Notebook Plasmid A and Plasmid B Characterization

ZONDAG 2-6-2019

WOENSDAG 12-6-2019

Security meeting + Benchling + Quartzy + Snapgene + Inventory check
We need: Antibodys for: Myc-tag and Flag-tag. Kanamycin.
Realization that we need to add terminators with PCR primers.

DONDERDAG 13-6-2019

Laboration planning, What needs to be done:

1. **Transformation of backbone into top10 ecoli cells (13/6 by group A)**

2. **Clone biobrick 2 (pBAD TetR) into backbone.** --> https://www.addgene.org/protocols/subcloning/
   a. amplifiy biobrick with PCR and add terminator --> https://www.addgene.org/protocols/pcr/
      - 2 µL Template DNA (10 ng-500 ng)
      - 5 µl 10X Taq buffer with $MgCl_2$
      - 1 µl dNTP mix (10 mM each nt)
      - 2.5 µL Forward Primer (10 µM stock)
      - 2.5 µL Reverse Primer (10 µM stock)
      - 0.2 µL Taq DNA Polymerase (5 units/µL)
      - 32.8 µL Sterile dH_2O (variable)
      - terminator sequence
   b. purify PCR product from gel
      I. eletrophoresis--> https://www.addgene.org/protocols/gel-electrophoresis/
   c. purify vector from cell culture --> miniprep
   d. preparation of vector and insert
      I. restriction enzyme digestion --> https://www.addgene.org/protocols/restriction-digest/
      gel purification of digested insert and vector (see electrophoresis above)

   e. ligation of vector and insert --> https://www.addgene.org/protocols/dna-ligation/
      I. Combine the following in a PCR or Eppendorf tube:
         - Vector DNA
         - Insert DNA
         - Ligase Buffer (1µL/10µL reaction for 10X buffer, and 2µL/10µL reaction for 5X buffer)
         - 0.5-1µL T4 DNA Ligase
         - H_2O to a total of 10µL
         - incubate

   f. transformation into host --> https://www.addgene.org/plasmid-protocols/bacterial-transformation/
   g. screening of selected colonies (with eye and PCR) (alternative restreak)
   h. Plasmid isolation with miniprep (for backup)

1. **Growth curves with arabinose and without**
   a. one with simply the *plasmid p15A Kan*

b. one with biobricks *plasmid p15A 1+2 Kan*
c. one without any plasmid

Plasmid A characterization

9.30-
Update: Max showed a transformation by heat shock and gave us competent top10 E.coli, Kanamycin plates, TSB medium. Told us that we need to remember to autoclave in time so that we have sterile material. We will autoclave pipet tips, Eppendorf tubes, water and glassware. Tomorrow morning at 9.

Protocol 1: Transformation by heat shock

1. Clean bench with ETOH
2. Cool down sterilized water
3. Take cells out of -80°C and thaw on ice for 15-20 min(Do not let them get warm)
4. Turn burner on for sterile environment
5. Take out Biobrick and follow the **BioBrick picking protocol** below
6. Add 1µl (50 ng) DNA in 7µl dH20 into sterile EpiTube mixed with 2µl KCM
7. Cool the mix on ice for 1 min
8. Add 10µl of Bacteria
9. Incubate for 30 min on ice
10. Heat shock for 1 min at 42°C
11. Incubate for 3 min on ice
12. Add 100 µl medium
13. Incubate for 1-2 hours at 37°C
14. Take 50µl transformed bacteria and plate them
15. Incubate the plate overnight at 37°C

BioBrick picking protocol

1. With a pipette tip, punch a hole through the foil cover into the corresponding well of the part that you want. Make sure you have properly oriented the plate. Do not remove the foil cover, as it could lead to cross contamination between the wells.
2. Pipette 10µL of dH2O (distilled water) into the well. Pipette up and down a few times and let sit for 5 minutes to make sure the dried DNA is fully resuspended. The resuspension will be **red**, as the dried DNA has cresol red dye. We recommend that you do not use TE to resuspend the dried DNA.
3. Transform 1µL of the resuspended DNA into your desired competent cells, plate your transformation with the appropriate antibiotic* and grow overnight.
4. Pick a single colony and inoculate broth (again, with the correct antibiotic) and grow for 16 hours.
5. Use the resulting culture to miniprep the DNA AND make your own glycerol stock (for further instruction on making a glycerol see this page). We recommend using the miniprepped DNA to run QC tests, such as restriction digests and sequencing.

https://www.addgene.org/protocols/bacterial-transformation/

VRIJDAG 14-6-2019

Lab notes

at 11:00 we did a restreak of the transformed bacteria from one colony, letting it incubate at 37 degrees celsius for 24 hours. further planning of the lab (see above)

Plasmid A characterization

9.30-
iGEM locker + General Meeting + Autoclave(liquid + cooling program) + Transformation + Plating
Transformation following **Protocol 1: Transformation by heat shock** and **BioBrick picking protocol**
Autoclaving again (plastic program) - for less water

ZATERDAG 15-6-2019

Resolved the terminator issue by finding a bi directional terminator in the original BioBrick and plan to add one more by plasmid PCR with Q5 in plasmid C, Tobi has designed primers. Sequence of added terminator is: 34: TERMINATOR: cgcaaaaaaccccgcttcggcggggttttttcgc.

WOENSDAG 19-6-2019

Order new Biobrick with Terminator + RBS + double stop codons
Use gBlocks DNA Fragment tool to check complexity.
Tomorrow: Order primers.

Also, overnight occulation in preparation of tomorrow's transformation.
Broth making and glycerol stock

DONDERDAG 20-6-2019

Transformation. Order primers. We didnt do transformation? right? did i forget something? "Tobi :D "
We made Glycerin stocks of the overnight culture:

- 500µl 50%glycerol in water
- 500µl Bacteria in culture medium

We mixed glycerin to a concentration of 50% and then added bacteria to the glycerin. After mixing by inverting until mix is homogenous it was stored at -20 in the lab fridge.

Plasmid isolation using the Plasmid isolation kit: Protokol is in the lab
We did for paralell aliqotes, stored in the +6 frige, concentration is not yet meassured. DNA was eluted in elution buffer, not in water.

Q: Can we use NanoDrop to determine DNA concentration before PCR gel electrophoresis?

PCR Protocol suggestion
PCR reactions (50 uL) contained HF buffer (1X), dNTPS (200 µM), forward primer, F_PCR (0.5 µM), reverse primer, R_PCR (0.5 µM), Dynazyme DNA polymerase (0.04 U/µl) and dH2O. PCR reactions were prepared on ice in two separate tubes. Bacterial cells from one purple colony were added to one tube and cells from a white colony were added to the other tube. The PCR protocol was: 1x [94 °C 5 min], 30 x [94 °C 20 s, 65 °C 30 s, 72 °C 1 min], 1x [72 °C 10 min], 1x [4 °C ∞].

Protocol 2: PCR mixture
*V1= V2*C2/C1 = 25 µL*1x/10x = 2,5 µL*

Total volume: 30 µl without DNA

Resuspended cell: 1 µl -> **x=1**
5xPCR - > **5x**
25mM MgCl2 - **2x** -> 4 mM
10mM dNTP - **0,5x** -> 0,2 mM

?DMSO (dimethyl sylphoxide) - **0,3x** -> 2%
Taq Polymerase (Go Taq)- **0,125x** -> 0,01x
MilliQ Water - **15,125x** -> 0,688x (68%)
?Primer forward - 0,5x? -> 0,23 mM?
?Primer reverse - 0,5x? -> 0,23 mM?

MAANDAG 24-6-2019

Things done today

- Restreak of C1757
- Overnight culture with C1757
- Made antibiotic stocks and plates.

Plan for today:
Digestion with restriction enzymes - https://www.addgene.org/protocols/restriction-digest/ and transformation tomorrow

Protocol for cloning 1: Controlled and Recommended
Take DNA - thaw on ice
Prepare restriction enzyme mixture in following order for both, vector and Insert seperatly:
Restriciton enzyme concentrations:
EcoR1: 12U/µl
PST1: 10U(u/µl)

Reaction mix:
Autoclaved water : enough for total volume of 20µl (15,88)
Buffer: 2µl
Acetyl BSA: 0,2µl
DNA(1µg/µl): 1µl
Add Restriction enyzyme 1: 0,5µl EcoR1: 0,42µl
Add restriction enzyme 2: 0,5 µl Pst1: 0,5µl
Final volume 20µl

Mix gently by pipetting up and down and then spin down the sample.
Incubate at 37°C for 1- to 4 hours (4 hours)
After incubation heat inactivation at 65°C to inactivate the restriction enzymes.

Gel purification (1 hr):
Take 5µl of digestion and mix with 1µl of loading dye.
At mix into gel and run gel.
After about 45 minutes stop the run and cut the bands out under UV light(Wear safety glasses against Uv!!!)
After the bands have been cut out, use the Gel extraction tool to reeluate the DNA, meassure the DNA concentration for following steps.

