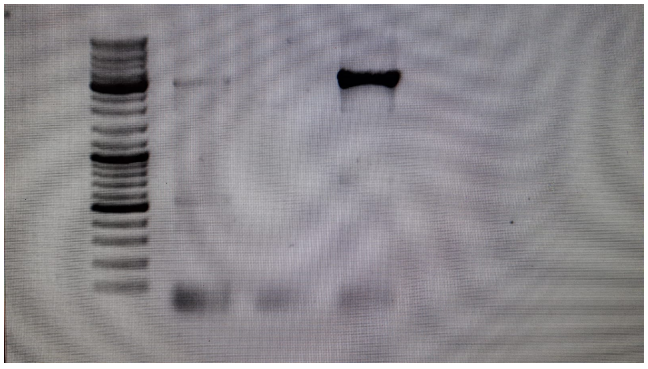
HiFI cloning

WOENSDAG 11-9-2019

Hifi Assembly protocoll
We linearized the vector AraC3 by PCR trying temperatures :65/68 and 72 with following result

grafik.png



At 72 °C PCR results in strong band of the right size and very few unspecific product. We PCR purified and ran the sample again with a possitve result and a concentration of 38.8ng/µl.
We then proceeded and prepared the hifi assembly for bb1.2 and 3 aswell as dor a negative controll(no enzyme) and the possitive controll from the kit.

AraC3+bb1.2

Optimal ng amounts according to NEB are:
Max total 0,2pmol for 2-3 inserts
25,10 fmol Vector (3224bp)= 50ng= (38.8ng/µl)= 1,29µl
50.2 fmol bb1.2_Amp (1380) = 46ng =(147ng/µl) = 0,32µl
Water: 8,39µl
Total: 0,0753 pmol

AraC3+bb3

Optimal ng amounts according to NEB are:
Max total 0,2pmol for 2-3 inserts
25,10 fmol Vector (3224bp)= 50ng = (38.8ng/µl)= 1,29µl
50.2 fmol bb3_Amp (1335) = 41.41ng =(76ng/µl) = 0,55µl
Water: 8,16
Total: 0,0753 pmol

Hifi mix was transofmred into competent cells and plated overnight, starting at 10 PM

ZATERDAG 14-9-2019

Redoing the ligation of AraC3 with BB3
This time i reduced the amount of template for vector linearization to0,1ng to reduce background transformation
PCR product will be shown here:
after purification i will follow the same recipie for ligation :

AraC3+bb3

Optimal ng amounts according to NEB are:
Max total 0,2pmol for 2-3 inserts
25,10 fmol Vector (3224bp)= 50ng = (27ng/µl)=1,85 µl
50.2 fmol bb3_Amp (1335) = 41.41ng =(76ng/µl) = 0,55µl
Water:
Total: 0,0753 pmol
10µl Hifi Master mix
Negative control:
reaction mix with water instead of enzyme
Of those ligations i will transform 1/2/3/5 µl both for experiment and negative controll, cause i think the amount of DNA used for transformation will also influence the background. This way i kind find the amoutn of DNA for transformation that does lead to thelowest background while still resulting in colonies
Negative con

Table39

	A	B	C	D	E
1		Reaction		negative	
2	Insert	0.55		0.55	
3	Vector	1.85		1.85	
4	Water	7.6		17.6	
5	Mastermix	10µl		0µl	
6					
7					
8					

Transformation

	A	B	C	D	E	F
1		Volume				positive
2	KCM	2	2	2	2	2
3	Water	7	6	5	3	3
4	DNA	1	2	3	5	5(1ng/µl)araC3

MAANDAG 16-9-2019

AraC3_BB1.2
25,10 fmol Vector (3224bp)= 50ng = (27ng/µl)=1,85 µl
50.2 fmol bb1.2_Amp (1380) = 42.80ng(+5 %) =(147ng/µl) = 0,31µl
Water: 7,84µl
Total: 0,0753 pmol
10µl Hifi Master mix

Arac3+OEbb1+OEbb2

25,10 fmol Vector (3224bp)= 50ng = (27ng/µl)=1,85 µl
50,20 fmol OEbb1 (1261bp)= 39.11(+2%)ng =(142,85 ng/ul)= 0,28µl
50,20 fmol OEbb2 (2019) = 62,62(+2%) ng = (169 ng/µl) =0,38µl
Total : 0,1256 pmol
Water: 7,49
10µl HIFI mix

negative:
25,10 fmol Vector (3224bp)= 50ng = (27ng/µl)=1,85 µl
50.2 fmol bb1.2_Amp (1380) = 42.80ng(+5 %) =(147ng/µl) = 0,31µl
Water: 17,84µl
Total: 0,0753 pmol
0 µl Hifi Master mix

