTS, 18/6/2024

in Lab: Philip, Noemie

ToDos:

- [x] Restart PACE -
- [x] qPCR analysis - redo
- [x] Sequencing analysis
- [x] make S2208 competent cells: start ON culture and ask for protocol: Mix&Go
- [x] Repreat Plaque assay for SIAH2 pool with new competent cells: Transformation

Plaque Assay:

- positive ctrl looks ok, pfu matches previous plaque assay
- siah2: no plaques -> competent cells might not have been so competent after all -> retry phage production with new competent cells
- also NightSeq od SIAH2 pool failed (makes sence when no phage replpication occured)
- Repeated transformation with new competent cells and ligation reaction from 14.06

Chemically competent S2208 cells:

- follow the Mix&Go protocol, but scaled down 10x
- Stored 2 aliquotes on our Box, the rest are in pricillas box

Restart TadA PACE

- Started new experimet
- P3b, Arabinose:40 , FLowrate 0.5 v/h
- seeded lagoon: to reach approx Cp 20

qPCR QC:

- run phage dilution curve (known titer) qPCR in Triplicates
- 1:5 dilutions from stock, inluding negative control only LB
- phage: P227, 6.8*10^10 pfu/mL
- Results: high background fluoresence (only clear signai until approx. Cp 20)
- Repeat qPCR with water sample with and without Primers tomorrow

Table4

	A	B	C	D	E	F
1		pfu/mL	Cp 1	Cp 2	Cp 3	Mean Cp
2	0	6.80e+10	7.41	7.38	7.24	7.34333333
3	1	1.36e+10	9.85	9.97	10.04	9.95333333
4	2	2.72e+9	12.07	11.93	12.04	12.0133333
5	3	5.44e+8	14.52	14.53	14.55	14.5333333
6	4	1.09e+8	16.92	16.96	17.34	17.0733333
7	5	2.18e+7	19.34	19.44	19.48	19.42
8	6	4.35e+6	21.54	21.91	21.95	21.8
9	7	8.70e+5	22.98	24.17	24.24	23.7966667
10	8	1.74e+5	23.2	25.53	25.68	24.8033333
11	9	3.48e+4	22.98	27.3	27.6	25.96
12	10	6.96e+3	23.18	28.53	27.62	26.4433333
13	11	0	23.15	18.25	29.18	23.5266667

DIMECRES, 19/6/2024

- [x] qPCR with water only and no primers (1: only water, 2: water and primers)
- [x] plaque assay SIAH2 (phage production day 2)
- [x] PACE: check medium, arabinose, qPCR
- [x] Sequencing Analysis Full Plasmid Seq levels 2
- [ ] Prepare update meeting
- [x] double check that CymRAM is part of the TU2-RFP KanR CymR backbone -> send for sequecning + PCR with agarose gel
- [x] check point mutation on luxAB
- [x] check point mutations on C term RNAP

in Lab: Noemie, Philip

Phage Production:

- Plaque assay of SIAH2 pool
- positive control: SIAH1 cl.2

TadA PACE: qPCR

Table5

	A	B	C	D	E
1		Cp 1	Cp 2	Cp 3	Mean Cp
2	Lagoon	12.97	13.31	13.16	13.1466666667
3	Chemostat	26.98	28.03	27.97	27.66
4	Firepol + Water + Primer	29.69	29.95	29.90	29.8466666667
5	Firepol + Water	-	-	-	-
6	Water	-	-	-	-
7	LB	-	-	-	-

Sequencing analysis: Full Plasmid Seq

- siah1 cl 2: no sequence, concentration was too low or quality not good enough -> FullPlasmid Seq does not work for sending phages!
- pES2008: length does not match - wrong plasmid
  - has an additional ori + amp resistance (on top of the ones that should be there)
  - Backbone (KanR + CymRAM) looks ok
  - TU1: RNAP N term ok
  - TU2: HsUBi1 ok
  - is missing TU 3 (UBCH5A) and TU4(gIII lux)
- pES2009: not sure if ok
  - backbone is missing the CymRAM - not needed for this plasmid, bcs none of the components have pCymR promoter -> but we need to check the backbone
  - luxAB E141D point mutation if lux gene: check if this is also present in previous sanger sequencing runs
- pES2010: not ok
  - backbone is missing the CymRAM, but this is needed as some promoters are pCymR
  - first TU is missing part of the promoter
  - E141D point mutation on lux gene
- pES2011:
  - backbone is missing the CymRAM, but this is needed as some promoters are pCymR
  - luxAB E141D point mutation on lux gene
- pES2003: not sure if ok
  - a bunch of point mutations in CGG RNAP-C, leading to AA changes (564 - 578) - check from where this issue comes
  - has an additional spacer in the backbone and a few point mutations on the backbone (not an issue, probably wrong sequence on addgene)

TU2-RFP KanR CymR: was checked with Sanger Sequencing on 31.05 and then CymRAM was part of the backbone. Also was CymRAM part of pES2008 - maybe mixture? 1. send for sequencing, 2. amplify and check on gel

luxAB: pES1026 was used to assemble all the constructs with the observed point mutations, there the sanger run was not long enough to cover this locations -> check this plasmid with sanger sequecning with different sequencing primer; this part comes from Lukass plasmid - ha did not observe this mutation on the fullplasmid seq of his plasmid

RNAP-C CGG: sequencing of pES1007 and pES0008 never covered this region -> send both to sequecning with reversed primer

Sent for sequencing: plasmid/primer

- TU2-A-RFP KanR CymRAM / 038
- pES1026 / 015
- pES1007 / 018
- pES0008 / 018

DIJOUS, 20/6/2024

in Lab: Noemie

Plaque assay:

- no SIAH2 phages: either cells are not competent of assembly failed
- check if cells are competent and repeat assembly

Sequencing results:

- TU2-A-RFP KanR CymRAM: CymRAM is present, does not seem to be a plasmid mixture?
- pES1026: Mutation observed in the level 2s is already part of this lvl 1 - error probably occured earlier
- pES1007: mutations observed in level 2s are already here
- pES0008: mutations observed in level 2s are already here

The mutations found in RNAP CGG is actually the WT RNAP (wich binds to the GAC promoter) -> probalby the two C termial RNAPs got switched up at some point -> check the the wt RNAP and if this is the case, switch back!

Transformations

- transformattion of TU2-A-RFP KanR CymRAM into our S2208 competent cells to check for transformation efficiency
- retransformation of pES0002, pES0019, pES0022, pES0008, pES0009, pES0013, pBPL3S2P2, TU1-A-RFP, TU2-A-RFP KanR CymRAM

qPCR: PACE

- ran qPCR also with Pricillas Primers, values are a bit higher, but not 35

Table6

	A	B	C	D	E	F	G	H
1		Primer	Cp 1	Cp 2	Cp 3	Mean Cp		
2	Lagoon	oLS1662/oLS1663	16.29	16.34	16.11	16.2466666667		
3	Chemostat	oLS1662/oLS1663	27.18	27.00	26.86	27.0133333333		
4	Firepol + Water + Primer	oLS1662/oLS1663	29.26	28.95	28.78	28.9966666667		
5	Lagoon	PW M13 fw&r	18.17	18.07	18.51	18.25		
6	Chemostat	PW M13 fw&r	30.42	29.68	28.26	29.4533333333		
7	Firepol + Water + Primer	PW M13 fw&r	31.28	30.85	30.86	30.9966666667		

Send for sequencing:

- pLS194g730: check if LuxA mutation is already on PLasmid we got or if it got intriduced during PCR
- pES0009: to verfiy the switchup between the two RNAP variants

PCR + Clean up

- of lux and gIII-lux, as described on 28.05
- of gIII-neg, as described on 10.06
- purified and stored on temp box (labelled with date!)

Repicked & performed colony PCR on remaining white colonies

- only for pES2009 and pES2010 white colonies were visible
- picked all of them (tot: 8) and performed 2 PCR reactions on each
    - with 018/019, Ta 62, 3 min elongation: amplify insert => shoud be 8.5 kb
    - with 038/001, Ta 68, 1 min 30 s: amplify backbone => should be 3.8 kb
- Ladder: GeneRuler 1kb, ran at 120 V for 45 min

Loading Scheme: (L) - (5 Variants of pES2009, loaded first I then B for each) - (3 Variants of pES2010, loaded first I then B for each)

WhatsApp Bild 2024-06-20 um 18.16.36_5337ec32.jpg

DIVENDRES, 21/6/2024

- [x] Are S2208 competent cells actually competent? Check plate
- [ ] set up GG assembly for SIAH2 with new SplitC SplitD plasmids
- [x] Troubleshoot Arabinose: keep PACE running or turn it off
- [ ] PACE qPCR: evtl. try a new batch of Firepol
- [x] pick single colonies of Transformants: either send for sequencing of colony PCR to check for correct plasmids
- [ ] check sequencing: verfiy mixup of the two RNAP variants, check lor luxA mutation in Lukas' Plasmid
- [x] Digestion Ligation of lux, gIII-lux, gII-neg for level 0 plasmids, consider sending PCR reaction for sequencing
- [x] Miniprep the two pES2010 clones, send them for sequencing with 038 AND 018 (single tube with 2 lables)
- [ ] plan assays for testing logic: luciferase, plaque assay, qPCR
- [ ] OD600 meausurement calibration

in Lab: Philip

Digestion of pBP, gIII-lux, lux, gIII-neg (PCR products from 20.6)

Restriction digest

Ligation:

T4 Ligation

Transformation + outgrowth:

Transformation

plated

Plate observations (plated on 20.6)

All plated bac grew

Fiew colonies even on chlor with nothing plated out (could be contaminated):

IMG_6087.jpg

S2208 seem to be competent, colonies grew, though not red -> picked two colonies and send for sequencing:
They are starting to turn red a couple of ours later now

IMG_6086.jpg

From the rest of the plates, 2 colonies were picked and send for E-coli night seq on plate

2 Liquid cultures of pES2010 send for night seq on same plate with each primer 18 and 38 (4 seperate wells)
(Done to fill night seq plate, the lab did not have enough night seq tubes)

Plate alyout (colonies were incoulated on 96-well plate (37°C) in same scheme)

image.png

	1	2	3
A	pBP L3S2P21 1 o017	pES0008_1 o017	pES2010_1_38 o038
B	pBP L3S2P21 2 o017	pES0008_2 o017	pES2010_1_18 o018
C	pBP L3S2P21 3 o017	pES0009_1 o017	pES2010_2_38 o038
D	pBP L3S2P21 4 o017	pES0009_2 o017	pES2010_2_18 o018
E	pES0002_1 o017	pES0013_1 o017	S2208_1 o019
F	pES0002_2 o017	pES0013_2 o017	S2208_2 o019
G	pES0019_1 o017	pES0022_1 o017	TU1-RFP_1 o017
H	pES0019_2 o017	pES0022_2 o017	TU1-RFP_2 o017

a

PACE
Arabinose started working, medium changed. Might be appropriate to change to P3c tmrw (or to 1v/h).
Samples taken and qPCR, new primers arrived, tested new and old primers (same old firepol aliquot)
Results:
Ran qPCR with old and new primers without phage, still got signal, **Fire Pol is likely the problem**. **I put new firepol at minus 20 frige next to new primers**. Old fire pol is still on bench (marked with cross) in case cou want to test.
Using new fire Pol and primers should resolve the problem as Lukas did validation with both

DISSABTE, 22/6/2024

- [x] Take 96 well plate and agar plates out of 37°C incuabtor
- [x] pick colonies from level 0 plates: colony PCR, start ON cultures
- [x] Keep PACEING (changed flow rate to 1v/h), qPCR
- [x] check sequencing: verfiy mixup of the two RNAP variants, check lor luxA mutation in Lukas' Plasmid
- [x] Check if arabinose is flowing (I marked the syringe level with tape, flow should be 3.36 mL/24h at 1 v/h))
- [x] sterile 2M Glucose solution

- [x] check RNAP which sequence is wich and correct all plasmids. -> change tube labelling and excell sheet
- [x] order more nightseq tubes (and full plasmid seq?)
- [x] level 1 for lucifeare is pcr was correct this time

in Lab: Gabriel, Noemie

Sequencing Analysis:

- pES0008 and pES0009 are indeed switched up
- luxA mutation is not from Lukas plasmid, got introduced during PCR => check on the level 0 plasmids

changed the labelling of the tubes and the excell sheet and the cloning spreadsheet: now every tube should contain what is specified on the google spreadsheet/cloning checklist

TadA PACE

- qPCR: Lagoon Cp is 16.05, Chemostat is 28.7, Water is 29.7 => used the new Firepol aliquit + new Primer aliquotes
- increased flow rate to 2 v/h
- arabinose is still running
- prepared 1L of 2M Glucose solution

Level 0 Assemblies for gIII-lux, lux, gIII-neg

- picked 8 colonies per plate
- ran colony PCR: Primers 017&018, Ta 59 C, Elongation 1 min 40 s
- Run gel: 120V 30 min
- prepare ON cultures of colonies with correct insert length

DIUMENGE, 23/6/2024

- [ ] set up GG assembly for SIAH2 with new SplitC SplitD plasmids
- [x] Keep PACEING (changed flow rate to 2 v/h), qPCR, check if arabinose is running
- [x] grow P3c from glycerol stock
- [ ] OD600 meausurement calibration
- [ ] plan assays for testing logic: luciferase, plaque assay, qPCR
- [x] Miniprep gIII-lux (pES0027), lux (pES0012), gIII-neg (pES0011) and send for sequencing

In lab: Gabriel, Pau

TadA PACE

- qPCR: Lagoon Cp is 17.68, Chemostat is 35.6, water is around 36 (new Firepol and new primers seem ok)
- Maintain flow rate to 2 v/h
- Arabinose still running
- Changed fresh medium bottle

Miniprep of Level 0 Assemblies

gIII-neg a (pES0011) --> 206.2 ng/ul
gIII-neg b (pES0011) --> 184.4 ng/ul
gIII-lux a (pES0027) --> 187.5 ng/ul
gIII-lux b (pES0027) --> 108.1 ng/ul
lux a (pES0012) --> 143.7 ng/ul (260/230 of 1.63)
lux b (pES0012) --> 185.5 ng/ul
lux c (pES0012) --> 216.9 ng/ul
lux d (pES0012) --> 213.9 ng/ul

all 260/230 and 260/280 ratios ok (unless indicated).
Sent for sequencing.
Not diluted to 100 ng/ul --> waiting for seq results.

DILLUNS, 24/6/2024

- [x] check NightSeq results from 21.06 and setup ON culutres if they are correct
- [ ] OD600 meausurement calibration
- [x] Keep PACEING (change to P3c, qPCR, arabinose)
- [x] set up GG assembly for SIAH2 with new SplitC SplitD plasmids

in Lab: Noemie

TadA PACE:

- qPCR
- prepared & attached new media (2L)
- changed strain to P3c
- reduced flow rate to 0.5 v/h
- arabinose is still running (syringe at 9 mL at 15:00)

NightSeq Results from 21.06:

- pBPL3S2P21: 1-4 all look fine => inocculate cl 1 (Well A1)
- pES0002: 1-2 both look fine => inocculate cl 1 (Well E1)
- pES0019: 1-2 both look fine (insert infront of the primer is a remainder of the cloning strategy) => inocculate cl 1 (Well G1)
- pES0008: 1-2 both look fine, but region where the WT and CGG variants differ is not covered => I assume these were taken before we confirmed the mixup & switched the labelling => inocculated cl 1 (Well A2) as pES0009
- pES0009: 1-2 both look fine, but region where the WT and CGG variants differ is not covered => I assume these were taken before we confirmed the mixup & switched the labelling => inoculated cl 1 (Well C2) as pES0008
- pES0013: 1-2 both look fine, but quality is better on 1 => inoculated cl 1 (Well E2)
- pES0022: 1-2 both look fine, but quality is better on 1 => inoculated cl 1 (Well G2)
- pES2010: 1-2 both have the last TU insert, cl 1 is missing CymRAM, backbone sequencing failed for cl 2 => both are wrong, did not inoculate ON cultures from them

- TU1-A-RFP: sequencing failed for 1, 2 seems to be a mixture (evtl. 1 and 2 got mixed) => restreak on a fresh Carb plate (Well H3 and G3) and pick again tomorrow
- TU2-A-RFP KanR CymRAM: problematic region not covered, pick 3 again to resend for sequecning (nightseq) & inoculated ON culutre direclty

SIAH2 phage production Day 1 Phage Production

- started GG assembly overnight
- freshly prepped SplitC and SplitD plasmids from Priscilla
- donor plasmid: SIAH2-SP Mut

Sequencing of Level 0 from 23.06. :

- added 2 additional reactions to ensure entire luciferase domain is covered

DIMARTS, 25/6/2024

- [x] Take ON cultures from the shaker and Miniprep (7x) -> replace the constructs in the box
- [x] check sequencing from TU2-A-RFP KanR CymRAM, prep one of the colones if correct (I picked 3, are in the shaker)
- [x] check Sequencing of level 0s -> if they look ok, construct lvl 1 s of lux/gIII-lux with WT T7 promoter and also with a constitutive promoter (we now only have gIII-lux and lux with the CGG promoter => as we now have all our constructs with the WT RNAP, instead of redoing all the Sub-RNAP constructs, we decided to switch the promoter insfromt of the gIII bzw luciferase. The constitutive lux is needed for testing our assays)
- [x] a few other lvl 1 s are also still missing (marked in blue on cloning spreadsheet) -> run reaction together with the ones mentioned in the previous point, with the newly prepped parts
- [x] TadA PACE: refill arabinose, qPCR, ...
- [x] Take plate with TU1-A-RFP out of the incubator, pick colonies for sequencing
- [x] SIAH2 phage production Day 2 (Assembly is cylcer slot 2)

in Lab: Philip

Sequencing analysis

Table7

	A	B	C
1	Sample	Sequencing	Comment
2	gIII-lux o017 a	no	
3	gIII-lux o017 b	no	Wierd, smth alligns
4	gIIIneg a	no	failed
5	gIIIneg b	no	no insert
6	lux a	yes	
7	lux b	yes	a couple of mutations
8	lux c	yes	best
9	lux d	yes	
10	gIII-lux o018 a	no	no inser
11	gIII-lux o018 b	no	no insert
12	TU2-A-RFP KanR CymRAM a	yes	all look good, small insertion in front of J23119 Promotor for all of the three
13	TU2-A-RFP KanR CymRAM b	yes	
14	TU2-A-RFP KanR CymRAM c	yes	

Miniprep (ON cultures)

Qiagen Miniprep

Picking TU1-A-RFP not possible because overgrown

picked from a fiew loci and reinocculated, plated out again.

PACE refilled arabinose

Make sure stepper motor is not screwed on too tightly and that no air gets in front of the filter when disconnecting the syringe.
qPCR

Phage production day 2

Phage Production

Pre-innoculated S2280 for ca 1h from cryostock (in fridge)

LVL1 assembly (on PCR machine slot 4)

pES0009/10 were reassmebled with the Lux that was assembled on 21.6 (because of mutated lux)

Other LVL1 that were still open

Table8

	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O
1	pES1018	p70_EGLN3_L2_CtermRNAP (GAC)	A	Amp	pTU1-A-RFP	pES0005	pES0013	Linker 2	pES0008	pBP-L3S2P21					
2	pES1019	p70_EGLN1_L2_CtermRNAP (GAC)	A	Amp	pTU1-A-RFP	pES0005	pES0014	Linker 2	pES0008	pBP-L3S2P21					
3	pES1043	CymR_EGLN3_L2_CtermRNAP(GAC)	A	Amp	pTU1-A-RFP	pES0019	pES0013	Linker 2	pES0008	pBP-L3S2P21					
4	pES1044	CymR_EGLN1_L2_CtermRNAP(GAC)	A	Amp	pTU1-A-RFP	pES0019	pES0014	Linker 2	pES0008	pBP-L3S2P21					
5	pES1045	CymR_asyn_L2_CtermRNAP (GAC)	A	Amp	pTU1-A-RFP	pES0019	pES0015	Linker 2	pES0008	pBP-L3S2P21					
6	pES1048	CymR_EGLN1_L4_CtermRNAP(GAC)	A	Amp	pTU1-A-RFP	pES0019	pES0014	pES0022	pES0008	pBP-L3S2P21					
7	pES1049	CymR_asyn_L4_CtermRNAP (GAC)	A	Amp	pTU1-A-RFP	pES0019	pES0015	pES0022	pES0008	pBP-L3S2P21					
8	pES1062	PCym_NtermRNAP_L1_Ub	A	Amp	pTU1-A-RFP	pES0019	pES0003	pES0020	pES0004	pBP-L3S2P21					
9	pES1009	Luciferase (CGG) B Part	B	Amp	pTU1-B-RFP	pES0017	pBP_BBa_B0034	-	pES0012	pBP-L3S2P21	ok	ok	ok	ok	ok
10	pES1010	Luciferase (GAC)	B	Amp	pTU1-B-RFP	pES0018	pBP_BBa_B0034	-	pES0012	pBP-L3S2P21	ok	ok	ok	ok	
11															
12															
13															

DIMECRES, 26/6/2024

- [x] Pick from newly streaked out TU1-A-RFP plate
- [x] Keep PACEing: qPCR; exchange madia bottle
- [x] level 1 assemblies: transformation
- [x] level 1 assembly: gIII-lux with WT promoter (18)
- [x] prepare fresh LB
- [x] cast gel, run PCR products
- [x] Reclone gIIIneg, gIIILux (troubleshoot sequencing)
- [x] Phage production day 3 (I pre-inoculated S2208 (ca 1h) from cryostock yesterday, they are in our fridge)

in Lab: Noemie

Level 1 assemblies: remaining gIII-lux with WT T7 promoter (from PCR product) and lux as both D and B parts

- ran GG protocol for 15 cycles

Table9

	A	B	C	D	E	F	G	H	I	J
1	pES1064	gIII-lux (D Part, CAG promoter)	D	Amp	pTU1-D-RFP	pES0018	pBP_BBa_B0034	-	pcr giiilux	pBP-L3S2P21
2	pES1065	gIII-lux (B part, CAG promoter)	B	Amp	pTU1-B-RFP	pES0018	pBP_BBa_B0034	-	pcr giiilux	pBP-L3S2P21
3	pES1066	Luciferase (CAG) D Part	D	Amp	pTU1-D-RFP	pES0018	pBP_BBa_B0034	-	pES0012	pBP-L3S2P21
4	pES1026	gIII-lux (D Part)	D	Amp	pTU1-D-RFP	pES0017	pBP_BBa_B0034	-	pcr giiilux	pBP-L3S2P21
5	pES1027	gIII-lux (B part)	B	Amp	pTU1-B-RFP	pES0017	pBP_BBa_B0034	-	pcr giiilux	pBP-L3S2P21

Transformation of the GG assemblies from yesterday and today

TadA PACE:

- exchanged chemostate waste bottle
- Mean Cp values: Lagoon 23.77, Chemostat 29.58, Water 33.97
- could be a phage contamination in the chemostat, keep monitoring chemostat over the next few days
- changed media bottle
- prepared fresh LB

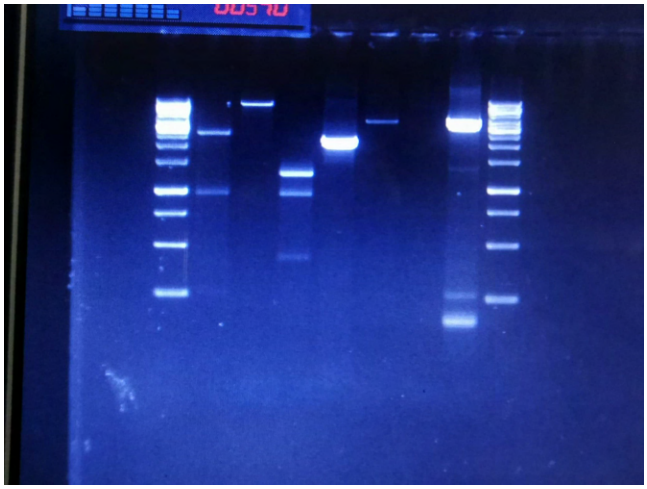
Picking TU1-A-RFP

- very dense, only a few very small single colonies
- inocculated ON culture & sent for sequencibg
- still very dense -> restreaked just in case colonies that i picke were too small

Level 0 gIII, gIII-lux, gIII-neg troubleshooting

- there is a SphI cut site in lux, this might decrease the efficiecy or lead to the lost of one part of the insert => reason why assembly might not work
- go from PCR products for lvl 1 assemlby, should also work ? bot not a longterm solution
- ran gel on the PCR products I found in the temp box
- Ladder: GeneRuler 1 kb
- Loading Scheme: gIII lux dig. - gIII neg dig. - lux dig. - lux pcr - gIII lux pcr - gIII neg pcr - gIII lux pcr
- Discussion
    - gIII lux: expected band at 3.3 kb (2.3 + 1 when cut with SphI), pcr looks fine, and digested shows the expected band
    - lux: expected band at 2.1 kb (1 + 1.1 when cut), pcr product looks fine, digested as well but has a unexpceted band at 300 bp
    - gIII neg: expected band at 1 kb, no such band observed on gel => check if template we use is actually what we think it is

WhatsApp Bild 2024-06-26 um 19.26.06_0ee34d71.jpg



Phage Production

S2208 culutre was in the incubator for 6 h(from fridge) and no growth was visible

reinocculated a new culutre & set it up ON instead

stored SIAH2 phage pool in the frisge

DIJOUS, 27/6/2024

in Lab: Noemie

Sequencing analysis: TU1-A-RFP both look fine

SDM: remove SphI sites for level 0 assembly of gIII lux

- Template: pLS-194g730
- Primers: 001, 002
- Ta 56, elongation for 2 min
- KLD Treatment: 1 uL PCR reaction, 5 uL KLD Buffer, 1 uL KLD Enzyme Mix, 3 uL Water, incubated at RT for 5 min
- Transformation: 2 uL of KLD reaction to 20 uL of cells, plated directly without recovery

TadA qPCR:

- lagoon mean Cp 23.78333333
- chemostat mean Cp 29.74
- Media control (from leftovers from media bottle that I exchanged yesterday) mean Cp 25.38
- Water mean Cp 33.97
- no settings changed, arabinose ist sill runnign

Sent to Microsynth: 48 sequencing reactions

- picked (2 from each plate) level 1s from 26.06 for NightSeq, for details (location &primers see pdf)
- inocculated small culutres in 96 well plate

- used multiple sequencing reactions for both to check for the issues we encoutnerd in the previous cloning rounds

Phage production Day 3

- plaque assay, with SIAH1 cl 2 as positive control
- made duplicates
- included a sample from the chemostat to check for phage contamination

DIVENDRES, 28/6/2024

in Lab: Noemie

TadA PACE:

- no phages visible in chemostat sample on plaque assay => no phage contamination
- qPCR:
  - Mean Cp Lagoon: 24.61
  - Mean Cp Chemostat: 30.82
  - Mean Cp LB: 31.66
  - Mean Cp Water: 32.01
- refilled media bottle & exchanged chemostat waste bottle
- sent lagoon sample for NightSeq
- we have a weird equilibrium of phage propagation and dilution rate of the lagoon: check for phage pfu in the lagoon with plaque assay & sent for sequencing, if everythings looks okey, increase the flowrate on monday.

Sequencing Analysis:

- splitting up two samples into one does not really work

Table10

	A	B	C
1	Construct	Clone A	Clone B
2	1009	luxB is missing a part	x (colony turned red eventually)
3	1010	luxB is missing a part (same as in 1009)	luxB is missing a part (same as in 1009)
4	1066	luxB is missing a part (same as in 1009)	-
5	1018	ok	ok, could be a mixture
6	1019	ok	ok, better quality
7	1043	ok	fwd reaction failed
8	1026	only 1/3 sequencing reactions worked -> prep and sequence the rest	only 1/3 sequencing reactions worked -> prep and sequence the rest
9	1027	mixture, but contains the correct one -> restreaked	x
10	1065	x	mixture, is also missing large parts of the luciferase
11	1044	sequecning failed (only very short strech)	sequecning failed (only very short strech)
12	1045	contains linker 2, but sequencing did not cover promoter -> we have to check for recombination after prep	contains linker 2, but sequencing did not cover promoter -> we have to check for recombination after prep
13	1048	one repeat of the operator is missing - recombination	sequecning failed (only very short strech)
14	1049	ok	x
15	1062	has a EGLN3?	x
16	1064	ok	x

Issues:

- luxB is missing luciferase (pES0012)
- repetitive sequence in the cumaric acid promoter leads to recombination during cloning: repick, colony PCR, retransfrom in NEB stable

Phage production

- we finally have SIAH2 phages! - Assmebly worcked with the new SplitC and SplitD plasmids and the new competent cells
- 3 plaques at and 20 plaques at 10^(-2) and 3 plaques at 10^(-3)
- 6.25 * 10^5 pfu/mL
- picked 3 single clones & inocculated ON cultures in LB (no antibiotics)

pLS-194g730 SDM

- colonies present on plate
- picked 3 & inocculuated ON cultures

DISSABTE, 29/6/2024

- [x] Minipreps
    - TU1 A RFP I (spun down, is in the fridge as pellet, blue rack), replace the old one on the box
    - Level 1 constructs from 28.06 (8x, in shaker), replace the wrong ones in the box (trash them)

        - send for sequencing (some samples that I split up for sequence did not sequence both reaction, resend the missing reaction just to make sure everything is correct)
    - LS-194g730 SDM culutres (3x) and send them for sequencing on Monday
- [x] Golden Gate Assemblies
    - Level 1 Assembly of luciferase with a **constitutive** promoter and with the cuminic acid promoter (something we can use as a positive control for luciferase assay & check our inducible system) - ! had issues with the plasmid 0012 yesterday, I would not trust it
    - Level 2 Assemblies (with the new Level 1 constructs) --> do on sunday
    - set up assemblies for the reactions that had recombination of operator sequence for noemie to take to bern (transformation in neb stable, should have less issues with recombination) - I will pick up the assembly reaction in the evening + also a Carb plate
- [x] 2nd plaque assay for SIAH2: with the 3 single clones, they are in the shaker (sterile filtration of supernatant this time), also send them for NightSeq. There is a relatively fresh, quite dense S2208 culture in the fridge (in the blue rack). You can use this to innoculate a culture, and then growing it to OD 0.5 will only take a few hours.
    - also perform plaque assay with samples from the lagoon and the chemostat, (with dilution series like we do with regular plaque assays, bcs media contains antibiotics)
- [x] PACE: qPCR, check liquid levels, arabinose
- [x] take pES1027 plate out of the incubator, pick colonies (2-3) -> consider doing colony PCR to confirm insert instead of sequencing if it is faster
- [ ] Troubleshooting: Find out why luxB is missing in our level 1 constructs

in Lab: Jakob, Pau

Miniprep (ON cultures):

Qiagen Miniprep

TU1-A-RFP: 296 ng/µL
pES1018: 758 ng/µL
pES1019: 801 ng/µL
pES1026 A: 188 ng/µL
pES1026 B: 613 ng/µL
pES1043: 287 ng/µL
pES1045: 211 ng/µL
pES1049: 205 ng/µL
pES1064: 711 ng/µL
LS-194g 730 SDM cl. 1: 217 ng/µL
LS-194g 730 SDM cl. 2: 163 ng/µL
LS-194g 730 SDM cl. 3: 147 ng/µL
--> concentrations adjusted to 100 ng/µL

TadA PACE:

- qPCR:
    - Mean Cp Lagoon: 28.55
    - Mean Cp Chemostat: 28.84
    - Mean Cp Water: 33.25

- Exchanged medium bottle
- Plaque assay for lagoon and chemostat

Phage production - SIAH2:

2 Plaque assay plates made and phages sent for sequencing

Sequencing:

phage production cl. 1-3: oLS-670.
pES1026 A and B: o018
pES1045: o017
LS-194g 730 SDM cl. 1-3: primer o004

Colony PCR for pES1027:

- picked 8 colonies
- 25 uL reaction
- initial cycle 98 °C for 10 min
- Primers 017&018, Ta 59 C, Initial denaturation 10 min, Elongation 1 min 40 s
- Run gel 1% agarose: 120V 40 min

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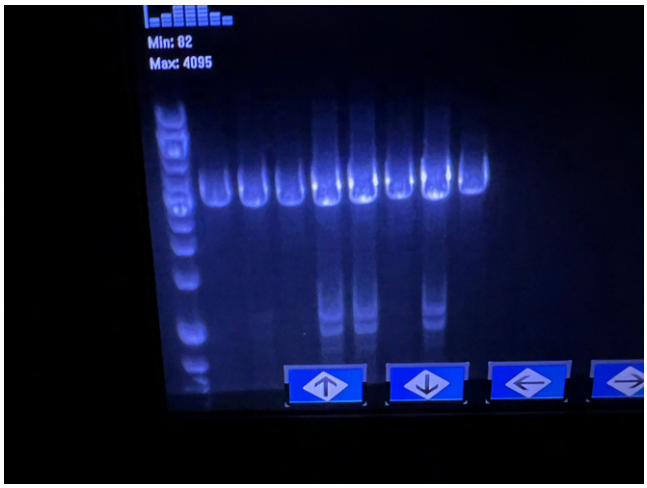


- prepare ON cultures of colonies 2, 3 and 4

Golden Gate Assembly:

EcoFlex Assembly

pES1044, pES1048, pES1062 --> 15 cycles

- For pES0020 (part of pES1062) we use the tube labelled as Linker 1

DIUMENGE, 30/6/2024

in Lab: Pau, Jakob

- [x] look at plaque assays
- [x] bring sequencing baggies to sequencing
- [x] miniprep pES1027 and prepare samples for sequencing
- [x] lvl2 assemblies with the new lvl1 construct
- [x] Transform lvl2 assemblies
- [x] Lux consec and inducable lvl 1 assemblies

Plaque assay:
positive control (8*10^10): ~40 plaques on -7 dilution --> 10^11 pfu/mL
clone 1: ~40 plaques on -7 dilution --> 10^11 pfu/mL
clone 2: ~15 plaques on -7 dilution --> 3.75*10^10 pfu/mL
clone 3: ~15 plaques on -7 dilution --> 3.75*10^10 pfu/mL

TadA PACE:

- Phage population seems to be very low according to plaque assay
  - Lagoon: 40 plaques on undiluted --> 10^4 pfu/mL
  - Chemostat: no plaques
- qPCR:
  - Mean Cp Lagoon: 24.08 --> 4*10^7 pfu/mL
  - Mean Cp Chemostat: 28.47
  - Mean Cp Water: 29
- Chemostat looks quite gross (biofilm, also already on 29.06.)
- arabinose syringe is working
- Exchanged waste bottle

Miniprep (ON cultures): Qiagen Miniprep
pES1027 cl. 2: 254 ng/µL
pES1027 cl. 3: 304 ng/µL
pES1027 cl. 4: 288 ng/µL
--> concentrations adjusted to 100 ng/µL
--> sent for sequencing

Golden Gate Assembly:
EcoFlex Assembly
pES2005, pES2008, pES2009, pES2010, pES2011 --> 30 cycles

- For pES2008, pES2009, pES2010, pES2011 --> we used the p1026b (which has been sent for sequencing but results are not in yet)

pES1067, pES1068, pES1069, pES1070 with lux constitutive and inducible promotors:

Constitutive and inducible lux

	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O
1	pES1067	Constitutive lux	A	Amp	pTU1-A-RFP	pBP-SJM910	pBP_BBa_B0034	-	pES0027	pBP-L3S2P21					
2	pES1069	Inducable lux (cymR)	A	Amp	pTU1-A-RFP	pES0019	pBP_BBa_B0034	-	pES0027	pBP-L3S2P21					

Transformation:

Transformation

pES2005, pES2008, pES2009, pES2010, pES2011 --> high efficiency protocol

Lab Journal - July

Project: iDEC 2024
Author: Jakob Wimmer
Entry Created On: 13 Jul 2024 09:12:13 UTC
Entry Last Modified: 08 Aug 2024 15:00:12 UTC
Export Generated On: 08 Oct 2024 09:23:30 UTC

DILLUNS, 1/7/2024

- [ ] (pick lvl2 colonies (pES2005, pES2008-2011) and send for sequencing (or ideally do colony PCRs to check for correct insertion))
  or just take the plates out (fridge) so they can be picked and sequenced together with the lvl1s on tuesday
- [ ] Transform lux lvl1 (pES1067 and 1069) --> GG assembly reactions are in PCR slot1 or probably in the common fridge, in J39 second shelf - labeled as 67 and 69
- [ ] TadA Pace - usual things

TadA PACE

qPCR done and Cp values entered into spreadsheet

- phage levels seem quite low

Level 1 transformations

pES1067 and pES1069 transformed into E. coli Transformation

Level 2 plates

pES2005 and pES2010 show potential positive colonies (put in fridge)
pES2008 and pES2009 showed no colonies at all: put back in incubator in case of slow growers
pES2011 only showed RFP expressing colonies

Colony PCR set up for 8 colonies each for pES2005 and pES2010 using primers 18 and 19 to amplify insert
PCR: Q5 Polymerase

pES2005: expected amplicon size ca. 4500 bp
pES2010: expected amplicon size ca. 8500bp

Transformation of pES1044, 1048, 1062 in NEB stable, grow ON at 30°C

DIMARTS, 2/7/2024

- [x] look at sequencing results of pES1026, pES1027, SIAH2 phages and pES1045
- [x] pick lvl2 colonies and lvl1 lux assemblies for sequencing, and lvl in neb stable

in Lab: Noemie

Sequencing Analysis

phage lagoon from 28.06.: sequencing failed ... probably phage titer is too low: Lukas suggested incubating S2208 with phages ON and then send thi sfor sequecning
pES1026: both cl. A and B look good (B was used for lvl2s and has higher concentration)
pES1027: clone 3 and 4 look good, but not everything is covered, clone 2 is mutated
pES1045: mixture, but correct plasmid is there -> retransformation to get rid of mixture, ordered primers for colony PCR to check for recombination in the promoter region
pLS-194g730 SDM: sequencing failed
SIAH2 phage clones: the arginine mutation We had previously is back: reason for this: I used the wrong plasmid as a input for phage production (it was called SP mut and I assumed it was the one we mutated, but it was the wrong one - see 24.06.) => phage production worked, but we need to repeat it (all of the 3 clones have this issue) sorry!

Colonies on plates with the level 1s were too small to pick -> left them at 30°C ON, pick and send for sequencing tomorrow

liquid ON culutres from 2010 also didn't grow, put back in shaker overnight (as they probably grow very slowly)

Phage lagoon sequencing failed, probably because phage titer was too low -> i let them propagate on SS208 in LB ON

Sent for sequencing:

- PvanCC, VanRAM
- LS-194g 730 SDM clones 1-3
- 3 colonies picked from the plate with pES2005 (I didn't inocculate a Liquid culture, but I marked the colonies I picked)

TadA PACE:

- qPCR: there seem to be phages, but still stagnant
- increased flow rate to 1 to increase selection pressure
- exchanged the tubidostat vessel to get rid of all the biofilm formation
- prepared a fresh bottle of LB

Golden Gate Assemblies:

- Level 1 Assembly of a random construct with PVan (just to get a construct of PVan with RBS, here the BB0034), ran the EcoFlex protocol, but only with 15 cycles + Transformation
  I ordered Primers to PCR the pVan + RBS with the correct overhangs
- Repeated Phage Production Day 1 for SIAH 2 (this time Donor SP is Siah2 SP A) -> run GG ON (slot 2)
- Level 1 assemblies of our constructs, but with the weak promoter J23108 -> might help with our issues of slow groth and reduce the burden on our cells => ran this assembly together with the Phage production assembly with Lukas's Protokol (not our standard level 1 assembly protocol)

Table11

	A	B	C	D	E	F	G	H	I	J
1	pES1071	weak_NtermRNAP_L3_Ub	A	Amp	pTU1-A-RFP	pBP-J23108	pES0003	pES0002	pES0004	pBP-L3S2P21
2	pES1072	weak_HsUba1_E1	B	Amp	pTU1-B-RFP	pBP-J23108	pBP_BBa_B0034	-	pES0001	pBP-L3S2P21
3	pES1073	weak_TuUba1_E1	B	Amp	pTU1-B-RFP	pBP-J23108	pBP_BBa_B0034	-	pES0006	pBP-L3S2P21
4	pES1074	weak_UBCH5A E2	C	Amp	pTU1-C-RFP	pBP-J23108	pBP_BBa_B0034	-	pES0007	pBP-L3S2P21
5	pES1075	weak_NtermRNAP_L1_Ub	A	Amp	pTU1-A-RFP	pBP-J23108	pES0003	Linker 1	pES0004	pBP-L3S2P21
6	pES1076	weak_EGLN3_L2_CtermRNAP(GAC)	A	Amp	pTU1-A-RFP	pBP-J23108	pES0013	Linker 2	pES0008	pBP-L3S2P21
7	pES1077	weak_EGLN1_L2_CtermRNAP(GAC)	A	Amp	pTU1-A-RFP	pBP-J23108	pES0014	Linker 2	pES0008	pBP-L3S2P21
8	pES1078	weak_asyn_L2_CtermRNAP(GAC)	A	Amp	pTU1-A-RFP	pBP-J23108	pES0015	Linker 2	pES0008	pBP-L3S2P21
9	pES1079	weak_EGLN3_L4_CtermRNAP(GAC)	A	Amp	pTU1-A-RFP	pBP-J23108	pES0013	pES0022	pES0008	pBP-L3S2P21
10	pES1080	weak_EGLN1_L4_CtermRNAP(GAC)	A	Amp	pTU1-A-RFP	pBP-J23108	pES0014	pES0022	pES0008	pBP-L3S2P21
11	pES1081	weak_asyn_L4_CtermRNAP(GAC)	A	Amp	pTU1-A-RFP	pBP-J23108	pES0015	pES0022	pES0008	pBP-L3S2P21
12	pES1082	weak_gIII-lux (D Part)	D	Amp	pTU1-D-RFP	pBP-J23108	pBP_BBa_B0034	-	pES0027	pBP-L3S2P21
13	pES1083	weak_gIII-lux (B part)	B	Amp	pTU1-B-RFP	pBP-J23108	pBP_BBa_B0034	-	pES0027	pBP-L3S2P21

DIMECRES, 3/7/2024

- [x] pick lvl 1s from plates: pES1044, 1048, 1062, pES1067 and pES1069 and send for sequencing (I took those with me to UniBern, so we can make it for sequencing on wednesday)
- [x] check on liquid cultrues 2010, send for sequencing if they grew --> nothing grew
  --> picked three new white cultures from pES2010
- [x] send lagoon phages for sequencing (from the liquid culutre with S2208)
- [x] put plate with PVan and pES1045 lvl 1 in the fridge until primers arrive
- [x] check microsynth sequencing results
- [x] TadA PACE: qPCR, check liquid levels --> not needed since PACE broke
- [x] Transformation of the Level 1 Assemblies from 2.7.
- [x] continue with phage production day 2
- [x] send one of the pLS-194g 730 SDM vairiant for sequencing, but with a primer that binds the backbone

in Lab: Pau, Jakob

Sequencing Analysis:

LS-194g 730 SDM: sequencing failed again -> could be that we have a wrong plasmid -> send one of them for sequencing with a primer that binds to the backbone

pES2005: clone 3 looks good (clone 1 and 2 might also be ok, but look weird)
VanRAM: we were given the wrong plasmid (has espP)
PVanCC: is ok

Sent for sequencing:
pLS-194g 730 SDM clone 1 with primer o017
propagated phages from lagoon: primer oLS-670.

Transformation:
Transformation
pES1071-1083 --> high efficiency protocol

Day 2 of phage production - transformation of SIAH2 assembly in S2208

Liquid o/n cultures:
picked pES2005 clone3 for miniprep + full plasmid sequencing
picked 3 pES2010 white cultures
picked 3 cultures from PVan and pES1045

TadA PACE:
put in new P3c culture and newly innoculated lagoon (see excel sheet for details)
set flow rate

Plates with level 1s:

- 1067 and 1064: still no visible colonies
- picked 3 colonies each from 1062, 1048, 1044 (1062 had both rather larger and very small colonies, and i tried to pick mainly the smaller ones)
- started 100 uL on cultures
- sequencing with AmpStart

DIJOUS, 4/7/2024

- [x] Phage production day 3
- [x] PACE things

In lab: Pau

Phage production day 3
Isolated supernatant from transformed S2208 o/n culture, 0.2um filtered.

Level 1 assemblies
All plates display some white colonies.
Put plates in fridge for picking tomorrow.

TadA PACE

Reactor broke down again. Sasha is on it.
Inoculate P3c o/n culture for OD600 calibration.

DIVENDRES, 5/7/2024

- [x] Pick lvl1 from plates (pES1071-pES1083) and do colony PCR/send for seq --> plates are in the fridge
- [x] Sequencing analysis of pLS-194g, propagated phages from lagoon
- [x] miniprep pES2005 and send for full plasmid seq --> liquid cultures in fridge
- [x] miniprep pES2010 and send for sequencing --> liquid cultures in fridge
- [x] miniprep PVan and pES1045 --> liquid cultures in fridge
- [x] PACE things --> OD600 calibration 0.2 to 1, 5 different

in Lab: Noemie

Sequecing Analysis

- pES1044: A and B look okey, C is wrong
- pES1048: A is ok, B and C sequencing failed/too short
- pES1062: C is ok, others not
- phage sample from lagoon, porpagated on S2208 -> still sequencing didn't work
- pLS-194g sequencing didn't work again-> check if it is the correct back bone by choosing sequencing primer that binds the backbone

=> inocculated overnight culutres for 1044 A, 1048 A, 1062 C

Minipreps: 2005, 2010, pVan, 1045

- trashed one culutres of the pVan bcs nothing grew and one of the 2010 culutres because pellet is red
- got very low yields for: two of the 1045 preps, and 2005 prep, not enough to send for sequencing
- sent two 2010, and one 1045 for sequencing

Transformants 3.7.24

- picked 2 colonies per plate, sent for NightSeq with o017
- prepared 96-well plate with 100 uL culutres, put in incubator ON

PACE:

- prepared serial dilution of the P3c ON culutre for reactor calibration (OD: 0.2, 0.4, 0.6, 0.8, 1)
- regrew dense p3c stock

PCR: pVan + RBS as level 0 insert

- Primer: 036, 041
- Template: set up 2 reactions, one with each prepped pVan level 1
- Ta: 59 C
- Elongation Time: 15 s

DISSABTE, 6/7/2024

- [x] take 96 well plate out of the incubator & move it into the fridge
- [x] miniprep 1044, 1048, 1062 culutres (shaker)
  --> 1062 did not grow, innoculated two new cultures
- [x] sequencing analysis
- [x] Phage production day 3 --> supernatant with phages is in the temp box (they are 0.2 um filtered), do phage assay
- [x] restart pace? (there is a saturated P3c in the shaker, dilute it & grow it again to OD 0.7 before putting it in the reactor; also: keep the rest of the culture as a fridge stock, so we don't have to grow from glycerol stock every time)
  --> PACE reactor is down over the weekend
- [ ] clone level 0 from pVan PCR product (pcr products are in the green rack in the fridge)
- [x] I took out the plates from the coldroom, because they were very wet & put them out to dry for the night, I f they are dry we can put them back in the cold room
- [ ] Look at lux stuff?

Miniprep (ON cultures):

Qiagen Miniprep

pES1044: 236 ng/µL

pES1048: 633 ng/µL

--> concentrations adjusted to 100 ng/µL

Sequencing Analysis:

pES1045 has recombination

Phage Production - Day 3: Plaque Assay:

two plates with plaque assay

Lvl 0 assembly - pVan:

NucleoSpin Gel and PCR Clean-up Restriction digest T4 Ligation Transformation

PCR clean-up --> eluted in 15uL, yields were rather low (40-50 ng/uL)

Restriction digest --> used all PCR clean-up fraction + backbone (pBP), performed PCR clean-up of digests --> yield low for pBP (7 ng/uL), high for PVan (70 ng/uL)

T4 ligation --> used all PCR clean-up fractions

Transformation --> 40 uL of competent cells, 10 uL of ligation product, high-efficiency protocol

DIUMENGE, 7/7/2024

- [x] miniprep pES1062 if it grew
- [x] pick culture 3 again from 2005 (put it back in incubator yesterday)
- [x] phage production day 4 - pick single plaques
- [x] Colony PCR of pES1045 plate (pick new colonies and test for recombination with primers 42 and 43)
- [x] Pick pES0028 colonies for o/n culture
- [x] Restart TadA PACE

In lab: Pau

Miniprep pES1062

Qiagen Miniprep

Yield: 97.4 ng/uL

--> did not dilute it further

Lvl 2 assembly pES2005 picking

Colony 3 from pES2005 grew again. Picked colony and culture O/N

Phage production day 4

Agar very dense and many agar-embedded colonies --> Lukas says it's okay

Put plates in the fridge to pick tomorrow or repeat.

SIAH2: 2 plaques at -6 / 9 plaques at -6

Control: 48 plaques at -6 / 23 plaques at -7

Estimated pfu/mL

- Control --> (23*10^7*1000/4 + 48*10^6*1000/4)/2 = 3.475 · 10^10 pfu/mL
- SIAH2 --> 1.375 · 10^9 pfu/mL

Colony PCR of pES1045 for recombination check

PCR: Q5 Polymerase

Picked 8 colonies

PCR --> primers 42, 43, Ta 61ºC, elongation 30s, final elongation 2min

Gel --> 1% agarose, 120V, 20 min

It's difficult to really determine the size, I forgot to add a negative control without recombination (e.g., pES1048 and pES1044). Repeat with those.

Lvl 0 assembly pES0028

Did not grow, put it back to the incubator.

TadA PACE restart

Restarted PACE (Echo)

Lagoon --> mixture of phages, see TadA PACE excel spreadsheet

Used the P3c culture from the fridge, diluted it a bit with fresh LB + antibiotics + glucose

Tomorrow: set culture of P3c in the morning, make clean start of everything except lagoon in the afternoon.

Changed fresh media bottle.

DILLUNS, 8/7/2024

- [x] Phage day 4 --> pick single plaques
- [x] NightSeq analysis from 5.7., inoculate 5 mL liquid cultres from the right ones

- [x] Send pES1062 for sequencing
- [x] Full plasmid seq for pES2005 if it grew
- [x] Sequencing analysis for pES2010
- [x] See if pES0028 grew, pick for o/n culture
- [x] TadA PACE stuff --> maybe prepare new bottle of fresh LB? --> PACE is broken again
- [x] play around with dye dilutions for a standard for PACE reactor calibration
- [x] check if we can assemble level 2 in a way we can finally test our logic this week

in Lab: Pau, Jakob

Sequencing Analysis:
pES2010 --> it's just pES1004 (how do they even live on Kan?)
pES1071 --> A1 good
pES1073 --> both good
pES1075 --> both good
pES1076 --> D2 probably good, but there is a unmatched sequenced stretch before the promoter
pES1077 --> both good
pES1078 --> G2 good
pES1079 --> A3 probably good, but there is a unmatched sequenced stretch before the promoter
pES1080 --> D3 probably good (quite some mistakes somewhat close to end of sequencing)
pES1081 --> both good
pES1082 --> G3 good

pES1072 --> both wrong
pES1074 --> both wrong
pES1083 --> both wrong

Miniprep: Qiagen Miniprep
pES2005: 201 ng/µL
--> concentrations adjusted to 100 ng/µL

Sequencing:
pES1062 sent for sequencing
pES2005 sent for full plasmid seq

O/N cultures:
picked three pES0028 --> colonies looked a bit weird though

Phage production day 4
Picked 3 plaques and left them in LB culturing O/N

TadA PACE
PACE broke down again and stuff got fried.
Prepared a new LB bottle

Golden Gate Assembly: EcoFlex Assembly

used 100 ng backbone instead, let ligate for 5 min at 16 °C instead

4 µL of assembly transformed on 100 µL bacteria

Table12

	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O
1	pES2008	AP1 (for plaque assay)	KanR (+CymRam)	pTU2-A-RFP KanR CymRAM	pES1001	pES1002	pES1004	pES1026	-	ok	ok				
2	pES2009	AP1 (for plaque assay)	KanR (+CymRam)	pTU2-A-RFP KanR CymRAM	pES1001	pES1003	pES1004	pES1026	-	ok	ok				
3	pES2010	AP1 (for plaque assay), inducible	KanR (+CymRam)	pTU2-A-RFP KanR CymRAM	pES1063	pES1002	pES1004	pES1026	-	ok	ok	ok			
4	pES2011	AP1 (for plaque assay), inducible	KanR (+CymRam)	pTU2-A-RFP KanR CymRAM	pES1063	pES1003	pES1004	pES1026	-	ok	ok				

DIMARTS, 9/7/2024

- [x] sequencing analysis pES1062
- [x] phage production - 2nd plaque assay
- [x] miniprep and send for seq pES0028
- [x] Check pES2008-2011 colonies (mark all visible colonies)
- [x] Repeat PCR + gel w/ primers 42 and 43 for pES1045 and pES1062 (and pES1048 as a positive control)
- [x] level 2 constructs with new weak plasmids
- [x] Make Kan and Spec plates

in Lab: Pau, Jakob

Sequencing analysis:

pES1062 --> is recombined

pick other colonies

Lvl 2 assemblies

All plates have colonies --> leave them in the incubator O/N so that "slow growers", which are probably the right ones, have time to grow.

Miniprep: Qiagen Miniprep

pES0028 cl.1: 241 ng/µL

pES0028 cl.2: 234 ng/µL

pES0028 cl.3: 231 ng/µL

--> all sent for sequencing

pES1071 A1: 239 ng/µL

pES1073 E1: 247 ng/µL

pES1075 A2: 229 ng/µL

pES1076 D2: 375 ng/µL

pES1077 F2: 278 ng/µL

pES1078 G2: ng/µL (culture innoculated today)

pES1079 A3: 288 ng/µL

pES1080 D3: 360 ng/µL

pES1081 F3: 264 ng/µL (--> possibly swapped with 1082 while doing minipreps, sent for sequencing)

pES1082 G3: 276 ng/µL
--> concentrations adjusted to 100 ng/µL

Second plaque assay (Phage Production **day 5)**

Phages were sterile filtered and sent for sequencing
Two plates with plaque assay for titer determination

- Control pos: P 227
- Reihenfolge (forgot to write it in the plate): SIAH2 1 - SIAH2 2 - SIAH2 3 - Control +

Recombination PCR w/ primers 42 and 43

Picked 4x colonies for pES1045, 4x colonies for pES1062, 2x colonies for pES1048 (as positive control)
PCR:

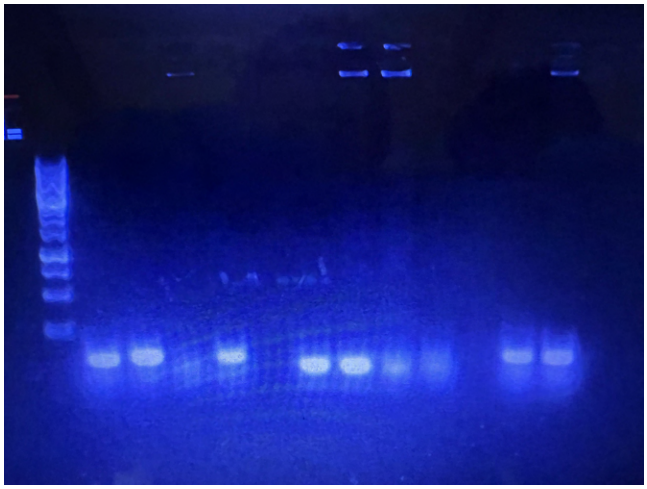
- 25 uL reaction
- initial cycle 98 °C for 10 min
- Primers 042&043, Ta 61 ºC, elongation 30 s
- Run gel 1% agarose: 120V 20 min

Expected bands:

- No recombination --> 129 bp
- Recombination --> 129 - 58 = 71 bp

Order: Genruler 1kb - pES1045 A1 - B1 - C1 - D1 - blank - pES1062 1 - 2 - 3 - 4 - blank - pES1048 1 - 2

IMG_5141.jpeg



Some pES1045 colonies show no recombination! We pick those colonies for o/n culture.

Golden Gate Assembly: EcoFlex Assembly

used 100 ng backbone instead, let ligate for 5 min at 16 °C instead - E2 is always still strong, since we did not get a plasmid for that
4 µL of assembly transformed on 100 µL bacteria

Table13

	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O
1	pES2012	AP1 (weak mix), inducible	KanR (+CymRam)	pTU2-A-RFP KanR CymRAM	pES1063	pES1073	pES1004	pES1026	-						
2	pES2015	AP2pos, asyn L4, (luciferase), weak	SmR	pTU2-a p15a SmR	pES1081	pES1009									
3	pES2016	AP2pos, EGLN3 L4, (luciferase), weak	SmR	pTU2-a p15a SmR	pES1079	pES1009									

DIMECRES, 10/7/2024

- [x] sequencing analysis pES0028 and pES1081
- [x] sequencing analysis SIAH2 phages and count pfu
- [x] miniprep pES1045 and pES1078
- [x] lab ämtchen
- [ ] presentation lab meeting tonight
- [x] colony pcr 62
- [x] plaque assay

in Lab: Noemie, Jakob

Sequencing Analysis:
pES1081 and pES1082 were swapped - need to redo assembly of pES2013-2015
pES0028 clone 1 and 2 are good, 3 might have insert --> put clone 1 into lvl 0 box
pES2005 full plasmid seq is correct (check a few mismatches --> linker muts just wrong sequence, lux muts are silent, some stuff in backbone)

Phage production:
Sequencing of all three clones is correct - going to use clone 1 going forward
Phage titer >10^11 pfu/mL for all of them
--> new plaque assay for just clone 1 with dilution -5 to -12

Minipreps

- pES1045 and pES1078
- sent for sequencing with primer 017
- stored in temp box, concentration not adjusted

Golden Gate Assembly: EcoFlex Assembly
for lvl2s: used 70 ng backbone instead, let ligate for 5 min at 16 °C instead - E2 is always still strong, since we did not get a plasmid for that
4 µL of assembly transformed on 100 µL bacteria
for lvl1s: 2 µL of assembly transformed on 50 µL bacteria

Table14

	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O
1	pES2015	AP2pos, asyn L4, (luciferase), weak	SmR	pTU2-a p15a SmR	pES1081	pES1009									
2	pES1084	pVan_EGLN3_L4_CtermRNAP(GAC)	A	Amp	pTU1-A-RFP	pES0028	pES0013	pES0022	pES0008	pBP-L3S2P21					
3	pES1085	pVan_asyn_L4_CtermRNAP(GAC)	A	Amp	pTU1-A-RFP	pES0028	pES0015	pES0022	pES0008	pBP-L3S2P21					
4	pES1086	pVan_gIII-lux (D Part)	D	Amp	pTU1-D-RFP	pES0028	pBP_BBa_B0034	-	pES0027	pBP-L3S2P21					
5	pES1087	pVan_NtermRNAP_L3_Ub	A	Amp	pTU1-A-RFP	pES0028	pES0003	pES0002	pES0004	pBP-L3S2P21					
6	pES1088	pVan_HsUba1_E1	B	Amp	pTU1-B-RFP	pES0028	pBP_BBa_B0034	-	pES0001	pBP-L3S2P21					
7	pES1089	pVan_TuUba1_E1	B	Amp	pTU1-B-RFP	pES0028	pBP_BBa_B0034	-	pES0006	pBP-L3S2P21					
8	pES1090	pVan_UBCH5A E2	C	Amp	pTU1-C-RFP	pES0028	pBP_BBa_B0034	-	pES0007	pBP-L3S2P21					
9	pES1091	pCym_HsUba1_E1	B	Amp	pTU1-B-RFP	pES0019	pBP_BBa_B0034	-	pES0001	pBP-L3S2P21					
10	pES1092	pCym_TuUba1_E1	B	Amp	pTU1-B-RFP	pES0019	pBP_BBa_B0034	-	pES0006	pBP-L3S2P21					
11	pES1093	pCym_UBCH5A E2	C	Amp	pTU1-C-RFP	pES0019	pBP_BBa_B0034	-	pES0007	pBP-L3S2P21					
12	pES1029	p70_EGLN3_L2_CtermRNAP(CGG)	A	Amp	pTU1-A-RFP	pES0005	pES0013	Linker 2	pES0008	pBP-L3S2P21					
13	pES1033	p70_EGLN3_L4_CtermRNAP(CGG)	A	Amp	pTU1-A-RFP	pES0005	pES0013	pES0022	pES0008	pBP-L3S2P21					
14	pES1060	CymR_EGLN3_L3_CtermRNAP (CGG)	A	Amp	pTU1-A-RFP	pES0019	pES0013	pES0002	pES0008	pBP-L3S2P21					
15	pES1055	CymR_EGLN3_L4_CtermRNAP(CGG)	A	Amp	pTU1-A-RFP	pES0019	pES0013	pES0022	pES0008	pBP-L3S2P21					
16	pES1031	p70_asyn_L2_CtermRNAP (CGG)	A	Amp	pTU1-A-RFP	pES0005	pES0015	Linker 2	pES0008	pBP-L3S2P21					
17	pES1035	p70_asyn_L4_CtermRNAP (CGG)	A	Amp	pTU1-A-RFP	pES0005	pES0015	pES0022	pES0008	pBP-L3S2P21					
18	pES1040	CymR_asyn_L3_CtermRNAP (CGG)	A	Amp	pTU1-A-RFP	pES0019	pES0015	pES0002	pES0008	pBP-L3S2P21					
19	pES1057	CymR_asyn_L4_CtermRNAP(CGG)	A	Amp	pTU1-A-RFP	pES0019	pES0015	pES0022	pES0008	pBP-L3S2P21					

Colony PCR

- picked 8 more colonies from plate
- PCR:
  - 12.5 uL reaction
  - initial cycle 98 °C for 2 min
  - Primers 042&043, Ta 61 ºC, elongation 30 s
  - Run gel 2% agarose: 120V 20 min

DIJOUS, 11/7/2024

- [x] pick cultures for night seq plate:
  - pES2008-2011
  - repick pES1072, 1074 and 1083 cultures
  - pES2012-2016
  - assemblies from wednesday
  - pES1062
- [x] Design new cloning strategy for Lvl 2 (Gibson)
- [x] Find/Order more Full Plasmid Seq labels, ask Pau --> found
- [ ] Test for solubility of SplitRNAP? Ask Max for the Paper

- [x] Sequence GG sites of the input plasmids for the level 2 assemblies (backbone, NOT 017/018/019, they often not cover GG sites)
- [x] next cloning troubleshooting steps:
  - [x] sequencing of level 1s and backbones
  - [x] assemble with esp3i instead of bsmsbi, is a isochizomer (cuts at 37 in cutsmart)
  - [x] assemble lvl 2s with just the backbone(rfp insers) to check protocol (both bsmbi and esp3i)

in Lab: Noemie, Jakob

Sequencing Analysis:
pEs1045 are all good --> put cl. 1 into lvl 1 box
pES1078 is good

Phage production - plaque assay:
SIAH2 cl. 1 has 9 plaques on -8 dilution --> Phage titer = 2.3*10^11 pfu/mL

Organization:
made a new box for lvl2 plasmids and for SIAH phages --> put stickers on the correct tubes, only use these --> after re-sequencing all level 1s we should also put a sticker on every confirmed plasmid

Assembly analysis:
all plates have colonies, slowest growing colonies:
pES1085 --> pVan asyn_RNAP, pES1087 --> pVan RNAP_Ub, pES1029 --> strong EGLN3_RNAP,
pES1031 --> strong asyn_RNAP, (pES1057, pES1055) --> pCym sub_RNAP
--> can't really see a pattern, maybe that fusion-constructs grow slower - but not sure whether this conclusion can be drawn

Sequencing:
picked pES2008-2011, pES1072, 1074 and 1083 cultures, pES2012-2016, assemblies from wednesday, pES1062

Troubleshooting Level 2 Assembly Reactions:

- Assembly of Level 2 construct with TU1A-D as inserts (should end up being 4 RFPs in a row)
  did this twice, one with Bsmbi (some protocol as ibefore at 42°C), other with Esp3I (isochizomer if Bsmbi) with the cycling protocol of the Bennet Lab blog
  run digest in the evening, stored at 4°C ON for Transfomation tomorrow
- sent level 1 and the TU1 backbone for sequencing with appropriate primers (see order sheet)

Solubility prediction:
https://protein-sol.manchester.ac.uk
average e. coli protein has score of 0.45
predicts good solubility for N-term RNAP - Ub (0.745),
predicts worse solubility for C-term RNAP - substrate fusions (0.38) --> that is better than the C-term on it's own though

DIVENDRES, 12/7/2024

- [x] Order Primers Gibson Assembly
- [x] Transformation of the two lvl 2 Assemblies

☑ Sequencing Analysis: NightSeq Plate
in Lab: Noemie, Jakob

Transformation of **Troubleshooting Level 2 Assembly Reactions:**

- Incubation on ice: 30 min
- Outgrowth: 1.5 hours

Quick Transformation Protocol of TU1A RFP in Stbl3 competent cells to see how efficiency is

Gibson Assembly Primers designed for pES2014, and ordered

Sequencing Analysis - Level 2s:

- pES2008
  - A: after the GG B site, it no longer matches (appears to be a J23119 promoter, which is both infront of KanR and CymR)
  - B: 018 covers Uba1, 038 read covers AmpR
- pES2009
  - A: only has first TU, has AmpR
  - 018 shows correct last TU but that after last GG site smth not expected has attached, 038 coveres AmpR
- pES2010
  - A: CymR is skipped -> we observed this before, but this time input plasmid shouldn't be an issue -> recombination bcs KanR and CymR have the same Terminator
  - B: only has the first TU
- pES2011
  - A seems to be a plasmid mix, 038 failed
  - B is missing CymR, seems to be a plasmid mix
- pES2012
  - A, B look the same: shows correct last TU but that after last GG site smth not expected has attached, 038 coveres AmpR
  - A is a plasmid mix
- pES2015
  - A: empty Backbone (we don't have red/white screening for this backbone)
  - B: 018 shows lac Promoter, which we only have infront of the RFP (should even be there, bcs no part in there has an RFP)
- pES2016
  - A: is a mixture, shows correct last TU but that after last GG site smth not expected has attached
  - B: shows correct last TU but that after last GG site smth not expected has attached

=> One main Issue is Recombination (seen in pES2010 Fullplasmid seq) => might also be an issue in general , bcs we havve a lot of sequences that repeat
=> But the also must be weird assemlies happening, bcs we see parts of lvl 1 assemblies in the lvl 2 constructs (maybe enzyme is not cuttign efficiently? ->wait for test comparison Esp3I and BsmBi)

Sequencing An alysis - Level 1s:

pES1029: B is correct, A is not
pES1031: B is correct, A is not
pES1033: both correct, innoculated B
pES1035: B is correct, A is not
pES1040: both not ok
pES1055: both correct, inooculated A
pES1057: both not ok
pES1060: B is correct, A maybe too
pES1062: both not ok
pES1072: both not ok
pES1074: is a D fragment instead of C --> not innoculated
pES1083: both not ok
pES1084: A is correct, B is not
pES1085: both not ok
pES1086: both not ok
pES1087: both not ok
pES1088: B is correct, A is not
pES1089: both correct, innoculated A
pES1090: both correct, innoculated A
pES1091: both correct, innoculated A
pES1092: both correct, innoculated B
pES1093: both correct, innoculated A
--> innoculated correct Level 1 plasmids

Innoculated Level 1 plasmids:
pES1029, pES1031, pES1033, pES1035, pES1055, pES1060, pES1084, pES1088, pES1089, pES1090, pES1091, pES1092, pES1093
also innoculated pTU1-D1-RFP and pTU-E-RFP

Failed Level 1 plasmids:
pES1057 put back in incubator to pick white colonies, pES1062 tubes put in incubator
other assemblies are probably not worth trying

Organization:
created proper labeling for our boxes and for our plasmids
Color coding system:
Ecoflex parts - Level 0 plasmids - Level 1 plasmids - Level 2 plasmids - Phages - Primers
printed labels for most things that we will make in the future should be in the drawer
use unlabeled tubes at your own risk

DISSABTE, 13/7/2024

- [x] miniprep level 1 plasmids
- [x] transform level 2s in stable cells, put at 30 °C --> Stable cells: A43 box in -80 °C (2nd box in 2nd row in 2nd column)
- [x] pick pES1040, pES1057 and pES1062 and innoculate level 2s

- [x] Sequencing Analysis: Economy Run Plate
- [x] assemble VanR - E fragment (pES1095) and lux - D1 fragment (pES1094)

in Lab: Jacob

Sequencing Analysis:
pTU1-A-RFP, pTU1-B-RFP, pTU1-C-RFP, pTU1-D-RFP --> are all correct
pES1001: correct
pES1002: sequencing looks weird towards end, possibly wrong
pES1003: correct
pES1004: correct
pES1007: correct
pES1008: correct
pES1009: correct
pES1010: correct
pES1011: correct
pES1012: correct
pES1013: correct
pES1014: correct
pES1017: correct
pES1019: actually has CGG RNAP --> relabeled to pES1030
pES1020: correct
pES1022: correct
pES1023: correct
pES1024: correct
pES1028: linker sequencing weird, might be difficult to sequence though
pES1043: actually has CGG RNAP --> relabeled to pES1051
pES1044: actually has CGG RNAP --> relabeled to pES1052
pES1047: correct
pES1048: actually has CGG RNAP --> relabeled to pES1056
pES1049: sequencing weirdly short (starts at wrong point)
pES1061: correct
pES1063: looks recombined (was not recombined in last sequencing though) --> therefore also in 2010 :(
pES1071: has many insertions in RNAP-Nterm (looked just fine in last sequencing though)
pES1073: correct
pES1075: RNAP-Nterm might have a deletion
pES1076: correct
pES1077: correct
pES1078: correct
pEs1079: correct
pES1080: correct
pES1081: correct
pES1082: correct

All of the RNAP-C fragments look super sketchy at the end of the sequencing, I think this is just because wrong sequencing, but I'm not 100% sure (08, 11, 13, 14, 19, 20, 22, 23, 24, 44, 47, 76, 78, 79, 80, 81)

Miniprep: Qiagen Miniprep

pTU1-D1-RFP: 166 ng/µL
pTU1-E-RFP: 146 ng/µL
VanRam TU: 188 ng/µL
pES1029: 421 ng/µL
pES1031: 814 ng/µL
pES1033: 477 ng/µL
pES1035: 568 ng/µL
pES1055: 244 ng/µL
pES1060: 315 ng/µL
pES1084: 240 ng/µL
pES1088: 387 ng/µL
pES1089: 448 ng/µL
pES1090: 177 ng/µL
pES1091: 277 ng/µL
pES1092: 426 ng/µL
pES1093: 132 ng/µL
--> concentrations adjusted to 100 ng/µL

Plates from 12.07.:
nothing is growing so far --> put them to 37 °C

- stbl3 competent cells show colonies -> they competent enough so we can use them

gIII-lux PCR:

- pLS-194g730 10 ng template
- 2 PCR Reactions: to get 1) Luciferase and 2) gIII transcriptionally linked to Luciferase
- Protocol: Ta 61C, Elongation for 1.5 min, 35 cycles
- clean up of PCR Reaction NucleoSpin Gel and PCR Clean-up

Table2

	A	B	C	D	E
1	PCR Reaction	Template	fwd Primer	rev Primer	
2	giii-lux	pLS-194g730	o009	o016	
3	lux	pLS-194g730	o015	o016	

Lvl 0 assembly - restriction digest:
Restriction digest --> digest at 37 °C for 5' and inactivation at 65 °C for 20 min --> PCR clean-up --> T4 ligation
Transformation --> 40 uL of competent cells, 10 uL of ligation product

Golden Gate Assembly/Level 0 ligation: EcoFlex Assembly --> transformed in stable cells

Table1

	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O
1	pES2009	AP1 (for plaque assay), (CGG), strong RNAP, strong tE1 & E2	KanR (+CymRam)	pTU2-A-RFP KanR CymRAM	pES1001	pES1003	pES1004	pES1026	-						
2	pES2021	AP1 (for plaque assay), (CGG), weak RNAP, pCym tE1 & E2	KanR (+CymRam)	pTU2-A-RFP KanR CymRAM	pES1075	pES1092	pES1093	pES1026							
3	pES1094	gIII-lux (D1 Part)	D1	Amp	pTU1-D1-RFP	pES0017	pBP_BBa_B0034	-	gIII-lux PCR product	pBP-L3S2P21					
4	pES1095	VanR	E	Amp	pTU1-E-RFP	VanR TU	-	-	-	-					
5	pES0012	Luciferase	4	ChlorA	pBP	PCR									
6	pES0027	gIII-lux	4	ChlorA	pBP	PCR									

Golden Gate Assembly: EcoFlex Assembly

Table3

	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O
1	pES2008	AP1 (for plaque assay), (CGG), strong RNAP, strong hE1 & E2	KanR (+CymRam)	pTU2-A-RFP KanR CymRAM	pES1001	pES1002	pES1004	pES1026	-						
2	pES2022	AP1 (for plaque assay), (CGG), strong RNAP, pCym tE1 & E2	KanR (+CymRam)	pTU2-A-RFP KanR CymRAM	pES1001	pES1092	pES1093	pES1026							
3	pES1062	PCym_Nterm RNAP_L1_Ub	A	Amp	pTU1-A-RFP	pES0019	pES0003	pES0020	pES0004	pBP-L3S2P21					
4	pES1063	pCymR_Nterm RNAP_L3_Ub	A	Amp	pTU1-A-RFP	pES0019	pES0003	pES0002	pES0004	pBP-L3S2P21					
5	pES1072	weak_HsUba1_E1	B	Amp	pTU1-B-RFP	pBP-J23108	pBP_BBa_B0034	-	pES0001	pBP-L3S2P21					
6	pES1074	weak_UBCH5A E2	C	Amp	pTU1-C-RFP	pBP-J23108	pBP_BBa_B0034	-	pES0007	pBP-L3S2P21					
7	pES1085	pVan_asyn_L4_CtermRNAP(CGG)	A	Amp	pTU1-A-RFP	pES0028	pES0015	pES0022	pES0008	pBP-L3S2P21					
8	pES1087	pVan_NtermRNAP_L3_Ub	A	Amp	pTU1-A-RFP	pES0028	pES0003	pES0002	pES0004	pBP-L3S2P21					

gIII-lux PCR:

- pLS-194g730 10 ng template --> made another 3 PCR reactions + 1 SDM PCR
- 2 PCR Reactions: to get 1) Luciferase and 2) gIII transcriptionally linked to Luciferase
- Protocol: Ta 61C, Elongation for 1.5 min, 35 cycles
- clean up of PCR Reaction NucleoSpin Gel and PCR Clean-up

Table4

	A	B	C	D	E
1	PCR Reaction	Template	fwd Primer	rev Primer	
2	giii-lux	pLS-194g730	o009	o016	
3	SDM	pLS-194g730	o001	o002	elongation time was too short, pcr product is almost 7 kb

Design (+order) of Primers to fix pES2009:

- Designed primers 054 and 055 to fix D141E mutation in LuxA
- Designed Primers (056-059) for Gibson assembly to insert CymRAM back into the back bone
- see excell sheet Primer tab for sequences and details on how I planned to do this

DIUMENGE, 14/7/2024

- [x] miniprep pES1040, pES1057, pES1062, pES2008 and pES2010
- [x] send pES1040, pES1057 and pES1062 for sequencing
- [x] pick pES1094, pES1095, pES0012, pES0027, pES2009, pES2021 and Level 2 troubleshooting test
- [x] transform assemblies in stable cells: 2008, 2022, 1062, 1063, 1072, 1074, 1085 and 1087 (tubes either still in thermo cycler or someone put them in box)
- [x] clean up gIII-lux PCR and potentially use it to assemble missing lvl1 fragments with gIII-lux (tubes either still in thermo cycler or someone put them in box)
- [x] clean up pLS-194g730 SDM and continue with SDM protocol (see June lab journal) (tubes either still in thermo cycler or someone put them in box)

in Lab: Gabriel, Michael

Miniprep and sequencing Qiagen Miniprep

pES1040 overnight cultures did not grow, 2 more set up
Only 1 of 2 pES1057 cultures grew, miniprepped, another culture inoculated

Nanodrop concentrations were pretty low:

pES2010: 154.2 ng/uL
pES1062A: 42.5 ng/uL
pES1057A: 44.5 ng/uL
pES1062B: 76.2 ng/uL
pES2008: 188.3 ng/uL

Colonies

pES2009 and pES2021 were put back into the incubator (no colonies)

PCR cleanups NucleoSpin Gel and PCR Clean-up

White precipitate seen in all PCR reactions before cleanup
gIII-lux: 69 ng/uL
pLS-194g739 SDM: 9 ng/uL -> elognation time of the pcr was too short, explains the low yield

gIII-lux Golden Gate assemblies EcoFlex Assembly

pES1068 and pES1070 show pES0012 as the insert (luciferase) but the title says gIII-lux, so they were set up with the gIII-lux PCR product

Table5

	A	B	C	D	E	F	G	H	I	J
1	**Name**	**Description**	**TU Type**	**Resistance**	**Backbone**	**Insert Part 1**	**Insert Part 2**	**Insert Part 3**	**Insert Part 4**	**Insert Part 5**
2	pES1065	gIII-lux (B part, CAG promoter)	B	Amp	pTU1-B-RFP	pES0018	pBP_BBa_B0034	-	gIII-lux PCR product	pBP-L3S2P21
3	pES1068	Constitutive gIII-lux	B	Amp	pTU1-B-RFP	pBP-SJM910	pBP_BBa_B0034	-	gIII-lux PCR product	pBP-L3S2P21
4	pES1070	Inducible gIII-lux	B	Amp	pTU1-B-RFP	pES0019	pBP_BBa_B0034	-	gIII-lux PCR product	pBP-L3S2P21
5	pES1083	weak_gIII-lux (B part)	B	Amp	pTU1-B-RFP	pBP-J23108	pBP_BBa_B0034	-	gIII-lux PCR product	pBP-L3S2P21
6	pES1086	pVan_gIII-lux (D Part)	D	Amp	pTU1-D-RFP	pES0028	pBP_BBa_B0034	-	gIII-lux PCR product	pBP-L3S2P21
7	pES1094	gIII-lux (D1 Part)	D1	Amp	pTU1-D1-RFP	pES0017	pBP_BBa_B0034	-	gIII-lux PCR product	pBP-L3S2P21

DILLUNS, 15/7/2024

- [x] miniprep pES 1040, pES1094, pES1095, pES0012, pES0027, pES2009, pES2021 and Level 2 troubleshooting test --> send for sequencing
- [x] send pES2008 and pES2010 for full plasmid seq
- [x] Pick colonies/overnight seq for 2008, 2022, 1062, 1063, 1072, 1074, 1085 and 1087
- [x] Transform pES1065, pES1068, pES1070, pES1083, pES1086 and pES1094 assemblies (left in thermocycler overnight, slot 6)
- [x] Continue with SDM protocol (see June lab journal) (PCR cleanup in temp storage) try with cleanded up pcr, protocol is with pcr product directly actually, rerun pcr
- [x] repick 1040, 1057, esp3i

in Lab: Michael, Noemie

Analysis Plates Level 2 Assembly BsmbiI vs Esp3I

- there are a lot more colonies on Esp3I sites -> Esp3i might be more efficient -> consider switching entire protocol to Esp3i (wait on sequencing results to confirm)
- ON cultres from BsmbI are way more red then the Esp3I, very weird, as both should have the same amount of RFP inside (turns out only the white colonies were picked, which are probably the ones that either recombined or assembly went wrong) -> repick again

Minipreps:

- 1040 ABC, 1057 ABC, 1049C didn't grow
- 1095 B had a red pellet, not prepped
- 0012A and 0012C were half spilled at step 7 -> still enough DNA
- sent for sequencing (economy run plate, foun dou that its cheaper than single tubes (>25) , so I sent some random samples just to fill the plate)

Nanodrop concentrations:

0012A: 34.8 ng/uL

0012B: 164.5 ng/uL

0012C: 56.1 ng/uL
0027A: 106.9 ng/uL
0027B: 208.8 ng/uL
0027C: 95.6 ng/uL
1094A: 311.1 ng/uL
1094B: 251.0 ng/uL
1095A: 135.3 ng/uL
1095C: 179.2 ng/uL
BsmBIA: 436.2 ng/uL
BsmBIB: 343.7 ng/uL
BsmBIC: 332.5 ng/uL
Esp3IA: 114.9 ng/uL
Esp3IB: 194.9 ng/uL
Esp3IC: 160.4 ng/uL

Transformation pES1065, pES1068, pES1070, pES1083, pES1086 and pES1094

- transformation in stbl3
- high efficency protocol
- recovery: 2 h, 37C

Picking/night-Seq: 2008, 2022, 1062, 1063, 1072, 1074, 1085 and 1087

- 2008 did not have colonies -> back in the incubator
- 2009 did not have colonies yesterday but was picked and sequenced today
- 2022 and 2021 (2021 two days in a row) did not have colonies either -> 2022 back in the incubator, cloning of 2021 was stopped (we will most likely not even need this)

Repeat Q5 Mutagenesis of pLS-194g730

- repeated, bcs pcr protocol was not the correct one before (see protocol on 27/06)
    - Template: pLS-194g730
    - Primers: 001, 002
    - Ta 56, elongation for 3 min

DIMARTS, 16/7/2024

- [x] sequencing analysis 15/07 NightSeq + MiniPrep correct ones
- [ ] Fix 2009: Gibson assembly (PCR, DpnI Digest, Gel extraction, Assembly Reaction, Transformation)
- [x] pick from plates from 15/07 and 13/7 (in incubator)
- [x] pick 2022 (from 14/7) for overnight seq (if colonies emerge)
- [x] continue protocol SDM pLS194g730 (PCR product in Fridge, KLD + Transformation)
- [x] sequencing analysis 15/07 Economy Plate

in Lab: Noemie, Michael

Colony picking

pES2022, pES1065, pES1068, pES1070, pES1083, pES1086 and pES1094
No colonies on any plate (at 10AM) -> put back in the incubator

Don't know why that assembly didn't work, Transfomration was probably okay, and assembly also looks fine -> maybe PCR didn't work?
I think we should always run a gel (and maybe even do Gel extraction if PCR band does not look super sharp) of our PCR product before using them in our assemblies! saves us multiple days when PCRs don't work

purified PCR reaction was sent for sequencing yesterday (too fill plate -> we'l see how it looks later today/early tomorrow

Sequencing Analysis
Economy Run:

- Only the start (maybe 30-40bp) was sequenced in 1057 and the sequencing stopped right at the usual recombination site -> we suspect recombination (at least partially)
- 1062A+B: recombination in the CymR promotor + multiple mutations in the linker
- 2010: okay (we already knew, this run was redundant)

NightSeq:

- 1062: A pES1007; B: recombined pCym
- 1063: A, B both Backbone with RFP (not assembled)
- 1072A: sequencing stopped after ~50bp
- 1072B: okay
- 1074A+B: both okay -> Prep A
- 1085: Backbone with RFP?, B has promoter SJM910 infront of Wheat Uba1 -> it aligns to pES1003
- 1087A is actually 1063 (pCym promotor) but its also recombined, B is backbone with RFP?
- 1094B is okay
- 1094A is a weird mix of different plasmids, has a chloramphenicol resistance (part of BP?)
- 2009 A, B are both pTU2-A-RFP (KanR, CymRAM)

Economy Run Plate:

- 0012 and 0027 both all 3 clones are empty Backbone, no insert at all
- 1094: A is okey, B has a G98Val luxAB
- 1095: C is okay
- the PCR products we sent were not sequenced (probably because in EconomyRun you have to specifiy if you send a PCR product or a plasmid, and I sent the entire plate as plasmid)
- Doesnt really work to compare BsmbI and Esp3i assembly, colony PCR would have been better (amplify insert & see how long it is) -> but in general, we had way more colonies with Esp3I

Gibson Assembly pES2009 (insert CymRAM)
PCR Backbone

- Template: pES2009 (-CymRAM), from Assembly in June
- Primer: 058, 059
- Ta: 66°C
- Elongation: 5 min
- Cycles: 25

PCR Insert

- Template: TU2-A-RFP (KanR + CymRAM)
- Primer: 056, 057
- Ta: 61°C
- Elongation: 30 s
- Cycles: 35

DpnI Digest of PCR products

- 0.5 uL of Enzyme added to PCR reaction
- incubation 15 min at 37°C

Gel extraction:

- 1% Agarose Gel
- Loading Dye: Purple Loading Dye
- Marker: Genruler 1kb
- 120V, 30 min

Expected Band length Insert: 833 bp
Expected Band length Backbone: 10.5 kb

no visible bands -> PCR failed

Repeated PCR reaction with different Aliquot of Polymerase, new Primer dilutions and new dNTPs aliquot

still no bands on the gel - I give up

Miniprep + Nanodrop
1072B, 1074A, 1094B and Esp3IA+B+C were miniprepped (all other sequences were incorrect)
concentrations:

- 1072B 342.8 ng/uL
- 1074A 199.2 ng/uL
- 1094B 373.8 ng/uL

SDM pLS194g730 KLD treatment and Transformation SDM with Q5 Mutagenesis + KLD Treatment

- Incubation on Ice for 30 min, 30 min recovery

Set up ON culture of S2208 in LB to use for Calibration of the PACE reactor tomorrow
unfortunalty, I lost our glycerol stock of S2208 when put it back into the -80 (I dropped it on the ground and then couldn't find it again) ....sorry :(...