Ligation (2 hr):
prepare ligation mix: (the amount of Vector and insert is to be calculated to a ration of 1:3 using the https://nebiocalculator.neb.com/#!/ligation

- Vector DNA
- Insert DNA
- Total DNA about 100ng
- Ligase Buffer (1µL/10µL reaction for 10X buffer, and 2µL/10µL reaction for 5X buffer) (What buffer do we have?)
- 0.5-1µL T4 DNA Ligase
- H_2O to a total of 10µL, mix gently (DO NOT under no circumstances vortex!!!)

Use noncut plasmid as control
Incubate 2 hours at room temperature.
Now do **transformation (2 hr)** into host, or store DNA at 6°C overnight, or -20 for longer.

DINSDAG 25-6-2019

Restriction digestion:
Dilute Insert to 10ng/µl (Stock solution in TE buffer)
Vector to 7ng/µl
Use 10µl of insert and 1µl of Vector for the digestion mix
Add amount of DNA:

- Vector: 10µl (70ng) This is according to addgen, for the ligation we plan to use 33ng
- Insert: 10µl (100ng) This is to little according to addgen, for ligation it is planned to use about 70ng (0,07µg)
- 2µl Buffer
- 0,2 µl BSA
- 0,42 µl EcoR1
- 0,50 µl pst1
- 6,73 µl dH2O

Fill up to 20µl
Incubate up to 4 hours on 37°c Start at 10:11 AM

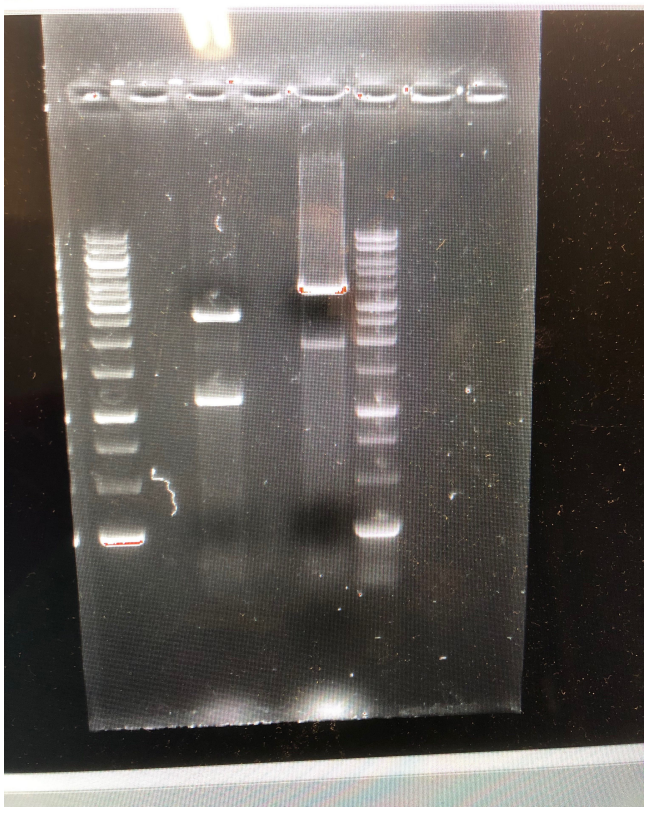
Load the 20µl Vector reaction with 5 µl of loading dye 5x
Heat inactivate the Insert reaction at 65°C for 15 minutes
Run gel for 30 minutes--> Gel didnt show any bands, we need to repeat experiment.
Gel extration protocol

Note: this did not work, nothing showed up in the gel :(
But the biobricks will arrive tomorrow ho
WE started overnight culture from the glycerin stock with plasmid

WOENSDAG 26-6-2019

Mini prep
digestion max 2 hours
Run digestion on Gel
See results

grafik.png



one can see the double digest with ecoR1 and Pst1 in the third row and the single digest in the 5th row
We Isolated the cut vector from the gel andpurified it by gel extraction, the concentration was about 11ng/µl

Competent cell protocol C1757
Overnight growth of a single colony in 10 ml TSB.
2. Dilute pre-inoculum 1/100 in 25 ml TSB. Incubate at 37°C until the
OD 600 readout is 0.4. After approximately 120-140 min.
3. Cool the culture on ice for 10 min.
4. Centrifuge (2 x 10) ml of each sample for 10 min at 4500 rpm, 4°C. Discard supernatant.
5. Resuspend in half the volume (5 ml) cold, sterile CaCl2 solution of 50mM.
6. Keep on ice for 15 min and centrifuge once more at 4500 rpm for 10 min at 4°C. Discard.
7. Resuspend in 1:20 of the volume (250 ul) of cold sterile 50mM CaCl2 .
8. Aliquot 200 µl of bacterial suspension in eppendorph tubes. Keep on ice.

Transformation
4. Thaw competent cells on ice (KCM and Plasmid prep)
5. Make a mix: 1 µl. 50 ng DNA, 2ul KCM and 7ul MilliQ
6. Add 10 ul bacterial cells
9. Transform samples in a water batch at 42°C for 2 min. Keep on ice for 5 min.
10. Ad 1 ml TSB broth containing 1x Kan. Incubate for 1 h at 37°C in an empty plate.
11. Plate on selective media.

Growth curve C1757
1. Inoculate one colony from each strain in 10 ml TSB. Grow overnight at 37°C.

2. Read the OD 600nm and dilute samples in 10 ml LD broth to 0.05 OD in a culturing flask.
3. Measure the OD 600nm until it reaches OD 0.2, (approx. 1-2h)
4. Prepare 2 flasks of each (TSB broth) total volume of 30 ml in each flask.
5. Take 1.5 ml from each sample in step 3. Centrifuge at 5000 rpm, 10 min. Remove supernatant and ad 1.5 ml TSB broth, wash twice more.
6. Dilute 1:500 in each of the 2 flasks. Repeat for each strain.
6. After more than 2h you can start reading the OD. Measure OD ever 30 min (10 times) from OD 0.2 (1 ml withdrawn for each measurement) (2 ml was saved in a 2ml eppendorph for WB analysis)
7. Plot time vs. OD.

OD measuremnt over time

Table3

	A	B
1	0	0.0004
2	60	0.0056
3	120	0.0027
4	150	-0.0018
5	180	

DONDERDAG 27-6-2019

Table4

	A	B	C	D	E	F	G	H	I
1		120	150	180	210	240	270	300	330
2	OD 1	0.0019	0.0025	0.0101	0.0297	0.0122	0.0103	0.0111	
3	OD 2								

1. Set up the following reaction in a microcentrifuge tube on ice.
   (T4 DNA Ligase should be added last. Note that the table shows a ligation using a molar ratio of 1:3 vector to insert for the indicated DNA sizes.) Use NEBioCalculator to calculate molar ratios.
   Ligation:
   - Vector DNA (11,5ng/µl) 33ng= 2,9µl
   - Insert DNA (5ng/µl) 68ng= 13,6 µl
   - Total DNA about 100ng
   - Ligase Buffer (10x) (2µL/20µL reaction for 10X buffer) = 2µl
   - H_2O to a total of 10µL, mix gently (DO NOT under no circumstances vortex!!!) = 0,5 µl
   - 0.5-1µL T4 DNA Ligase (1U/µl) = 1µl

   ** The T4 DNA Ligase Buffer should be thawed and resuspended at room temperature.*
2. Gently mix the reaction by pipetting up and down and microfuge briefly.
3. For cohesive (sticky) ends, incubate at 16°C overnight or room temperature for 10 minutes.
4. For blunt ends or single base overhangs, incubate at 16°C overnight or room temperature for 2 hours *(alternatively, high concentration T4 DNA Ligase can be used in a 10 minute ligation)*.
5. Heat inactivate at 65°C for 10 minutes.

6. Chill on ice and transform 1-5 µl of the reaction into 50 µl competent cells.

Control: Vector + Ligase
Reaction: Vector + insert+ ligase

Transformation:
we are using 5µl DNA from our cloning. this should be about 25µg total DNA
5µl DNA + 3µl H20+ 2µl KCM
added 10µl Bacteria.
Started incubation at 14:33
Will plate them at 16:33

VRIJDAG 28-6-2019

Mini prep kit (Protocol for high yields)
Do a PCR (sequence it) and run a gel
1905 IDT sequence

PCR primers
https://bitesizebio.com/9945/how-to-calculate-a-dna-primer-concentration/

F_primer: 26,9 nmol - - Add 100 µl nuclease free water (26,9 nmol/100 µl) x (1000 pmol/nmol)= 269 pmol/µl = 269 µM
Add **269 µl** nuclease free water (26,9 nmol/269 µl) x (1000 pmol/nmol)= 269 pmol/µl = 100 µM

R_primer: 32.5nmol - - Add 100 µl nuclease free water (32,5 nmol/100 µl) x (1000 pmol/nmol)= 325 pmol/µl = 325 µM
Add **325 µl** nuclease free water (32,5 nmol/325 µl) x (1000 pmol/nmol)= 325 pmol/µl = 100 µM

Add nuclease free water for correct concentration

Creating the PCR machine protocol "igem amp"
Calculating elongation time and annealing temperature with biolabs and thermo fischer webside

Plasmid A Characterization
We checked the plates.
Ligation with only vector grew on antibiotic fr

Plated colonies after transformation with cloned Plasmid

	A	B	C	D	E
1		Kanamycin		Antibiotic free	
2		Grew	Didnt grow	grew	didnt grow
3	ligation vector+insert	x(after 48 hours) 5 colies	x		
4	ligation only vector		x	x (white)	
5	P15A	x Red			
6					
7					
8					
9					

MAANDAG 1-7-2019

What Ayleen Has done today:

- Aliqutoe of LB broth to 4 flasks
- restreak of coloy 1 and 2 of Top10 p15A-2 on two seperate KM plates
- made an overnight culture of coloy 1 and 2 of top 10 p15A-2 in 10 ml LB broth and 10 mikroL KM
- Transformation of plate 6 well 4A and 4C into top10 cells according to transformation protocol . 4A--> plasmid B1C3, 4C--> plasmid B3C5

medium added in 100 mikroL: LB broth. Plated on Cml plates overnight . negative control was NOT made.