VRIJDAG 20-9-2019

Transformation of AraC3_1.2 aswell as AraC3_1+2 led to low background.
colonies were scr

Arac3+OE1.2+OEbb3
25,10 fmol Vector (3224bp)= 50ng =(27ng/µl)= 1,85µl
50,20 =30%=(65)fmol OE1.2 (1351bp)= 54.72ng =(95ng/ul)= 0,576µl
50,20 =50%= (100,4)fmol OEbb2 (1311) = 81,34 ng = (44ng/µl) =1,84µl
Total 190,4 fmol
Water 5,73
MM 10µl
Negative control:
25,10 fmol Vector (3224bp)= 50ng =(27ng/µl)= 1,85µl
50,20 =30%=(65)fmol OE1.2 (1351bp)= 54.72ng =(95ng/ul)= 0,576µl
50,20 =50%= (100,4)fmol OEbb2 (1311) = 81,34 ng = (44ng/µl) =1,84µl
Total 190,4 fmol
Water 15,73
MM 0µl

Since the gel shows unspecific bands i guesed the real concentration to be 30% and 50% lower for OE1.2 and OE3 respectively, and therefore calculated 30 and 50% higher amount of moles. I am still below the maximum of 200fmol for hifi cloning. The controll as allways will be the reaction mix without enzyme. A positive transofmration controll will be AraC3

Sequencing of
AraC3_1+2: Primers:
- VR
- Seq2R
- Seq1F
- Seq1R
- VF2

12µl per reaction = 60µl at 40-100ng/µl

AraC3_1.2: Primers
- VR
- Seq1R
- VF2

12µl per reaction: 36µl at 40-100ng/µl

Primers to send:
Seq2R_HFIFI: 10µM 50µl
Seq1F_HIFI: 10µM 50µl
Seq1R_HIFI :
10µM 50µl

MAANDAG 23-9-2019

Arac3+OE1.2+OEbb3
Colony PCR
Primers:
- Seq3R
- Seq1F

Amplicon : 1223 bp
Elongation Time 1min 30 sec
Anealing temp: 95,3°C

Sequencing results for **Arac3+OEbb1+OEbb2** came back #18 has almost no mutations and no stopcodons, which should render it functional. However some gaps need to be sequenced againt o make sure.
I made two liquid cultures, 15 ml TSB 15µl Chloramhenicol, 15µl Ecoli containing SP, and added 300µl of a 50x 10%(20%?) Arabinose solution to one of the flasks.

Sequencing of **AraC3_1.2** showed a mutation in the C protein, creating a stopcodon and therefore rendering it nonfunctional, we have to screen more colonies, since the cloning worked other succesfull clones should have plasmids without fatal mutations.

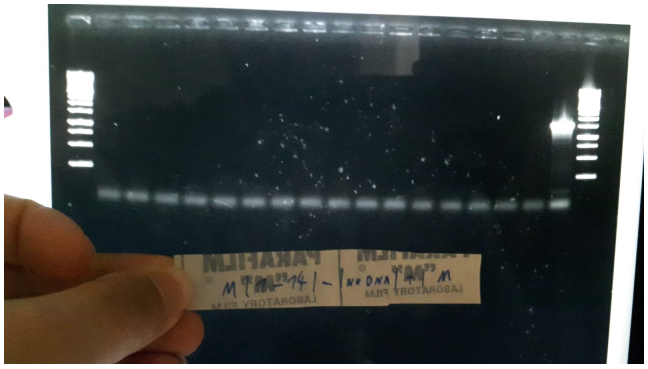
DINSDAG 24-9-2019

Use SeqP1R to sequence the missing gap of RFP in **Arac3+OEbb1+OEbb2 (Done)**

AraC3_1.2
We need to screen the AraC3_1.2 colonies for more possitives since #6 has a mutation.
Screening will be done with P1F and VR which should result in a Amplicon of 770 bp(1min). #6 can be used as positive controll.

We need to screen more Model plasmid since none of the 14 screened colonies has the right insert.
We took colonys from the AraC_1.2 Plate 6-22 and MP plate 15-27. AraC_1.2 and MP PCR was done in two machines, one at 1min elongation and the other at 2min 10 sec elongation time.