DIMECRES, 17/7/2024

- [x] When Primers arrive: Gibson Assembly of 2014
- [x] assmble pES2017-2020 (once we have pES1094 and pES1095)
- [x] Pick colonies that weren't growing yesterday. If no colonies are present: re-transform

--> wait for 1070, 1083 and 1086 sequencing results - otherwise the whole assembly might have just been unsuccessfull

- [x] Lab duty (Box for Tipboxes was full yesterday nightl but it was too late to bring them)
- [x] continue SDM pLS194g730 (plate in the incubator, also PCRreaction is in the temp storage, if no colonies appear, run a gel to confirm that pcr worked in the first place)
- [x] Calibration for the PACE reactor (ON culture of S2208 is in the shaker)
- [x] new glycerol stock of S2208 --> 15% Glycerol Stock frozen
- [x] I did the sequencing analysis of the EconomyRun plate yesterday, but I didn't move the correct plasmids from temp storage to level 1 box
  --> moved 1095, swapped out 1094

When Level 2 Constructs are grown, or Level 1 constructs with pCym (both plates and liquid culutres) put them at 30°C to prevent recombination

Plates:

pES2022, pES2008 and pLS194g730 --> no colonies, put back in incubator
pES1070, pES1083 and pES1086 --> one colony each, picked and sent for NightSeq
pES1065, pES1068 and pES1094 --> no colonies (do we no already have 1094?)

Golden Gate Assembly: EcoFlex Assembly

Table6

	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O
1	pES2017	AP1 (for plaque assay), (CGG), weak RNAP, pVan hE1 & E2	KanR (+CymRam)	pTU2-A-RFP KanR CymRAM	pES1071	pES1088	pES1090	pES1094	pES1095						
2	pES2018	AP1 (for plaque assay), (CGG), weak RNAP, pVan tE1 & E2	KanR (+CymRam)	pTU2-A-RFP KanR CymRAM	pES1071	pES1089	pES1090	pES1094	pES1095						
3	pES2019	AP1 (for plaque assay), (CGG), weak RNAP, weak tE1, pVan E2	KanR (+CymRam)	pTU2-A-RFP KanR CymRAM	pES1071	pES1073	pES1090	pES1094	pES1095						
4	pES2020	AP1 (for plaque assay), (CGG), weak RNAP, strong tE1, pVan E2	KanR (+CymRam)	pTU2-A-RFP KanR CymRAM	pES1071	pES1003	pES1090	pES1094	pES1095						

Gibson Assembly pES2014

PCR Insert

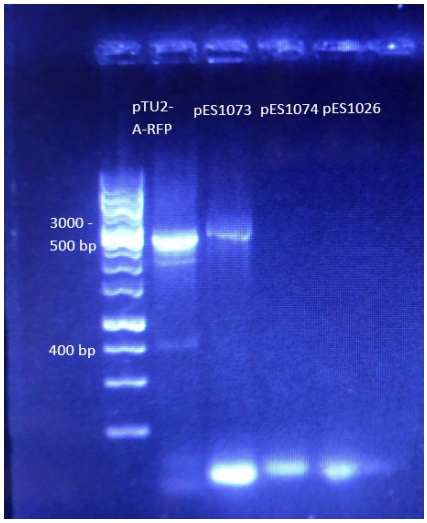
- Template: TU2-A-RFP (KanR + CymRAM) / pES1073 / pES1074 / pES1026
- Primer: 044, 045 / 048, 049 / 050, 051 / 052, 053
- Ta: 72 °C / 66 °C / 68 °C / 61 °C
- Elongation: 1.5 min / 1.5 min / 30 s / 1.5 min
- Cycles: 35

DpnI Digest of PCR products

- 0.5 uL of Enzyme added to PCR reactions
- incubation 15 min at 37°C

Gibson assembly was not done because we don't have a correct pES1063 plasmid. We still ran PCRs with the Gibson primer with the backbone and all other inserts and will load them on a gel after digestion:

image.png



The PCR and digestion of the backbone worked, for 1073 partially and for 1074 and 1026 only partially.
I think it is most likely that I (Michael) made a mistake with the PCR (I pipetted into a wrong tube at one point and had to prepare a seperate master mix for 1074 and 1026 - both samples that didn't work). I will repeat the PCR when I will do Gibson for the next time (probably tomorrow)

Gel extraction:

- 1% Agarose Gel
- Loading Dye: Purple Loading Dye
- Marker: Genruler 1kb
- 120V, 30 min

Expected Band length Insert: 833 bp
Expected Band length Backbone: 10.5 kb

Sequencing Analysis - Full Plasmid Seq:

- pES2008 is completely correct - the miniprep is a mixture (~11kb and ~5.5kb) according to microsynth
- pES2010 is partly correct - CymR and pCym are recombined though

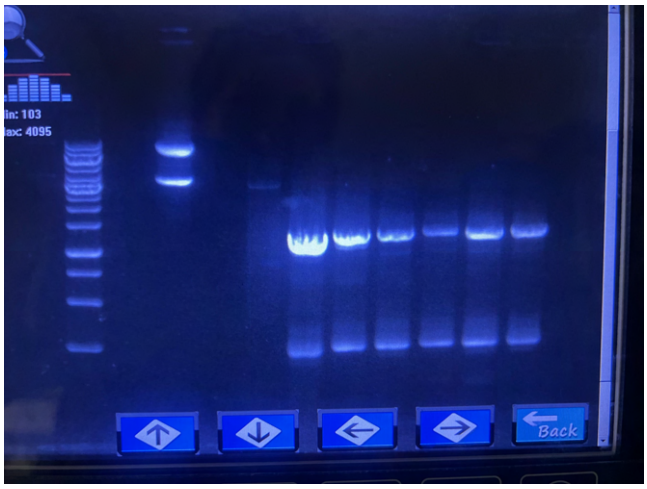
PCR - BsmB1 vs Esp3I:

- 10 ng template from BsmB1 A-C and Esp3I A-C
- Primers: o018, o019
- Protocol: Ta 62C, Elongation for 2.5 min, 35 cycles
- expected fragment size: 4.6 kb

Run all PCR products, the PCR product of pLS194g730 and 10 µL of pES2008 on a 1% agarose gel:

Loading Scheme: Ladder - empty - pES2008 - empty - pLS194g730 SDM - BsmB1 A - BsmB1 B - BsmB1 C - Esp3I A - Esp3I B - Esp3I C

IMG_5186.jpeg



Transformation:
pES2017-2020 --> 5 µL each transformed in 25 µL Stable Cells
pES2008 unpurified --> 5 µL transformed in 40 µL Stable Cells
pES2008 purified --> 3 µL transformed in 40 µL Stable Cells
0.3 µL pES2008 unpurified + 1 µL pES1008 / 1014 / 1055 / 1076 / 1081 transformed in 10 µL S2060 each
--> put everything to 30 °C

Testing our logic:
We got a small alliquote of competent S2060 (without MP) from Lukas. Also we got a cryo-stock of non-competent S2060 (without MP) and innoculated a culture to make a competent batch.

DIJOUS, 18/7/2024

Super-most important:

- [x] look at pES2008 plates and see if they already grew (at 30°C) - didn't grow
- [x] Restart PACE (autoclave tubing, dry autoclave run is at 11AM, wet autoclave is at 2PM --> when you autoclave to media tube in the lb media, this will be autoclaved only in the afternoon and will be very hot when it comes back, it will need a few hours to cool down)

Other things:

- [x] sequencing analysis pES1070, pES1083 and pES1086 --> miniprep if correct --> cultures did not grow
- [x] check pES2017-2020 plates and pick + nightseq if they grew - didn't grow
- [x] make competent S2060
  o/n culture is in shaker -> use ON culture to inoculate larger culture without antibiotics, OD at inocculation shouldn't be higher than 0.01 -> then let it grow to OD 0.5, monitor OD during growth (do not go higher)
  use the Mix&Go Kit to make the competent cells (it is located in the large fridge in the pace room, small yellow box on one of the upper shelfs), protocol should also be in the box or otherwise online

- [x] Gibson Assembly 2014: either redo 1063 and/or order Primers for a different Nterm RNAP
- [x] Prepare fresh LB

Depends on results from wednesday:

- [x] Fix 2009: Gibson assembly (PCR, DpnI Digest, Gel extraction, Assembly Reaction, Transformation) - Troubleshoot PCR bcs it didn't work twice

In lab: Michael, Pau

Colony Picking of transformed lvl2 assemblies

- No colonies on the 30 °C incubator plates (pES2017-pES2020 and pES2008 transformants) -> check again tomorrow
- pLS194g730 SDM plate from two days ago (16.7) doesn't have any colonies :/. Maybe wait one more day and then try to redo the SDM
- 2008 and 2022 from 14.7 don't have any colonies either (only 1 red colony). -> re-assemble and transform 2022. re-assembly of 2008 not necessary as we have a working clone now

Restart TadA PACE

Autoclaved all tubes and tube + LB media.
Prepared new LB media w/ antibiotics and glucose.
Restarted PACE (Echo)
Lagoon --> mixture of phages, see TadA PACE excel spreadsheet
Used the P3c culture from the fridge.

Phage propagation for TadA PACE

Given that we have little left of the more recent phage samples, we have decided to propagate them to inoculate the lagoon.

1. Made liquid culture of the culture of S2208 from 09.07 that was in the fridge, together with a tube containing only LB as a negative control against contamination of our LB, and let it grow to an OD of 0.55
2. Inoculated 10 uL of 02.07 phage in 5mL of LB (qPCR pfu/mL = 1.57·10^7, desired pfu/mL ~ 10^4 (according to this Liu lab paper)) at 37ºC shaker O/N
3. Tomorrow, pellet cells (8000 g for 2 min), filter supernatant w/ 0.22 um filter, and store at 4ºC
4. Perform plaque assay to determine phage titer (and thus the presence of phages)

(Note from 21.07) --> improvements to this protocol have been made, see 21.07

GG assembly of pES1063

EcoFlex Assembly

Table7

	A	B	C	D	E	F	G	H	I	J
1	pES1063	pCymR_Nterm RNAP_L3_Ub	A	Amp	pTU1-A-RFP	pES0019	pES0003	pES0002	pES0004	pBP-L3S2P21

30x cycles, reaction O/N --> do transformation tomorrow

Competent S2060 w/ Mix&Go Kit

O/N culture cells inoculated in 50 mL LB in 150 mL Erlenmeyer at 10:30h. 26ºC, 240 rpm (as recommended in protocol) until OD of 0.4-0.6.

OD at 12:30h --> 0.07

OD at 14:00h --> 0.07

OD at 15.30h --> 0.3

Start Mix&Go at 16.45h. They stay in freezer for ~3h until we put them in the -80ºC --> should not have affected competence.

Lvl 1 assemblies pES1070, pES1083, pES1086

None of the cultures grew... put plates back in the 30ºC incubator and let 37ºC liquid cultures shaking o/n

Sequencing results:

- pES1083 and pES1086 don't align at all (online annotation tools didn't help to find whats on it), 1086 is also only 75 nt long and a mix of different sequences
- pES1070 is only 145 nt long, a mix of different sequences and doesnt align to anything.

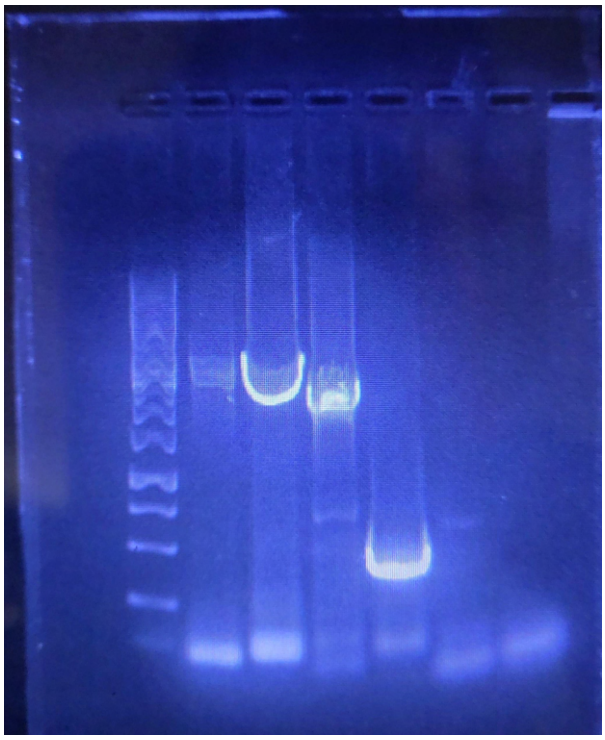
Gibson Assembly PCR of 2009 backbone and insert + 1074 and 1026 (for 2014)

PCR Backbone

- Plasmids: pES2009 (-CymRAM) / TU2-A-RFP (KanR + CymRAM) / 1074 / 1026
- Primer: 058, 059 / 056, 057 / 050, 051 / 052, 053
- Ta: 66°C / 60°C / 61°C / 68°C
- Elongation: 5 min/ 30 s / 30 s / 3.5 min
- Cycles: 30 / 35 / 35 / 35

Gel order: 1073, 1026, TU2-A-RFP (with 2014 overhangs), 1074, TU2-A-RFP (with 2009 overhangs), 2009-CymR

gel.jpg



1073 has a very weak band -> probably no yield in the gel extraction later, 2009 failed again (-> it must be the primers, all the other components of the PCR worked for other products)
I will extract the DNA from the gels tomorrow

Lvl 2 assemblies pES2021 and pES2022 with Esp3I
As advised, I followed the Esp3I protocol from https://wiki.rice.edu/confluence/display/BIODESIGN/Golden+Gate+Assembly

cycling conditions

	step	temp	time
1	initial digestion	37 °C	10 min
2	digestion (30x)	37 °C	1.5 min
3	annealing + ligation (30x)	16 °C	3 min
4	digestion	45 °C	5 min
5	digestion and ligase inactivation	50 °C	10 min
6	inactivation	60 °C	10 min

We are running out of TU2-A-RFP (KanR + CymRAM), there is only 1-2 uL left. -> we will transform the backbone when we do the other transformations

DIVENDRES, 19/7/2024

- [x] look at pES2008 plates and see if they already grew (at 30°C)
  --> if pES2008 purified grew --> pick and send for night seq twice (with primer o038 and o018)
  --> if pES2008 unpurified grew --> do colony PCR (with primers o018 and o001) to find colonies with correct pES2008 and send those colonies for night seq
  --> if co-transformed plates grew, send them for night seq twice (with primer o017 (lvl1 exclusive) and KanStart primer (lvl2 exclusive, in custom primer list))
- [x] check pES2017-2020 plates and pick + nightseq if they grew
- [x] Check pES1070, pES1083, pES1086 o/n cultures (37ºC shaker) and plates (30ºC incubator)
- [x] Run a gel with assemblies 1063, and 2017-2022 before transformation
- [x] Transform pES1063
- [x] Transform pTU2-A-RFP (KanR + CymRAM) for miniprepping
- [x] Transform 2017-2020 + 2021 and 2022
- [x] Gel extraction of the 2014 Gibson PCR products
- [x] Phage propagation day 2 --> in the morning, inoculate 2x 0.5 mL of fridge S2208 in 5 mL LB and incubate 37ºC to make plaque assay
- [x] PACE stuff --> PACE reactor broke
- [x] assemble 1070 in benchling to check the sequencing of 1070

In lab: Michael, Pau

Colony Picking

- still no colonies on 2017 - 2020 (pVan plasmids) --> re-transform today
- still no colonies on the cotransformed plates
- no colonies on gel purified 2008 but we have colonies on unpurified 2008

We will run a colony PCR with different 2008 colonies to (hopefully) get colonies that only have the correct (~11kb) plasmid and none of the incorrect (~5.5kb plasmid)

Colony PCR of "unpurified pES2008"

PCR: Q5 Polymerase

We saw some colonies in the unpurified pES2008. To check which colonies have the right insert, we used primers o018 and o019.
PCR:

- 25 uL reaction
- initial cycle 98 °C for 10 min
- Primers 018&019, Ta 62 ºC, elongation 4 min 30 s, 30x cycles
- Run gel 1% agarose: 120V 40 min

Expected bands:

- Correct backbone + insert = 8.6 kb

Order: Genruler 1kb - pES2008 colony 1 to colony 8

IMG_20240719_164002.jpg

Seems that all colonies have a band at around 1.2kb and one at about 3.5kb...

- 1.2kb might correspond to the backbone (pTU2-A-RFP KanR+CymR)
- 3.5kb --> matches with pES1026 and pES1002

From full plasmid seq we got that bacteria have two plasmids, one 11.5kb and one 5.5kb. The 5.5kb could actually be pES1026 or pES1002.

Idea: extract gel bands to figure out what it actually is.

Gel extraction of the 2014 Gibson PCR + digestion products

- 1073: 6.4 ng/uL (9uL left -> not enough for Gibson assembly, we need 100-200 ng of each insert)
- 1074: 123.5 ng/uL
- 1026: 50.7 ng/uL
- TU2-A-RFP (CmR+KanR): 20 ng/uL

Now 1063 and 1073 are the only parts missing for the 2014 Gibson assembly. After 1063 is confirmed and the sequence is miniprepped, we can run the PCR + digest together with 1073 and then assemble 2014

TadA PACE

PACE reactor broke again. Turned it off until Sasha looks at it again.

Phages from the last day before it broke for the first time (02/07) are being propagated and could be used to seed the new lagoon once PACE is fixed.

Phage propagation for TadA PACE

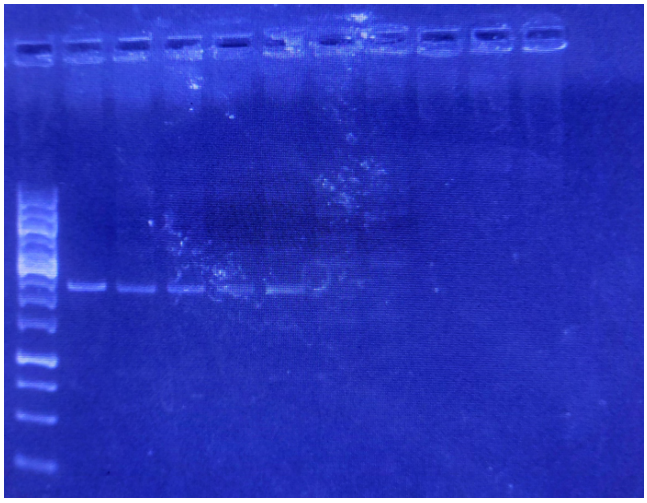
Set a culture of S2208 in the morning.
Pelleted o/n cultures of S2208 with phages and filtered 0.22 um. Filtered phages are in the temp box until plaque assay is analyzed.
Positive control: P 227

(Note from 21.07) --> improvements to this protocol have been made, see 21.07

Gel before transformation:

Took 1 uL of assembly product for the gel which should be around 45-50 ng of total DNA
order: ladder, 1063, 2017, 2018, 2019, 2020, 2021, 2022

image.png



1063 and 2017-2020 have fragments at around 2500 bp which could be inserts (like 1090, 1093)? No DNA was detected in 2021 and 2022. We still decided to proceed with the transfection because we suspect that only a small fraction was assembled and gel red is not sensitive enough to detect such a small ammount of DNA. If the transformation works, our hypothesis might be true (edit on 23.7.: it worked.)

Transformation of pES1063, pES2017-2020, pES2021, pES2022

Transformation

We tried to re-transform pES2017-2020 with what was left in the fridge, and transformed newly assembled pES1063, pES2021 and pES2022
High-efficiency transformation
5 uL reaction in 100 uL Stbl3 (box: A44)
Incubated at 30ºC in a zipper bag.

Troubleshooting of the 3 failed PCR attempts for the Gibson assembly of 2009 (= reinsertion of CymR): The pTU2-A-RFP (CymR+KanR) plasmid we have probably has a 5bp insertion in a non-coding region which happens to be exactly where the forward primer was supposed to bind. This could be one reason the PCR didn't work. The sequence for a primer that accounts for this insertion would be tagaagcggccgcgaattcctgctcgtagtttaCCGGATcctttaca (I ordered it, o056 v2).
Also to extend the ends of a DNA fragment, we should maybe lower the annealing temp. after the first few cycles. Initially, only one part of the primer binds to the DNA but as soon as the ends have been extended, the primer is complementary to 20 bp more and has much lower annealing temp.

DISSABTE, 20/7/2024

- [x] pick colonies of 1063, 2017-2022 (if they grew) --> picking tomorrow
- [x] Phage propagation day 3 --> plaque counting
- [x] 1070, 1083, 1086 had only one colony and bad sequencing results (1085 and 1087 were never successfully assembled too)
  -> PCR product of gIII-lux today and assembly tomorrow
- [x] Inoculate P3c to start TadA PACE tomorrow
- [x] Do gel extraction to understand what we have in "pES2008 unpurified" transformants
- [x] Gel extraction of miniprepped pES2008 and transformation of purified lvl2 plasmid
- [x] Pick pES2008 and pES2008+lvl1 co-transformants

In lab: Gabriel, Pau

Phage propagation of lagoon phages for TadA PACE --> re-start at day 0
pfu/mL is pretty low (plaques at 0 and -1 only) --> could be because I grew S2208 wo/ antibiotics and they lost the gIII plasmid.
Just to make sure, we start new cultures from the glycerol stock at the -80ºC in LB+Carb. Tomorrow we will inoculate phages.

Lvl1 and lvl2 assemblies from 19.07 and pES2008+lvl1 co-transformants --> status
pES1063 and pES2017-pES2022 from 19.07 --> some plates have some small colonies, we leave it O/N at 30ºC and pick tomorrow so that colonies are bigger
pES2008 purified --> no growth
pES2008 + pES1008 --> one colony!! --> could be the backbone though, as suggested by the colony PCR of "unpurified pES2008" transformed colonies --> we do gel extraction of the gel that we ran yesterday to see what is in the "unpurified pES2008" colonies
Other pES2008 + lvl1 co-transformants --> no growth

To check what is in the single colony that grew in pES2008 + pES1008, we perform colony PCR with the following primers:

- Primers o016 and o019 --> will amplify specifically for pES2008 with a band width of 8.4kb (and won't amplify backbone or level 1s)
- Primers o018 and o019 --> will amplify pES2008 (8.6 kb), and we expect a band of 3.2-3.5 kb for whatever level 1 there is (hopefully pES1008, but could also be something else as we saw yesterday that the "pES2008 unpurified" has something that amplifies with that same band length), thus we will need to confirm with seq. Finally we will also see if there is transformed backbone, which would give a signal of about 1.2 kb.

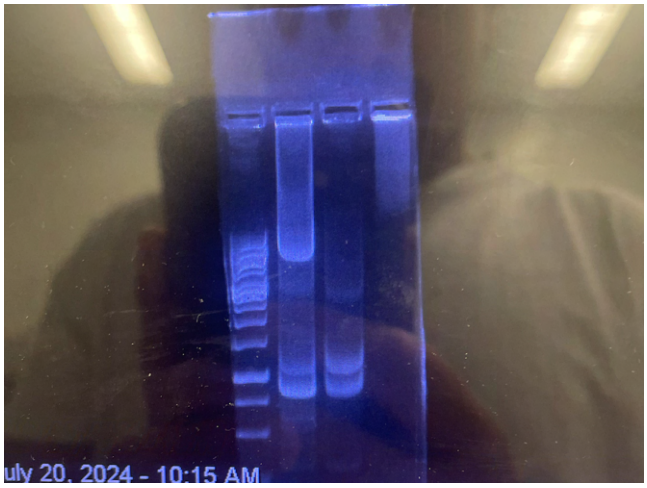
PCR:

- Initial denaturation at 98ºC for 5 minutes

- Tm 57ºC (for both), Elongation time 2 min 30 s
- Ran 1% agarose gel at 120V for 40 min

Order: Ladder - o016&o019 - o018&o019

Screenshot 2024-07-21 at 12.22.22.png



We see a band of the expected size at o016&o019 which should correspond to pES2008, but also two bands at 800bpb and 1kb --> it could be due to non-specific amplification, so we will perform a PCR with untransformed Stbl3 cells tomorrow.

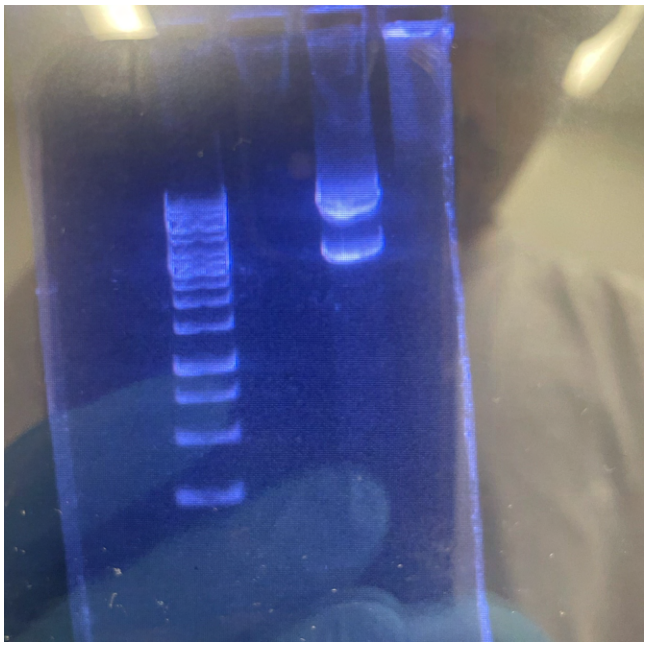
At o018&o019 we see the same two bands --> again, no band of 8.6kb that should correspond to pES2008. The other two bands might be backbone and pES1008, but also some other lvl1 --> we should design specific primers for lvl1s

pES2008 mixture re-extraction and transformation
We run a gel again with 25 uL of minippreped pES2008 and perform gel extraction of the correct band.
Gel bands look exactly the same as last time (see 17.07). We proceed with gel extraction of the uppermost band, which should correspond to pES2008.

NucleoSpin Gel and PCR Clean-up

Ladder - empty - pES2008 unpurified miniprep

Screenshot 2024-07-21 at 12.23.57.png



Yield: 6.7 ng/uL --> will transform tomorrow

Gel extraction of the "unpurified pES2008" transformation colony PCR

NucleoSpin Gel and PCR Clean-up

Extraction of colonies 3 to 5 in the same tube.

Yields:

- Band at 1.2kb --> 28 ng/ul
- Band at 3.5kb --> 17 ng/ul

Sent for sequencing with primer o017.

gIII-lux PCR:

- PCR Reaction to get gIII transcriptionally linked to Luciferase using 0.5uL of pLS-194g730 and primers o009&o016
- Protocol: PCR: Q5 Polymerase reaction volume 50uL, initial denaturation 30s, Ta 61ºC, Elongation time 2 min, 30 cycles, final elongation of 2 min
- clean-up of PCR Reaction NucleoSpin Gel and PCR Clean-up

Table8

	A	B	C	D
1	PCR Reaction	Template	fwd Primer	rev Primer
2	giii-lux	pLS-194g730	o009	o016

DIUMENGE, 21/7/2024

- [x] Produce gIII-lux and then re-assembly of pES1070, pES1083, pES1085, pES1086, and pES1087
- [x] Phage production day 1
- [x] Picking pES1063 and pES2017-2022 (whatever grew) and make o/n cultures
- [x] Transformation of purified pES2008
- [x] Send purified bands from "unpurified pES2008" transformant colonies for sequencing
- [x] Decide what do to with the co-transformant pES2008+pES1008 --> design specific primers for lvl1s?

Phage propagation for TadA PACE - day 1

Protocol (based on this Liu lab paper):

1. Dilute o/n culture of S2208 (or some relatively fresh culture from the fridge) in 5 mL LB+Carb and let it grow to an OD of 0.4-0.6
2. Infect with desired phage sample (desired pfu/mL ~ 10^4) and incubate for 16-20 hours at 37ºC shaker
3. Next day, pellet cells (4500 g for 5 min), filter supernatant w/ 0.22 um filter, and store at 4ºC
4. Perform plaque assay to determine phage titer (and thus the presence of phages)

S2208 cells were diluted and grown to an OD of 0.5 before inoculating 10 uL of phage samples from 02.07, 01.07, and 30.06.

pES2008 co-transformations

pES2008 + pES1076 shows one colony

Purified pES2008, pES2008+pES1055, pES2008+pES1081 --> no colonies

Colony PCR PCR: Q5 Polymerase

Colony PCR done of new cotransformation colony, along with yesterday's single colony again

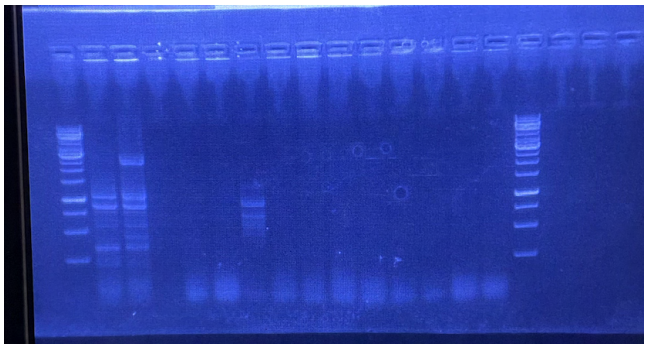
- Primer pair 16 and 19 used to test for insert (annealing 57 C, elongation 2:40)
- primer pair 18 and 19 used to see level 1s (annealing: 50 C, elongation 2:40)

Additionally, both primer pairs were tested with untransformed Stbl3 cells, since there is consistently a band for all colony PCRs around 1.2 kb regardless of primer pair, to see if this may be genomic amplification

8 colonies transformed with unpurified pES2008 were also ran with primer pair 16 and 19 to see if they indeed contain pES2008, to start ON cultures to get more pES2008

Order: ladder - o016&o019 for pES2008+pES1008 - o016&o019 pES2008+pES1076 - Stabl3 control - o018&o019 pES2008+pES1008 - o018&o019 pES2008+pES1076 - Stabl3 control - 8x (o018&o019 "pES2008 unpurified" transformants) - ladder

:(



Lanes 1-3
Expected product for first two bands: around 8.4 kb, not seen. Unspecific amplification seen
Third lane is negative control, no amplification seen as expected

Lanes 4-6
Expected product for lanes 4 and 5: around 8 kb, and around 3.2 kb, no amplification seen
Lane 6 is negative control, unspecific amplification seen

Lanes 7-14
Expected band: around 8.4 kb, not seen

We made a o/n liquid culure of both co-transformants which we could re-analyze with more specific primers, and we have transformed cells again with purified pES2008 plasmid hoping it works.

Lvl1 and lvl2 assemblies and pTU2-A-RFP (KanR+CymRAM) transformations from 19.07
pES1063 --> many white colonies --> pick 3 colonies for culturing
pES2021 and pES2022 --> many red colonies but some white colonies --> pick 3 colonies each for culturing
pTU2-A-RFP (KanR+CymRAM) --> many red colonies --> pick 3 colonies for culturing

Production of gIII-lux for lvl1 assemblies
Ran 5 uL in a 1% agarose gel at 120V for 40min

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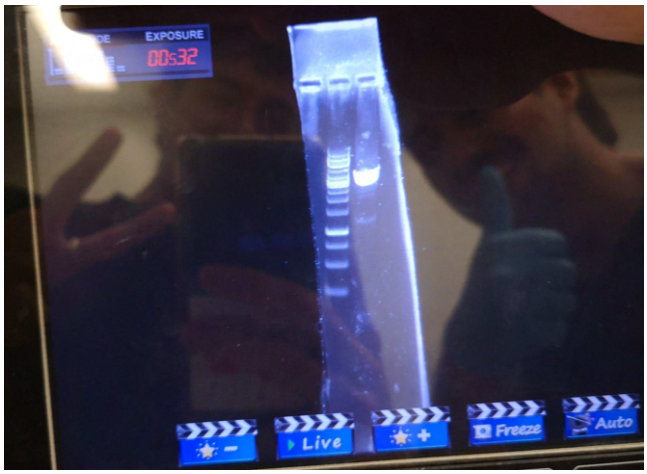


Expected band at 3.4 kb --> we observe that band --> proceed to clean-up

NucleoSpin Gel and PCR Clean-up

Yield --> 86.9 ng/uL --> left in lvl0 box

--> we proceed to assembly of lvl1s.

Assembly of pES1070, pES1083, pES1085, pES1086, and pES1087

EcoFlex Assembly

Table9

	A	B	C	D	E	F	G	H	I	J
1	**Name**	**Description**	**TU Type**	**Resistance**	**Backbone**	**Insert Part 1**	**Insert Part 2**	**Insert Part 3**	**Insert Part 4**	**Insert Part 5**
2	pES1070	Inducible gIII-lux	B	Amp	pTU1-B-RFP	pES0019	pBP_BBa_B0034	-	gIII-lux PCR product	pBP-L3S2P21
3	pES1083	weak_gIII-lux (B part)	B	Amp	pTU1-B-RFP	pBP-J23108	pBP_BBa_B0034	-	gIII-lux PCR product	pBP-L3S2P21
4	pES1085	pVan_asyn_L4_CtermRNAP(CGG)	A	Amp	pTU1-A-RFP	pES0028	pES0015	pES0022	pES0008	pBP-L3S2P21
5	pES1086	pVan_gIII-lux (D Part)	D	Amp	pTU1-D-RFP	pES0028	pBP_BBa_B0034	-	gIII-lux PCR product	pBP-L3S2P21
6	pES1087	pVan_NtermRNAP_L3_Ub	A	Amp	pTU1-A-RFP	pES0028	pES0003	pES0002	pES0004	pBP-L3S2P21

Transformation of purified pES2008 Transformation

We use the purified band that we extracted from yesterday's gel with a yield of 6.9 ng/ul

- 12 ul used since yield was very low and probably highly contaminated

Growing at 30 degrees

Suggestion: label liquid cultures with what antibiotics are in the media (e.g., LB + Carb)

DILLUNS, 22/7/2024

- [x] Miniprep pES1063, pES2021, pES2022 and send for seq, miniprep pTU2-A-RFP(KanR+CymRAM) (do we need to send for seq as well?)
- [x] See if there are colonies in pES2017-pES2020, otherwise repeat assembly
- [x] See if there are colonies in the other pES2008+lvl1 co-transformants
- [x] Restart PACE --> I propagated phages from the last 3 days of PACE (i.e., 02.07, 01.07, and 30.06). Today you can pellet cells, filter supernatant that contains the phage, and use these phages for seeding the lagoon. You can also do a plaque assay parallely just to confirm that there are phages
  --> also, there is an almost fresh P3c culture in the fridge. It is an O/N culture so it is dense, so a possiblity is to take out a bit to make a new liquid culture that could be kept in the fridge as a stock (Sasha said he would like that instead of picking from the -80ºC every time) and dilute the rest in a bit of LB + Antibiotics + Glucose (there is a bottle in the fridge) to start PACE
- [x] Transform pES1070, pES1083, pES1086, pES1087 from o/n assembly

Note: old plates in the fridge have been moved inside labeled bags to the 4ºC room to make space. Newer plates remain in the fridge so they are more in hand.

in Lab: Noemie

Minipreps: pES1063, pES2021, pES2022, pTU2-A-RFP(KanR+CymRAM)

Plates from 30°C Incubator

- pES2017-2020 (from 19.07): there are very few very small colonies on each plate -> picked a few and sent them for sequencing
- pES2008 + lvl1 co transformants: no colonies, moved plates to the fridge,
- pES2008 from 21.7.: no colonies yet, put it back into incubator
- 1 colony on pLS724g240 plate -> picked

Send for Sequencing:

- preps of pES1063 a-c, pES2021 a-c, pES2022 a-c, pTU2-A-RFP (KanR+CymRAM)
- pES2008+1008 // pES2008+1076: sent for Sequencing (NightSeq) with Primers: KanStart, 017
- 2 clones each from pES2017-2020 and one from pLS724g240

TadA PACE: Restart

- put new P3c culture, restarted Pace (but without changing the tubing etc, put a chemostat sample on the plaque assay to chekc for phage contamination)
- seeded with a mixture of 500 uL of each of the 3 propagated phage pools (30.6., 1.7., 2.7.) -> don't know titer, so I just put same amount of each
- turned off reactor after 1 h of runing, because something is wrong with OD measurement, the turbidostat washed out almost completely und reactor didn't stop punping media through

Plaque Assay:

- chemostat, and propagated phages 30.6., 1.7., 2.7.
- unfortunatly forgot pos. ctrl

Transform pES1070, pES1083, pES1086, pES1087, high efficiency protocol with 1 h recovery

DIMARTS, 23/7/2024

- [x] Sequencing analysis of the 3.5 kb and the 1.2 kb band that appeared in pES2008 unpurified colony PCR
- [x] Sequencing EconomyRun and NightSeq from 22.7.
- [x] Once we have cotransformed cells --> we can start testing our logic --> innoculate cultures of cotransformed cells with SIAH phages and let grow o/n
  --> have negative control transformed with empty backbone instead of pES2008 and also innoculate with phages
  --> negative control innoculated with wrong phages
  --> positive control with S2208 innoculated with SIAH phages
  --> do qPCR (and I would suggest to also do a plaque assay in paralel)
- [ ] Fix 2009: Gibson assembly (PCR, DpnI Digest, Gel extraction, Assembly Reaction, Transformation) - Troubleshoot PCR bcs it didn't work thrice -> new primer was ordered by Michael as far as I know => Once gibson assembly 2009 have worked: Fix 2009: Q5 Mutagenesis to remove lux mutation (with the product of the Gibson Assembly if the assembly works)
- [x] Make cuminic acid stock solution (100 mM) -> Lorze fragen
- [x] See if there are colonies in the pES2008 we transformed on 21.07
- [x] look transformation pES1070, pES1083, pES1086, pES1087 plates, in 37C incubator
- [x] PACE: Prepare new tubing & clean glasware for autoclaving
- [x] send lvl2 again economyrun? change primer
- [x] 1063 plate & pick again -> small colonies, run 1063 assembly on gel? colony PCR
- [x] 2014 Gibson Assembly: change insert 1063 to something else we already have -> order primers

in Lab: Noemie, Michael

Plaque Assay:

- there were phages in the chemostat -> exchange tubing and glassware (titer: around 4 * 10^6 pfu/mL)
- phage pool 30.6., propagated: 1.25 * 10^11 pfu/mL
- phage pool 1.7., propagated: 3 * 10^10 pfu/mL
- phage pool 2.7., propagated: 2 * 10^10 pfu/mL

Sequencing Analysis:

- pES2008 unpurified low band and high band: sequencing failed for both -> possible reason: 017 primer was used, which binds level 1 backbone -> as these are level 2 constructs, the primer shouldn't bind -> it is actually good that sequencing didn't work
  another reason why the couldn't sequence could be that the sample was very impure
- EconomyRun for all Level 2s failed with KanStart Primer -> send again with 018 -> or the constructs are completely wirong don't have the Kanamycin resistance? but that would be weird bcs they grow on the Kan plates
- TU2-A-RFP KanR CymRAM: ok
- 1063 a-c: all 3 show recombination in the primer -> why? we now use the Stbl3 cells + growth at 30°C? -> liquid cultures for Minipreps were still grown at 37°C -> search for plate

When I looked at the plate there were 2 differentl ysized colonies -> we observed before that in this case the small colonies are likely to be unrecombined, so the small colonies should have been picked (the ones that were marked were the larger ones) -> do colony PCR

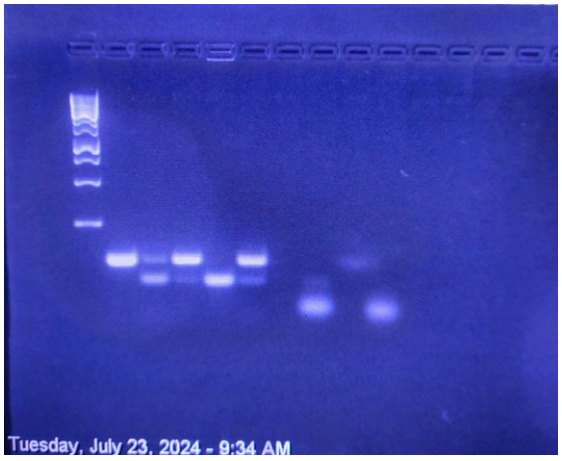
- NightSeq 2017, 2018, 2019, 2020, SDM all failed -> why? -> maybe concentration was too low bcs the colonies were quite small -> grow the ON culutres and prep them, and sen dfor sequencing afterwards
- Contransformations:
    - pES2008/pES1008 + o017: sequencing shows UBA1, sequence is on 2008, but does not confirm presence of 1008
    - pES2008/pES1008 + KanStart: Plasmid 2008 with CymRAM is present (covers This, not much more of the plasmid) -> but read is super low quality and ver noisy
    - pES2008/pES1076 + o017: shows pES1076 is present
    - pES2008/pES1076 + KanStart: Plasmid 2008 with CymRAM is present (covers This, not much more of the plasmid) -> but read is super low quality and ver noisy

  I would be careful and keep these low qulity/noisy sequencing results in mind, but they do show that the two plasmids we need (might) be in the cells -> try a first Assay to test our logic!

1063 Colony PCR and inoculation

We did a colony PCR with 5 pES1063 colonies and primers 42/43 to check for recombination in the promotor. Having a recombined promotor yields a smaller PCR product as seen in the 2% gel below
(1kb ladder, 5* 1063 colonies, empty, TU2-A-RFP (KanR+CymR) Gibson PCR, 2009-CymR Gibson PCR)

20240723_1063gel.jpg



Since the first, third and fifth colony probably have the non-recombined promotor, they were inoculated in a O/N culture in the 30°C shaker (labelled 1063A, 1063B, +1063C)

2009 Gibson Assembly attempt nr. 4

- Another unsuccessful attempt to extend the ends of 2009-CymR and CymR of the backbone for a Gibson assembly. It did not work again, as seen in the gel above.
- I used the same protocol and temperatures as before with two changes:

- the TU2-A-RFP (KanR+CymR) F primer was replaced with a new primer that contains the insertion that we have in our sequenced plasmid. However, I did not compare the melting temp. of this primer to the reverse primer before ordering, and they were 10°C apart (mean temp. was chosen for PCR) which might have reduced the efficiency by a lot
- Additionally, I adjusted the annealing temperatures: After 3 cycles with previous annealing temps (assuming partial primer annealing), the temperature was increased to 72°C for the remaining cycles because at this point we assume that the ends should have been extended successfully. This would reduce the probablilty of getting off-target products. However it was a bit useless, since we still have no product and maybe still a little bit off-target products (blurry bands).

I have no idea how to fix this. We could try to go much lower with the annealing temperatures? maybe longer annealing time for longer primers? I will also check for secondard structures of the primers

Phage propagation assay with the Strain 2060 + pES2008 + pES1076

- Starting phage titer: calculated to 10^6 pfu/mL (diluted first 1:100)
  - SIAH 1: 1* 10^11 pfu/mL -> dilute 1:100 & add 1 uL
  - SIAH2: 2.3* 10^11 pfu/mL -> dilute 1:100 & add 0.5 uL
  - TadA 2.7.: 2*10^10 pfu/mL-> dilute 1:100 & add 5 uL
- Set up liquid culutres of the strain, monitor OD, adjust it to OD 0.1 and then aliquot it in the deep-well-plate
- 1 mL culture volume in Deep Well plate
- 3 Replicates of every condition
- seal with a breathable cover, incubation O/N shaking at 37°C

Table10

	A	B	C	D	E	F	G	H	I
1	Condition Nr	S2060 + pES2008 + pES1076	S2208	SIAH1 phages (1:100)	SIAH2 phages (1:100)	TadA phages from pool 2.7. (1:100)	Media		Wells
2	1	1 mL culture, OD 0.1	-	1 uL			LB + Kan + Carb		A1-3
3	2	1 mL culture, OD 0.1	-		0.5 uL		LB + Kan + Carb		B1-3
4	3	1 mL culture, OD 0.1	-			5 uL	LB + Kan + Carb	neg. ctrl to check for gIII expression in absence of E3	C1-3
5	4	-	-	1 uL			LB + Carb	neg. ctrl in LB to determine starting titer	D1-3
6	5	-	-		0.5 uL		LB + Carb	neg. ctrl in LB to determine starting titer	E1-3
7	6	-	1 mL culture, OD 0.1	1 uL			LB + Carb	pos. ctrl	F1-3
8	7	-	1 mL culture, OD 0.1		0.5 uL		LB + Carb	pos. ctrl	G1-3
9	8	1 mL culture, OD 0.1					LB + Kan + Carb	pos. ctrl to see if cells grow	H1-3
10									

Plate pES2008 from 21.08: 4 colonies grew -> Picked all 4 (growth at 30°C for the liquid cultures as well) with Primers -> we ran out of sequencing labels, I just had 1 left (only sent that one, for the others prep the ON cultures and send those with an appropriate primer)

Plates pES1070, pES1083, pES1086, pES1087 from 23.08: colonies were still very small, kept then in incubator a bit longer

Cumic acid stock:

- 164.2 g/mol; 0.1 M solution = 16.4g/L = 16.4 mg/mL
- 1000x (100mM) cumic acid stock is in the small fridge in our lab (dissolved in 50/50 water and ethanol)

Primer design for level Gibson assembly

- Assembly of 2014 couldn't be finished because 1063 always recombined -> we'll make the same level two plasmid but replace 1063 with other Nterm_RNAP-Linker-Ub combinations. The new plasmids will be pES2023 and pES2024
- The reverse primer has an annealing temp of 71°C

Table15

	primer name	target plasmid	sequence	annealing Temp. of complimentary part (NEB)	length (nt)
1	o060	pES1071	attcgcggccgcttctagaCGTCTCAATCTCTATCTGACAGCTAGC	68°C	46
2	o061	pES1075	attcgcggccgcttctagaACGTCTCAATCTCTATCTGACAGCT	67°C	44

PACE: prepared tubing for autoclaving

DIMECRES, 24/7/2024

- [x] Additional Lab Duty 22.7.- 26.7.: Prepare Glassware for autoclaving -> when clean glasware comes back after washig (usually it is put either in the hallway or near tatjanas bench), cover openings with aluminium foil, put autoclaving tape with room number, and bring them to the place for autoclaving
- [x] Pick pES1070, pES1083, pES1086, pES1087 (plates) --> picked for o/n cultures, no NightSeq labels (have been ordered) so miniprep tomorrow
- [x] check if ON culutres grew (in the 30C shaker) -> prep pES2017-2020, SDM prep& send for sequencing (Nightseq didn't work, consider changing primer) --> did not grow, put back to incubator
- [x] Sequencing Analysis of the 2008 purified clones from 21.7. (ON cultures are in the shaker) -> I only sent 1 for NightSeq bcs we ran out of sequencing labels
- [x] Phage propagation assay in s2060+pES2008+pES1076 (on the robot): --> do qPCR (and I would suggest to also do a plaque assay in paralel)
- [ ] Sequencing analysis pES2021, 2022

In lab: Michael, Pau

O/N culture picking and miniprep Qiagen Miniprep

all 2017-2020 cultures and pLS194g730 SDM didn't grow but the four 2008 purified cultures grew. yield:

- purified 2008.1: 125.1 ng/uL
- purified 2008.2: 681.4 ng/uL
- purified 2008.3: 508.6 ng/uL
- purified 2008.4: 566.8 ng/uL

2008.2 - 2008.4 were sent for sequencing with KanStart.
2020 and SDM were re-inoculated but with only one colony since there wasn't more on the plate. 2017 - 2019 didn't have any more colonies on the plate :/

Sequencing analysis

- Nightseq purified 2008.1 (KanStart primer): There are mutations in the linker (as expected) but also in the ps70/RBS part. Also, the whole CymRAM region is missing which makes us assume that it was recombined out.

S2060 pES2008+pES1076 qPCR

Negative control (LB+SIAH1/2) was contaminated with cells

Perform qPCR with primers oLS1662 and oLS1663 as described in qPCR: Phage Titer
Made duplicates for each replicate and included LB+MasterMix and MasterMix-only negative controls.

Results:

Table16

	Cells	Phage	Cp
1	S2060 + pES2008 + pES1076	SIAH1	11.71
2		SIAH2	14.64
3		TadA	23.82
4	LB (contaminated)	SIAH1	17.74
5		SIAH2	19.17
6	S2208	SIAH1	7.00
7		SIAH2	6.69
8	S2060	-	26.45
9	LB	-	32.96
10	Master mix	-	32.11

Without LB+phage as a control we cannot know what the initial concentration of phages is and thus how much it propagated in our cells. Because the negative control got contaminated, we have to repeat the experiment. We will add the following controls:

1. Filter SIAH phages again 0.22um

2. Add negative control with LB + TadA
3. Add Kan to the negative controls with LB --> should not affect our readings and at least if the contamination comes from outside (e.g., LB bottle, S2208 cells in the SIAH phage samples) we would reduce risk of contamination
4. Add wells with only LB to discard cross-contamination / the LB being contaminated to start with
5. Position negative controls on the other side of the plate to reduce risk of cross-contamination even more
6. Add condition with only pES2008 transformants --> first we need to transform S2060 cells with only pES2008. We will wait until we have the sequencing results tomorrow to make sure that pES2008 is right again and then proceed with transformations of pES2008-only and co-transformations

For now, we repeat the experiment with considerations 1 to 5.
Note: because the filters are big, they are not suited to filter low volumes. I manage to get 100-200uL of the filter but before losing the rest of the sample I just pull it back and return the unfiltered sample to the original tube. Thus, the new tubes with handwritten "SIAH 1 (or 2) phages" corresponds to the newly filtrated phages.

Phage propagation assay with the Strain 2060 + pES2008 + pES1076 (attempt 2)

- Starting phage titer: calculated to 10^6 pfu/mL (diluted first 1:100)
    - SIAH 1: 1* 10^11 pfu/mL -> dilute 1:100 & add 1 uL
    - SIAH2: 2.3* 10^11 pfu/mL -> dilute 1:100 & add 0.5 uL
    - TadA 2.7.: 2*10^10 pfu/mL-> dilute 1:100 & add 5 uL
- Set up liquid culutres of the strain, monitor OD, adjust it to OD 0.1 and then aliquot it in the deep-well-plate
- 1 mL culture volume in Deep Well plate
- 3 Replicates of every condition
- seal with a breathable cover, incubation O/N shaking at 37°C
- Applied previously mentioned considerations, see updated table:

Table17

	A	B	C	D	E	F	G	H	I
1	Condition Nr	S2060 + pES2008 + pES1076	S2208	SIAH1 phages (1:100)	SIAH2 phages (1:100)	TadA phages from pool 2.7. (1:100)	Media		Wells
2	1	1 mL culture, OD 0.1	-	1 uL			LB + Kan + Carb		A1-3
3	2	1 mL culture, OD 0.1	-		0.5 uL		LB + Kan + Carb		D1-3
4	3	1 mL culture, OD 0.1	-			5 uL	LB + Kan + Carb	neg. ctrl to check for gIII expression in absence of E3	C1-3
5	4	-	-	1 uL			LB + Kan	neg. ctrl in LB to determine starting titer	A9-12
6	5	-	-		0.5 uL		LB + Kan	neg. ctrl in LB to determine starting titer	B9-12
7	6	-	-	-	-	5 uL	LB + Kan	neg. ctrl in LB to determine starting titer	C9-12
8	7	-	1 mL culture, OD 0.1	1 uL			LB + Carb	pos. ctrl	F1-3
9	8	-	1 mL culture, OD 0.1		0.5 uL		LB + Carb	pos. ctrl	G1-3
10	9	1 mL culture, OD 0.1					LB + Kan + Carb	pos. ctrl to see if cells grow	H1-3
11	10	-	-	-	-	-	LB + Kan	neg. ctrl of LB	D9-12

S2060 pES2008+pES1076 plaque assay

Plaque assay following protocol described in Phage Production using fresh S2208 cells from fridge culture.

4x plates:

- Plate 1: S2060+pES2008+pES1076 + SIAH1 - "" SIAH2 - Control
- Plate 2: LB + SIAH1 - LB + SIAH2 - Control (LB+phage controls were contaminated, so will have to repeat)
- Plate 3: S2208+SIAH1 - S2208+SIAH2 - Control
- Plate 4: S2060 + TadA phages + S2060 + Control

Control: P 227 (fridge)

DIJOUS, 25/7/2024

- [x] Additional Lab Duty 22.7.- 26.7.: Prepare Glassware for autoclaving -> when clean glasware comes back after washig (usually it is put either in the hallway or near tatjanas bench), cover openings with aluminium foil, put autoclaving tape with room number, and bring them to the place for autoclaving
- [x] check if ON culutres grew (in the 30C shaker) -> prep pES2017-2020, SDM prep& send for sequencing (Nightseq didn't work, consider changing primer)
- [x] Miniprep p1063, pES1070, pES1083, pES1086, pES1087
- [x] As soon as 1063 is mini prepped and the sequence is confirmed (), do a PCR with the Gibson primers (o046 and o047) and digest according to the Gibson protocol. Do the same for 1073 (primers o049 and o050). Run the digests on a Gel -> Then do the Gibson assembly of 2014 (all other parts of 2014 are in the temp. storage box)
- [x] Phage propagation assay in s2060+pES2008+pES1076 (on the robot): --> do qPCR + plaque assay
- [x] If sequencing results for purified+miniprepped pES2008 are correct --> repeat co-transformations --> they are not correct...
- [x] Prepare new LB (small and big bottle)
- [x] PACE: Assemble Reactor, restart PACE (phage pools, propagated., and P3c liquid culture stock are in the fridge -> use the liquid culutre stock to set up a new culutre & use this to start PACE)
- [x] Lab Duty: Bring Pipette Tip boxes for recycling + Cardboard recycling
- [x] Repeat phage propagation assay with other "negative control phages"--> P 227

Lvl1 and lvl2 o/n cultures

Grew: pES1063 A+C, pES1070 C, pES1083 A-C, pES1085 A-B, pES1086 A, pES1087 C --> miniprep

Some show a very mild growth, so one more day in the 30ºC should do the job --> put them back in the 30ºC incubator.

SDM didn't grow and the plate also doesn't have any colonies left (I left it in the incubator for one night)

Miniprep, Nanodrop and Sequencing

- 1063 A: 35.1 ng/uL
- 1063 C: 61.1 ng/uL
- 1070 C: 70.4 ng/uL
- 1083 A: 45.6 ng/uL
- 1083 B: 96.2 ng/uL
- 1083 C: 34.5 ng/uL
- 1085 A: 66.6 ng/uL
- 1085 B: 107.6 ng/uL
- 1085 C: 80.1 ng/uL
- 1086 A: 76.6 ng/uL

- 1087 C: 42.2 ng/uL

Everything was sent for for sequencing. Since the concentrations are so low (many bacterial cultures were not super dense yet, maybe there was any other issue with the miniprep?), only little volume is left in some tubes (min. 8 uL)

Sequencing Analysis of 2008, 2021 and 2022

- All four purified 2008 samples that were sequenced had insertions and other mutations in the promotor/RBS region (look like a recombined or mutated pCym promotor) and a CymRAM deletion (the Nterm_RNAP_L_ubiquitin part looks fine, but its probably useless if the promotor doesn't work)

I have no idea how to proceed

- 2021 and 2022 sequences didn't exist on Benchling, I created them but the sequence also only aligns partly and looks very weird and doesn't align where it should. It might be consisting of other parts than we expect but I didn't figure it out

Gibson PCR, Gel and Assembly

As suggested in the lab meeting, I retried the PCR with the gibson primers but alwas with at least 5°C lower than the calculated annealing temp.

For primers that had annealing temps far apart, I used a temperature that was slightly below the annealing temperature of the primer with the lower temp.

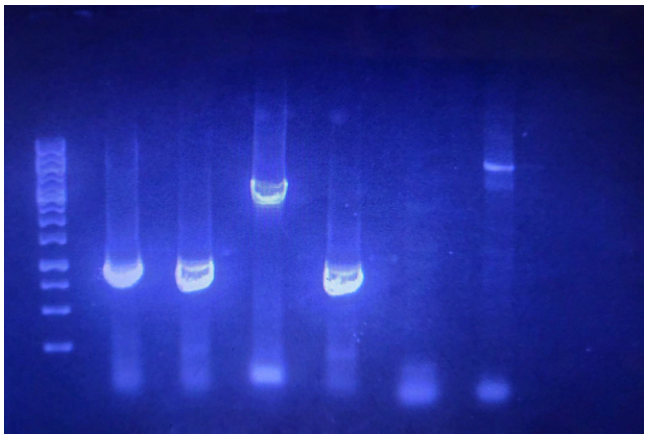
Also I increased the annealing time from 15 to 20s since some primers are very long

end extension PCR

	part	primer	annealint temp (°C)	extension time	E
1	1063 (A)	46,47	59	1 min	
2	1071	60,47	65	1 min	
3	1075	78,47	65	1 min	
4	TU2-A-RFP (KanR + CymR)	56v2, 57	55	30s	
5	2009	58, 59	61	5 min	

order: 1063, 1071, 1073, 1075, backbone, 2009-CymRAM. I left one lane free between every sample to avoid cross-contamination when cutting out the bands

20240725_gel_gibson.jpg



I will assemble 2014 and 2 variants of this plasmid (with 1071 and 1075 instead of 1063) tmr after extracting the DNA from the gel
The backbone fragment for 2009 didn't work again, even though I put the annealing temp. very low (55°C). I'm confused

S2060 pES2008+pES1076 qPCR (attempt 2) - results
Negative control with LB+phages looks clean!
Perform qPCR with primers oLS1662 and oLS1663 as described in qPCR: Phage Titer
Made duplicates for each replicate and included LB+MasterMix and MasterMix-only negative controls.

Results:

Table18

	Cells	Phage	Cp
1	S2060 + pES2008 + pES1076	SIAH1	15.72
2		SIAH2	18.84
3		TadA	26.86
4	LB	SIAH1	23.59
5		SIAH2	25.97
6		TadA	31.13
7	S2208	SIAH1	6.56
8		SIAH2	6.69
9	S2060	-	27.50
10	LB	-	30.45
11	Master mix	-	29.79

Results indicate that SIAH1/SIAH2 are propagating in our cells, but TadA also showed an increase from a Cp below limit of detection (~30) to 27, so we cannot be sure that there isn't random assembly of split polimerase subunits. However, it is also weird that LB-TadA shows a Cp below the detection limit... Finally, plaque assay showed that the S2060-only condition had phages. Improvements:

- Separate the wells with S2060-only from the S2208 positive control condition to avoid phage cross-contamination
- Change TadA phage for another negative control --> using P 227, the phage we use for positive control in plaque assays (I think it comes from the CRISPR experiments so it will do the job as negative control
- Increase starting OD to 0.4-0.6, as is suggested in this Liu lab paper

S2060 pES2008+pES1076 qPCR (attempt 3) - set-up

- Starting phage titer: calculated to 10^6 pfu/mL (diluted first 1:100)
  - SIAH 1: 1* 10^11 pfu/mL -> dilute 1:100 & add 1 uL
  - SIAH2: 2.3* 10^11 pfu/mL -> dilute 1:100 & add 0.5 uL
  - P227 2.7.: 6.8*10^10 pfu/mL-> dilute 1:100 & add 1.5 uL
- Set up liquid culutres of the strain, monitor OD, adjust it to OD 0.4-0.6 and then aliquot it in the deep-well-plate
- 1 mL culture volume in Deep Well plate
- 3 Replicates for every condition
- seal with a breathable cover, incubation O/N shaking at 37°C
- Applied previously mentioned considerations, see updated table:

Table19

	A	B	C	D	E	F	G	H	I
1	Condition Nr	S2060 + pES2008 + pES1076	S2208	SIAH1 phages (1:100)	SIAH2 phages (1:100)	P 227 (1:100)	Media		Wells
2	1	1 mL culture, OD 0.1	-	1 uL			LB + Kan + Carb		A1-3
3	2	1 mL culture, OD 0.1	-		0.5 uL		LB + Kan + Carb		D1-3
4	3	1 mL culture, OD 0.1	-			1.5 uL	LB + Kan + Carb	neg. ctrl to check for gIII expression in absence of E3	C1-3
5	4	-	-	1 uL			LB + Kan	neg. ctrl in LB to determine starting titer	A9-12
6	5	-	-		0.5 uL		LB + Kan	neg. ctrl in LB to determine starting titer	B9-12
7	6	-	-	-	-	1.5 uL	LB + Kan	neg. ctrl in LB to determine starting titer	C9-12
8	7	-	1 mL culture, OD 0.1	1 uL			LB + Carb	pos. ctrl	F1-3
9	8	-	1 mL culture, OD 0.1		0.5 uL		LB + Carb	pos. ctrl	G1-3
10	9	1 mL culture, OD 0.1					LB + Kan + Carb	pos. ctrl to see if cells grow	F9-12
11	10	-	-	-	-	-	LB + Kan	neg. ctrl of LB	D9-12

S2060 pES2008+pES1076 plaque assay (attempt 2) - set-up

Results from previous phages show more phages in the negative (contaminated) control than in the co-transformants+phages. Also, it shows phages in the control where there were only co-transformant cells without phages. To make sure that our original liquid culture of co-transformants are not contaminated with phages, we will perform plaque assay with this culture.

Plaque assay following protocol described in Phage Production using fresh S2208 cells from fridge culture.

4x plates:

- Plate 1: S2060+pES2008+pES1076+SIAH1 - ""+SIAH2 - ""+TadA - Control
- Plate 2: LB+SIAH1 - LB+SIAH2 - LB+TadA - Control
- Plate 3: S2208+SIAH1 - S2208+SIAH2 - Control
- Plate 4: S2060 (from deep-well) - S2060 original culture - LB (from deep-well)- Control

Control: P 227 (fridge)

DIVENDRES, 26/7/2024

- [x] 2014 Gel extraction and Gibson assembly
- [x] Sequencing analysis
- [x] Lab Duty: Bring Pipette Tip boxes for recycling + Cardboard recycling
- [ ] Additional Lab Duty 22.7.- 26.7.: Prepare Glassware for autoclaving -> when clean glasware comes back after washig (usually it is put either in the hallway or near tatjanas bench), cover openings with aluminium foil, put autoclaving tape with room number, and bring them to the place for autoclaving
- [x] check if ON culutres grew (in the 30C shaker) -> prep pES2017-2020, SDM prep& send for sequencing (Nightseq didn't work, consider changing primer)
- [x] PACE: Assemble Reactor, restart PACE (phage pools, propagated., and P3c liquid culture stock are in the fridge -> use the liquid culutre stock to set up a new culutre & use this to start PACE)
- [x] As soon as our economy stickers arrive, put 10 stickers in Lukas' drawer
- [x] pasteurize OD standards (for calibrating PACE reactors)
- [x] Phage propagation assay in s2060+pES2008+pES1076 (on the robot) attempt 3: --> do qPCR + plaque assay
- [x] Count plaques for plaque assay attempt 2
- [x] Miniprep colonies that grew
- [x] transform 2008, 2009 and 2014

Sequencing Analysis:

pES1063: A: very short sequencing, C: recombined pCym -> trashed

pES1070: pCym recombined, big chunks missing -> trashed

pES1083: A and B are good, C has an insertion -> C trashed

pES1085: all look good (still re-inoculated a new O/N culture with 1085 because we have very little total DNA)

pES1086: does not allign, restriction site is A instead of D, might be another plasmid

pES1087: has a large insertion in RNAP (10 nt)

Liquid Cultures --> minipreped

pLS-724g230 SDM, pES1087A, pES1087B and pES2020 grew and were miniprepped

--> we discarded the other tubes

yields:

- 1083A: 138.6 ng/uL
- 1087B: 124.5 ng/uL
- SDM: 20 ng/uL
- 2020: 27 ng/uL

I don't understand what went wrong and why two plasmids have such low yields. I followed the protocol carefully and even pre-warmed the columns before eluting. 20 ng/uL is not even enough for sequencing, so have to hope that other liquid colonies grow until tomorrow. sorry :(
Since getting 2020 was such a pain (grew super slowly, transformation and inoculation failed multiple times), we decided that we will transform it anyways into S2060 and then send it for night-seq and in the best case we have a lvl2 plasmid thats already in the right strain.

Gel extraction and Gibson assembly NucleoSpin Gel and PCR Clean-up

- 1063: 60.6 ng/uL
- 1071: 55.5 ng/uL
- 1073: 22.5 ng/uL
- 1075:64.0 ng/uL
- 2009-CymR: 9.7 ng/uL (expected, very weak band)

Gibson Assembly

I assembled 2014 with 1071 instead of 1063 since the integrity of 1063 couldn't be confirmed (very short sequencing)

Gibson assembly reaction

	component	final concenctration (pM)	added volume (uL)
1	TU2-A-RFP (KanR + CymR)	20	2.5
2	1071	40	0.5
3	1073	40	1
4	1074	40	0.5 (of 5x dilluted DNA)
5	1026	40	3.75
6	NEBuilider HiFi DNA Assembly Master Mix (2X)		8.25

Incubated at 50°C for 1h
Estimated concentration of the assembled plasmid if 100% of the backbones contain inserts: 137.5 ng/uL. Realistically it's probably 50-100ng/uL
1026 is almost empty -> make new 1026 with overhangs if this doesnt work

Plaque Assay Analysis (attempt 2):
Plate 1: have some colonies on -3 (SIAH1) and -2 (SIAH2)

Plate 2:have more colonies on -3 (SIAH1) and -2 (SIAH2)
Plate 3: positive controls look good (all grew)
Plate 4: negative controls look good (did not grow)
--> wait for more results, but seems like the phages did not propagate

S2060 pES2008+pES1076 qPCR (attempt 3) - results

Negative control with LB+phages looks clean!

Perform qPCR with primers oLS1662 and oLS1663 as described in qPCR: Phage Titer

Made triplicates for each replicate and included LB+MasterMix and MasterMix-only negative controls.

Results:

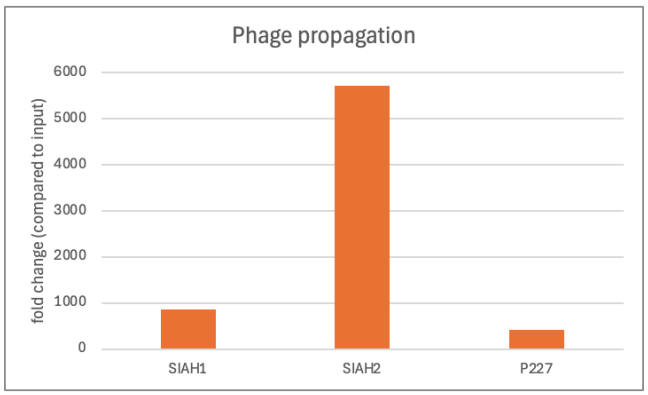
Table20

	Cells	Phage	Cp
1	S2060 + pES2008 + pES1076	SIAH1	13.65
2		SIAH2	16.32
3		P227	18.02
4	LB	SIAH1	23.41
5		SIAH2	28.80
6		P227	26.72
7	S2208	SIAH1	6.9
8		SIAH2	6.3
9	S2060	-	25.09
10	LB	-	28.07
11	Master mix	-	28.94

Phage propagation:

- SIAH1 --> 867-fold
- SIAH2 --> 5700-fold (but initial CT value weirdly low), might be lower
- P227 --> 415-fold

image.png



S2060 pES2008+pES1076 plaque assay (attempt 3) - set-up

Plaque assay following protocol described in Phage Production using fresh S2208 cells from fridge culture.

4x plates:

- Plate 1: S2060+pES2008+pES1076+SIAH1 - ""+SIAH2 - ""+P227 - Control
- Plate 2: LB+SIAH1 - LB+SIAH2 - LB+P227 - Control
- Plate 3: S2208+SIAH1 - S2208+SIAH2 - Control
- Plate 4: S2060 (from deep-well) - S2060 original culture - LB (from deep-well)- Control
- Plate 5: Test - Like Plate 3 but with S2060+pES2008+pES1076 inside of agar instead of S2208

Control: undiluted P227 (fridge)

PACE reactor assembly and start:

initial phage concentration in the lagoon: 200'000 PFU/ml

Gel:

to check pES2021 and pES2022, to purify pES2008 --> decided to check all Level 2s we have --> loaded 10 µL each

0.8% agarose

Order:

Marker - empty - 2008 (green) - 2003 (green) - 2005 (green) - 2008 (undated) - 2008.1 pure - 2008.2 pure - 2008.3 pure - 2008.4 pure - 2009 (green) - 2010 (undated) - 2010 (6.7.) - 2010 (15.7.) - 2011 (undated) - 2021 a-c (22.7.) - 2022 a-c (22.7.)

IMG_5256.jpeg

Gel analysis:

- unpuriefied pES2008 has 2 bands as expects --> one at 5.5 kb, one at higher up --> is not as clean this time though
- pES2003 is at 5.5 kb as expected
- pES2005 is at 7.5 kb as expected
- undated pES2008 might be too small
- pES2008 pure 1-4 could are all at correct size --> have mutated promoter though?
- pES2009 has correct size, but also has an impurity
- undated pES2010 also has correct size
- pES2010 from 05.07. and 16.07. are too small
- undated pES2011 is has correct size
- all pES2021 and pES2022 are too small

--> undated are probably the ones from 13.06. --> 3/3 assemblies on that day worked (except for cym recombination)
--> did we do something different that day? must have been the first time we used updated protocol (only other time we succesfully assembled was on the 08.07 (we used more backbone and longer annealing time then, but only 1/4 was successful))

pES2008 purification:
as little agar as possible, spun away ethanol at 17.9k g, preheated elution buffer to 50 °C
upper band: 14.4 ng/µL --> slightly better than the last few times, looked like there was an actual curve, can try transforming with this
lower band: 17.2 ng/µL --> discarded

pES2008 issue - Plan B:
set up 35 mL of S2060+pES2008/1076 liquid culture --> can midiprep and gel extract

pES2009
We figure that fixing pES2009 is actually not necessary. We don't need the CymR, since we don't have a gene in the pCym and we don't need the Lux SDM since we are not using the luciferace assay anyways.

Transformation:

Transformed 20 µL of competent S2060 with:

rest of first pES2008 gel purification, pES2009, pES2014, pES2020

co-transformation of pES2009 + pES1008/1014/1028/1031/1033/1035/1076/1081

--> EGLN3 and asyn with linker 2/3/4 and strong promoter each + EGLN3 L2 weak promoter + asyn L4 weak promoter

--> put plates at 30 °C

--> is it maybe wrong to do recovery at 37 °C?!

Plates:

made new Kan and Kan+Carb plates

DISSABTE, 27/7/2024

- [x] check Plaque assay --> maybe redo it or everything?
- [x] Sequencing analysis of 1087A and B
- [x] check plates from transformation --> no colonies yet (09:00), back at 30 °C
- [ ] Plan plan C (Level 2 that can not recombine --> different promoters and terminators)
- [x] keep TadA PACE going
- [x] clean bench and pipettes well with DNA remover
- [x] Transform Lukas' plasmid and pES2008, pES2014 in Stbl3
- [x] Repeat plaque assay: spin down plate first
- [x] double check all primers & place order

in Lab: Noemie, Jakob

Sequencing Analysis:

pES1063 C: pCym is recombined

pES1087: B is correct, C might be a mix

Plaque Assay Analysis (attempt 3):

Plate 1: have some colonies on -3 (SIAH1), -2 (SIAH2) and -1 (P227)

Plate 2: drops flew into each other --> can't be used

Plate 3: positive controls look good (all grew)

Plate 4: negative controls look good (did not grow)

Midi Prep:

https://www.mn-net.com/media/pdf/ed/82/0f/Instruction-NucleoBond-Xtra.pdf

only had 35 mL of culture (co-transformed S2060 - 2008/1076)

resuspended in 50 µL --> 470 ng/µL

--> Gel purification - elution in µL --> 341 ng/µL

Order: M (gene ruler mix) - midi - midi - M

IMG_5259.jpeg

TadA PACE:

- qPCR:
  - Mean Cp Lagoon: 23.96
  - Mean Cp Chemostat: 28.96

qPCR:

callibration curve for SIAH1, SIAH2 and P227 phage stocks - dilutions of 10^0 to 10^-7 were measured, only one well per dilution though.
We can calculate regression curves from this data and extrapolate Phage Titer from deltaCp values
I put the raw data and my calculations into the iDEC PACE folder.

image.png

Plaque assay (attempt 4) - set-up

is the LB + phages plate was useless, repeated plaque assay with the samples from 26/07

Plaque assay following protocol described in Phage Production using fresh S2208 cells from fridge culture.