Plasmid A characterization

Question for meeting: How about using a high copy number plasmid for assembling of the biobricks, and once they are assebled we transfer them into a medium copy plasmid? This way we get higher plasmid concentrations for cloning. Concentration with Plasmid we used for cloning was high enough when we took a lot of cells (80ng/µl in a medium copy number 10-12 copys, but high copy 100-300 definetly leads to higher concentrations. could also be usefull to amplify biobricks that have been cloned into plasmids.

Liquid culture of positve cells
Transformation with http://parts.igem.org/Part:pSB3C5 Chloramphenicol medium copy plasmid , Plate 6 Well 4C
Colony PCR
    Postive: Transformed cells (Insert 2052 bp)
    Negative: Original P15A plasmid Insert size (1069bp)
Gelelectrophoresis
Liquid culture of white cells of P15A-2 cloning

II. PCR screen of colonies
First calculate how much of each component you need for the PCR reaction. Use the initial concentrations, the final concentrations and the final reaction volumes to calculate these. Fill out the form below and show the lab assistants. This should be done during the first or the second day of the lab course.
Prepare an ice bucket.

Add the PCR-reaction components to two separate 1.5 mL Eppendorf tubes (marked "1" resp. "2"), check of what you added, and put on ice:

Pick one marked

from the agar plate and resuspend it in 20µL MQ in a sterile 1.5 mL tube. Use a small pipette tip for this and pipette up and down gently to mix and release the cells from the tip. Add 2 µL from the resuspended cells to the tube marked "1" (the rest will be used later for inoculation).

Thermocycling conditions for "colony pcr taq"(name) PCR:
For the insert PCR - primers called backbone forward and reverse.
https://www.neb.com/protocols/0001/01/01/taq-dna-polymerase-with-standard-taq-buffer-m0273

colony pcr taq pol

	A	B	C
1	STEP	TEMP	TIME
2	Initial Denaturation	94°C	7 min
3	25 Cycles	94°C 58°C 68°C	25 seconds 40 seconds 1 minute/kb
4	Final Extension	72°C	7 minutes
5	Hold	4°C	

PCR 2 - Max feedback

	A	B	C
1	STEP	TEMP	TIME
2	Initial Denaturation	95°C	10 min
3	25 Cycles	95°C 53°C 72°C	30 seconds 40 seconds 1 minute/kb
4	Final Extension	72°C	7 minutes
5	Hold	4°C	

Primer annealing optimal temperature calculation:
https://www.thermofisher.com/se/en/home/brands/thermo-scientific/molecular-biology/molecular-biology-learning-center/molecular-biology-resource-library/thermo-scientific-web-tools/tm-calculator.html

Table2

	A	B	C	D	E	F	G	H	I	J	K
1	ID #1	Sequence #1	Molecularweight g/mol	Extinctioncoefficientl/(mol˙cm)	Tm°C	ID #2	Sequence #2	Molecularweight g/mol	Extinctioncoefficientl/(mol˙cm)	Tm°C	AnnealingTemperature°C
2											
3											
4	Primer#1	GAAGCCTGCATAACGCGAAG	6160.1	200600.0	62.9	Primer#2	GGCGTATCACGAGGCAGAAT	6191.1	202600.0	63.4	57.9
5	Annealing temperature lower than 45°C is not recommended.										

Colony PCR
Go tag buffer 10µl x
MgCl 5µl x
Nukleotide mix 1µl x
0,5 µl Forward x (1µl accidently)
0,5µl Reverse x (1 µl accidently)
0,25µl goTag x
2µl BActeria x
30.75 µl MQ H20 x

negazitive controll
Go tag buffer 10µl x
MgCl 5µl x
Nukleotide mix 1µl x
0,5 µl Forward x
0,5µl Reverse x
0,25µl goTag x
32.75 µl MQ H20 x
positive controll

Go tag buffer 10µl x
MgCl 5µl x
Nukleotide mix 1µl x
0,5 µl Forward x
0,5µl Reverse x
0,25µl goTag x
1µl P15A x
31.75 µl MQ H20/ x

----------second try----------

Colony PCR
Go tag buffer 10µl x
MgCl 5µl x
Nukleotide mix 1µl x
5 µl primer mastermix
0,25µl goTag x
2µl BActeria x

26.75 µl MQ H20 x

negazitive controll
Go tag buffer 10µl x
MgCl 5µl x
Nukleotide mix 1µl x
5 µl primer mastermix
0,25µl goTag x
28.75 µl MQ H20 x

positive controll
Go tag buffer 10µl x
MgCl 5µl x
Nukleotide mix 1µl x
5 µl primer mastermix
0,25µl goTag x
1µl P15A x
27.75 µl MQ H20/ x

DINSDAG 2-7-2019

Ayleen:::::

- Restriction digestion:

Dilute biobrick 1 and 3 to 10ng/µl (Stock solution in water) --> add 100 microL autoclaved MilliQ
do not dilute vector--> conc is 50ng/microL---> take 3 microL to get 150ng

5 eppitubes--> 3 for biobrick, 1 for vector, 1 neg control w/o any DNA
Use 3X 10µl of insert and 3µl of Vector for the digestion mix
Add amount of DNA:
Vector: 3µl (150ng) T*his is according to addgen, for the ligation we plan to use 33ng*
Insert: 3 X 10µl (100ng) T*his is to little according to addgen, for ligation it is planned to use about 70ng (0,07µg)*
2µl Buffer
0,2 µl BSA
0,42 µl EcoR1
0,50 µl pst1
13.88 µl milliQ for vectors, and 6.88 µl for biobrick vectors
Fill up to 20µl
Incubate up to 2 hours on 37°c Start at 10:11 AM

- Made Chloramphenicol stock by diluting 0.26 g Chl in 10 ml 70% etOH to get 26 mg/ml Chl. Aliquotes in 1 ml epitubes stored in -20 degrees fridge.

Jannah:::
Heat inactivate the Insert reaction at 65°C for 15 minutes
For each insert - make a mix of 8 ul solution add *Add 2 µl of loading dye 5x*
For the vector - make a mix with 16 ul solution add 4 ul of loading dye 5x
Load 20 *µl of the vector and 10 µl of the inserts in the gel*
Run gel for 30 minutes--> Gel didnt show any bands, we need to repeat experiment.
Gel extration protocol

Competent cell protocol C1757

Overnight growth of a single colony in 10 ml TSB.
2. Dilute pre-inoculum 1/100 in 25 ml TSB. Incubate at 37°C until the
OD 600 readout is 0.4. After approximately 120-140 min.

3. Cool the culture on ice for 10 min.
4. Centrifuge (2 x 20) ml of each sample for 10 min at 4500 rpm, 4°C. Discard supernatant.
5. Resuspend in half the volume (10 ml) cold, sterile CaCl2 solution of 50mM.
6. Keep on ice for 15 min and centrifuge once more at 4500 rpm for 10 min at 4°C. Discard.
7. Resuspend in 1:20 of the volume (500 ul) of cold sterile 50mM CaCl2 .
8. Aliquot 200 µl of bacterial suspension in eppendorph tubes. add 10% of 50%glycerol. Keep on ice.

Rebecca
Growth curve

Overnight culture
Diluted cells to 0.05 OD (dilute less next time)
Grew cells until ~0.6 OD
No washing
Diluted cells to 0.05 OD - Time 0

NB- 30 ml -0.05
LB - 30 ml - 0.05
TSB 1 - 30 ml - 0.05
TSB 2 - 30 ml - 0.05
TSB 2 - 15 ml - 0.05
TSB 2- 0.005 dilution

PS. At 210 min - bad dilution step and incorrect OD measurement.

Table6

	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O
1		15	30	45	60	75	90	105	120	135	150	180	195	210	270
2	NB	0,034	0,08	0,102	0,138	0,199	0,238	0,384	0,416	0,505	0,695	0,854	1,38	1,25	2,07
3	LB	0,08	0,077	0,138	0,198	0,202	0,256	0,306	0,432	0,526	0,787	0,976	1,5	1,01	2,33
4	TSB 30 1	0,044	0,072	0,11	0,138	0,213	0,373	0,413	0,564	0,645	0,899		1,12	1,3	2,17
5	TSB 30 2	0,042	0,07	0,108	0,158	0,164	0,248	0,349	0,479	0,592	0,938	1,2	1,25	1,43	2,22
6	TSB 15	0,016	0,092	0,096	0,135	0,172	0,267	0,328	0,434	0,551	0,787	0,917	1,35	1,08	
7	TSB 30 0.005		0,013	0,002	0,018	0,047	0,049	0,052	0,076	0,098	0,124	0,127	0,196	0,245	

Plasmid A characterization

Colony PCr results

Band in positive is 3000 bp, it shoud be 1259. Where does this descrepancy come from?