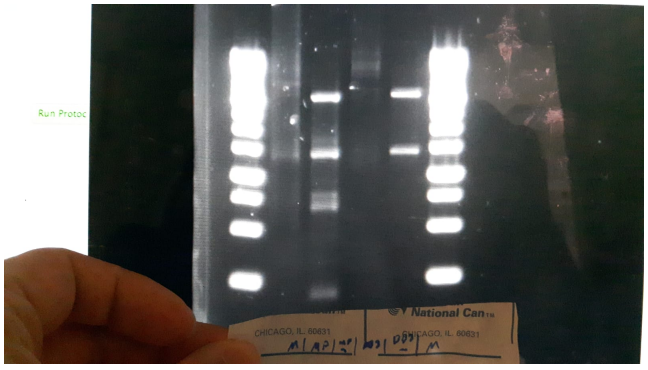
MP with Seq3R_Seq1F.png



Difficulty is that we dont have a possitive controll for the PCR reaction, therefore we use P1F and VR, like we do for 1.2 screening. This Amplifies the region between BB1 and VR, containing BB3. Possitive colonies should have an amplicon of 2028 bp(2Min10sec).

I also tested the ligation of the MP and AraC3_BB3, they both seemed to have worked, due to the disappearence of the Insert bands.

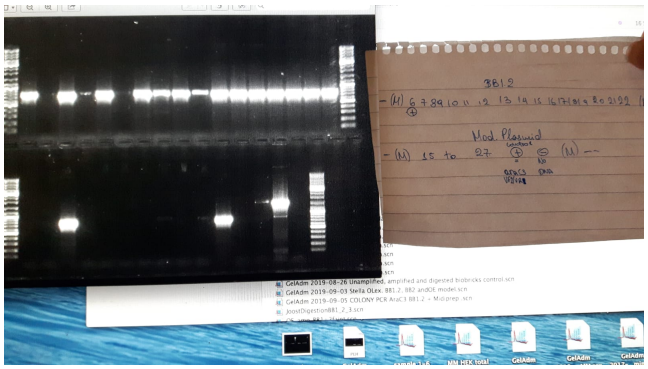
Ligation mix of MP and AraC3_BB3.png



DONDERDAG 26-9-2019

To do list:
Screen AraC3_bb3 plates for positives.
Send positive AraC3_BB1.2 for sequencing-

AraC3_BB1.2 screening with Seq1F and VR(many positives 770bp)/MP with Seq1F and VR(no positives).png



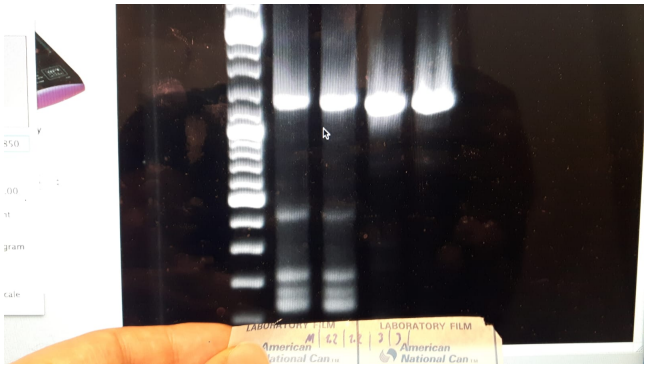
Do PCR for OE1.2 and OE3
Redo the ligation for MP
Take SP and delete bb1 and bb2 to create AraC3_bb1 and AraC3_bb2 respectively.

PCR for amplified1.2 and amplified BB3
TM 65°C
Elongation time 45sec
Max_primer_BB1/BB1.2
Tobi_BB3

Q5 Biobrick amplification reaction mix

	A	B	C	D	E	F	G
1	Recepie		Biobrick	negative control	STEP	TEMP	TIME
2	Q5 High-Fidelity 2X Master Mix	12.5 µl	12,5µl	12,5µl	Initial Denaturation	98°C	30s
3	10 µM Forward Primer	1.25 µl			25 Cycles	98°C 66°C 72°C	10 seconds 30 seconds 30 seconds/kb= 1 min
4	10 µM Reverse Primer	1.25 µl	MM 1,25 ml	MM 1,25µl	Final Extension	72°C	2 minutes
5	Template DNA	10ng/µl	1µl	0µl	Hold	4°C	
6	Nuclease-Free Water	to 25 µl	10,25µl	11,25µl			