7x plates:

- Plate 1-3 (for each biological replicate): S2060+pES2008+pES1076+SIAH1 - ""+SIAH2 - ""+P227
- Plate 4-6: LB+SIAH1 - LB+SIAH2 - LB+P227

--> repeated for each biological replicate

- Plate 7: Dilution series of SIAH1, SIAH2, P227 stocks to check concentration again: diluted 1:100 before starting dilution for plaque assay

qPCR Phage propagation (attempt 4):

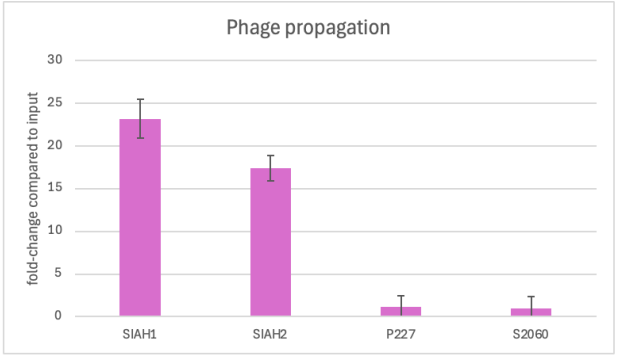
spined down plate before taking sample for qPCR (and plaque assay) -> remove cells to hopefully reduce background (this was not done yesterday)

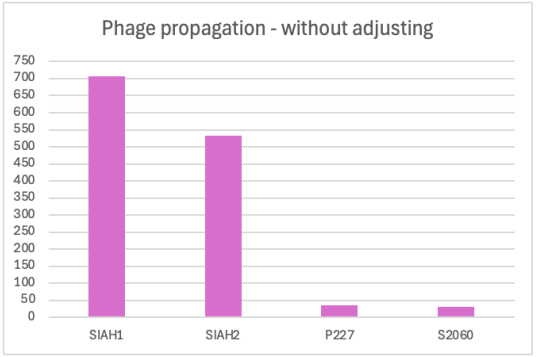
Table21

	Cells	Phage	Cp
1	S2060 + pES2008 + pES1076	SIAH1	14.47
2		SIAH2	17.38
3		P227	19.50
4	LB	SIAH1	23.94
5		SIAH2	26.43
6		P227	25.17
7	S2208	SIAH1	6.4
8		SIAH2	7.37
9	S2060	-	25.59
10	LB	-	30.52
11	Master mix	-	31.14

much better and cleaner results!
It seems like the S2060 cells alone already decrease the Cp by ~5 compared to LB alone. In the left graph we adjusted for this!
I put the raw data and my calculations into the iDEC PACE folder.

image.png





Transformation:
We transformed the pES2014 gibson assembly, the pES2008 purification (from 26.07.) and the pES2008 purification (from 27.07.) into DH5a and Stbl3 cells each (20 µL).
Incubation at 30 °C.

Cleaned both benches & wiped all pipettes with DNAaway

DIUMENGE, 28/7/2024

- [x] check plates from co-transformation from 26.07.
- [x] check plaque assay
- [x] check PACE (qPCR), propaaate pphages in 2208 for sequencing
- [x] minprep 1085 A, B, pLS724g240SDM (in the fridge)
- [x] prepare LB for autoclave (for PACE medium) + put it in cold room

in Lab: Noemie, Gabriel

TadA PACE:

- Arabinose is not flowing, also I feel the levels of the media and waste bottles did not change much from yesterday, I put tape to measure liquid level
- inocculated ON cultures of S2208 + phage pool from today and also with phages from 25.5 to send them for Sequencing
- qPCR
  - Lagoon: 20.61
  - Turbidostat: 31.18
  - LB: 32
  - Water: 32

Miniprep: 1085 A, 1085 B

- pLS724g240SDM peelt was red -> shouldn't be -> probably conatimination of one of our TU+ backbones

check plates:

- Transformation from 27/7 did not grow yet -> put them back
- piecked & set up 2009, 2009+1008, 2009+1033. 2009+1035, 2009 +1081 ON culutres
- other plates from 26/7 did not grow

Plaque Assay Analysis (attempt 4):

- unlike the qPCR; the plaque assay does not show the phage propagation in the cells containing our logic

Plaque assay from 27/07 - #Plaqes/Dilution

	A	B	C	D	E	F	G	H	I	J
1		SIAH1			SIAH2			P227		
2		R1	R2	R3	R1	R2	R3	R1	R2	R3
3	LB + phage	5/-3	20/-2	8/-3	3/-3	15/-2	16/-2	10/-2	10/-2	5/-2
4	S2060 + pES2008 + pES1076 + phage	3/-3	12/-2	3/-2	30/-1	7/-1	15/-1	2/-2	25/0	25/0

DILLUNS, 29/7/2024

- [x] Send pES2008 from midiprep for sequencing (to check whether it is a plasmid mix)
- [ ] As soon as Gibson assembly of 2014 is confirmed, change the plasmid on Benchlin, in the cloning checklist and in the excel sheet (replace the 1063 insert with 1071)

- [x] check PACE (qPCR)
- [x] check pES2008 and pES2014 transformations from 27.07. -> in 30°C incubator
- [x] pick up contransformats and 2009 from shaker**: prep 2009 a**nd send contrasformatns for sanger /or do colony pcr?
- [x] S2208 + TadA phages -> spin down and send for NightSeq
- [x] send 1085 AB for sequencing
- [x] work on calibration curves (in PACE folder)

in Lab: Noemie

PACE:

- arabinose still not flowing, rest looks fine -> sasha fixed it
- sent the propagated phages from 25.5 and 28.7. for sequencing
- prepared new tubing & LB for SIAH PACE
- qPCR Cp values
  - Lagoon: 14.3
  - Chemostat: 26.7
  - Water: 26.8
  - LB: 26.9

  -> possible Master mix or Primer contamination - I marked the aliquots we used today with a red X -> maybe use a new aliquot from now on & would be great if you could run tomorrow's qPCR with both aliquots in parallel to compare

Discussion of our Data with Lukas

- The best negative control would be a inactivated SIAH, but we don't have that - next best would be a phage with a insert with a similar size as SIAH -> better to use TadA than P227 as a control => I think we should do some reading to see if there are any known mutations that completely inactivate SIAH1/SIAH2
- usually, plaque assay is the ground truth
- do an activity-dependent plaque-assay (as Jacob did on the 26.) -> use stock from SIAH1, SIAH2, TadA -> bring them to the same titer -> do a plaque assay with those -> if our logic works, we should definitely see this here
  - according to the plate from Jacob on the 26., SIAH phages do seem to propagate better, but from what we see, the initial starting titers were probably not the same
- not really an idea why we don't see phage propagation in the plaque assay

check plates/liquid culutures:

- 2014 gibson assembly - nothing grew after 2 nights at 30°C -> put the plates in 37°C for another night just in case
- pES2008 purification (from 26.07.) and the pES2008 purification (from 27.07.) both had colonies - 2 different sizes, very big ones and tiny ones - likely that the correct ones are the small ones -> picked 3 each for NightSeq and inoculated ON cultures
- pES2009 liquid culutre was not very dense -> I took a sample for sequencing, but put the culutre back in the incubator for another night & prep tmr

Cotransformations

- all grew, sent them for sequencing (2 reactions each with different primers to confirm the presence of both plasmids)
- made glycerol stocks of all our contransformed strains

Check S2060+pES2008+pES1076 for phage contamination

- we saw incresed Cp for just the strain in qPCR runs in the previous days -> possible phage contamination?
- spun down liquid culture from S2060+pES2008+pES1076, took supernatant and added it to an S2208 culture with approx. OD of 0.5 -> incubation overnight (goal: check tomorrow with qPCR if phages are present in the supernatant - if there would have been a phage contamination, the phages would have propagated and should be present in really high titers after ON incubation)

Activity dependent plaque assay

- 2 plaque assays: once with S2208 top agar and S2060+pES2008+pES1076
- phages: SIAH1 stock, SIAH2 stock, TadA stock from 30.6. propagated => diluted them all to reach 1*10^11 pfu/mL as a starting point for the dilution series

I moved the boxes containing the Level 0, Level 1, Level 2 plasmids to the -20°C freezer in the main lab (under Priscillas/Tatjanas bench)

qPCR calibration curves

- added trendlines and equaltions
- plotted theoretical concentration vs Cp
- compare Cp values from 27/7 with those curves: calcuated phage titers

DIMARTS, 30/7/2024

- [x] LB with tubing for SIAH pace is prepared, but I put it in the cold room ON -> **put it for autoclaving before the wet run**
- [x] prepare fresh LB for TadA PACE
- [x] TadA qPCR: possible Master mix of Primer contamination - I marked the aliquots we used on 29/07 with a red X -> maybe use a new aliquot from now on & would be great if you could run qPCR once with both aliquots in parallel to compare
- [x] do some literature research & figure out if there are known mutations that inactivate SIAH1/SIAH2 -> if yes: order primers
- [x] troubleshoot 2014 gibson assembly - why didn't it work? (plate is now in 37°C)
- [x] Michael: re-measure OD of pasteurized milk probes
- [x] sequencing analysis economy run: move correct plasmids from temp to regular box
- [x] check for phage contamination of S2060+pES2008+pES1076: run qPCR (infected S2208 is in the shaker)
- [x] count plaques on activity dependent plaque assay (is in 37°C incubator)
- [x] nightseq analysis
  - [x] tadA phages: check the sequence of the TadA, what mutations do we have?
  - [x] contransformations: check if both plasmids are present, if one of them is not correct, trash the liquid culutres in the fridge and the glycerol stock -> for the ones that are correct -> do phage propagation assay and/or activity-dependent plaque assay
  - [x] pES2009: prep liquid culture if it looks fine

in Lab: Gabriel, Michael

Sequencing results pES2008 and pES1085

- pES2008 results were wrong
- pES1085B had no signal, will resend for sequencing
- pES1085A had a very short sequencing run (ca 200 bp) which matches the lvl1 backbone and beginning of insert, but does not contain much of the insert. Should be ok but a bit weird, will resend for sequencing

Preparations for SIAH PACE

ca 2 m tubing to be autoclaved, PACE LB from yesterday put in to autoclave.
Liquid culture started for S2060, placed in 37 C incubator

Activity dependent placque assay

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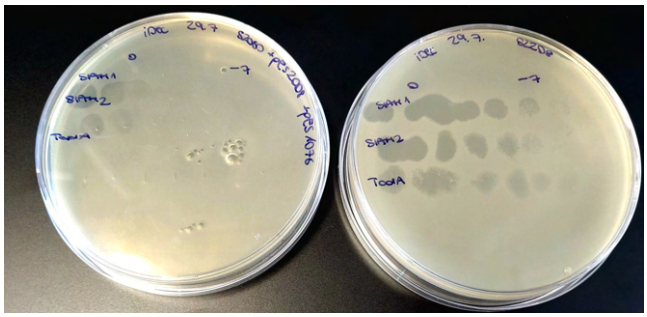

left: activity-dependent plaque assay, right plaque assay with S2208 strain
it's weird that we dont have single, quantifiable plauqes but overall it looks promising. We're still repeating the assay

> Sasha: Lukas's comment was that bacterial layer looks not dense enough, might be the reason we don't see individual pfus. Potentially that's because our strain grows slower? in that case might make sense to use higher OD when doing plaque assay?

Table22

	A	B	C	D
1		SIAH1	SIAH2	TadA
2	S2208	35/-7	4/-7	>dl/-7
3	S2060 + pES2008 + pES1076	x/-3	x/-2	empty

Gibson assembly Troubleshooting

- we had PCR products for all inserts and backbones so the Gibson assembly could only have been unsuccesful because of non-matching overhangs or incorrect volumes in the Gibson assembly mix.

- there is a mismatch between primer 45 and primer 77 (where the backbone and 1071 should join). this is because we initially replaced 1063 (the original part of 2014) with 1071 and only ordered 1 new forward primer (for 1071) since it looked like the complementary region between the backbone reverse primer and the new forward primer are the same as with 1063. It turns that the overhang of the backbone reverse primer reaches so far into the sequence of 1063 that it's not complementary anymore -> We can't reuse the backbone reverse primer for the assembly with 1071 and I ordered a nes primer (079)

TadA qPCR

System set up with new primers and PCR mix
Primers and polymerase used on 29/7 tested separately by setting up the system again
It seems that the cells were not dense enough for the assay
We will repeat

Cp of TadA qPCR samples

	A	B	C	D
1		New Mix	Old primers	Old polymerase
2	Phage	13.12	15.8	16.26
3	Chemostat	25.76	26.55	26.35
4	LB control	28.9	29.68	29.2

qPCR for phage contamination of S2060 +2008 + 1076

- S2208+supernatant from S2060 + 2008 + 1076: 24.14
- S2060 + 2008 + 1076: 28.45

Literature Research on mutations that inactivate SIAH1 and SIAH2

- SIAH1: C41S and C44S
- SIAH2: C80S and C83S
- these mutations drastically reduced SIAH1 and SIAH2 activities in these papers [1, 2]. We can't quantify how much the activity was reduced since activity of targets downstream of SIAH1/2 (i.e degraded proteins leading to expression changes) were measured in the papers

NIght-Seq Analysis

- 2009 looks good anywhere (in the single transformed and co-transformed strains)
- the lvl 1 strains were not sequenced (just NNNs) -> we assume the lvl 1 plasmid might have been kicked out because both have the same ori and the bacterial culture of night-seq samples is without anitbiotics
    - Resent for sequencing with primers only for lvl1
- 1085A looks good and moved to level 1 box
- TadA:

    - No mutations on TadA, but mutations seen on intein
- We decided to re-sequence the colonies (NightSeq of 7 uL of the liquid culture) but this time with the AmpStop primer. If we have the ampicillin resistance gene, it must be our lvl 1 plasmid (it has been sequenced and validated before)

TadA PACE

media change and waste discarded

DIMECRES, 31/7/2024

- [x] when Primers arrive: run PCRs (Tas are in the google sheets) -> for the assemblies designed for KLD: run a part of the sample on a gel to check if the PCR worked, bcs we only have enough KLD-Mix for one try, we can't repeat
- [x] Prep 2009 liquid culture
- [x] check if 2009 co-transformed liquid cultures grew
- [x] count plaque assay
- [x] Find out issue with level 1s in cotransformants (ongoing)
- [x] keep TadA PACE running & start SIAH PACE (don't forget that the media is different from TadA PACE)
- [x] lab duty

In the lab: Gabriel, Michael

PCR reaction for KLD mutagenesis

Primers and annealing temperature

	primers	template DNA	annealing temp.
1	060, 061	1076	70 °C
2	062, 063	1076	69 °C
3	061, 064	1076	69 °C
4	065, 066	1076	69 °C
5	067, 068	1076	69 °C
6	069, 066	1076	69 °C
7	070, 061	1076	69 °C
8	071, 072	EGLN3 or 1076	63 °C

2.5 min extenstion time (with 1kb per 30s), 35 cycles

Master mix PCR: Q5 Polymerase

PCR master mix

		1x	10x
1	dNTPs	1 µL	10 µL
2	5X Q5 buffer	5 µL	50 µL
3	Q5 polymerase	0.25 µL	2.5 µL
4	H2O	10.25 µL	102.5 µL
5			

each tube: 16.5 µL mastermix + 1.25 uL forward and 1.25 uL reverse primer (10 µM) + 1 µL of dilluted (10ng/uL) DNA
for reactions 1-7: add 15 µL more H2O, **for reaction 8:** add 5 µL GC enhancer (total volume = 25 µL per tube)

Gel with the PCR products

order: ladder, 1, 2, 3, 4, 5, 6, 7, 8, empty, positive control (pure 1076 plasmid)

Mutagenesis PCR product gel

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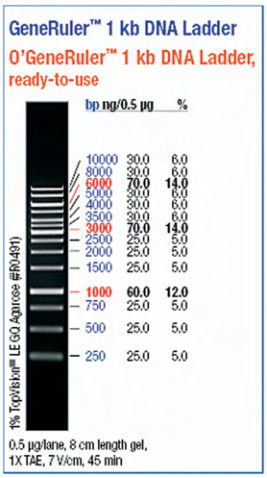


- We have 200 - ~1500bp off-target products in all reactions, but mostly in 4,5, and 6.
- The peptide sequence (8) should be 125 bp long and is below the 250 mark -> should be correct.
- Reactions 1-3 and 7 are sligthly higher than the positive control plasmid (which is expexted since the PCR products are linear)
- primer 67 (reaction 5) has one off-target binding site (with 7 mutations) at position 2206 which points in the reverse direction. The product of this amplification would have around 1.5kb
- All other primer should not have off-targets according to Benchling

KLD reaction and transformation of mutated 1076 plasmids

SDM with Q5 Mutagenesis + KLD Treatment

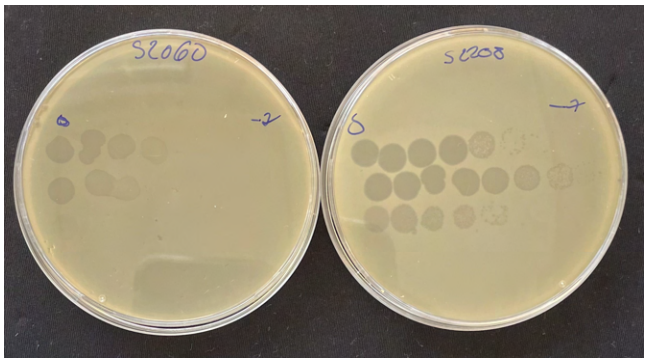
- We decided to proceed with PCR reactions 1,2,3, and 7
- We scaled the KLD reaction down to 0.5 uL PCR product, 2.5 uL KLD Buffer, 0.5 uL KLD Enzyme Mix and 1.5 uL water per reaction

Mastermix: (4.5x): 11.25 uL KLD buffer, 2.25 uL KLD enzyme Mix and 6.75 uL water
4.5 uL mastermix + 0.5 uL DNA per sample

- Transformation with the high-efficiency protocol Transformation into stbl3 cells
- I decided to also transform 2020, 2021 and 2022 (seuqencing unsuccesful in the past) while I was on it anyways

Plaque assay
Top: SIAH1
Middle: SIAH2
Bottom: TadA

Plaque assay 30.07



TadA seems to show lowest activity in S2208, but the S2060 plaque assay looks promising, though no plaques can be counted

TadA PACE qPCR qPCR: Phage Titer

qPCR TadA 31.07

	A	B
1	**Sample**	**Avg. CP**
2	Turbidostat	18.4
3	Chemostat	27.8
4	Neg ctrl	28.6

Minipreps of Level 1 cotransformants and pES2008, pES2009
Sequencing with ampstop was delayed a day due to label issues
We will also miniprep a sample of the cotransformants to send purified plasmids for sequencing /run gels

Yields:

Minipreps 31.07

	A	B
1	**Sample**	**Yield (ng/uL)**
2	2009+1008	382
3	2009+1033A	544
4	2009+1033B	384
5	2009+1033D	308
6	2009+1035A	456
7	2009+1035B	404
8	2009+1081A	338
9	2009+1081B	353
10	2008	357
11	2009	245

cotransformants were sent for sequencing using AmpStart primer from microsynth to confirm the presence of a level 1 plasmid, as there was none seen in nightseq from yesterday

2008 and 2009 were moved to the level 2 box

SIAH1 pace

Setup for SIAH1 pace began according to How to PACE

Supplements for medium (0.8x):

- Carb
- Kan
- no glucose

Chemostat is 30 mL of pS2060 pES2008 + pES1076

The lagoon was inoculated with **10^7 PFU of SIAH1 phages** in 2 mL LB (from 03.06).

flow rate: 1 v/h

DIJOUS, 1/8/2024

Lab Journal - August

Project: iDEC 2024

Author: Michael Bohl

Entry Created On: 01 Aug 2024 06:30:48 UTC

Entry Last Modified: 03 Oct 2024 17:35:28 UTC

Export Generated On: 08 Oct 2024 09:23:36 UTC

DIJOUS, 1/8/2024

- [x] mutagenesis PCR or reactions 4,5,6

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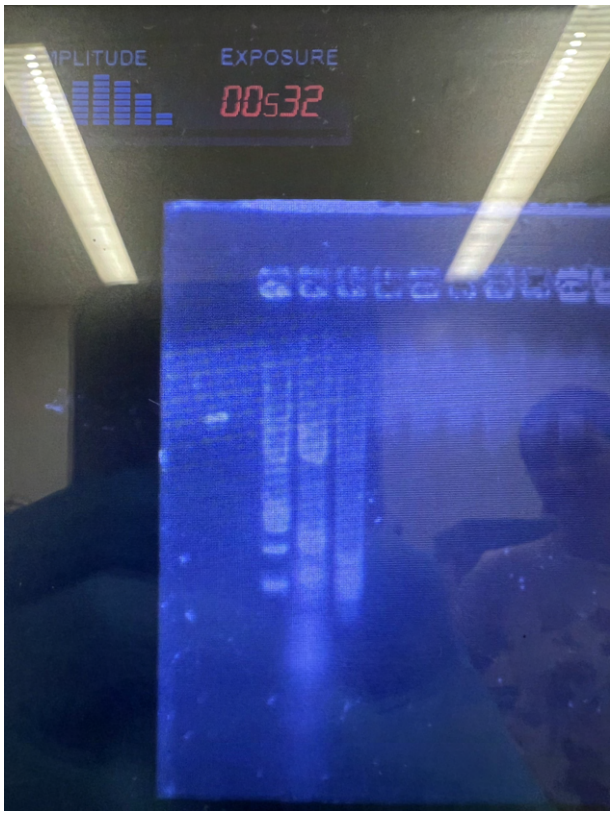


C

- [x] Gel extraction of the PCR products
- [x] qPCR of TadA PACE and SIAH pace
- [x] check if the colonies in the 30°C shaker grew (discard the ones with bad sequences)
- [x] check the plates in the 30° incubator. If they grew, pick and innoculate O/N culture

in Lab: Michael, Gabriel

All O/N cultures in the incubator grew (now in the fridge). As soon as we have the sequencing results we will decide what to do with them

Most plates in the 30°C incubator have none or tiny colonies. I put them back and put 1076 in the 37°C incubator since it neither contains CymRAM or pCym, nor is it a lvl2 plasmid that is prone to recombination.

mutagenesis PCRs PCR: Q5 Polymerase

As discussed in the lab meeting yesterday, we will increase the extenstion time for the mutagenized 1076 to hopefully get more full-size products.

mutagenesis + end-extension PCR

	primer pair	template DNA	annealing temp.	extension time
1	065, 066	1076	69 °C	3 min
2	067, 068	1076	69 °C	3 min
3	069, 066	1076	69 °C	3 min
4				35 cycles

Mastermix for 4 50 uL reactions: 4 uL dNTPs, 40 uL 5X reaction buffer, 2uL Q5 polymerase, 130 uL water
in every tube: 1 uL DNA (10 ng/uL), 2.5 uL primer F, 2.5 uL primer R, 44uL mastermix
Samples were run un a gel, the 5kb band was extracted but the yields were extremely low (9, 15 and 24 ng/uL) :((
-> not worth the KLD treatment since we only have a few uL left

- repeated the mutagenesis PCR, I think we should proceed with the unpurified PCR products since it's very unlikely in my opinion that the off-target PCR products have an antibiotic resistance: the beta-lactamase gene is around 800 bp long and around 800 bp away from the closest primer -> side products with antibiotic resistances should be at least 1.6kb long but all side products we can see on the gel are smaller than ~1-1.2 kb

SIAH1 PACE

Reactor crashed overnight :(new liquid culture started with backup bacteria to restart the system
started with 10^7 PFU/mL in the lagoon

Samples taken at 16:30, 17:30 and 18:30 (Box H9 in fridge) to run qPCR tomorrow

TadA and SIAH1 qPCR

Samples were still taken from the TadA and SIAH1 lagoons and chemostat to qPCR

Results:

Table1

	A	Cp value
1	LB (neg ctrl)	29.52
2	SIAH1 lagoon (today)	21.60
3	SIAH1 chemostat (today)	33.33
4	SIAH1 lagoon (yesterday, after ~3h of PACE)	23.246
5	TadA lagoon	28.3
6	TadA chemostat	39.08
7	TadA phages from 30.7 (pos ctrl)	15.04

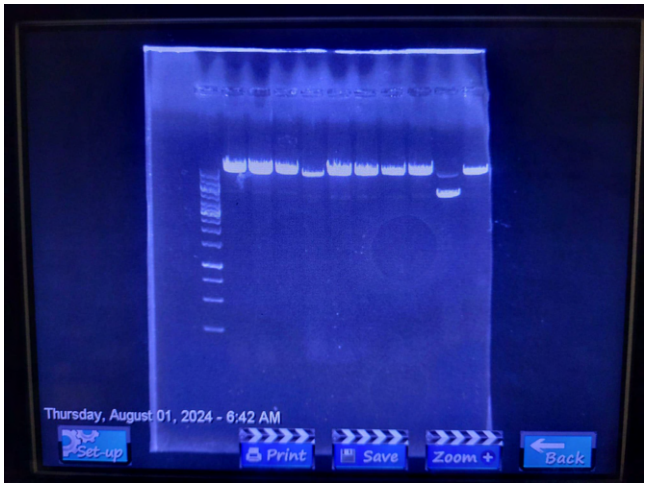
- TadA phages seem to be washed out.
- Also, todays SIAH results are meaningless since the reactor broke (there was no flow during the night and the phages could even propagate after yesterday)
- Nevertheless, the Cp value of yesterday's sample from the SIAH lagoon looks promising.

Digest and gel of 2009 cotransformants Restriction digest

2009 cotransformants were digested with XbaI to run on a gel to try to identify a band corresponding to the level 1 plasmid.
Digested in a 10 ul system and heat inactivated for 20 mins at 65 C
All 10 ul ran on gel

Cotransformants from left to right (all with pES2009): +1008, +1033A, +1033B, +1033D, +1035A, +1035B, +1081A, +1081B
Two right-most lanes are pES2008 and pES2009 as controls

pES2009 cotransformants, XbaI digested



Expected size level 2: 11kb
Expected size level 1: 5kb

All cotransformants show strong band at 11 kb corresponding to the level 2 plasmid. There is also a very weak band around 5 kb corresponding to the level 1 plasmids. As it is very weak, we think that the level 1 plasmid is being kept at the smallest concentration possible to confer amp resistance and otherwise being removed due to incompatibility with level 2. Explains why the sequencing came back with NNNN. Will continue with plaque assay to see if it is still viable for phage propagation

pES2008 looks weird in this, should resequence

Plaque assay of pES2009 cotransformants

The following cotransformants were grown from liquid cultures in fridge to run a plaque assay

- pES2009+1008
- pES2009+1033A
- pES2009+1035A
- pES2009+1081B

These were grown to an OD of ca 0.6 and plated with a dilution series of SIAH1, SIAH2 and TadA phages

DIVENDRES, 2/8/2024

- [x] KLD treatment of the PCR products (in the thermocycler, slot 1)
- [x] pick 3 colonies and inoculate liquid cultures of plates in the 30° incubator and on the bench (2020-22, 1073 mutants)
- [x] After KLD treatment, transform mutagenized 1073 plasmids (the ones that didn't work at first and the ones that didn't grow in the incubator
- [x] Gibson end-extension PCR of the backbone (if primers arrive), Gel extraction/PCR cleanup if there's only one band
- [x] Gibson assembly of 2014
- [x] Night-Seq and Economy Run analysis of the co-transformed strains: check if both plasmids are present
  - [ ] if one of them is not correct, trash the liquid culutres in the fridge and the glycerol stock
- [x] qPCR with SIAH1 phages (samples in box H9 in fridge), 2009 phages from the overnight propagation assay (does TadA PACE still make sense?)
- [x] check plaque assays
- [ ] 2009 phage propagation assay, if we get evidence for succesful cotransformation of 2009
- [x] test transformation efficiency

in Lab: Michael, Jakob

Plaque Assay:

none of the co-transformant plates show phage growth
the positive control looks good

qPCR:

Table2

	A	Cp value
1	LB (neg ctrl)	28.64
2	SIAH1 - 10^9 positive control	11.93
3	SIAH1 - 10^7 positive control	18.00
4	SIAH1 chemostat (02.08., 09:30)	28.68
5	SIAH1 lagoon (01.08, 16:30)	21.06
6	SIAH1 lagoon (01.08, 17:30)	19.62
7	SIAH1 lagoon (01.08, 18:30)	19.45
8	SIAH1 lagoon (01.08, 22:00)	20.88
9	SIAH1 lagoon (02.08., 09:30)	21.67
10	TadA lagoon	23.62
11	TadA chemostat	28.07

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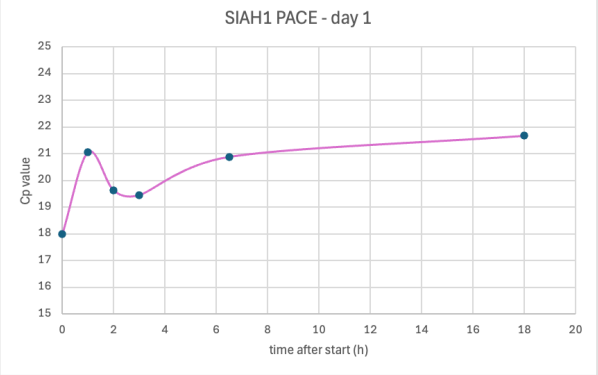
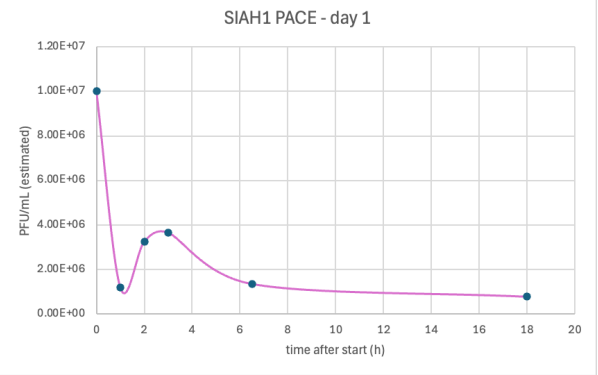




Sequencing Analysis - pES2009 cotransformants:

Nightseq:

- pES1033 B, C (worst) and D all look good, A is nothing
- pES1035 A sequencing is short (200 nt), B is nothing
- pES1081 A sequencing is short (100 nt), B is nothing

KLD:

of 1076 muts 4, 5, 6 --> scaled down KLD reaction (0.5 uL PCR product, 2.5 uL KLD Buffer, 0.5 uL KLD Enzyme Mix and 1.5 uL water)

Transformation of KLD and Efficiency determination:

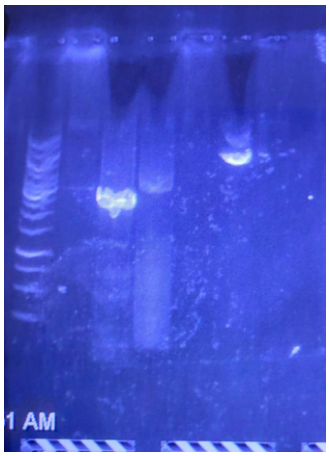
high efficiency protocol
transformed 2 µL of KLD reaction in 20 µL Stbl3 cells each
also transformed pES2009 and pTU2-A-RFP for test of transformation efficiency
--> diluted each to 10 ng/µL and 1 ng/µL and transformed 2µL in either 20 µL Stbl3 or 20 µL DH5a
--> recovery with 80 µL, spun down and resuspended in 30 µL
--> after recovery made dilutions of transformed bacteria, 25 µL each - 1:10, 1:50, 1:100

Gibson end-extension PCR:

Table3

	primers	template	ann. temp.	ext. time
1	44,79	TU2-A-RFP (KanR+ CymR)	70 °C	1.5 min
2	52,53	1026	61 °C	2.5 min

gel_2.8.24.jpg



1kb ladder, empty, backbone, 1026, empty, 2008 (we were curious if and why 2008 has a second band that's a few kb smaller than the original plasmid)

1026 band was very week. We saw the same double band on 2008 as yesterday and didn't figure out what it was (this 2008 is the latest one and is supposed to be purified)

NucleoSpin Gel and PCR Clean-up PCR cleanup yields: 135.7 ng/uL 1026 and 106.2 ng/uL backbone

- note: 260/230 ratios were extremely high (>1000), DNA must have been super dirty.
- all the insert/backbone amounts were re-calculated (https://nebiocalculator.neb.com/#!/dsdnaamt)

Gibson Assembly

assembled 2014 with 1071 (instead of 1063, which is recombined)

Gibson assembly reaction

	component	required amount (pM)	length (kb)	added amount (mass and corresponding volume)
1	TU2-A-RFP (KanR + CymR)	0.2	2.8	357.3 ng / 3.5 uL
2	1071	0.4	1	246.4 ng / 4.8 uL
3	1073	0.4	3.3	813.1 ng / 36.1 uL
4	1074	0.4	0.6	147.8 ng / 1.2uL
5	1026	0.4	3.5	862 ng / 6.4 uL
6	NEBuilider HiFi DNA Assembly Master Mix (2X)			51.7

- I scaled the whole reaction 10X down (otherwise it woult use too much reagents and we don't have enough 1073)
- -> 5.2 master mix and 10.4 total reaction
- Incubated at 50°C for 1h
- with 100% efficiency, we would have around 180 ng of plasmid in the whole reaction. In reality, definitely less (maybe 100ng?) -> I would suggest to use a third (3 ul) to transform

O/N cultures:
picked 8 cultures from pES2008 plate (from 26.07. purification) for minpreps

DISSABTE, 3/8/2024

- [x] check pES1076 muts
- [x] calculate transformation efficiency
- [x] Economy Run analysis of the co-transformed strains
  - [ ] if one of them is not correct, trash the liquid culutres in the fridge and the glycerol stock
    --> none are correct
- [x] make competent S2060+MP and competent S2060/2008/1076
- [x] make S2060+MP glycerol stock
- [x] qPCR for SIAH1 PACE
- [x] minprep pES2008s
- [x] check Lukas' cells in the incubator

in Lab: Jakob
Tranformation efficiency:

Table5

	Condition	undiluted (CFU)	1:10 (CFU)	1:50 (CFU)	1:100 (CFU)
1	Stbl3 cells, 2009, 20 ng	8	1	0	0
2	Stbl3 cells, 2009, 2 ng	0	1	0	0
3	DH5a cells, 2009, 20 ng	>200	29	3	1
4	DH5a cells, 2009, 2 ng	>100	6	5	2

--> pES2009 determination worked well:
Stbl3: 4.5 * 10^2 CFU/µg DNA
DH5a: 7.6 * 10^5 CFU/µg DNA
--> DH5a competency is on the low and but is ok, Stbl3 competency is very low --> will make new competent patch
(--> for the pTU2-A-RFP I used the backbone with the wrong resistance, so nothing grew on the Kan-Plates,
I can't find the correct KanR-CymR backbone though - I think it's not correctly labeled anymore, new minprep was done on 22.7 --> please put red label on again)

pES1076 SDM
all three transformants have colonies
picked 2 colonies each for miniprep and sequencing

Sequencing analysis:
Economy run for pES2009 co-transformations --> all seqeuencings failed
--> AmpStart was used again, maybe it is actually mutated and we can not use it for these, potentially need to send for sequencing again with AmpStop? or make sure they are ok in another way?

Miniprep:
only 3/8 picked pES2008 cultures grew
also miniprepped pES2060/2008/1076 (to transform pES2060+MP cells if we do not have confirmed pES2008 yet)
Yields:
pES2008 A: 310 ng/µL
pES2008 B: 232 ng/µL
pES2008 C: 301 ng/µL
pES2008/1076: 139 ng/µL
--> diluted to 100 ng/µL

ran all preps on a gel:
M - 2008 A - 2008 B - 2008 C - 2008/1076

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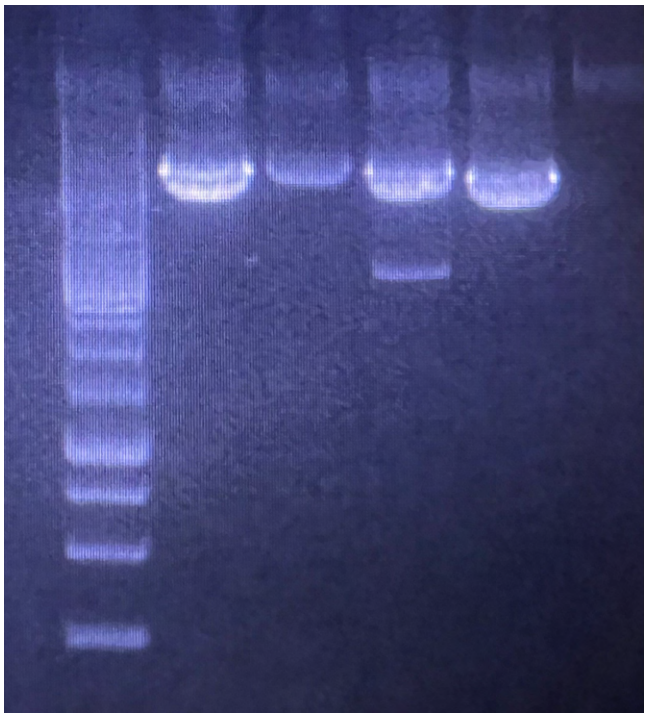

--> height of the pES2008 band seems to be about right, but I must have switched up the 2008 C and 2008/1076 samples while minipreping (ran a second gel with same results) --> repeat miniprep of 2008/1076 and run another gel --> actually also only showed one band --> discarded both pES2008 C and 2008/1076
--> will send 2008 A for full plasmid seq

SIAH1 PACE - qPCR:

repeated all samples and took new sample at 09:00,
reactor broke down at some point over the night though - don't know exactly when.
TadA reactor is shut off, but tested samples anyways just to check

Morning qPCR

	A	Cp value
1	LB (neg ctrl)	27.48
2	SIAH1 - 10^7 positive control	18.99
3	SIAH1 chemostat (03.08., 09:00)	27.35
4	SIAH1 lagoon (01.08, 16:30)	21.13
5	SIAH1 lagoon (01.08, 17:30)	19.88
6	SIAH1 lagoon (01.08, 18:30)	19.69
7	SIAH1 lagoon (01.08, 22:00)	20.80
8	SIAH1 lagoon (02.08., 09:30)	21.53
9	SIAH1 lagoon (02.08., 15:15)	13.53
10	SIAH1 lagoon (03.08., 09:00)	10.28
11	TadA lagoon (03.08.)	23.08
12	TadA chemostat (03.08.)	25.20

I wanted to see whether SIAH1 might have increased its catalytic activity (because the Cp escalated so much), I innoculated 1 mL LB and 1 mL S2060/2008/1076 with the 2 µL original SIAH1 phage (10^9 pfu/mL) or 2 µL of SIAH1 (02.08., 15:15, 10^9 pfu/mL) and let it propagate for 3h. --> also prepared that SIAH1 15:15 phage for sequencing

Afternoon qPCR

	A	Cp value
1	LB (neg ctrl)	28.40
2	SIAH1 10^7 pfu/mL	18.45
3	SIAH1 10^9 pfu/mL	11.96
4	SIAH1 lagoon (03.08., 11:30)	13.25
5	SIAH1 lagoon (03.08., 12:30)	13.04
6	SIAH1 lagoon (03.08., 13:30)	13.54
7	SIAH1 lagoon (03.08., 14:30)	13.57
8	SIAH1 10^9 + LB	20.62
9	SIAH1 10^9 + S2060/2008/1076	18.78
10	SIAH1 02.08. (10^9) + LB	23.90
11	SIAH1 02.08. (10^9) + S2060/2008/1076	21.46

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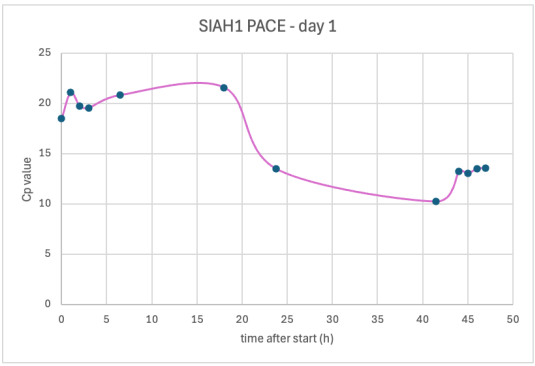

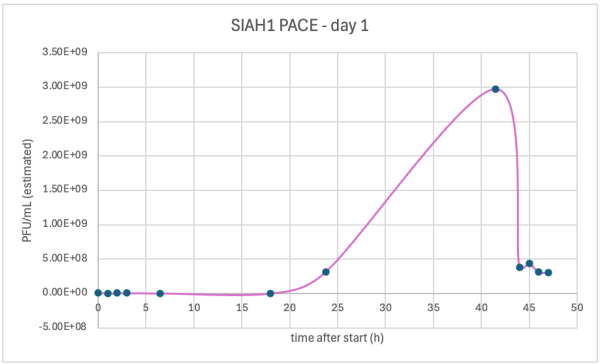

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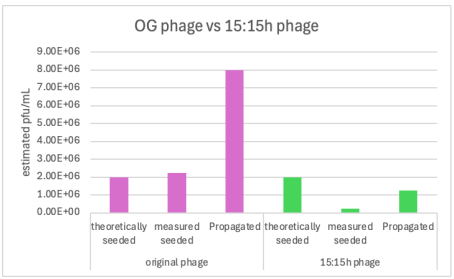

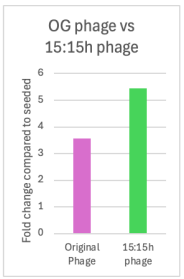

Competent Cells

made chemically competent cells with the mix & go kit --> Stbl3 cells, S2060 + MP, S2060/2008/1076,
10 mL culture for 1 mL competent cells, 10 aliquotes of 100 µL each
also I made a new box for all of our competent cells and labeled our cryo stock box

DIUMENGE, 4/8/2024

- [x] miniprep pES1076 SDMs and send for sequencing (or run gel first)
- [x] potentially test newly competent cells
- [x] transform gibson assembly (in a PCR tube the fridge labelled with G) with newly competent Stbl3 cells
- [x] do o/n propagation with pES2009 (not sure whether it makes sense since the sequencing is so weird) -> did culutres of the strain -> moved experiment to tmr
- [x] pES2023 assembly
- [ ] co-transform S2060+MP cells with 2008 or 2009 and 1076 muts (might want to wait until we confirm sequence of 1076 muts and new 2008 prep?) - why not use S2060?

in Lab: Noemie

pES2023 Assembly (aka pES1076 in level 2 Backbone)

- molar ration 2:1 for insert A, 5:1 for insert B (bcs of small size)

- Cycling Protocol: Bennett Lab with Esp3I, long protocol: 20 min 37°C, 25x (1.5 min 37°C, 3 min 16°C), 5 min 45°C, 10 min 50°C, 10 min 65°C

Table4

	A	B
1	pTU2-a p15a SmR	0.5 uL
2	pES1076	1.4 uL
3	pTU1-B	0.2 uL
4	T4 Ligase Buffer	1 µl
5	T4 Ligase	0.5 µl
6	Esp3I	0.5 µl
7	Water	5.9 µl

PACE:

- both reactors seem to be running
- Arabinose is disconected from TadA PACE
- liquid levels of all bottles: there is enough media/space in waste bottle until tomorrow
- took a sample from chemostat and lagoon from both PACE reactors at 11:30
- qPCR:

Table6

	A	B	C	D	E
1	Sample	Cp values			Mean Cp
2	TadA lagoon	26.52	26.64	26.70	26.62
3	TadA turbidostat	29.10	29.19	28.88	29.0566666667
4	SIAH lagoon	13.17	13.28	13.21	13.22
5	SIAH turbidostat	29.52	29.74	30.07	29.7766666667
6	LB	30.32	30.11	29.48	29.97
7	Water	31.15	31.44	30.89	31.16

TadA does not seem to increase in titer, also sequencing last week showed that no mutations have occured in the TadA -> might worth discussing if we want to check if the MP is inside the strain. Also, for large parts of the evolution the Arabinose syringe did not work properly. Mutagensis might not properly work/induced in the system

SIAH: phage titer seems constant and also quite high -> I don't know the current flow (probably still 1 v/h), but for washout flow rate should be increased tomorrow

pES1076 SDMs

- cast 1% Agarose gel
- Miniprepped mutation reaction 5,6 according to protocol, Nandrop concentration written on tube (not adjusted)
- I messed up the preps for the two clones of SDM 4 (I forgot to add P2 before adding N3) -> preps failed -> I put fresh LB + Amp into the tubes and let them grow again ON
- loaded 5 uL on gel -> ran for 20 min at 120 V
- Loading order: GeneRuler 1 kb - mut 5 cl. 1 - mut 5 cl. 2 - mut 6 cl. 1 - mut 6 cl. 2

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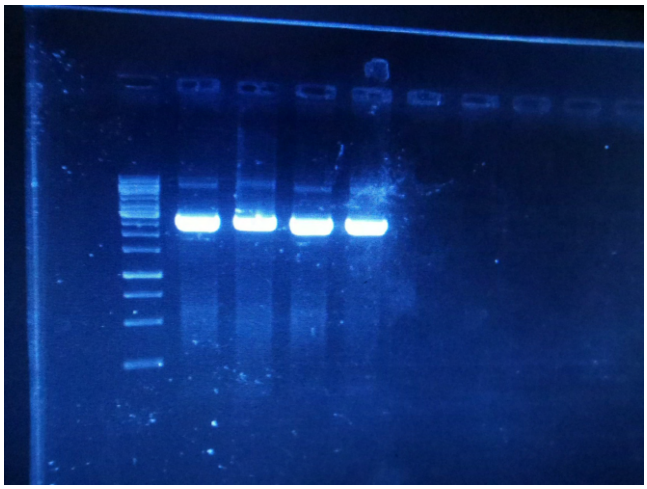


expected band: should be around 5 kb
observed very bright band a around 2.5 kb and only a very faint band at 5 kb
did not continue with cotransformation, wait for sequencing first, maybe this helps with finding out what this bright band is

ON Propagation of pES2009 contransformants

- I set up cultures of the 4 strains needed for the Assay (see Table below). I inoculated them from Glycerol stocks (bcs I didn't find liquid cultures for all of them in the fridge). Unfortunately, they didn't grow enough during the day to reach OD 0.5 -> I left them ON in the incubator -> moved the experiment to tomorrow

Transformations:

1. Determine Transfromation Efficinecy of new competent cells: transformed either 2 ng or 20 ng of pTU1-A-RFP in 20 uL of the competent cells (Stbl3)
   recovery: added 80 µL of LB
   made dilutions: 1:0, 1:5, 1:10, 1:50, 1:100, 1:500, 1:1000 -> plated 10 uL each on LB+Carb plates
2. Gibson Assembly 2014 in new Stbl3 cells, plate entire reaction on LB + Kan
3. pES2023 Assembly in new Stbl3 cells, plate entire reaction on LB + Sm

DILLUNS, 5/8/2024

- [x] Sequencing Analysis
- [x] bring down sequencing tubes from fridge (1 full plasmid seq, 1 night seq)
- [x] prep 2 pES1076 SDM 4 liquid culutres, send them for sequencing (together with the SDM preps from yesterday, that are in the temp storage box, 4 tubes)
- [x] PACE: qPCR (i **booked a slot on the qPCR machine for us from 12:00-14:00**; what is going on with TadA pace?, increase SIAH flow rate (2 vol/h)
- [x] check plates from transformation:
  - [x] calculate transformation efficiency,
  - [x] pick colonies from 2014, 2023
- [x] innoculate pES2020-2022 and pES1076 mut depending on night seq results
- [x] lab duty: pipette tip boxes recycling
- [ ] run PCRs & Gibson assembly: EGLN3 as 35aa (in same environment as in pES0176) -> see cloning spreadsheet primers 071-074 => gel extract => gibson assembly
- [x] phage ON propagation assay that I planed yesterday (dilute ON culutres to OD 0.1 -> let them grow to OD 0.5 before starting the assay)

in Lab: Michael, Noemie

PACE

- Sasha: increased SIAH flow rate to 2v/h
- Sasha: TadA reactor seems to have crashed over the weekend
- put a orange tape to mark liquid levels on each bottle (at around 4)

Chemostat and Lagoon samples (for SIAH1 and TadA) were taken at 9:05 and phage titers were determined with qPCR

Table8

	sample	Cp
1	LB	26.51
2	TadA chemostat	26.35
3	tadA lagoon	24.22
4	SIAH1 chemostat	26.49
5	SIAH1 lagoon	10.88
6	10^7 PFU SIAH phages (pos ctrl.)	17.56

Miniprep of 1076 mutant 4:
colony 1: 354.2 ng/uL
colony 2: 409.6 ng/uL

Gibson Assembly of pES1076
PCR w/ Q5 (50 uL reactions)

Table9

	A	B	C
1		Reaction 1	Reaction 2
2	Primers	071, 072	073, 074
3	Template	pES1076	pES1076
4	Ta	63	70
5	Elongation TIme	30s	2.5 min

followed by DpnI Digest (15 min at 37°C)
I ran a small volume on a gel - expeced band sizes: 0.1 kb for R1, 4.3 kb for R2
bands looked fine (correct size and only one band) - I only briefly looked at the gel, forgot to take a picture, sorry!
purified the PCR reactions & stored them in the temp storage box (they are labeled: RX puri, 5.8.)

Transformations 04/08
pES2014 had no colonies ->put back in incubator for another Day
pES023: picked colonies & send for NightSeq
Transformation Efficiency of fresh Stbl3 batch

- 20 ng DNA Transformed in 20 uL of cells: 6 * 10^5 CFU/ ug DNA
- 2 ng DNA Transformed in 20 uL of cells: 1 * 10 ^6 CFU/ug DNA

Sequencing Analysis

- 2020 A sequencing is very short (300bp) and doesn't properly align and has very low quality, B sequencing failed (only 30 bp) => kinda makes sense that sequencing looks weird bcs the primer used should not even bind this plasmid
- 2021 A, B same issue as above
- 2022 A, B same issue as above
- 1076 mut1 A an ori and dCas9 was sequenced -> smth very wrong is in that tube ,B has very poor sequencing quality (probably mixutre) and run breaks off before the mutagensized region
- 1076 mut2 A ok, B has a deletionwhere the mutagenesis should have happened
- 1076 mut3 A sequnecing broke of very early, B ok
- 1076 mut7 A sequencing failed, B ok
- SIAH1 phages: sequencing only 30 bp and very niosy
- unknown_or_1081_AmpStop aligns neither to 1081 nor 1008, idk what this is

The primer used to sequence the Level 2s was poorly chosen (017 doesn't even bind Level 2 constructs) => I would send them again for sequencing with a better suited Primers

1076 muts: inocculated mut2 A, mut3 B, mut7 B. For mut1 picked new colonies

ON Propagation of pES2009 contransformants

- ON cultures were diluted to OD 0.1, then grew again to OD 0.5

- S2208 and S2060 + pES2009 + pES1081A grew to approx 0.8 and then diluted back down to 0.4
- S2060 + pES2009 + pES1033B, S2060 + pES2009 + pES1035A grew to OD 0.4-0.5
- unfortunatly the volume of the cultures was too small to fill the entire plate, I diluted the cultures to OD 0.4 to maximise the volume, but there still wasn't enough
    - the following wells are empty/not enough volume: D11, F6, F7, G7, H3
- plate was covered with a breathable seal and grown ON at 37°C in the shaker

Table7

	A	B	C	D	E	F	G	H	I	J	K
1	Condition Nr	S2060 + pES2009 + pES1033B	S2060 + pES2009 + pES1035A	S2060 + pES2009 + pES1081A	S2208	SIAH1 phages (1:10)	SIAH2 phages (1:10)	TadA from 30.6. (1:10)	Media		
2	1	1 mL culture, OD 0.5			-	10 ul			LB + Kan + Carb		
3	2	1 mL culture, OD 0.5			-		5 ul		LB + Kan + Carb		
4	3	1 mL culture, OD 0.5			-			8 ul	LB + Kan + Carb		
5	4		1 mL culture, OD 0.5			10 ul			LB + Kan + Carb		
6	5		1 mL culture, OD 0.5				5 ul		LB + Kan + Carb		
7	6		1 mL culture, OD 0.5					8 ul	LB + Kan + Carb		
8	7			1 mL culture, OD 0.5		10 ul			LB + Kan + Carb		
9	8			1 mL culture, OD 0.5			5 ul		LB + Kan + Carb		
10	9			1 mL culture, OD 0.5				8 ul	LB + Kan + Carb		
11	10	-			-	10 ul			LB + Kan		
12	11	-			-		5 ul		LB + Kan		
13	12	-			-	-	-	8 ul	LB + Kan		
14	13	-			1 mL culture, OD 0.5	10 ul			LB + Carb		
15	14	-			1 mL culture, OD 0.5		5 ul		LB + Carb		
16	15				1 mL culture, OD 0.5			8 ul	LB + Carb		
17	16	1 mL culture, OD 0.5							LB + Kan + Carb		
18	17		1 mL culture, OD 0.5						LB + Kan + Carb		
19	18			1 mL culture, OD 0.5					LB + Kan + Carb		
20	19	-	-	-	-	-	-	-	LB + Kan		

Plate Layout of Prpagation Assay (Condition Number)1

	1	2	3	4	5	6	7	8	9	10	11	12
A	1				5				9			
B	2				6							
C	3				7							
D	4				8				10			
E									11			
F	13				16				12			
G	14				17							
H	15				18				19			

DIMARTS, 6/8/2024

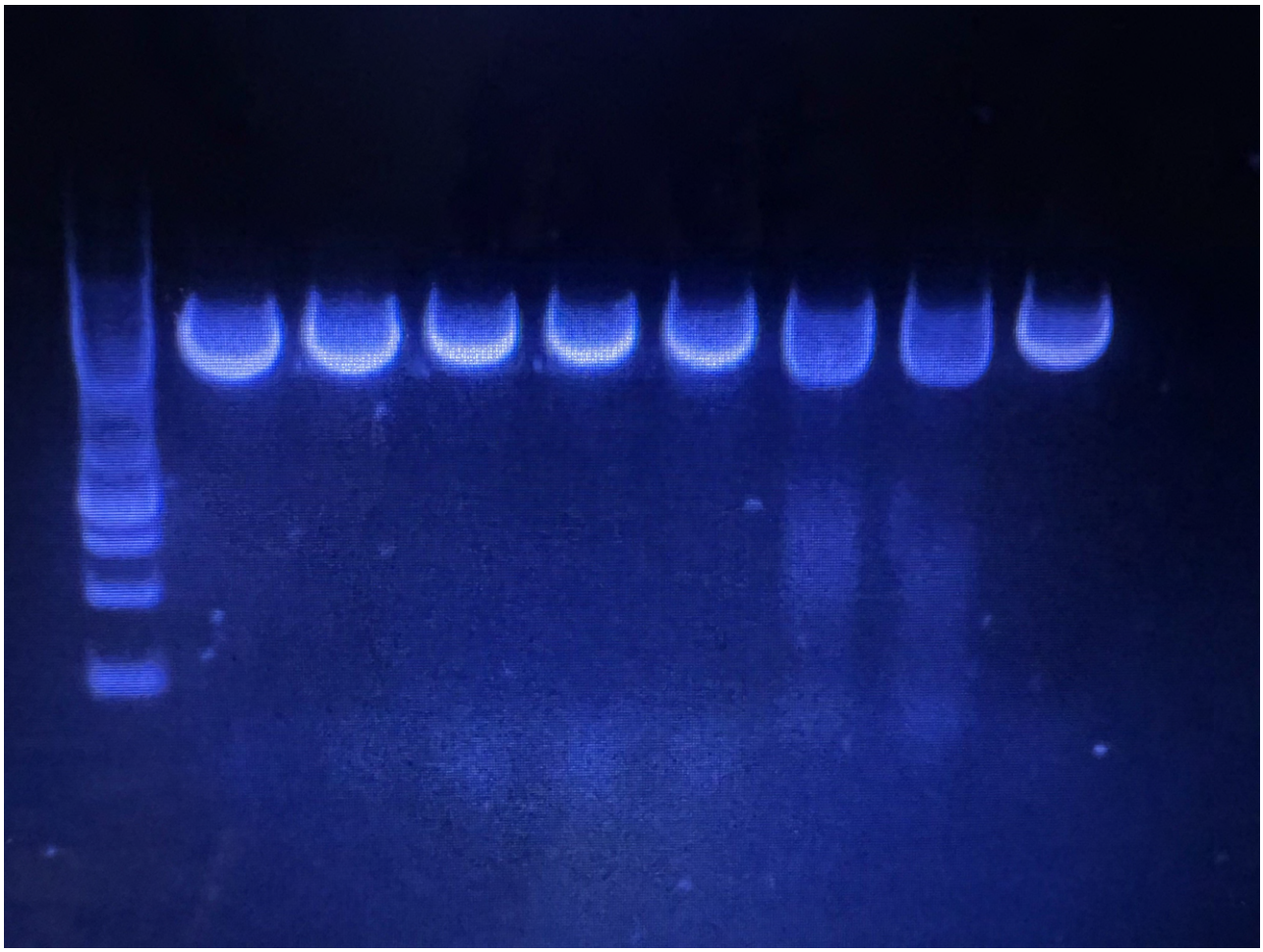
- [x] co-transform S2060 cells with 2008 or 2009 and 1076 muts (wait until we confirm sequence of 1076 muts and new 2008 prep?)
- [x] Miniprep O/N cultures of the confirmed 1076 mutants and of 2023 if the NightSeq results are positive
- [x] Maybe try to retransform 2014 if there are still no colonies on the plate --> still no colonies, discarded
- [x] SIAH PACE
- [x] qPCR Overnight phage propagation (deep well plate is in the shaker)
- [x] sequencing analysis
- [x] finish Gibson Assembly pES1096: EGLN3 35 aa peptide (the same as pES1076, just a short peptide instead of the entire EGLN3) -> purified PCR products are stored in the temp box, labeled RX puru 5.8., continue with Assembly reaction

In the lab: Jakob&Michael

Sequencing Analysis:
pES1076 mut 1: wrong
pES1076 mut 4: 2 is good, 1 has a big insert
pES1076 mut 5: 1 is good, 2 has big insert
pES1076 mut 6: 1 is good, 2 has wrong mutation and an insert
--> should run them on a gel to check they are actually the right size though, since mix from PCR was used
SIAH1 02.08 15:15: perfect SIAH1 without mutations
pES2020: both wrong, empty backbone
pES2021: both wrong, empty backbone
pES2022: both wrong
pES2023: A and C nothing, B wrong

Ran all pES1076 muts on a gel --> all look to be of similar size, although mut 5 and mut 6 are potentially slightly shorter? Size can't really be determined because marker is unclear, seems reasonable though

IMG_5327.jpeg



Minipreps:

pES1076 mut1.1: 260 ng/µL 77

pES1076 mut1.2: 383 ng/µL 135

pES1076 mut2: 181 ng/µL 39

pES1076 mut3: 188 ng/µL 42

pES1076 mut7: 499 ng/µL 192

pES2023 A: 6 ng/µL

pES2023 B: 15 ng/µL

pES2023 C: 7 ng/µL

--> volumes adjusted to 100 ng/µL

Golden Gate Assembly:

EcoFlex Assembly

100 ng Backbone, 100 ng of each insert

With BsmB1

30 cycles of 1 min at 42 °C and 3 min at 16 °C

5 min at 60 °C

Table12

	A	B	C	D	E	F	G	H	I	J	K	L	M	N
1	pES2023	AP2 (weak_EGLN3_L2_CtermRNAP(CGG))	SmR	pTU2-a p15a SmR	pES1076	pTU1-B Dummy								
2	pES2023 (mut1)	AP2 (weak_EGLN3_L2_CtermRNAP(CGG))	SmR	pTU2-a p15a SmR	pES1076 (mut1) - used unconfirmed cl. 2	pTU1-B Dummy								
3	pES2023 (mut2)	AP2 (weak_EGLN3_L2_CtermRNAP(CGG))	SmR	pTU2-a p15a SmR	pES1076 (mut2)	pTU1-B Dummy								
4	pES2023 (mut3)	AP2 (weak_EGLN3_L2_CtermRNAP(CGG))	SmR	pTU2-a p15a SmR	pES1076 (mut3)	pTU1-B Dummy								
5	pES2023 (mut4)	AP2 (weak_EGLN3_L2_CtermRNAP(CGG))	SmR	pTU2-a p15a SmR	pES1076 (mut4)	pTU1-B Dummy								
6	pES2023 (mut5)	AP2 (weak_EGLN3_L2_CtermRNAP(CGG))	SmR	pTU2-a p15a SmR	pES1076 (mut5)	pTU1-B Dummy								
7	pES2023 (mut6)	AP2 (weak_EGLN3_L2_CtermRNAP(CGG))	SmR	pTU2-a p15a SmR	pES1076 (mut6)	pTU1-B Dummy								
8	pES2023 (mut7)	AP2 (weak_EGLN3_L2_CtermRNAP(CGG))	SmR	pTU2-a p15a SmR	pES1076 (mut7)	pTU1-B Dummy								

SIAH PACE:

Table10

	sample	Cp
1	LB	30
2	SIAH1 chemostat	29.69
3	SIAH1 lagoon	12.63
4	SIAH 10^7	18.94

Even at flowrate of 2v/h the SIAH1 phage does not get washed out --> we swapped out the lagoon to TadA phages - the propagated phages from 22.07. were used to innoculate with 2 mL of 10^7 PFU/mL at roughly 19:00. The flow rate was kept at 2 v/h, if the phage is washed out, it might make sense to repeat the washout at a lower flowrate. To swap the lagoon all of the needles and the final tube to the waste were swapped (the tube was not sterile but rinsed with EtOH).
The Media bottle was swapped at roughly 19:00. 1.6 mL carb and 0.8 kan were added to 2L of LB.

Phage propagation assay:

Table11

	sample	Ct - first run	Ct - 2nd run
1	S2060/2009/1033 + SIAH1	15.94	14
2	S2060/2009/1033 + SIAH2	18.82	17.2
3	S2060/2009/1033 + TadA	25.18	27.15
4	S2060/2009/1033	23.86	29.78
5	S2060/2009/1035 + SIAH1	16.68	15.47
6	S2060/2009/1035 + SIAH2	19.97	17.49
7	S2060/2009/1035 + TadA	23.77	27.75
8	S2060/2009/1035	23.5	29.21
9	S2060/2009/1081 + SIAH1	11.53	no signal
10	S2060/2009/1081 + SIAH2	14.87	no signal
11	S2060/2009/1081 + TadA	24.06	25.71
12	S2060/2009/1081	22.43	28.40
13	S2208 + SIAH1	15.86	16.31
14	S2208 + SIAH2	19.33	19.47
15	S2208 + TadA	25.47	28.38
16	LB + SIAH1	19.79	19.56
17	LB + SIAH2	19.36	20.24
18	LB + TadA	24.3	29.25
19	LB	23.1	29.87

Mastermix: NA (no signal)

- first run: The LB and everything else aside from the mastermix could be contaminated, positive controls too look weird (Cp should be around 6)
- second run: worked with the flame, also took directly from the deep-well plate (maybe contamination happened while spinning down the 96-well plate)) -> no contamination but positive controls still look very suspicious -> we have to repeat the assay
- Calculations below should be taken with a grain of salt (something was fishy with this assay and we have no good indicator of how well TadA propagated)

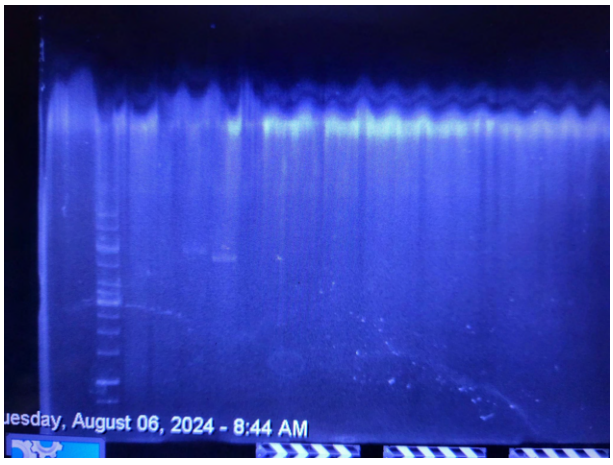
Fold change / overnight propagation rate (average of both qPCR)

	transformant	SlAH1	SlAH2	TadA
1	2009/1033	14.4	3.46	1.68
2	2009/1035	8.63	2.1	2.03
3	2009/1081	306.55	266.87	3.7

2014 Gibson Assembly product PCR + Gel:

- Mastermix: (5x 25 uL reactions): 25 uL buffer, 1.25 Q5, 2.5 dNTPs, 6.25 primer 018, 6.25 primer 019, 83.75 H2O
- 24 uL mastermix + 1uL DNA per tube sample
- annealing temp.: 61°C, ext. time: 4 min (8kb)
- used a mix of the unassembled interts/backbone as neg. ctrl and 2009 and 2008 as positive control
- 2008 and 2009 each showed the expected band respectively but 2014 had no band-> assembly didn't work again?? The primers are definitely correct and we got PCR products
- In the gel below, the visible bands are 2008 and 2009, since the gel imaging machine had a frozen screen, I tried to visualize the gel with the UV light were gels are usually cut. I assume that the big blur in the top part could from DNA contamination frim the UV light.

20240806_172018 (002).jpg



Gibson Assembly of EGLN3 peptide plasmid Gibson Assembly

- decided do it with a molar 10:1 insert/backbone ratio as recommended here https://www.neb.com/en/tools-and-resources/usage-guidelines/guidelines-for-using-nebuilder-hifi-dna-assembly
- 32 ng (400 fmol) insert, 106ng (40 fmoi) backbone (as recommended in the prorocol)
- dilluted the insert 1:4
- incubated at 50°C for 15 min

Table13

	part	added amount
1	insert (EGLN3 peptide)	1.6 uL (1:4 dilluted)
2	backbone (1076 vector)	0.57 uL
3	water	0.33 uL
4	NEB Gibson Assembly Master Mix (2X)	2.5 uL

Transformation:
pES2023 assembly --> 5 µL in 50 µL DH5a
pES2023 mut1-7 --> 5 µL in 50 µL DH5a
pTU1-B-Dummy --> likely <3 µL in 30 µL DH5a
pES2014 Gibson assembly --> 2.5 µL in 30 µL DH5a
pES1076 pep gibson assembly --> 2.5 µL in 30 µL DH5a
Co-Transformation of pES2009 and pES1076 mut2-7 in (2 µL each) in 20 µL S2060
Co-Transformation of pES2009 and pES1076 mut2-7 in (2 µL each) in 20 µL S2060

DIMECRES, 7/8/2024

- [x] Put LB and LB Agar out from fridge for autoclaving (before 13:30)
- [x] check plates in the incubator
  - [x] innoculate liquid cultures of the transformations that worked and send for nightseq

  Note: I saw that you tried to transform TU1-B-Dummy, but that wont work, bcs this is just annealed oligos with overhangs & not a plasmid
- [x] PACE maintainance, qPCR (there is a dilution of TadA phages in the temporary storage that matches the innoculation concentration to compare to in qPCR)
- [x] Make Kan, Spec and Carb Plates --> with new agar bottles
- [x] Assembly suggestion:

  assemble level one plasmids of E1, E2, RNAP-Ub that have all different terminators and promoters (we have different promoters and terminators from the kit in the lvl 0 box, but the plasmids still need to be planned - I think Noemie mentioned that the stronger terminators should be in the back once assembled to the lvl2 plasmid)
  --> we can use these lvl1 to assemble lvl2s that should not be able to recombine
  Note: shouldn't the stronger terminators should be in the front?
  We didn't find any other terminators in the lvl0 box than the one we used

In the lab: Gabrial + Michael
Sequenicing Analysis:
pES1076 mut1.2 is correct 🥳 - label (printed sticker should be around) and put in level 1 box
--> it might have a deletion in the RNAP - somewhat close to end of sequencing though, so I am not sure
--> also use this for co-transformation
pES2008 full plasmids seq is perfect - I also edited the few things in the benchling sequence that were always wrong! 🍾

Transformations

All 2009 cotransformants showed colonies

2008 + 1076 muts cotransformants did not show growth yet, left in the 30 C incubator

Single transformants worked

All sent on a nightseq plate

We re-assembled 2017-2020 (with BsmbI) EcoFlex Assembly

(Tara helped. If it works now pls employ her)

Phage propagation assay

Layout

- column 1-3: added SIAH1 (10 uL, 1:10 dilluted, from 3.6)
- column 5-7: added SIAH2 (3 uL, 1:10 dilluted, from 9.7)
- column 9-11: added TadA (10 uL, 1:10, from 30.6)
- we decided to use 10x more since all TadA Ct values were too high last time)
- column 12: no phages

Note: Cotransformant 2009+1035 did not grow past OD 0.29, still used for assay

Plate Layout

	1	2	3	4	5	6	7	8	9	10	11	12
A	S2060 - 2008/1076				S2060 - 2008/1076				S2060 - 2008/1076			
B	S2060 - 2008/1008				S2060 - 2008/1008				S2060 - 2008/1008			
C	S2060 - 2009/1033				S2060 - 2009/1033				S2060 - 2009/1033			
D	S2060 - 2009/1035				S2060 - 2009/1035				S2060 - 2009/1035			
E	S2060 - 2009/1081				S2060 - 2009/1081				S2060 - 2009/1081			
F												LB
G	LB				LB				LB			LB
H	S2208				S2208				S2208			LB

qPCR

Values were very weird with the switched lagoons

- Cp of 11 in the lagoons on both runs
- Cp of 28 in the positive control phages used to innoculate

DIJOUS, 8/8/2024

- [x] qPCR of the O/N propagation assay
- [ ] pick and innoculate 2008 + 1076 mutants and send for NightSeq

- [x] Sequencing analysis of the NightSeq plate
- [x] Miniprep if correct: 2014, 1096, 2009, 2023-2030
- [x] order primers for introducing mutations into SIAH1/2 -> better negative control than TadA
- [x] Transform 2017-2022 into DH5a/Stbl3, 1097 (1076 mut1) into S2060

in Lab: Gabriel, Michael

- updated naming scheme: 1076 muts are now pES1097-1103 and 2023 muts are now pES2024-2030 (see cloning excel file)

qPCR for phage propagation assay

- I used the same TadA phages as previously (from 30.6) but apparently there are no phages in this tube.
  - All samples with TadA phages have Ct values of at least 30 -> the data is pretty much useless and we have to repeat with other phages.
- Also, the PFUs don't seem to be right on either SIAH1 or SIAH2 as we have consistently less SIAH2 phages everywhere
- Even without a negative control, it looks like phages propagate worse in 2009 bacteria compared to 2008

Ct values

	strain	SIAH1	SIAH2	TadA	fold change - SIAH1	fold change - SIAH2	G
1	S2060 - 2008/1076	15.6911111	17.8755556	30.8466667	14.3645859142	31.7056181594	
2	S2060 - 2008/1008	16.9188889	17.6066667	30.4111111	6.1333135204	38.2014501564	
3	S2060 - 2009/1033	18.66	19.7633333	30.6033333	1.834714522	8.5675867818	
4	S2060 - 2009/1035	18.7766667	21.3233333	31.5366667	1.6921868752	2.905706326	
5	S2060 - 2009/1081	16.7866667	21.3733333	31.11	6.7219923464	2.8067273015	
6	S2208 + phage	11.8683333	17.1622222	30.8311111	203.2656044152	51.9841533668	
7	LB + phage	19.5355556	22.8622222	30.8488889			

- Ct of LB without phages: 32.21

Colony Picking

- 2008/1076mut co-transformants didn't grow

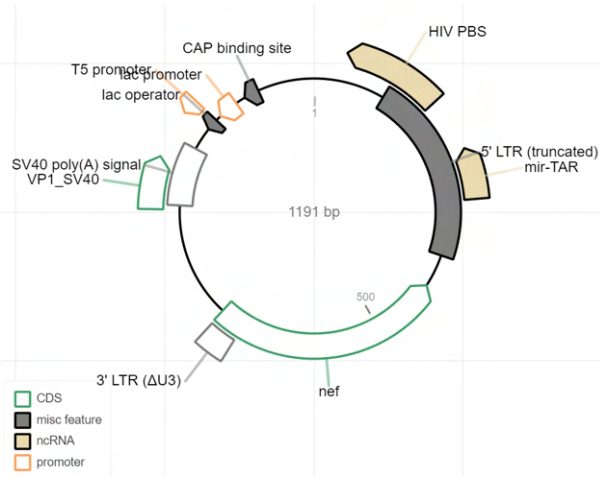
Sequencing analysis for 2023 and cotransformants

Overnight cultures did not grow for all 2023 mutants and for 2009 cotransformants. There was either an issue with selective plates or with antibiotics in the ON cultures. Sequencing data will be allow for conclusion

- We had not a single correct lvl2 sequence on our NightSeq plate (we selected the oO19 primer that was sent to Microsynth on 28.6) -> we have no confirmed co-transformants and no confirmed lvl 2 EGLN3 plasmids
  - The sequences do not align to any of the lvl 2 constructs we sent, but they align to each other within a sample (not between samples even if it is the same lvl2)
  - either the primer that was sent to microsynth was incorrect and contaminated (we got some sequences, they just don't align to anything) -> we will test this today

    - alternatively, the spectonomycin plates did not contain antibiotics and the Kan+Carb plates only containes Carb -> we will also test this
    - or the primer binds to something on the E coli genome and is sequenced during night seq
- some EGLN3 muts (1097-1103) were confirmed, but most had no signal (just NNN) -> doesn't matter anyways since nothing grew and 2009 wasn't confirmed anywhere
- sequencing the lagoon from yesterday did not give any insights whether we had TadA or SIAH phages in our PACE (2 samples that were sequenced with primer OLS-760 - and were just NNNs)
- All 1096 is correct
- We tried to auto-annotate the sequences we got from 2014 (3 independent colonies) and realized that **we probably have some mammlian vector contamination in our DNA/in our bacteria** (see below). No idea how it got in there, maybe in the gel chambers or during gel extraction???
- sequenced 2023 plasmids contain fractions of other random yeast/bacterial elements and sometimes a restriction enzyme (e.g. eco47RI), could be other engineered plasmids with resistances that contaminated our samples

image.png



- Also, next time don't use primer 019 when checking co-transformants as it also binds to lvl1 backbones (pTU1-A-RFP)

DIVENDRES, 9/8/2024

- [x] mutagenesis PCR of SIAH1 and SIAH2 phage plasmids (use primers 80-83 if they arrived)
    - [x] KLD treatment, transformation
- [x] pick, innoculate, and sequence colonies from yesterday
- [x] analyze sequnecing results
- [x] trash everything in the temp box that says "Gibson" or "2014" - we don't know which part of the assembly is contaminated with this mammalian vector
- [x] check if all 2009 and 2008 cotransformants (2008+1076, 2008+1008, 2009+1033, 2009+1035, 2009+1081) are in the fridge for a propagation assay tomorrow. If any clones don't have any liquid culture left in the fridge, re-innoculate a new culture (ideally with nightseq) --> all co-transformants are available
- [x] transform competent S2060/2008/1076 with mutagenesis plasmid

In lab: Jakob, Gabriel

SIAH1 SP and SIAH2 SP - SDM PCR:
SIAH1 reaction with 67 °C annealing, SIAH2 with 69 °C
10 ng Template, 35 cycles
repeated SIAH2 at 68 °C since the first one looked weird on the gel (the second one also did, but we ended up using that one)
--> KLD treated

Transformation:
2 µL of each KLD reaction in 20 µL DH5a each, recovery with 80 µL LB for 1 h
1 µL of DP6 (mutagenensis plasmid) in 30 µL competent S2060/2008/1076 and 30 µL DH5a, recovery with 70 µL LB+Gluc for 1 h

TadA Phage Propagation:
TadA phages from 30.07 were tested on plaque assay by Lucas (11 plaques on -6, 3 plaques on -7) --> ~5*10^9 PFU/mL
5 µL of this phage used to innoculate 5 mL of S2208 for o/n propagation

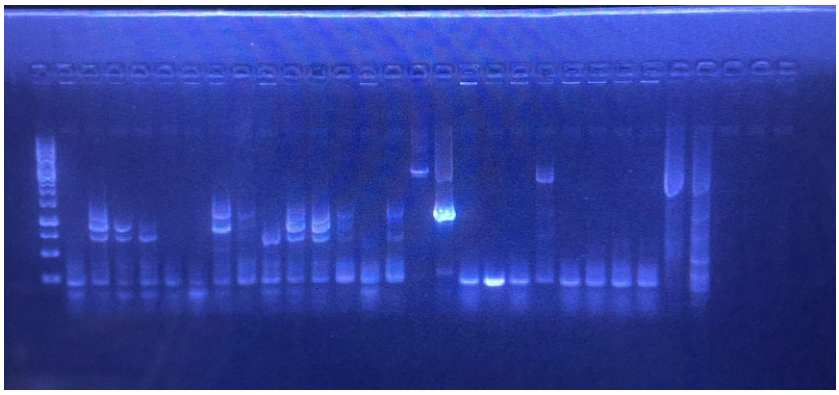
Transformations from 8.8
Single transformants of assemblies and 2023-2030 showed colonies (not many), Some double transformants with 2009 also showed colonies

Since there is no nightseq tonight we ran a colony PCR for 4 colonies per successful transformation to see if it is worth continuing with the plasmids over the weekend. If colony PCR is positive we will set up ON cultures to prep and cotransform over the weekend.

Colony PCR:
Primers 18 and 19
annealing temp 62 C
elongation time 1.5 min

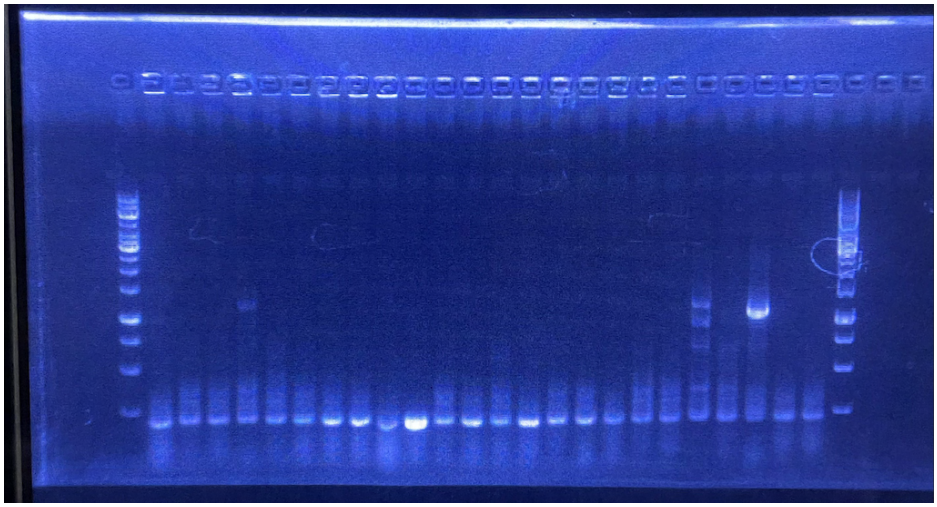
Gel1:
1-4: 2017
5-8: 2018
9-12: 2019
13-16: 2020
17-20: 2023
21-24: 2024
25: SIAH1 SDM PCR
26: SIAH2 SDM PCR

Screenshot 2024-08-09 at 16.26.57.png



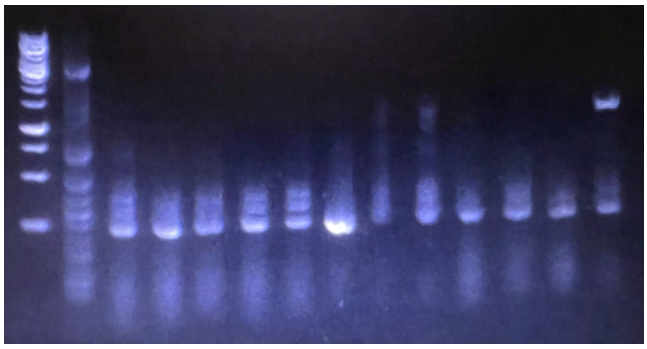
Gel 2:
1-4: 2028
5-8: 2027
9-12: 2029
13-16: 2030
17-20: 2009+1101
21-24: 2009+1102

Screenshot 2024-08-09 at 16.26.47.png



Gel 3:
1: SIAH2 SDM PCR
2-12

IMG_5334.jpeg



The colony PCR shows that most of the constructs have empty backbones. The 2017-2020 assemblies show bands similar to the control in well 16 of gel 1 (empty TU2-A-KanR) and the 2023-2030 show signal around 250 bp, which aligns with the length of an empty pTU2-a-SmR. The exception being 2023 clone 4 (lane 20 gel 1), which shows a good band

ON culture was strated for this culture to be miniprepped.