Thermocycling conditions for "colony pcr taq"(name) PCR:

For the BioBrick amplification - primers called amplification forward and reverse.

PCR trial 1 - TC- Plus

	A	B	C
1	STEP	TEMP	TIME
2	Initial Denaturation	98°C	30 s
3	25 Cycles	98°C 68°C 72°C	10 seconds 30 seconds 30 seconds/kb= 1 min
4	Final Extension	72°C	2 minutes
5	Hold	4°C	

PCR trial 2 - Thermo Hybaid

	A	B	C
1	STEP	TEMP	TIME
2	Initial Denaturation	98°C	30s
3	25 Cycles	98°C 66°C 72°C	10 seconds 30 seconds 30 seconds/kb= 1 min
4	Final Extension	72°C	2 minutes
5	Hold	4°C	

PCR reaction for Biobrick amplification

	A	B	C	D
1	Q5 High-Fidelity 2X Master Mix	12.5 µl	12,5	
2	10 µM Forward Primer	1.25 µl		
3	10 µM Reverse Primer	1.25 µl	MM 1,25 ml	
4	Template DNA	10ng/µl	1µl	0µl
5	Nuclease-Free Water	to 25 µl	10,25	11,25

WOENSDAG 3-7-2019

Ayleen:

- Gelpurification of digested test Biobricks BB1, BB2, BB3 and vector
- Nanodrop of each to mesure concentration

Table5

	A	Conc ng/µl
1	V	5,6
2	V2	5,1
3	BB1	2,7
4	BB1.2	6,0
5	BB2	4,9
6	BB2.2	5,6
7	BB3	2,9
8	BB3.2	2,5
9		

Ligation:

- Vector DNA (5,6ng/µl) 33ng= 5,9µl
- Insert DNA BB1 (2,7ng/µl) 68ng= 25,2 µl
- Insert DNA BB2 (4,9 ng/µl) 68ng= 13,9 µl
- Insert DNA BB3 (2,9ng/µl) 68ng= 23,4 µl
- *Total DNA about 100ng*
- Ligase Buffer (10x) (2µL/20µL reaction for 10X buffer) = 4 µl
- **H_2O** to a total of 40µL, mix gently (DO NOT under no circumstances vortex!!!) =

  - BB1--> 3,9 µl **H_2O**
  - BB2--> 15,2 µl **H_2O**
  - BB3 --> 5,7 µl **H_2O**
  - V neg --> 29,1 µl **H_2O**
- 0.5-1µL T4 DNA Ligase (1U/µl) = 1µl

Control: Vector + Ligase
Reaction: Vector + insert+ ligase
Incubate 2 hours at room temperature.

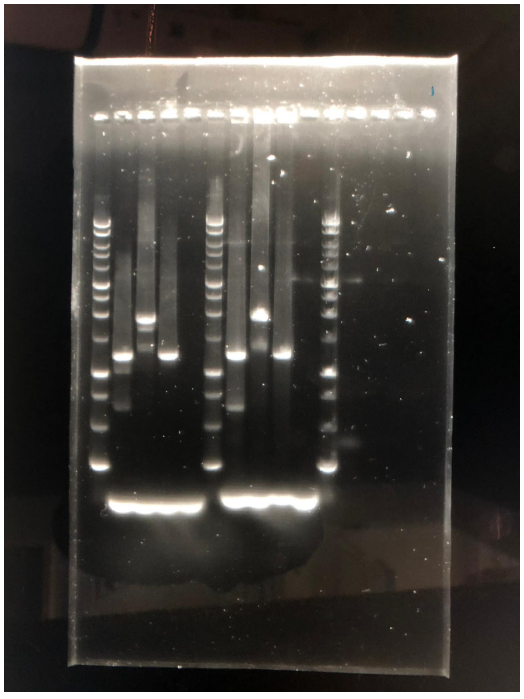
Transformation made by Rebecca
Original transformation protocol

we are using 5µl DNA from our cloning. this should be about 25µg total DNA
5µl DNA + 3µl H20+ 2µl KCM
added 10µl Bacteria.
Started incubation at 14:33
Will plate them at 16:33

Rebecca
Miniprep and plasmid isolation of plasmids C1B3 (HCN) and C355 (LCN)
Made chloramphenicol plates
Transformation of C1757 with the following two plasmids and no plasmid as negative control.

Plasmid A characterization

grafik.png



PCR reaction result, left at 68°C and right at 66°C
We are going to proceed with Amplification of the Real biobricks at 66°C

Table31

	A	B	C	D
1	Recepie		Biobrick (4)	negative control
2	Q5 High-Fidelity 2X Master Mix	12.5 µl	12,5	
3	10 µM Forward Primer	1.25 µl		
4	10 µM Reverse Primer	1.25 µl	MM 1,25 ml	
5	Template DNA	10ng/µl	1µl	0µl
6	Nuclease-Free Water	to 25 µl	10,25	11,25

Table3 - BioBrick amplification

	A	B	C
1	STEP	TEMP	TIME
2	Initial Denaturation	98°C	30s
3	25 Cycles	98°C 66°C 72°C	10 seconds 30 seconds 30 seconds/kb= 1 min
4	Final Extension	72°C	2 minutes
5	Hold	4°C	

DONDERDAG 4-7-2019

Troubleshooting with Max.
New ligase
Buffer?
Amplification
Adding overhangs to bricks
Analyze that DNA is present after gel purification (Both for vector and insert)
Increase amount of total DNA for ligation

Calculate how much we get after gel purification, expected 200-400ng
next time digest higher amount of plasmid.

We can take PCR diractly for digestion!!!!

Vector+insert+ligase
Vector+Ligase
vector

Amplicon Gel purification
Vector Gel purification
Amplicon digestion (No purification
Ligate Purified vectors with non purified digested amplicons

Plan tomorrow 5/7

1. Make a gel
2. Take 5 ul from PCR tubes(tot 25 ul) , to load on the gel
3. 1, 1pbad, 2, 3, neg, vb3, vb5,
4. Take 1 ul for nanodrop
5. Restriction digestion directly in the PCR (or with the PCR solution)
6. Digest all of it, 40 ul digestion, 05ul PstI = 0.5U

EcoRI: 12 U/ul
PstI: 10 U/ul

Reaction mix:
Autoclaved water : enough for total volume of 20µl (15,88)
Buffer: 4µl
Acetyl BSA: 0,4µl
DNA(1µg/µl): 18µl
Add Restriction enyzyme 1: 0,5µl EcoR1: 0,42µl
Add restriction enzyme 2: 0,5 µl Pst1: 0,5µl
Final volume 40µl

Buffer: 2µl
Acetyl BSA: 0,2µl
DNA(1µg/µl): µl
Add Restriction enyzyme 1: 0,5µl EcoR1: 0,42µl
Add restriction enzyme 2: 0,5 µl Pst1: 0,5µl
Final volume µl

After digestion all the vector(cut with Not1) and 2µl of each brick were loaded on a gel, also Vectors(Cut with EcoR1/Pst1 and purified by gelpurification) were loaded, to check if after gel purification there is still DNA. The Not1 cut vectors will be gel purified from the gel, the Bricks and the Ecor/Pst cut vectors are just for controll.
After the gel run and the purification of the Not1 cut vector we will ligate the bricks into the double digest vectors and the Not1 will be selfligated.

Plasmid A characterization
To do:
1. C1B3 C3B5 Plasmid digestion
2. Plasmid purification by gelelectrophoresis
3. PCR purification by gelelectrophoresis (Maybe directly use the Gel extraction kit?)
4. Gel prep wiith Bricks and Plasmids

5. Store over

Put a control plate in the incubator
Digestion:
151ng/µl C1B3
256ng/µl C3B5
Reaction mix:
Autoclaved water : enough for total volume of 20µl (15,88)
Buffer: 2µl
Acetyl BSA: 0,2µl
DNA(1µg/µl): 1µl
Add Restriction enyzyme 1: 0,5µl EcoR1: 0,42µl
Add restriction enzyme 2: 0,5 µl Pst1: 0,5µl
Final volume 20µl

Table32

	A	B	C	D
1		C1B3	C3B5	
2	Water	10,28	12,98	
3	Buffer	2	2	
4	BSA	0,2	0,2	
5	DNA	6,6µl(151ng/µl)= 1µg	3,9µl(256ng/µl)=1µg	
6	EcoR1	0,42	0,42	
7	Pst1	0,5	0,5	

VRIJDAG 5-7-2019

Notes from Nerea

Updated Max's protocol to make TOP10 competent cells (find update version on drive). Used this protocol to make C1757 competent cells that Rebecca had cultured overight on liquid media.