PCR product of PCR for amplified1.2 and amplified B B3 .png



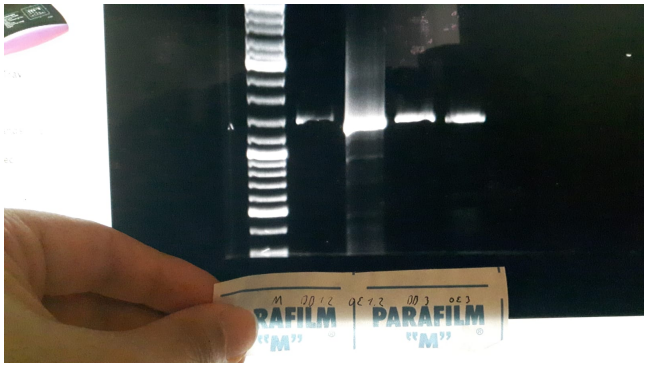
One of each BB was used for PCR purification and subsequent OE PCR

PCR for OE1.2 and OE3
OE_1.2_1.2+3 67°C
OE_3_1.2+3 69°C
Elongation time 45 sec

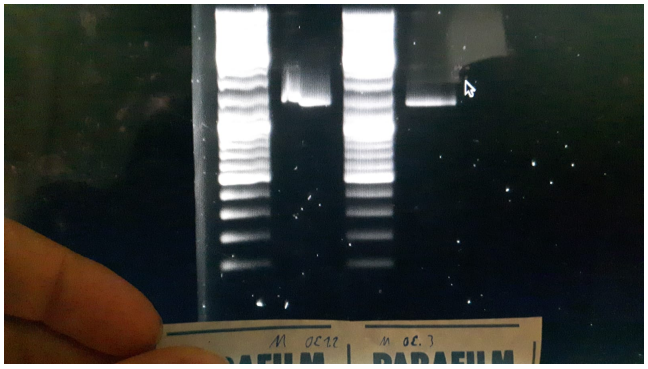
One of each was used in Gelpurification(hope it works this time, i am using washbuffer and elution buffer from the PCR kit, since they seemd to work. Wahbuffer of the PG kit didnt contain any enthanol(didnt smell like it))

This does not work, for some reason OE1.2 is shorter then OE 3, I think this is because the PCR purified BB1.2 and 3 contain unspecific bands leading to some kind of shit.
I will gel purify the second BB1.2 and 3tomorrow. The gel purified BB1.2 and 3 will then be used as template for OE PCR.
If this doesnt work fuck it, why am I doing this anyway.

Gel purified BB1.2 and BB3 and OE 1.2 and 3



Gel purified OE1.2 and 3



ZATERDAG 28-9-2019

Arac3+OE1.2+OEbb3

25,10 fmol Vector (3224bp)= 50ng =(27ng/µl)= 1,85µl
50,20 fmol OE1.2 (1351bp)= 41.91ng =(17,4ng/ul)= 2,41µl
50,20 fmol OEbb2 (1311) = 40,67 ng = (12,3ng/µl) =3,31µl
Total 125,5 fmol
Water 2,43
MM 10µl
Negative control:
25,10 fmol Vector (3224bp)= 50ng =(27ng/µl)= 1,85µl
50,20 fmol OE1.2 (1351bp)= 41.91ng =(17,4ng/ul)= 2,41µl
50,20 fmol OEbb2 (1311) = 40,67 ng = (12,3ng/µl) =3,31µl
Total 125,5 fmol
Water 12,43
MM 0µl

Screening of the MP
PRimers:
P1F
VR
Amplicon: 2028
Anealing temp 59.3

MAANDAG 30-9-2019

Sequencing

AraC3_BB1.2_20	3 sequences =36µl
AraC3_BB1.2_21	3 sequences=36µl
AraC3_BB3_4	3 sequences=36µl
AraC3_BB3_7	3 sequences=36µl
MP_8	4 sequences=48µl
MP_13	4 sequences=48µl

New primer to send: P3R 50µl of 10µM solution
All the other primers are at microsynth

DONDERDAG 3-10-2019

Plan: screen colonies BB3 - send to sequencing

THURSDAY - site directed mutagenisis - PCR 3 temperatures -

Deltetion Primers

Delete BB1 R	**GTTGAACAGTACGAACGTGCCGAGG**	Snapgene: 64°
Delete BB1F_II	**AGCTAGCACTGTACCTAGGACTGAGCTAGC**	NEB: 72° Snapgene: 65°
Delete BB2 R_II	GCAAATAATCAATGTTGGCCGGCTTGACG	Snapgene: 65°
Delete BB2_F_II	ACTACGCTGACGCTTCTTAATAACTGTAACAGAGC	NEB: 72° Snapgene: 64°

Following the protocol from NEB, but using our regular q5 mastermix, since we only have 10 reactions which will be saved until the construct with the correct RBS arrives.
https://international.neb.com/protocols/2013/01/26/q5-site-directed-mutagenesis-kit-quick-protocol-e0554

Temperatures tried: 65°,68°,72°

Cycling conditions: Q5 regular protocol, but elongation time 2:50 min

Stela used the pcr products from 72°C and went on with the ligation and transformation protocol.