DISSABTE, 10/8/2024

- [x] check the single 2023 culture we innoculated
- [ ] **assemble pES2023-2030 again, somehow successfully this time** 😞
- [x] pick SIAH1 SDM SP and SIAH2 SDM SP (did not grow)
- [x] Dummy B production to attempt 2023-2030 assemblies again, peptide sequence from 1076 can also be included now - run a gel first to confirm length of dummy
- [x] pick DH5a + DP6 culture
- [x] pick S2060/2008/1076/DP6 (did not grow)
- [x] put LC for propagation assay in the incubator (they are already prepared on the bench)
- [x] SIAH2 KO PCR run on gel

in Lab: Noemie, Gabriel

SIAH1 and SIAH2 SDM SP

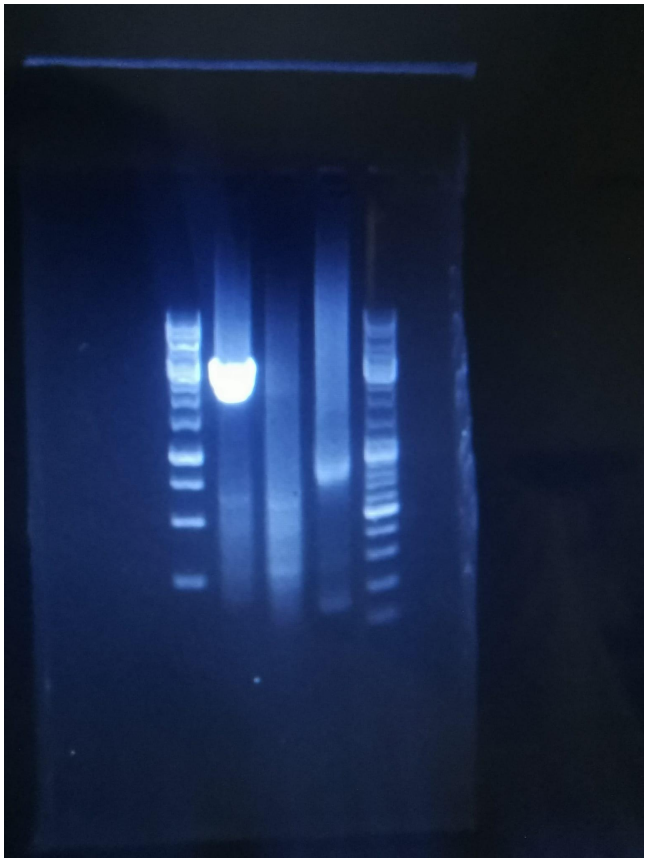
Did not grow, put back in incubator, but should repeat PCR as some of the bands looked werid yesterday.
Other transformations also did not grow.

PCR repeated with same conditions as yesterday

Dummy B PCR from twist combined 2

Ran all 3 PCRs on a gel, 120V, 25 mi
Expected bands: around 3 kb for SIAHs, 160 bp for Dummy B
Loading order: GeneRuler 1kb - SIAH1 SDM + SIAH2 SDM - Dummy B PCR - Generuler Mix

WhatsApp Bild 2024-08-10 um 14.20.22_ee67c37a.jpg



- SIAH1 SDM looks good -> continued with KLD treatment & Transformation (High Efficiency Protocol)
- SIAH2 SDM didn't work again: Tm that was used does not account for the mismatches, repeat PCR with reducing the annealing Temperature
    - Repeated PCR: Template: 2 ng SIAH2-SP, Primers: 082, 083, Elongation time: 1 min 45 s, Tm: 64°C

  Note: During reaction, the cycler had an error and stopped the reaction, I didn't notice for a while, but afterwards I just restarted the machine and continued the protocol
- Dummy B: weak band at around 750 bp could correspond to the Template, weak band at the correct length -> DpnI digest (1 h, 37C) followed by purification over spin column
    - I would run a gel before using this
    - stored it in the level 1 box

pES2023 culture grew

- miniprepped, 40 ng/ul (rather low, but was also a low copy number plasmid)
- stored it in the temp box until sequencing

Plaque Assay:

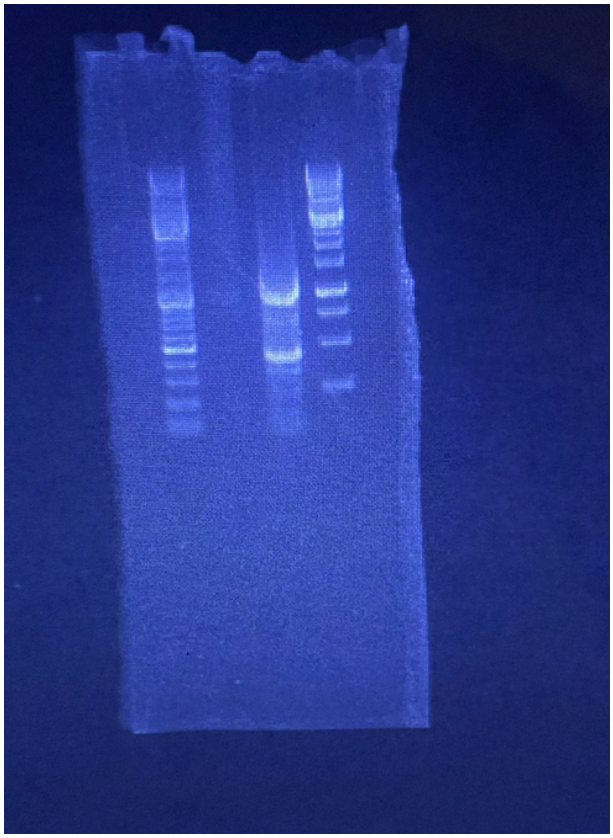
- spun down & filtered TadA phages that were propagated overnight on S2208
- plaque assay: TadA, SIAH1 and SIAH2 that we have stored in the box, each in triplicates to more accuratly determine the titer
- I used the SIAh 1 and SIAH2 with the printed labled (not the handwritten ones)

- I stored the TadA phage that we used for this now in the SIAH box, next to the SIAH1 and SIAH2 phage tube that should be used for the overnight propagation assays
- incubation ON at 37°C

Gel run2

Running order: gene ruler mix, dummy B2 purification, SIAH2 SDM, Generuler 1kb

Screenshot 2024-08-10 at 19.55.29.png



- Dummy TU1-B shows no band: repeat the pcr with way less Template (1ng) -> ran overnight
- SIAH2 knockout: PCR didn't work again, none of the two bands are at our expected length

DIUMENGE, 11/8/2024

- [x] miniprep DH5a + DP6
- [x] Take Priscillas Plates out of the incubator (3 phage spooting plates) & put them in our fridge
- [ ] count plaques and determine titer of SIAH1, SIAH2, TadA (and update labelling on the Tube, the amounts used for ON propagation in the Benhcling protocol)
- [x] Overnight propagation assay + activity dependent plaque assay - cultures from glycerlos stock did not grow :(
- [x] check plates trafos
- [ ] Run qPCR on turbidostat to see why cells died + dilutions
- [x] Co transform 2008 with 1076 muts in S2060
- [x] Make more Dummy B for 2023-2030

- [x] Set up 2023-2030 assemblies
- [x] evtl BB digestion + gel to check cutting efficiency

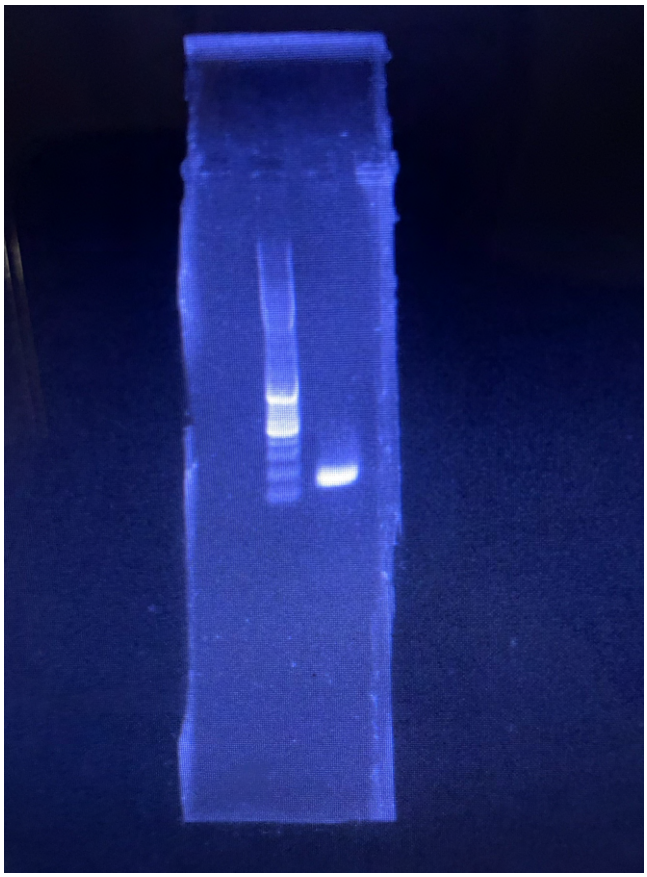
ON colonies

Of the cotransformants for phage propagation, only 2008/1076 grew. We already have propagation and activity dependent assays
they were inocculated from glycerol stocks, and incubated in the shaker for > 12h

Dummy B

PCR ran overnight will less DNA, loaded on gel
order: generuler mix, Dummy B

Screenshot 2024-08-11 at 09.30.58.png



Purified with nucleospin kit and eluted in 20 ul, yield 48 ng/ul

SIAH1 KO

Transformation successful, colonies picked to be prepped and sequenced

DP6 minipreps

yields:
DP6 a: 368
DP6 b: 258

In temp storage

Cotransformations

50 ul aliquots of competent S2060 co-transformed with:

- 2008+1008
- 2008+1035
- 2008+1081
- 2008+1076
- 2008+1096
- 2008+1097
- 2008+1098
- 2008+1099
- 2008+1100
- 2008+1102
- 2008+1103

plasmids for transformation were pre-mixed and added to cells together (maybe this will help), also a lot of DNA was added (80-100 ng per plasmid)
retransformed 2008+1076 again as positive control (to check for F-pilus loss later in propagation assays)

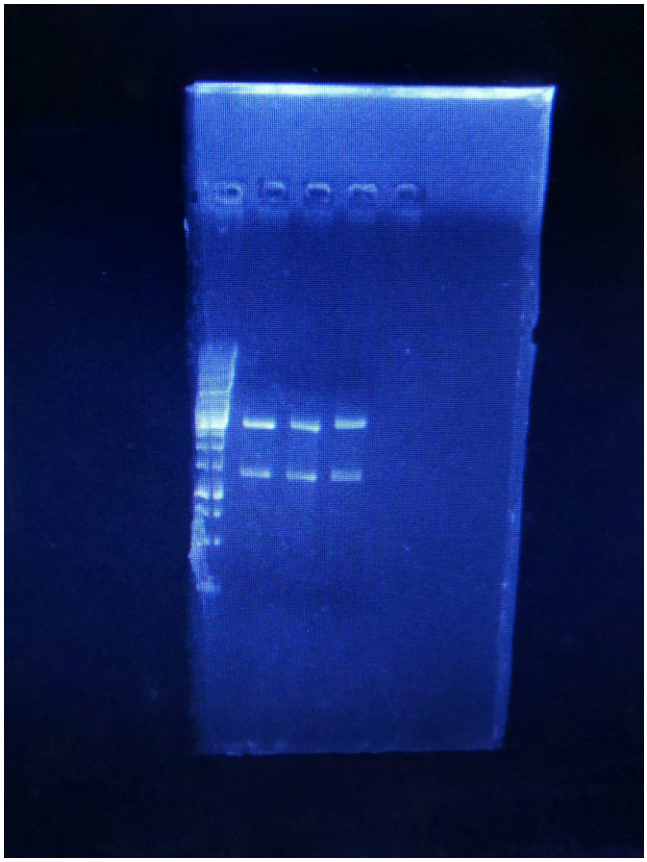
Golden Gate Assemblies pES2023-2031:

- Master Mix: 60 uL Water, 10 uL T4 Ligase Buffer, 5 uL Dummy-B (48 ng/uL), 5 uL pTU2-a p15a SmR, 5 uL BsmbI, 5 uL T4 Ligase
- 5:1 ratio for Dummy-B (higher excess is recommended for small inserts)
- 2:1 ratio insert:vector
- Added 100 ng corresponding Level 1 insert (1076, 1096-1103)
- Cycling Protocol: Lukas' GG BsmbI protocol (runs 19 h overnight)

Digestions: to check the activity of Esp3I and BsmbI on our plasmids -> to rule out enzyme activity for the issues with cloning

- digested 200 ng TU2-A-RFP with 0.5 uL of ESp3I/BsbI respectively in T4 Ligase Buffer (10 uL Reaction volume)
- incubation 1 h (37°C for Esp3I, 42°C and 55°C for BsmbI)
- Loaded on 1% Agarose Gel, 120 V, 40 min -> Order: GenRuler 1 kb - Esp3I 37°C - BsmbI 42°C - BsmbI 55°C
- Expected bands:
    - undigested plasmid: 3.2 kb
    - digested plasmid: 1.1 kb + 2.1 kb

WhatsApp Bild 2024-08-11 um 17.49.00_52a182a8.jpg



All 3 samples seem to be fully digested. The ladder almost ran off the gel, but there are only 2 clear band in each sample. The Enzymes don't seem to be the issue for our cloning issues.

qPCR:

- TadA dilution series/Siah dilution series/Bravo Turbidostat
- first run failed (all Cp values 33/34) -> reason: the 150 Reaction calculations on the sheet above the bench are not correct (too little water) -> repeat the run
- second run was inconclusive - even in wellls were there was atleast master mix, the machine gave no signal at all (not even background signal, which we usally observed when we have only master mix)

DILLUNS, 12/8/2024

- [x] send pES2023 for sequencing (prepped on 10.8, stored in temp box)
- [x] Send purified DP6 for sequencing (temp box)
- [x] send purified pcr product dummy b for sequencing
- [x] Miniprep and sequence SIAH1 KO colonies in shaker
- [x] Assemble SIAH1 SDM SP (KO) with split C and split D plasmid (phage production protocol, we have aliquots of split C and split c in our box) +send for sequencing to check if mutagenesis worked
- [x] Transform 2023-2030 assemblies

- [x] Order more BsmbI
- [x] lab duty: empty pipette tip box box is almost full

In the lab: Jakob, Michael

Miniprep:
3/4 SIAH1 KO SP cultures grew --> miniprepped:
SIAH1 KO SP cl. A: 313 ng/µL
SIAH1 KO SP cl. B: 372 ng/µL
SIAH1 KO SP cl. C: 312 ng/µL
concentrations adjusted to 100 ng/µL

Co-transformations:
nothing grew yet, left plates in incubator --> quite some colonies started growing by the afternoon
O/N cultures were picked and cultures sent for sequencing:
pES2008+pES1081
pES2008+pES1096
pES2008+pES1097
pES2008+pES1098
pES2008+pES1099
pES2008+pES1102
pES2008+pES1103
Aside from that the pES2009 co-transformants were also innoculated from the cryo stocks

Sequencing:
sent pES2023, DP6 cl.A and cl. B, Dummy B PCR and SIAH1 KO SP cl.A to cl. C for sequencing (labels borrowed from Schwank Lab)

qPCR:
calibration curve of SIAH1, SIAH2 and TadA phages
3 replicated dilutions of each phage from -0 to -7 and 3 technical replicates per sample
Results:

Table14

	A	B	C	D
1	Dilution	SIAH1 (average Cp)	SIAH2 (average Cp)	TadA (average Cp)
2	10^0	7.33	7.70	15.83
3	10^-1	9.76	11.35	19.20
4	10^-2	13.45	15.18	23.38
5	10^-3	17.00	19.05	26.85
6	10^-4	20.39	22.76	28.69
7	10^-5	23.79	26.12	28.90
8	10^-6	26.84	28.25	29.01
9	10^-7	28.46	29.17	28.71
10	LB	29.48		
11	MM	29.52		

From these measurements I interpolate, that the Phage titer of SIAH2 is actually 7.1*10^10 PFU/mL.
The Phage titer of TadA seems to be 2*10^8 PFU/mL. These are the concentrations we should work with in the future.
I also made a dilution of each phage based on these values to 10^8 PFU/mL

qPCR Calibration - SIAH1.pdf

Phage production - SIAH1 KO SP - Day 1:

Phage Production

Assembly of phage plasmid from SIAH1 KO SP --> one per purified clone

Transformation:
transformation of 3 µL pES2023-2031 in 50 µL DH5a each
transformation of 2 µL pES2008 in 50 µL S2060/DP6
transformation of 2 µL pES2008 and 2 µL pES2023 (unconfirmed) in 50 µL S2060/DP6

DIMARTS, 13/8/2024

- [x] pick S2060/DP6/pES2008, send for night Seq and propagate for competency --> let's call this D2668
- [x] pick pES2023-2031 and send for nightseq
- [x] also check for potential pES2008 co-transformant slow growers
- [ ] transform SIAH1-KO selection plasmid in S2208 (tubes are in slot 1, labelled A,B,C = assemblies from different colonies with SIAH1 ko plasmid), wait for sequencing results first
- [x] O/N propagation assay of 2008 + co-transformants
- [x] check pES2009 cotransformants from stocks

- [ ] whenever there is some time - start with presentation for meeting on wednsedday

in Lab: Jakob

Sequencing Analysis:

- DP6: cl. A has short sequence that does not start from primer binding site, cl. B has no sequencing
  --> clone A was re-sequenced and now looks to be correct
- SIAH1 KO SP: Sequencing starts ~150-200 bp too late, right at SDM site --> very suspicious, I will run them on a gel today
- pTU1-Dummy-B: likely conatins pTU1-Dummy-B
  also contains Dummy D, and Ps70 promoter --> could this be the template, as the signal strength drops quite a lot, gel from 11.08 also confirms that only Dummy B was amplified
- pES2023: nothing was sequenced

O/N cultures:

pES2009 + pES1033, pES1035, pES1081 all grew from cryo stock
pES2009 + pES1008 did not grow (also at some point people thought it contained pES2008 instead of pES2009 for some reason)

pES2008 + pES1081, pES1096, pES1098, pES1099 and pES1102 all grew --> wait for NightSeq
pES2008 + pES1097 and + pES1103 did not grow --> reinnoculated liquid cultures

Transformations:

pES2008+pES1076 has cultures now,
pES2008+pES1100 has one culture
--> picked two cultures from pES1076 and one of pES1100

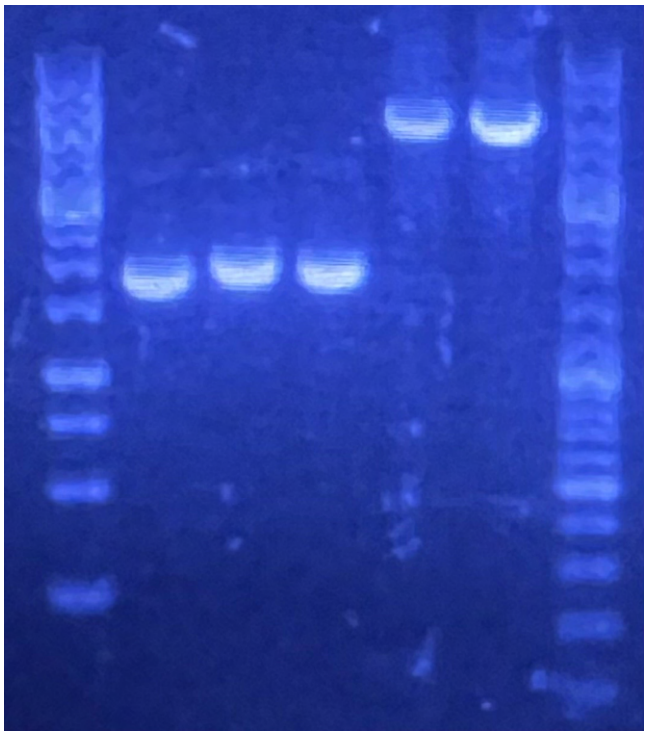
pES2023-2031 all have cultures --> all have cultures, will perform colony PCR
(assembly contained the weird pTU1-Dummy-B, but theoretically this should not have an effect)
D2668 does not have any cultures yet
S2060/DP6/2008/2023 does not have any cultures yet

Colony PCR:

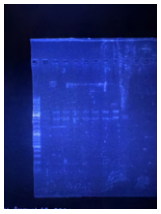
3 cultures picked per plate from pES2023-2031, if available a large culture was picked for the third reaction
Primers: o018 and o019, positive control: pES2003

Gel 1:
Generuler 1kb - SIAH1 KO SP cl. A - cl. B - cl. C - DP6 cl. A - cl. B - Generuler Mix - Assembly 2023 - 24 - 25 - 26 - 27 - 28 - 29 - 30 - 31

IMG_5345.jpeg



a



--> SIAH1 KO SP bands are at ~2kb instead of 3kb

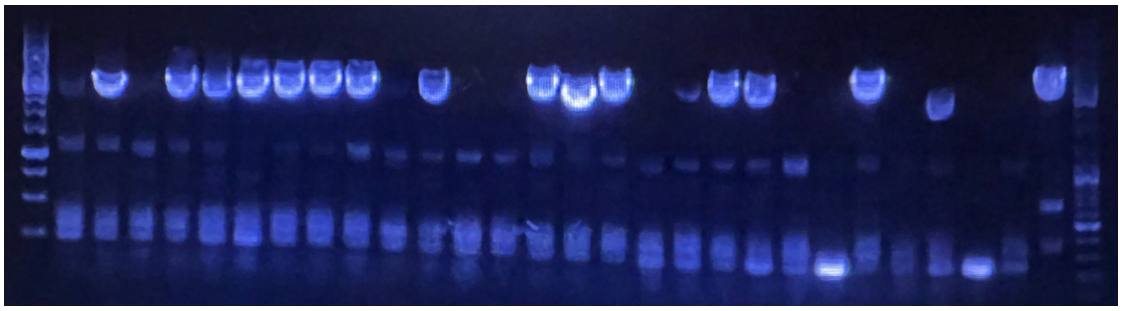
--> DP6 looks like it is lower than expected (8.6kb) but ladder is quite blurry up there

--> can't see much on the gel for the assemblies (only had some weak bands, none at 5kb)

Gel 2:

Generuler 1kb - 23 - 24 - 25 - 26 - 27 - 28 - 29 - 30 - 31 - 2003 - Generuler Mix

IMG_5347.jpeg



--> picked o/n cultures and nightseq:
pES2023 B, pES2024 AB, pES2025 AB, pES2026 B, pES2027 B, pES2028 A, pES2029 AB, pES2030 C, pES2031 DEF

NightSeq Analysis:
pES2008 confirmed in all colonies,
all lvl1s also confirmed in co-transformants:
pES1096, 1097, 1098, 1099, 1103

pES1102 is also confirmed but might have two other mutations in EGLN3 aswell
The pES1081 is actually pES1076

--> I picked 2 more cultures of pES1102 in case the mutations are confirmed
--> I also repicked pES1097 and pES1103 since they did not grow (A is the one that was confirmed by sequencing) and also picked 2 more cultures for each (B and C)
--> made cryo stocks for pES1096, 1098, 1099 and 1102

ON Propagation of pES2009 contransformants

- ON cultures were diluted to OD 0.3, grown to OD 0.5-0.65 and finally diluted to OD 0.5
- S2060/2009/1033 and S2060/2009/1035 only grew to OD 0.3 and were used like that, also some bacteria was spilled in the shaker with those samples, so their data should generally be taken with a grain of salt, S2060/2009/1081 was diluted to OD 0.3
- S2208 were diluted to OD 0.5 from OD 0.84
- 10 µL of the 10^8 PFU/mL dilutions were used to innoculate 1 mL of bacteria --> 10^6 PFU/mL starting concentration
- plates were covered with a breathable seal and grown ON at 37°C in the shaker

Plate 1 - Layout

	1	2	3	4	5	6	7	8	9	10	11	12
A	S2060 - 2009/1033				S2060 - 2009/1033				S2060 - 2009/1033			S2060 - 2009/1033
B	S2060 - 2009/1035				S2060 - 2009/1035				S2060 - 2009/1035			S2060 - 2009/1035
C	S2060 - 2009/1081				S2060 - 2009/1081				S2060 - 2009/1081			S2060 - 2009/1081
D												
E	**SIAH1 samples**				**SIAH2 samples**				**TadA samples**			**no phage samples**
F												
G												
H	LB without phages											

Plate 2 - Layout

	1	2	3	4	5	6	7	8	9	10	11	12
A	S2060 - 2008/1076 (newly transformed)				S2060 - 2008/1076 (newly transformed)				S2060 - 2008/1076 (newly transformed)			S2060 - 2008/1076 (newly transformed)
B	S2060 - 2008/1096				S2060 - 2008/1096				S2060 - 2008/1096			
C	S2060 - 2008/1098				S2060 - 2008/1098				S2060 - 2008/1098			S2060 - 2008/1098
D	S2060 - 2008/1099				S2060 - 2008/1099				S2060 - 2008/1099			
E	)				S2060 - 2008/1102				S2060 - 2008/1102			
F	**SIAH1 samples**				**SIAH2 samples**				**TadA samples**			**no phage samples**
G	LB				LB				LB			
H	S2208				S2208				S2208			

DIMECRES, 14/8/2024

- [ ] troubleshoot SIAH KO SDMs
- [x] do phage propagation qPCR
- [ ] do phage propagation with S2060/2008/1097 and S2060/2008/1103 (they are in the shaker in the second shacker room)

  --> those are the samples that were spilled :(-> look for plates and repick if possible or retransform
  - [ ] potentially also include S2060/2008/1100 if it grew and send it for sequencing -> no, retransform
  - [x] make cryo-stocks for these strains
  - [x] check spilled on es again (sequencing)

- [ ] repeat co-transformation of pES2008 with pES1101 (and potentially with pES1100 if it did not grow) + check what others are we missing atm?
- [x] doublecheck pES1102 sequencing and potentially send other clones for sequencing
- [x] check S2060/DP6/2008 plate (did not grow yesterday)
- [x] label DP6 cl.A tube (re-sequencing confirmed it's correct)

in Lab: Noemie

qPCR from overnight propagation assay

- plate 2 well G11 contaminated
- 105 wells in total => for 1-2 technical replicates: we only have 192 wells bqs of Polymerase shortage
  - Plate 1: 1 Technical replicate
  - Plate 2: 2 Technical replicates
- Potential Issue with pES1102 cotranformant: I don't think there is an Issue there, the "mutations" seen in sequencing are in areas with low quality and in nucleotide repeats
- `Data`

check plates:

- S2060/pES2008/DP6 did not grow
- S2060/DP6/pES2008/pES2023 did not grow
- I put them back in the Incubator
- If they don't grow, we could try to retransform and plate them on LB plates with less antibiotics (e.g only half)

Phage Propagation Assay

- inocculated S2060/2008/1097 A and S2060/2008/1103 A (here, sequences are confirmed) with starting OD 0.2 -> let grow until OD reached 0.5 -> the ones that overshot were diluted back down to OD 0.5
- As these were in the Beaker where some of the other cultres got spilled, I sent them for sequening again to check of we have contamination. I kept clones B and C in the fridge in case sequencing shows that these tubes got contaminated. Fresh colonies were picked & are in the shaker if this would be the case.
- As we saw today that TadA titer is too high, I reduced the amount of TadA used to 2 uL (SIAH1 and SIAH2 remained at 10 uL)
- incubation at 225 rpm o/n at 37°C

Phage ON propagation assay - 14.8.

	1	2	3	4	5	6	7	8	9	10	11	12
A	1103 + SIAH1			LB + SIAH1								
B	1103 + SIAH2			LB + SIAH2								
C	1103 + TadA			LB + TadA								
D	1097 + SIAH1			S2208 + SIAH1								
E	1097 + SIAH2			S2208 + SIAH2								
F	1097 + TadA			S2208 + TadA								
G	LB			S2208								
H												

Co-transformations in compentent S2060 cells

- 2008 + 1101/1100 (bcs these didn't grow/only few colonies in the previous try)
- 2008 + 1035/1082 (to get the asyn constructs also with human E1, so far we only teste the wheat one)
- premixed the two plasmids in equimolar ratio (total of 300 ng per shot) before adding to the cells, transformedn in 50 ul shots, plated after 2.5 h of recovery

SIAH1 Knockout:

- initial PCR reaction looked fine (sizewise), but the gel was a bit blurry -> repeated the pcr reaction
  - Template: 1 ng SIAH1-SP // Primer: 080, 081
  - Ta: 67°C, Elongation 90 s
  - storen in the fridge o/n

set up 200 ul liquid cultures from glycerol stock to start liquid culutres tommorow mornign to repeat the phage propagation assay from 13.8.

DIJOUS, 15/8/2024

Color code:

high Priority
medium priority
first thing in the morning

- [x] repeat phage propagation assay from 13.8, add the new ones from yesterday, reduce TadA input - do we repeat the entire assay or only the mutagenized EGLN3?
  - [x] inocculate 1:100 liquid culutres (from the small cultures in the incubator)
- [x] create EGLN3 mutants as peptide (either PCR + Gibson from the mutant plasmids or mutagenize the Peptide with the SDM primers-> depends a bit if we have enough KLD or not)
- [x] check sequencing of 2008+1097/1103 -> if it looks good (& we have enough Polymerase for qPCR) do qpcr of propagation assay (it is at 37°C) ; if not, repeat propagation assay with new culture

- [x] check pES2023-31 sequencing and miniprep o/n cultures (pellets are in the freezer) - if we have time, show Tara how to miniprep
  - [x] co-transform S2060/DP6 with pES2023-31 and pES2008 if they are correct (! add glucose to plates, reducing antibiotic concentration)
- [x] cotransformations (! 100 mM of glucose is needed in the medium/plates when working with DP6)
  - [x] pace strain with alphasynuclein: add DP6 -> discuss if we use 2008 or 2009
  - [x] repeat the transformations with DP6 that didn't work this week
  - [x] transform DP6 into competent S2208 cells (for drifting SIAH1)
- [x] check plates in the incubator (cotransformations) -> very small colonies, we wait another day
- [ ] find or clone a negative control substrate plasmid (maybe design a primer that mutates the 3 lysines in the ELGN3 peptide plasmids)
- [ ] prepare PACE (for alpha-synuclein): prepare tubing for autoclaving, prepare enough media, pregrow bacterial culture
  - [ ] prepare an extra set of tubing for autoclave - so we have a backup and don't have to wait a day if we need to restart the reactor
- [x] SIAH1 KO: run a gel/ purify? depends on gel results - KLD treatment + transformation (pcr reaction is in the fridge, in the green rack) -> big smear and no bands on the gel
- [ ] Low priority but maybe useful --> in cloning checklist have a list for the co-transformants that we have

in Lab: Noemie, Michael

Phage propagation Assay - repeat 13/8

- diluted o/n cultures 1:100 in appropriated LB + antibiotics (total volume of 15 mL per culture)
- put in shaker 37°C at 11:00
- at 14:15 the ODs were still very low (0.06 - 0-12). All tubes aside from S2208 (OD 0.84) were brought back in the incubator

Set up PCRs for creating EGLN3 mutants as peptides (slots 4-6)

- used the gibson assembly approach for all variants execpt for FIADVEA (becase primer for pcring the peptide out of the plasmid covers the back of the degron
- for FIADVEA, the KLD approach was used
- all reactions were 25 ul reaction, except for 76 (50 ul reaction)

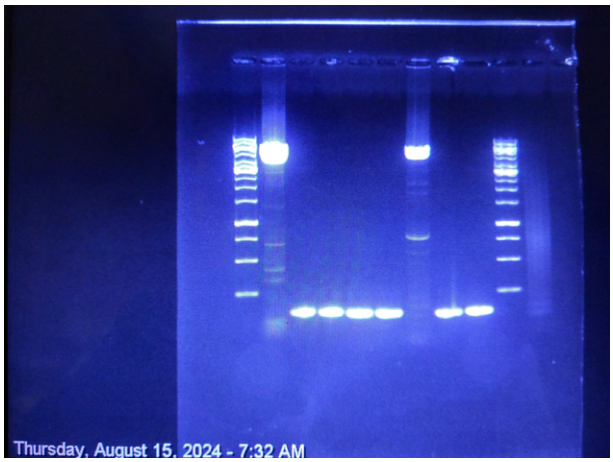
PCRs 15.8.

	A	B	C	D	E	F	G	H	I
1	Reaction Label	97	98	99	100	102	103	76	101
2	Template	pES1097	pES1098	pES1099	pES1100	pES1102	pES1103	pES1076	pES1076
3	Primers	071/072	071/072	071/072	071/072	071/072	071/072	073/074	067/068
4	Ta	63	63	63	63	63	63	70	69
5	Elongation Time	30s	30s	30s	30s	30s	30s	2.5 min	3 min

All PCR products (+SIAH1 ko SDM) were digested with Dpn1 and 5ul PCR product was run on a gel

order: GeneRuler 1kb ladder, 1076, 1097, 1098, 1099, 1100, 1101, 1102, 1103, GeneRuler 1kb ladder, SIAH1 ko SDM

20240815_160832 (002).jpg



All bands have the expected size and the PCR products were directly purified (no gel extraction since the off-target bands were very weak)

- 1101 was treated with KLD
- After column purification: 1103 had a A260/230 value was 0.02

tomorrow: assmebly

- Gibson for 97, 98, 99, 100, 102, 103 with 76
- KLD with 101, SIAH
- Transformation

Phage Propagation Assay from 14.8.

- Polymerase for qPCR has not arrived yet - moved plate to the fridge, we cam measure it the in the next few days
- Sequencing of the ON culutres used to set up the assay: 2008 confirmed in both, both level ones also confirmed - all good, we can continue with these strains

Sequencing Analysis of level 2 Assemblies:

- pES2023 B ok
- pES2024 A ok, B ok
- pES2025 A ok, B ok
- pES2026 B ok
- pES2027 B ok
- pES2028 A ok
- pES2029 A ok, B ok
- pES2030 C ok
- pES2031 D E F all empty backbone

Co-transformations:

poured agar plates: with 0.6/0.8x antibiotics & 100 mM Glucose

10 plates Kan + Spec + Chlor 9
1 plate Kan + Chlor
2 plates Kan + Carb + Chlor

- S2060/DP6 with pES2023-31 and pES2008 (2031 sequencing was incorrect -> only to 2030)
- S2060/DP6/2008 -
- S2060/DP6 + 1035/1081 + 2008/2009
- S2208 with DP

All plates are in the 37°C incubator

Miniprep:

- pES2023: 91.9 ng/uL
- pES2024: 71.0 ng/uL
- pES2025: 128.5 ng/uL
- pES2026: 91.6ng/uL
- pES2027: 111.5 ng/uL
- pES2028: 110.1 ng/uL
- pES2029: 110.3 ng/uL
- pES2030: 103.4 ng/uL

O/N propagation assay

- All bacteria were grown to an OD of 0.44 - 0.52
- As before 10^8 PFU/ml phages were used: 10uL of SIAH1, 10uL of SIAH2 and 2uL of TadA (TadA is probably higher concentrated)

Plate design:
row 1-3: SIAH1, row 5-6: SIAH2, row 9-11: TadA

Plate 1

	1	2	3	4	5	6	7	8	9	10	11	12
A	S2060 - 2008/1099				S2060 - 2008/1099				S2060 - 2008/1099			
B	S2060 - 2008/1096				S2060 - 2008/1096				S2060 - 2008/1096			
C	S2060 - 2008/1097				S2060 - 2008/1097				S2060 - 2008/1097			
D	S2060 - 2008/1098				S2060 - 2008/1098				S2060 - 2008/1098			
E	S2060 - 2008/1102				S2060 - 2008/1102				S2060 - 2008/1102			
F	LB + phage				LB + phage				LB + phage			
G												
H									LB without phages			

Plate 2

	1	2	3	4	5	6	7	8	9	10	11	12
A	S2060 - 2008/1076				S2060 - 2008/1076				S2060 - 2008/1076			
B	S2060 - 2009/1081				S2060 - 2009/1081				S2060 - 2009/1081			
C	S2060 - 2009/1035				S2060 - 2009/1035				S2060 - 2009/1035			
D	S2060 - 2009/1033				S2060 - 2009/1033				S2060 - 2009/1033			
E												
F	S2208				S2208				S2208			
G												
H												

DIVENDRES, 16/8/2024

- [x] Prepare PACE Reactor for a-Synuclein
  - [x] prepare tubing for autoclaving, prepare enough media,
  - [x] prepare an extra set of tubing for autoclaving - so we have a backup and don't have to wait a day if we need to restart the reactor
- [x] Phage propagation assay: qPCR from 15.8. and 14.8. (fridge) - if qPCR Polymerase has arrived
- [x] Gibson assembly and transformations of EGLN3 mutants peptide (97, 98, 99, 100, 102, 103 with 76)
- [x] transform Dpn-treated 1101
- [x] Cotransformations: check plates in the incubator from 14.8. and 15.8.
  - [x] pick if they grew (plates from 14.8. grew, nothing from 15.8 yet)
  - [x] check if correct plasmids are present (sequencing with appropriate primers that cover regions of interest)
- [x] Negative control substrate: find or clone a negative control substrate plasmid
- [x] SIAH1 KO Troubleshoot
- [ ] Collect strains & establish a numbering system: in cloning checklist have a list of the co-transformants that we have

In the lab: Michael

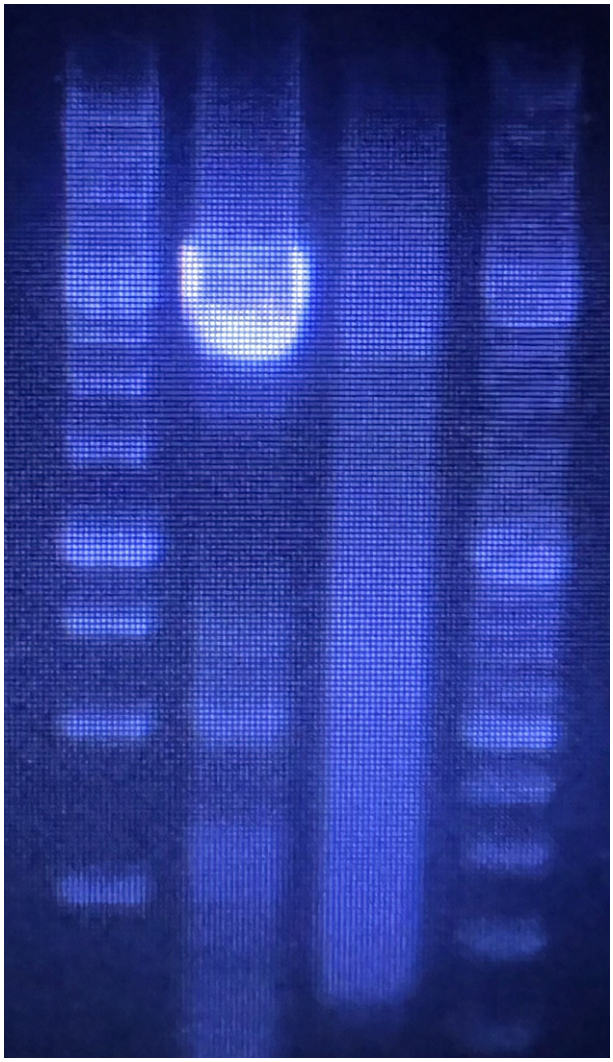
Negative control substrate

- I made 2 SDM pimers (084, 085) to exchange 3 lysines on 1096 (the plasmid that contains the 35 aa EGLN3 peptide) -> the resulting plasmid is pES1104 and its peptide can't be ubiquitinated
- The primers yield a peptide sequence that can be gibson assembled with the backbone on our bench labelled as 1076 Gibson (it's the 1076 backbone with extended ends from 073 and 074 primers)
- I accidently made a mistake in the 085 primer: it ends with TCTT instead of TATT which will just cause a silent mutation instead of a substitution with Arginine. -> I cancelled the order when I noticed it a few hours later.
- Microsynth sent us two e-mails: One where they said this primer is cancelled and one where they das the order could not be cancelled because the primer is already in production. This means we will only have the correct primer pair after one day later.

SIAH 1 ko SDM PCR

- I repeated the SDM PCR (80, 81, and 82, 83 primers) with lower annealing temp (62 °C) and longer extension times (2 min). The previously used temperatures and extension times should be fine in theory but this is everything we can do at the moment. The primers shouldn't form dimers or secondary structures and there is no obvious reason why this didn't work
  --> ran both PCRs on a gel -- GR 1kb - SIAH1 KO - SIAH2 KO - GR Mix:

IMG_5364.jpeg



  - SIAH2 KO PCR is nothing, SIAH1 KO has a band at ~3 kb as it should but there are also other bands--> since it already did not work once like that, I tried gel purifying --> yield: 31 ng/µL (but curve looked really bad)
  - Our SIAH2 SP DNA could maybe be different from the sequence on Benchling, otherwise it woudln't make sense that primers with one mismatch don't bind, even at very low annealing temperatures

PACE

- propared two sets of tubing and two 2L LB bottles (without antibiotics or glucose) and autoclaved them

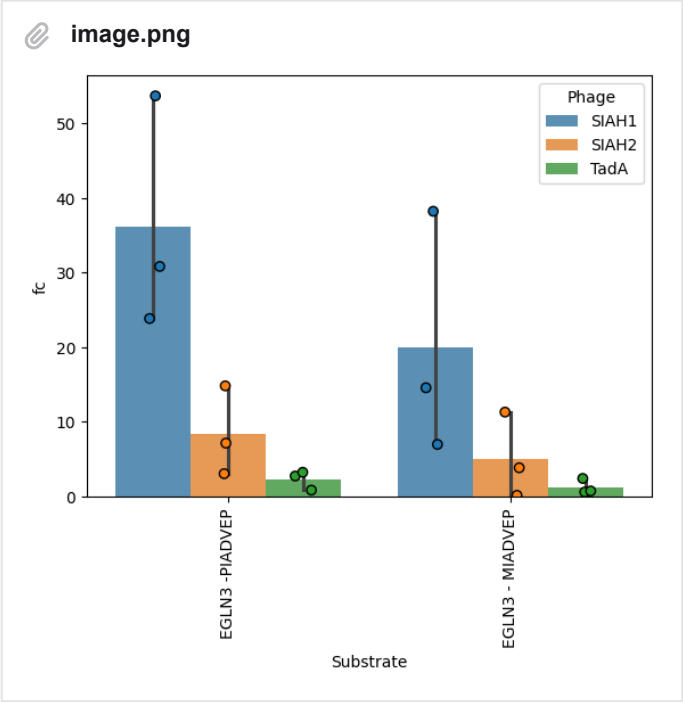
qPCR of Phage propagation assays from 14.08 and 15.08

- I did a Mastermix for 200 samples -> 10uL primer needed but the OLS-62 had only 8.5-9.5 uL left - qPCR should still work fine but if we see higher Cp values we know why
- oLS-1662 and 1063 primers are empty now --> aliquotes were refilled

These qPCR plots are also inserted on 19.8 (because they were generated then) but I will also put them here for the sake of clear chronological order

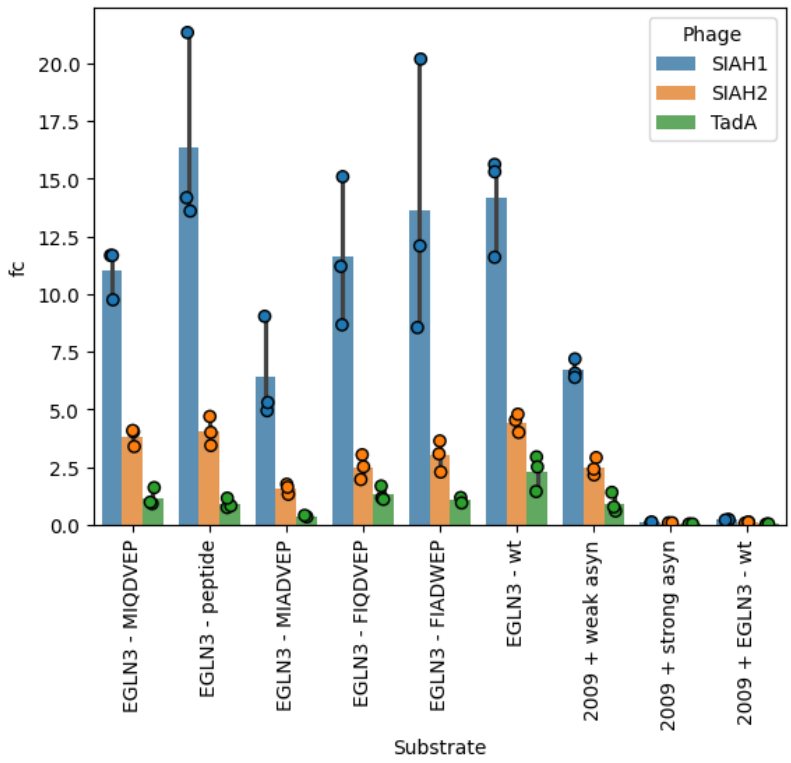
propagation assay 14.8

image.png



propagation assay 15.8

image.png



Gibson Assembly

minimum amount of DNA per assembly: 1.5 pmol

Table15

	Assembly name	backbone	insert	backbone ammount	insert ammount
1	pES1105	1076 - Gibson	1097 - Gibson	0.05 pmol / 1 uL	0.5 uL
2	pES1106	1076 - Gibson	1098 - Gibson	0.05 pmol / 1 uL	0.5 uL
3	pES1107	1076 - Gibson	1099 - Gibson	0.05 pmol / 1 uL	0.5 uL
4	pES1108	1076 - Gibson	1100 - Gibson	0.05 pmol / 1 uL	0.5 uL
5	pES1109	1076 - Gibson	1102 - Gibson	0.05 pmol / 1 uL	0.5 uL
6	pES1110	1076 - Gibson	1103 - Gibson	0.05 pmol / 1 uL	0.5 uL

- every reaction contained 1 uL backbone, 0.5 uL insert, 1 uL H2O, 2.5 uL mastermix
- using the same volume (0.5 uL) for all inserts will yield different insert:backbone ratios since the DNA concentrations are different. The insert:backbone ratios start from 2.2 : 1 (in 1005) and go up to 13 : 1 (for 1099)
- Incubatd at 50°C for 20 min

Transformation: Transformation

- transformed of each Gibson Assembly (1105-1110) and also 1111 KLD.
- 3 µL assembly of each in 30 µL DH5a, high efficiency protocol

O/N cultures:

- Innoculated co-transformant plates from 14.8 and sent them for sequencing

DISSABTE, 17/8/2024

Color code:

high Priority
medium priority
first thing in the morning

- [x] Cotransformations: check plates in the incubator from 15.8 and 16.8
  - [x] pick if they grew
  - [x] check if correct plasmids are present (colony PCR or sequencing with appropriate primers that cover regions of interest for the strains that aren't directly going into PACE)
  - [ ] re-transform colonies that didn't grow (if there are any. Note: 2 empty Chlor + Carb + Kan + Gluc plates are still on the bench in case you need to re-transform)
  - [x] set up overnight propagation assays --> tomorrow
  - [x] If S2208 + DP6 grew: pick, confirm (colony PCR) and put SIAH1 in it (to start drifting the SIAH1 sequenece)

- [x] Sequencing Analysis of co-transformants (S2208 2008 w/ 1081, 1101, 1100, 1035) --> because it is NightSeq, we won't have the results until monday, however I will perform the propagation assay anyways tomorrow (today, cultures were not grown too much because the shaker wasn't shaking, so I let them shake during the day and put in the fridge in the aftrernoon to refresh them tomorrow)
  - [x] If the sequence is correct, set grow the cultures to OD 0.5 and start phage propagation assay

- [x] PACE:
  - [x] pick 2008/2009+aysn colonies for PACE, do colony PCR (or sequencing but then we have to wait)
  - [x] if they look correct -> innoculate to OD 0.6 and start PACE (lagoon and chemostat glass might look slightly dirty but it's autoclaved and is fine. The other glassware was washed and will be autoclaved) --> first we need to do the drifting of SIAH1

- [x] refill oLS-1662 and 1063 primer aliquotes (we ran out of them)
- [x] make assemblies of pES1104 - pES1111 in Benchling
- [x] Cloning:
  - [ ] APs with different promotor strengths for the various components (e.g. strong/weak & maybe also inducible?) for both AP1 and AP2
  - [x] a-synuclein constructs as level 2
- [x] Clean up our fridge compartment (decide which plates and tubes to keep) --> plates with colonies were stored at 4ºC room in the labeled bags.

- [ ] Collect strains & establish a numbering system: in cloning checklist have a list of the co-transformants that we have
- [x] Add 1104-1111 to clonning checklist in benchling

In lab: Gabriel, Pau

Transformants and co-transformants from 15.08 and 16.08

What did grow:

- DH5a + pES1109 to pES1111 --> pick for o/n culture, tomorrow miniprep, monday send for seq
- S2060/DP6 + 2008 - S2060/DP6 + 2008 + 1081 - S2060/DP6 + 2009 + 1081, S2060/DP6 + 2009 + 1035 --> pick for o/n culture, do colony PCR, and if looking good they can be used to start PACE with one of the co-transformants (first, talk to Sasha about phage drift)
- S2208 + DP --> pick for o/n culture, do colony PCR, and if good tomorrow add SIAH1 to start drift
- S2060/DP6 + 2008 + 2023 - S2060/DP6 + 2008 + 2024 - S2060/DP6 + 2008 + 2026 - S2060/DP6 + 2008 + 2027 - S2060/DP6 + 2008 + 2028 - S2060/DP6 + 2008 + 2029 --> pick for o/n culture, do colony PCR, if good then use for phage propagation assay

What did not grow:

- S2060/DP6 + 2008 + 1035
- S2060/DP6 + 2008 + 2025 - S2060/DP6 + 2008 + 2030

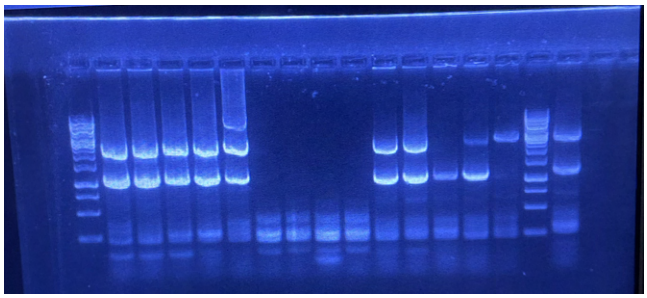
Colony PCR (see above for which colonies were used)

Primers 18,19
Ta: 62 (NEB calculator)
Extension time: 4 mins

2x 1% agarose gel

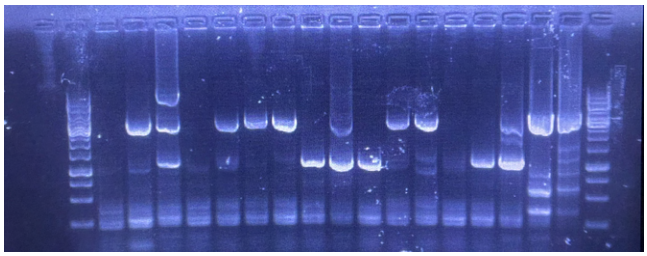
Order Gel 1:
1kb ladder
1-3: DP6+2009+1081
4-6: DP6+2009+1035
7-9: blank
10-12: DP6+2008+1081
13-15: DP6+2008+2023
1kb ladder

Colony PCR 1



Order Gel 2
1kb ladder
16-18: DP6+2008+2024
19-21: DP6+2008+2026
22-24: DP6+2008+2027
25-27: DP6+2008+2028
28-30: DP6+2008+2029
31,32: Control (2003)
1kb ladder

Colony PCR 2



Weird bands seen. 1 at 3 kb which corresponds wiht substrate (lvl1 or lvl2) but no band at 8 kb, however there are consistent bands at 1 kb. Sequences and binding checked. It is not from the drift plasmid, and did not show up on positive control with pES2003. Not seen through all samples, so probably not genomic amplification.

Results: gels look pretty bad... However, colonies grew, and all plasmids transformed had been previously confirmed with sequencing. An alternative is to miniprep and run gel to see the bands directly without amplification. Could be that plates were bad, or antibiotic concentrations too low as well.

We do an O/N culture of one replicate per transformation. We take the colonies corresponding to the positions: 1, 4, 5, 11, 15, 18, 21, 24, 27, 30. Tomorrow we will do minprep and run a gel.

Generate lvl2 constructs for a-synuclein

EcoFlex Assembly

Golden Gate Assemblies pES2032 and pES2033:

- 5:1 ratio insert:Dummy-B (higher excess is recommended for small inserts), e.g., 0.4uL/reaction(stock at 48 ng/ul)
- 2:1 ratio insert:vector, i.e., 0.5uL/reaction
- Added 100 ng corresponding Level 1 insert (1035, 1081), i.e., 1 uL/insert
- Cycling Protocol: Lukas' GG BsmbI protocol (runs 19 h overnight)

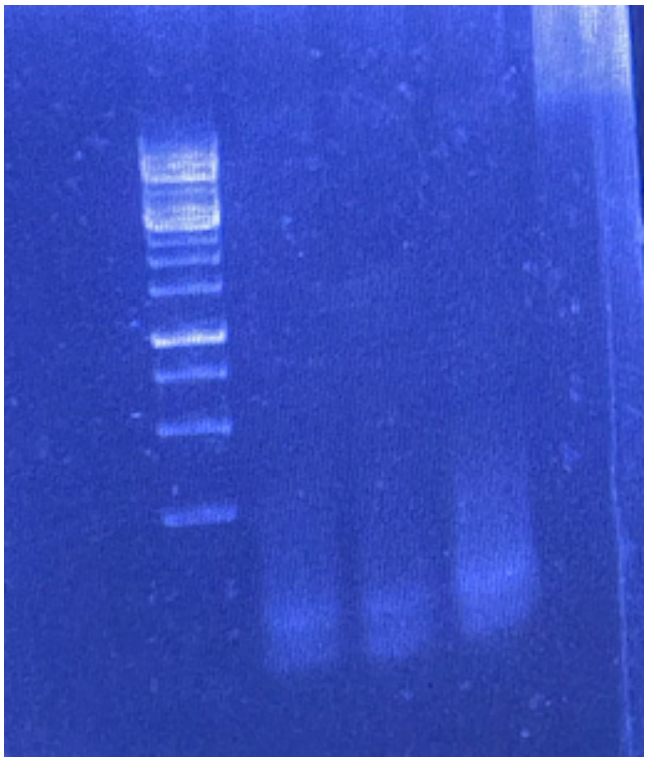
S2208+DP6 check

PCR: Q5 Polymerase

Colony PCR with primers o009 and o010 and then run gel. Should yield a band of 1kb aprox.

Ta: 70ºC (according to NEB), elongation time: 30s.

Colony PCR DP6



Result: somehow it did not work, repeat tomorrow.

DIUMENGE, 18/8/2024

high Priority

medium priority

first thing in the morning

- [x] Miniprep o/n cultures with S2060+DP6 co-transformants and run colony PCR --> o/n cultures did not grow
- [x] Miniprep o/n cultures of pES1105-pES1111 + Lukas' tubes

- [x] Do phage propagation assay with all co-transformant (lvl2+lvl2 co-transformants, 2008+1101/1081/1035/1110, ...) liquid cultures (glucose for those with DP6!) --> not done as o/n cultures did not grow
- [x] Start phage drift with o/n culture of S2208+DP6 --> set o/n culture of S2060+DP6 from -80 stock
- [x] Transform pES2032 and pES2033 DH5a (the one that is not in A43, it has the blue line)
- [x] Repeat co-transformations with S2060+DP6 cells
- [x] Prepare o/n culture of non-competent S2060+DP6 from stock in< -80 --> it's in the shaker

In Lab: Gabriel, Pau

ON cultures did not grow

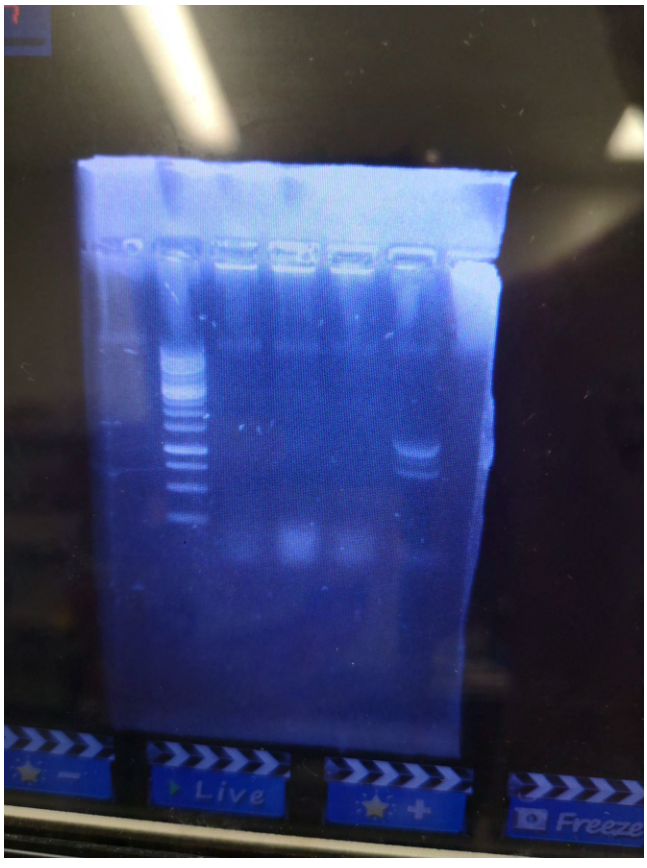
Should retransform all samples. We run a colony PCR again for S2208+DP6 with a positive control.

S2208+DP6 check

PCR: Q5 Polymerase

Colony PCR with primers o009 and o010 and then run gel. Should yield a band of 1kb aprox.
Ta: 70ºC (according to NEB), elongation time: 30s
Gel order: GenRuler 1kb - 3x S2208+DP6 colonies + positive control (DP6 plasmid)

IMG_20240818_122444.jpg



Result: the S2208+DP6 did not work. However, we spoke with Lukas and this is what he told us:

DP6 drift

Spoke with lukas, he says drifing in S2208 with DP6 is not necessary as the DP6 has inducible gIII (tetracycline). The combination of 2 gIII-containing plasmids may be a burden on the cell, and explain why the transformation of DP6 into S2208 was not very successful.

This should also mean we can drift in any cell with DP6 as long as we induce gIII with tetracycline.

Given that right now we have no co-transformant with the DP6 confirmed, we decide to start a o/n culture of S2060+DP6 from the -80 stock. Tomorrow, this can be used to start the phage drift in the PACE reactor.

Miniprep pES1105-1111
pES1105A 261.6 ng/uL
pES1105B 246.6
pES1105C 188.9
pES1106A 247.4
pES1106B 215.4
pES1106C 213.4
pES1107A 246.3
pES1107B 343.4
pES1107C 205.1
pES1108A 307.8
pES1108B 228.6
pES1108C 262.6
pES1109A 346.1
pES1109B 238.1
pES1109C 276.1
pES1110A 217.0
pES1110B 258.4
pES1110C 232.5
pES1111A 361.9
pES1111B 331.8
pES1111C 401.4
--> diluted to 100 ng/uL

Transformation of pES2032 and pES2033
5uL assembly reaction in 100uL DH5a competent cells.
High-efficiency protocol, 1h recovery time.

Repeat co-transformation
Transformation
Transformed using the high efficiency protocol with 30uL of competent cells and 1h recovery time. Added a 300ng of plasmid mix at 1:1 ratio (plasmids were mixed beforehand) and cells were plated on agar with Chlor + 100mM glucose + corresponding antibiotics. Because using 3 antibiotics, working concentration was set to 0.6x. Streaked plates were left on the 37ºC incubator o/n.

Given that there weren't many competent cells left, I prioritized the transformation of:

- S2060/DP6 with pES2008+pES1035 (Kan + Carb + Chlor + glucose)
- S2060/DP6 with pES2008+pES1081 (Kan + Carb + Chlor + glucose)
- S2060/DP6 with pES2008+pES2023-30 (Kan + Spec + Chlor + glucose)
    - For pES2028+pES2030, I used an unlabeled tube that was together with the rest of the labeled "S2060/DP6" competent cell tubes, so I hope it's the same

Did not co-transform with pES2009 given that during the previous meeting we talked about using human E1 instead of tE1.

DILLUNS, 19/8/2024

high Priority
medium priority
first thing in the morning

- [x] Inoculate a PACE 50mL tube with S2060+DP6 (there is a o/n culture in the shaker) to start phage drift in the afternoon. The rest of the S2060+DP6 cells shoud be used to make new competent cells
- [x] Make S2060+DP6 competent cells
- [x] Start phage drift with S2060+DP6 and tetracyclin --> see yesterday's notes on what Lukas told us for more info
- [x] Send minipreps for pES1105 to pES1111 for sequencing --> sorry didn't have time to do it yesterday, the miniprepped eppendorfs are on the small fridge in a white eppendorf rack
- [x] Check if co-transformations grew: -> no / very tiny colonies
    - [ ] pick if they grew
    - [ ] check if correct plasmids are present (colony PCR or sequencing with appropriate primers that cover regions of interest for the strains that aren't directly going into PACE)
    - [ ] re-transform colonies that didn't grow (if there are any. Note: 2 empty Chlor + Carb + Kan + Gluc plates are still on the bench in case you need to re-transform)
    - [x] Start o/n phage propagation assay with all co-transformants:
        - [ ] Lvl2+lvl2 co-transformants --> add glucose as they contain DP6
        - [x] Liquid cultures in the fridge with 2008+1101/1081/1035/1100 --> we sent for sequencing on Friday, confirm beforehand
- [x] Design primer to be able to sequence lvl2+lvl2 co-transformants --> maybe we could make a primer for SmR resistance cassette, similar to how we have the one for KanR.
- [x] Make LB Agar - I made two 1L bottles

In the lab: Michael

Colony picking + innoculation for drift and O/N propagation assay

- No colonies on any plates yet (at 9:30), one colony on pES 2033
- S2060 + DP6 (and all 2008+EGLN3 variants) bacteria were innoculated 1:20 in LB + antibiotics (and glucose) at 10:00. At 13:20, S2060 + DP6 cells had an OD of 0.61 and were put into a chemostat flask

Competent cells

- I made S2060 + DP6 competent cells in 200 uL aliquotes. Should be enough for the rest of the project

SIAH1 drift

- assembled and started the PACE reactor with S2208 + DP6 (OD 0.6) and 10^5 PFU/mL in the lagoon
- 100 mL 2 M glucose, 1 mL 1000x chloramphenicol and 2 mL 1000x tetracycline were added to the medium

Sequencing Analysis

- 2008 + 1035A and B are perfect in both plasmids. 1035C very low signal in 2008 and potentially multiple point mutations in 2008
- all sequences of 2008 + 1081 colonies look good
- 1100A and 11001A have a point mutation in the polymerase in 2008 but look good otherwise
- 2008 + 1100 and 1101 B and C look good in both plasmids

New sequencing SmR Primer

- made primer o086 for sequencing plasmids containing SmR in Benchling. I ordered it to be stored at Microsynth.

qPCR data analysis from last week

- used Sasha's Script to make plots for the phage propagation/qPCR results from 14.8 and 15.8
- Again, it looks like the the propagation isn't really motif-dependent :/ Also two of the 2009 cotransformant assays didn't work (somehow LB had a higher Cp in the bacteria, maybe an issue with mutli-channel pipette accurracy)

plot from 15.08

image.png

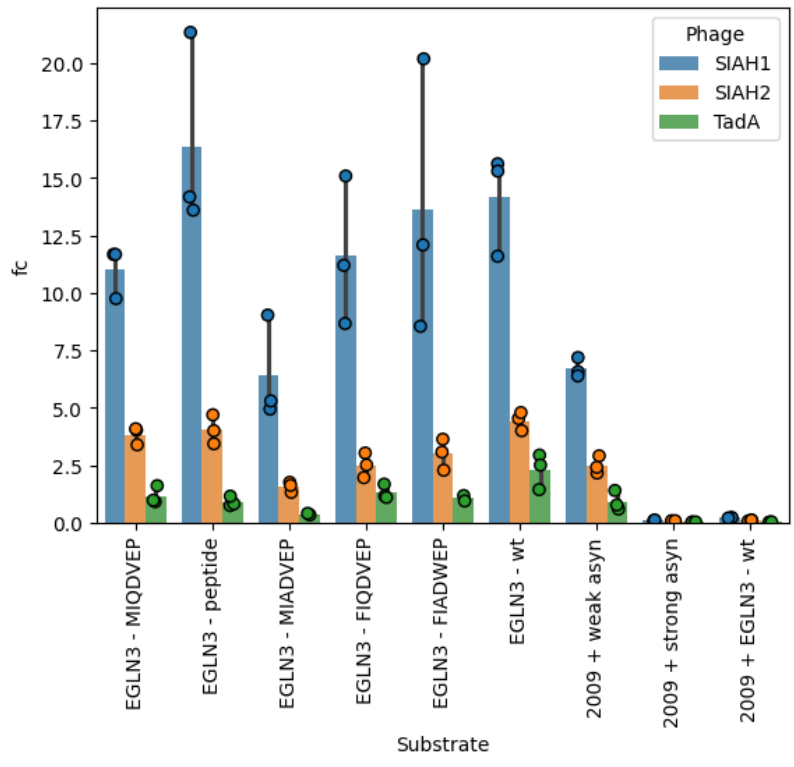


Table16

	A	B	C
1	**Substrate**	**Phage**	**fc**
2	2009 + EGLN3 - wt	SIAH1	0.213555349032212
3	2009 + EGLN3 - wt	SIAH2	0.098167893928574 4
4	2009 + EGLN3 - wt	TadA	0.023287056643 1042
5	2009 + strong asyn	SIAH1	0.094814915452 9502
6	2009 + strong asyn	SIAH2	0.076280248810 9034
7	2009 + strong asyn	TadA	0.024331837089 9640
8	2009 + weak asyn	SIAH1	6.712459648136622
9	2009 + weak asyn	SIAH2	2.492672387677700
10	2009 + weak asyn	TadA	0.921079221256652
11	EGLN3 - FIADWEP	SIAH1	13.60344749677285
12	EGLN3 - FIADWEP	SIAH2	2.995118138527717
13	EGLN3 - FIADWEP	TadA	1.059845636556850
14	EGLN3 - FIQDVEP	SIAH1	11.64834662122203
15	EGLN3 - FIQDVEP	SIAH2	2.501433492411277
16	EGLN3 - FIQDVEP	TadA	1.311590332500222
17	EGLN3 - MIADVEP	SIAH1	6.423248476177946
18	EGLN3 - MIADVEP	SIAH2	1.565741382033914
19	EGLN3 - MIADVEP	TadA	0.372524818699286
20	EGLN3 - MIQDVEP	SIAH1	11.03053587244148
21	EGLN3 - MIQDVEP	SIAH2	3.832950650833526

22	EGLN3 - MIQDVEP	TadA	1.16480119195150
23	EGLN3 - peptide	SIAH1	16.3666730417718
24	EGLN3 - peptide	SIAH2	4.04186718089284
25	EGLN3 - peptide	TadA	0.906088895723572
26	EGLN3 - wt	SIAH1	14.1677234967862

O/N propagation assay from 14.08:

image.png

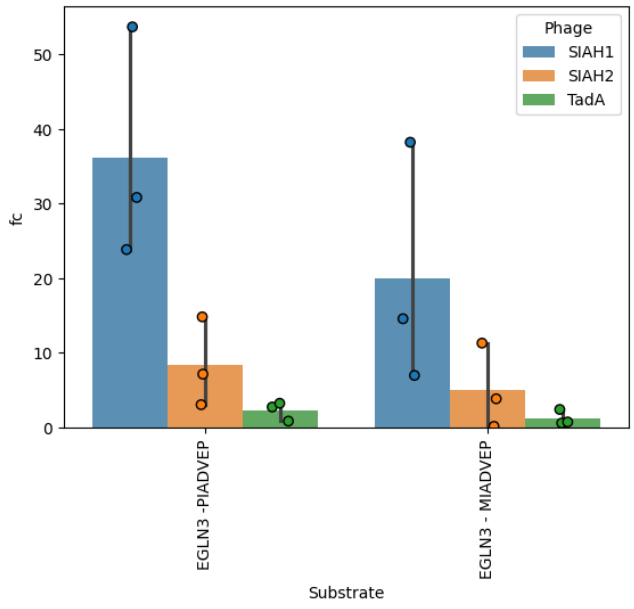


Table17

	A	B	C
1	**Substrate**	**Phage**	**fc**
2	EGLN3 - MIADVEP	SIAH1	19.9153480939401
3	EGLN3 - MIADVEP	SIAH2	5.1176698080 5046
4	EGLN3 - MIADVEP	TadA	1.2702507975 3260
5	EGLN3 - PIADVEP	SIAH1	36.089679402 6560
6	EGLN3 - PIADVEP	SIAH2	8.3531199734 1525
7	EGLN3 - PIADVEP	TadA	2.2981656441 0682

- Absolute fold changes differ between motifs but in my opinion only within the range of standard fluctuations

Overnight propagation assay

Plate layout

- Column 1-3: SIAH1,
- Column 5-7: SIAH2,
- Column 9-11: TadA
- innoculated 750 uL bacteria of OD 0.4-0.5 and 10 uL of 10^8 PFU/mL phage per well
- Unfortunately, three strains, 2008 + 1081/1035/1103, did not grow and I noticed too late. Instead of not including them in the assay at all, I decided to dillute them directly from a dense culture to ~ OD 0.5. So we have to take the data of this assay with a grain of salt.

Plate #1

	1	2	3	4	5	6	7	8	9	10	11	12
A	S2060 - 2008/1096				S2060 - 2008/1096				S2060 - 2008/1096			
B	S2060 - 2008/1097				S2060 - 2008/1097				S2060 - 2008/1097			
C	S2060 - 2008/1098				S2060 - 2008/1098				S2060 - 2008/1098			
D	S2060 - 2008/1099				S2060 - 2008/1099				S2060 - 2008/1099			
E	S2060 - 2008/1100				S2060 - 2008/1100				S2060 - 2008/1100			
F	S2060 - 2008/1101				S2060 - 2008/1101				S2060 - 2008/1101			
G	S2060 - 2008/1102				S2060 - 2008/1102				S2060 - 2008/1102			
H	S2060 - 2008/1103				S2060 - 2008/1103				S2060 - 2008/1103			

Plate #2

	1	2	3	4	5	6	7	8	9	10	11	12
A	S2060 - 2008/1076				S2060 - 2008/1076				S2060 - 2008/1076			
B	S2060 - 2008/1081				S2060 - 2008/1081				S2060 - 2008/1081			
C	S2060 - 2008/1035				S2060 - 2008/1035				S2060 - 2008/1035			
D	S2060 - 2009/1081				S2060 - 2009/1081				S2060 - 2009/1081			
E	S2060 - 2009/1035				S2060 - 2009/1035				S2060 - 2009/1035			
F	LB				LB				LB			
G												
H	S2208				S2208				S2208			

DIMARTS, 20/8/2024

- [x] Replace bacteria in the PACE reactor, these ones seem dead
  - [x] innoculate cryostock O/N
- [x] SDM PCR with 1076 to remove lysines (with primers 084 and 085 if they arrive)
  - [x] run it on a gel
  - [x] Gibson assembly
  - [x] transformation

- [ ] check structure of RNAP and potential ubiquitination sites
- [x] transform SIAH1 KO phage
- [x] Phage propagation qPCR
  - [ ] qPCR data analysis -> Sasha said he would do it
- [x] check if we have a negative selection plasmid
- [x] Check if co-transformations grew -> only one co-transformed colony grew
    - [x] pick if they grew
    - [x] check if correct plasmids are present (sequencing with appropriate primers that cover regions of interest for the strains that aren't directly going into PACE)
    - [x] re-transform colonies that didn't grow
    - [ ] Start o/n phage propagation assay with all co-transformants:
      - [x] Lvl2+lvl2 co-transformants --> add glucose as they contain DP6

- [x] Collect strains & establish a numbering system: in cloning checklist have a list of the co-transformants that we have
- [x] make glycerol stocks of confirmed co-transformants (especially liquid cultures in the fridge with pES2008 + 1035/1081/1101/1100, sequencing results should be in today)
- [x] lab duty: trash cardboard and pipette boxes

In the lab: Michael

qPCR of the last O/N propagation assay

- I ran the qPCR with all wells from the O/N propagation assay + PACE drift samples

The qPCR looks a bit suspicious: TadA propagatied as well as SIAH2.
Note: As mentioned before, the EGLN3 - PIADVEP culture didn't grow to OD 0.5 but was dilluted

image.png

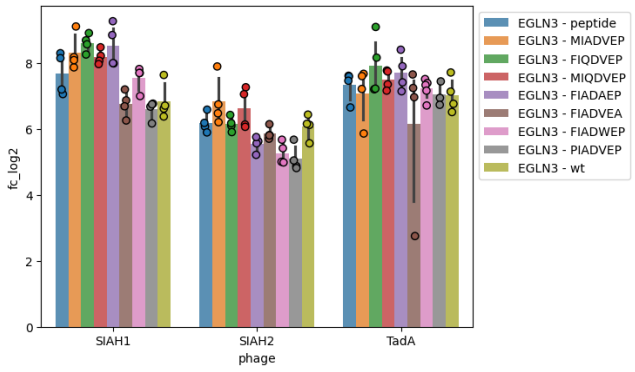
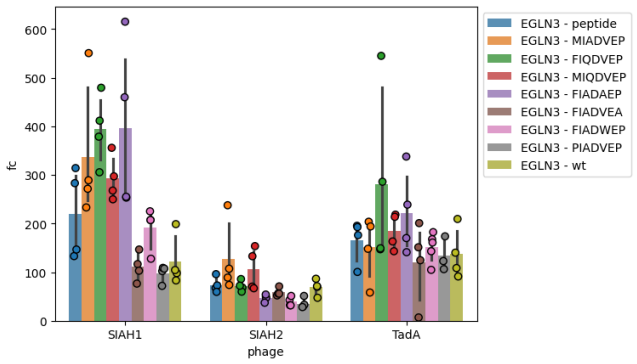


image.png



- The noise in the data is obviously very amplified because the absolute fold changes are super high this time
- even though the negative control might have been contaminated with SIAH, I think it's reasonable to conclude that changing our EGLN3 degron doesn't affect propagation

Colony picking

- No colonies on almost all co-transformants -> plates trashed, I will retransform today
- Only very few 2032 and 2033 colonies grew and only one colony on S2060 2008/DP6/1026 grew
- Picked the colonies that grew (2032, 2033, 2008/DP6/2026) and sent them for NightSeq with my new primer (SpecStart) + KanStart for the co-transformant. I didn't find any suitable primer for verifying DP6 -> maybe colony PCR tomorrow

Drift PACE

- The bacteria in the PACE chemostat died. I did a qPCR with chemostat and lagoon samples and they were almost identical (Cp ~11) -> the chemostat is contaminated with phages
- We have to find the source of the contamination before we restart everything. Our current hypothesis is that it's from the cryostock but the little ammount of phages would of course not be detectable -> I innoculated some S2060/DP6 in a liquid culture to check if we have phages in the culture
- qPCR fresh O/N culture and culture I used for innoculation the PACE reactor tomorroe

Cryostocks

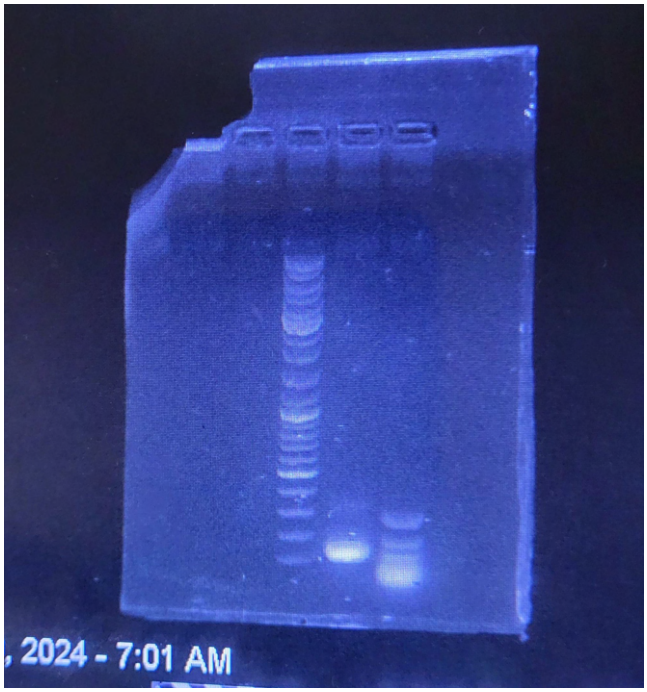
- I made cryostocks of every liquid culture we had (S2060+2008+1081/1033/1096/1097/1098/1099/1100/1101/1102/1102 and 2009+1081/1035/1033)
- 600 uL bacteria (OD 0.5 - 1) with 600 uL 50% glycerol
- While I put them in the freezer I realized that I made some cryostocks that we already have. We can trash the duplicates I made in case the box should get full at some points

Negative control substrate

- The 1096 SDM primers (084 and 085) arrived and I ran a PCR with them PCR: Q5 Polymerase
- 35 cycles, 70°C annealing temp (I lowered it due to the mutations in the primer), 30s elongation time, 1 uL 10ng/uL DNA of 1096
- I ran 5 uL of the PCR product on a gel and it looked perfect (expected size is 125 bp)

order: GeneRuler Mix ladder/ SDM product/ GeneRuler Ultra Low Range ladder

20240820_153836 (002).jpg



- I gel purified the rest of the PCR product and got a Nanodrop 54.8 ng/uL concentration but a very low A260/230 ratio (0.05)
- Gibson assembly Gibson Assembly
  - 2.5uL Master mix
  - 1uL H2O
  - 0.5 uL backbone (1076 with overlap)
  - 1uL insert (purified PCR product)
  - 50°C for 20 min
- KLD treated SIAH1-ko phage plasmid SDM with Q5 Mutagenesis + KLD Treatment

Transformation Transformation

- re-transformed 2008 + 2024-2029 in S2060/DP6 bacteria
- SIAH1 ko phages in 50 uL Dh5a
- negative control substrate (1104) in 50 uL DH5a
- I premixed the DNA for all transformations and followed the high efficiency protocol

New naming scheme for our co-transformants

- I used Max's template to get an overview of our glycerol stocks and started a new naming (S2060 + DP6 + 2008 + 1076 is getting too long)

- Our current glycerol stocks are labelled the long way but we should start labelling future stocks with this scheme
- The new strains have 6 digits
  - 1. digit: original strain. 6 for S2060 and 8 for S2208 (We need to define the meaning other numbers if we should ever keep other strains)
  - 2. and 3. digit: AP2 plasmid. 08 for 2008, 09 for 2009, etc.
  - 4 - 6. digit: AP1 plasmid: 076 for 1076, 101 for 1001, etc.
  - If the strain also contains the drift plasmid, the name should be appended by a "D"
- All our stocks are listed here
- Ignore this, the naming scheme was updated - see below

DIMECRES, 21/8/2024

- [x] qPCR of liquid S2060/DP6 cultures to detect phages (2 tubes in the 37°C shaker in the room with the ice machine) - run them on Taras plate in the afternoon
  - [ ] no phages in the bacteria -> grow S2060+DP6 to OD 0.6
  - [ ] restart the PACE reactor (drift)
- [x] Sequencing analysis of the EGLN3 peptide plasmids
  - [x] move tubes from temp. box to plasmid box
- [x] NightSeq Sequencing analysis of 2032/2033 and 2008+2026+DP6, check the liquid cultures - delay at microsynth; will come tomorrow
  - [ ] Find a way to confirm DP6: slower growth when no glucose (drop assay)? pBAD sequencing primers from microsynth ? sequencing woth 009 or 010? design sequencing primer to sequence forward from CmR?
- [ ] Negative Selection plasmids: what do we already have & what do we still need to do here?:
- [x] Check if co-transformations grew
  - [x] pick if they grew
  - [ ] check if correct plasmids are present (sequencing with appropriate primers that cover regions of interest for the strains that aren't directly going into PACE)
  - [ ] Start o/n phage propagation assay with all co-transformants:
    - [ ] Lvl2+lvl2 co-transformants --> add glucose as they contain DP6
    - [ ] Liquid cultures in the fridge with 2008+1101/1081/1035/1100 --> we sent for sequencing on Friday, confirm beforehand
- [x] relabel glycerol stocks and trash the doubles
- [x] put fresh LB to be autoclaved (with tubing)
- [x] set up culuters for time course experiment tomorow
- [ ] design primers for NLRP3 (it should arrive this week) - as soon as NLRP3 arrives, start cloning
- [ ] SIAH1 KO - when approach doesn't work again redesign and reorder asap
- [x] Cloning: (when sequencing shows that the assemblies with Lukas' protokol works): AP1 with different promoter strengths (strong/weak and maybe inducible? promoters as level 2, also asyn constructs as level 2)
  - PCR gIII neg -> construct 1006 for negative selection later
  - AP1 with all components under weak promoter -> 2032
  - have constructs so we can directly compare EGLN3 and asyn (same promter and same linker) -> e.g. with 1078

in Lab: Noemie, Michael

- We tested the bacterial culture that were used for PACE drift, the S2208/DP6 cryostock and the strains of the last O/N propagation assay for phage contamination with qPCR and they were all phage-free

Sequencing Analysis:

- 1110 is is asynuclein with Linker 4 (1081)
- The other plasmids look fine and only have mutations were signal quality is very low
- These clones look the best: 1105C, 1106A, 1107A, 1108A, 1109A, 1110C (1110 was sequenced as 1111)

NightSeq

- The data will come delayed (tomorrow) but I will re-innoculate the colonies I picked because the Spectinomycin I added to the liquid culture was actually Streptomycin (I took it from another freezer and the box was mislabelled) and the cultures didn't grow

Remarks to naming scheme for **co-transformants**

- how do we differentiate if the AP2 we use is a level 2? now there are only 3 numbers, where we can't see the difference btw level1 and level2 and it could occur that different strains end up with the same number
- new sheme: AP1 full 4 digit numebr - AP2 last 3 digits - 00 when no other plasmids and DP bzw MP when mutagensis plasmid is added

PCR (2 50 uL reactions)

- Template: p231.1 (should be pDB016?) - ca. 1 ng Template per reaction
- Primers: 009, 010
- Conditions: Ta 61 °C and elongation for 40 s
    - ran a gel, PCR didnt' work
    - used the wrong template plasmid: the correct template would be #99220 -> rerun pcr (rest stays the same) - didn't run the gel yet, stored the reaction in the fridge over night

Co-transformants in the incubator:

- I picked the few colonies that grew (1104, 2023, 2026, 2027, 2028) and innoculated them in O/N cultures
- We still have no colonies on the SIAH1 ko phage
- It was too late for dropping of the samples for NightSeq -> will miniprep and send for sequening tomorrow

Cloning of plasmids pES2032-pES2036 with BSmbI, assemle with Lukas' protocol overnight

- rational: these are AP1s with different promoter stregnths for the different components (E1, E2)

DIJOUS, 22/8/2024

- [x] NightSeq Sequencing analysis of 2032/2033 (? - we have a labelling clash here - these two plasmids were not on the cloning spreadsheet, so when I (Noemie) did new assemblies today, I assigned these numbers to different constructs -> they will need to be renamed when they are confirmed, but I don't know what sould be on there tbh) and 2008+2026+DP6, check the liquid cultures - delay at microsynth; will come tomorrow
    - [x] Find a way to confirm DP6: slower growth when no glucose (drop assay)? pBAD sequencing primers from microsynth ? sequencing woth 009 or 010? design sequencing primer to sequence forward from CmR?