Started by making competency solution as follows:

(Stock solutions) --> I keep them at room temperature in my bench

$MgSO_4$ --> 6.02 g disolved in 50mL MiliQ H20
$MgCl_2$ --> 10.40 g disolved in 50 mL MiliQ H20

(Competency solution)

80mL broth
1mL MgSO4 stock solution
1mL MgCl2 stock solution
10 g PEG6000

Calibrated the pH meter as showed in the protocol at Max's lab
Adjusted competency solution using HCl and NaOH until 6.1pH was reached
Put on the autoclave machine and wait 2 hours

Measuered OD from Rebecca's culture

Diluted 100microL of culture (1 and 2) into 900 microL of LB
Used 1 mL of LB to calibrate the OD

We obtained the following OD (we had to dilute them 1/10 so that the OD would fit in the machine, then you just need to mulitply the OD x10 to obtain the real OD of the sample without being diluted:

C1 (culture 1): 4.3
C2 (culture 2): 4.27

We diluted again the cultures to obtain a final concentration of 10 mL with an initial desired OD of 0.2. Therefore, we had to dilute as following:

4.3/ 0.2 = 21.5
4.27/0.2 = 21.35

10/21.5 = 0.46
10/21.35= 0.46

We take 460 microL of each culture and dilute it in 10mL of broth.

Incubate in shaker @ 37 C fro 1.5h
Measure OD at 1.5h and keep measuring until we reach OD of 0.6

make cells competent

- cool cultures in ice bath in cooling room as soon as they reach $OD_{600}$of 0.6
- refill cultures to centrifuge tubes (after emptying H_2O, of course)
- Take 3.125mL of the cell culture
- spin at 3000 rcf and 4°C for 5 min
- carry rotor to cold room
- discard supernatant and resuspend culture in 12.5 mL competency solution in total
- add 7 mL of 85% glycerol
- cool on ice for 10 min
- aliquot per 100 uL in sterile 1.5 mL Eppendorf vials
- store vials at -80°C

Calculate how much we get after gel purification, expected 200-400ng
Calculation:
We started the digest with 1000ng everything was loaded on a gel and purified, after purification and elution in 50µl Elution buffer we get
8,6ng/µl C1B3 and 15,4ng/µl of C3B5 which corressponds to 430 and 770ng of purified digested vector.
Start : 1000ng
End 430-770ng

We started the digestion of the Vectors B3 and B5 with 1µg

Work for today:
Rebecca: Take Biobrick amplicons and digest them
Tobi: Digest Biobricks from gel purification and digest them with rebecca

After digestion we clone
next time digest higher amount of plasmid.
We can take PCR diractly for digestion!!!!

Vector+insert+ligase
Vector+Ligase
vector

Amplicon Gel purification
Vector Gel purification
Amplicon digestion (No purification
Ligate Purified vectors with non purified digested amplicons

Ligation:

1. Set up the following reaction in a microcentrifuge tube on ice.
   (T4 DNA Ligase should be added last. Note that the table shows a ligation using a molar ratio of 1:3 vector to insert for the indicated DNA sizes.) Use NEBioCalculator to calculate molar ratios.
   Ligation:
   - Vector DNA (50ng)
   - Insert DNA (68ng)
   - Total DNA about 100ng
   - Ligase Buffer (10x) (2µL/20µL reaction for 10X buffer) = 2µl
   - H_2O to a total of 10µL, mix gently
   - 0.5-1µL T4 DNA Ligase (1U/µl) = 1µl
   - (DO NOT under no circumstances vortex!!!)

Cloning

	A	B	C	D	E	F	G
1		Brick 1-pbad Orange 118ng/µl	Brick3 Orange 130ng/µl	Brick1-pbad blue 118ng/µl	Brick3 blue 110ng/µl	C3 8,3	C5 7,8
2	Vector 8,6ng/µl	50ng= 8,8µl	50ng= 8,8µl	50ng= 8,8µl	50ng= 8,8µl	50ng= 6	50ng= 6,4
3	Insert	98ng= 1µl	95ng= 1µl	1µl	95ng= 1µl		
4	Ligase Buffer	2µl	2µl	2µl	2µl	2µl	2µl
5	Water	7,2	7,2	7,2	7,2	11	10,6
6	Ligase	1µl	1µl	1µl	1µl	1µl	1µl

** The T4 DNA Ligase Buffer should be thawed and resuspended at room temperature.*

1. Gently mix the reaction by pipetting up and down and microfuge briefly.
2. For cohesive (sticky) ends, incubate at 16°C overnight or room temperature for 10 minutes.
3. For blunt ends or single base overhangs, incubate at 16°C overnight or room temperature for 2 hours *(alternatively, high concentration T4 DNA Ligase can be used in a 10 minute ligation)*.
4. Heat inactivate at 65°C for 10 minutes.

5. Chill on ice and transform 1-5 µl of the reaction into 50 µl competent cells.

Clonation:
20µl DNA +5µl KCM
positive control: B1C3 1µl+7µlwater+2µl KCM

MAANDAG 8-7-2019

Strategy to finally get some results

First we repeat the Amplification : Brick1 and 2 give multiple bands but also contain the right fragments.
We will run the PCR and isolate the right bands of 1 and 2 by gel extration. After that we run all biobricks with a second primer pair, which i checked using NCBI Primer blast, to have basicly no off targets.
Hetero dimer, homo dimer and hairpins should all be molten away at conditions above 53°C, but please check for yourself using IDT oligo analyzer. Set the Mg concentration on 2mM.
By doing this second Amplification PCR we should be able to create good pcr results, showing only one band in the gel.
With those amplified biobricks we can then proceed.
I also want to sequenc our Bricks after those steps, to check how to cloning/restiction sites on both ends look.

Dependend on what kit we have, i think it is NEB assembly, we can design primers for this cloning strategy.
With NEB assembly we should be able to fascilitate the cloning progress.
Alternativly we could try overlab extention PCR to connect Bricks togheter and once we have one construct we use NEB assembly to insert it into a vector

Today we need to design primers and order them. They should arrive in two days, so the same day the PCR purification kit arrives.
Both PCRs can be done in one day, with sequencing results at the next day(Thursday?)
WE also need to start using a uniform nomenclature. I propose:
SD= single Digested
DD= double digest
GP =Gel purified
PP= PCR purified
E= EcorR1
P= PST1
N=Not1
X=Xba1
S= Spe1

The following nomenclature is used for naming new strains:
The bacterial strain - the plasmid name (inserts added:

Top10-p15A(Kan)
Woud be the name of Top10 cells transformed with the Kanamycin vector (containing the RFP biobrick from the kit)

Top10-b1C3(*BB1*, Cam)
Would be the name of Top 10 cells transformed with the high copy number vector b1C3 containg the insert Biobrick 1 and has a chloramphenicol resistance gene

C1757-b1C5(*BB1-BB2*, Cam)
Is the name of Anders C-strain E.coli transformed with the low copy number vector containing biobrick 1 and biobrick 2, chlorampenicol resistance
.....

Those should be writen at the white bord in the lab.

Right now its difficult to distinguish between all the different epi tubes, which is also my fault because i didnt name them clearly enough.

Primer List:

Primer for amplification_round 1 and 2.txt

PCR
run on gel 1µl from reaction ü 1µl dye
clean up using pcr clean up
do two measurments at nanodrop
take specific amount of DNA + buffer according to protocol enough for digest
Digest 4 µg of vector so we have enough for

DINSDAG 9-7-2019

Joost and Ayleen PCR purification

centrifuge set at 1 min 13000 rpm

purifying BB 1.2 and 3 from PCR reaction with 64 and 65 degreees.
added 120 µl PB buffer to each tube.
centrifuge,
750 µl wash buffer
centrifuge
50 µl Buffer EB
30 µl EB added waited 1 minute and then centrifuge again
centrifuge in eppitube
measure nano drop:::

Nano drop of purification of BB1.2 and BB3

	A	ng/uL	ng/uL
1	BB1.2 64°	15,6	14,6
2	BB3 64°	15,0	15,3
3	BB1.2 65°	17,6	16,3
4	BB3 65°	17,5	17,1

Ayleen : transformed AraC from plat5 well M18, into top10 cells, positive control B3C5 vector, neg control water --> followed transformation protocol
the M18 well was suspended with 10 µl water and leftf in room temp. the water evaporated and well was resuspended in 10 µl and stored in -21 after 24 hours.

WOENSDAG 10-7-2019

Ayleen:::

PCR purification purified BB 1.2 and 3
24 µl x 5 = 120 µl PB buffer added
centrifuge lid flew off and ripped caps off so contiuned with 1.2 and 3 from the Blue PCR machine

Nano drop:

Table7

	ng/uL	ng/uL	C	D
1	BB1.2	21.6	21.0	average: 21.3
2	BB3	17.1	16.8	average: 16.95

Gel purification of vector B3C5. I ran on gel. total volume 10 µl added 2.5 µl 5x loading dye
ran gel 110 volt around 1 h.

measured OD of "overnight" culture (was incubetd from 9:30 - 16:00) of B1C3 1 and 2
B1C3_1 >>> 10x dilution>>> OD: 0.023 and 0.038
B1C3_2>>>> 10x dilution>>> OD: 0.061 and 0.062

B1C3_1>>>> No diltion >>>> OD: 0.418 and 0.435
B1C3_2>>>> No diltion >>>> OD 0.825 and 0.790

Did PCR mix with BB1,2,3
running PCR during the night

Joost:::
Digested 400 ng of BB1.2 and BB3.
Nanodrop:

Table8

	A	B	C	D	E
1	Sample	measurement 1	measurement 2	mean ng/ul	Leftover volume (ul)
2	1: BB1.2	4.5	7.1	5.8	46
3	3: BB1.2	32.3	94.4		45
4	2: BB3	10.3	38.5		46
5	4: BB3	7.9	11.1	9.5	47

Expected yield was around 8 ng/ul. Therefore, sample 2 and 3 were deemed unreliable.