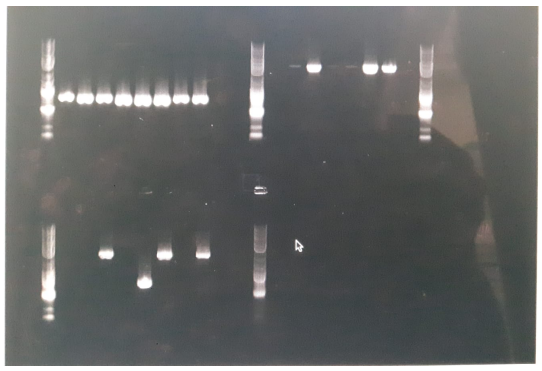
Deltion was succesfull, now we need to screen the colonies for correct clones.
Screening:

Machine 1: elongation time: 1min
AraC3_BB1
primers:
seq1F
VR
Amplicon: 782bp
Anealing temp: 59.3

Machine 2: elongation time 2:30
AraC3_BB2:
primers:
Seq2R
VF2
Amplicon: 2329
Anealing temp 59.3

Screening of the MP
PRimers:
Seq 1F
VR
Amplicon: 2028
Anealing temp 59.3

grafik.png



Tree colonies of each were taken.

MAANDAG 7-10-2019

Sequencing of colonies
AraC3_BB1:
Primers:
VR
Seq1R-HIFI

AraC3_BB2
Primers:
VR
Seq2R-HIFI

MP
Primers:
Seq3F-II need to send
Seq1R-II

Ara3_BB1.2
Primers
VR
Seq1R

Ara3_BB3
Primers
VR
P3R

AraC3:
Primers
VF2

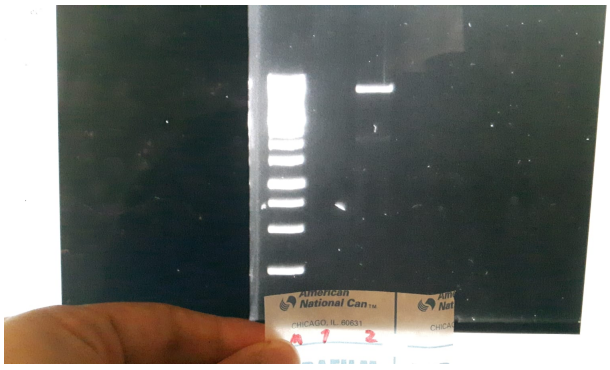
DONDERDAG 10-10-2019

Adding RBS site to BB1 and BB2
BB1:
Primers:
Anealing temp 72°C
Elongation time: 2:30

BB2:
Primers:
Anealing temp 72°C
Elongation time: 2:50

Put both in the same machine at 72°C and elongation time 3 minutes
Elongation time withQ5 is 30sec/KB

image.png



PCR only worked with BB2, so I proceeded with BB2. cloning was done, controll was lysed bacteria, positive was arac3 plasmid

VRIJDAG 11-10-2019

We send some colonies of BB2 for sequencing bei ENS

Today stella did mutagenisis of BB1, with succesfull PCR. She did cloning and saturday we can take the colonies.

WOENSDAG 16-10-2019

thermo fisher construct is here
I dilute in 50µl of water to a concentration of 100ng/µl
I will use: Amplicilin plate
2µlDNA
2µlKCM
6µl DNA

Poitive controll: Chloramphenicol plate
AraC3:
2µlDNA

2µlKCM
6µl water

negative control Ampicilin plate
2µlKCM
8µl water

Working timeline:
Do Colony PCR with
Seq1F
Seq2R
at 59.3 °C
Amplicon 1451bp (1min 45 sec)

grow over night:
- the positive selected colonies
- RFP control
- ask for gfp control

at 8 am dilute to 0.5 OD and induce with 0.2 Arabinose until 2 PM
Meassure at FACS