- [x] Transformations of the Assembly reactions 2032-2036
- [x] Time course experiment: 2 plates (one in the shaker & taking measurements, one on the robot for luciferase readout) - EGLN3 wt & EGLN without VxP motif + controls - run 6 biological replicates (4x2 for luciferase plate, one set with 10x phage)
- [x] Co-transformants: Miniprep samples and send them for sequencing (or do colony PCR? 2 reactions per sample, with primers specific to only one of the plasmids), which ones didn't grow? should we retransform those?
- [x] SIAH1 KO - try to phosphorylate and ligate purified PCR product -> pcr und run a gel of the assembled reaction -> reorder primers if his doesn't work
- [x] Run gel on PCR reaction from yesterday
  - [x] when it looks okay, assemble 1006
- [ ] Set up propagation assay with the AP1s with different stringencies (pES2032-pES2035): have weak RNAP N-term, and varying expression of E1 and E2
- [ ] Start SIAH PACE on a target: a-syn? but as a level 2 construct (different ori)? - when using a level 2, make a phage propagation assay in parallel, to see effect between level 1 and level 2
- [ ] Confirm leakyness of RNAP subunit hybridisation: clone an AP without E2
  - [x] order Dummy C Primers (annealed oligos?) bcs we don0t have any other C part we can use
- [x] Confirm leakyness of gIII expression: transform a strain with only AP1 (e.g. pES2008) - run propagation assay to see how this affects phage propagation
- [x] retransform pES1004 (tube in our box is basically empty)
- [x] double check NLRP3 primers & order them (Noemie)
- [x] clone a level 2 plasmid with pES1087 as Ntrem RNAP (inducible, pVan) - don't forget that we need the gIII lux as a D1 part (pES1094), and add the repressor as an E Part
- [x] colony PCR of 2038, 2039 and colonies that didn't grow in liquid culture (Michael)

in Lab: Noemie, Michael

Time course experiment

- grew the strains S2208, 1076-08-00, 1100-08-00 to around OD 0.3-0.5, then they were diluted to match the exact OD 0.3
- for TadA I used 2 uL (SIAH1 and SIAH2 remained at 10 uL)
- Plate 1 was incubation at 225 rpm at 37°C, samples for qPCR were taken at the times intervals below (6 Biological replicates) - starting volume was 1 mL in each well
- Plate 2 was used for luciferase readout in parallel (2x4 Biological replicates) here: one set of replicates was induces with 10x phage)

Plate 1: Propagation in Deep Well Plate

	1	2	3	4	5	6	7	8	9	10	11	12
A	1076-08-00	1076-08-00	1076-08-00	1100-08-00	1100-08-00	1100-08-00	1078-08-00		1100-08-00		S2208	
B	Siah1	Siah 2	TadA	Siah1	Siah 2	TadA						
C												
D												
E												
F												
G	LB SIAH1			LB SIAH2								
H	LB TadA			S2208 -S1	S2208- S2	S2208 Tad						

Start: 11:40

Samples Taken -20 uL- from left half of the plate (the ones on the right are only there to check for phage contamination in the initial cultures at the very end) at timepoints:

12:10 Timepoint 1: Plate 1 Rows 1-6

12:41 Timepoint 2: plate 1 rows 7-12

13:45 Timepoint 3; Plate 2 Rows 1-6

14:45 Timepoint 4: plate 2 rows 7-12

16:45 Timepoint 5: Plate 3 rows 1-6

18:45 Timepoint 6: plate 3 rows 7-12

Plate 2: Phage propagation - Luciferase readout

	1	2	3	4	5	6	7	8	9	10	11	12
A	1076-08-00						1100-08-00					
B	Siah 1		Siah 2		TadA		Siah 1		Siah 2		TadA	
C	1x	10x	1x	10x	1x	10x	1x	10x	1x	10x	1x	10x
D												
E	S2208 +SIAH1			LB SIAH1								
F	S2208 + SIHA2			LB SIAH2								
G	S2208 + TadA			LB TadA								
H	1076-08-00		1100-08-00		S2208							

GE:

ran a gel with yesterdays PCR reaction (1% Agarose, 120 V, 40 min)

pcr product is present -> pcr purifiacation

GG Assembly: 2037 and 1006

- used 200 ng of the gIII PCR product (instead of pES0011)
- used Esp3I instead of BsmBI for the assembly of 2037 (so I can run them together in the same cycler)
- Lukas's 37°C protocol, 18 h

Plasmid names

-> renamed the plasmids that were assembled on the weekend (ex-2032, 2033, which contain asyn in lvl2 backbone) to pES2038/2039 because of a naming clash

NightSeq Sequencing Analysis

- All sequencing files we got were empty (NNN).
- All but one samples were sequenced with the new SpecStart primer - maybe there's an issue with the new primer
- -> I will run a colony PCR with 2038 and 2039 (ex-2032/33). If we see bands it means that the primer was the issue.
- one sample that was supposed to have a KanR-containing lvl2 plasmid (2026-08-DP) had also no sequencing signal. It also didn't grow in the liquid culture with kanamycin, chloramphenicol, spectinomycin and glucose. -> maybe the antibiotics were too low/ unevenly distributed on the agar plate?

Miniprep and Sequencing

- Miniprepped 3 liquid cultures of 1081-08-DP (A,B,C) and 1031-08-DP (A.B,C), 1104 (EGLN3 peptide without lysines) and 2032+2033 colonies (that grew on a Spectinomycin plate, in Spectonomycin liquid cultures) but couldn't be confirmed by NightSeq (NNNs).
- I didn't manage to send to colonies I picked for NightSeq yesterday, so I sent them for EconomyRun miniprepped sequencing today. If they look good, then keep the remaining liquid cultures in the fridge
- Miniprepping 2032+2033 was more to check if there was an issue with NightSeq / the SpecStart primer and to find out why theses colonies grow on spectinomycin but have no SpecStart sequencing signal.
- yields:
  - 1081-08-DP A:118 ng/uL, B:560.7 ng/uL, C: 120 ng/uL. 1033-08-DP A:377.3 ng/uL, B: 452.0 ng/uL, C: 1240 ng/uL.
  - pES1104: 1148.0 ng/uL, nothing (4 ng/uL) in 2032+2033

- I send the DNA of 1081-08-DP A,B and 1031-08-DP A,B for sequencing with 3 primers (017, KanStart, and pBAD_f for DP6)
- I also send DNA from 2030 for sequencing with the SpecStart primer, just to see if it works

Colony PCR

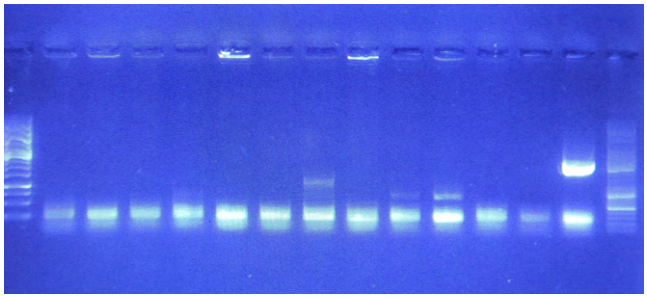
rationale: We have multiple S2060+DP+2008+2023-29 colonies growing on agar plates with antibiotics. However, when I picked them, none of them grew in liquid cultures with (0.8x) kan, chlor, spec and glucose (I'm 100% sure about the antibiotics in the LB).
I assume either the plates don't have the antibiotics (or not enough) I thought they have. Before we sequence these colonies we have, I want to check with a colony PCR whether it's even worth all the money (8 strains x 2 colonies x 3 plasmids).
To make matters even more complicated, 2038 and 2039 grew on Spec plates, in liquid cultures with spectonomycin but couldn't be sequenced in NightSeq with SpecStart -> also loaded them on a gel to see whats going on

- primers: 018, 019,
- 62 °C annealing temp (NEB calculator)
- 4 min elongation time (to see all plasmids on the gel), 32 cycles
- initial denaturation 1 min longer than usual
- I ran the gel only for 25 min but decided not to continue after having a first look

order: (23 is DNA from 2023-08-DP, 24 from 2024-08-DP, and so on)

1kb ladder, 23, 24, 25, 25, 26, 27, 28, 29, 30, 2031, 2032, 2031 miniprepped, 2032, empty backbone (pos ctrl), ladder (gel seems distorted on the right border)

image.png



expected bands: ~ 4kb for 2023-2030 and ~ 8 kb for 2008

- Same result as 5 days ago: no signs of correct plasmids in our colonies. -> I trashed the plates. (We already have a batch of plates with "similar" 2023-2030 co-transformants in the fridge that didn't grow in the liquid culture and didn't have bands, so it doesn't make sense to accumulate more of them)
- It doesn't make sense to me. The only way to explain this is if there was no kanamycin and spectonomycin on the agar plate and random bacteria grew there (the gel didn't test for DP6 and chloramphenicol)

SIAH Drift

- there are no clean autoclaved glass botttles remainging: prepared fresh glass bottles with storbar for autoclaving (12 for turbidostat, 4 for lagoon) - will be ready to use tomorrow
- I also cleaned up all the other old glass bottles with old bacterie
- pregrew S2060+DP6 in Chlor. (30 mL culutre) to OD 0.5

Primer Design

NLRP3:

- has 2 interesting regions with VxP motif and Lysines nearby (one at position 200 and one at position 707) - see Philips presentation on NLRP3 for details
- Desinged 3 sets of primers - the amplify the sequence and attach the correct overhanges for Golden Gate cloning - they have the Part 2 overhangs (Level 1 Assemblies, or, also Level 0 if needed)

- for a 35 aa peptide in the region of the VxP at position 200 (087/088)
- for a 35 aa peptide in the region of the VxP at position 707 (089/090)
- for the entire NLRP3 protein (091/092)

Dummy for TU1-C

- oligo for oligo annealilng, will result in the Part C overhangs, with 45 bp of rnadpm DNA in between

SIAH1 KO:

- redesigned the primers with the NEBCloner this time, maybe now it works better

Transformation:

- Assembly reactions from 21/08: in DH5a
- SIAH1 KO: in DH5a
- pES1004 retransformation in DH5a
- pES2008 in S2060
- plated after 1h recovery

DIVENDRES, 23/8/2024

To Dos:

- [x] Sequencing analysis
  - [x] if 1104 is correct -> put sticker on and move to the temp box
  - [x] if 1081-08-DP / 1033-08-DP are correct -> make glycerol stocks and consider using these strains for propagation assays or PACE in the future (pBAD primers are for DP6)
  - [x] trash liquid cultures that are not okay
  - [x] Check sequence of pES2038 and pES2039 (in microsynth labeled as pES2032 and pES2033)
- [x] Check plates of transformations of 22/08
  - [x] pick colonies if they grew (for: 1004 is a retransformation, pciking a single one should be enough. if the sequence of 1004 DNA is confirmed, picking 1004 is not necessary)
  - [x] S2060 + 2008: Propagation assay to Confirm leakiness of gIII expression --> did not grow, repeat transformation
  - [x] SIAH 1 KO: IN case this doesn't work again, I redesigned & ordered new primers, they should arrive in the next few days --> it grew, picked for o/n cultures
  - [x] Co-transform and set up propagation assay with the AP1s with different stringencies (pES2032-pES2035): have weak RNAP N-term, and varying expression of E1 and E2 --> some did not grow, repeat assembly when pES1004 is available again
- [x] SIAH1 PACE:
  - [x] start Drifting (Tubing should be autoclaved tomorrow morning, LB with tubing is already autoclaved, pregrown culutre of S2060 is in the fridge (50 mL Falcon, OD 0.6)
  - [x] prepare LB
- [x] Transformation of Assemblies (running Overnight: one is fpr the negative selection constructs, the other is an inducible Ntrem RNAP, the tubes are labeled 1006 and 2037)
- [ ] Assemble in Benchling --> pES2024 onwards

in Lab: Pau

Sequencing analyses

2008+2026+DP

- KanStart (for 2008) --> empty
- o086 --> empty (although confirmed that o086 does not work)

pES1104 (o017, aligned sequence results to pES1096 and checked for mutations in lysines) --> K6N (A-->C), K11R (A-->G), K24N (A -->T), mutations are correct!

- Diluted to 100ng/uL, relabeled and moved to lvl1 box

pES2030 w/ SmR o086 primer --> empty, seems like the primer does not work

1033-08-DP6

- KanStart (for 2008) -->
  - A: 1kb sequence, looks good --> keep this one
  - B: shorter sequence, also looks good
- o017 (for 1033)
  - A: looks good, few mutations only at the end on low quality regions
  - B: looks good, few mutations only at the end on low quality regions
- pBAD (for DP6)
  - A: looks good
  - B: looks good, some mutations only at the beginning (quality of mutated bases is good tho)
- I keep replicate A

1081-08-DP6

- KanStart (for 2008)
  - A: empty
  - B: looks good, few mutations only at the end on low quality regions
- o017 (for 1081)
  - A: looks good, shorter sequence
  - B: looks good, some mutations at the last 100bp but all are low quality bases so should be okay
- pBAD (for DP6)
  - A: short sequence, looks good
  - B: looks good
- I keep replicate B

I do glycerol stocks for 1033-08-DP6 A and 1081-08-DP6 B.

Check plates with transformations

S2060+pES2008 (for leaky gIII evaluation) --> did not grow, will repeat transformation today

pES2032-pES2034 --> only red colonies

pES2035 and pES2036 --> some white colonies, picked for o/n culture (did not send for nightseq as results would arrive monday anyway)

pES1004 --> many colonies, picked two for miniprepping

DH5a containing SIAH1 KO SP --> grew, pick for on culture

Transformation of pES2037 and pES1006 Transformation

5uL assembly reaction in 100uL DH5a competent cells.

High-efficiency protocol, 2h recovery time.

Transformation of pES2008+S2060 Transformation

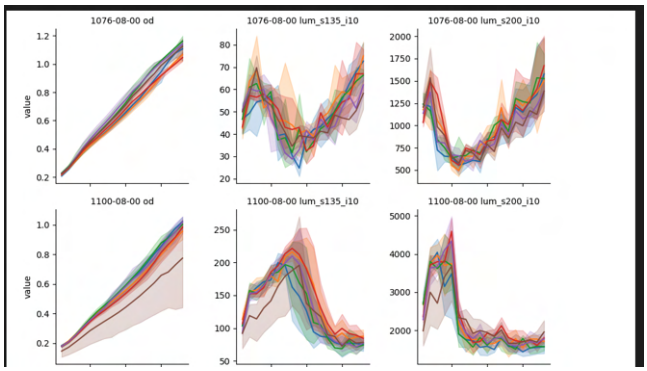
2uL of pES2008 in 50uL of competent S2060. Plate contains 100mM glucose, Chlor, and Kan
High-efficiencly protocol, 2h recovery time.

SIAH1 drift

- assembled and started the PACE reactor with S2060 + DP6 (OD 0.6) and 10^5 PFU/mL in the lagoon (100uL of the 10^8 pfu/mL diluted SIAH1)
- For 1L media: 50 mL 2 M glucose, 0.5 mL 1000x chloramphenicol and 1 mL 1000x tetracycline were added to the medium

Luciferase readout:

image.png



No signal detected.

DISSABTE, 24/8/2024

- [x] Miniprep pES2035, pES2036 and pES1004 and run gel to check for correct plasmids
- [x] Miniprep SIAH1-KO SP for sequencing, once mutations are confirmed start phage assembly with split c and split d
- [x] Once pES1004 is miniprepped, repeat pES2032 to pES2034 (table is made below)
- [x] Check plates for transformations from yesterday
  - [x] S2060 + 2008: Propagation assay to Confirm leakiness of gIII expression --> did not grow
- [x] Co-transform and set up propagation assay with the AP1s with different stringencies (pES2032-pES2035): have weak RNAP N-term, and varying expression of E1 and E2 --> today: re-transform pES2032 to pES2034 and minprep pES2035
- [ ] Check GG assembly of pES2032-pES2035 --> it says sticky ends are incompatible?
- [x] When NLRP3 and Primers arrives: start cloning --> NLRP3 arrived, I put it in the fridge, waiting for primers
- [x] When oligos fpr Dummy B arrive: Confirm leakyness of RNAP subunit hybridisation: clone an AP without E2 --> not here yet
- [x] SIAH1 PACE
  - [x] Check phage levels
  - [x] Change media, waste, etc.
  - [x] Start SIAH PACE on target once we have the co-transformed strain
- [x] Make new plates and co-transform 2023 (and maybe 2024-2029) with 2008 into S2060/DP6

In lab: Pau

SIAH1 drift PACE qPCR

Performed qPCR for lagoon, chemostat, o/n culture of S2060+DP6, LB, and water. qPCR: Phage Titer

Table19

	Condition	Cp
1	Lagoon	26.76
2	Chemostat	26.54
3	o/n culture	26.41
4	LB	26.79
5	Water	27.11
6	Blank	26.92

From these results, seems like the phage has washed out. Maybe the starting concentration was not enough? Depending on tomorrows qPCR results, we will have to reseed the lagoon.

Miniprep for pES2035, pES2036, pES1004 and SIAH1 KO SP Qiagen Miniprep

Eluted in 50uL EB. Yields:
pES1004 270.6 ng/uL
pES2036 A 268.8
pES2036 B 224.6
pES2036 C 273.3
pES2035 A 243.4
pES2035 B 315.4
SIAH1 KO SP A 311.9
SIAH1 KO SP B 371.1
SIAH1 KO SP C 342.1

Diluted to 100 ng/uL, will send for sequencing tomorrow.

Picking yesterday's transformations pES1006 and pES2037 in DH5a and pES2008 in S2060

pES1006 --> almost all white colonies, picked o/n cultures
pES2037 --> mostly red colonies, picked white colonies for o/n cultures
pES2008 in S2060 --> did not grow again, will transform pES2008 in S2060+DP6 in case the problem is with the S2060 competent cells.
--> could not have grown because of chlor in plate

GG assembly pES2032 to pES2034 EcoFlex Assembly

BSmbI, assemble with Lukas' protocol (GG BsmbI) overnight

Table18

	A	B	C	D	E	F	G
1	pES2032	KanR (+CymRam)	pTU2-A-RFP KanR CymRAM	pES1071	pES1072	pES1074	pES1026
2	pES2033	KanR (+CymRam)	pTU2-A-RFP KanR CymRAM	pES1071	pES1002	pES1074	pES1026
3	pES2034	KanR (+CymRam)	pTU2-A-RFP KanR CymRAM	pES1071	pES1072	pES1004	pES1026

Repeat co-transformation S2060+DP6 with pES2008 + pES2023-29

Transformation

Transformed using the high efficiency protocol with 50uL of competent cells and 1h recovery time. Added a 300ng of plasmid mix at 1:1 ratio (plasmids were mixed beforehand) and cells were plated on agar with Chlor + 100mM glucose + Spec + Kan. Because using 3 antibiotics, working concentration was set to 0.6x. Streaked plates were left on the 37ºC incubator o/n.

- S2060/DP6 with pES2008+pES2023-30 (Kan + Spec + Chlor + glucose)

Additionally, because the S2060 + pES2008 transformation hasn't worked twice, I transformed pES2008 in S2060+DP6 (in case it is a problem with the S2060 competent cells?). Agar plates were prepared with Chlor + 100mM glucose + Kan in a 1x working concentration. 2uL of pES2008 were added to 50uL competent cells.

- S2060/DP6 with pES2008 (Kan + Chlor + glucose)

Plates incubated at 37ºC

DIUMENGE, 25/8/2024

- [ ] Time Course Assay: run qPCR --> 96w plates is in the fridge
- [x] Check GG assembly of pES2032-pES2036
- [x] When NLRP3 and Primers arrives: start cloning --> NLRP3 arrived, waiting for primers
- [x] When oligos fpr Dummy B arrive: Confirm leakyness of RNAP subunit hybridisation: clone an AP without E2
- [x] Co-transform and set up propagation assay with the AP1s with different stringencies (pES2032-pES2035): have weak RNAP N-term, and varying expression of E1 and E2 --> today, transform pES2032-34 and send pES2035 for sequencing
- [x] Send miniprepped pES2035, pES2036, pES2037, pES1006, and SIAH1 KO SP for sequencing
- [x] SIAH1 drift PACE
  - [x] Check phage levels
  - [ ] Change media, waste, etc.
  - [ ] Start SIAH PACE on target once we have the co-transformed strain
- [ ] make glucose 2M stock

- [x] Transform pES2008 in DH5a bc we are running out
- [x] Transform yesterday's assemblies (pES2032-34) in DH5a
- [x] Check transformations 2023-08-DP to 2029-08-DP and 2008-DP
- [x] Miniprep pES2037 and pES1006

In lab: Gabriel, Pau

SIAH1 drift PACE qPCR

Performed qPCR for lagoon, chemostat, LB, and water. qPCR: Phage Titer

SAH1 drift 25.08

	Condition	Cp
1	Lagoon	27.21
2	Chemostat	27.14
3	LB	27.39
4	Water	28.74
5	Blank	27.59

Results: no detectable phage levels in any condition (Cp around 27 for all of them). However, the fresh media consumption rate is very low and the incubator cylinders are not warm to the touch, so it might be that bacteria are barely growing. We unplug and plug again the reactor to see if it resets properly. We also decide to inject 0.5 mL of SIAH1 phages at 10^9 pfu/mL (x100 times as what was seeded on day 0) to the lagoon with an insulin needle. Finally, we connect the sampling robot to the lagoon waste to detect phage titers over time.

Update to that: temperature crashed only at 1pm today, which doesn't explain why the media consumption rate has been so low...

Tomorrow we will include a positive control of the injected phages for the qPCR to make sure the problem does not lie with the reagents.

Miniprep pES2037 and pES1006

Qiagen Miniprep Elution volume 50uL. Yields:

pES2037 A 251.0 ng/uL
pES2037 B 267.1
pES2037 C 200.5
pES1006 A 203.9
pES1006 B 248.8
pES1006 C 157.1
--> Diluted to 100 ng/uL and sent for sequencing.

Plates with pES2023 to pES2029 + pES2008 and only pES2008 in S2060/DP6 transformations

Nothing grew :(I leave it 24h more and if not repeat co-transformation when more pES2008 is available.

Sequencing info

All eppies are in the temp box in the fridge, including newly transformed/miniprepped pES1004 which was used yesterday for assemblies pES20232-pES2034.

Primers used:

pES1004 A o017 --> sent for seq just to make sure there are no problems with the plasmid, although it should be okay.

pES1006 A-C o017

pES2035 A-B o019

pES2036 A-C o019

pES2037 A-C o019

SIAH1 KO SP A-C o018

Order 4167673

Transformation of pES2032-2034 assemblies + pES2008 because we a re running out

5 uL of the GG reaction or 1 uL of pES2008 were added to 75uL DH5a cells. High efficiency protocol. Recovery time of 1h.

Transformation

Plates incubated at 37ºC.

Time course assay qPCR

A single master mix was prepared for all reactions.

Reactions for plate 3 did not look good, many wells missing Cp values (though the mastermix was the same), maybe sample evaporated? (plate was well sealed). Will repeat qPCR for plate 3 tomorrow.

Plates 1 and 2 seem to show that SIAH1/2 propagates faster in S2060 than TadA.

DILLUNS, 26/8/2024

- [x] SIAH1 drift PACE
  - [x] Check phage levels
  - [x] Change media, waste, etc.
  - [ ] Start SIAH PACE on target once we have the co-transformed strain
- [x] Co-transform and set up propagation assay with the AP1s with different stringencies (pES2032-pES2035): have weak RNAP N-term, and varying expression of E1 and E2 --> today, send pES2032-pES2035 for sequencing
- [x] When NLRP3 and Primers arrives: start cloning --> primers and NLRP3 arrived!
- [x] When oligos fpr Dummy C arrive: Confirm leakyness of RNAP subunit hybridisation: clone an AP without E2
- [x] Make new plates and repeat co-transformation 2023 (and maybe 2024-2029) with 2008 into S2060/DP6 --> waiting for pES2008 confirmation

- [x] Check transformed plates from 24/08 and 25/08.
- [x] Drop the bag with sequencing goodies in the mailbox
- [x] make glucose 2M stock
- [x] Check yesterday's transformations (pES2032-pES2034 and pES2008)

Update on SIAH1 drift PACE

According to Lukas, PACE pumps stopped working at some point of the night. He restarted it as soon as he arrived to the lab, but that means bacteria overgrew in the chemostat. We perform qPCR to make sure there is something left in the lagoon. That also means that the sampling robot did not sample anything, as pumps were not working.

SIAH1 drift 26/08

	Condition	Cp
1	Lagoon	25.66
2	Chemostat	28.82
3	Positive control phages	14.83
4	LB	29.03
5	Water	29.85
6	Blank	29.07

Results: there seems to be something in the lagoon, but the signal is very weak. The positive control worked. The pumps failing might have something to do with it, so we will measure again tomorrow.

To make sure PACE ran smoothly o/n, a mark was made to the lagoon waste.

Transformed plates from 25/08 for pES2032-pES2034 and pES2008

There are 1-2 colonies per plate. We pick them for o/n culture and send them for nightseq in a plate together with transformations from 24/08.

Co-transformant plates from 24/08 with 2023-08-DP to 2029-08-DP and 2008-DP

After two days in the incubator, some colonies start appearing. We pick them for o/n culture and send them for nightseq. Liquid cultures for 2023-08-DP to 2029-08-DP were prepared with 0.6x working concentrations of antibotics (Spec+Kan+Chlor).

For differentiating 2023-2029 from 2008 in the co-transformants, we designed a new primer targeting the SpecR start. The previous primer (o086) had a Tm of 59ºC, whereas Microsynth recommends around 48ºC. We designed a new primer named o097 and ordered it together with the nightseq, which will probably delay a bit the results. For pES2008, given that it is a bit urgent to have it confirmed to use it again, we sent a single nightseq tube.

Sequencing information:

- pES2008 --> o019
- pES2032 to pES2034 --> o019
- 2008-DP6 --> o019 (for 2008) + pBAD (for DP6)
- 2023-08-DP6 to 2029-08-DP6 --> KanStart (for 2008) + pBAD (for DP6) + o097

Sent as nightseq plate, order 4168597
pES2008 (o019) sent as tube, order 4168623

NLRP3 PCR with primers o087/o088 and o089/o090
Using NRLP3 sequence from Twist and designed primers from Microsynth.
Rationale:

- Primers o087/o088 amplify a 35aa peptide in the region of the VxP at position 200 and add the correct overhangs for GG cloning
- Primers o089/o090 amplify a 35aa peptide in the region of the VxP at position 707 and add the correct overhangs for GG cloning
- Both generated peptides have the Part 2 overhangs (Level 1 assemblies, and also Level 0 if needed)

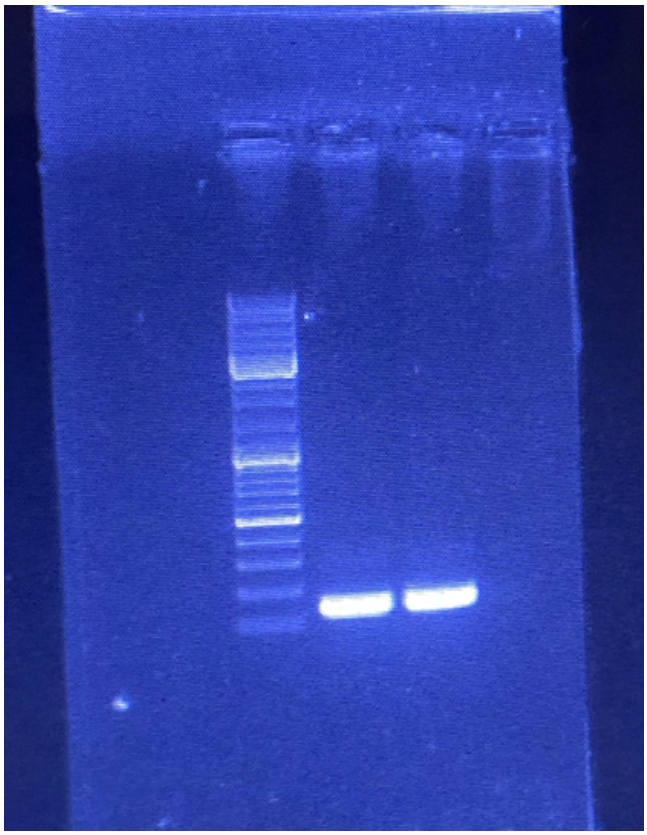
There are also primers o091/o092 that amplify the entire NLRP3 protein in case we need that at some point.

NLRP3 peptide 200
Primers 87 and 88
Ta 61 C
Elongation time 40 sec

NLRP3 peptide 707
Primers 89 and 90
Ta 61 C
Elongation time 40sec

Loading order: genemix ruler, NLRP3 peptide 200, NLRP3 peptide 707
Expected length ca. 150 bp

Screenshot 2024-08-26 at 16.35.17.png



Both PCR reactions seem to have worked, can be cleaned up and used for cloning

NLRP3 fragment cleanup
yields:
NLRP3_200: 73 ng/ul
NLRP3_707: 82 ng/ul

Dummy for TU1-c
To generate the Dummy for TU1-C, oligos o093 and o094 have been designed. They generate Part C overhangs with 45bp in between.

Annealing protocol using T4 ligase and T4 PNK used.

Cloning pES1112 and pES1113 NLRP3
Rationale: to have lvl1s with the two NLRP3 fragments, the generated peptides with the overhangs and the same backbone/inserts as for pES1076 were used, which worked before for EGLN3. See cloning checklist for more information.

Cloning pES2040 and pES2041 dummy TU1-C
Rationale: to remove E2 from the system and be able to detect the leakiness of gIII, the annealed dummy TU1-C and the same backbone/inserts as for pES2008 and pES2009 were used.

DIMARTS, 27/8/2024

- [x] Repeat qPCR for time course assay for plate 3, as for some reason some values were not measured by the machine
- [x] SIAH1 drift PACE
  - [x] Check through grafana if PACE ran smoothly o/n, also check the mark on the lagoon waste
  - [x] Check phage levels
  - [x] Change media, waste, etc. and mark arabinose and lagoon waste
  - [x] Create spreadsheet for PACE
  - [x] Start PANCE (liquid culture + phages in the shaker)
  - [ ] Start SIAH PACE on target once we have the co-transformed strain
- [x] Co-transform and set up propagation assay with the AP1s with different stringencies (pES2032-pES2035): have weak RNAP N-term, and varying expression of E1 and E2 --> depends on pES2032-pES2035 being confirmed, which will be most probably today for pES2035 and tomorrow for pES2032-pES2034), then they need to be co-transformed (eppies in the temporary storage in the fridge) with different substrates and tested
- [x] Sequencing analysis of orders 4167673 and 4168526 and 4168623
  - [x] If SIAH1-KO did not work, repeat with new primers - worked
- [x] Transform pES2040 and pES2041 (pES2008 and pES2009 but no E2)
  - [ ] Once pES1112 and pES1113 are confirmed, proceed to clone them into lvl2s. => we needed to reorder the primers to introduce start codon
  - [ ] Co-transforming pES1112 and pES1113 with pES2008 can also be an option for a propagation assay
  - [ ] Once pES2040 and pES2041 are confirmed, perform phage propagation assay to determine E2 dependence
- [x] Miniprep single transformants and miniprep a part of the liquid culture of co-transformants to run on a gel (save 600uL for glycerol stock once confirmed)
  - [x] Pick new pES2032 and pES2034 or re-transform/re-assemble
    => pES2034 all colonies are red, retransform
    => pES2032 no more colonies, retransform
    also 35
    - [ ] next time do colony pcr before sequencing
- [x] Phage propagation assay pES2008 S2060+DP6
- [ ] Make new plates and repeat co-transformation 2030 with 2008 into S2060/DP6
- [x] Run gel: pES2037, 2036, 2035, 2008A-C, 2033
- [x] start Phage production SIAH1 KO - SP A
- [x] book qPCR for tomorrow morning (phage propagation + pace)
- [ ] Book trian London-Basel

In lab: Noemie, Pau

SIAH1 drift PACE

PACE seems to have run smoothly overnight. Performed qPCR for lagoon, chemostat, positive control phages (same SIAH1 used to seed PACE), LB and water.
New spreadsheet was started to record PACE: SIAH1 DP drift PACE (folder iDEC PACE on Drive)

SIAH1 drift 26/1

	Condition	Cp
1	Lagoon	23.51
2	Chemostat	29.7
3	Positive control phages	14.87
4	LB	29.72
5	Water	30.80
6	Blank	29.93

Results: phages seem not to be washed out but are hardly replicating.
Given that phage propagation looks very limited, we decide to perform PANCE in parallel. For that, S2060-DP6 from a liquid culture in the fridge are grown to a an OD of 0.4-0.6 and PANCE tube is prepared as follows.

- Media: 1x Chloramphemicol, 2x Tetracycline, 100 mM Glucose, 40 mM Arabinose
- Strain: S2060 + DP6 , 1:100 from liquid culutre
- Phages: 10^6 pfu/mL SIAH1
- started 50 mL culutre over night

As a control, an identical PANCE was run but without Tetracycline, to make sure the addition of Tet is making a difference. The rationale behind is to see if tetracycline is being the problem in our PACE for why phages are not being washed out but still do not replicate a lot.

Liquid cultures from yesterday's picks
No co-transformant grew, I leave them in the shaker for longer. Some cultures showed growth in the afternoon and put in the fridge. The rest were left shaking o/n
pES2032 A and pES2034 A grew red, so we have to pick new colonies if there are, otherwise re-assemble / re-transform.
pES2008 grew well and so were miniprepped
2008 DP6 B grew well and 1.5mL of the culture were miniprepped.
2008 DP6 A had some growth but was left in the shaker a bit longer.

Sequencing analysis
1004 --> there are two mutations in the ori but otherwise all fine
1006 --> A and B ok, C has a huge deletion --> keep B
2035 --> sequencing started at two completely wrong parts of the plasmid. A looks bad, B looks fine for what was amplified but maybe there is no RNAP which is downstream of where the primer binds? --> check plasmid size in a gel
2036 --> all are NNN --> have to repeat
2037 --> aligns perfectly, but as the first TU is so long, ot not even covers the entire first TU -> I would either send it for sequencing again or run it on a gel (! when I assembled this sequence in benchling, I noticed that 1094 is A D part according to benchling, but a D1 part according to the cloning spreadsheet. I don't know which of both is right, bsc sequencing does not cover the GG front GG site - I assumed the cloning spreadsheet is correct, because also in the lab journal it is mentioned that it is a D1 part -> I changed the GG overhangs in the benching sequence to D1)

SIAH1 KO-SP --> A and B have two point mutations at 180 and 189 (T --> A), however B has many point mutations along SIAH1 sequence and A has two point mutations

2008 C: ok, but only covers N-term-Ub. Still, we dilute them to 100ng/uL and put them in the lvl.2 box

SIAH1 KO: Started phage production Day 1 Phage Production

NightSeq Plate:

- 2034 A: does not align, backbone with mRFP
- 2033 A: only has the first TU
- 2032 A: has backbone with mRFP
- 2008 A: ok
- 2008 B: ok

Table20

	A	B	C	D	E	F	G	H
1	Strain	KanStart	pBAD	o097				
2	2029-08-DP6 A	Sequencing failed	ok	should Sequence the SmR Level 2, but all sequences we have show AraC and CloDF13 ori => matches the MP6 plasmid (even though we should have the DP6 plasmid in there)				
3	2029-08-DP6 B	Sequencing failed	ok					
4	2028-08-DP6 A	Sequencing failed	ok					
5	2028-08-DP6 B	Sequencing failed	ok					
6	2027-08-DP6 A	Sequencing failed	ok					
7	2027-08-DP6 B	Sequencing failed	ok					
8	2026-08-DP6 A	Sequencing failed	ok					
9	2026-08-DP6 B	Sequencing failed	ok					
10	2025-08-DP6 A	Sequencing failed	ok					
11	2025-08-DP6 B	Sequencing failed	ok					
12	2024-08-DP6 A	Sequencing failed	ok					
13	2024-08-DP6 B	Sequencing failed	ok					
14	2023-08-DP6 A	Sequencing failed	ok					
15	2023-08-DP6 B	Sequencing failed	ok					
16	2008-DP6 A	Sequencing failed	ok					
17	2008-DP6 B	Sequencing failed	ok					

Results for primer o097, which targets SmR of 2023 to 2029, are yet to arrive.

Minipreps

Liquid cultures 2008 A-C, 2033 were miniprepped

Phage propagation Assay: pES2008 S2060+DP6

- objective: test for leakyness of gIII expression
- inocculated fresh pES2008 S2060+DP6 culutres & grew to OD 0.5

Phage Propagation: test for gIII leaky expression

	1	2	3	4	5	6	7	8	9	10	11	12
A	pES2008 S2060+DP6 SIAH1			S2208 + S1	LB + SIAH1		pES2008 S2060+DP6					
B	pES2008 S2060+DP6 SIAH2			S2208 + S2	LB + SIAH2		LB					
C	pES2008 S2060+DP6 TadA			S2208 + TA	LB + TadA							
D												
E												
F												
G												
H												

Gel Electrophoresis:

1% Agarose Gel, 120 V, 40 min

Loading order: GeneRuler Mix - 2008 A - 2008 B - 2008 C- 2033 - 2035 A - 2035 B - 2036 A - 2036 B - 2036 C- 2037 A - 2037 B - 2037 C - GeneRuler Mix

Expected Band lengths:

- 2008: 11.2 kb -> all three have bands at the expected size
- 2033: 11.3 kb -> bright band is too short, the faint one has the correct size
- 2035: 11.3 kb -> too short
- 2036: 5 kb -> size matches
- 2037: 12.2 kb -> too short

IMG_20240827_145646.jpg

Transformation of pES2032 to pES2035 and pES2040-2041

Given that pES2032 to pES2035 did not work, we transformed them again with previous assembly preparation.

Additionally, we transform pES2040 and pES2041 which were assembled overnight.

Transformation was performed in DH5a using the high-efficiency protocol. Recovery time 1h. Transformation .

Time course assay qPCR attempt 2

Wells where qPCR machine indicates uncertainty: K6, C24, H11, H24

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DIMECRES, 28/8/2024

- [x] SIAH1 drift PACE
  - [x] Check through grafana if PACE ran smoothly o/n, also check the mark on the lagoon waste and arabinose
  - [x] Check phage levels with qPCR, also from the samples taken by the sampling robot (I think we can also stop it by now) SIAH1 DP drift PACE
  - [x] Change media, waste, etc. and mark arabinose and lagoon waste
  - [ ] Start SIAH PACE on target once we have the co-transformed strain
- [x] Analysis qPCR 27.08 (Time Course) sheet 2 of this spreadsheet
- [x] SIAH1 drift PANCE --> continue by seeding new tube
  - [x] also check phage titer by qPCR
- [x] Check yesterday's transformatons of 2032-2035 and 2040-2041 - > nothing grew yet, let them grow for another day
- [x] Sequencing: check if NightSeq
- [x] qPCR of phage propagation expermient 27.8
- [x] SIAH 1 KO phage production
- [ ] design gibosn assembly phage shock promoter
- [x] running out of split C and Split D -> retransform
- [x] send night seq of pace lagoon
- [x] send 2036 for sequencing again after prep (bcs size matcehs on gel, sequencing nightseq failed)
- [ ] Co-transform and set up propagation assay with the AP1s with different stringencies (pES2032-pES2035): have weak RNAP N-term, and varying expression of E1 and E2 --> depends on pES2032-pES2035 being confirmed

in Lab: Noemie

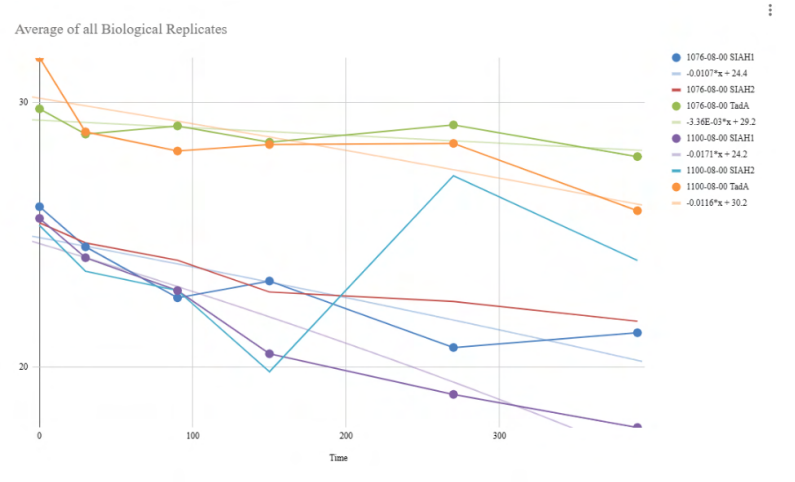
qPCR:

- sampling robot plate (well A1-H8): a lot of wells were emtpy, don't know how informitive this will be (reason for this is that reactor broke down during the night & sampler continued running
- SIAH1 drift PACE turbidostat and lagoon samples
- SIAH1 drift PANCE: +tet and -tet samples
- phage propagation assay (leaky gene III expresson?)

Analysis qPCR timecourse (using Pau's Spreadsheet)

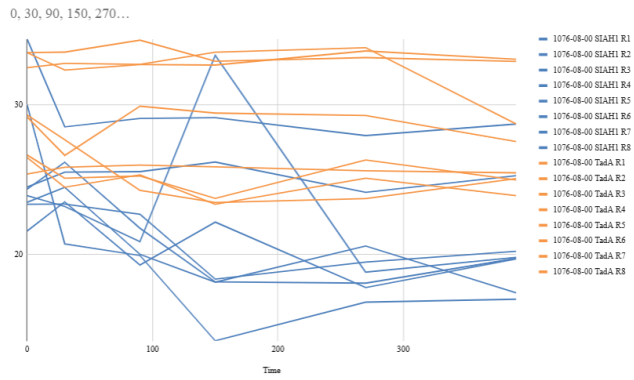
- I couldn't add the std dev for the individual points on Google sheets, but they are huge

image.png



I plotted all the individual biological replicates (here only for the canonical substrate, and onlx SIAH1 and TadA)

image.png

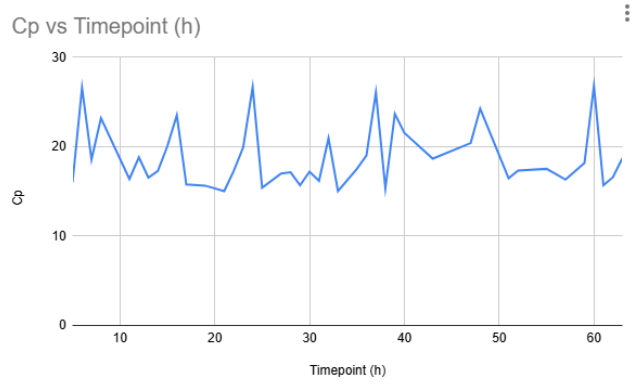


SIAH1 drift PACE

- autosampler: a lot of wells are empty
- pace broke down during the night, restarted this morning
- we had page washout => I saw in a different strain that we have MP6 instead of DP6 => this could explain why we have no propagation => I didn't reseed, will wait for this until tomorrow to see if we have MP or DP
- sent a sample from the chemostat for Nightseq to check for MP/DP

Data from the autosampler: I removed all the empty wells plotted Cp over time

image.png



SIAH1 drift PANCE

qPCR Cp values:

- - tet: 17.61, stdev 0.03
- + tet: 19.37, stdev 0.11
    - they are too close together, with tet we should get way higher titers (should max out), again here we could have the MP6/DP6 mixup issue => I sent a aliquot of the cultre we used for sequencing

I restarted PANCE, the same as yesterday, but this time a took a phage sample before starting overnight incubation to have a starting point

NightSeq with new SmR F primer (o097) finally arrived -> iI inserted the results in the table from yesterday
doesn't give much information about the level 2 we have inside: all reads show araC +CloDF13 ori (whihc we both don't have on the p15a levle 2 backbone; and also, it isnt' the drift plasmid => matches perfectly the MP6 plasmid - do we have the MP instead of DP6?)

Phage Shock Promoter

Plasmid sequence I got from Lukas (phage shock promoter infront of TadA) :

pTPH412 (plasmid mit Phage Shock Promoter) => is on addgene

the psp was not annotated, I used this paper (referenced in the Paper from where this plasmid is from) to identify the psp

Phage Production Day 2:

- did the transformation, but the tube was not properly in the thermocycler. Should still work, GG should even work when incubater at RT, but if phage production doesn't work out, this might be a possible reason (! also, we only have one shot of competent S2208 left, we need to make more if we have more phages to make)
- retransforedn SplitC and SplitD plasmid, as we are running out

Sequencing: sent 2036 A and B (prepped yesterday) for sequencing

DIJOUS, 29/8/2024

High Priority:

- [x] **pES2037 (inducible Nterm RNAP):** pick colonies, colony pcr and liquid culutre

- [x] double check overlaps/assembly

- [x] **SIAH1 drift**
  - [x] MP/DP mixup? yes

  PACE
  - [x] Check through grafana if PACE ran smoothly o/n, also check the mark on the lagoon waste and arabinose
  - [x] Check phage levels with qPCR SIAH1 DP drift PACE
  - [x] Change media, waste, etc. and mark arabinose and lagoon waste
  - [ ] Start SIAH PACE on target once we have the co-transformed strain (wait for 2036 sequencing) ok
    - [x] do cotransformation

  PANCE
  - [ ] continue by seeding new tube
  - [x] check phage titer by qPCR
  - [ ] keep an aliquit from fresh culutre to get initial titer for qPCR the next day

- [x] Check yesterday's transformatons of 2032-2035 and 2040-2041
  - [ ] Once pES2040 and pES2041 are confirmed, perform phage propagation assay to determine E2 dependence
  - [ ] Once at least pES2032 is confirme, perform phage propagation assay to determine if weak expression of RNAP, E1, and E2 reduce Cp
- [x] SIAH1 KO phage production day 3
- [x] prep SplitC and SplitD
- [ ] phage shock promoter: design gibosn assembly (to put psp in front of Nterm-Ub)
- [ ] When NLRP3 new primers arrive: assemble pES1112 and pES1113 => they have arrived
- [ ] Make new plates and repeat co-transformation 2030 with 2008 into S2060/DP6
- [ ] Make new S2060 competent cells --> can be done Friday by Jakob and Pau
- [ ] Make new S2060+DP6 compentent cells (we got new S2060 cells from Lukas with DP6), I grew them in culutre
- [ ] For 2023-08-DP6 2029-08-DP6 , perform PCR of liquid cultures (if they grew) with primers 071 and 022 (they ampliy linker sequence) together to check for the presence of 2023 to 2029 specifically
  - [ ] Some cultures already grew and are in the fridge: 2023A and B, 2024 B, 2028 A

in Lab: Noemie

SIAH1 Drifting

- the PACE and PANCE strain we are using right now are not with DP6 but MP6 (confirmed by sequencing)=> also, all **competent cells we have that are labelled with S2060+DP6 are actually S2060+MP6!**
- this explains why we have washout in PACE all the time
- I got S2060+DP6 cells from Lukas again, which I pre grew, but it didn't grow enough during the day. I put it in the fridge overnight to prevent it overgrowing. please take it out again in the morning & srtat growing it again
- qPCR:

Table21

	A	B	C
1		Average Cp	StdDEV
2	PACE Lagoon	23.25	1.0053854982
3	PACE Turbidostat	28.8366666667	0.1955334583
4	PANCE -tet initial titer	23.5533333333	0.1040833
5	PANCE -tet final titer	no value	-
6	PANCE + tet initial iter	no value	-
7	PANCE + tet final titer	23.1666666667	0.3557152419
8	Water	31.26	0.2165640783

for the inital titer of PANCE +tet and the final titer of PANCE -tet, I didn't get any value for all three measurements - isn't really relevant though as we are using the wrong strain to drift anyways

didn't passage the PANCE, didn't restart PACE (as strain didn't grow), we can restart tomorrow then

Sequencing:

- pES2036: A looks okey, but sequencing quality is rather low, there is a 20 bp deletion in the backbone (not in a relevant area)
- cotransformed pES2036 + pES2008 in S2060 + DP6 (this time an old aliquot from Lukas which actually should be DP and not MP)

Colony PCR of 2037 (all white colonies)

- Primers 018/019
- Ta: 62°C, Elongation 4 min
- ran 2008 as positive control
- Results: none of the 2037 colonies show the correct insert (all too short)

=> we need to reasssemble

Transformation pES2032-2035 and 2040-2041

- 2032, 2035, 2040 grew -> I picked, sent for sequencing & inocculated liquid cultures (I tried a new primer, which sequences from the luciferase -> should cover 3 TUs, instead of only 1 or 2 when we use 018 or 019)

Phage Production

- Did the day 3 plaque assay (phageas are not sequenced yet), positive control: SIAH1
- prepped Split C and Split D (not sequenced yet, stored them in the temp box)

Set up new GG Assemblies: for 2037, 2033, 2034, 2040, 2041, 2032

- with BsmBI , cycling Protocol from Lukas over night

DIVENDRES, 30/8/2024

High Priority:

- [ ] **pES2037 (inducible Nterm RNAP): Transformation (Assembly is running in slot 6)**
  Consider doing pcr on the assembly reaction (use 1 ul of reaction as template, then do the same protocol as for colony pcr) to see if the final plasmid has assembled - either use primers that only amplify when the plasmid has assembled or make sure extension time is long enough
  Transform in a larger volume of cells (50-100 uL), use the cells with the highest Efficiency & spin them down before plating
- [x] **SIAH1 drift**
  - [x] **first thing in the morning: take the new S2060+DP6 from the fridge and start growing it again** (make sure that you take the one with yesterdays date, all the older ones we have laying around probably have the MP and not the DP

  PACE
  - [x] Check through grafana if PACE ran smoothly o/n, also check the mark on the lagoon waste and arabinose
  - [x] restart PACE with the new strain which actually has DP6
  - [x] Check phage levels with qPCR SIAH1 DP drift PACE - not really necessary today, as we are still running with the wrong strain
  - [x] Change media, waste, etc. and mark arabinose and lagoon waste
  - [x] Start SIAH PACE on target once we have the co-transformed strain: I cotransformed, it is growing at 37°C atm --> no colonies yet

  PANCE
  - [x] start PANCE again with the new strain
  - [x] check phage titer by qPCR (tomorrow)
  - [x] store an aliquot from fresh culutre once everything has been inoculated (and store in fridge) to get initial titer for qPCR the next day

Remaining To Dos:

- [x] Check 2032-2035 and 2040-2041
  - [x] Check sequencing results
  - [x] Once pES2040 and pES2041 are confirmed, transform into S2060 and perform phage propagation assay to determine E2 dependence
  - [x] Once at least pES2032 is confirmed, transform into S2060 and perform phage propagation assay to determine if weak expression of RNAP, E1, and E2 reduce Cp
- [x] SIAH KO phage production
  - [x] Check plaque assay, pick single plaque (& send for sequencing tomorrow)
  - [x] I reprepped Split C and D, but they are not sequenced yet (currently stored in temp box, send for sequenceing and then move to freezer)

Note: A few things went wrong during the assembly (tube was not placed correclty in the cycler, we ran out of SplitD), so when we don't have any phages just repeat

- [x] When NLRP3 new primers arrive: assemble pES1112 an dpES1113 => they have arrived, I put them in the Primer box, not diluted yet
- [x] Make new plates and repeat co-transformation of 2023-2030 with 2008 into S2060/DP6 --> once we have competent S2060-DP6
- [x] Make new S2060 competent cells --> today start o/n culture from glycerol stock
- [x] Make competent S2208 --> today start o/n culture from culture in the fridge
- [x] Make new S2060+DP6 compentent cells (we got new S2060 cells from Lukas with DP6) --> today start o/n culture from liquid culture in the shaker
  --> turns out they are MP6 aswell
- [x] For 2023-08-DP6 2029-08-DP6 , perform PCR of liquid cultures (if they grew) with primers 071 and 022 (they ampliy linker sequence) together to check for the presence of 2023 to 2029 specifically
- [x] Miniprep yesterday's cultures and run on a gel

in Lab: Pau, Jakob

SIAH KO phage production:
Plaque assay can not really used, half of the plate did not grow properly, no plaques can be picked
Lucas suggested instead of using our new Split C/D preps we should ask Priscilla for an aliquote she made from a cryo-stock, to be on the safe side
also there is no good sequencing primer for the these plasmids, so we would need to send the plasmids for full-plasmid sequencing

SIAH1 SP drift PACE
PACE was stopped.
We autoclave PACE components.
Lukas' aliquote proved to be MP6 as well. Thus, he gave us a new aliquote that is most likely DP6, but we will only know by Monday. In any case, we inoculate a o/n culture and will start PACE and PANCE tomorrow.

NLRP3 PCR with primers o098/o088 and o099/o090
Using NRLP3 sequence from Twist and designed primers from Microsynth.
Rationale:

- Primers o087/o088 amplify a 35aa peptide in the region of the VxP at position 200 and add the correct overhangs for GG cloning
- Primers o089/o090 amplify a 35aa peptide in the region of the VxP at position 707 and add the correct overhangs for GG cloning

<u>NLRP3 peptide 200</u>
Primers 98 and 88
25uL reaction, Ta 70 C for 20 sec, 35x cycles
Elongation time 40 sec

<u>NLRP3 peptide 707</u>

Primers 99 and 90
25 uL reaction, Ta 63 C for 20 sec, 35x cycles
Elongation time 40sec

1% agarose gel 120V 30 min. Loading order: genemix ruler, NLRP3 peptide 200, NLRP3 peptide 707
Expected length ca. 150 bp

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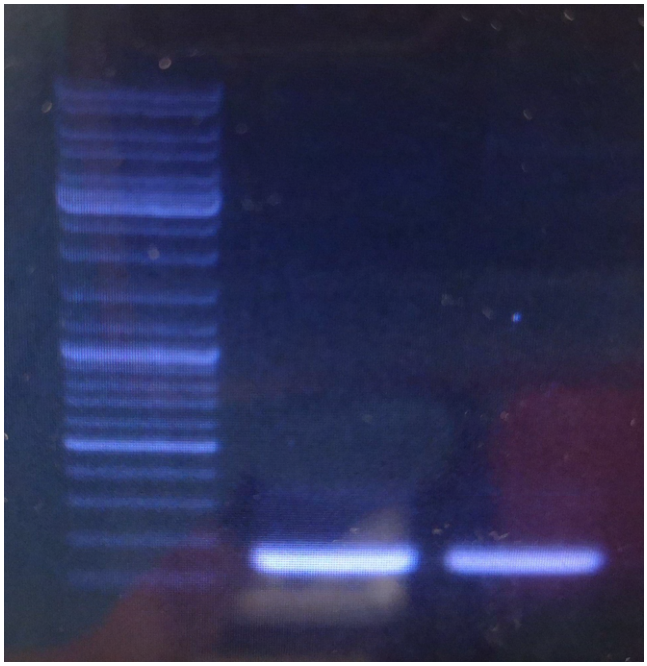

Bands look fine. I perform gel extraction

NLRP3 fragment cleanup
yields:
NLRP3_200: 29.7 ng/uL
NLRP3_707: 11.9 ng/uL

Cloning pES1112 and pES1113 NLRP3
Rationale: to have lvl1s with the two NLRP3 fragments, the generated peptides with the overhangs and the same backbone/inserts as for pES1076 were used, which worked before for EGLN3. See cloning checklist for more information.

GG assembly using Lukas' PCR protocol. Added 2uL of NLRP3_200 and 4uL of NLRP3_707 and water to a final volume of 11uL.

Table22

	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P	Q	R	S	T	U	V	W	X	Y	Z
1	pES1112	weak_NLRP3_200_L2_Cter mRNA(CGG)	A	Amp	pTU1-A-RFP	pBP-J23108	NLRP3_200	Linker 2	pES0008	pBP-L3S2P21																
2	pES1113	weak_NLRP3_707_L2_Cter mRNA(CGG)	A	Amp	pTU1-A-RFP	pBP-J23108	NLRP3_707	Linker 2	pES0008	pBP-L3S2P21																

Update: almost runnng out of Linker 2, I decide to ony do pES1112 and amplify Linker 2 before doing pES1113.

PCR to check 2023-08-MP to 2029-08-MP

Rationale: these co-transformants were sent for NightSeq but KanStart shows no result (thus cannot confirm the presence of pES2008) whereas the newly designed primer o097 unexpectedly binds to MP6. The presence of MP6 was confirmed with pBAD. To test for the presence of the other two lvl2 plasmids, we decide to perform PCR of the liquid cultures with the following primer combinations:

- Primers 071 and 022 amplify the linker sequence and should be able to tell the presence of pES2023-2029 with band size of 300bp
- Primers 018 and 019 amplify both pES2008 and pES2023-2029 and should yield sizes of 8.6kb (pES2008) and 3.2kb (pES2023-2029).

PCR reaction:

- Dipped the tip in the liquid culture and then in the PCR tube
- Initial denaturation of 10min
- 071 022 Tm of 63ºc
- 018 019 Tm of 71ºC

1% agarose gel, 120V 20 min. Order: 2023-08-MP6 to 2029-08-MP6 w/018&019 - 2023-08-MP6 to 2029-08-MP6 w/022&071 - GeneRuler Mix

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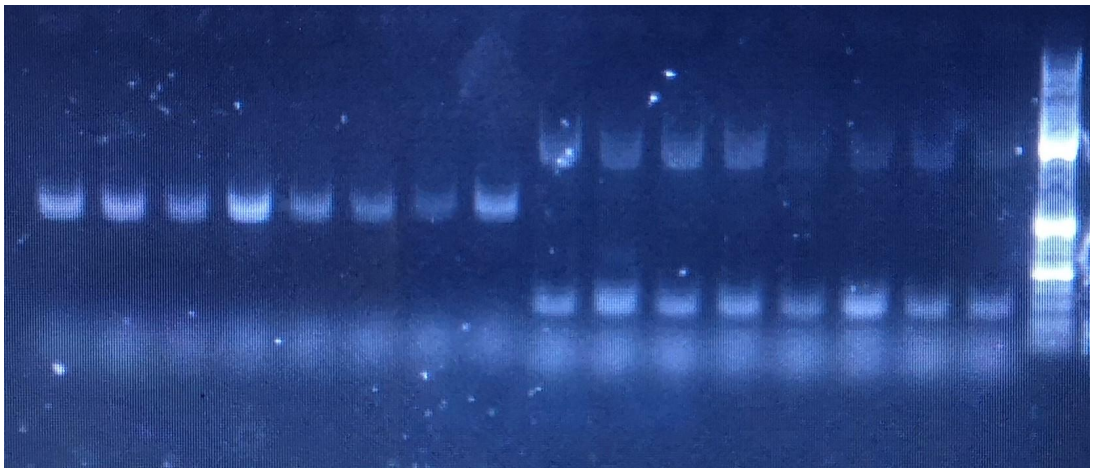


Result: for 018&019 we see one band at around 1.5kb, and for 022&071 we see one band around 300bp as expected and one at around 3kb.
Interpretation: cells are transformed with pES2023 to pES2029

Making competent cells:

prepared o/n cultures of:
S2060,
S2060+DP6 --> we got a new aliquote from Lucas that are most likely DP6, but we will only know by Monday
S2208

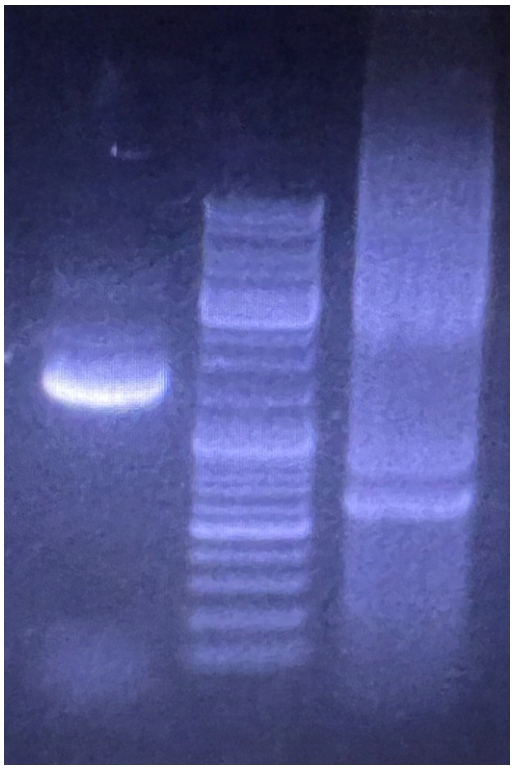
Sequencing Analysis and Minipreps:

none of the sequencings worked --> all iquid cultures except for pES2040 had RFP, discarded
Miniprep yield - pES2040: 410 ng/µL --> diluted to 100 ng/µL

pES2037 PCR:
First reaction: 1 µL asssembly reaction, 25 µL reaction, Primers o018 and o019, 62 °C, 5 min elongation time,
Second reaction: 1 µL asssembly reaction, 25 µL reaction, Primers o009 and o019, 67 °C, 2.5 min elongation time,

Gel: pES2040 - Gene ruler mix - pES2037 PCR with o009
The expected size fort pES2040 would've been 10.7 kb, actual is 1.2 kb, no idea what that could be, discarded it
actually pES2037 with o009 and o019 could not have worked, should have been o009 and o018, oops

IMG_5395.jpeg



Transformation:
Assemblies from 29.08: pES2032, 2033, 2034, **2037**, 2040, 2041
transformed 5 µL each in 100 µL DH5a, recovery with 200 µL LB

2036-08-MP6 co-transformation:
some colonies grew by the afternoon,
--> picked 5 clones, can colonie PCR tomorrow if they grew
--> put plate back at 37 °C to see if slow growers show up

DISSABTE, 31/8/2024

High priority:

- [x] PACE
  - [x] restart PACE with the new strain which actually has DP6 (probably)
  - [x] Change media, waste, etc. and mark arabinose and lagoon waste
  - [ ] Start SIAH PACE on target once we have the co-transformed strain: I cotransformed, it is growing at 37°C atm
- [x] PANCE
  - [x] start PANCE again with the new strain
  - [x] store an aliquot from fresh culutre once everything has been inoculated (and store in fridge) to get initial titer for qPCR the next day
- [x] Oligo anneal linker 2 (pES0021) as we are running out
- [ ] Continue with assembly of pES1113
- [x] Transform pES1112

Other to-dos:

- [x] Make competent cells
  - [x] prepare larger liquid cultures in the morning (50 mL each), let grow till 0.4-0.6 and make competent S2208, S2060, S2060+DP6
- [x] phage shock promoter: design gibosn assembly (to put psp in front of Nterm-Ub) (sequence is uploaded on benchling)
- [x] Check transformation of 2036-08-MP6, if positive we can use it to start SIAH PACE on target
- [x] Run gel with PCR products of liquid cultures for 2023-08-MP6 to 2029-08-MP6
- [x] Once pES2040 and pES2041 are confirmed, transform into S2060 and perform phage propagation assay to determine E2 dependence
- [x] Once at least pES2032 is confirmed, transform into S2060+DP6 and perform phage propagation assay to determine if weak expression of RNAP, E1, and E2 reduce Cp
- [x] colony PCR 2036-08-MP6 --> none of the picked colonies grew

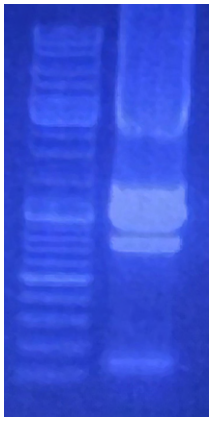
Competent Cells:
innoculated 50 mL of LB with 3-5 mL of the o/n culture of S2060 and S2208,
80 mL innoculated of S2060+DP6 (enough to also start PACE)
competent cells aliquoted in ~300 µL

Colony PCR:
colony PCR of pES2032, 2033, 2034, 2037, 2040, 2041
put the plates in the fridge for a bit since some of the colonies look like they will turn red,
picked 2 colonies each, 3 for pES2032, 2033, 2037 and 2041
ran PCR once with primers o003 and o018 (62 °C, 1 min)

Gels:
Number 1:
Gene ruler mix - pES2037 (9.5 kb expected)

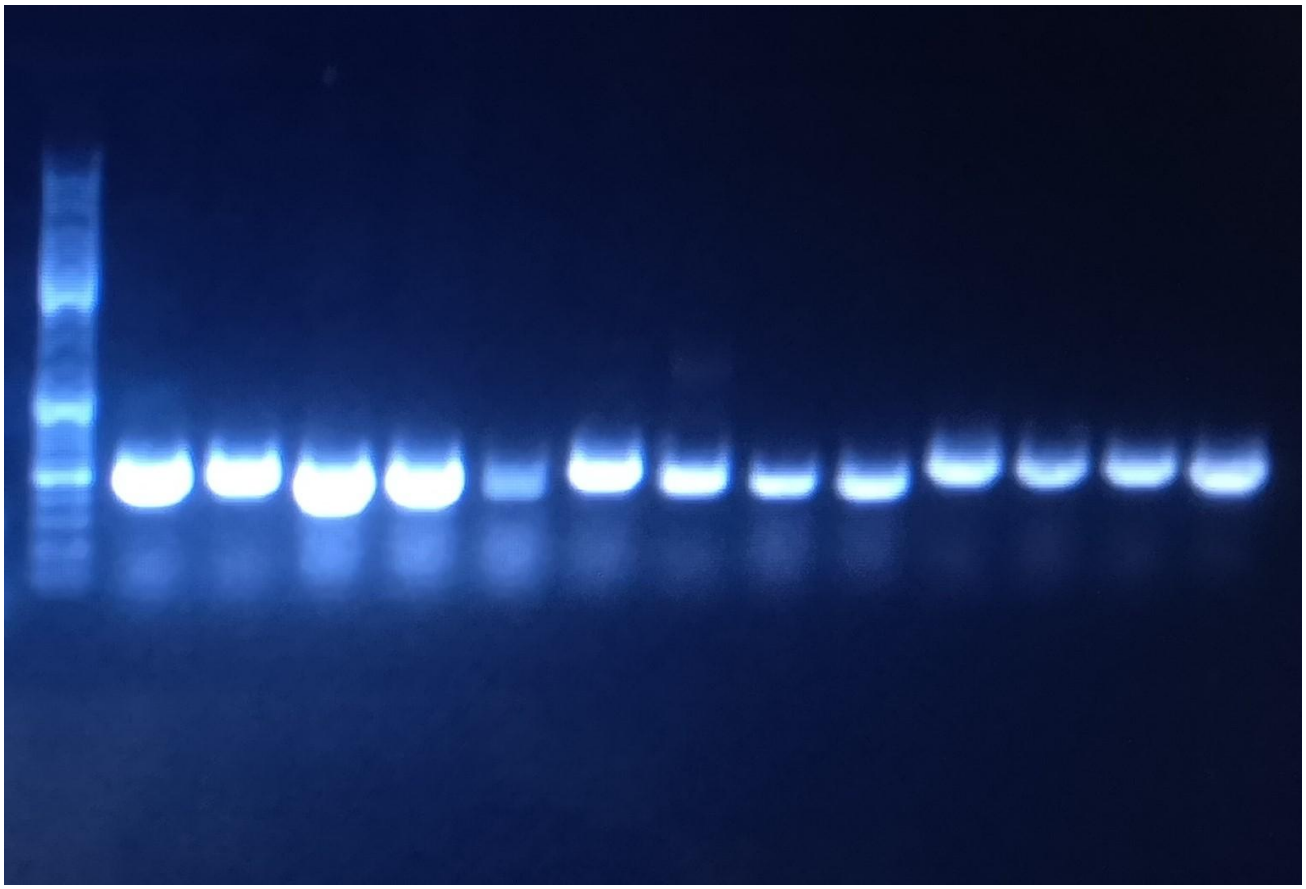
most prominent band around 1.2 kb, which is the empty backbone --> red colonies,
a higher band at ~2.5-3 kb, could be any of the level 1 plasmids, there does not seem to be amplification of fully assembled plasmid

:(



Number 2:
Gener ruler mix - pES2032 1 - 2 2033 1 - 2 - 2034 1 - 2 - 2037 1 - 2- 3- 2040 1 - 2 - 2041 1 - 2
Expected band size: 550 bp; except for 2037 --> 1.4 kb
all bands are at roughly 500 bp, which is fine for all but pES2037, although I actually expected to see more negative results because of RFP
--> no idea, why red colonies give 500 bp band? --> put plates back at 37 °C, should maybe just pick white colonies tomorrow,
I think all assemblies failed

jakob's gel.jpg



Oligo annealing - Linker 2
50 µL o022 and 50 µL o023 combined
Primer concentration: 100 µM, Total reaction volume: 100 uL

Heat at 94 °C for 2 minutes, turn off heat block, leave them in heat block until cooled back down to room temperature
Concentration: 1069 ng/µL --> adjusted to 100 ng/µL

2036-08-MP6 co-transformation:
none of the picked cultures grew o/n, some new colonies showed up on the plate though,
I picked some of the new colonies into the same tubes, no high hopes though

pES1113 assembly --> continuated from 31.08 with old Linker 2 (there was still 1uL left, new Linker 2 is now in the box)

pES1112 transformation Transformation
100uL DH5a cells and 10uL GG assembly. 1h recovery time. Plated in LB Agar+Carb and left at 37ºC.

PACE SIAH1 SP drift

- assembled and started the PACE reactor with S2060 + DP6 (o/n culture refreshed in the morning) and 10^6 PFU/mL in the lagoon (100uL of the 10^9 pfu/mL diluted SIAH1, supposing lagoon fits 10 mL)
- For 1L media: 50 mL 2 M glucose, 0.5 mL 1000x chloramphenicol and 1 mL 1000x tetracycline were added to the medium
- PACE started running at 16h

PANCE SIAH1 SP drift:

S2060-DP6 from a liquid culture in the fridge are grown to a an OD of 0.4-0.6 and PANCE tube is prepared as follows.

- Media: 1x Chloramphemicol, 2x Tetracycline, 100 mM Glucose, 40 mM Arabinose
- Strain: S2060 + DP6 , 1:100 from liquid culutre
- Phages: 10^6 pfu/mL SIAH1
- started 50 mL culutre over night

As a control, an identical PANCE was run but without Tetracycline, to make sure the addition of Tet is making a difference.

DIUMENGE, 1/9/2024

High priority:

- [ ] **troubleshoot pES2037 assembly --> by far most important thing to do**
- [ ] keep PACE running
  - [x] Check phage titer by qPCR
  - [x] Change media, waste, etc. and mark arabinose and lagoon waste
  - [ ] Start SIAH PACE on target once we have the co-transformed strain: check o/n liquid culture
- [ ] PANCE:
  - [x] check phage titer by qPCR, including sample from initial phage titer (fridge)
  - [ ] store an aliquot from fresh culutre once everything has been inoculated (and store in fridge) to get initial titer for qPCR the next day
  - [ ] Passage to continue PANCE
- [x] Pick pES2032-2034, 2040 and 2041 if white colonies present
- [x] make carb plates

Other To Dos:

- [ ] double check phage shock gibson assembly primers 100-104 (first time I did it) - order once ok
  (should work on pES1001 + pTPH412, or pES2008 (100 and 104))
- [x] Transform 1113
- [x] Check transformation of 2036-08-MP6 (liquid cultures in shaker), if negative throw them out

In lab: Gabriel, Pau

SIAH1 SP drift PACE

In the morning when we arrive the reactor had stopped running. The lagoon is filled up, however, which means PACE kept running smoothly until the lagoon was filled and at some point after that stopped working...
I restart PACE by unplugging and plugging again.

qPCR:

SIAH1 PACE drift 01/09

	Condition	Cp
1	Lagoon	24.79
2	Chemostat	30.58
3	Positive control phages	16.12
4	LB	30.62
5	Water	32.07
6	Blank	30.815

Interpretation: phages did not replicate too much, probably because PACE broke down. Let's see tomorrow.

SIAH1 SP drift PANCE

qPCR:

SIAH1 PANCE 01/09

	Condition	Cp
1	PANCE+Tet	14.99
2	PANCE No Tet	22.63
3	PANCE+Tet initial titer	28.19
4	PANCE No Tet initial titer	28.125
5	LB	30.62
6	Water	32.07
7	Blank	25.51

Results: phages propagated very well and the addition of Tet makes a great difference. However, both phages replicated a bit (when compared to the initial titer), maybe because TetR is a bit leaky?

PANCE passaging: we calculate how many pfu/mL there are in the PANCE Erlenmeyer based on the Cp from the qPCR and then determine how many uL of phages we need to seed a new 50mL Erlenmeyer to a final concentration of 10^6 pfu/mL (based on this paper by Liu). Then, the new Erlenmeyer is prepared with fresh media containing 1x Chloramphemicol, 2x Tetracycline, 100 mM Glucose, and 40 mM Arabinose. About 400uL of o/n S2060-DP6 culture are added together with the phages.

pES2037 troubleshooting

Isothermal reaction will be left overnight at 37 C

Colonies

ON cultures set up for 2032-2035, 2040, 2041. 2037 seemed to start showing growth, 1 colony was ready for picking, plate put back in incubator. 1112 was also picked

Minipreps for 2024-08-MP6 to 2029-08-MP6 to run a gel

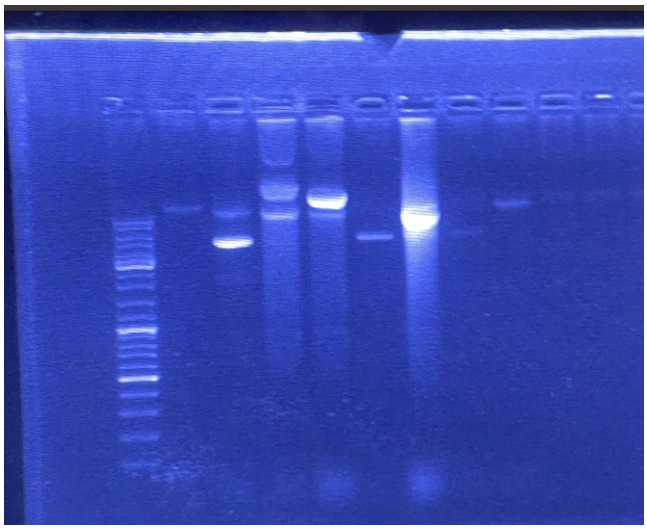
I miniprep whatever cultures had grown and run a gel.

I forget to take aside a bit of culture for a glycerol stock... thus, I picked again from the spots where I picked the first time, hoping there might be still some cells.

Ran on an 1% agarose gel, 120V.

Expected bands: 11.2kb (2008), 6.6kb (MP6) and 5.2kb (2024-2029).

Screenshot 2024-09-01 at 16.57.09.png



Transformation of pES1113

Transformation High-efficiency protocol, 100uL DH5a cells and 9uL GG assembly, 1h recovery.

Co-transformation of 2036-08-DP6 and 1113-08-DP6

High-efficiency protocol, 100uL competent S2060-DP6 cells and 2uL 1:1 mix, 1h recovery. Plates had a 0.6x working concentration of antibiotics.