- Initiated digestion of 800 ng BB1.2 and BB3 purified by Ayleen.

Nanodrop results of vector B1C5 DD GP (By Rebecca)

Table9

	A	B	C	D	E
1	Sample	Measurement 1	Measurement 2	Mean ng/ul	Leftover volume (ul)
2	Tube 1	3.0	2.9	2.95	48
3	Tube 2	2.0	3.4	2.7	48

DONDERDAG 11-7-2019

Joost:::

Digested backbone B1C3

Reaction 1: EcoRI + PSTI -> 8000 ng in one reaction. Reaction volume of 125 ul
Reaction 2: NOTI -> 2000 ng in one reaction. Reaction volume 40 ul

---

Nanodrop B1C3 (leftovers of digestion) + BB1.2 & BB3 (that I had digested yesterday.)

Table10

	A	B	C	D	E	F
1	Sample	M1	M2	Mean (ng/ul)	Volume left	ng left
2	B1C3	129.8	127.1	128.45	55	7064.75
3	BB1.2	10.8	9.1	9.95	45	447.75
4	BB3	3.9	4.1	4.0	45	180

Gel purification B1C3
NOT1 digested material could be purified, the EcoR1/PST1 did not seperate properly, and most material was not digested. We assume that this is because of the large volume of digestion mix. Therefore the second digestion will be repeated on the leftover material from the digestion that was ran today, this time in smaller aliquotes.

Ayleen:::
Gel purified BB 1, 2, 3
PCR amplification of BB1, 2, 3 with 10 µl from each solution

Plasmid A characterization

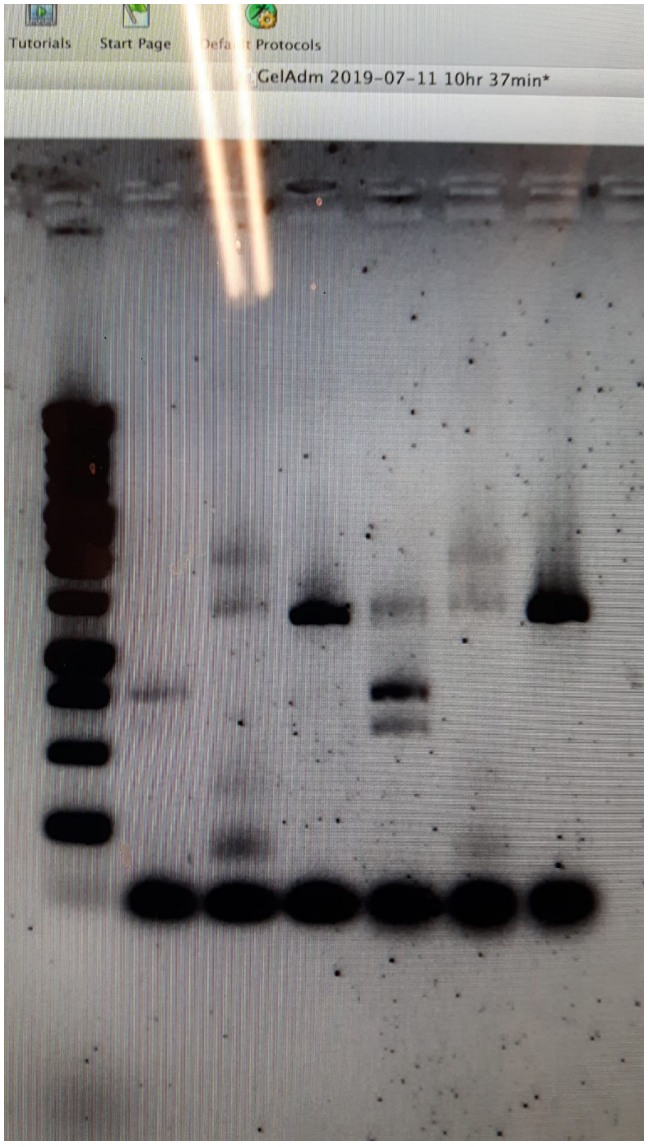
I ran a PCR with the amplification primers using following PCR protocol in the orange and the blue machine.

Table33

	A	B	C
1	STEP	TEMP	TIME
2	Initial Denaturation	98°C	30s
3	25 Cycles	98°C 66°C 72°C	10 seconds 30 seconds 30 seconds/kb= 1 min
4	Final Extension	72°C	15 minutes
5	Hold	4°C	

Results:

L_Brick1_2_3(orange)_1_2_3(blue).png



The blue machine gave the desired bands for biobrick 1, 2 and 3

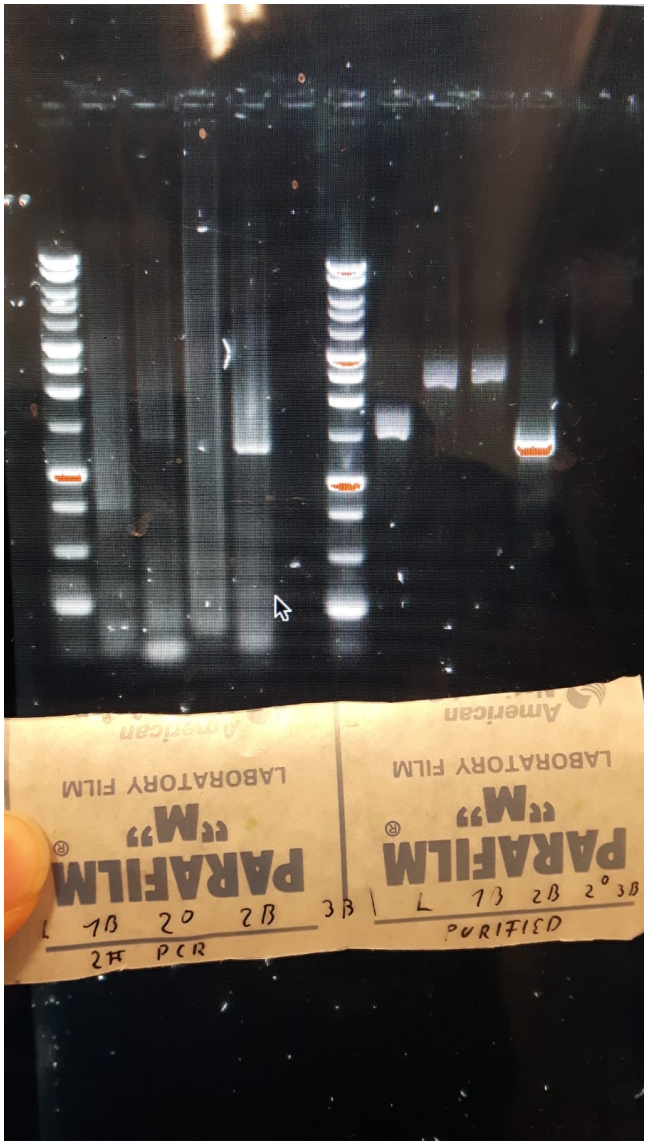
As a next step we took 4µl of PCR 1_2_3 (blue) and 2(orange) for a PCR using amplification primer 2 with following Programm on 67°C.
The rest(20µl) was used for Gel purification and will be run on a gel togheter with the Second PCR.

Table34

	A	B	C
1	STEP	TEMP	TIME
2	Initial Denaturation	98°C	30s
3	25 Cycles	98°C 67°C 72°C	10 seconds 30 seconds 30 seconds/kb= 1min 10 sec
4	Final Extension	72°C	5 minutes
5	Hold	4°C	

Result of the #2 PCR and Gel purification

grafik.png



We ran a PCR with Amplification Primer set 2# over night.