DILLUNS, 2/9/2024

- [ ] ~~keep PACE running~~
  - [ ] ~~Check phage titer by qPCR (fill this Excel)~~
  - [ ] ~~Change media, waste, etc. and mark arabinose and lagoon waste~~
  - [ ] ~~Start SIAH PACE on target once we have the co-transformed strain: check o/n liquid culture~~
- [x] PANCE:

- [ ] check phage titer by qPCR, including sample from initial phage titer (fridge) and fill this Excel
- [ ] store an aliquot from fresh culutre once everything has been inoculated (and store in fridge) to get initial titer for qPCR the next day
- [ ] Passage to continue PANCE

- [x] Put LB bottles from cold room out for autoclaving
- [x] make sure to double check with Lucas that DP6 from friday is actually DP6
- [ ] SIAH KO phage production - repeat assembly once we have split C & D aliquote from Priscilla
- [ ] Once pES2040 and pES2041 are confirmed, transform into S2060 and perform phage propagation assay to determine E2 dependence
- [ ] Once at least pES2032 is confirmed, transform into S2060+DP6 and perform phage propagation assay to determine if weak expression of RNAP, E1, and E2 reduce Cp
- [x] Miniprep ON cultures and send for sequencing
- [x] Check 2037 plate in incubator to see if more coloneis grew (send for nightseq)
- [x] Check if o/n cultures of 2024-08-MP6 to 2029-08-MP6 grew (it might take more than one day)
- [x] Transform isothermal assembly

PACE

reactor died. Wait for new reactor to be ready then restart

PANCE

SIAH1 SP drift PANCE

qPCR:

SIAH1 PANCE 01/1

	Condition	Cp
1	PANCE	14.71
2	Initial	25.69
3	LB	30.18
4		

Phage shows similar rate of propagation as previous step, calculated amount necessary for re-inoculation at 10^6 pfu/ml: 247 ul

Minipreps and sequencing

All ON cultures for sequencing grew, additionally pES1087, pES1113, and two pES2037 colonies were sent for nightseq.

Assembly transformation

isothermal 2037 assemblies transformed into DH5a

Lab Journal - September

Project: iDEC 2024
Author: Gabriel Cervera
Entry Created On: 03 Sep 2024 08:19:39 UTC
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DIUMENGE, 1/9/2024

1wh High priority:

- [ ] **troubleshoot pES2037 assembly --> by far most important thing to do**
- [ ] keep PACE running
  - [x] Check phage titer by qPCR
  - [x] Change media, waste, etc. and mark arabinose and lagoon waste
  - [ ] Start SIAH PACE on target once we have the co-transformed strain: check o/n liquid culture
- [ ] PANCE:
  - [x] check phage titer by qPCR, including sample from initial phage titer (fridge)
  - [ ] store an aliquot from fresh culutre once everything has been inoculated (and store in fridge) to get initial titer for qPCR the next day
  - [ ] Passage to continue PANCE
- [x] Pick pES2032-2034, 2040 and 2041 if white colonies present
- [x] make carb plates

Other To Dos:

- [ ] double check phage shock gibson assembly primers 100-104 (first time I did it) - order once ok
  (should work on pES1001 + pTPH412, or pES2008 (100 and 104))
- [x] Transform 1113
- [x] Check transformation of 2036-08-MP6 (liquid cultures in shaker), if negative throw them out

In lab: Gabriel, Pau

SIAH1 SP drift PACE

In the morning when we arrive the reactor had stopped running. The lagoon is filled up, however, which means PACE kept running smoothly until the lagoon was filled and at some point after that stopped working...
I restart PACE by unplugging and plugging again.

qPCR:

SIAH1 PACE drift 01/09

	Condition	Cp
1	Lagoon	24.79
2	Chemostat	30.58
3	Positive control phages	16.12
4	LB	30.62
5	Water	32.07
6	Blank	30.815

Interpretation: phages did not replicate too much, probably because PACE broke down. Let's see tomorrow.

SIAH1 SP drift PANCE

qPCR:

SIAH1 PANCE 01/09

	Condition	Cp
1	PANCE+Tet	14.99
2	PANCE No Tet	22.63
3	PANCE+Tet initial titer	28.19
4	PANCE No Tet initial titer	28.125
5	LB	30.62
6	Water	32.07
7	Blank	25.51

Results: phages propagated very well and the addition of Tet makes a great difference. However, both phages replicated a bit (when compared to the initial titer), maybe because TetR is a bit leaky?

PANCE passaging: we calculate how many pfu/mL there are in the PANCE Erlenmeyer based on the Cp from the qPCR and then determine how many uL of phages we need to seed a new 50mL Erlenmeyer to a final concentration of 10^6 pfu/mL (based on this paper by Liu). Then, the new Erlenmeyer is prepared with fresh media containing 1x Chloramphemicol, 2x Tetracycline, 100 mM Glucose, and 40 mM Arabinose. About 400uL of o/n S2060-DP6 culture are added together with the phages.

pES2037 troubleshooting

Isothermal reaction will be left overnight at 37 C

Colonies

ON cultures set up for 2032-2034, 2040, 2041. 2037 seemed to start showing growth, 1 colony was ready for picking, plate put back in incubator. 1112 was also picked

Minipreps for 2024-08-MP6 to 2029-08-MP6 to run a gel

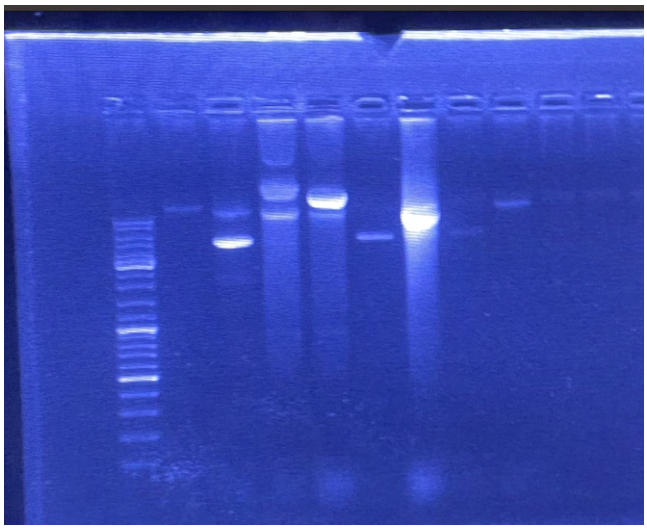
I miniprep whatever cultures had grown and run a gel.

I forget to take aside a bit of culture for a glycerol stock... thus, I picked again from the spots where I picked the first time, hoping there might be still some cells.

Ran on an 1% agarose gel, 120V.

Expected bands: 11.2kb (2008), 6.6kb (MP6) and 5.2kb (2024-2029).

Screenshot 2024-09-01 at 16.57.09.png



Transformation of pES1113

Transformation High-efficiency protocol, 100uL DH5a cells and 9uL GG assembly, 1h recovery.

Co-transformation of 2036-08-DP6 and 1113-08-DP6

High-efficiency protocol, 100uL competent S2060-DP6 cells and 2uL 1:1 mix, 1h recovery. Plates had a 0.6x working concentration of antibiotics.

DILLUNS, 2/9/2024

- [ ] ~~keep PACE running~~
  - [ ] ~~Check phage titer by qPCR (fill this Excel)~~
  - [ ] ~~Change media, waste, etc. and mark arabinose and lagoon waste~~
  - [ ] ~~Start SIAH PACE on target once we have the co-transformed strain: check o/n liquid culture~~
- [x] PANCE:

- [ ] check phage titer by qPCR, including sample from initial phage titer (fridge) and fill this Excel
- [ ] store an aliquot from fresh culutre once everything has been inoculated (and store in fridge) to get initial titer for qPCR the next day
- [ ] Passage to continue PANCE

- [x] Put LB bottles from cold room out for autoclaving
- [x] make sure to double check with Lucas that DP6 from friday is actually DP6
- [ ] SIAH KO phage production - repeat assembly once we have split C & D aliquote from Priscilla
- [ ] Once pES2040 and pES2041 are confirmed, transform into S2060 and perform phage propagation assay to determine E2 dependence
- [ ] Once at least pES2032 is confirmed, transform into S2060+DP6 and perform phage propagation assay to determine if weak expression of RNAP, E1, and E2 reduce Cp
- [x] Miniprep ON cultures and send for sequencing
- [x] Check 2037 plate in incubator to see if more coloneis grew (send for nightseq)
- [x] Check if o/n cultures of 2024-08-MP6 to 2029-08-MP6 grew (it might take more than one day)
- [x] Transform isothermal assembly

PACE

reactor died. Wait for new reactor to be ready then restart

PANCE

SIAH1 SP drift PANCE

qPCR:

SIAH1 PANCE 01/1

	Condition	Cp
1	PANCE	14.71
2	Initial	25.69
3	LB	30.18

Phage shows similar rate of propagation as previous step, calculated amount necessary for re-inoculation at 10^6 pfu/ml: 247 ul

Minipreps and sequencing

All ON cultures for sequencing grew, additionally pES1087, pES1113, and two pES2037 colonies were sent for nightseq.

Assembly transformation

isothermal 2037 assemblies transformed into DH5a

DIMARTS, 3/9/2024

- [ ] PANCE:
  - [ ] check phage titer by qPCR, including sample from initial phage titer (fridge) and fill this Excel

- [ ] store an aliquot from fresh culutre once everything has been inoculated (and store in fridge) to get initial titer for qPCR the next day
- [ ] Passage to continue PANCE

- [ ] SIAH KO phage production - repeat assembly once we have split C & D aliquote from Priscilla
- [x] ~~Check if o/n cultures of 2024-08-MP6 to 2029-08-MP6 grew (it might take more than one day)~~
- [x] Sequencing analysis
- [ ] Miniprep positive colonies
- [x] Make Kan plates
- [x] When primers arrive: gibson assembly of 2008 to add pVan elements
- [x] Redo 2032,33,34,35 and 40/41 assemblies
- [ ] repick 2025-2029 2008 MP6 cotransformants
- [x] Assemble level 2 with NLRP3 peptide 200

Sequencing analysis: Economy run

All sequences look weird apart from 1112

2032: Does not align where it is supposed to, looks like it has pES1074 (how's that possible?)

2033: NNN

2034: Does not align where it is supposed to

2037: NNN

2040: NNN

2041: NNN

1112: a and c are positive

Will reset assemblies overnight.

Sequencing Analysis: Nightseq

2037 looks like shit

1087 is positive

1113 b is positive

Isothermal assemblies

Growth seen for both BsmBI and Esp3I assembly, though many seem to be turning red

Colonies picked for nightseq (all that didn't look red)

Assemblies (2032-2035, 2040, 2041, 2043)

Table1

	A	B	C	D	E	F
1	**Assembly**	**Backbone**	**TU1**	**TU2**	**TU3**	**TU4**
2	2032	pTU2-A-RFP-KanR-CymRAM	pES1071	pES1072	pES1074	pES1026
3	2033	pTU2-A-RFP-KanR-CymRAM	pES1071	pES1002	pES1074	pES1026
4	2034	pTU2-A-RFP-KanR-CymRAM	pES1071	pES1072	pES1004	pES1026
5	2035	pTU2-A-RFP-KanR-CymRAM	pES1071	pES1002	pES1004	pES1026
6	2040	pTU2-A-RFP-KanR-CymRAM	pES1001	pES1002	Dummy C	pES1026
7	2041	pTU2-A-RFP-KanR-CymRAM	pES1001	pES1003	Dummy C	pES1026
8	2043	pTU2-a-p15a-SmR	pES1112	DummyB		

S2060 2036-2008-DP6

Colonies present, ON culture set up to prepare for phage propagation tomorrow

PANCE

Table2

	Sample	Cp
1	PANCE	16.79
2	inital titre	22.88
3	LB	31.23

Spreadsheet pfu calculations are wrong.

Diluted 1:1000 to get to Cp 25.

Miniprep

1113 and 1087 miniprepped. yields:

1113: 450 ng/ul
1087: 280 ng/ul

DIMECRES, 4/9/2024

- [ ] PANCE:
  - [x] check phage titer by qPCR, including sample from initial phage titer (fridge) and fill this Excel
  - [x] Order more oLS1662
  - [ ] store an aliquot from fresh culutre once everything has been inoculated (and store in fridge) to get initial titer for qPCR the next day
  - [ ] Passage to continue PANCE
- [ ] Once pES2040 and pES2041 are confirmed, transform into S2060 and perform phage propagation assay to determine E2 dependence
- [ ] Once at least pES2032 is confirmed, transform into S2060+DP6 and perform phage propagation assay to determine if weak expression of RNAP, E1, and E2 reduce Cp
- [x] Transform GG assemblies
- [ ] Manual 2037 assembly using new 1087
- [x] Start propagation of S2060 2036+2008+DP6 --> today it didn't grow, let's wait until tomorrow. We also pick more colonies
- [x] When primers arrive: gibson assembly of 2008 to add pVan elements
- [x] Pick more 2036-08-DP6
- [x] When Priscilla gives us the aliquot of SplitC/SplitD, proceed with phage production --> she hasn't sent it for seq yet

In lab: Gabriel, Pau

PANCE SIAH1 SP drift

First qPCR showed everything positive, including a blank containing only master mix. Repeat in new plate, with new FirePol (the aliquot I had used was running out anyways), new primer aliquotes (label new tubes with orange sticker, previous aliquotes were also running out). Again, everything was positive, so we decided to repeat the assay with water from Lukas. It came out positive again...

We inoculate PANCE with phages from yesterday and will try to solve this issue tomorrow.

Gibson assembly to add pVan+VanR elements to pES2008

Gibson Assembly

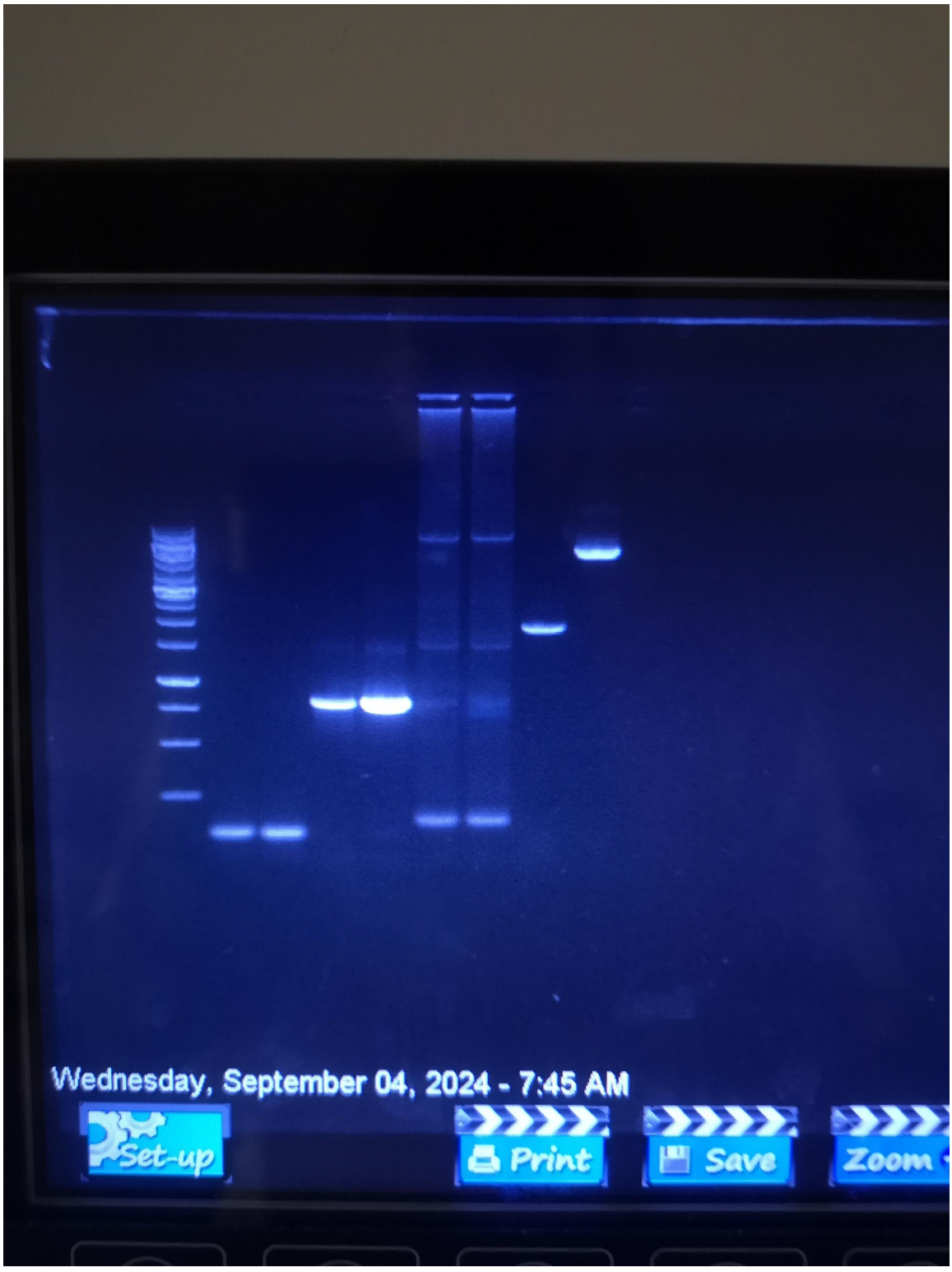
Primers and templates for initial PCR (PCR: Q5 Polymerase) :

- Vector: pES2008 105&106, Ta: 59, extension time 4:30
- Insert 2 (VanRAM): pES1095 (VanR) 107&108, Ta: 72, extension time 0:45
- Insert 1 (pVAN): pES0028 109&110, Ta: 62, extention time 0:30

Added 0.5uL of template DNA to the PCR reaction.

Modification: ran 5uL of the reaction in the gel to confirm sizes (10.4 kb, 850 bp, and 72bp, respectively) and once confirmed digested the rest of the reaction volume with 1ul DpnI
Gel (1% agarose, 120V, 30min) order: GeneRuler 1kb - L2 - L1 - V - pES2037 old - pES2037.7 (these two are to check for a miniprepped transformation and have nothing to do with this Gibson assembly)

IMG_20240904_162130.jpg



Result: We see bands for insert 1 and insert 1 and insert 2, but pES2008 shows an additonal band to the expected 10kb. For that reason, we decide to run the rest of the PCR reaction and perform gel extraction of the correct band.

NucleoSpin Gel and PCR Clean-up

Yield for gel extraction proved to be pretty bad (< 3 ng/uL)... we set a new PCR reaction o/n for pES2008 w/primers 105&106 (vector)
For insert 1 and insert 2, we proceed with DpnI digestion.

Transformation of pES2032-pES2035 and pES2040-pES2043
High-efficiency protocol. 75uL of DH5a competent cells. 5uL of GG assembly reaction.

ON sequencing
2 of the 2037 colonies sent for nightseq may be positive, will miniprep and resend for sequencing because signal was shit, will also run gel.
Additionally, we still continue with Gibson assembly.

The two pES2037 colonies were miniprepped and sent for sequencing with primer o018 to amplify the VanR region.

Picking 2036-08-DP6
Given that the o/n culture did not grow, three more colonies were picked today. Antibiotic concentrations were set at 0.6x.

Vetor PCR
PCR of pES2008 was redone with primers 105 and 106. Ta increased to 61, extension time increeased to 5:00. Left overnight

DIJOUS, 5/9/2024

- [x] PANCE:
  - [x] check phage titer by qPCR, including sample from initial phage titer (fridge) and fill this Excel
  - [x] store an aliquot from fresh culutre once everything has been inoculated (and store in fridge) to get initial titer for qPCR the next day
  - [x] Passage to continue PANCE
- [ ] Once pES2040 and pES2041 are confirmed, transform into S2060 and perform phage propagation assay to determine E2 dependence
- [ ] Once at least pES2032 is confirmed, transform into S2060+DP6 and perform phage propagation assay to determine if weak expression of RNAP, E1, and E2 reduce Cp
- [x] Once pES2037 is confirmed, co-transform with different substrates
- [x] Start propagation of S2060 2036+2008+DP6
- [ ] When Priscilla gives us the aliquot of SplitC/SplitD, proceed with phage production --> she said once it is sent for seq and confirmed, she will give us an aliquote
- [ ] Manual 2037 assembly using new 1087
- [x] Repeat pES2043 transformation in SmR plate
- [ ] Generate RNAP-C term with inducible Van
- [x] send 2032-2035, 2040,2041 for nightseq
- [x] Order new Economy run labels

Sequencing analysis of pES2037

Both seem to have VanRAM which technically means it is correctly assembled. There is a big indel/mismatch region at the end of the sequencing but that could just be due to the algorithm trying to align bad quality basepairs. We will proceed with sending pES2037.7, which seemed to have almost the right size in gel, to full-plasm seq. Additionally, we will start co-transformation of pES2037

Order: Generuler 1kb ladder - pES2037 old - pES2037.7

insert image from gel

Result: again, pES2037 old is smaller than expected, but pES2037.7 matches in size.

Co-transformation with pES2037 (yet to be confirmed!) and different lvl1 and lvl2 substrates
We perform the follow combinations:

- 2023-37-DP6 --> lvl2 EGLN3 (full sequence)
- 1076-37-DP6 --> lvl1 EGLN3 (full sequence)
- 1096-37-DP6 --> lvl1 EGLN3 (35aa peptide)
- 1112-37-DP6 --> lvl1 NLRP3 (peptide 200, 35aa)
- 1113-37-DP6 ·--> lvl1 NLRP3 (peptide 707, 35aa)
- 1081-37-DP6 --> lvl1 a-syn (full sequence)
- 2036-37-dp6 --> lvl2 a-syn

Kan+Spec+Chlor+Glucose: x2 (1 plate)
Kan+Carb+Chlor+Glucose: x5 (3 plates)

High efficiency protocol, 60 uL S2060-DP6 competent cells, 1h recovery.

Repeat transformation of GG assemblies for pES2040, pES2041, and pES2043
We the 5 uL of the GG assembly from 03.09 for transformation of 100uL DH5a. High efficiency protocol, 1h recovery.

2036-08-DP6 co-transformant o/n cultures
Colonies picked yesterday grew. We miniprep 1mL of the liquid culture and run a gel.
Order: Generuler 1kb ladder - 2036-08-DP6 A - B - C

insert gel photo

Result: for replicate A we can observe three bands at the expected sizes, namely for pES2036 (5 kb), pES2008 (11.2 kb), DP6 (8.6 kb). We proceed to make a phage propagation assay with 2036-08-DP6 A. We also make glycerol stock for this strain.

Phage propagation assay with 2036-08-DP6

- ON cultures of 2036-08-DP6 and S2208 (provided by Tara) were diluted, grown to 0.9 and 0.8 OD and diluted again to OD 0.4.5 and 0.4 respectively
- 10 µL of the 10^8 PFU/mL dilutions were used to innoculate 1 mL of bacteria --> 10^6 PFU/mL starting concentration

    - 2uL of TadA (TadA is probably higher concentrated)
- LB is a control for cross-contamination and does not contain antibiotics
- Cells without phages is a control for no phage contamination in our stock (here, media does contain the right antibiotics)
- Phages without cells are a control for initial titer and are in LB+antibiotics so that nothing can grow in there
- plates were covered with a breathable seal and grown ON at 37°C in the shaker

Plate Layout

	1	2	3	4	5	6	7	8	9	10	11	12
A	2036-08-DP6 + SIAH1				SIAH 1 (no cells)				S2208 + SIAH1			
B	2036-08-DP6 + SIAH2				SIAH 2 (no cells)				S2208 + SIAH2			
C	2036-08-DP6 + TadA				TadA (no cells)				S2208 + TadA			
D												
E	2036-08-DP6 no phages								S2208 no phages			
F	LB								LB			
G												
H												

Gel for pES2008 PCR for 2044 Gibson assembly

PCR failed, primers designed and ordered. primers can be seen attached to final plasmid pES2045 (new gibson) , the backbone is now split into two 5-6 kb fragments to make amplification easier, and the pVan insert has been lengthened to make it more streamlined for gibson assembly

PANCE

This time we decided to change FirePol aliquote and results were finally okay once again.
qPCR data:

Table3

	A	B
1	PANCE	14.33
2	initial titre	28.3
3	LB	29.20
4	Water	29.98

Passage 31 uL of phage into a new PANCE Erlenmeyer

Inducible C-term polymerase assemblies

plasmids 1084 and 1085 have pVan infront of substrate-RNAP

VanRAM TU was assembled into pTU1-B-RFP, in order to build an AP2 that has pVan-substrate-RNAP as TU1 and VanRAM as TU2

pTU1-A-RFP was nowhere to be found, culture set up for miniprepping

PCR of 1076 to generate EGLN3 peptide for pVAN addition

Primers 71,72 at Ta 72 and 30sec elongation

No gel was run, clean up yield: 75 ng/ul

DIVENDRES, 6/9/2024

- [x] PANCE:
  - [x] check phage titer by qPCR, including sample from initial phage titer (fridge) and fill this Excel
  - [x] store an aliquot from fresh culutre once everything has been inoculated (and store in fridge) to get initial titer for qPCR the next day
  - [x] Passage to continue PANCE
- [ ] Once pES2040 and pES2041 are confirmed, transform into S2060 and perform phage propagation assay to determine E2 dependence
- [ ] Once at least pES2032 is confirmed, transform into S2060+DP6 and perform phage propagation assay to determine if weak expression of RNAP, E1, and E2 reduce Cp
- [ ] If full plasmid pES2037 sequencing results come out wrong, consider Manual 2037 assembly using new 1087
- [ ] Quantify propagation assay
- [ ] Start cultures for cotransformants
- [ ]

Sequencing analysis

2033 was positive

2034,2035,2040 were all negative

- 2034 showed NNN
- 2035 seems to only contain 1 TU, or is a level1
- 2040 does not align at all

Minipreps

miniprep of 2033 and pTU1-A-RFP. yields:

- pES2033: 894 (very high, absroption curve looked good, but will send for sequencing to make sure nothing sus)
- pTU1-A-RFP: 295 (diluted to 100)

Placed in corresponding boxes

Full plasmid seq

pES2037 looks mostly fine, however the TU containing gIII and luxAB is inserted backwards, the sequence and regulatory regions seem to be fine though, so the transformants will be carried forward into ON cultures to prepare for propagation assay.

pES2037-xxx-DP6 cotransformations

- 1076: colonies present, liquid cultures prepared
- 1081: colonies present, liquid cultures prepared
- 1096: colonies present, liquid cultures prepared
- 1112: few colonies present, liquid culture prepared
- 1113: few colonies present, liquid culture prepared
- 2023: no colonies, left to grow another day
- 2036: no colonies, left to grow another day

Liquid cultures were put into fridge, Tara will put them in incubator around 10:00 in the morning tomorrow and they will be picked up around 16:00/17:00 to avoid overgrowth and put back in fridge to begin propagation assay on Sunday

pES2040-2043 transformations

- pES2040: 1 red colniy, and 1 white that is too small to pick. Will leave in incubator overnight
- pES2041: 1 colony that is too small to pick. Will leave in incubator overnight
- pES2043: A few colonies that may be turning red. Will leave in incubator overnight

Phage propagation 2036-08-DP6 qPCR

image.png

	1	2	3	5	6	7	9	10	11
A	24.10	23.73	23.98	24.14	24.10	23.96	8.77	9.39	9.19
B	23.91	24.03	24.46	24.46	24.31	24.33	9.17	9.12	10.48
C	25.04	25.24	25.96	25.81	26.44	26.50	11.89	12.61	12.28
D	16.43	16.51	16.89	NaN	NaN	NaN	27.27	27.52	27.22
E	30.06	30.86	31.28	NaN	NaN	NaN	28.77	28.94	29.64
F	31.49	30.57	32.20	NaN	NaN	NaN	29.03	29.67	30.19

image.png

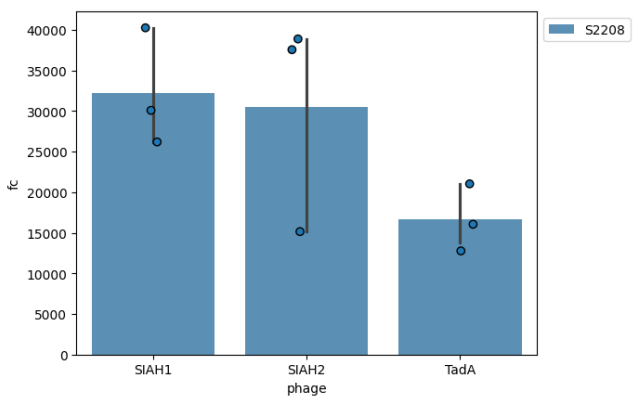
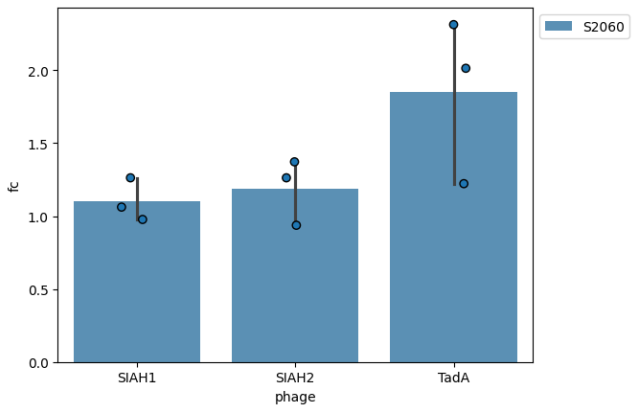


image.png



S2060-2036-2008-DP6 cells do not seem to propagate phages, but it is weird that the fold change is basically the same. Test should be done to see whether the cells are functional by repeating the experiment with tetracycline to induce drift of the cells

PANCE qPCR

Table4

	Sample	Cp
1	PANCE	16.61
2	initial tire	27.34
3	LB	31

Phage will be diluted 1:1000 to passage PANCE

Transformation of pTU1-B-VanRAM into DH5a

5 ul of assembly mix was transformed into 50 ul of DH5a cells via heat shock

recovered for 1 hr at 37 C

Plated on carbenicillin

DISSABTE, 7/9/2024

- [x] Take sample from PANCE
- [x] Take out 2037-xxx-DP6 liquid cultures and put into fridge
- [x] Pick colonies from transformations in incubator
- [x] Take out Tara's qPCR

Assemblies to prepare inducible N-Terminal polymerase

labelled PCR tubes in thermocycler slot 1

- 1: pE1116
- 2: pES1117
- 3: pES1118
- 4: pES1119
- 5: pES1115

pES2040,2041,2043

Liquid cultures prepared

2037-xxx-DP6

cultures did not grow

DIUMENGE, 8/9/2024

short summary:

- started a propagation assay with van-inducible AP1 (3027) with different substrates induced vs uninduced conditions
- GG assemblies of lvl 2 plasmids with weak RNAP, E1, and E2 promotors (2032-35)
- transformation of van-inducible AP2 plasmids (with EGLN3, NRLP3 and asyn)
- maintained SIAH1 drift PANCE

- [x] PANCE:
  - [x] check phage titer by qPCR (sample taken yesterday in SIAH1 PACE box), including sample from initial phage titer (fridge) and fill this Excel
  - [x] store an aliquot from fresh culutre once everything has been inoculated (and store in fridge) to get initial titer for qPCR the next day
  - [ ] Passage to continue PANCE

- [x] Start propagation assay with 2037 cotransformants (in fridge), do induced vs. uninduced conditions
- [ ] Miniprep liquid cultures for pES2040,2041,2043
- [x] Transform assemblies in thermocycler
- [x] Redo assemblies for 2032-2035, and 2040-2043

- [x] Check if 2037 cotransformant liquid cultures grew in shaker, set up new ones from plates in fridge (labelled with green tape)

in lab: Michael

new 2037 co-transformants

- I picked fresh 2037 co-transformant colonies from plates in the fridge and innoculated them in a liquid culture with 0.6x Carb + Kan + Chlor + 100mM Gluc (to avoid the loss of the F-pillus)

PANCE

- did a qPCR with samples from yesterday (7.9) and initial titer from 6.9
- I resuspended the newly ordered 1662 primer in 1320 uL H2O as the microsynth datasheet said

Cp values

	Sample	Cp
1	PANCE	15.68
2	initial titer	26.58
3	LB	27.813

- dillute the phages 1:1000
- I forgot to pre-grow 50 mL S2208+DP6 in the morning and realized too late (shortly before I had to leave)
  -> will dillute the phages tomorrow. Sorry, it's my first time doing this.

Propagation assay

- all 5 liquid cultures of 2037 co-transformants grew to ODs around 2
  -> dilluted 1:20 in LB + 0.6 Carb + 0.6x Kan + 0.6x Chlor + 100 mM Gluc at 11:30
- grown to OD 0.48-0.64 at 14:00 -> stored on ice until the assay was set up
- 10 uL SIAH1 and SIAH2, 2 uL TadA phages in the respective wells
- bacteria were induced with 100 uM vannilic acid (added 10 uL of a 10 mM dillution to the wells). I used this paper as reference
- A full 12mL tube didn't have sufficient volume for doing two triplicates: uninduced cells will be tested in triplicates and induced cells with duplicates

Plate design

Plate 1

	A	B	C	D	E	F	G	H	I	J	K	L
1	1076-37-DP6 - uninduced + SIAH1				1076-37-DP6 - uninduced + SIAH2				1076-37-DP6 - uninduced + TadA			
2	1081-37-DP6 - uninduced + SIAH1				1081-37-DP6 - uninduced + SIAH2				1081-37-DP6 - uninduced + TadA			
3	1096-37-DP6 - uninduced + SIAH1				1096-37-DP6 - uninduced + SIAH2				1096-37-DP6 - uninduced + TadA			
4	1112-37-DP6 - uninduced + SIAH1				1112-37-DP6 - uninduced + SIAH2				1112-37-DP6 - uninduced + TadA			
5	1113-37-DP6 - uninduced + SIAH1				1113-37-DP6 - uninduced + SIAH2				1113-37-DP6 - uninduced + TadA			
6	LB + SIAH1				LB + SIAH2				LB + TadA			
7	S2060 + SIAH1				S2060 + SIAH2				S2060 + TadA			
8	S2208 + SIAH1				S2208 + SIAH2				S2208 + TadA			

Plate 2

	A	B	C	D	E	F	G	H	I	J	K	L
1	1076-37-DP6 + van + SIAH1			1076-37-DP6 + van + SIAH2			1076-37-DP6 + van + TadA			1076-37-DP6 - no phage		
2	1081-37-DP6 + van + SIAH1			1081-37-DP6 + van + SIAH2			1081-37-DP6 - van + TadA			1081-37-DP6 - no phage		
3	1096-37-DP6 + van + SIAH1			1096-37-DP6 + van + SIAH2			1096-37-DP6 + van + TadA			1096-37-DP6 - no phage		
4	1112-37-DP6 + van + SIAH1			1112-37-DP6 + van + SIAH2			1112-37-DP6 + van + TadA			1112-37-DP6 - no phage		
5	1113-37-DP6 + van + SIAH1			1113-37-DP6 + van + SIAH2			1113-37-DP6 + van + TadA			1113-37-DP6 - no phage		
6												
7												
8												

Transformation

- the PCR tubes of the assemblies in the thermocycler (pES1116, pES1117, pES1118, pES1119, pES1115) were partially melted and there was only around 5 uL in every tube
- transformed everything into 100 uL competent DH5a bacteria (like it was done the last time)
- High efficiency protocol, 1h recovery.
- plated on Carb plates

GG Assemblies

- with Esp3i
- We ran out of T4 ligase and I opened the backup stock

Golden Gate Assembly

	assembly	backbone	TU 1	TU 2	TU 3	TU 4
1	pES2032	pTU2-A-RFP KanR CymRAM	pES1071	pES1072	pES1074	pES1026
2	pES2033	pTU2-A-RFP KanR CymRAM	pES1071	pES1002	pES1074	pES1026
3	pES2034	pTU2-A-RFP KanR CymRAM	pES1071	pES1072	pES1004	pES1026
4	pES2035	pTU2-A-RFP KanR CymRAM	pES1071	pES1002	pES1004	pES1026

incubation in slot 1 overnight (long protocol)

Miniprep

- didn't have enough time for this. -> will do it tomorrow

DILLUNS, 9/9/2024

short summary:

- qPCR of the propagation assay with AP1 plasmids that have van-inducible RNAP
  - Result: No phage propagation compared to control and no difference between van-induced and uninduced
  - the same O/N propagation assay was repeated
- transformants with lvl 1 plasmid (AP2) with van-inducible substrate expression (pES116-119) didn't grow -> re-assembly
- continued making SIAH1 KO phages (Priscilla gave us her splitC&D aliquots)
- miniprepped AP1 plasmids (2041&42) without E2 and sent for sequencing to test E2 dependence at some point
- maintained PANCE

- [x] PANCE:
  - [x] dillute phages 1:1000 in fresh culture
  - [x] store an aliquot from fresh culutre once everything has been inoculated (and store in fridge) to get initial titer for qPCR the next day
  - [x] Passage to continue PANCE

- [ ] Gibson Assembly of 2045 (2008 + 1095) if primers arrive - primers didn't arrive
- [x] qPCR of O/N propagation assay
- [x] Make SIAH1 ko phages if Priscilla confirms the sequence of split c&d
- [x] Transformation of GG assemblies (pES2032-2035)

- [x] Miniprep pES2040, 2041, 2043 and send for sequencing
- [x] make primers with lvl 0 overhangs for cloning EGLN3 peptide into a pVAN-inducible lvl 1
- [x] check transformed bacteria in the incubator, pick and send for NightSeq colonies if they grew - didn't grow
- [x] re-assemble pES1115-1119

- [x] NightSeq of the co-transformants used for the propation assay
- [ ] reorder T4 ligase (we're using the backup rn)
- [x] figure out what 2042 is - I identified the TU and entered them in the cloning spreadsheet. Pls don't forget to enter the parts as soon as you design a plasmid

in lab: Michael, Jakob

bacteria with assemblies that were transformed yesterday (pES1115-1119) didn't grow -> re-assemble them today

PANCE

Given the qPCR results (see yesterday), the previous phages were dilluted 1:1000 at 12:00

Miniprep

yields:

- 2040.1: 1381.2 ng/uL
- 2040.2: 470.4 ng/uL
- 2041.1: 468.9 ng/uL
- 2041.2: 1486.6 ng/uL
- 2043.1: 6.9 ng/uL -> won't be sequenced but I keep the empty tube of the liquid culture just in case
- 2043.2: 213.5 ng/uL

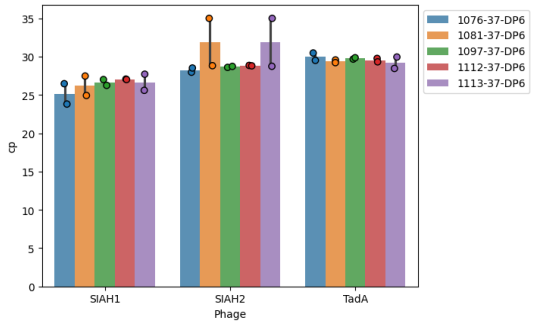
all samples were dilluted to 100 ng/uL and sent for sequencing (except 2043.1) with the 018 primer

qPCR results

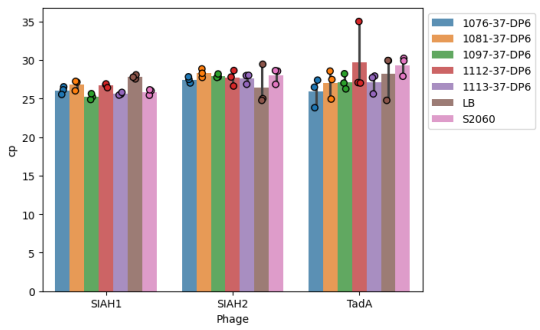
- The data looks weird. For example do some empty wells have Cp values of 26 whereas a third or so of all wells with samples have a Cps of 35+. From the few wells that have reasonable Cp values, it looks like the phages propagate equally in presence or absence of vanillic acid. How is this possible??
- We repeat the qPCR

Results of the 2nd attempt:

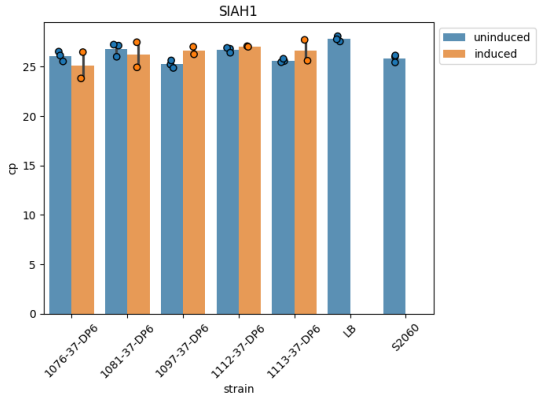
Induced



Uninduced



SIAH1



- looks like there isn't any particularely noteworthy propagation under any condition, let's wait for the rerun of the assay and the sequence of the cultures
- I'm already pe-growing new cultures in case we have to repeat the assay today

SIAH1 KO phage production

- Priscilla gave me aliquots of SplitC and SplitD
  - I put a green sticker on them and put them in the lvl 2 box
- I used the sample in the lvl 2 box labeled A SIAH1 KO SP. It did not have a date, nor a concentration -> pls label with the date if anybody who was involved sees it
- GG assembly overnight with Lukas' long protocol

Golden Gage Assembly repeat

Lukas' Protocol

Table5

	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P	Q	R	S	T	U	V	W	X	Y	Z
1	pES1115	vanR_partB	B	Amp	pTU1-B-RFP	vanR TU					ok															
2	pES1116	pVan_EGLN3_L2_CtermRNAP(CGG)	A	Amp	pTU1-A-RFP	pES0028	pES0013	Linker 2	pES0008	pBP-L3S2P21	ok															
3	pES1117	pVan_asyn_L4_CtermRNAP(CGG)	A	Amp	pTU1-A-RFP	pES0028	pES0015	pES0022	pES0008	pBP-L3S2P21	ok															
4	pES1118	pVan_NLRP3_200_L2_CtermRNAP(CGG)	A	Amp	pTU1-A-RFP	pES0028	NLRP3_200_frag	Linker 2	pES0008	pBP-L3S2P21	ok															
5	pES1119	pVan_NLRP3_700_L2_CtermRNAP(CGG)	A	Amp	pTU1-A-RFP	pES0028	NLRP3_700_frag	Linker 2	pES0008	pBP-L3S2P21	ok															

Phage propagation - repeat

bacteria all had OD between 0.4 and 0.6 and were diluted to 0.4

Plate 3

	A	B	C	D	E	F	G	H	I	J	K	L
1	1076-37-DP6 - uninduced + SIAH1				1076-37-DP6 - uninduced + SIAH2				1076-37-DP6 - uninduced + TadA			1076-37-DP6 - uninduced - no phage
2	1081-37-DP6 - uninduced + SIAH1				1081-37-DP6 - uninduced + SIAH2				1081-37-DP6 - uninduced + TadA			1081-37-DP6 - uninduced - no phage
3	1096-37-DP6 - uninduced + SIAH1				1096-37-DP6 - uninduced + SIAH2				1096-37-DP6 - uninduced + TadA			1096-37-DP6 - uninduced - no phage
4	1112-37-DP6 - uninduced + SIAH1				1112-37-DP6 - uninduced + SIAH2				1112-37-DP6 - uninduced + TadA			1112-37-DP6 - uninduced - no phage
5	1113-37-DP6 - uninduced + SIAH1				1113-37-DP6 - uninduced + SIAH2				1113-37-DP6 - uninduced + TadA			1113-37-DP6 - uninduced - no phage
6	LB + SIAH1				LB + SIAH2				LB + TadA			LB - uninduced - no phage
7	S2060 + SIAH1				S2060 + SIAH2				S2060 + TadA			S2060 - uninduced - no phage
8	S2208 + SIAH1				S2208 + SIAH2				S2208 + TadA			S2208 - uninduced - no phage

Plate 4

	A	B	C	D	E	F	G	H	I	J	K	L
1	1076-37-DP6 + van + SIAH1			1076-37-DP6 + van + SIAH1 + tetracyclin	1076-37-DP6 + van + SIAH2				1076-37-DP6 + van + TadA			1076-37-DP6 - no phage
2	1081-37-DP6 + van + SIAH1			1081-37-DP6 + van + SIAH1 + tetracyclin	1081-37-DP6 + van + SIAH2				1081-37-DP6 - van + TadA			1081-37-DP6 - no phage
3	1096-37-DP6 + van + SIAH1			1096-37-DP6 + van + SIAH1 + tetracyclin	1096-37-DP6 + van + SIAH2				1096-37-DP6 + van + TadA			1096-37-DP6 - no phage
4	1112-37-DP6 + van + SIAH1			1112-37-DP6 + van + SIAH1 + tetracyclin	1112-37-DP6 + van + SIAH2				1112-37-DP6 + van + TadA			1112-37-DP6 - no phage
5	1113-37-DP6 + van + SIAH1			1113-37-DP6 + van + SIAH1 + tetracyclin	1113-37-DP6 + van + SIAH2				1113-37-DP6 + van + TadA			1113-37-DP6 - no phage
6												
7												
8												

Primer design

- designed and ordered primers for adding lvl 0 overhangs to the EGLN3 peptide.
- The forward primer is quite long (57nt) but it wasn't possible to get matching annealing temps otherwise (because of the different GC contents)

DIMARTS, 10/9/2024

short summary:

- qPCR of yesterday's propagation assay with AP1 plasmids that have van-inducible RNAP
  - Very noisy results, the data is all over the place and we would need to repeat the qPCR but it doesn't matter because **we found out there is no RNAP-Ubiquitin fusion on 2037 (the van-inducible AP1 we have been using). This explains why we had no propagation** and no difference between induced and uninduced cells
- So we still have no functional, inducible AP1 plasmid
- We made a Gibson assembly of 2045 which basically clones a pVan promotor and VanR into 2008 -> should be functionally equivalent to 2037
- Transformed SIAH1 KO SP, Gibson assembly of 2045, and van-inducible AP2s (assembled yesterday)
- The sequences of AP1s without E2 ligases (2040+41) were not correct and had mayor deletions/recombinations --> picked new colonies and sent for Nightseq
- maintained PANCE

- [x] qPCR of O/N propagation assay
- [x] Gibson Assembly of 2045 (2008 + 1095) if primers arrive (end-extension PCR, gel, assembly)
- [x] order phage shock primers 104 + 100 for 2008 and 102 + 103 for pTPH412

- [x] PANCE:
    - [x] check phage titer by qPCR (sample taken yesterday in SIAH1 PACE box), including sample from initial phage titer (fridge) and fill this Excel
    - [x] store an aliquot from fresh culutre once everything has been inoculated (and store in fridge) to get initial titer for qPCR the next day
    - [x] Passage to continue PANCE
- [ ] Transformations
    - [ ] SIAH1 KO phage assembly
    - [ ] pES1115-1119 (assembled yesterday)
    - [ ] Gibson Assembly of 2045
    - [ ] co-transform 2040, 2041 and 2043 if the sequences are correct

- [x] Sequencing analysis
    - [ ] move samples from the temp box -> trashed
    - [x] make glycerol stocks of co-transformants that are correct
      --> none are, because 2037.7 is not correct, made glycerol stocks with warning
    - [x] trash liquid cultures 2037 co-transformants with the green sticker in the fridge if the ones that were sequenced are correct
- [x] reorder T4 ligase

in lab: Jakob, Michael

PANCE

- 1 mL sample of the PANCE reaction was taken at 10:00

Cp values:

Table8

	sample	Cp
1	initial titer	23.9
2	PANCE	14.6
3	LB	26.8

- 1:1000 dillution and passage at 14:05
- aliquot of fresh culture stored in the fridge
- We have phage contamination (Cp 26) in either our shared primers or firepol (Sasha and Lukas had the same Cps in neg. controls). On this qPCR plate with the propagation assay, no values were above 27.

Sequencing Analysis

pES2040 - both sequencings show E1, missing everything in between, but sticky ends match Lvl2 plasmid

pES2041.1: shows E1, missing everything in between, but sticky ends match Lvl2 plasmid
pES2041.2: shows expected sequence starting from o018 primer
pES2043: nothing sequenced
--> Will run a gel with 2040, 2041 and 2043 to verify the size (only pES2041.2 looks promising)
gel looks shit --> we picked new colonies (1 each) from the 2040 and 2041 plates and sent them for NightSeq

Co-transformations - Sequencing Analysis
for 1076-37-DP6, 1081-37-DP6, 1096-37-DP6, 1112-37-DP6, 1113-37-DP6
KanStart sequencing shows E1 for all five co-transformants
--> there is no RNAP in 2037.7, the phage propagation could not have worked
DP6 is good in all five, all level 1 plasmids are present

qPCR - Phage propagation
--> we now know that the phage can not propagate, because there is no RNAP-N-term

Gibson Assembly end-extension PCR:

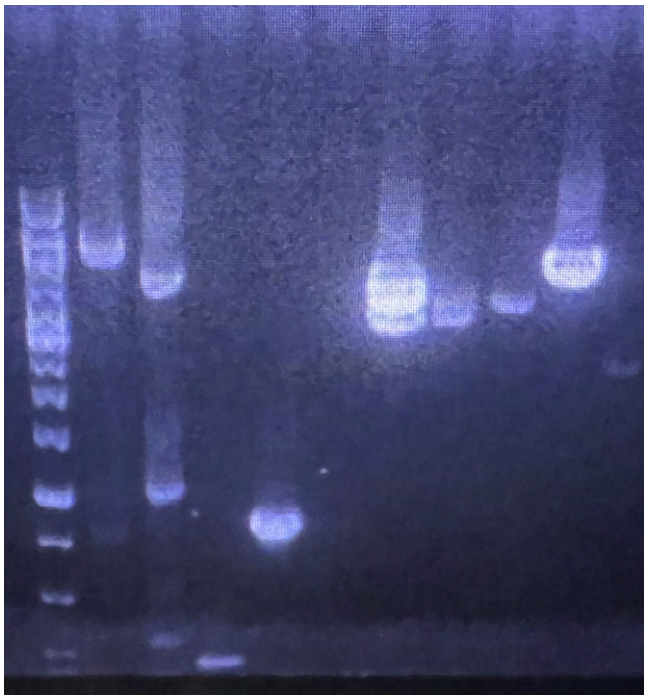
- annealing temp was calculated with the complementary parts of the primers
- 50 uL reactions with 10 ng template each PCR: Q5 Polymerase

end extension PCR for 2045 Gibson Assembly

	template	primers	annealing temp (NEB)	elongation time
1	2008 part 1	111, 106	65 °C	3 min (~5.5/6.2kb)
2	2008 part 2	105, 112	62 °C	3 min (~5 kb)
3	1095 (VanR)	107, 114	72 °C	1 min (~850bp)
4	pES0028 (pVan)	113, 110	62 °C	30 s (~200 bp)

There are two possible PCR products for 2008 part 1 One with and one without CymRAM. Both are fine since we don't need CymRAM
Loading Scheme: Gener Ruler 1 kb - GA 2008 part 1 - GA 2008 part 2 - GA VanR - GA pVan - mastermix (neg ctrl) - pES2040.1 - pES2040.2 - pES2041.1 - pES2041.2 - pES2043 (to double check the plasmid integrity)

IMG_6830.jpeg



- Gibson PCR products have the expected size
- Sequencing (last 4 lanes): none of the plasmids (pES2040-2043) are even close to the expected size
- PCR products were purified (no gel purification)
- rationale: we had very bad yields with gel extractions in the past and the off-target band (2008 part 2) does very likely not contain a Kan-resistance and will be selected out (I checked all primer off-target sites and none are within 1000 bp distance of the KanR gene)

Gel purification yields

- 0028: 113 ng/uL
- 1095: 141 ng/uL
- 2008 part 2: 58 ng/uL
- 2008 part 1: 106 ng/uL

Gibson Assembly

values were slightly rounded (+- 3%)

1:2 ratio of backbone parts:inserts

Table6

	part	molar amount	DNA mass required	added volume
1	2008 part 1	50 fmol	200 ng	3.5 uL
2	2008 part 2	50 fmol	170 ng	1.6 uL
3	pES0028	100 fmol	13 ng	0.15 uL (dilluted)
4	pES1095	100 fmol	53 ng	0.4 uL
5	NEBuilider HiFi DNA Assembly Master Mix			10 uL (2 aliquots)
6	ddH2O			4.35 uL

- incubation for 1h at 50 °C

Golden Gate Assembly

repeat assembly of pES2037, since we do not actually have the correct plasmid --> set up three reactions: 1 for 37 °C isothermal, one for Lukas' protocol and one for the original protocol

Table7

	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P	Q	R	S	T	U	V	W	X	Y	Z
1	pES2037	AP1 pVAN RNAP (CGG), weak hE1, weak E2	KanR (+CymRam)	pTU2-A-RFP KanR CymRAM	pES1087	pES1072	pES1074	pES1094	pES1095																	

Transformation

5 µL of assemblies of pES1115-1119 in 100 µL DH5a each,
10 µL of Gibson assembly in 200 µL DH5a,
2 µL of SIAH1 KO SP (from 27.08.) into competent 30 µL S2208 cells
retransformation with 5 µL of pES2032-2035 in 100 µL DH5a

Sequencing

pES2040 and pES2041 two new culters were picked each and one each was sent for nightseq
We ordered new NightSeq labels

DIMECRES, 11/9/2024

short summary:

- picked yesterdays transformants (pVan inducible AP1&AP2) made a colony PCR (which looked promising for some colonies) and sent the samples for sequencing
- If the sequences are correct, we can miniprep and co-transform the plasmids tomorrow and start with a van-inducible AP1+AP2 phage propagation assay on Friday
- Transformed some NLRP3-AP2s into S2060+DP6 to start PANCE (this is not promising but out of despair)
  - EDIT: we didn't do PANCE enventually
- Plaque assay of SIAH1 KO phages to determine titer

- stopped SIAH drift PANCE
- [x] PANCE
  - [x] take an aliquot of the culture
  - [x] qPCR
  - [x] do we still need to continue running it? - Sasha said no
- [x] pick pES2045 colonies
- [x] pick colonies of all freshly transformed bacteria
- [x] NiqhtSeq sequencing analysis of 2040 + 2041
- [x] assemble the plasmid with 2008+psp on Benchling and add it to the cloning spreadsheet
- [x] check if pES2032-2035 or pES1115-1119 grew - they grew
- [ ] transform pES2037 variants --> used 1087 is not correct, trash them
- [x] miniprep 2043.1 if it grew and send for sequencing

in lab: Jakob and Michael

11.9 PANCE

	Sample	Cp
1	initial titer	24.58
2	PANCE	23.64
3	LB	27.53

- Looks like the phages were not really propagating overnight (maybe somwthing with the O/N culture was off)
- PANCE was stopped anyways since it was already running for 10 days (8 passages)
- The phage samples were sent for sequencing

Sequencing Analysis
pES1087 that was used yesterday could not be sequenced
--> will load it on the colony PCR gel and look for other 1087 tube, but assemblies from yesterday are probably trash
--> they re-sequenced it, an now it looks correct --> let's go diving for the assemblies in the trash

pES2040.3 --> very short sequencing at the correct location
pES2041.3 --> very short sequencing at the correct location
--> none of the cultures grew anyways though

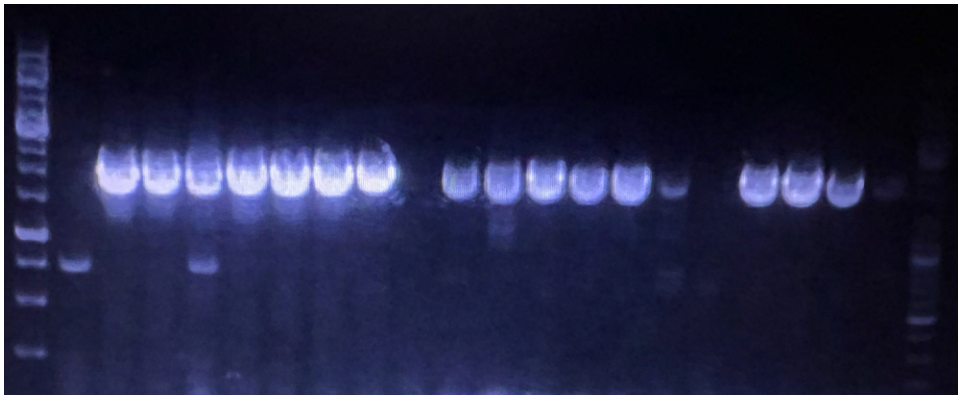
Colony PCR
pES2045 - 8 colonies, 2 reactions each, pES1115-pES1119 - 4 colonies each (20), pES2032-pES2035 - 3 colonies each (12)
--> 48 samples overall

pES2045: o054 and o018 at 62 °C, 1 min elongation; o019 and o018 at 62 °C, 4.5 min elongation
pES1115-1119: o017 and o018 at 59 °C, 1.5 min elongation

pES2032-pES2035: o054 and o018 at 62 °C, 1 min elongation

Gel 1:
GR 1kb - pES2045 o054 1-8 - pES2032 1-3 - pES2033 1-3 - pES2034 1-3 - pES2035 1-3 - GR Mix

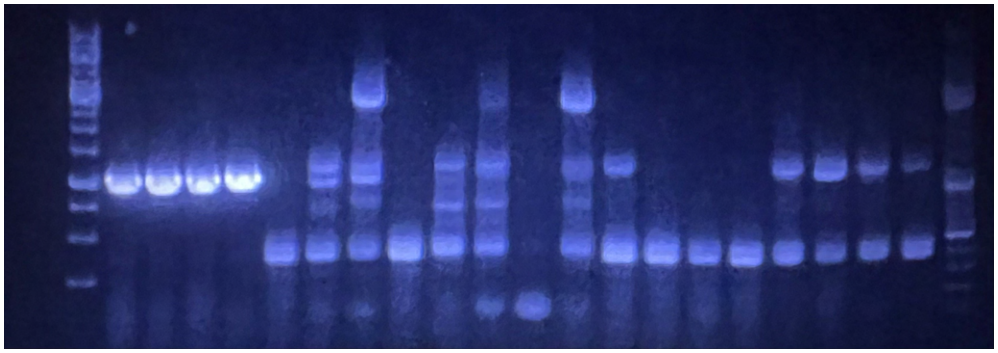
IMG_6836.jpeg



expected band size: 2 kb
--> pES2045 2-3 and 5-8 look good, pES2032 2-3 are good, pES2033 1-3 are good, pES2034 3 is good, pES2035 1 is good
--> for pES2045 wait for other PCR still

Gel 2:
GR 1kb - pES1115 1-4 - pES1116 1-4 - pES1117 1-4 - pES1118 1-4 - pES1119 1-4 - GR Mix

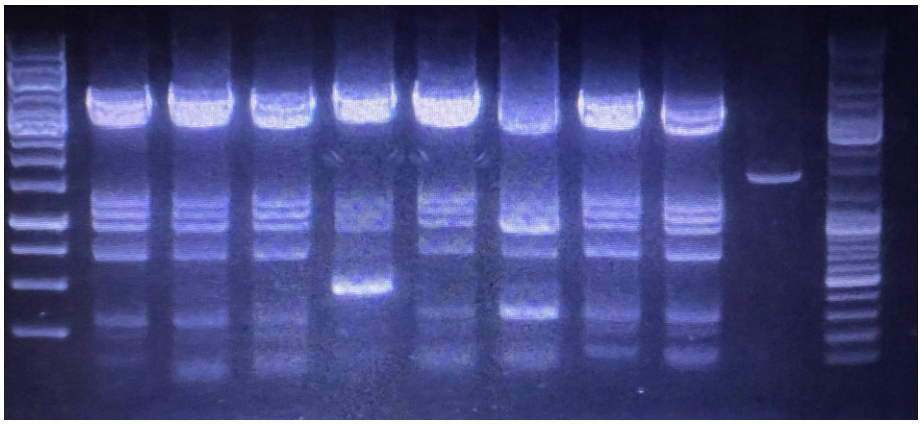
IMG_6832.jpeg



expected band size:
pES1115 - 1 kb --> all 4 look good
pES1116 - 3.2 kb --> clone 3 could be promising
pES1117 - 3 kb --> clone 2 or 4 could be promising
pES1118 - 2.6 kb --> none look good
pES1119 - 2.6 kb? --> none look good

Gel 3:
GR 1kb - pES2045 o019 1-8 - pES1087 - GR Mix

IMG_6838.jpeg



expected band size:
pES1087: 3.1 kb --> looks to be around < 2 kb, let's not use the pES2037 from yesterday
pES2045: 9 kb --> the bands seem to be around 5-6 kb, does not look good, but these primers are not a great combination, might just be another binding site

- started another 2045 Gibson assembly (just as a backup) if the sequencing results look bad we already have new colonies with 2045 that we can pick

NightSeq
picked cultures based on the colony PCR and sent for NightSeq,
5 cultures of pES2045, 2 each of pES2032-2035, pES1115-1119 and pES1087

Miniprep
2043.1 (initial miniprep had only 9 ng/uL but culture regrew)
yield: 32.1 ng/uL. Again suspiciously low but we'll try to sequence it anyways

Transformations

- 2008 + 1112/1113 into S2060+MP6
- backup 2045 assembly + 1112/1113 into S2060+MP6
- backup 2045 assembly in DH5a
- one of the 2037 assemblies (we could not recover the other tubes)

high efficiency protocol Transformation

Phage Production Day 3
2 plaque assays from phages
SIAH1 10^8 dilution as positive control

Gibson Assembly Repeat - pES2045

Table9

	part	molar amount	DNA mass required	added volume
1	2008 part 1	50 fmol	200 ng	3.5 uL
2	2008 part 2	50 fmol	170 ng	1.6 uL
3	pES0028	100 fmol	13 ng	0.15 uL (dilluted)
4	pES1095	100 fmol	53 ng	0.4 uL
5	NEBuilider HiFi DNA Assembly Master Mix			10 uL (2 aliquots)
6	ddH2O			4.35 uL

Golden Gate Assembly Repeat - pES2040-2043

assembly using Lukas' protocol,

also repeated all three 2037 assemblies, since 2 of the tubes got lost

Table10

	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P	Q	R	S	T	U	V	W	X	Y	Z
1	pES2040	AP1 (removed E2), (CGG), strong RNAP, strong hE1	KanR (+CymRam)	pTU2-A-RFP KanR CymRAM	pES1001	pES1002	Dummy C	pES1026	-																	
2	pES2041	AP1 (removed E2), (CGG), strong RNAP, strong IE1	KanR (+CymRam)	pTU2-A-RFP KanR CymRAM	pES1001	pES1003	Dummy C	pES1026	-																	
3	pES2043		SmR	pTU2-a-p15a-SmR	pES1112	DummyB																				
4	pES2037	AP1 pVAN RNAP (CGG), weak hE1, weak E2	KanR (+CymRam)	pTU2-A-RFP KanR CymRAM	pES1087	pES1072	pES1074	pES1094	pES1095																	

DIJOUS, 12/9/2024

short summary:

- The sequencing results of the van-inducible plasmids are a bit disappointing
  - All van-inducible Nterm clones (2045) align 5kb later than they should -> we have a long deletion/recombination
  - Most van-inducible Cterm contained random vectors (probably from other lab members) that happen to have the same resistance. Only van-inducible Cterm ELGN3 looked correct although it seems to be a mix of different plasmids
  - The liquid cultures with weak constitutive Nterm promotors all turned red overnight -> empty backbone was innoculated and sequenced
- The sequencing results of the phage drift look as expected. With longer drift, we have a higher diversity in the phage samples.
  - A more quantitative analysis of the diversity of the phage library will follow in the upcomming days
- We have SIAH1 KO phages now. 3 Plaques were picked to confirm the sequence
- We reassembled the van-inducible Cterm plasmids (EGLN3, asyn, NLRP3) but this time also with linker 3 and 4 instead of just L 2.
- The newest 2037 (pvan Nterm) assemblies were transformed again, we'll se how it goes this time

- [x] miniprep correct plasmids from NightSeq
  - [ ] if pES2045 is correct --> use it for co-transformation, most important thing!

- [x] if pES1115-1119 are correct --> assemble lvl2 plasmids
- [ ] if pES2032-2035 are correct, co-transform them with pES2008

- [x] if pES2043 is correct, co-transform it with pES2045 --> not correct
- [x] Pick and sequence SIAH1-KO phages
- [x] use primers 115 and 116 (if they arrive) to add lvl 0 overhangs to EGLN3 peptide and assemble a lvl 1 TU with a pVan promotor and the peptide

- [x] Check SIAH1 drift sample sequences
- [x] retransform 1074 (running out of it)
- [x] transform pES2040-2043 reasembly
- [x] transform pES2037 assemblies
- [x] assemble pvan-inducible AP2s (like 1116-1119) but with linker 3 and 4 instead of 2, just to have them as a backup)
- [ ] clean bench with DNA away

in lab: Jakob, Michael

Sequencing Analysis - Cloning

- pES2043 - NNN again -> trashed

- pES2045 - difficult to say what is wrong, but sequencing does not start where it would be expected (it aligns at around 10kb instead of 5kb)

- pES1115 - A looks good, B has nothing
- pES1116 - A looks like a mixture, B sequencing is short
- pES1117 - A is a yeast gene, B is M13 vector
- pES1118 - A is a plasmid vector (pSC101), B has T7 promoter
- pES1119 - A is a plasmid vector (pSC101), B is M13 vector
  --> prep pES1115 and pES1116, clean everything

- pES1087 - A is correct, B is T7 promoter and random protein --> since the other stock we have was confirmed by sequencing, it was not minipreped though

- all liquid cultures from 2032-2035 - except 2035B - turned red -> only have the backbone
  --> pES2032-2035 as expected are all backbone, except 2035B, which matches pES1004

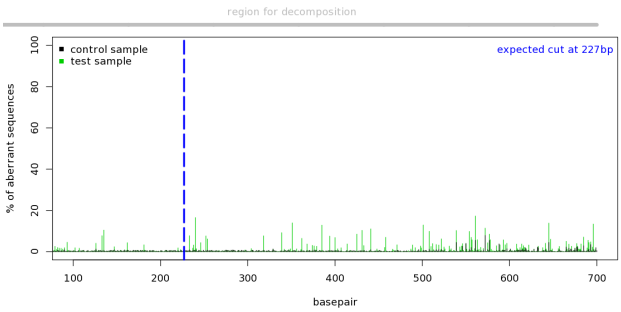
- co-transformants
  DP6 is there, o017 was wrong promoter to use, KanR sequencing shows nothing in clone C and Amp resistance in D

Sequencing Analysis - SIAH1 drift

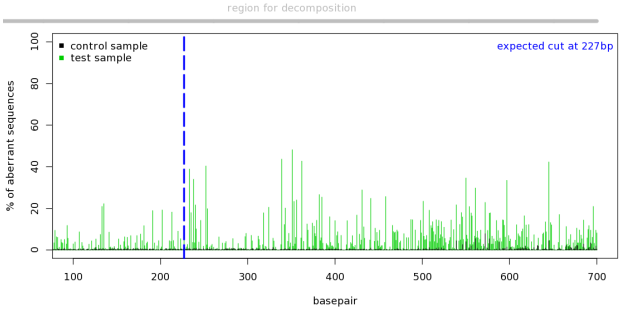
all SIAH1 phages were still sequenced correctly, but the signal becomes less and less clear, as the passage number and therefore the drift time increases
--> shows that the phages drifted, but we can't really conclude by how much
--> should we pick single plaques for sequencing or use another sequencing methode to get a more quantitative assesment?

I used TIDE - a tool thats normally used to quantify Cas9 cut indels - to quantify the diversity in the sequencing signal of our drifted phages. It showed that the sequencing signal gets more and more mixed with more passages

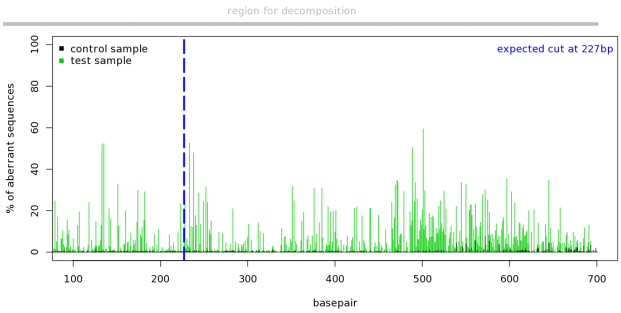
passage4



passage6



passage8.png



control = sequence of WT SIAH1. Ignore the "expected cut site" line (The tool is actually for another purpose)

SIAH1 ko phages

- We have 7.5*10^5 PFU on the plaque assay (30 plaques * 10^2 dillution * 1000/4mL)

20240912_103724 (002).jpg

- pick 3 phage plaques and propagate them o/n on S2208
- after 5 hour propagation, 75 µL phages were taken out, spun down and 50 µL sent for sequencing

Golden Gate Assembly

as a backup, we start to assemble the pVan inducable substrates also with linkers 3 and 4

Table11

	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P	Q	R	S	T	U	V	W	X	Y	Z
1	pES1120	pVan_EGLN3_L3_CtermRNAP(CGG)	A	Amp	pTU1-A-RFP	pES0028	pES0013	pES0002	pES0008	pBP-L3S2P21																
2	pES1121	pVan_asyn_L3_CtermRNAP(CGG)	A	Amp	pTU1-A-RFP	pES0028	pES0015	pES0002	pES0008	pBP-L3S2P21																
3	pES1122	pVan_NLRP3.200_L3_CtermRNAP(CGG)	A	Amp	pTU1-A-RFP	pES0028	NLRP3_200_frag	pES0002	pES0008	pBP-L3S2P21																
4	pES1123	pVan_NLRP3.700_L3_CtermRNAP(CGG)	A	Amp	pTU1-A-RFP	pES0028	NLRP3_700_frag	pES0002	pES0008	pBP-L3S2P21																
5	pES1124	pVan_EGLN3_L4_CtermRNAP(CGG)	A	Amp	pTU1-A-RFP	pES0028	pES0013	pES0022	pES0008	pBP-L3S2P21																
6	pES1125	pVan_asyn_L2_CtermRNAP(CGG)	A	Amp	pTU1-A-RFP	pES0028	pES0015	Linker 2	pES0008	pBP-L3S2P21																
7	pES1126	pVan_NLRP3.200_L4_CtermRNAP(CGG)	A	Amp	pTU1-A-RFP	pES0028	NLRP3_200_frag	pES0022	pES0008	pBP-L3S2P21																
8	pES1127	pVan_NLRP3.700_L4_CtermRNAP(CGG)	A	Amp	pTU1-A-RFP	pES0028	NLRP3_700_frag	pES0022	pES0008	pBP-L3S2P21																

Golden Gate Assembly - Inducable substrate

also set up pure digests for pES2048 stepwise assembly

Table12

	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P	Q	R	S	T	U	V	W	X	Y	Z
1	pES2048	AP2_pVan_EGLN3_L2_CtermRNAP(CGG)	SmR	pTU2-a p15a SmR	pES1116	pES1115																				
2	pES1117	pVan_asyn_L4_CtermRNAP(CGG)	A	Amp	pTU1-A-RFP	pES0028	pES0015	pES0022	pES0008	pBP-L3S2P21	ok															
3	pES1118	pVan_NLRP3.200_L2_CtermRNAP(CGG)	A	Amp	pTU1-A-RFP	pES0028	NLRP3_200_frag	Linker 2	pES0008	pBP-L3S2P21	ok															
4	pES1119	pVan_NLRP3.700_L2_CtermRNAP(CGG)	A	Amp	pTU1-A-RFP	pES0028	NLRP3_700_frag	Linker 2	pES0008	pBP-L3S2P21	ok															

Adding lvl 0 overhangs to the EGLN3 peptide

50 uL PCR PCR: Q5 Polymerase

- template: 10ng 1096
- primers 1115/1116

- annealing temp: 65 °C
- elongation time: 30s

Colony PCR - pES2037

in case pES2045 is not correct we keep going for this in parallel

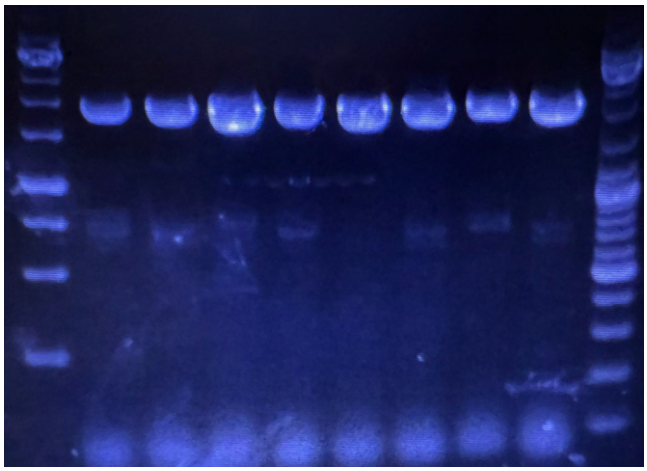
8 colonies, 2 reactions each

o054 and o018 at 62 °C, 1 min elongation; o019 and o018 at 62 °C, 4.5 min elongation

Gel 1:

GR 1kb - pES2037 o054 1-8 - GR mix

IMG_6842.jpeg



expected band: 2.8 kb

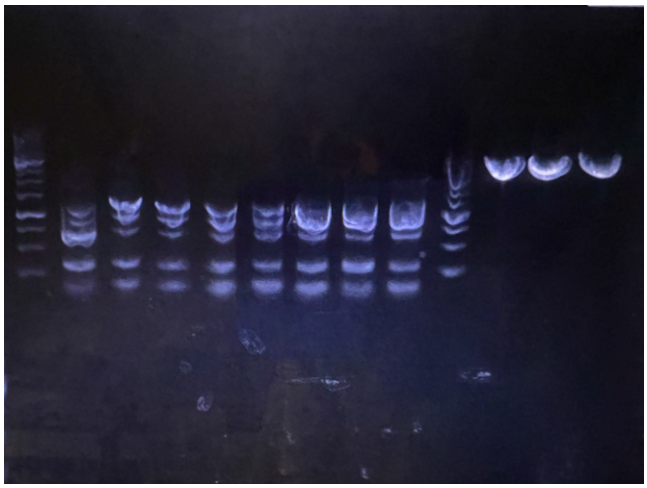
--> bands are all at 2-1.5 kb, which could be due to a secondary binding site, o054 might have been a bad primer choice

--> let's wait for second gel results to decide on

Gel 2:

GR 1kb - pES2037 o019 1-8 - pES2045 1-3 - GR mix

IMG_6844.jpeg



expected band: 9.5 kb --> no band even close to that size, sent colonies for sequencing anyways, pES2045 also looks way too small

Miniprep

pES1115 A: 55ng/µL
pES1116 A: 43ng/µL
pES2045 C: 1224 ng/µL
pES2045 D: 789 ng/µL
pES2045 E: 679 ng/µL

Transformation

100 µL of DH5a in pES1074 tube
5 µL each of pES2040-2043 in 100 µL DH5a
10 µL of pES2037 assemblies (old and Lukas) in 100 µL DH5a

New NightSeq Samples

We sent the following colonies for NightSeq

- 2 colonies of a 2045 backup assembly + transformation (already started the process yesterday, just in case 2045 would fail
- 3 picked SIAH1 KO phages
- 7 colonies of newly assembled 2037
- re-picked 2 new colonies on the plates of 1117, 1118, 1119 (the ones we sequenced looked bad but maybe some colonies on the plate are still correct)

DIVENDRES, 13/9/2024

short summary

- We found out why the Gibson assembly of the van-inducible Nterm (2045) didn't work (an off-target end-extension PCR product, that was apparently not as benign as assumed)
- We retried the end-extension PCR to gel extract and assemble again

- We assembled new APs for the whole logic but they have more similar sizes (compared to the large AP1 and small AP2 we have rn). Every part is van-inducible
- In addition to re-assembling pvan inducible Cterm plasmids, we picked more colonies and sequenced them yesterday -> most were correct this time -> we have van-inducible EGLN3, aysn, and NRLP3_700
- We tried to assemble Nterm under phage shock promotor (2042) but the gel extraction of the Gibson assembly parts yielded basically nothing. -> will retry tomorrow
- Our freshly made SIAH1 ko phages were sequenced and are actually TadA :(- seems like we have contamination
    - We will restart the Phage production process

- [ ] use phage shock primers to clone phage shock promotor into 2008
    - [x] PCR
    - [x] DpnI treatment + Gel
    - [ ] Gibson assembly
    - [ ] Transformation
- [x] Assembly pES2049-2051
- [x] retry 2045 Gibson assembly - this time with gel purification
- [x] Pick transformations that grew (colony PCR?)
- [x] continue assembly of 1128 - 1130
    - [x] gel/PCR purify EGLN3 peptide
    - [x] transformation of lvl 1 assembly
- [x] Sequencing analysis
    - [x] Miniprep everything that's correct
- [x] Miniprep pES2032-2035 and send for sequencing
- [x] Check if we still have full plasmid seq labels (it appeared to me that we have none left) - new labels ordered
- [ ] do plaque assays -> not necessary anymore

in lab: Michael, Jakob

Gibson end extension PCR PCR: Q5 Polymerase

- lowered melting temps (will gel-extract anyways)

PCR

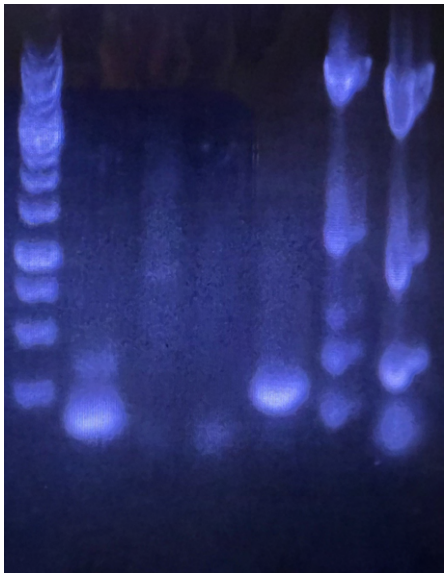
	template (1 ng)	primers	anealing temp	elongation time	slot	comment
1	2008	105, 112	59 °C	3 min (~5 kb)	2	Gibson 2045 (part 1)
2	2008	111, 106	62 °C	3 min (~5.5/6.2kb)	4	Gibson 2045 (part 2)
3	2008	100, 112	59 °C	3 min (~5 kb)	2	Gibson for PSP (part 1)
4	2008	111,104	62 °C	3 min (~5.5/6.2kb)	4	Gibson for PSP (part 2)
5	pTPH412	102, 103	71 °C	30 s (230 bp)	5	Gibson for PSP

- We are re-trying to assemble 2045 but this time we will gel extract (mabye the off target product with correct overhangs caused the deletion in the 2045 assembly last time)
- I realized later that 62 °C annealing temp is correct for 2008 part 2 for 2045 but not for 2008 part 2 2042 (PSP)
  -> PCR will be redone

Gel

GR 1kb - EGLN3 - 2008 V2 - 2008 P2 - PSP - 2008 V1 - 2008 P1

IMG_6847.jpeg

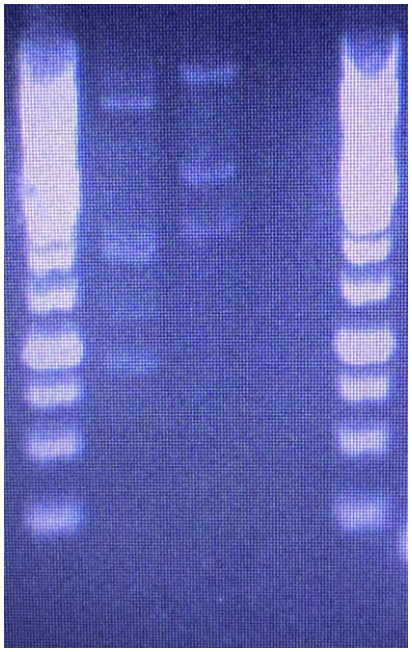


- EGLN3 peptide: expected band size -> gel extraction -> moved to temp box
- 2008 part 2 (for pvan and psp): bands are missing
- PSP: correct band size -> PCR purified and ready for assembly