Table35

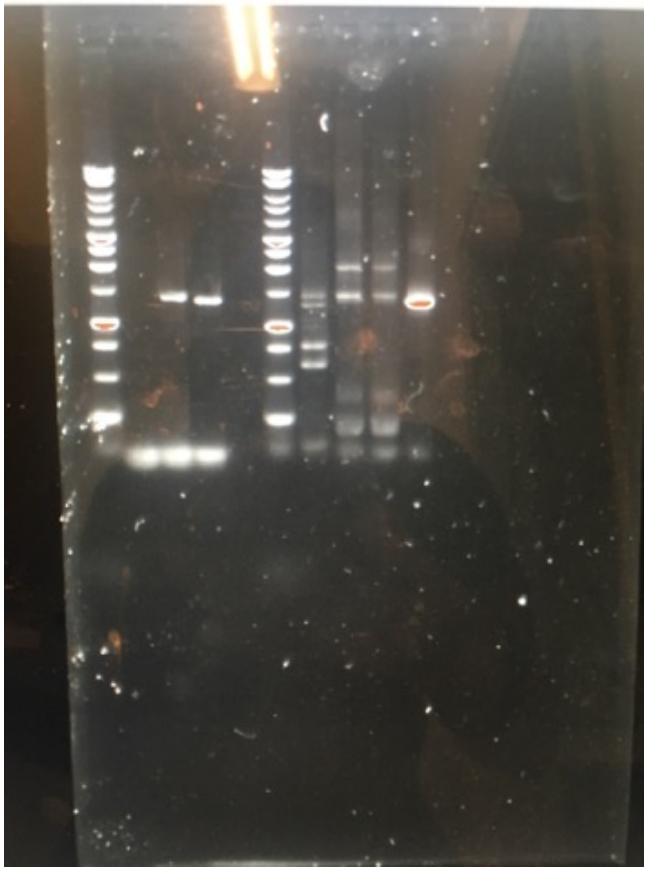
	A	B	C
1	STEP	TEMP	TIME
2	Initial Denaturation	98°C	30s
3	25 Cycles	98°C 67°C 72°C	10 seconds 30 seconds 30 seconds/kb= 1min 10 sec
4	Final Extension	72°C	5 minutes
5	Hold	4°C	

VRIJDAG 12-7-2019

Ayleen::::

Made new TAE buffer 2 L
made new gels and casted gels.
Ran neg control , BB 1.2 and BB3 , empty then ladder and PCR product of BB 1, 2, 2orange ,3

image.png



Do two reaction in 50µl one with 10µl template and one with 1µl template

nanodrop on Plasmid purifcation of B1C3

Table11

	ng/uL	B	average
1	138,2	139,6	138,9
2	139,2	136,3	137,75
3	114,6	108,4	111,5
4	184,0	150,9	167,45
5	143,6	141,2	142,4

---

nanodrop of BB1.2 and BB3 amplification

Table12

	ng/uL	ng/uL	ng/uL	average
1	bb1.2	15,4	16,2	15,8
2	bb3	20,3	19,4	19,85
3				

loaded gel with ligation B1C5 DD, lig 1:3 B1.2, lig 1:5 B1.2, lig 1:3 B3, lig 1:5 B3.
2 µl of liagtions + 2 µl dye.
125 volt 33 min

Ligation
ligation of BB1.2 and BB3 in C3 vector

Table13

	A	B	C	D
1		Neg	C3 BB1.2	C3 BB3
2	T4 buffer	4	4	4
3	T4 ligase	1	1	1
4	Vector	5	5	5
5	Insert	0	7.5	7.37
6	H2O	10	1.5	2.63
7	20 ul			

MAANDAG 15-7-2019

Ayleen::::

Transformation of AraC from plate 4, well 21E into top10 cells. followed transformation protocol. Did not do neg/pos control.
the plasmid the AraC/pbad is in is B1C3. We use this and add our biobricks in this plasmid. We do not need to cut out araC and add to our plasmid. we use this plasmid now.
the resuspended DNA is stored in -20. the transformed bacteria were also stored in -20 before moved to a fridge instead, this was a mistake, might have killed them.

Digestion of Biobricks
Digest 200 ng of each

Table14

	A	B	C	D	E	F
1		1.2	3	1	2	Neg
2	Buffer	2				
3	BSA	0.2				
4	PstI	0.5				
5	EcoRI	0.42				
6	DNA	8.09	8.13	4.12	1.15	-
7	H2O	8.79	8.75	12.16	15.73	16.88
8	Total 20 ul					

PCR Amplification - Q5 PCR 50 ul Reaction mix

Table15

	A	B	C
1		Biobricks	Neg
2	Q5 Master Mix	25	
3	P1	2.5	
4	P2	2.5	
5	DNA	1	0
6	H2O	19	20

Ligation

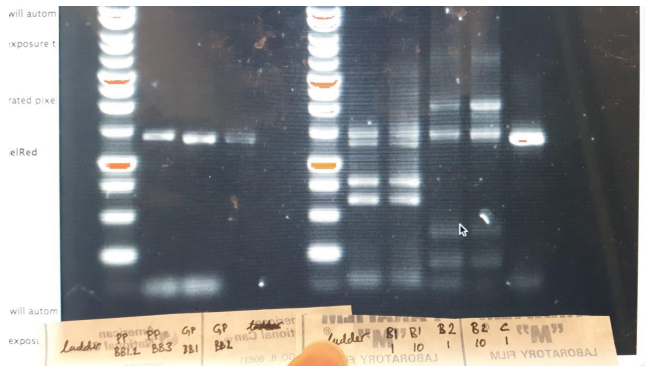
Vector conc. 12.05 ng/ul

Table16

	A	B	C	D	E	F
1	1:3	BB1.2	BB3	BB1	BB2	Neg
2	T4 Buffer	4				
3	T4 Ligase	1				
4	Vector	4.15	4.15	4.15	4.15	4.15
5	Insert	10	9.8	8.8	14.9	-
6	H2O	0.85	1.05	2.05	4.05	10.85

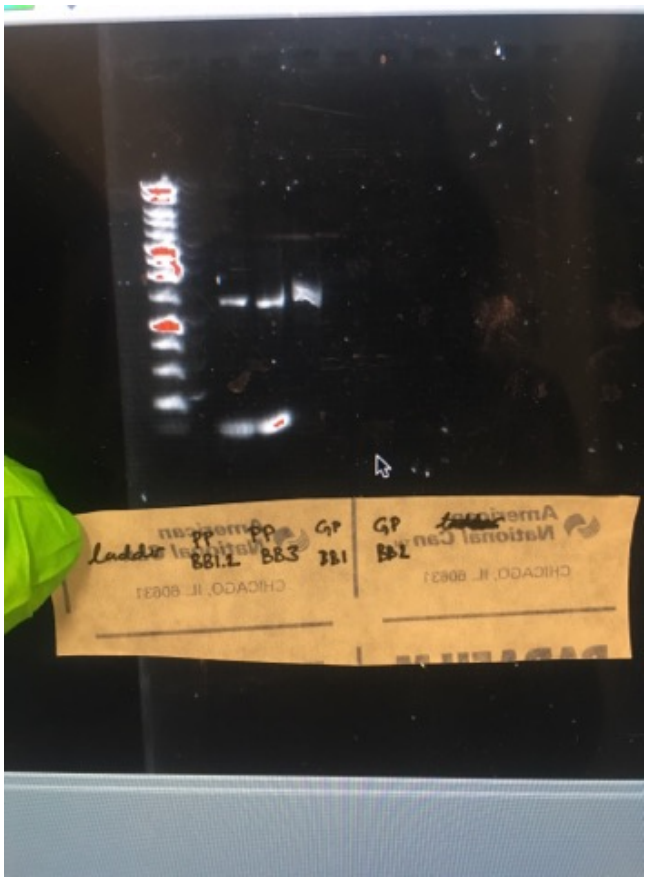
Ran gel on pp bb1.2 bb3, gp bb1 and bb2 and pcr product bb1, bb1.10. bb2, bb2.10 and C, run on 120 volt 25 min

image.png



reedoing pp bb1.2 and bb3; gp bb1 and bb2

image.png



there seems to be no band for GP BB2 in both tries. missing after gelpurification.

Today we transformed with AraC3
We did second PCR
On the right side you can see the PCR in 50µl toal volume with 1 and 10µl DNA template
the lower concentrated PCR gives less unspecific bands, but still to many.

DINSDAG 16-7-2019

ayleen:::
made a PCR solution of all biobricks (1, 1.2, 2, 3, neg)

Digestion of 1.2 and 3 with XbaI and PstI - 400 ng was digested
Final concentration is 400ng/20ul = 20ng/ul
Tubes were incubated at 37C for 3 hours

Table17

	A	B	C	D
1		BB1.2	BB3	neg
2	Buffer H	2	2	2
3	BSA	0.2	0.2	0.2
4	PstI	0.5	0.5	0.5
5	XbaI	0.5	0.5	0.5
6	DNA	16.2	16.3	0
7	H2O	0.6	0.5	16.8

Transformation

Table18

	A	B	C	D
1		Ligated Vector+ Biobricks + dig neg	Postive control	Negative control
2	KCM	2	2	2
3	DNA	5	1	0
4	H2O	3	7	8

Colony PCR

Table19

	A	B	C
1		Colonies	Neg
2	5x Gotaq Green buffer	10	
3	MgCl2	8	
4	dNTPs	1	
5	Backbone_P_F	5 (10 uM)	
6	Backbone_P_R	5 (10 uM	
7	Gotaq Enzyme	0.25	
8	DNA	1	0
9	H20	19.75	20.75

at 16:00 anali and i will do an overnigght culture of transformation of AraC which was succesful and restreak. Anali restreak B1c3 empty vector.