- 2008 part 1 (for pvan and psp) probably correct bands but many off-targets -> gel extraction -> low concenration for pvan (35 ng/uL) but insufficient for psp assembly -> repeat this PCR tomorrow

GR 1kb - pES1115 digest - pES1116 digest - p15-a SMR - GR 1kb

IMG_6848.jpeg



expected sizes: pES1115 - 860 bp, pES1116 - 3 kb, backbone - 2.2 kb
--> there does not seem to be backbone and way too little of the other pieces, although the correct sizes seem to be present - won't proceed with gel extraction, but if the other assemblies fail, we should repeat this with waaaay more digestion product

Golden Gate Assembly

to have to similarely sized APs instead of a small and a big one
pES2049-2051 are assembled using both our old and Lukas' protocol

Assembly 1

	A	B	C	D	E	F	G	H	I	J
1	pES2049	AP2 pVan_EGLN3_L4_CtermRNAP(CGG), vanRAM, gene3	SmR	pTU2-A p15a SmR	pES1116	pES1115	Dummy C	pES1026		
2	pES2050	AP1 pVAN RNAP (CGG), strong tE1, strong E2, no g3	KanR (+CymRam)	pTU2-A-RFP KanR CymRAM	pES1087	pES1003	pES1004	Dummy D		
3	pES2051	AP1 weak RNAP (CGG), strong tE1, strong E2, no g3	KanR (+CymRam)	pTU2-A-RFP KanR CymRAM	pES1071	pES1003	pES1004	Dummy D		

Assembly 2

	A	B	C	D	E	F	G	H	I	J
1	pES1128	pVan_EGLN3_peptide_L2_CtermRNAP(CGG)	A	Amp	pTU1-A-RFP	pES0028	EGLN3_peptide	Linker 2	pES0008	pBP-L3S2P21
2	pES1129	pVan_EGLN3_peptide_L3_CtermRNAP(CGG)	A	Amp	pTU1-A-RFP	pES0028	EGLN3_peptide	pES0002	pES0008	pBP-L3S2P21
3	pES1130	pVan_EGLN3_peptide_L4_CtermRNAP(CGG)	A	Amp	pTU1-A-RFP	pES0028	EGLN3_peptide	pES0022	pES0008	pBP-L3S2P21

Assembly 3

	A	B	C	D	E	F	G	H	I	J
1	pES2052	AP2 pVan_asyn_L4_CtermRNAP(CGG), vanRAM, gene3	SmR	pTU2-A p15a SmR	pES1117	pES1115	Dummy C	pES1026		
2	pES2053	AP2 pVan_NLRP3-700_L2_CtermRNAP(CGG), vanRAM, gene3	SmR	pTU2-A p15a SmR	pES1119	pES1115	Dummy C	pES1026		
3	pES2054	AP2_pVan_asyn_L4_CtermRNAP(CGG)	SmR	pTU2-a p15a SmR	pES1117	pES1115				
4	pES2055	AP2_pVan_NLRP3-700_L2_CtermRNAP(CGG)	SmR	pTU2-a p15a SmR	pES1119	pES1115				

Sequencing Analysis

SIAH1 KO sequencing - all phages are TadA phages

pES1117 - C is a mixture, D looks good, but also contains some mutations/is a mixture

pES1118 - C is short, D is nothing

pES1119 - C is short, D is good

pES2037 - 6 are RFP, one is nothing, 1 has E2

pES2045 - G is nothing, F has VanRAM but is wrong exactly after site of primers

- The KanStart sequencing results have a short overlap with the 019 results from yesterday. Its seems that the plasmid contains pvan and VanRAM but only gene3 (no RNAP, E1,E2)

Miniprep

pES2032 A: 463 ng/µL

pES2032 B: 619 ng/µL

pES2033 A: 574 ng/µL

pES2033 B: 662 ng/µL

pES2034 A: 670 ng/µL

pES2034 B: 330 ng/µL

pES2035 A: 302 ng/µL
pES2035 B: 610 ng/µL
pES1117 D: 180 ng/µL
pES1119 D: 113 ng/µL
--> adjusted to 100 ng/µL
--> sent for sequencing

Transformation
5 µL of pES2049-2051 (old protocol) assembly in 100 µL DH5a
5 µL of pES1117-1119 re-assembly in 50 µL DH5a
5 µL of pES1120-1130 in 50 µL DH5a
5 µL of pES2048 (old and lukas) in 100 µL DH5a
5 µL of pES2037 iso in 100 µL DH5a

DISSABTE, 14/9/2024

short summary

- re-did the PCR + Gibson assembly of the phage shock Nterm plasmid (2042)
  - this time the gel extraction was succesful -> Gibson assembly was transformed
- The newest Gibson assembly of van-inducible Nterm (2045, this time with gel instead of PCR extraction) was transformed
- AP1s and AP2s with a more evenly balanced logic instead of one big and one small plasmid (2049-2051) were transformed
- tried to re-do GG assemblies of the most important AP1s but in seperate steps: 1. digestion, 2. gel extraction 3. ligation.
  - Gel extraction didn't work (yields < 10ng)

- [x] Sequencing analysis pES2032-2035 (+2037 colony), if they are not good I think we should stop trying to clone them
- [x] transform pES2049-2051 lukas' protocol
- [ ] do colony PCRs of all of the transformations from yesterday
  --> i did not put the plates in the incubator - let's hope the colonies grow till the evening
- [x] use phage shock primers to clone phage shock promotor into 2008
  - [x] PCR of 2008 part 1 and part 2 (phage shock promotor is already ready and purified)
  - [x] Gel
  - [x] Gibson assembly
  - [x] Transformation
- [x] Gibson assembly of 2045

in Lab: Jakob

Sequencing Analysis
pES2032 - A shows RNAP but then jumps to end, B shows E1
pES2033 - A shows E1, B shows gene three
pES2034 - A shows EGLN3, B shows nothing
pES2035 - A shows RNAP but then jumps to end, B shows E1
--> seems like single TUs are being ligated into the lvl2 backbone

pES2037 - empty backbone
pES1116 - is good
pES1117 - overall structure looks ok, but the RNAP does seem mutated, still a mixture
pES1119 - overall structure looks ok, substrate is NLRP3 but does not match benchling sequence

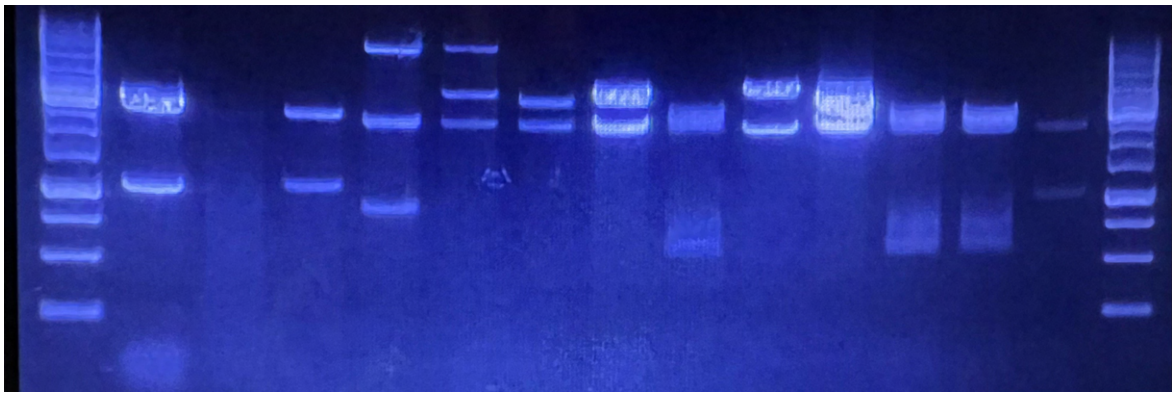
Co-transformants
made glycerol stocks of 1112-08-DP6 and 1113-08-DP6, marked them as unconfirmed
did not send them for sequencing, since we will almost certainly not use them (unspecific RNAP assembly)

Miniprep
pES1074 A: 221 ng/µL
pES1074 B: 271 ng/µL
pES1118 E: 200 ng/µL
pES1118 F: 164 ng/µL
pES2008:694 ng/µL
pES2037 old: 249 ng/µL (in 30 µL)
pES2037 Luk: 124 ng/µL (in 30 µL)
pES2043: 536 ng/µL (in 30 µL)
--> adjusted to 100 ng/µL

Golden Gate Assembly
set up separate digestion reactions + gel extraction
Digestion reactions: 8.5 µL of plasmid, 1 µL of NEB buffer, 0.5 µL of BsmB1 --> 2 hours at 55 °C, inactivation 20 min at 80 °C
digested fragments:
pTU2-A-RFP (KanR), pTU2-a SmR, pTU2-A SmR, 1003, 1004, 1026, 1072, 1074, 1087, 1094, 1095, 1115, 1116, 1117, Dummy C, Dummy D

Gel 1:
GR 1kb - pTU2-A-RFP KanR (2.9 kb) - p15a-a-RFP (2.2 kb) - p15a-A-RFP (2.3 kb)- pES1115 (880 bp) - pES1116 (3 kb) - pES1117 (2.8 kb) - pES1003 (3.3 kb) - pES1004 (600 bp) - pES1026 (3.5 kb) - pES1072 (3.3 kb) - pES1074 A (600 bp) - pES1074 B - pES1087 (1 kb) - GR 1kb

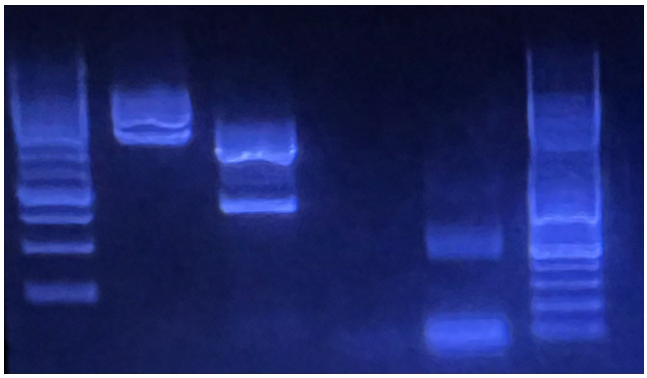
IMG_6859.jpeg



--> pTU2-A-RFP KanR (upper band), p15a-a-RFP (missing?) - p15a-A-RFP (upper band)- pES1115 (lowest band) - pES1116 (middle band) - pES1117 (upper band) - pES1003 (upper band) - pES1004 (lower band) - pES1026 (upper band) - pES1072 (upper band) - pES1074 A (lower band) - pES1074 B (lower band) - pES1087 (lower band, very weak)

Gel 2:
GR 1kb - pES1094 (3.5 kb) - pES1095 (870 bp) -Dummy C (20 bp) - Dummy D (20 bp) - GR mix

IMG_6860.jpeg



--> pES1094 (upper band) - pES1095 (lower band) -Dummy C (very weak band, lower) - Dummy D (lower band)
--> gel extraction yields were super low (<= 10 ng/µL), so I am not going to proceed with the ligation - huge waste of time :(

Gibson Assembly

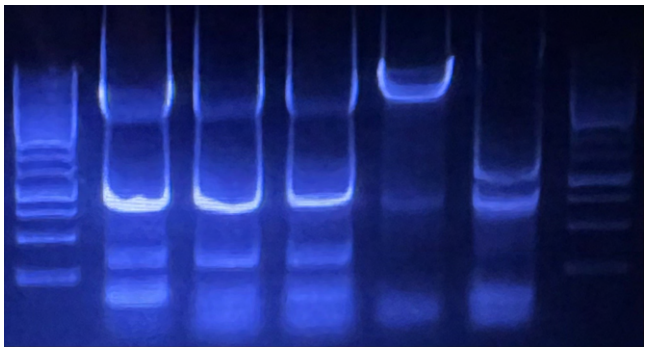
Gibson PCR for 2042

	template	primers	elongation time	annealing temp
1	2008 (part 1)	100/112	3 min (~5 kb)	62 °C
2	2008 (part 2)	111/104	3 min (~5.5/6.2kb)	55 °C
3	2008 (as one part)	100/104	5 min (11kb)	55 °C

--> run on a gel

GR 1kb - 2008 V1 yesterday - 2008 P1 yesterday - 2008 P1 - 2008 P2 - 2008 P1,2 - GR 1kb

IMG_6860.jpeg



--> extracted from the upper band of 1-4

--> yield: 2008 V1: 72 ng/µL, 2008 P1: 128/59 ng/µL, 2008 P2: 73 ng/µL, nothing for the full 2008 fragment

Gibson Assembly of 2045

	part	molar amount	DNA mass required	added volume
1	2008 part 1	50 fmol	100 ng	3 uL / 1.4 µL
2	2008 part 2	50 fmol	85 ng	1.5 uL
3	pES0028	100 fmol	7 ng	0.6 µL (1:10)
4	pES1095	100 fmol	26 ng	1.8 µL (1:10)
5	NEBuilider HiFi DNA Assembly Master Mix		5 uL (2 aliqots)	

2:2:1 insert:vector ratio

Gibson Assembly of 2042 (2008 w/ PSP)

	part	molar amount	DNA mass required	added volume
1	2008 part 1 (with psp overhangs)	25 fmol	100 ng	0.8 µL / 1.7 µL
2	2008 part 2 (with psp overhangs)	25 fmol	85 ng	1.2 µL
3	PSP	125 fmol	18 ng	0.9 µL (1:10)
4	NEBuilider HiFi DNA Assembly Master Mix			5 uL (2 aliquots)
5	ddH2O			0.5 uL

5:1 insert:vector ratio

Transformation
5 µL of pES2049-2051 (Lukas' protocol) in 100 µL DH5a
5µL of pES2052-2055 (Lukas' protocol) in 100 µL DH5a
5 µL of pES2042 and pES2045 in 100 µL DH5a
retransformation with 1 µL of pES1072, pES1115 and pES1116 in 30 µL DH5a (also kept empty tubes)
--> for all Spec plates I also streaked out one with less bacteria, since they seem to get overgrown

DIUMENGE, 15/9/2024

short summary

- Did a colony PCR with 2 colonies of the most important assemblies (to either pre-select for sequencing or continue with unconfirmed assemblies)
- Most colonies didn't have the expected bands :(-> innoculated fresh ones for those that couldn't be pre-selected
- re-transformed what didn't grow (or totally overgrew)
- Restarted the SIAH1 KO phage production (we had TadA contamination last time

- [x] Restart SIAH KO phage production
- [x] look at all of the plates in the incubator (I forgot to incubate the plates on friday - sorry, by saturday evening I think colonies are beginning to grow)
  - [x] potentially do colony PCRs to pre-select colonies
  - [x] pick colonies
    (I started a plate with 0.5 mL cultures, we could then send them for NightSeq on Monday and use them on tuesday (inocculate with 0.5 mL and miniprep on the same day, can start using on tuesday then with confirmed sequences),
    or we could already make big cultures on sunday, miniprep on monday and send them for sequencing and already use them in parallel while unconfirmed - would save us a day but I am not sure what makes more sense)
  - [x] retransform assemblies that did not grow at all

in lab: Michael

what grew:

- pES1115, pES1116, pES1118, pES1120, pES1122-pES1130
- pES1072
- pES2037 isothermal (only 2 white colonies, the rest was red),
- pES2049, pES2050, pES2051, pES2052, pES2053
- pES2055 (only 1 pickable colony)
- pES2048 Lukas protocol

Colonies that could not be picked

- 2048 old protocol (overgrown, no single colonies pickable)
- 2054 (overgrown, no single colonies pickable)
- All 2050 colonies were red, on another plate with 2050 there was only one single colony
- 1121 didn't grow
- The most recent Gibson assemblies 2042 and 2045 didn't grow -> back in the incubator -> 2042 grew later and was picked
- Many plates were totally overgrown and had only 1-3 colonies that were seperated from the remaining blurr on the plate
- generally, everything that was plated on Spec plates totally overgrew (second time now -> I assume our spec is partrially degraded (either the stock or during the preparation of the plates) and everything grows on the plate or it gets degraded
    - will make 3 new spec plates with 2x concentration and test with neg. ctrl strain

Colony PCR

picked 2 colonies each (to either pre-select before sending for sequencing or co-transform already)

PCR: Q5 Polymerase Primers 018/019, 25 uL reactions, 62 °C

1% gel, 120V, 35-40 min

Gel1:

GR mix / 1118A / 1118B / 1120A / 1120B / 1122A / 1122B/ 1123A / 1123B / 1124A / 1124B / 1125A / 1125B / 1126A / 1126B / 1127A / 1127B / 1128A / 1128B / GR mix

20240915_163511 (002).jpg

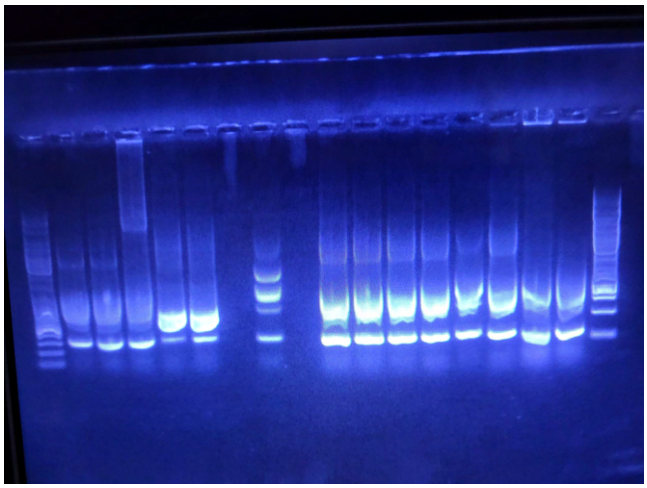
expected sizes: 2.5, 2.5, 3.1, 3.1, 2.5, 2,5, 2.5, 2,5, 2.5, 2,5, 3.1, 3.1, 2.8, 2.8 2.5, 2,5, 2.5, 2.5 (kb)

- 1118B, 1123B, 1128A+B (and maybe 1127B?) have weak bands of the correct size
- the remaining colonies are missing the corect band

Gel2:

GR mix / 1118E / 1118F / 2043 / 1074A / 1074B / empty /SIAH1 KO SP / empty / 2048L.A / 2048L.B / 2049A / 2049B / 2050o.A /2050o.B / 2051o.A / 2051o.B /GR 1kb

20240915_171058 (002).jpg

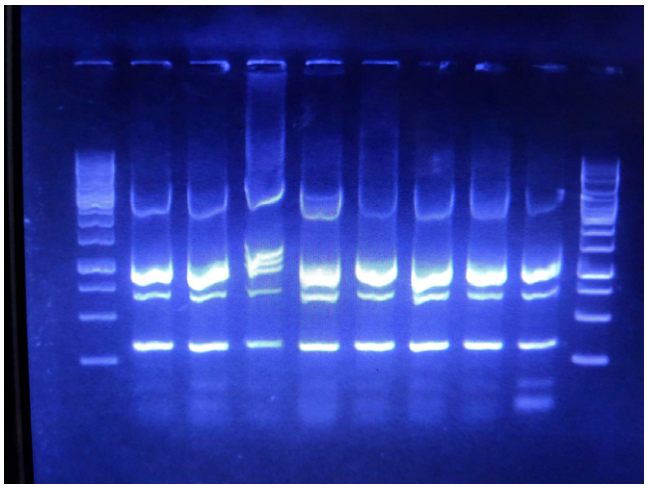


expected sizes: 2.5, 2.5, 2.7, 2.7, 0.7, 0.7, -, 3.0, - 7.6, 7,6, 5.1, 5.1, 5.1, 5.1

- 1118 E/F seem and 1074 A/B could be correct although there is no defined band but blurr at the expected size
- SIAH1 KO SP probably correct (correct + some unexpected bands)

Gel 3:
2037 iso A / 2037 iso B / 2037 old (DNA) / 2037 old (colony) / 2037L (DNA) / 2037L (colony) / 2045.9 (from 11.9) /2045.10 (from 11.9)

20240915_171354 (002).jpg



expected size: 9.6 kb everywhere but 7.6 kb for the last two bands

- None of the expected bands are visible (maybe the amplification products are just too long?)
- There is a blurr aproximately at the right size for 2037 old DNA

Re-transformation (assemblies that couldn't be picked)

- 50 uL DH5a
- pES1121
- pES2048/49/52/53/54/55 on plates with 2X spec

Phage assembly

- Since the last SIAH1 KO phage production attempt was unsuccesfull, we started again from day 1.
  Phage Production
- The SIAH1 KO SP I used did not have a date but it should be from 27.8. I wil run it on a gel to check the size

DILLUNS, 16/9/2024

- [x] Pick newly re-transformed colonies
- [x] Continue SIAH1 KO production: take out assembly in slot 4 and transform into S2060
- [x] re-transform 2045 Gibson assembly if it didn't grow since Saturday
- [x] Miniprep liquid cultures of pES2042 and some of pES2037 --> 2042 did not grow
- [x] Co-transformations of 2048-08-MP6 if 2048 transformation worked (maybe also co-transform 2037old protocol DNA, had a blurr at approximately the right size in the colony PCR)
- [x] make new Dummy D
- [x] make small LB bottles
- [x] assemble recently designed plasmids in Benchling (incl. but not limited to 2052/53/54/55)
- [x] Trash old plates in the fridge (or bring them to the cold room) --> cold room
- [x] make new chloramphenicol stock (1000x concentration = 20 mg/mL -> 1000mg / 50 mL EtOH). powder is in the fridge in the PACE room

Colony PCR

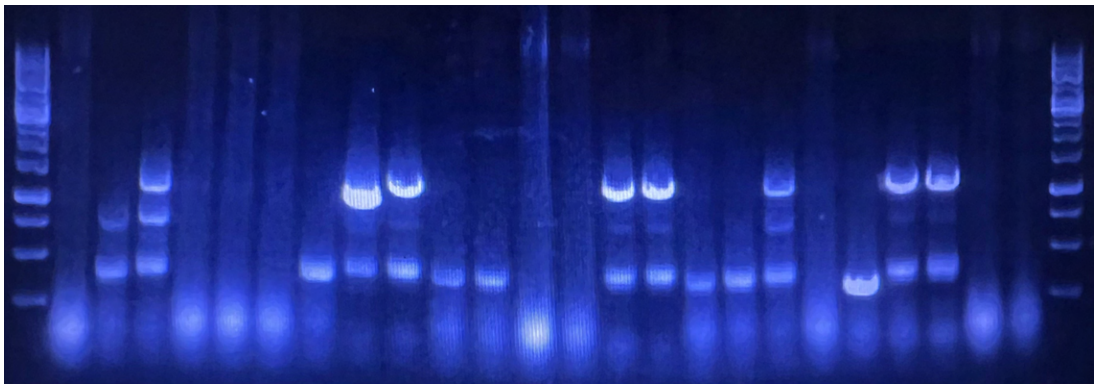
picked 2 colonies each pre-select before sending

PCR: Q5 Polymerase

For level 1 plasmids: Primers 017/018, 12.5 uL reactions, 59 °C, 1.5 min elongation (2.5 kb), 3 colonies picked
For level 2 plasmids (except pES2054/2055): Primers 001/038, 12.5 uL reactions, 68 °C, 1.5 min elongation (3.3 kb), 4-5 colonies picked
For pES2054/2055: Primers 018/019 , 12.5 uL reactions, 62 °C, 2 min elongation (4 kb), 5 colonies picked
1% gel, 120V, 25 min

Gel 1:
GR 1kb / 1118 1-3 / 1120 1-3 / 1121 1-3 / 1122 1-3 / 1123 1-3 / 1124 1-3 / 1125 1-3 / 1126 1-3 / GR 1kb

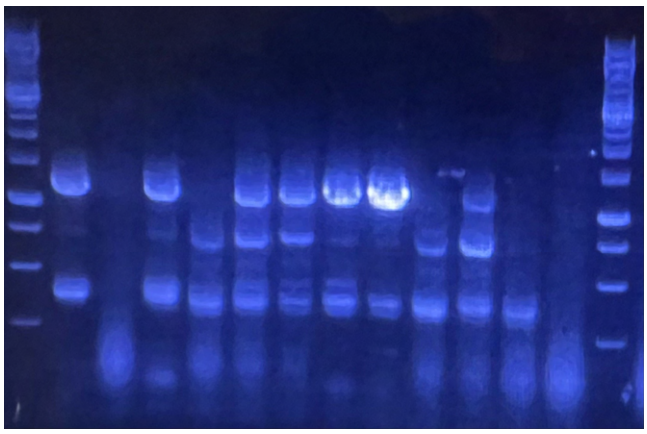
IMG_6881.jpeg



the expected size would be be 2.5-3 kb, the empty backbone should have 1.3 kb
the picked colonies were definetly not red, so I don't really know how to interpret that

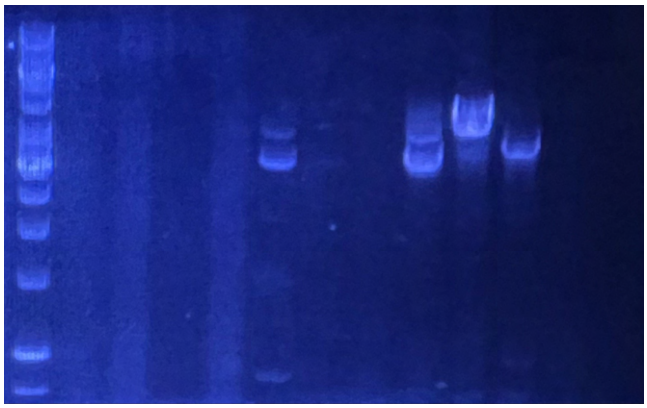
Gel 2:
GR 1kb / 1127 1-3 / 1128 1-3 / 1129 1-3 / 1130 1-3 / GR 1kb

IMG_6882.jpeg



Gel 3:
GR 1kb / 2042 1-3 / 2045 / 2048 1-4 / 2049 1-4

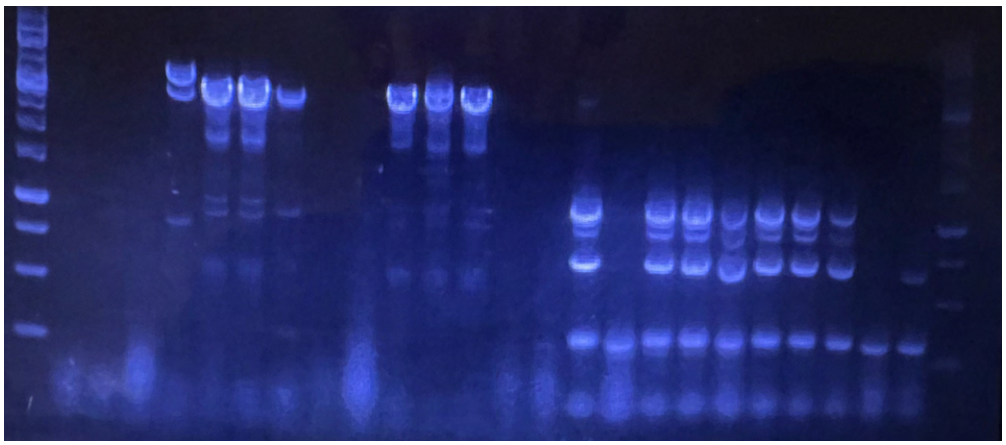
IMG_6884.jpeg



no bands for pES 2042 and pES2045 --> will not send them for sequencing, but will re-transform the assemblies
pES2048 1 and 4 have a band above 3 kb and 3.5 kb (3.8 expected), pES2049 1 (>3.5 kb) and 2 (3 kb) have bands (3.3 kb expected)

Gel 4:
GR 1kb / 2051 1-4 / 2052 1-5 / 2053 1-5 / 2054 1-5 / 2055 1-5 / GR 1kb

IMG_6886.jpeg



pES2051 4 has two bands at 3 kb band 3.5 kb , overlooked that the pirmer combination was wrong for that one (does not have gIII), possible off-target binding site for o001 should yield 3.4 kb band, so 4 could be promising anyways
pES2052 1-3 have bands at ~3. kb (3.3. expected) --> small colonies, 4-5 were large
pES2053 1-3 have bands at ~3. kb (3.3. expected) --> small colonies, 4-5 were large
pES2054 and pES2055 have bands at 250 bp, at 600 bp and at ~1 kb, epected would be ~4 kb (only 2054 1 has a band even close to that size), empty backbone would have 250 bp --> maybe colonies will turn red and we can then select
--> o018 and o019 combination is bad as always

Golden Gate Assembly

assembly using old protocol but using the one hour digestion in the end

(30 cycles of 1 min at 42 °C and 5 min at 16 °C, 60 min at 55 °C, 20 min at 80 °C) --> will call this "new protocol" if we use it again

--> tube was melted really badly, was in old thermocycler

I repeated the assembly after producing Dummy D, since we basically had none for the first assembly

Table13

	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P	Q	R	S	T	U	V	W	X	Y	Z
1	pES2050	AP1 pVAN RNAP (CGG), strong IE1, strong EZ, no g3	KanR (+CymRam)	pTU2-A-RFP KanR CymRAM	pES1087	pES1003	pES1004	Dummy D		ok																

Dummy PCR

50 uL reactions, 59 °C, 20s elongation, 20 ng template

NucleoSpin Gel and PCR Clean-up

Dummy B: 50 ng/µL, Dummy D: 55 ng/µL --> should be more than enough, since they are so short

PCR for Twist_Combined_2

	A	B	C	D
1	Descriptiion	Template	Primer 1	Primer 2
2	Dummy TU1-B	Twist_combined_2	o028	o029
3	Dummy TU1-D	Twist_combined_2	o030	o031

NightSeq plate

Bildschirmfoto 2024-09-16 um 17.54.48.png

	1	2	3	4	5	6	7	8
A	pES11184 o017	pES1123A o017	pES1127A o017	pES1115A o017	pES20485 o019	pES20521 o019	pES20548 o019	pES2037E o019
B	pES11185 o017	pES11234 o017	pES11274 o017	pES1116A o017	pES20491 o019	pES20522 o019	pES20556 o019	pES2037F o019
C	pES1120A o017	pES11244 o017	pES1128A o017	pES1172A o017	pES20492 o019	pES20523 o019	pES20557 o019	pES2037G o019
D	pES11204 o017	pES11245 o017	pES11284 o017	pES2042A o019	pES20495 o019	pES20531 o019	pES20558 o019	pES2037Luk o019
E	pES11214 o017	pES1125A o017	pES1129A o017	pES2042B o019	pES2050cont o019	pES20532 o019	pES2037A o019	pES2040A o019
F	pES11215 o017	pES11254 o017	pES11294 o017	pES2045 o019	pES20514 o019	pES20533 o019	pES2037B o019	pES2040B o019
G	pES1122A o017	pES1126A o017	pES1130A o017	pES20481 o019	pES2051A o019	pES20546 o019	pES2037C o019	x
H	pES11224 o017	pES11264 o017	pES11304 o017	pES20484 o019	pES2051B o019	pES20547 o019	pES2037D o019	x

Miniprep

pES2037 Luk: 177 ng/µL
pES2037 old: 99 ng/µL
--> diluted to 100 ng/µL

Transformation

5 µL of pES2042/2045/2050 in 100 µL DH5a
5 µL of SIAH1 KO splitC/D in 30 µL of S2208
2 µL of pES2037 o/L + 2 µL of pES1076 in 100 µL S2060+DP6

Primer order

I ordered primers that should work better for colony PCRs, since they have similar Tm.
o117 binds the same location as o019 but has the same Tm as o018,
o118 bind ubiquitin (forward), o119 binds C-term RNAP (forward) and o120 binds E2 (forward)
--> this should allow do perform better colony PCRs selective for the assembled plasmid

DIMARTS, 17/9/2024

short summary

- We finally have combinations of plasmids to make the whole logic van-inducible (both C- and Nterm)
  - we already co-transformed the plasmids and will pick and sequence tomorrow
- Full plasmid sequences are not confirmed yet
- Many level 1 assemblies worked as well - soon we will have all substrate-linker combinations under a pvan promoter
- continued SIAH1 KO phage production - in 2 days we will know whether it worked
- Figured that some NLRP700 constructs are actually NLRP_200 -> started lvl 0 PCR and reassembly of NLRP3_700

- [x] sequencing analysis, miniprep correct plasmids
  - [x] see if an inducable logic can be put together from correct assemblies --> it can
  - [x] if pES2037 luk or pES2037 G are correct --> pick cultures and prepare for propagation assay
    --> they are not correct
  - [x] miniprep pES1115, pES1116 and pES1072 if they are correct --> pES1116 is not correct
- [x] transform second pES2050 assembly (with new Dummy D)
- [x] make Kan and Carb plates
- [x] make a plaque assay for SIAH1 KO phages
- [x] repeat assembly of pES1128-1130, pES2048, pES2050

In lab: Michael and Jakob

Sequencing Analysis

- 1118 E&F: don't align - can also not be annotated -> contamination with Amp resistance?
- 2043: doesn't align to 2043 either - is some other AP1 plasmid (strong promotor, Nterm RNAP, L3, ubiquitin)
- 2037 Lukas' protocol: NNN
- 2037 old protocol: aligns perfectly but 5 kb too late at gene 3 :/ (maybe we have the same deletion as previously)
- 1074 A&B align, both have a T813C mutation (in the ori) - this mutation has also been there previously
- 2008 re-transform: aligns perfectly

NightSeq

pES2037 - A and D show E2; B, C, E and G show g3, F shows VanRAM --> maybe all of them are single TU inserts --> we stop trying to get this plasmid
pES2040 - B looks good from what is sequenced, A shows the backbone
pES2042 - A is NNN, B is super short
pES2045 - is NNN
pES2048 - 1, 4 and 5 are RFP
pES2049 - 1 is NNN, 2 and 5 look good
pES2050 - looks promising (but well might have been contaminated)
pES2051 - all look good, sequenicng is a bit short though
pES2052 - 3 looks good, 1 is NNN, B is RFP
pES2053 - assembly is good, but the substrate is actually NLRP3-200 (expected 700)
pES2054 - 6 and especially 7 look promising, 8 is NNN
pES2055 - all look good, but the substrate is actually NLRP3-200 (expected 700)

pES1118 - 4 and 5 look okay, but they're a mixture and both have a 1bp deletion in the Cterm (although signal quality is low at this point), not necessary though, since the pES1119 that we have is actually pES1118
pES1120 - 4 and A look good
pES1121 - 4 is empty backbone, 5 is short sequencing in RNAP
pES1122 - A looks good, 4 substrate is messed up
pES1123 - 4 and A look good, but Linker 4 isn't correct in benchling -> should be linker 3
pES1124 - 4 and 5 look good
pES1125 - 4 and 5 look good
pES1126 - 4 looks good, A is mutated
pES1127 - 4 looks good, A is NNN
pES1128/1129/1130 - have weak promoter instead of pVan

pES1115 - generally looks ok, but like a mixture
pES1116 - is another plasmid
pES1072 - looks good

Innoculation

- in the morning: 2050, 2051A, 2040B, 2042B, 2048/49, 2052/53/54/55 -> miniprep and transform in the evening
- in the afternoon: 1120.4, 1122 A, 1123 A, 1124 4, 1125 4, 1126 A, 1127 4
- in the evening: innoculated 2051 1 and 4

NightSeq

- picked new colonies of 2042, 2045, 2048, 1121, 1116 and sent them for sequencing.
- Also re-sequenced 2049, 2050, 2053, 2054, 2055 from the other side (018 primer) to get more assurance that we're working with the right DNA

Miniprep

pES1115 A: 178 ng/µL
pES1072 A: 267 ng/µL
--> diluted to 100 ng/µL

pES2040 B: 320 ng/µL
pES2049 3: 56 ng/µL
pES2049 4: 106 ng/µL
pES2049 5: 5 ng/µL
pES2050: 295 ng/µL
pES2051 A: 18 ng/µL
pES2051 B: 26 ng/µL
pES2052 6: 89 ng/µL
pES2053: 87 ng/µL
pES2054 6: 155 ng/µL
pES2054 7: 168 ng/µL
pES2055 x: 117 ng/µL
pES2055 x: 54 ng/µL
--> used them for co-transformations and afterwards only kept pES2050, will miniprep the same samples again tomrrow

GG assembly

Table14

	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P	Q	R	S	T	U	V	W	X	Y	Z
1	pES1119	pVan_NLRP3.700_L2_CtermRNAP(CGG)	A	Amp	pTU1-A-RFP	pES0028	NLRP3_700_frag	Linker 2	pES0008	pBP-L3S2P21	ok	ok	ok	ok	ok											
2	pES1128	pVan_EGLN3_peptide_L2_CtermRNAP(CGG)	A	Amp	pTU1-A-RFP	pES0028	EGLN3_peptide	Linker 2	pES0008	pBP-L3S2P21	ok	ok														
3	pES1129	pVan_EGLN3_peptide_L3_CtermRNAP(CGG)	A	Amp	pTU1-A-RFP	pES0028	EGLN3_peptide	pES0002	pES0008	pBP-L3S2P21	ok	ok														
4	pES1130	pVan_EGLN3_peptide_L4_CtermRNAP(CGG)	A	Amp	pTU1-A-RFP	pES0028	EGLN3_peptide	pES0022	pES0008	pBP-L3S2P21	ok	ok														
5	pES2050	AP1 pVAN RNAP (CGG), strong tE1, strong E2, no g3	KanR (+CymRam)	pTU2-A-RFP KanR CymRAM	pES1087	pES1003	pES1004	Dummy D		ok																

NLRP3-700 PCR

50 ng NRLP3 sequence from Twist as template, primers o090 and o099, 100 uL reaction, 63 °C, 40 s elongation
PCR Clean-up yield: 105 ng/µL

Phage production

spun down bacteria of SIAH1 KO phage production and made two plaque assays, positive control is SIAH1 10^11 PFU/mL stock

Co-transformation

2 µL of pES2008 with 2 µL of pES2049/2052/2053/2054/2055 in 100 µL S2060+DP6
2 µL of pES2051 with 2 µL of pES2049/2052/2053 in 100 µL S2060+DP6
2 µL of new pES2050 with 2 µL of pES2049/2052/2053 in 100 µL S2060+DP6
1 µL of new pES2050 assembly with 2 µL of pES2049/2052/2053 in 100 µL S2060+DP6
--> two different versions of pES2049, pES2051 and pES2054-55 were used
5 µL of new pES2050 assembly in 150 µL DH5a
5 µL pES2048 assembly in 150 µL Dh5a

DIMECRES, 18/9/2024

short summary

- Sequencing was delayed by one more day (we picked and innoculated but will only know by tomorrow)

- Did a restriction digest and ran the product on a gel - everything looked fine besides 2050
- Sent 2049-2053 for full-plasmid seq
- SIAH1 KO phages grew - we picked 3 phages and sent them for sequencing
- Transformed pES1128-1130 + pES1119 assemblies + re-did co-transformations with 2051
- Tried to Gibson assemble plasmids with phage shock promotor but gel extraction failed - will retry again

- [x] check what we can do with successful co-transformants --> 2050>2051>2008
  - [ ] potentially do phage propagation and prepare for time-course on thrusday
  - [ ] check reverse sequencing of the used plasmids
  - [x] if promising, send them for sequencing
- [x] if nightseq looks promising, send pES2049, pES2050, pES2051, pES2052, pES2053 for full-plasmid seq
  --> base decision on digest gel
- [x] continue SIAH1 KO phage production
- [x] pick co-transformants, innoculate and conduct phage propagation assay
- [x] NightSeq analysis of newly innoculated cultures
- [x] If 2042 and 2045 sequences look bad, do 2008 part 2 Gibson PCR again and try to assemble one last time
- [x] pick cultures of pES2050 unless otherwise confirmed --> all colonies were red
- [x] do lab duty

in lab: Jakob, Michael

SIAH1 KO phage production

41 plaques on -7 dilution --> phage concentration was 10^8 PFU/mL
picked 3 plaques and innoculated S2208 in the morning
2 plaque assays in the evening after 5 hours propagation and phages sent for sequencing

Miniprep

pES2040 B: 164 ng/µL
pES2042 A: 63 ng/µL
pES2042 B: 182 ng/µL
pES2045 A: 900 ng/µL
pES2048 A: 72 ng/µL
pES2048 6: 82 ng/µL
pES2048 7: 56 ng/µL
pES2049 2: 154 ng/µL
pES2049 3: 258 ng/µL
pES2049 4: 63 ng/µL
pES2051 A: 560 ng/µL
pES2051 B: 457 ng/µL
pES2051 1: 324 ng/µL
pES2051 4: 322 ng/µL
pES2052 3: 317 ng/µL
pES2052 6: 364 ng/µL
pES2053 6: 335 ng/µL
pES2054 6: 183 ng/µL

pES2054 7: 234 ng/µL
pES2055 3: 358 ng/µL
pES2055 4: 222 ng/µL
pES2055 5: 354 ng/µL
pES2055 7: 263 ng/µL
pES2055 8: 308 ng/µL

pES1116 B: 416 ng/µL
pES1121 A: 165 ng/µL
pES1121 B: 86 ng/µL

pES1120 4: 601 ng/µL
pES1122 A: 424 ng/µL
pES1123 A: 432 ng/µL
pES1124 4: 970 ng/µL
pES1125 4: 463 ng/µL
pES1126 A: 340 ng/µL
pES1127 4: 348 ng/µL
--> diluted to 100 ng/µL

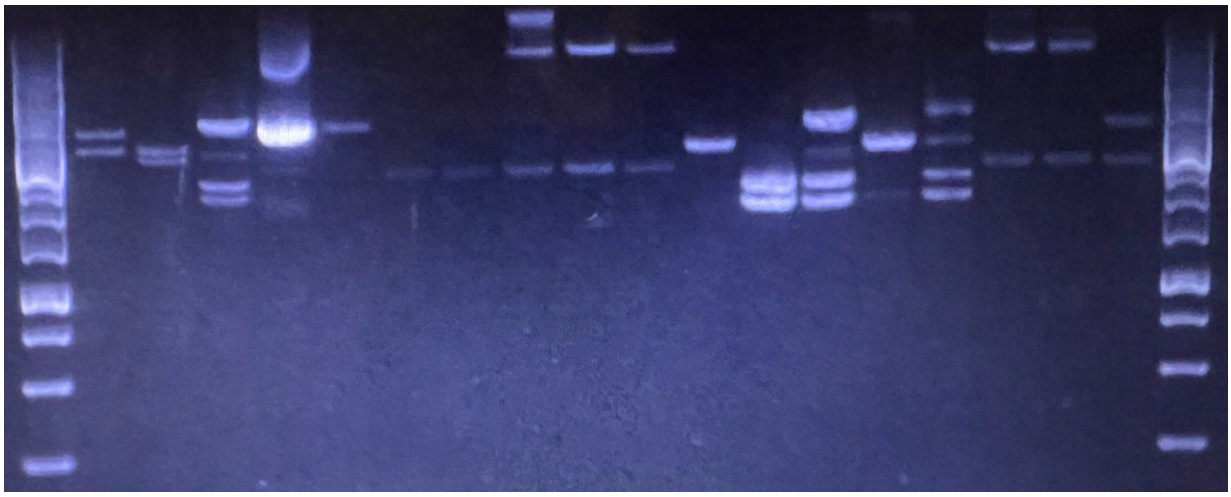
NightSeq Analysis
got delayed by one day

Restriction Digest
Since NightSeq is delayed and we want some additional assurance that the plasmids we're working with are correct, we're doing a restriction digest

- 2050 and 2052: with BamHI (cuts weak promotor) and SexAI ;)
- 2040, 2042, 2045, 2048, 2049, 2050, 2051, 2052, 2053, 2054, 2055: BsaI

Gel 1 - BsaI digests:
GR 1kb - 40 B (7.9&2.9kb) - 42 A (8.5&2.9kb) - 42 B - 45A (8.5&3kb) - 48 A (7.4&2.3kb) - 48 6 - 48 7 - 49 2 (7.5&2.3kb) - 49 3 - 49 4 - 50 (5&2.9kb) - 51 A (5&2.9kb) - 51 B - 51 1 - 51 4 - 52 3 (7.2&2.3kb) - 52 6 - 53 6 (6.8&2.3kb) - GR 1kB

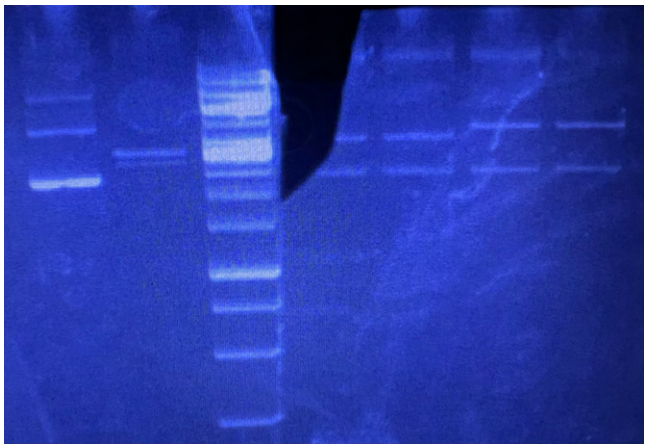
IMG_6896.jpeg



40.B has a band at ~4-5 kb and one at 5-6 kb
42.A has two bands at rougly ~5-6 kb, 42.B has two bands at 2.5-3kb, one at ~4kb and one at ~6kb
45 has a abnd at ~5-6 kb and one at 8-10 kb
48.6 and 48.7 both seem to have a band at ~3kb, 48.A has a band at 5-6 kb
49.3 and 49.4 both have bands at ~3 kb and at 8-10 kb, 49.2 has one even higher band, probably uncut plasmid
50 only seems to have one band at ~4-5 kb
51.4 has bands at ~2.3 kb, 2.6 kb, ~4kb and ~5-6 kb, 51.B is the same, 51.1 is missing the highest band, 51.A is missing both upper bands
52.3 and 52.6 both have bands at ~3 kb and very high at probably 8-10 kb
53.6 has bands at ~3 kb and 6 kb --> not exactly what was expected
--> i think everything is a bit smaller than I estimated at first, since the ladder is a bit weird
--> pES 2040-2048 do not look promising, but also are not super important
--> pES2050 unfortunatelly does not look good
--> pES2049, pES2051, pES2052 and pES2053 look really good

Gel2 - BsaI, BamHI and gibson PCR:
54 6 (3.7&2.2kb) - 54 7 - 55 7 (3.3&2.2kb) - 55 8 - GR 1kb - 2056 PCR - 2045 PCR - GR 1kb - 50 p (5.3&2.3 kb) - 51 A p (3 bands)

gel is mirrored, PCR products have been cut out



54 and 55 have bands at ~2.5 kb and ~4 kb, 54 bands look bigger --> these plasmids look really good
discerning pES2050 and pES2051 did not work, none of the bands match what we expected --> actually the upper band in 2051 could be uncut plasmds

Co-transformations
except for the co-transformations with pES2050 none of the other variants grew well, makes sense for the pES2050 assembly and pES2051, since the miniprep had low concentration --> will repeat pES2051 co-transformations

2 colonies each were picked of the pES2050 + 2049(4)/2052(6)/2053(6) --> they will be sent for sequencing and the corresponding plasmids will be sent for full-plasmid sequencing
A few of the other co-transformant also have colonies, which were picked as a back-up, in case the pES2050 co-transformations do not work afterall, 12 might be very important

Table15

	Number	Strain	C
1	1	2053-08-DP6	A
2	2	2053-08-DP6	B
3	3	2049(4)-08-DP6	A
4	4	2055-08-DP6	A
5	5	2054-08-DP6	A
6	6	2054-08-DP6	B
7	7	2055-08-DP6	B
8	8	2053-51(A)-DP6	A
9	9	2053-51(B)-DP6	B
10	10	2049(4)-51(B)-DP6	A
11	11	2049(4)-51(B)-DP6	B
12	12	2049(2)-50-DP6	A
13	13	2049(2)-50-DP6	B

Transformation

5 µL of pES1128-1130 assembly in 100 µL DH5a
5 µL of each pES1119 assembly in 100 µL DH5a
5 µL of pES2050 assembly in 100 µL DH5a
2 µL of pES2051 A/B and 2 µL of pES2049(2)/2052(6)/2053 in 100 µL S2060+DP6

Phage shock promotor Gibson assemblies

- backup strategy in case our logic fails
- 2042 (we already have all parts)
- 2045 (2008.2 is missing -> PCR)
- 2056 (=2050 with psp instead of pvan, new plasmid) - stopped later since 2050 is incorrect

Gibson end-extension PCR

	template	primers	temp	elongation time
1	2008 (part 2 for 2045)	111/106	62 °C	3 min (~5.5/6.2kb)
2	2050 (for 2056)	100/104	55 °C	4min (7.8kb)

gel-extraction didn't work - no yield :/. Will repeat on Friday

Phage shock promotor as lvl 0 part

- made and ordered primers to get phage shock promotor with lvl 0 overhangs (121/122)

DIJOUS, 19/9/2024

- [x] co-transform again with 2008 and 2048/2054/2055 as backup
- [x] see whether we can somehow make new pTU2-A-RFP with KanR but without CymRAM
  --> we try to assemble a pES2037 like plasmid in the SmR backbone
  --> this has a different ori compared to the lvl1 plasmids, so we could then use it with the different pVan substrates directly
  --> pES2044
- [x] Do phage propagation with time course of pVan induction
  --> use cultures
- [x] assemble pES2048 again, once we have pES1116 --> unless otherwise confirmed --> pES2048A seems to be fine, today we send it for seq from the reverse side, however we still do another assembly as a backup

- [x] run pES2051 A/B/1/4 on a really nice gel --> not done bc they look like shit in sequencing results
- [x] miniprep 2049-08-DP6, 2054-08-DP6 and 2055-08-DP6 co-transformants and run them on a gel
- [x] make competent S2060+DP6
- [x] test tetracyclin resistance and F-pilus in our S2060+DP6

- [x] Economy Seq and NightSeq Analysis
- [x] assemble lvl2 plasmids from correct lvl1s (1120-1130) --> if pES2050 and pES2051 both turn out to be wrong, maybe consider changing them in the cloning checklist to instead make 2 TU plasmids (pES2057-pES2067)
- [x] if co-transformations don't look good, see if one of the backups could work
- [x] pick colonies from yesterdays transformation

- [x] innoculate pES2049.4 again for miniprepping
- [x] tidy up fridge
- [x] check whether pES2050 assembly turns red --> is does turn red

Sequencing Analysis
pES2049 looks perfect
pES2050 is probably trash
pES2051 (4 and A) both are probably missing the E1
pES2052 both look perfect
pES2053 probably misses gIII, looks like pES2055
pES2054 looks perfect

SIAH KO phages :
all clones are perfect

Co-transformations:
pES2050 is E2 inserted into KanR backbone,
pES2049 and pES2052 were not sequenced from the SmR_Start primer,
pES2053 was sequenced at the correct location and looks fine, but EcoSeq showed that it does not contain gIII anyways
--> results don't matter much, since we already knew that pES2050 was not ok, mixed results on usability of o097

Other NightSeq:
pES1116: looks good --> labeled and diluted, use for pES2048 assembly
pES1121: both are phage plasmids
pES2042: A looks veeery promising, B is missing large parts
pES2045: backbone looks fine, but pVan is not correct
pES2048: A looks promising, is sequenced correctly,
pES2049: clone 5 reverse seq looks bad, but we already have a 2049 that looks nice anyways
pES2050: confirms that it is E2 in KanR backbone
pES2053: confirms that it is missing gIII (same as pES2055)
pES2054: both clone 6 and 7 are perfect
pES2055: both clone 7 and 8 are perfect

Running 2049-08-DP6, 2054-08-DP6, and 2055-08-DP6, pES2042, and pES2048 on a gel
Rationale: to check that co-transformants have all three plasmids, 1.5mL of o/n culture were miniprepped and run on a 1% agarose gel, 40 min, 120V. Additionally, miniprepped pES2042 and pES2048 are run to check for correct plasmid size
Expected bands:

- pES2042 --> 11.4kb
- pES2048 --> 6.2kb
- 2049-08-DP6 --> 9.7kb (2049) + 11.2kb (2008) + 8.6kb (DP6)
- 2054-08-DP6 --> 5.9kb (2054) + 11.2kb (2008) + 8.6kb (DP6)
- 2055-08-DP6 --> 5.5kb (2055) + 11.2kb (2008) + 8.6kb (DP6)

Order: GeneRuler Mix - 2049-08-DP6 - 2054-08-DP6A - 2054-08-DP6 B - 2055-08-DP6 A - 2055-08-DP6 B - 2042 - 2048

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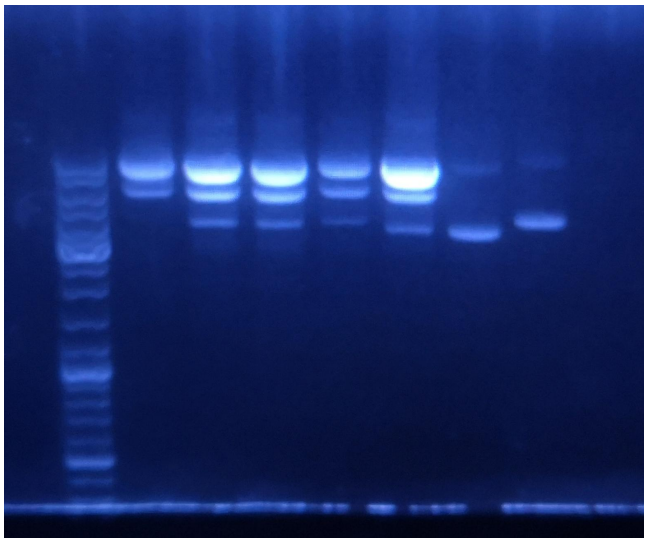


Result: bands are overall a bit shifted downwards, otherwise we see three bands for each (for 2049-08-DP6, 2049 and DP6 have a pretty similar size) so they are probably all good. Let's see sequencing results tomorrow. Band size is as expected for 2048 but not really for pES2042...

Culture picking and Sequencing

picked 2 cultures each of pES1119, pES1127, pES1028 and pES1030 (o017 sequencing)
picked a few new pES2053 cultures (o018 sequencing)
sent co-trans cultures 3, 4 and 5 for NightSeq with KanStart and o097
pES2048 A sent with o018 (Economy sequencing)

Plaque Assays

both were unreadable, but the concentration seems to be high
all SIAH1 KO phage sequencing looks perfect --> we will use clone A going forward

The phages were loaded onto thour qPCR plate 1:1, 1:100 and 1:10000 diluted --> Cp = 7.14 / 13.21 / 20.02
--> according to the calibration curve we once did, this should be equ1al to roughly 10^11 PFU/mL
Given that sequencing results for SIAH1 KO were perfect, we repeat the plaque assay as in Phage Production to determine phage titer

Make S2060+DP6 competent cells

o/n culture was inoculated in fresh 50mL w antibiotics and protocol Mix&Go was followed.

Co-transformation of pES2049/2054/2055 with pES2008 in S2060-DP6

Rationale: in case the co-transformants that we used for plaque assay are not correct for some reason, we repeated the co-transformation (backup). Note that again pES2049 was used instead of pES2048 because the latter is not yet assembled (pending on confirmation of pES1116). Thus, these co-transformants will have two copies of gene III (one in pES2049, one in pES2008).

High-efficiency protocol, 4uL of 1:1 ratio pre-mixed plasmid DNA in 100uL competent cells.

Phage propagation assay

we use Vanillic acid concentrations of 0 µM, 10 µM, 100 µM and 500 µM to induce the Subs-L-RNAP (same amount of ethanol, 1%)
the induction began roughly 30 min before the phages were added at RT
100 µL samples were taken after 0.5 h, 1 h, 2 h and 4 h --> put back for o/n propagation
seeding OD for our bacteria was 0.2, after growing for 20 min from o/n culture and being diluted, 1 mL per well
both S2208 and S2060-DP6 were seeded at OD 0.1
10 µL SIAH1 10^8 and 2 µL TadA 10^8 were added to each well (one plate per phage type)

Deep Well Plate Layout (one for each phage)

	A	B	C	D	E	F	G	H	I	J	K	L
1	2049-08-DP6 + 0 Van			2049-08-DP6 + 10 µM Van			2049-08-DP6 + 100 µM Van			2049-08-DP6 + 500 µM Van		
2	2054-08-DP6 + 0 Van			2054-08-DP6 + 10 µM Van			2054-08-DP6 + 100 µM Van			2054-08-DP6 + 500 µM Van		
3	2055-08-DP6 + 0 Van			2055-08-DP6 + 10 µM Van			2055-08-DP6 + 100 µM Van			2055-08-DP6 + 500 µM Van		
4												
5												
6	S2060+DP6 + 0 Van + tetracyclin			2049-08-DP6 + 0 Van + tetracycline			2054-08-DP6 + 0 Van + tetracycline			2055-08-DP6 + 0 Van tetracycline		
7	S2060+DP6 + 0 Van			S2060+DP6 + 10 µM Van			S2060+DP6 + 100 µM Van			S2060+DP6 + 500 µM Van		
8	S2208 + 0 Van			S2208 + 10 µM Van			S2208 + 100 µM Van			S2208 + 500 µM Van		

Golden Gate Assembly

Rationale: we try to assemble a pES2037-like plasmid in the SmR backbone. This has a different ori compared to the lvl1 plasmids, so we could then use it with the different pVan substrates directly. Plasmid labeled as pES2044

Assembly pES2044 using new protocol (see 16.09) and with Lukas'.
Rest of plasmids were assembled using new protocol only.

Table16

	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P	Q	R	S	T	U	V	W	X	Y	Z
1	pES2044	AP1 pVAN RNAP (CGG)_ strong tE1, strong E2	SmR	pTU2-A p15a SmR	pES1087	pES1003	pES1004	pES1094	pES1095																	
2	pES2048	AP2_pVan_E GLN3_L2_CtermRNAP(CGG)	SmR	pTU2-a p15a SmR	pES1116	pES1115																				
3	pES2057	AP2_pVan_E GLN3_L3_CtermRNAP(CGG)	SmR	pTU2-a p15a SmR	pES1120	pES1115																				
4	pES2059	AP2_pVan_N LRP3.200_L3_CtermRNAP(CGG)	SmR	pTU2-a p15a SmR	pES1122	pES1115																				
5	pES2060	AP2_pVan_N LRP3.700_L3_CtermRNAP(CGG)	SmR	pTU2-a p15a SmR	pES1123	pES1115																				
6	pES2061	AP2_pVan_E GLN3_L4_CtermRNAP(CGG)	SmR	pTU2-a p15a SmR	pES1124	pES1115																				
7	pES2062	AP2_pVan_asyn_L2_CtermRNAP(CGG)	SmR	pTU2-a p15a SmR	pES1125	pES1115																				
8	pES2063	AP2_pVan_N LRP3.200_L4_CtermRNAP(CGG)	SmR	pTU2-a p15a SmR	pES1126	pES1115																				
9	pES2064	AP2_pVan_N LRP3.700_L4_CtermRNAP(CGG)	SmR	pTU2-a p15a SmR	pES1127	pES1115																				

DIVENDRES, 20/9/2024

- [x] Gibson assembly: 2042, 2045
  - [x] gel extraction
- [ ] do time course phage propagation assay where phages are added after different amounts of time of van induction --> phage propagation assay we did yesterday has pVan+phages added at the same time and tested for different concentrations, now we want to know what van induction time is best. Here we want to add Vanilic acid at different timepoints (e.g., 30 min, 1h, 2h), then add phages at the same time and measure phage titer by qPCR after 4 hours. See more details below
  --> whether this is relevent depends on the results of thursday --> otherwise troubleshoot

- [x] Take a new sample from the phage propagation assay from yesterday that is still in the shaker. As a reference, t=24h would be at 12h aprox
- [x] transform pES2044 in DH5a
- [x] also transform the other assemblies aswell
- [x] make lvl 0 overhangs for PSP if primers arrive
- [x] check pES1119, pES1128-1130 and pES2053 sequencing and miniprep correct cultures (have green dot on top, left shaker - middle row)
- [x] Check co-transformation of 2049-08-DP6, 2054-08-DP6, and 2055-08-DP6 (plate in incubator) --> these co-transformants were made in case the co-transformants that were used for yesterday's phage propagation assay didn't work (nightseq was sent yesterday, and gel showed that...)
- [x] Look at plaque assay do determine phage titer for SIAH1 KO - greater than 10^7
- [x] See if the o/n culture of S2060+DP6 with Tet grew --> Lukas is unsure if these cells lost the plasmid with TetR
- [x] Sequencing analysis

in lab: Michael

qPCR results of the phage propagation assay after 22h

- no propagation in any strain but in the positive control (ask Sasha for the specific data)
- Will redo the assay once we have fresh co-transformants

Sequencing Analysis

EconomyRun

- 2048 A: looks good, only one silent mutation -> co-transform with 2008

NightSeq

- 2053: all colonies look okay-ish (have a few mutations at positions with low signal quality and could be a mix). But 2053.2 looks good
- 1119: both A and B are NNN
- 1128: A: empty backone / red colony. B: wrong promoter (pJ231119), full EGLN3 instead of the peptide
- 1129 A and B wrong promoter (pJ231119) , wrong linker (L2)
- 1130 A wrong promoter (pJ231119) , wrong linker (L2), full EGLN3 instead of the peptide. B: something with TetR

2049.4/8 cultures are red but weren't event sent for sequencing

- co-transformants: 2008 is present in all colonies
  - 2054: NNN
  - 2055: NNN
  - 2049: DP6 got sequenced
- Either its just the sequencing primer or all plasmids with Spec resistance got lost

Full plasmid Seq

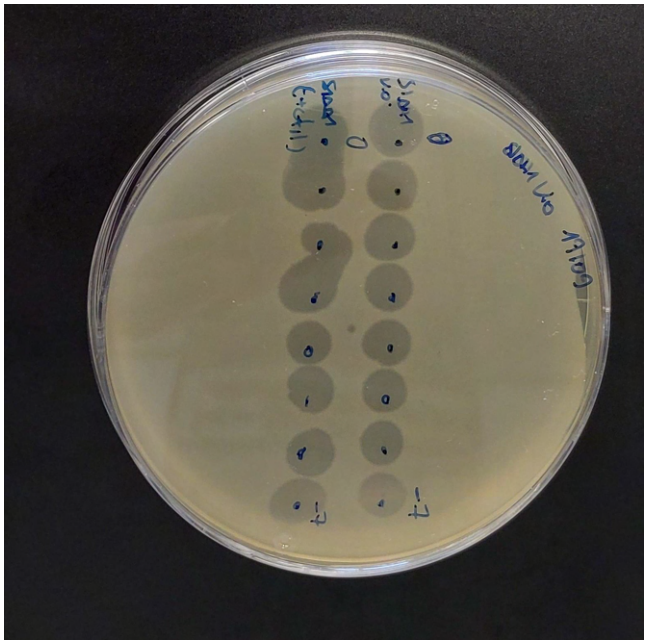
- both 2049.2 and 2049.4 look good. They have a silent mutation in VanR and two mutations in the ori (idk the impact of these)
- 2050: only E2 and ori got sequenced
- 2051: is missing CymRAM (=irrelevant) and E1

- 2052 looks good (same mutations as 2049)
- 2053 (old one) is missing g3-lux (our plasmid should be funcionally equivalent to 2055 then)

Plaque Assay

- Phage titer can still not be determined as its much higher than 10^7 (see plaque assay below)
- Re-did the assay but from 10^5 dillution to 10^-12

plaque assay



Gibson + level 0 overhang PCR

Table17

	template	primers	C	D
1	phage shock promotor plasmid from Lukas	121 /122	57 °C	30 s
2	2008 (part 2 for 2045)	111/106	62 °C	3 min (~5.5/6.2kb)

2008 part 2 was run on a gel and extracted: yield: 48.2 ng/uL
phage shock promotor also had the expected size and was PCR extracted -> in lvl 0 box now

Phage propagation assay Phage Propagation Assay

- Dilluted co-transformant cultures in 0.6x Kan + Chlor, 1.2x Spec + 100mM glucose to pre-grow them to OD 0.5

- Eventually didn't start the O/N propagation assay since these specific clones don't really seem to work for some reason
    - Sasha suggested picking new colonies (ideally fresh co-transformants) and trying again with them

Transformation Transformation

- 100 uL DH5a, 2X spec plates (new assemblies): - 2048 assembly was not transformed since we got positive sequencing results
    - 2044 Lukas protocol
    - 2044 old protocol
    - 2057
    - 2059
    - 2060
    - 2061
    - 2062
    - 2063
    - 2064
- 50 uL S2060+DP6
    - 2048 + 2008
    - 2049 + 2008
    - 2055 + 2008

High efficiency protocol

Phage shock promotor Gibson assemblies

- backup strategy

2042 assembly (5:1:1)1

	part	amount	mass	volume
1	2008 part 1	30 fmol	93 ng	1.6 uL
2	2008 part 2	30 fmol	120 ng	1.7 uL
3	PSP insert	150 fmol	23 ng	1.1 uL (1:10)
4	NEBuilider HiFi DNA Assembly Master Mix			5 uL
5	H2O			0.6 uL

2045 assembly (3:3:1:1)1

	part	ammount	mass	volume
1	2008 part 1	30 fmol	93 ng	2.5 uL
2	2008 part 2	30 fmol	120 ng	2.5 uL
3	pES0028	100 fmol	15 ng	1.4 (1:10)
4	pES1095	100 fmol	55 ng	0.4 uL
5	NEBuilider HiFi DNA Assembly Master Mix			10 uL
6	H2O			3.2 uL

- everything was prepared but the assembly reaction wasn't started yet (no time)
- PCR tubes are in our freezer and are ready to be put in the thermocycler

DISSABTE, 21/9/2024

- [x] check SIAH1 KO plaque assay and determine phage titer
- [x] pick new co-transformants 48/49/55-08-DP6. (The old ones didn't seem to work and backup co-transformants didn't grow)
- [x] pick & innoculate colonies of all succesful transformants
  - [x] verify size (either colony PCR or let it grow, miniprep + gel)
  - [ ] re-transform assemblies that didn't grow (if there are any) - use 2X spec
- [x] Transform Gibson assembly for pES2042 and pES2048
- [ ] order oLS 1663 primer (we're running out soon)
- [x] reassemble + transform 1119
- [x] miniprep 2053
- [x] Phage propagation assay with 2054-08-DP6 (fresh co-transformant the shaker) or wait one more day and do it with all strains.

In lab: Pau

Picking transformations from 19.09 and 20.09

2049-08-DP6, 2054-08-DP6 and 2055-08-DP6 from 19.09 --> new colonies for 2049 and 2055, picked for o/n culture, plate in the fridge

2049-08-DP6, 2054-08-DP6 and 2055-08-DP6 from 20.09 --> only one colony grew for 2055, picked and plate back to the incubator

2044 old and new --> picked 3 colonies for each, plate in the fridge

2057, 2060, 2061, 2062, and 2064 --> no colonies, looks like some might be about to appear, put back to the incubator

2059 --> one colony, picked and put back to the incubator

2063 --> 3 colonies picked and put back to the incubator

Transformation of pES2042 and pES2045 (Gibson assembly)

After 1h incubation at 50ºC, Gibson assemblies were transformed in 100uL DH5a following high-efficiency protocol, 1h recovery.

Miniprep of pES2053 and 2054-08-DP6 A/B

Yield for pES2053 --> 142 ng/uL.

Minipreps were run on a gel (1% agarose, 120V, 40 min). Expected bands:

- pES2053: 9.1 kb
- 2054-08-DP6: 5.9kb (2054) + 11.2kb (2008) + 8.6kb (DP6)

Order: GeneRule 1kb - pES2053 + 2054-08-DP6 A - 2054-08-DP6 B

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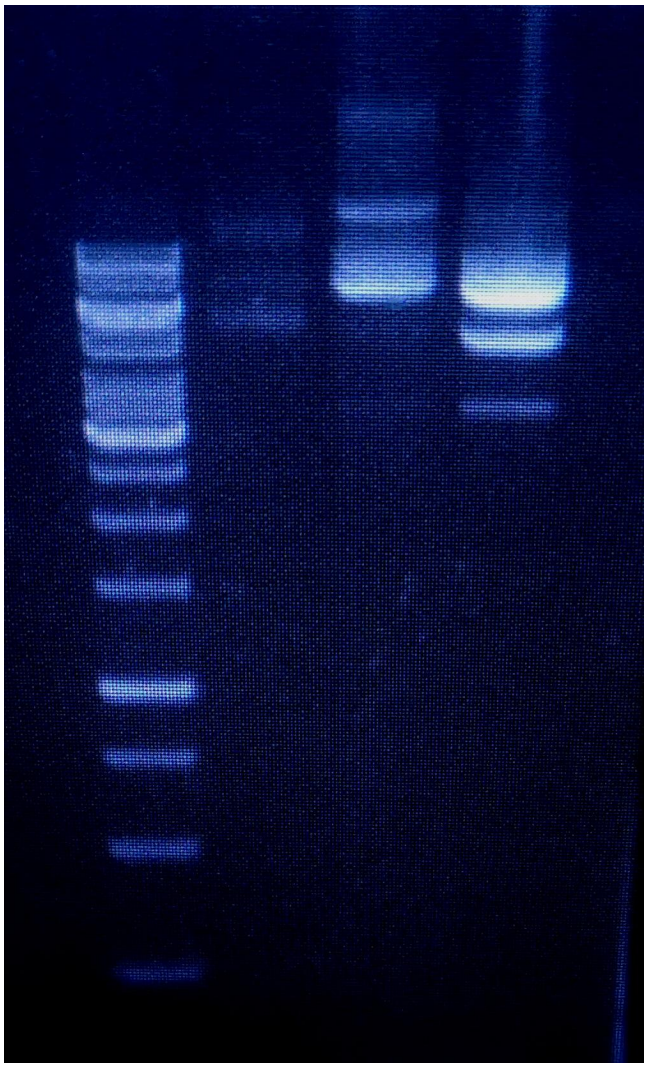


Results:

- pES2053 --> we observe three bands (it's dimmer bc some amount got lost during loading), one band at 11kb, one at 8kb, and one at 5kb. The band at 8kb could be the right one, but still the other two bands are confusing, loading more to do gel extraction might be necessary
- 2054-08-DP6 A --> one band at 11kb, one at 9kb, one at 7ish kb, it looks pretty okay but should be confirmed with sequencing
- 2054-08-DP6 B --> clear bands at 6kb, 4kb, and 3kb, not nice

Phage propagation assay with 2054-08-DP6 (new co-transformant)
Used Vanillic acid concentrations of 0 µM, 10 µM, 100 µM and 500 µM to induce the Subs-L-RNAP
the induction began roughly 15 min before the phages were added at RT
100 µL samples were taken after 0.5 h, 1 h, 2 h and 4 h, and finally left shaking overnight to take a sample after 24h.
seeding OD for our bacteria was 0.1 (S2060-DP6), 0.17 (S2208) and 0.18 (2054-08-DP6), after growing for 1h30min from o/n culture, no dilution.
10 µL SIAH1 10^8 and 2 µL TadA 10^8 were added to each well (one plate per phage type)
NOTE: for the SIAH 1 plate, wells F10-12 (i.e., S2060-DP6+ 0 Van + Tet) are in E10-12!!

Deep Well Plate Layout (one for each phage)1

	A	B	C	D	E	F	G	H	I	J	K	L
1	2054-08-DP6 + 0 Van									S2060+DP6 + 0 Van		
2	2054-08-DP6 + 10 Van									S2060+DP6 + 10 µM Van		
3	2054-08-DP6 + 100 Van									S2060+DP6 + 100 µM Van		
4	2054-08-DP6 + 500 Van									S2060+DP6 + 500 µM Van		
5	LB (no phage)				LB + antibiotics + phages (initial titer)					LB (no phage)		
6	2054-08-DP6 + 0 Van + tetracycline									S2060+DP6 + 0 Van + tetracyclin		
7	LB (no phage)									LB (no phage)		
8	S2208 + 0 Van			S2208 + 10 µM Van			S2208 + 100 µM Van			S2208 + 500 µM Van		

Incubation started at 12:45h.

SIAH1 KO phage production plaque assay
SIAH1 KO --> 27 plaques at dilution -9 --> concentration of 6.75*10^12 --> labelled as such
Positive control --> 9 plaques at -9 --> concentration of 2.2*10^12 (x20 than expected)

Assembly of pES1119
GG assembly with BsaI using Lukas' protocol.

Table18

	A	B	C	D	E	F	G
1	pES1119	pTU1-A-RFP	pES0028	NLRP3_700_frag	Linker 2	pES0008	pBP-L3S2P21

DIUMENGE, 22/9/2024

- [x] Michael: redo PSP with lvl 0 overhangs
- [x] Take 24h sample for phage propagation assay of 2054-08-DP6 (see layout below) and run qPCR of everything
- [x] If picked cultures grew, perform another phage propagation assay
  - [x] 50mL red-cap tube of S2060-DP6 in fridge

- [x] Transform pES1119 (reaction in slot 2)
- [x] Miniprep o/n cultures
  - [ ] Lvl2 transformants for sequencing
  - [x] Co-transformants can be ran on a gel in addition to be sent for sequencing
- [x] Following Lukas' advice (see below), perform co-transformations in S2060 competent cells and use these for phage propagation assay

in lab: Gabriel, Michael

Lukas is trying the following for his own strains:

1. Co-transformation of plasmids in S2060 (without DP6) --> selection can be performed with Tet as it is in the F-plasmid of the S2060 strain
   a. The co-transformant strain can be used already for phage propagation assay, as for this DP6 is not required
   b. Parallely, sequential transformation can be tested, although I think in one week that will be timewise impossible
2. Make these cells competent and transform them with DP6

The idea is that somehow DP6 is giving problems, so it might help to transform it as last thing.

Layout for picking samples for 24h:
NOTE: for the SIAH 1 plate, wells F10-12 (i.e., S2060-DP6+ 0 Van + Tet) are in E10-12!!
LB without phages was picked from E1-3.

Layout is also in the drive

Table19

	A	B	C	D	E	F	G	H	I	J	K	L
1	2054-08-DP6 0uM SIAH1	2054-08-DP6 0uM SIAH1	2054-08-DP6 0uM SIAH1	2054-08-DP6 10uM SIAH1	2054-08-DP6 10uM SIAH1	2054-08-DP6 10uM SIAH1	2054-08-DP6 100uM SIAH1	2054-08-DP6 100uM SIAH1	2054-08-DP6 100uM SIAH1	2054-08-DP6 500uM SIAH1	2054-08-DP6 500uM SIAH1	2054-08-DP6 500uM SIAH1
2	S2060-DP6 0uM SIAH1	S2060-DP6 0uM SIAH1	S2060-DP6 0uM SIAH1	S2060-DP6 10uM SIAH1	S2060-DP6 10uM SIAH1	S2060-DP6 10uM SIAH1	S2060-DP6 100uM SIAH1	S2060-DP6 100uM SIAH1	S2060-DP6 100uM SIAH1	S2060-DP6 500uM SIAH1	S2060-DP6 500uM SIAH1	S2060-DP6 500uM SIAH1
3	2054-08-DP6 Tet SIAH1	2054-08-DP6 Tet SIAH1	2054-08-DP6 Tet SIAH1	S2060-DP6 Tet SIAH1	S2060-DP6 Tet SIAH1	S2060-DP6 Tet SIAH1	LB + SIAH1	LB + SIAH1	LB + SIAH1	LB	LB	LB
4	S2208 0uM SIAH1	S2208 0uM SIAH1	S2208 0uM SIAH1	S2208 10uM SIAH1	S2208 10uM SIAH1	S2208 10uM SIAH1	S2208 100uM SIAH1	S2208 100uM SIAH1	S2208 100uM SIAH1	S2208 500uM SIAH1	S2208 500uM SIAH1	S2208 500uM SIAH1
5	2054-08-DP6 0uM TadA	2054-08-DP6 0uM TadA	2054-08-DP6 0uM TadA	2054-08-DP6 10uM TadA	2054-08-DP6 10uM TadA	2054-08-DP6 10uM TadA	2054-08-DP6 100uM TadA	2054-08-DP6 100uM TadA	2054-08-DP6 100uM TadA	2054-08-DP6 500uM TadA	2054-08-DP6 500uM TadA	2054-08-DP6 500uM TadA
6	S2060-DP6 0uM TadA	S2060-DP6 0uM TadA	S2060-DP6 0uM TadA	S2060-DP6 10uM TadA	S2060-DP6 10uM TadA	S2060-DP6 10uM TadA	S2060-DP6 100uM TadA	S2060-DP6 100uM TadA	S2060-DP6 100uM TadA	S2060-DP6 500uM TadA	S2060-DP6 500uM TadA	S2060-DP6 500uM TadA
7	2054-08-DP6 Tet TadA	2054-08-DP6 Tet TadA	2054-08-DP6 Tet TadA	S2060-DP6 Tet TadA	S2060-DP6 Tet TadA	S2060-DP6 Tet TadA	LB + TadA	LB + TadA	LB + TadA	LB	LB	LB
8	S2208 0uM TadA	S2208 0uM TadA	S2208 0uM TadA	S2208 10uM TadA	S2208 10uM TadA	S2208 10uM TadA	S2208 100uM TadA	S2208 100uM TadA	S2208 100uM TadA	S2208 500uM TadA	S2208 500uM TadA	S2208 500uM TadA

Liquid cultures

Single transformations:

- 2044: All old were negative (RFP), new spec ones grew
- 2059: A grew
- 2063: A, B, C grew

plates

- 2042 and 2045 were plated on wrong antibiotic (Spec) -> re-transform today
- 2059 colonies were re-picked (miniprep had no yield)

Cotransformants

- 2049-08-DP6: All grew
- 2055-08-DP6: All grew

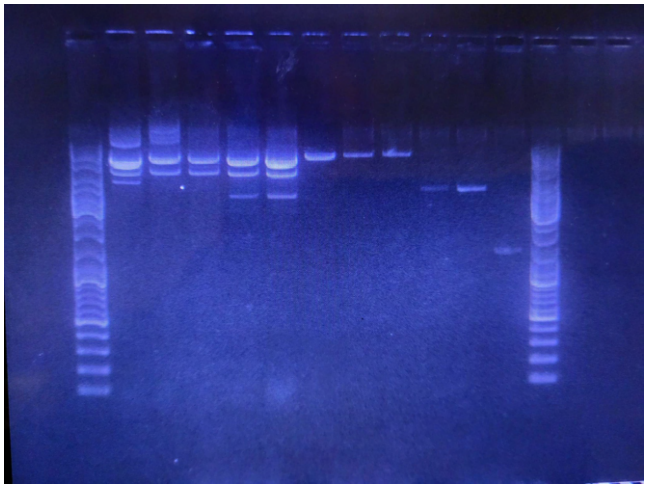
Miniprep

...

Gel to check co-tansformants and miniprep

order: ladder / 2049-08-DP6 A / B / C/ 2055-08-DP6 A / C / 2044 A/ B/C / 2063 A/ B C / ladder

20240922_163533 (002).jpg



- The ladder I used was labelled with 1kb but it looks more like GR mix
- bands are generally slightly lower but this is expected since plasmids (especially when supercoiled) move a bit faster through the gel than linear DNA

expected sizes:

- 2008: 11kb (present in all tested co-transformants)
- 2049: 9.7 kb (present in A,B,C)
- 2055: 5.5kb (present in A, C)
- DP6: 8.6kb (present in all tested co-transformants)

2049-08-DP6 A and 2055-08-DP6 A + C seem correct
2044: all three seem correct (11.6 kb)
2063: 5.6 kb: A and B can be sequenced tomorow

propagation assay

took 10 uL of 10^8 PFU/mL phages per well

the titer of SIAH1 KO was multiplied by 20 (since in the plaque assay the positive control had 20x more phage than expected). We can adjust the titer after the qPCR
2049 B and 2055 A was used (bands looked okay)

Table20

	A	B	C	D	E	F	G	H	I	J	K	L
1	2049-08-DP6 + SIAH1 + 100 uM van				2049-08-DP6 + TadA + 100 uM van				2049-08-DP6 + SIAH1 KO + 100 uM van			2049-08-DP6
2	2054-08-DP6 + SIAH1 + 100 uM van				2054-08-DP6 + TadA + 100 uM van				2054-08-DP6 + SIAH1 KO + 100 uM van			2054-08-DP6
3	2055-08-DP6 + SIAH1 + 100 uM van				2055-08-DP6 + TadA + 100 uM van				2055-08-DP6 + SIAH1 KO + 100 uM van			2055-08-DP6
4	2049-08-DP6 + SIAH1 + 500 uM van				2049-08-DP6 + TadA + 500 uM van				2049-08-DP6 + SIAH1 KO + 500 uM van			2049-08-DP6 + 500 uM van
5	2054-08-DP6 + SIAH1 + 500 uM van				2054-08-DP6 + TadA + 500 uM van				2054-08-DP6 + SIAH1 KO + 500 uM van			2054-08-DP6 + 500 uM van
6	2055-08-DP6 + SIAH1 + 500 uM van				2055-08-DP6 + TadA + 500 uM van				2055-08-DP6 + SIAH1 KO + 500 uM van			2055-08-DP6 + 500 uM van
7	LB + SIAH 1				LB + TadA				LB + SIAH 1 KO			
8	S2208 + SIAH 1				S2208 + TadA				S2208 + SIAH 1 KO			

Transformations Transformation

into 100 uL DH5a:

- 1119
- 2060
- 2061
- 2062
- 2064
- 2042
- 2045

Since 2044 looked good on the gel, I already co-transformed 2044 A with

- 1116, 1117, 1118, 1076, 1081, 1104, 1112 into 75 uL S2060 +DP6
- 116, 117, 118 into 5 uL S2060 (without DP6 - see Lukas' suggestion)

High efficiency protocol

DILLUNS, 23/9/2024

- [x] propagation assay qPCR
- [x] Send for sequencing:
  - [x] NightSeq of co-transformants 49(A) /55(A,B,C)-08-DP6 + fresh co-transformants from yesterday (XX-44-DP6)
  - [x] 1119
  - [x] 2060, 2061, 2062, 2064
  - [x] 2042, 2045
  - [x] 2044 (especially A)
- [x] Send miniprepped 2054-08-DP6 A/B for sequencing and if right make new glycerol stocks and throw away the ones I did for the previous co-transformants (the ones we used for the phage propagation assay on 19.09) --> gel shows A as okay-ish and B as bad

- [x] Make new Spectinomycin stock --> Lukas had a frozen 50mL aliquote that we thawed
- [x] try gel-extraction of 2053 and transform it
- [x] assemble 2044 in Benchling
- [x] put sticker on DNA in the green rack or temp box and move to lvl 2 box:
  2048A, 2049.2 or 4, 2052(6), 2054(6/7), 2055(7/8)
- [x] dillute all miniprepped samples to 100 ng/uL
- [x] order oLS 1663 primer (we're running out soon)
- [x] For the report: figure out the qPCR machine specific machine settings, including readout wavelength = , cycling temperatures, and also add citation for the polymerase Roche LightCycler 480 II,
- [x] Transform S2060 with pES2008 and pES2044 for negative control
- [x] figure out what happened to 2048-08-DP6 -> was picked today and sent for sequencing

In lab: Pau, Michael

NightSeq Plate

- 2048-08-DP6 A
- 2045 A/B/C (019)
- 2042 A/B/C (019)
- 1116-44-00 A/B (017)
- 1117-44-00 A/B
- 1118-44-00 A/B
- 1104-44-DP6 A/B
- 1116-44-DP6 A/B
- 1117-44-DP6 A/B
- 1118-44-DP6 A/B

EconomyRun

- 2044A (018, 019)
- 2044B (019)
- 2044C (019)
- 2063A (019)
- 2063B (019)
- 2048-08-DP6 A
- 2049-08-DP6 A
- 2054-08-DP6 A
- 2055-08-DP6 A/C

Full Plasmid Seq

- 2044 A

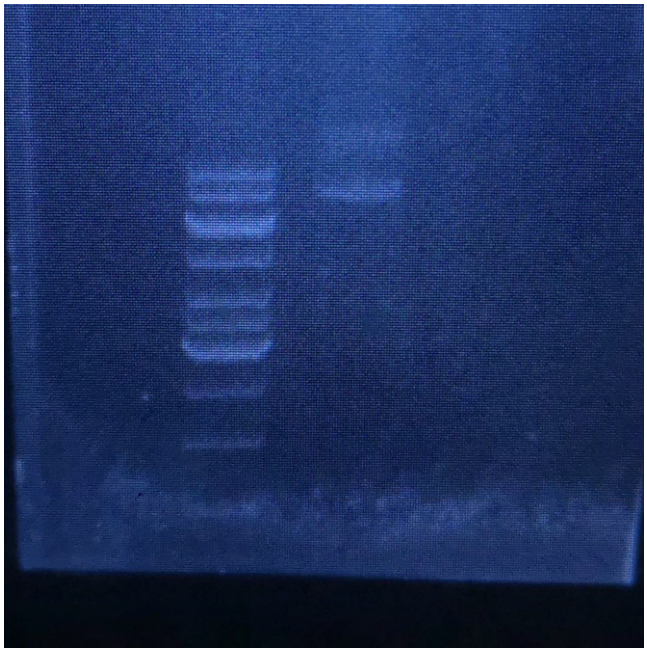
- Innoculated all picked colonies with respective antibiotics
- prepared 49/54/55-08-DP6 for the assay tomorrow
- important: always grow xx-44-00 strains with Tetracycline (to ensure we keep the F-plasmid at all times)
- ordered / trashed most the tubes standing on the green rack on the bench

qPCR for phage propagation assay --> layout and results here

- in summary, we see no substrate/phage specific propagation but since we didn't have a tetracycline control, we don't know whether is because the bacteria lost the F-plasmid or because the logic doesn't work

Gel extraction for pES2053

IMG_20240923_142737_edit_48698498835277.jpg



Band at 9.1kb should be the correct one. Performed extraction with yield of 10.7 ng(uL, so it's unusable.

Transformation of pES2044 and pES2008 in S2060

As a negative control for phage propagation assay, pES2044 and pES2008 were transformed in competent S2060 and grown o/n in a liquid culture (no colony selection), with the assumption that growth is going to be pretty clonal anyways. This way, they should be ready for phage propagation assay tomorrow.

DIMARTS, 24/9/2024

- [x] propagation assay
  - [x] pre-grow liquid cultures: 2049-08-DP6, 2054-08-DP6, 2055-08-DP6, 1116-44-00, 1117-55-00. 1118-44-00
- [x] Sequencing Analysis
  - [x] Economy
  - [x] NightSeq
- [x] if sequences are correct, miniprep 2045, 2060, 2061, 2063, 2064 and 2050
- [ ] when co-transformants are confirmed -> make glycerol stock
- [x] send 2059 for NightSeq (if it grew) -> miniprepped and no yield -> maybe no plasmid?

- [x] phage shock AP2s
  - [x] PCR
  - [x] purification
  - [x] assembly
  - [x] transformation and co-transformation
- [x] Phage propagation assay with 1116-44-00 to 1118-44-00. Negative control of pES2044 in S2060 has been transformed o/n as a liquid culture and can be used here. There is also a 2008 in S2060 in case it is needed. A "positive control" culture consisting of 1076-08-00 has also been prepared from glycerol stock
- [x] potentially do some more co-transformations (2044 with different linkers) as backup strategy

in lab: Michael

Sequencing Analysis (Economy)

- 2044: A (= the clone used for all co-transformations) NNN with 019 primer and very poor signal and only 27 bp with the 018 primer :/ - Signal quality was very low though
- However the sequence of 2044 was confirmed in NightSeq results
- B and C align but have multiple short (1-4 bp) deletions in the RNAP - it's hard to say whether thats a sequencing artifact or not but it appears unlikely to mee that we see no substitution mutations but multiple deletions in the Nterm RNAP which we have already sequenced many times without seeing any of this
- re-sequencing of 2044 C showed no deletions but still mutations at points of low signal quality -> artifact

- 2063A: NNN, B: not 2063 but has pVan and EGLN3 (unknown linker)
- co-transformants
  - 2008 confirmed in 54-08-DP6 and 55-08-DP6 but not in 49-08-DP6
  - AP1 only confirmed in 2055-08-DP6 A, other AP1s are only NNN.

Note that

- 1. the signal quality was apparently very low in most samples according to microsynth
- I sent 500-700 ng DNA per sample which is normally more than enough but considering this is a mix of 3 plasmids, I should have sent more
- 2. the primer used for AP1s was a new primer that should theoretically work but wasn't confirmed yet

Considering the following facts

- the individual plasmids of the co-transformants were sequence confirmed
- the co-transformants were miniprepped and had 3 bands at ~ correct size (a bit lower due to plasmid)
- the co-transformants grew in antibiotics on both the plate and on liquid cultures

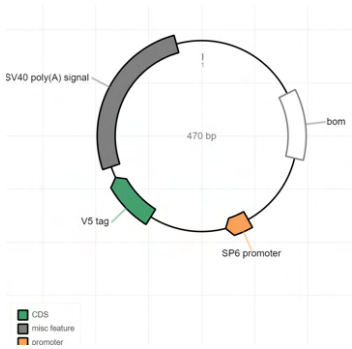
I assume that the error is on the sequencing side and not due to the lack of the correct plasmids in our co-transformants. The only alternative hypothesis would be that our strains are contaminated with plasmids that are different to ours but have a similar size and the same resistance

- I will still miniprep our co-transformants again and run them on a gel (this time with restriction digest) just to make sure I'm not insane (spoiler: this increased the confusion even more)

NightSeq

- all co-transformants have 2044 and there are no mutations that occur systematicly in all samples (only at low quality positions)
- 1116 matches everywhere but the substrate is not EGLN3 (maybe EGLN3 peptide?) - I figured out too late that its actually 1125 (no idea how this got in there?)
- 1117 and 1118 matches pretty much perfectly in all co-transformants
- 2045 A and C look very good -> miniprep and load on a gel
- 2042 A and C look very good -> miniprep and load on a gel
- 2050 A and B are both empty backbones although only 2050A turned red
- 1119A some random plasmid (see below), 1119B empty backbone
- 2060 A + B correct, 2061, 2062, 2064 empty backbones

1119A annotation



- some DP6 co-transformants (less important right now) didn't grow: 2048-08-DP6A/B, 1118-44-DP6 B, 1104-44-DP6 A/B -> trashed

Phage shock AP2s:

1. PCR: PCR: Q5 Polymerase template: PSP plasmid we got from Lukas, primers 123/124, 54C, 20s
   PCR failed - no band -> retry PCR
   2. attempt: band with the right size
2. PCR purification. NucleoSpin Gel and PCR Clean-up yield: 84 ng/uL -> PSP is pES0030
3. GG assembly of 1131-1134 EcoFlex Assembly

insert 2

	assembly	backbone	insert 1	insert 2	insert 3	insert 4	insert 5
1	pES1131	pTU1-A-RFP	pES0030	pES0013	pES0002	pES0008	pBP-L3S2P21
2	pES1132	pTU1-A-RFP	pES0030	pES0015	pES0002	pES0008	pBP-L3S2P21
3	pES1133	pTU1-A-RFP	pES0030	NLRP3_200_frag	pES0002	pES0008	pBP-L3S2P21
4	pES1134	pTU1-A-RFP	pES0030	NLRP3_700_frag	pES0002	pES0008	pBP-L3S2P21

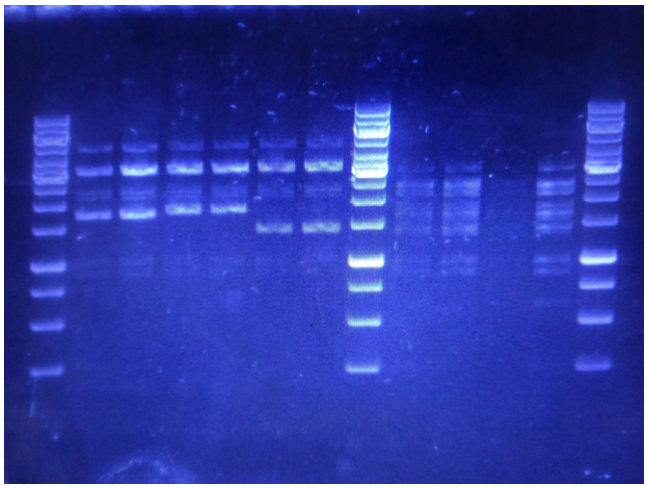
- transformed into 100 uL DH5a
- high efficiency protocol plated but without recovery due to time reasons (should still work since the assembly has Amp resistance)

Restriction Digest (to confirm co-transformants)

- Context: this was before we had NightSeq results
- SphI enzyme should cut every plasmid (2044, 2008, 2049, 2054, 2055, DP6) exactly once
- 1h incubation at 37 °C

GR 1kb ladder, 1116-08-00A, 1116-08-00B, 1117-08-00A, 1117-08-00B 1118-08-00A, 1118-08-00C, GR 1kb ladder, 55-08-DP6A, 55-08-DP6C, 49-08-DP6A, 54-08-DP6A, GR 1kb ladder

20240924_140303 (002).jpg



gel looks pretty bad - none of the AP1s (lvl 2 plasmids) are present
we don't expect the 49/54/55-08-DP6 plasmids to be cut multiple times as visible in the gel -> probably contaminated with another plasmid? F-Plasmid gets cut?

- Gel results were eventually disregarded since NightSeq looked okay

Miniprep Qiagen Miniprep

- Since Sequencing results of 2060A, 2042 A/C and 2045 A/C looked very good -> samples were miniprepped
- Loading these plasmids on a gel however showed that the bands run far too low (3-6kb) -> big deletions
  (didn't paste this unimportant gel picture to make the lab journal less laggy)

- 2060 A was not run on the gel but its a Cterm plasmid which generally work pretty well and sequencing was fine
- 2059 grew in liquid culture but had no yield (??) The last two clones didn't even grow in liquid culture

Propagation Assay:

- 10 uL phages with a titer of 10^8 PFU/mL
- all strains were dilluted and pre-grown to OD 0.2-0.22
- positive controls: S2208, DP6-containing strains with tet, 76-08-DP6
- negative controls: bacteria without phage, S2060 + 2044 + van, S2060 + 2008, LB + phage
- started at 21:05

Plate1

	A	B	C	D	E	F	G	H	I	J	K	L
1	2049-08-DP6 + SIAH1				2049-08-DP6 + SIAH1 KO				2049-08-DP6 + TadA			2049-08-DP6
2	2054-08-DP6 + SIAH1				2054-08-DP6 + SIAH1 KO				2054-08-DP6 + TadA			2054-08-DP6
3	2055-08-DP6 + SIAH1				2055-08-DP6 + SIAH1 KO				2055-08-DP6 + TadA			2055-08-DP6
4	2049-08-DP6 + SIAH1 + 500 uM van				2049-08-DP6 + SIAH1 KO + 500 uM van				2049-08-DP6 + TadA + 500 uM van			2049-08-DP6 + 500 uM van
5	2054-08-DP6 + SIAH1 + 500 uM van				2054-08-DP6 + SIAH1 KO + 500 uM van				2054-08-DP6 + TadA + 500 uM van			2054-08-DP6 + 500 uM van
6	2055-08-DP6 + SIAH1 + 500 uM van				2055-08-DP6 + SIAH1 KO + 500 uM van				2055-08-DP6 + TadA + 500 uM van			2055-08-DP6 + 500 uM van
7	LB + SIAH1				LB + SIAH1 KO				LB + TadA			
8	S2208 + SIAH 1				S2208 + SIAH 1 KO				S2208 + TadA			

Plate2

	A	B	C	D	E	F	G	H	I	J	K	L
1	116-44-00 + SIAH1				116-44-00 SIAH1 + KO				116-44-00 + TadA			116-44-00
2	117-44-00 + SIAH1				117-44-00 + SIAH1 KO				117-44-00 + TadA			117-44-00
3	118-44-00 + SIAH1				118-44-00 + SIAH1 KO				118-44-00 + TadA			118-44-00
4	116-44-00 + SIAH1 + 500 uM van				116-44-00 + SIAH1 KO + 500 uM van				116-44-00 + TadA + 500 uM van			116-44-00 + 500 uM van
5	117-44-00 + SIAH1 + 500 uM van				117-44-00 + SIAH1 KO + 500 uM van				117-44-00 + TadA + 500 uM van			117-44-00 + 500 uM van
6	118-44-00 + SIAH1 + 500 uM van				118-44-00 + SIAH1 KO + 500 uM van				118-44-00 + TadA + 500 uM van			118-44-00 + 500 uM van
7	S2060 + 2044 + SIAH1 + 500 uM van				S2060 + 2044 + SIAH1 KO+ 500 uM van				S2060 + 2044 + TadA + 500 uM van			
8	S2060 + 2008 + SIAH1				S2060 + 2008 + SIAH1 KO				S2060 + 2008 + TadA			

Plate3

	A	B	C	D	E	F	G	H	I	J	K	L
1	2049-08-DP6 + SIAH1 + Tet				2049-08-DP6 + SIAH1 KO + Tet				2049-08-DP6 + TadA + Tet			
2	2054-08-DP6 + SIAH1 + Tet				2054-08-DP6 + SIAH1 KO + Tet				2054-08-DP6 + TadA + Tet			
3	2055-08-DP6 + SIAH1 + Tet				2055-08-DP6 + SIAH1 KO + Tet				2055-08-DP6 + TadA + Tet			
4												
5	76-08-DP6 + SIAH1				76-08-DP6 + SIAH1 KO				76-08-DP6 + TadA			
6												
7												
8												

New co-transformation Transformation

- idea: test more / longer linkers for 2044 + substrates and test constitutively expressed EGLN3 (1079) with 2044
- 1120/22/23/24 + 2044 (A)
- 1079 + 2044 (A)
- 1104 + 2044 (A)
- transformed into 100 uL S2060 (no DP6)
- plated on Carb + Spec plates (will add tet as soon as its in the liquid culture)
- high efficiency protocol

DIMECRES, 25/9/2024

- [x] qPCR of the phage propagation assay
- [x] make glycerol stocks
  - [x] pES2044 and pES2008 in S2060
  - [x] 1116-44-00 (which is actually 1125-44-00), 1117-44-00, 1118-44-00
  - [x] 1125-44-DP6, 1117-44-DP6 (A), 1118-44-DP6 (A)
  - [x] 2049-08-DP6, 2054-08-DP6, 2055-08-DP6
- [x] Pick and innoculate colonies from the plates
- [x] pick and NIghtSeq of transformants (1131-34)
- [x] Clean up the mess (all the liquid cultures) on the bench
- [x] do lab duty
- [x] make LB
- [x] miniprep pES2050 B and pES1119 C and send them for sequencing
  --> pES2050 pellet was red after all - trashed
- [x] assemble pES1114 (PSP_NtermRNAP)
- [x] put sticker on confirmed sequencing tubes + dillute to 100 ng/uL
- [x] innoculate culture for terminators lvl 0s

in lab: Jakob, Michael

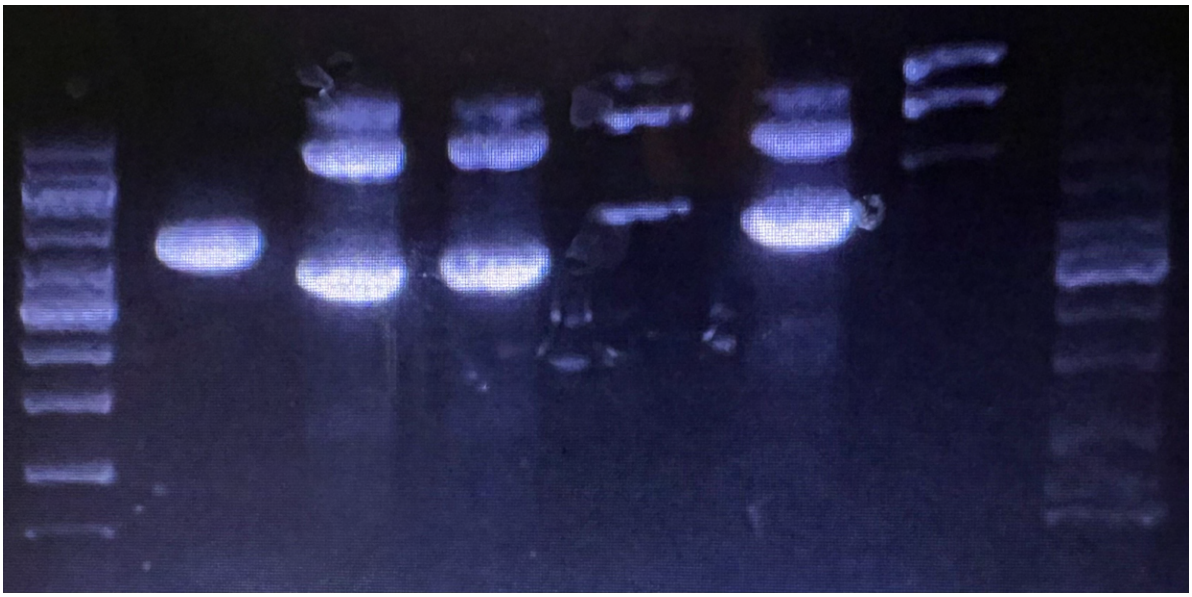
qPCR of the propagation assay

- disclaimer: I used 4 different aliquots of Firepol since we're running out right now. One of them was marked with an X (whatever that means).

Miniprep

pES1119 C: 495 ng/µL --> diluted to 100 ng/µL
1112-44-DP A: 556 ng/µL
1112-44-DP B: 394 ng/µL
1079-44-DP A: 174 ng/µL
1079-44-DP B: 445 ng/µL
2048-08-DP: 267 ng/µL
--> cultures were filled with corresponding media again and put back in incubator, in case we decide to use them
--> run on a gel: GR 1kb - 19 - 12 A - 12 B - 79 A - 79 B - 48 GR mix

IMG_6919.jpeg



pES1119(4.5 kb) looks correct - after second thought it probably runs too high, let's see what sequencing says,
1112-44-DP (4.5 kb, 8.7 kb, 11.6 kb) looks promising, 1112 runs a bit low,
1079-44-DP (5.1 kb, 8.7 kb, 11.6 kb) looks very good,
2048-08-DP (6.2 kb, 8.7, 11.3 kb) looks very good
--> sent pES1119, 1112-44-DP A, 1079-44-DP B and 2048-08-DP for sequencing

Innoculation - Lvl 0 terminators

innoculated 5 ml liquid cultures of pBP-L3S2P21/L3S1P51/L3S1P11/L3S1P32

Golden Gate Assembly

standard protocol + 30 min digest (37 °C), 30 min inactivation (80°C)

Table21

	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P	Q	R	S	T	U	V	W	X	Y	Z
1	pES1114	PSP_NtermRNAP_L3_Ub	A	Amp	pTU1-A-RFP	pES0030	pES0003	pES0002	pES0004	pBP-L3S2P21																

New Propagation assay

since the S2060 seem to be super sus, we decided to test SIAH1 propagation on our cells
innoculated 3 mL cultures of:

S2060 comp. cells from 31.08., S2060 comp. cells first patch,
S2060 iDEC cryo stock, S2060 Lukas' comp. cells,
S2208, 1076-08-00, 1117-44-00, LB
used 1.5 mL of each culture for the propagation assay
--> innoculated each culture with 10 µL of SIAH1 - 10^8 PFU/mL phages 2 hours after the start, let phages propagate o/n

Co-transformation
in Lukas' S2060
pES2044 + 1084/1117/1033/1035/1126/1127 --> 1 µL each in 30 µL of competent cells
put tetracycline in the agar aswell
also transformed 10 µL pES1114 in 300 µL DH5a

DIJOUS, 26/9/2024

- [x] pick pES1114 cultures
- [x] assemble and transform constitutive level 2s with VanRAM (pES2069 and pES2070)
- [x] miniprep lvl 0 terminators
- [x] assemble and transform psp lvl1 variants and missing constitutive variants (pES1135-1154, pES1121)
- [x] do qPCR of S2060 propagation test
  - [x] pick other co-transformants, if our S2060 is fine afterall
    --> decided to pick, since our S2060 does currently not seem to be the problem
  - [ ] pick S2060 lukas co-transformations if our S2060 is not fine
- [x] check pES11131-1134 sequencing
  - [x] miniprep pES1131-1134
  - [ ] co-trasform if sequencing is alright --> with pES2044 (wait for S2060 test results)
    --> sequencing looks bad
- [x] check pES1119 and co-transformation sequencing
- [x] test Van toxicity on propagation in 1076-08-00

Key To Dos from the lab meeting

- Test propagation with very low van induction (<= 10 uM) --> once negative control is fixed
- propagation with 1076-08-00 and van induction (to test van toxicity) --> today
- lumminescence assay --> not possible

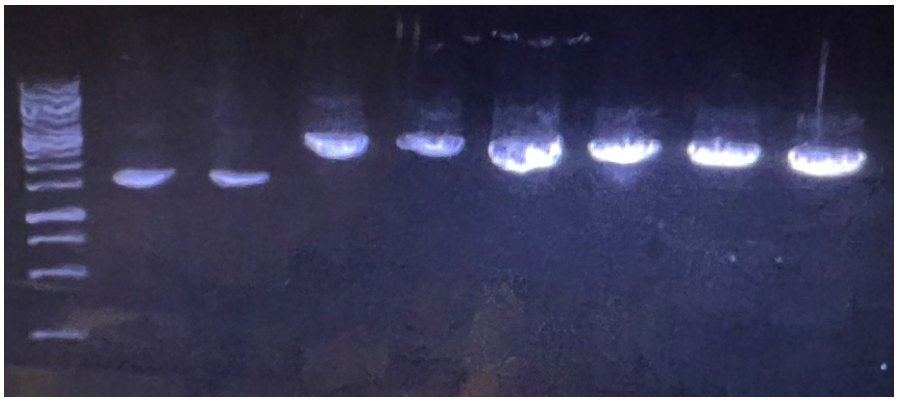
Sequencing Analysis
Economy Seq:
pES1119 is NNN
1112-44-DP: confirmed --> made a glycerol stock
1079-44-DP: confirmed--> made a glycerol stock
2048-08-DP: pES2008 is confrimed, was sent with wrong primer for pES2048 --> made a glycerol stock and sent for NightSeq with primer 119

NightSeq:
S2060: NNN --> neither contain DP6

1122-44-00 A: confirmed --> made a glycerol stock
1079-44-00 A: confirmed --> made a glycerol stock
1104-44-00: part of pES2044 is sequenced instead of pES1104

pES1131: A is NNN, B is short, C is LacZ --> all of them were red anyways
pES1132: A is LacZ, B is E2
pES1133: A and B are NNN, C is LacZ
pES1134: A and B are NNN, C is LacZ
--> seems weird, I'll run the minipreps on a gel:
GR 1kb - 32 A (5 kb) - 32 B - 33 A (4.7 kb) - 33 B - 33 C - 34 A (4.7 kb) - 34 B - 34 C

IMG_6931.jpeg



--> pES2032 seem to be 1.5 kb, pES2033 and 2034 are 3-3.5 kb --> trash all of them, repeat assembly

Miniprep
--> all pES1131 cultures turned red --> picekd 3 new colonies that looked white
pES1132 A: 209 ng/µL
pES1132 B: 254 ng/µL
pES1133 A: 321 ng/µL
pES1133 B: 228 ng/µL
pES1133 C: 611 ng/µL
pES1134 A: 370 ng/µL
pES1134 B: 315 ng/µL
pES1134 C: 566 ng/µL

pBP L3S2P21: 153 ng/µL
pBP L3S1P11: 175 ng/µL
pBP L3S1P32: 255 ng/µL
pBP L3S1P51: 151 ng/µL
--> diluted to 100 ng/µL

Golden Gate Assembly - Level 1

Table23

	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P	Q	R	S	T	U	V	W	X	Y	Z
1	pES1033	p70_EGLN3_L4_CtermRNAP(CGG)	A	Amp	pTU1-A-RFP	pES0005	pES0013	pES0022	pES0008	pBP-L3S2P21	ok	ok	ok	ok	ok											
2	pES1119	pVan_NLRP3.700_L2_CtermRNAP(CGG)	A	Amp	pTU1-A-RFP	pES0028	NLRP3_700_frag	Linker 2	pES0008	pBP-L3S2P21	ok	ok	ok	ok	ok											
3	pES1121	pVan_asyn_L3_CtermRNAP(CGG)	A	Amp	pTU1-A-RFP	pES0028	pES0015	pES0022	pES0008	pBP-L3S2P21	ok	ok														
4	pES1131	PSP_EGLN3_L3_CtermRNAP(CGG)	A	Amp	pTU1-A-RFP	pES0030	pES0013	pES0002	pES0008	pBP-L3S2P21																
5	pES1135	p70_NLRP3.200_L2_CtermRNAP(CGG)	A	Amp	pTU1-A-RFP	pES0005	NLRP3_200_frag	Linker 2	pES0008	pBP-L3S2P21																
6	pES1136	p70_NLRP3.200_L3_CtermRNAP(CGG)	A	Amp	pTU1-A-RFP	pES0005	NLRP3_200_frag	pES0002	pES0008	pBP-L3S2P21																
7	pES1137	p70_NLRP3.200_L4_CtermRNAP(CGG)	A	Amp	pTU1-A-RFP	pES0005	NLRP3_200_frag	pES0022	pES0008	pBP-L3S2P21																
8	pES1138	p70_NLRP3.700_L2_CtermRNAP(CGG)	A	Amp	pTU1-A-RFP	pES0005	NLRP3_700_frag	Linker 2	pES0008	pBP-L3S2P21																
9	pES1139	p70_NLRP3.700_L3_CtermRNAP(CGG)	A	Amp	pTU1-A-RFP	pES0005	NLRP3_700_frag	pES0002	pES0008	pBP-L3S2P21																
10	pES1140	p70_NLRP3.700_L4_CtermRNAP(CGG)	A	Amp	pTU1-A-RFP	pES0005	NLRP3_700_frag	pES0022	pES0008	pBP-L3S2P21																
11	pES1141	weak_EGLN3_L3_CtermRNAP(CGG)	A	Amp	pTU1-A-RFP	pBP-J23108	pES0013	pES0002	pES0008	pBP-L3S2P21																
12	pES1142	weak_asyn_L3_CtermRNAP(CGG)	A	Amp	pTU1-A-RFP	pBP-J23108	pES0015	pES0002	pES0008	pBP-L3S2P21																
13	pES1143	weak_NLRP3.200_L3_CtermRNAP(CGG)	A	Amp	pTU1-A-RFP	pBP-J23108	NLRP3_200_frag	pES0002	pES0008	pBP-L3S2P21																
14	pES1144	weak_NLRP3.700_L3_CtermRNAP(CGG)	A	Amp	pTU1-A-RFP	pBP-J23108	NLRP3_700_frag	pES0002	pES0008	pBP-L3S2P21																
15	pES1145	weak_NLRP3.200_L4_CtermRNAP(CGG)	A	Amp	pTU1-A-RFP	pBP-J23108	NLRP3_200_frag	pES0022	pES0008	pBP-L3S2P21																
16	pES1146	weak_NLRP3.700_L4_CtermRNAP(CGG)	A	Amp	pTU1-A-RFP	pBP-J23108	d	pES0022	pES0008	pBP-L3S2P21																
17	pES1147	psp_EGLN3_L2_CtermRNAP(CGG)	A	Amp	pTU1-A-RFP	pES0030	pES0013	Linker 2	pES0008	pBP-L3S2P21																
18	pES1148	psp_asyn_L2_CtermRNAP(CGG)	A	Amp	pTU1-A-RFP	pES0030	pES0015	Linker 2	pES0008	pBP-L3S2P21																
19	pES1149	psp_NLRP3.200_L2_CtermRNAP(CGG)	A	Amp	pTU1-A-RFP	pES0030	NLRP3_200_frag	Linker 2	pES0008	pBP-L3S2P21																
20	pES1150	psp_NLRP3.700_L2_CtermRNAP(CGG)	A	Amp	pTU1-A-RFP	pES0030	NLRP3_700_frag	Linker 2	pES0008	pBP-L3S2P21																
21	pES1151	psp_EGLN3_L4_CtermRNAP(CGG)	A	Amp	pTU1-A-RFP	pES0030	pES0013	pES0022	pES0008	pBP-L3S2P21																
22	pES1152	psp_asyn_L4_CtermRNAP(CGG)	A	Amp	pTU1-A-RFP	pES0030	pES0015	pES0022	pES0008	pBP-L3S2P21																
23	pES1153	psp_NLRP3.200_L4_CtermRNAP(CGG)	A	Amp	pTU1-A-RFP	pES0030	NLRP3_200_frag	pES0022	pES0008	pBP-L3S2P21																
24	pES1154	psp_NLRP3.200_L4_CtermRNAP(CGG)	A	Amp	pTU1-A-RFP	pES0030	NLRP3_700_frag	pES0022	pES0008	pBP-L3S2P21																

Golden Gate Assembly - Level 2

Table22

	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P	Q	R	S	T	U	V	W	X	Y	Z
1	pES2069	AP1 strong RNAP (CGG), strong tE1 , strong E2	SmR	pTU2-A-p15a SmR	pES1001	pES1003	pES1004	pES1094	pES1095																	
2	pES2070	AP1 weak RNAP (CGG), strong tE1 , strong E2	SmR	pTU2-A-p15a SmR	pES1071	pES1003	pES1004	pES1094	pES1095																	

O/N cultures

picked 3 cultures each of pES1114 and pES1131 (yesterdays cultures turned red),
sent them for NightSeq

S2060 propagation assay - qPCR

first run/rerun
LB: 27.0 / 30.2
LB + tet + phages: 22.8 / 23.4
S2060 from cryo stock: 15.7 / 14.8
S2060 old competent stock: 15.3 / 15.0
S2060 31.08. competent stock: 13.3 / 13.4
S2060 Lukas' competent stock: 14.5 / 14.4
1117-44-00 (uninduced): 14.0 / 14.6
1076-08-00: 14.6 / 14.8
S2208: 17.6 / 8.7
--> Lukas thinks something is contaminated
--> we sent S2060 old comp, S2208 and SIAH1 phages for NightSeq with

Repeat same qPCR, once with differen H2O, once with primer aliquote from Lucas and once with differen Hotfire
--> none of the qPCR components were contaminated, as all of the variants had the same result - the LB negative control was slightly contaminated thoug, and the S2208 results were off in the first run

Van toxicity test
innoculated 3 cultures each of 1076-08-00 and S2208, control of LB+Kan+Carb+Tet (3 mL, innoculated with 150 µL of o/n culture),
20 µL of SIAH1 10^8 PFU/mL added to each tube,
Varying concentrations of vanillic acid added (10 µM, 100 µM and 500 µM)
--> let phages propagate for 5 hours before taking out 1 mL each for qPCR, then put back in incubator

qPCR Results:
LB+500: 24.8
S2208+10: 9.3
S2208+100: 9.1
S2208+500: 9.1
1076-08+10: 18.6
1076-08+100: 19.0
1076-08+500: 18.6
--> Van does not seem to prevent propagation of phages in propagation capable cells

Phage contamination test
since one of our hypotheses is, that our phages could be contaminated with proper M13 phages with gIII, we again test our phage aswell as P227 and TnpB on our S2060 cryo, on Lukas' S2060, on S2208 and LB.
2mL cultures were seeded with 10 µL of o/n culture, 10 µL of SIAH1 / TnpB (1:10^3) or 100 µL of P227 (1:10^4)

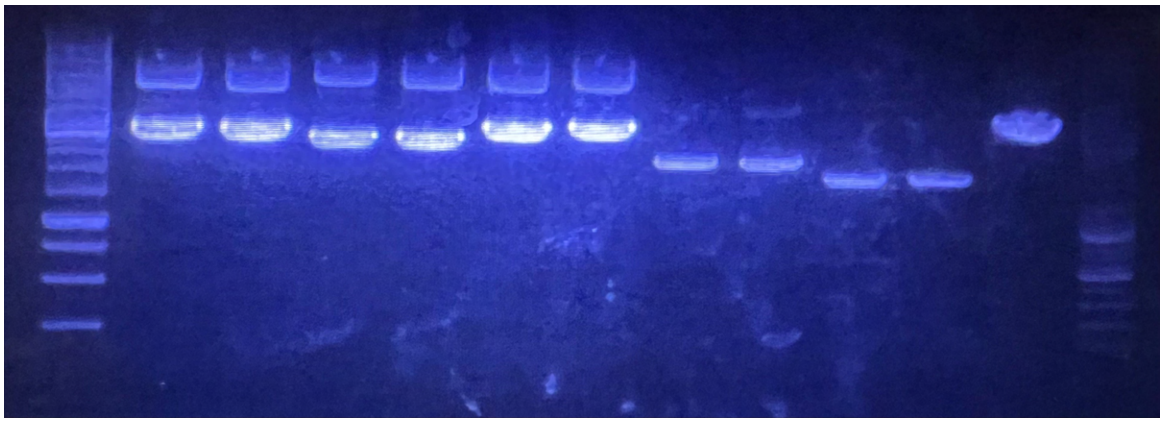
Transformations
5 µL of each transformation on 60 µL of DH5a
10 µL of Lvl1 assemblies in 100 µL DH5a

DIVENDRES, 27/9/2024

- [x] figure out what is happening
  --> partly, we are doing our best
- [x] miniprep pES1114 and pES1131
  --> send them for EconomySeq
- [ ] assemble pES2068 with pES1114
- [x] check plates of pES1135-1154 and pick colonies

Co-transformations
I prepared cryo-stocks of the 1120/1123/1123-44-00 co-transformations that we picked yesterday - once we get the sequencing results, we will need to label them to show that they are confirmed
The rest of the cultures was miniprepped and loaded onto a gel - the other minipreps also were:
GR1 kb - 20 A - 20 B - 23 A - 23 B - 24 A - 24 B - 14 A - 14 B - 14 C - 31 A - 31 B - 31 C - GR mix

IMG_6974.jpeg



--> expecting the plasmids to run smaller on the gel, pES1114 could be correct, pES1131 C looks promising and all co-transformants look good

Miniprep

pES1114 A: 166 ng/µL
pES1114 B --> was red
pES1114 C: 140 ng/µL
--> adjusted to 100 ng/µL
pES1131 D: 198 ng/µL
pES1131 E: 161 ng/µL
pES1131 F: 342 ng/µL

Golden Gate Assembly

Assembled once with pES1114 A and once with pES1114 C (labelled as pES2068 A/B). New GG protocol (iDEC --> GG BsmBI)

Table24

	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P	Q	R	S	T	U	V	W	X	Y	Z
1	pES2068	AP1 PSP RNAP (CGG), strong tE1, strong E2	SmR	pTU2-A p15a SmR	pES1114	pES1003	pES1004	pES1094	pES1095																	

Phage contaminaton test - qPCR

I additionally also added sampes of each bacteria without phages onto the plate

Average Cp

	S2060 cryo	S2060 cryo	S2060 Lukas	S2208	LB
1	w/o phages	28.5	29.2	29.0	28.9
2	SIAH1	16.3	16.1	8.7	24.9
3	TnpB	13.2	12.6	9.4	21.7
4	P227	19.8	13.6	10.3	21.9

Lukas suggested running our qPCRs with Ta = 53 °C instead
--> overall the results look the same, but the negative controls are cleaner, so we will use this protocol from now on

Average Cp1

	S2060 cryo	S2060 cryo	S2060 Lukas	S2208	LB
1	w/o phages	31.2	31.4	31.2	31.8
2	SIAH1	15.7	15.4	7.9	24.0
3	TnpB	12.6	11.9	8.6	20.1
4	P227	18.0	12.9	9.5	20.6

S2060 troubleshooting - propagation assay
we collected our current results to investigate different hypotheses for how they could explain our results or be disproven by them
https://docs.google.com/presentation/d/1jjO7OcZpnvN4IfQVqw9jkugSDbfUcIer/edit?usp=share_link&ouid=101049355695909805953&rtpof=true&sd=true

Lukas gave us three older stocks of S2060 for testing, to see wheter they also propagate phages in the same way
--> one stock of electrocompetent S2060 without date (S2060 electro), one competent stock from 09.2021 (S2060 sept21) and one cryo stock from 01.2021 (S2060 jan21)

We perform another o/n propagation on 2 mL of different bacterial cultures, innoculated with 15 µL of SIAH1 (10^8 PFU/mL)
As a negative control we also add DH5a to our assay
To see whether seeding density affects the result a lot, we also included samples where only 1 µL of SIAH1 (6*10^6 PFU/mL) was added.

Well1

	1	2	3	4
A	DH5a + SIAH1	S2060 electro + SIAH1	S2060 sept21 + SIAH1	S2060 jan21 + SIAH1
B	S2060 cryo (innoculated with 50 µL of previous o/n propagation on S2060 cryo)	S2060 cryo + SIAH1	LB + SIAH1	S2208 + SIAH1
C	LB (innoculated with 50 µL of previous o/n propagation on S2060 cryo)	S2060 cryo + 1 µL SIAH1	LB + 1 µL SIAH1	S2208 + 1 µL SIAH1

Plaque Assays
Lukas also hypothesized, that the propagation that we measure is just the initial amplification of the first patch of phages, but that the offspring does not have gIII --> made 2 plaque assays from the samples of 26.09. o/n propagation

Each plate has a 0 to -7 dilution of the phages propagated on:
S2060 cryo, S2208 and LB

2 more plaque assays with S2208 propagation from 26.09. and 1076-08-00 propagation from 25.09.

DISSABTE, 28/9/2024

- [x] do qPCR of the propagation assay on different older S2060 strains
  --> variables: S2060 strain, SIAH1 innoculation amount, double o/n

- [x] miniprep pES2069 and 2070 or repick if they become red (looked like they might)
- [x] miniprep pES1135-1154 colonies (as many as possible, repick cultures that become red)
- [ ] make more NLRP3-200 (PCR)
- [x] repeat assembly of pES1132-1134
- [x] Transform pES2068

In lab: Pau, Gabriel

qPCR for phage propagation assay of different older S2060 strains
See Excel.

Plaque assay results
While SS208-derived phages show growth on all dilutions, whereas phages

Sequencing analysis
pES1114 A/C --> both are good, A is better
pES1131 --> bad

Assembly of pES1131-1134

Table25

	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P	Q	R	S	T	U	V	W	X	Y	Z
1	pES1131	PSP_EGLN3_L3_CtermRNAP(CGG)	A	Amp	pTU1-A-RFP	pES0030	pES0013	pES0002	pES0008	pBP-L3S2P21																
2	pES1132	PSP_asyn_L3_CtermRNAP(CGG)	A	Amp	pTU1-A-RFP	pES0030	pES0015	pES0002	pES0008	pBP-L3S2P21																
3	pES1133	PSP_NLRP3.200_L3_CtermRNAP(CGG)	A	Amp	pTU1-A-RFP	pES0030	NLRP3_200_frag	pES0002	pES0008	pBP-L3S2P21																
4	pES1134	PSP_NLRP3.700_L3_CtermRNAP(CGG)	A	Amp	pTU1-A-RFP	pES0030	NLRP3_700_frag	pES0002	pES0008	pBP-L3S2P21																

Transformation pES2068A/C

Transformed 5uL into 20uL DH5a competent cells.

Cultures from cryo stocks

Thawed the following strains twice (one will be inoculated with phages tonight, one will be used for phage propagation assay tomorrow morning). For strains with vanillic acid promoters, three tubes were inoculated, one for 0 uM, one for 10 uM, one for 100uM. All strains were grown with appropriate antibiotic including Tet.

- S2060
- S2208
- 0000-08-00
- 0000-44-00
- 1076-08-00
- 1035-08-00
- 1102-08-00 (disrupted EGLN3)
- 1079-44-00
- 1117-44-00
- 1118-44-00
- 1122-44-00
- 1125-44-00

Additionally, the following cultures were co-transformed and will be picked tomorrow early in the morning:

- 2008 + 1084
- 2008 + 1102
- 2008 + 1151A/B
- 2008 + 1152A/B
- 2008 + 1153A/B
- 2008 + 1154A/B
- 2044 + 1084
- 2044 + 1102
- 2044 + 1151A/B
- 2044 + 1152A/B
- 2044 + 1153A/B
- 2044 + 1154A/B

O/N phage propagation assay in liquid cultures

Previously mentioned liquid cultures were split into two and infected with 10^4 pfu/mL of SIAH1 or TadA, respectively. Strains with vanillic acid promoters were supplemented with either 0uM, 10uM, or 100uM vanillic acid.

DIUMENGE, 29/9/2024

- [x] passage o/n propagation assays
- [ ] do qPCR after second propagation

Double propagation assay / PANCE

out of the innoculated cultures, only the ones with pES2044 grew, most likely due to an antibiotic mismatch
the o/n propagations of these cultures that were induced with 100 µM Van did not grow / died (except for 1079, which does not have inducible Cterm-RNAP)

The following cultures were passaged:
1079-44-00 0/10/100 Van
1117-44-00 0/10 Van
1118-44-00 0/10 Van
1122-44-00 0/10 Van
1125-44-00 0/10 Van
0000-44-00
--> the bacteria was spun down and the phages passaged 1:20

5 different conditions were used to control for different variables:

- co-cultures innoculated with phages from o/n propagation in co-cultures
  --> double o/n samples, like PANCE with one passage
- S2060 cultures innoculated with phages from o/n propagation in co-cultures
  --> to control for background of first propagation round
- LB innoculated with phages from o/n propagation in co-cultures
  --> to measure o/n propagation result
- co-cultures innoculated with LB phage innoculated control
  --> to control for left over phage population from initial phage innoculation
- S2208 with phages from o/n propagation in co-cultures
  --> as a positive control and to potentially amplify differences in phages after o/n propagation

--> propagation was run for 6 hours

100 µM Van propagation Assay

since the bacteria that was induced with 100 µM Van did not grow or even died, we tried another propagation assay with cultures 1117-44-00, 1118-44-00, 1122-44-00 and 1125-44-00.
The cells were grown o/n without induction. They were then diluted 1:2 and induced with 100 µM Van and infected with 10^6 PFU/mL SIAH1 phages.
As a negative control the cultures were also infected uninduced and phages were added to LB.
--> propagation was run for 5 hours

Plaque Assay

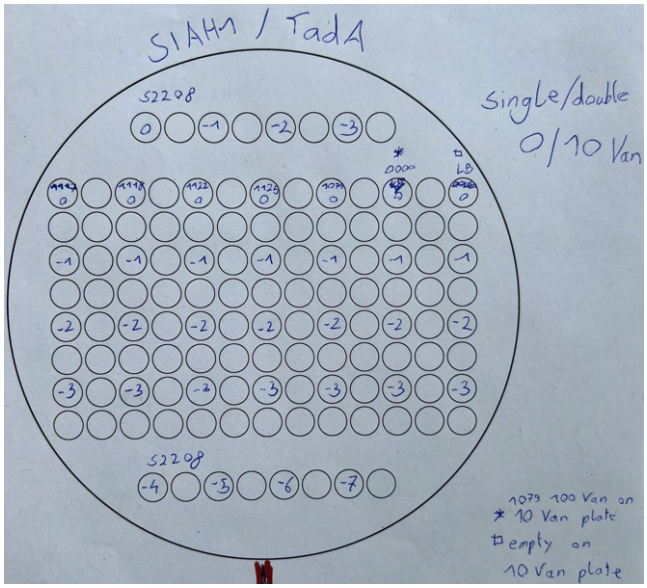
Plaque assays were made with the samples of the o/n propagation, dilutions of 0 to -3 were put on for each sample and the S2208 propagation was used as a positive control in dilutions of 0 to -7.
Two plaque assays for SIAH1 (0 and 10 Van) and two for TadA (0 and 10 Van)

Samples:

1117-44-00, 1118-44-00, 1122-44-00, 1125-44-00, 1079-44-00, 0000-44-00 and LB

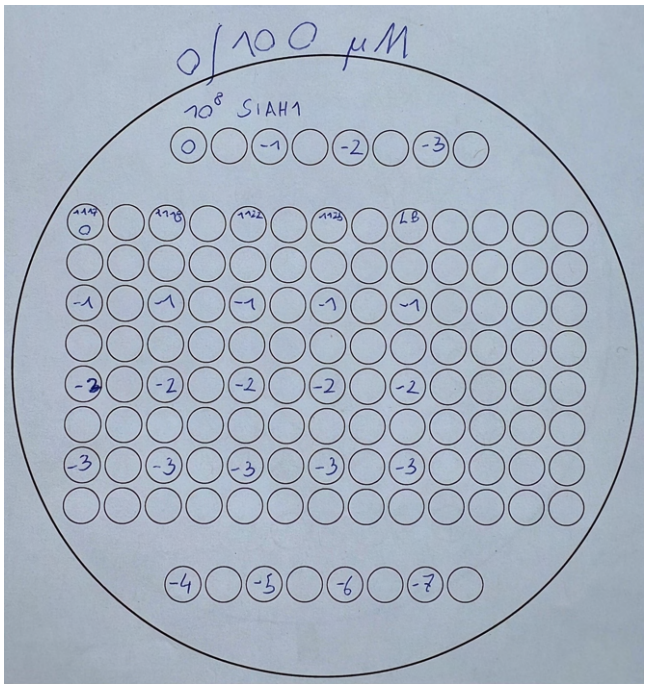
--> the same plaque assays were also made from the double o/n propagations of the same samples

Layout 1



Another 2 plaque assays were prepared for the 100 µM Van propagation --> one for 0 Van and one for 100 Van

Layout 2



C-term RNAP toxicity confirmation assay

to confirm that it is actually the Cterm RNAP that is toxic to the bacteria, we innoculated cultures and let them grow from the same OD

- 2048-08-DP6, 2054-08-DP6 and 2055-08-DP6 have only Cterm RNAP inducable and 3 different substrates
- 0000-08-00 as a negative control
- 0000-44-00 and 1079-44-00 only have Nterm RNAP inducable

--> cultures were grown from cryostock and diluted to OD 0.1
--> each culture was induced with either 0, 10, 50 or 100 µM Van, and either infected or not infected with phages (10^6 PFU/µL) for the 10 µM condition

Layout

	0 µM Van	10 µM Van	50 µM Van	100 µM Van	10 µM Van + SIAH1
1	0000-08-00	0000-08-00	0000-08-00	0000-08-00	0000-08-00
2	2048-08-DP6	2048-08-DP6	2048-08-DP6	2048-08-DP6	2048-08-DP6
3	2054-08-DP6	2054-08-DP6	2054-08-DP6	2054-08-DP6	2054-08-DP6
4	2055-08-DP6	2055-08-DP6	2055-08-DP6	2055-08-DP6	2055-08-DP6
5					
6	0000-44-00	0000-44-00	0000-44-00	0000-44-00	0000-44-00
7	1079-44-DP6	1079-44-DP6	1079-44-DP6	1079-44-DP6	1079-44-DP6

Sequencing

picked 2 colonies from each co-transformation and sent for NightSeq:
1084-44-00, 1102-44-00, 1151-44-00, 1152-44-00, 1153-44-00, 1154-44-00, 1102-08-00, 1151-08-00, 1152-08-00, 1153-08-00, 1154-08-00
--> with phage shock inducible CtermRNAP
--> picked cl. A from side A and clone B from side B

also picked the only white culture from both pES2069 and pES2070 and sent for sequencing,
picked 2 cultures from pES0002 and 5 cultures of pES2068 (phage shock NtermRNAP)

also prepared EcoSeq plate for the final Lvl1 assemblies (pES1135-1154)

DILLUNS, 30/9/2024

- [ ] start PANCE
- [x] check plaque assays
- [ ] bring sequencing orders down before 17:30
- [x] put out LB Agar bottles for autoclaving
- [x] check sequencing of 1120/1123/1123-44-00 co-transformations and make stickers for the cryo-stocks if they are correct

Sequencing Analysis

pES1114 --> was already confirmed, but A being good is confirmed, B confirmed to be empty backbone, C sequencing failed

pES1131 --> were already shown to be wrong

2048-08-DP6 sequencing with o118 looks weird, if we want to use it we should send it with KanStart or something again
Co-transformations of 1120-44-00, 1123-44-00 and 1124-44-00 are all good

Sequencing for glll for both S2060 and SIAH1 failed, confimring it is not presen, S2208 was sequenced
SIAH1 sequencing confirms it is still good

Overnight Propagation - Plaque Assay

single o/n

	Strain	0 µM Van + SIAH1	10 µM Van + SIAH1	0 µM Van + TadA	10 µM Van + TadA
1	LB	2.5*10^4	/	2.25*10^4	/
2	0000-44-00	<250	/	2.5*10^3	/
3	1117-44-00	<250	4.5*10^4	uncountable	<250
4	1118-44-00	250	2.75*10^4	0.5*10^3	<250
5	1122-44-00	<250	2.5*10^4	2*10^3	<250
6	1125-44-00	10^3	2.75*10^4	4.25*10^3	<250
7	1079-44-00	750	3.5*10^4	1.75*10^3	4*10^4

double o/n

	Strain	0 µM Van + SIAH1	10 µM Van + SIAH1	0 µM Van + TadA	10 µM Van + TadA
1	LB	1.25*10^3	/	2.25*10^3	/
2	0000-44-00	<250	/	<250	/
3	1117-44-00	<250	2.25*10^3	<250	5.25*10^3
4	1118-44-00	<250	2*10^3	<250	<250
5	1122-44-00	<250	0.75*10^3	<250	<250
6	1125-44-00	<250	0.5*10^3	<250	1.5*10^3
7	1079-44-00	<250	0.75*10^3	<250	<250

100 µM Van induction vs no induction (on dense culture) - Plaque Assay

Table26

	Strain	0 µM [PFU/mL]	100 µM [PFU/mL]
1	LB	2.75*10^6	/
2	1117-44-00	3*10^4	4*10^6
3	1118-44-00	7*10^4	3.5*10^6
4	1122-44-00	5.75*10^4	10^6
5	1125-44-00	3*10^5	3.5*10^6

--> for a graph see google drive
--> concluding from this data, we se that if there is propagation on our bacteria it has to be at a range of 1-1.5x propagation, realistically the data could suggest that the Van induced bacteria die and the phages therefore don't waste themselves on bacteria that can not propagate them, the difference between TadA and SIAH1 looks promising thoug, although both TadA plates have a big questionable outlier

C-term RNAP toxicity confirmation assay
seems like none of the strains that we tested actually carred about the induction - they seem to grow equally well - will add the data to the drive soon

Miniprep
pES2068 A: 26 ng/µL
pES2068 B: 19 ng/µL
pES2068 C: 152 ng/µL
pES2068 D: 9 ng/µL
pES2069 D: 118 ng/µL
pES2070 D: 167 ng/µL

Co-transformation
1014-08-00, 1029-08-00, 1031-08-00, 1120-08-00, 1131-08-00, 1147-08-00, 1148-08-00, 1084-69-00, 1117-69-00, 1151-69-00, 1152-69-00
1 µL of each plasmid in 30 µL S2060, A clone of all unconfirmed plasmids was used

Golden Gate Assembly
standard protocol

totally the last assemblies, I swear

	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P	Q	R	S	T	U	V	W	X	Y	Z
1	pES1155	p70_NtermRNAP_L4_Ub	A	Amp	pTU1-A-RFP	pES0005	pES0003	pES0022	pES0004	pBP-L3S2P21																
2	pES1156	p70_NtermRNAP_L2_Ub	A	Amp	pTU1-A-RFP	pES0005	pES0003	Linker 2	pES0004	pBP-L3S2P21																
3	pES1157	pVan_NtermRNAP_L4_Ub	A	Amp	pTU1-A-RFP	pES0028	pES0003	pES0022	pES0004	pBP-L3S2P21																
4	pES1158	pVan_NtermRNAP_L2_Ub	A	Amp	pTU1-A-RFP	pES0028	pES0003	Linker 2	pES0004	pBP-L3S2P21																

Lab Journal - October

Project: iDEC 2024

Author: Jakob Wimmer

Entry Created On: 30 Sep 2024 11:30:43 UTC

Entry Last Modified: 08 Oct 2024 09:24:28 UTC

Export Generated On: 08 Oct 2024 09:24:41 UTC

DIMARTS, 1/10/2024

- [x] start PANCE
- [x] transform pES1131-1134
- [x] transform pES1155-1158

Sequencing Analysis

pES0002: both look good, miniprep both and mix them

pES2068: only clone C looks good, send for full plasmid seq and discard the others

pES2069 D: sequencing looks good, send for full-plasmid seq

pES2070 D: fwd sequencing is missing, could still send for full-plasmid seq, but I don't think we will use it anyways

"Final Assemblies":

pES1033: has Linker2, is pES1018 --> trash

pES1119: clone A looks good, B was not sequenced

pES1121: clone A looks like a mixture, but I'll just keep it anyways, B is sequenced at wrong location and short

pES1131: clone A looks good, clone B is sequenced at wrong location

pES1135: both are correct, kept A

pES1136: both are correct, kept A

pES1137: B is correct

pES1138: both are NNN

pES1139: both are NNN

pES1140: B is correct

pES1141: both are correct, kept B

pES1142: B is correct

pES1143: are actually pES1144, kept A

pES1144: both are NNN, but have it from pES1144

pES1145: both are correct, kept A

pES1146: A is correct

pES1147: A is correct

pES1148: are actually pES1149, kept A

pES1149: A is E2, B is gIII, but have it from pES1148

pES1150: both are NNN

pES1151: A is correct, B has asyn and has another promoter

pES1152: A is short, B misses asyn

pES1153: B is correct, A misses substrate

pES1154: both are correct, kept A

pES2070: is NNN

Co-transformations:
the co-transformants were sent for sequencing with o018 instead of o118, will need to send again to confirm Level 2 plasmid
1084-44-00: both clones actually contain pES1111, a plasmid we do not have
1102-44-00: actually contain pES1084, must have gotten swapped at some point
1151-44-00: clone A looks good, clone B seems to actually be pES1081
1152-44-00: clone A is sequenced in wrong location, clone B is missing asyn --> also seen in EcoSeq
1153-44-00: clone A is missing NLRP3, clone B looks good
1154-44-00: both look good

1102-08-00: sequencings are short or in wrong location
1151-08-00: sequencings seem to be random stuff
1152-08-00: sequencings failed
1153-08-00: sequencings are super short
1154-08-00: sequencings failed

Co-transformations
picked two colonies each of the co-transformations that were done yesterday and sent them for sequencing (24-well plate in incubator)

Transformations
have no more DH5a, transformed in S2060 instead
pES1132-1135 --> 5 µL of assembly each in 50 µL S2060
pES1155-1158 --> 5 µL of assembly each in 100 µL S2060

Golden Gate

Table1

	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P	Q	R	S	T	U	V	W	X	Y	Z
1	pES1138	p70_NLRP3.700_L2_CtermRNAP(CGG)	A	Amp	pTU1-A-RFP	pES0005	NLRP3_700_frag	Linker 2	pES0008	pBP-L3S2P21																
2	pES1139	p70_NLRP3.700_L3_CtermRNAP(CGG)	A	Amp	pTU1-A-RFP	pES0005	NLRP3_700_frag	pES0002	pES0008	pBP-L3S2P21																
3	pES1143	weak_NLRP3.200_L3_CtermRNAP(CGG)	A	Amp	pTU1-A-RFP	pBP-J23108	NLRP3_200_frag	pES0002	pES0008	pBP-L3S2P21																
4	pES1148	psp_asyn_L2_CtermRNAP(CGG)	A	Amp	pTU1-A-RFP	pES0030	pES0015	Linker 2	pES0008	pBP-L3S2P21																
5	pES1150	psp_NLRP3.700_L2_CtermRNAP(CGG)	A	Amp	pTU1-A-RFP	pES0030	NLRP3_700_frag	Linker 2	pES0008	pBP-L3S2P21																
6	pES1152	psp_asyn_L4_CtermRNAP(CGG)	A	Amp	pTU1-A-RFP	pES0030	pES0015	pES0022	pES0008	pBP-L3S2P21																

PANCE
For PANCE we will have duplicates of each sample and will run it in parallel at two passage stringencies
Additionally, multiple strains will be used. For Van inducible strains, we will propagate at both 10 µM and 1 µM Vanillic Acd
1008-09-00, 1033-09-00, 1035-08-00, 2048-08-DP6, 2054-08-DP6, 2055-08-DP6, 1084-44-00, 1117-44-00, 1120-44-00, 1123-44-00, 1124-44-00, 1151-44-00, 1152-44-00, 1151-08-00, 1152-08-00
--> 1033-09-00 and 1035-08-00 will also be innoculated with TadA as a negative control

PANCE Layout - Half

	A	B	1 µM induction	1 µM induction	10 µM induction	10 µM induction
1	2048-08-DP6	2048-08-DP6	2054-08-DP6	2054-08-DP6	2055-08-DP6	2055-08-DP6
2	1152-08-00	1152-08-00	1084-44-00	1084-44-00	1084-44-00	1084-44-00
3	1151-08-00	1151-08-00	1117-44-00	1117-44-00	1117-44-00	1117-44-00
4	1008-09-00	1008-09-00	1120-44-00	1120-44-00	1120-44-00	1120-44-00
5	1033-09-00	1033-09-00	1123-44-00	1123-44-00	1123-44-00	1123-44-00
6	1035-08-00	1035-08-00	1124-44-00	1124-44-00	1124-44-00	1124-44-00
7	1033-09-00 TadA	1033-09-00 TadA	1151-44-00	1151-44-00	1151-44-00	1151-44-00
8	1035-08-00 TadA	1035-08-00 TadA	1152-44-00	1152-44-00	1152-44-00	1152-44-00

the layout will be on the plate twice, from the second day onward
the cultures were diluted from the tube 1:5 (200 µL bacteria + 800 µL LB+antibiotics)
10 µL from 10^6 phage dilution and 10 µL of 1/0.1 mM Vanillic Acid were added

Daily Protocol - PANCE

All needed phage and vanillic acid dilutions are in the fridge in the PANCE October box.
LB with the correct antibiotics aswell as aliquotes thereof are in the fridge and colorcoded.
All needed bacteria are in the fridge.

Preparation Bacteria

1. Fill 96-well plate with LB as depicted in the color code of the layout, 800 µL per well
2. add 200 µL of bacteria to the correct well
3. put the plate in the shaker to warm up and get into log-phase

qPCR

1. prepare 250x MasterMix and fill the entire top half of the qPCR plate
2. load 2 µL of each sample using the 12 channel pipette, technical replicates should be next to each other
3. run the qPCR

Passaging

after the bacteria had some time to grow, passage from the old to the new PANCE plate using the 8 channel pipette
--> use the current lower and higher stringency

DIMECRES, 2/10/2024

- [x] passage PANCE
- [x] pick pES1132-1135 and pES1155-1158 colonies and send them for NightSeq
- [x] transform missing variants

PANCE

passaged 1:10 and 1:50, low stringency is always left, high stringency is right

qPCR results:

9th row is MsterMix, nothing sticks out particularely

Table2

	A	B	C	D	E	F	G	H	I	J	K	L
1	30.16	30.33	30.19	30.27	30.45	30.52	30.19	29.93	30.21	30.27	27.76	30.1
2	22.09	22.67	22.69	22.61	22.49	22.2	22.74	22.68	23.24	23.17	23.28	23.62
3	22.71	22.08	22.79	22.71	21.04	21	20.58	20.62	27.82	27.91	27.95	27.6
4	22.14	21.96	22.46	22.33	22.99	22.72	22.51	22.68	24.27	24.07	24.13	24.19
5	21.49	21.67	22.08	22.25	24.86	25.31	23.86	23.96	27.92	28.51	28.05	28.05
6	21.2	21.54	21.88	21.75	22.51	22.36	22.17	21.98	24.05	24.65	25.08	24.96
7	21.78	21.72	22.1	22.19	22.97	22.95	23.43	23.05	20.51	20.45	20.85	20.84
8	22.05	22.07	21.94	21.81	22.81	23.07	22.78	23.06	23.26	22.73	22.88	22.78
9	32.68	33.27	32.7	32.95	32.65	32.2	32.87	32.6	32.78	32.91	32.73	33.28

DIJOUS, 3/10/2024

- [x] passage PANCE
- [x] pick missing variants --> also sent for NightSeq
- [x] miniprep pES1132-1135 and pES1155-1158
- [x] assemble pES2071-2074
- [x] co-transform with pES1132-1135 --> used cl. A and B with pES2008, 2044, 2069

PANCE

when I arrived the PANCE plate was on our bench, not in the shaker, maybe I never even put it in the shaker in the first place
I will passage like normal and then put both plates into the shaker again --> should maybe run qPCR of both tomorrow and then decide from which one to continue passaging

passaged 1:10 and 1:50

qPCR

low stringency

	A	B	C	D	E	F	G	H	I	J	K	L
1	32.64	32.65	32.75	32.81	32.36	32.79	32.6	33.05	32.52	32.46	32.31	32.01
2	26.45	25.83	24.88	25.04	25.12	25.48	25.14	24.86	25.82	26.02	25.82	25.92
3	25.12	25.68	26.08	25.87	23.85	23.76	23.11	23.18	29.81	30.17	30.83	30.74
4	24.84	24.52	24.69	25.02	24.15	24.33	25.44	25	25.89	26.02	25.92	26.1
5	24.55	24.45	23.77	23.58	27.48	27.73	26.22	26.44	31.91	31.74	31.33	31.32
6	23.79	23.85	24.63	24.55	25.17	25.73	25.15	24.89	27.35	27.17	27.51	27.72
7	24.08	23.95	24.05	23.98	25.87	25.65	25.72	25.49	23.65	23.24	23.83	23.35
8	24.36	24.45	23.83	23.91	25.69	25.91	25.55	25.79	25.82	25.7	25.55	25.98

high stringency

	A	B	C	D	E	F	G	H	I	J	K	L
1	33.61	33.45	33.91	33.61	33.24	32.86	33.74	33	33.69	34.45	34.95	28.26
2	29.27	27.76	30.02	28.2	27.89	27.97	27.5	28.7	27.21	27	34.44	26.88
3	28.68	27.86	27.99	28.17	27.2	26.82	25.67	25.67	32.73	32.75	34.33	32.11
4	26.57	27.34	25.69	27.64	27.62	29.19	26.65	26.76	28.15	28	34.43	26.45
5	25.93	26.63	27.92	28.05	29.44	29.71	28.77	27.58	32.91	32.91	34.12	31.83
6	26.96	26.7	27.44	27.09	26.98	26.87	25.92	27.05	28.72	28.73	34.76	27.33
7	25.96	26.12	26.8	26.46	27.94	26.88	27.98	27.96	25.77	25.27	35.01	25.22
8	25.94	26.16	26.17	25.63	27.44	26.8	29.58	29.52	28.53	28.11	34.88	27.77

--> can't conclude anything yet really, I am hopefull about the phage shock promoter though
--> all of the strains with DP6 seem to have lost F-pilus

DIVENDRES, 4/10/2024

- [x] passage PANCE
- [x] do qPCR of Day 3 AND Day 2 (I didn't put it in the shaker probably)

If there is time, otherwise I will do it on Saturday:

- [x] sequencing analysis

In lab: Pau

Sequencing analysis

pES1132: is also substrate less, misses the asyn, second assembly where this happens --> discard the co-transformants
pES1133: cl. B has an insertion in the promoter (maybe it is a sequencing mistake, not sure how big of a problem that is), cl. A actually contains NLRP3-700, it is pES1134
pES1134: cl. A looks good, keep that co-transformation
pES1155: both look perfect
pES1156: both look perfect
pES1157: does not look ok
pES1158: A might have a deletion in RNAP but otherwise looks good, B is empty backbone

1014-08-00: both have pES2008, no lvl1 sequenced
1029-08-00: cl. B is good, A is missing lvl1
1031-08-00: Lvl1 not confirmed
1120-08-00: pES2008 not sequenced, Lvl1 not confirmed
1131-08-00: Lvl1 not confirmed
1147-08-00: cl. A looks good
1148-08-00: Lvl1 not confirmed

1084-69-00: both are good, use cl. A
1117-69-00: both are good, use cl. A
1151-69-00: both are good, use cl. A
1152-69-00: not good

Older co-transformants:

2048-08-00, 1152-08-00 (with wrong pES1152) and 1151-08-00 now have pES2008 confirmed

1151-44-00 and 1152-08-00 (with wrong pES1152) now have pES2044 confirmed

PANCE

Performed qPCR for days 2 and 3.

Passaged PANCE BUT got the layout wrong (this is ment for Jakob, wait for me for tomorrow before taking samples hahah)

Re-run results of Day 2:

Low stringency

	A	B	C	D	E	F	G	H	I	J	K	L
1	30.98	32.09	32.88	32.77	33.1	32.32	32.76	31.94	32.25	32.13	32.34	33.16
2	25.82	25.1	24.56	25.22	25.28	24.85	24.88	24.9	22.78	22.59	23.12	22.66
3	25.24	25.47	25.85	24.91	22.44	21.99	22.36	21.71	29.72	29.69	30.22	27.51
4	24.13	24.03	24.21	23.92	23.99	24.14	24.35	24.69	23.2	22.97	23.17	22.85
5	23.83	23.67	23.79	23.48	24.57	24.49	23.58	23.57	30.95	30.89	28.83	30.42
6	23.45	22.75	24.15	22.71	25.63	24.64	24.85	22.49	24.72	22.91	24.87	23.93
7	19.75	20.64	21.06	22.13	24.99	25.32	23.74	25.55	16.88	19.47	17.83	20.46
8	21.63	21.51	21.58	21.23	25.75	25.11	24.77	25.97	23.78	25.08	24.23	25.63

High Stringency

	A	B	C	D	E	F	G	H	I	J	K	L
1	32.77	33	33.25	33.09	31.13	32.57	32.45	31.84	33.02	32.79	29.82	30.8
2	25.47	27.01	27.24	29.3	28.22	27.63	26.23	26.54	23.99	24.12	24.23	24.72
3	26.6	27.49	28.9	27.14	23.98	23.52	23.96	23.66	31.86	30.5	29.68	30.32
4	26.64	26.69	26.23	25.87	28.46	27.44	25.44	25.93	23.34	23.3	23.74	23.46
5	24.66	25.01	26	25.94	26.19	25.58	25.98	25.81	31.59	31.51	31.47	31.68
6	25.35	25.23	25.49	25.77	26.9	24.87	27.24	24.85	24.76	24.33	24.59	24.17
7	20.64	21.62	21.56	21.81	25.64	27.51	26.22	29.21	21	20.87	21.47	21.77
8	21.72	20.82	21.12	20.44	24.77	27.72	26.75	26.84	25.55	27.95	26.84	28.02

Results of Day 3:

Low Stringency_4

	A	B	C	D	E	F	G	H	I	J	K	L
1	32.26	33.49	32.95	32.75	33.01	33.15	33.03	32.76	34.46	32.46	33.51	33.19
2	28.58	27.23	28.58	29.57	28.56	29.71	27.89	29.78	22.59	22.97	22.29	22.54
3	27.96	28.12	29.97	28.47	23.95	22.94	23.29	23.35	29.31	28.59	28.16	28.75
4	25.77	26.98	25.57	26.57	19.28	23.25	22.57	24.78	30.15	19.08	30.67	19.22
5	25.84	28.97	24.89	24.97	22.3	22.44	22.07	22.55	30.89	30.79	30.78	28.87
6	22.83	23.47	24.66	24.17	29.13	29.28	27.84	28.41	21.46	21.11	21.73	21.82
7	19.57	19.58	21.46	21.21	28.51	29.79	29.6	28.76	21.09	20.59	21.77	21.51
8	19.87	19.73	19.95	19.61	29.98	29.54	28.73	27.61	28.93	29.49	29.8	28.55

High Stringency_4

	A	B	C	D	E	F	G	H	I	J	K	L
1	32.65	32.79	32.98	32.81	32.89	33.68	34.05	33.29	33.18	32.49	32.71	31.96
2	32.05	33.03	32.23	29.15	32.25	32.21	32.51	32.55	25.26	25.75	25.68	26.16
3	32.03	30.97	32.81	32.97	26.57	25.45	26.02	25.06	31.79	31.57	28.99	30.21
4	31.23	31.85	29.78	32.62	25.12	30.78	26.03	28.88	32.6	22.11	31.86	21.14
5	31.27	28.77	27.46	32.45	25.3	25.03	25.76	24.06	32.05	32.61	30.53	
6	27.18	30.24	27.88	30.64	31.07	32.54	32.49	31.64	23.49	23.05	23.58	23.31
7	22.7	22.7	23.61	23.35	32.89	32.74	32.56	29.7	24.74	25.45	26.05	25.01
8	22.81	22.63	24.35	24.02	29.78	31.68	32.52	31.95	31.95	31.6	31.68	32.23

DISSABTE, 5/10/2024

- [x] passage PANCE --> will restart tomorrow instead
- [x] do qPCR of Day 3 AND Day 2 (I didn't put it in the shaker probably)
- [x] transform pES2071 A, 2072 A and 2074 A
- [x] pick 1131, 1133 B and 1134 A co-transformants, don't pick 1132 co-transformants
  --> will actually only pick 1131 for now, can pick if we ever need pES1131
- [x] transform pES2071 A, 2072 A and 2074 A

PANCE - Day 4

Low Stringency - 5

	A	B	C	D	E	F	G	H	I	J	K	L
1	32.32	32.47	32.08	33.05	32.72	32.8	32.43	32.94	32.71	32.55	33.32	33.19
2	31.73	31.84	29.83	31.81	31.03	31.6	28.25	30.81	24.91	25.02	23.03	23.29
3	32.46	31.78	29.81	31.76	27.03	26.86	25.99	26.49	31.49	31.62	30.8	31.3
4	30.97	31.31	30.29	28.11	30.85	30.3	30.6	30.18	21.23	20.91	20.67	20.53
5	26.52	26.23	25.47	26.04	23.61	23.46	24.74	23.19	32.47	31.83	32.59	32.13
6	23.34	23.53	25.94	25.93	32.47	31.91	29.91	28.72	23.81	23.34	24.45	24.28
7	14.41		17.48	16.91	31.74	32.01	32.95	31.56	23.02	22.52	22.2	21.45
8	16.89	16.89	18.65	18.32	30.86	30.5	31.64	30.79	31.48	32.1	31.78	31.47

High Stringency - 5

	A	B	C	D	E	F	G	H	I	J	K	L
1	32.2	32.77	32.89	33.1	33.02	33.12	32.77	32.75	33.07	33.45	32.54	32.27
2	32.73	32.98	33.28	32.74	32.85	32.93	32.63	32.46	31.72	31.12	31.09	29.99
3	33.04	32.55	32.94	32.82	33.19	32.1	32.62	32.55	32.47	33.4	32.39	32.63
4	32.07	32.99	32.61	33.14	32.64	32.76	33.5	33	23.85	23.45	24.13	23.55
5	32.57	32.65	29.28	31.6	29.68	27.06	27.82	29.66	33.06	32.86	33.09	33
6	29.29	28.88	31.13	31.84	33.2	33.06	31.8	32.42	26.25	26.19	27.63	26.63
7	16.48	13.77	20.48	19.62	32.94	33.25	33.3	32.96	29.64	28.16	29.98	31.25
8	20.63	20.21	21.63	21.5	32.64	32.81	32.69	32.72	33.17	32.86	29.73	33.13

--> for some reason the TadA control is the one that is washing out the least
--> SIAH1 on the same bacteria is also not completely washed out yet, but has a much higher Cp
--> some of the pVan inducible cultures look promising

--> we will start a second round of PANCE over the next week, swapping out some culutres and keeping some of the same
--> we will do a TadA control for every strain this time

DIUMENGE, 6/10/2024

- [x] restart PANCE --> whole layout once with SIAH1 and once with TadA
  (just so you know, pES2069 is like pES2008 but with the Spec Backbone, so it has a different ori than the lvl1 and works with pVan inducible lvl1s)
- [x] pick pES2071, pES2072 and pES2074

In the lab: Pau

PANCE attempt 2 - Day 1

Here, we work with biological duplicates and two passage stringencies ("low" is 1:10 dilution from the previous plate, "high" is 1:50 dilution from the previous plate).

Today, PANCE was set up:

- The cultures were diluted from the tube 1:5 (200 µL bacteria + 800 µL LB+antibiotics) with 10 µL of 1/0.1 mM Vanillic Acid and incubated for 1h.
- 10 µL from 10^6 phage dilution were added.

PANCE Layout for columns 1-6, repeat same layout for columns 7-12

	1 µM induction	1 µM induction	10 µM induction	10 µM induction	no induction	no induction
1	1084-44-00	1084-44-00	1084-44-00	1084-44-00	1029-08-00	1029-08-00
2	1117-44-00	1117-44-00	1117-44-00	1117-44-00	1033-09-00	1033-09-00
3	1120-44-00	1120-44-00	1120-44-00	1120-44-00	1035-08-00	1035-08-00
4	1124-44-00	1124-44-00	1124-44-00	1124-44-00	1147-08-00	1147-08-00
5	1131-44-00	1131-44-00	1131-44-00	1131-44-00	LB	LB
6	1151-44-00	1151-44-00	1151-44-00	1151-44-00	S2060	S2060
7	1084-69-00	1084-69-00	1084-69-00	1084-69-00	1131-69-00	1131-69-00
8	1117-69-00	1117-69-00	1117-69-00	1117-69-00	1151-69-00	1151-69-00

NOTE (for Gabriel): ignore the second half of the two plates (columns 7-12), I added stuff there by mistake. We usually fill the whole plate because one half is "low stringency" (we make a 1:10 passage) and the other half is "high stringency" (we make a 1:50 passage), but for the first day we are not passaging so only half of the plate is necessary.

Daily Protocol - PANCE

All needed phage and vanillic acid dilutions are in the fridge in the PANCE October box.
LB with the correct antibiotics aswell as aliquotes thereof are in the fridge and colorcoded.
All needed bacteria are in the fridge.

Preparation Bacteria

1. Fill 96-well plates with LB as depicted in the color code of the layout, 800 µL per well (helps to use the automatic multi-channel pipette). One plate is SIAH1, one plate is TadA
2. add 200 µL of bacteria to the correct well
3. put the plate in the shaker to warm up and get into log-phase

qPCR

1. prepare 250x MasterMix and fill the entire top half of the qPCR plate
2. load 2 µL of each sample using the 12 channel pipette, technical replicates should be next to each other (we do not spin down the deep-well plate)
3. run the qPCR

Passaging

after the bacteria had some time to grow, passage from the old to the new PANCE plate using the 8 channel pipette
--> use the current lower stringency (columns 1-6) and higher stringency (columns 7-12) layout

DILLUNS, 7/10/2024

- [x] Passage PANCE
- [x] Perform qPCR for PANCE day 1 (shaker 37ºC for plates, two plates)

PANCE attempt 2

Did protocol as highlighted above, but only for 1:20 passaging of phages. This way, one side of the plate has TadA and one side has SIAH1 phages

DIMARTS, 8/10/2024

- [ ] Sequencing analysis of pES2071, 2072, and 2074
- [ ] miniprep picked colonies (shaker 37ºC for tubes, 8 tubes) --> colonies were sent for nightseq yesterday