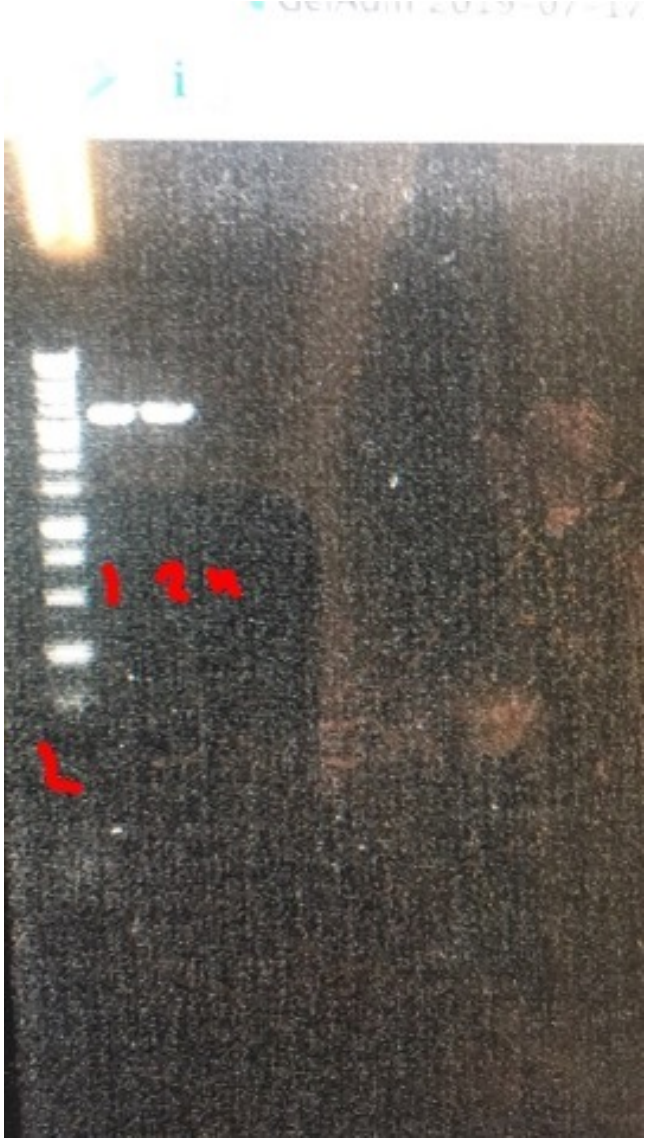
overnight culture 10 ml tsb 10 mikrL Chl. 2 e flasks.

WOENSDAG 17-7-2019

plasmid prep on AraC3 and B1C3BB3 , B1C3SD

digestion of 2x2mikrograms with Spei and pstI and buffer B, incubation 5 hours in 37 degrees.
run gel on digestion.

image.png



flask 1 and 2 and negative control.

DIGESTION OF BIOBRICKS (AraC3 VECTOR)

Digestion of 1.2 and 3 with XbaI and PstI - 400 ng was digested
Final concentration is 400ng/20ul = 20ng/ul
Tubes were incubated at 37C for 3 hours

Table22

	A	B	C	D
1		BB1.2	BB3	neg
2	Buffer H	2	2	2
3	BSA	0.2	0.2	0.2
4	PstI	0.5	0.5	0.5
5	XbaI	0.5	0.5	0.5
6	DNA	16.2	16.3	0
7	H2O	0.6	0.5	16.8

,

DIGESTION OF BACKBONE (AraC3 VECTOR)

PlP 1.1 - 139.35ng/ul

PlP 2.3 - 164.85 ng/ul

Digest 4 ug = 2ug + 2ug, 2ug=2000ng

Digested AraC3 conc. 2000ng/20ul = 100ng/ul

Table20

	A	B	C	D
1		1	2	Neg
2	Buffer B	4		
3	BSA	0.4		
4	PstI	1		
5	SpeI	1		
6	DNA	12.1	12.1	0
7	H2O	1.5	1.5	13.6
8	20 ul			

Ligation AraC3

The insert amounts are double because I forgot that digested inserts were 20ng/ul and not 10ng/ul.

Table21

	A	B	C
1		1.2	3
2	T4 buffer	4	4
3	T4 Ligase	1	1
4	Vector	0.5	0.5
5	Insert	6.1 -> 3.05	5.9
6	H2O	8.4	8.6

DONDERDAG 18-7-2019

Colony PCR
iGEM Vector primers

Table23

	A	B	C
1		Colonies	Neg
2	5x Go green buffer	10	
3	MgCl2	4	
4	dNTPs	1	
5	Forward Primer	2.5	
6	Backward primer	2.5	
7	Gotaq enzyme	0.25	
8	DNA (1 colony in 10ul)	1	-
9	H2O	28.75	29.75

Which program ?

Growth curve

1. Inoculate cells in 10 ml TSB with 10ul Chloramphenicol, grow overnight 37C.
2. Dilute overnight cultures to OD 0.2 in 10 ml TSB with 10ul Chloramphenicol and grow until OD 0.6.
3. Dilute cultures to OD 0.05 in 30 ml TSB with 30 ul of chloramphenicol (and 0.2% Arabinose) and grow for 5 hours.
4. Take samples (1.5 ml) at 6 time points.

Growth curve was performed with BB1, BB1.2, BB1.2 Ara, BB3, C3SD and Top10 cells. BB1.2 colonies were not red so it was not the correct colonies

MAANDAG 22-7-2019

Milda:::

Plasmid A construction:
Today I did digestion of biobrick BB3 (concentration 24.6 ng/ul). In total 200 ng were digested.

Protocol:
Buffer H 2 ul
BSA 0.2 ul
PstI 0.5 ul
XbaI 0.5 ul
DNA 8.1 ul
H20 8.7 ul

Incubate at 37 degrees for 2 hours. Inactivate at 65 degrees for 10 minutes.

Joost:::

Digestion of C3 BB1 with PstI and SpeI
2000 ng devided over 2x1ug reactions
Incubation of 3:20 h at 37 degrees. Heat inactivated at 65 degrees for 10 minutes.

Prduct ran on 1% agarose, single clear band visible around expected height of 3400 bp for each of the samples.

Ligation of C3 BB1 and BB3 (Milda)
Reaction preformed at 16 degrees o/n in duplicate with empty vector/no insert as negative control.
Vector:Insert ration of 1:3
vector: 1 ul (50 ug)
Insert: 2.85 ul (56.91 ug)

DINSDAG 23-7-2019

23/7 - BB1,2 + AraC combination

Table24

	A	B	C
1		BB 1.2	Negative
2	T4 buffer	4	4
3	T4 Ligase	1	1
4	Vector	0.3	0.3
5	Insert	5	-
6	H2O	9,7	14,7

Table25

	A	B	C	D	E	F
1						
2	AraC3	3280 bp	0.023 pM	0.050 ug	50 ng	
3	1.2	1333 bp	0.069 pM	0.061 ug	61 ng	
4	1.2	1333 bp	0.115 pM	0.1 ug	100 ng	

insert: 20 ng/ul
vector: DD AraC3 2 - 100ng/ul - 0,3ul

Milda:::

Plasmid A construction:
Continuing work with BB3 and C3 BB1 ligation. Heat inactivated ligation reaction. Then did the transformation, using 5 ul (about 25 ng of DNA) of the ligation mixture (with 2 ul of KCM and 3 ul of H20) (x2 replicates), ligation negative control (5 ul reaction with 2 ul of KCM and 3 ul of H20) as well as 1 ul of C3 plasmid as positive control (with 2 ul of KCM and 7ul of H20), and negative control (only 2 ul of KCM and 8 ul of H20). We plated the two replicates in two ways: either 50 ul or 25 ul of the cells, on chloramphenicol marker plates (7 plates in total).

Stella::::
Ligation AraC , 1.2

WOENSDAG 24-7-2019

lisa and Ayleen::

Double Digestion of BB1 with xbal and pst1

24/7 - BB1 + AraC combination

Table26

	A	B	C
1		BB 1	Negative
2	T4 buffer	4	4
3	T4 Ligase	1	1
4	Vector	0.5	0.3
5	Insert	7.8	Negative sample digestion
6	H2O	6.7	6,7

insert: 155.8 ng (5:1) - 400 ng in 20 ul - 20 ng/ul - 7.8 ul
vector: 100 ng/ul - 0.5

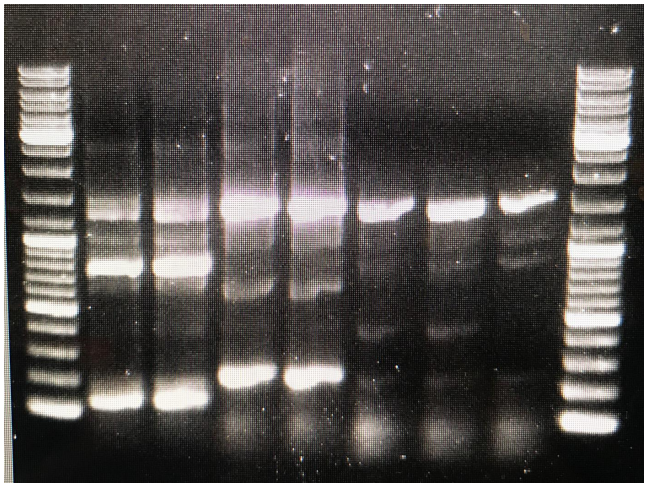
Tobi:
we amplified and purified BB2 by PCR and PCR purification with a final concentration of 220ng/µl
we took digested BB1 and AraC3 nd ligated overnight

Milda:::

Plasmid A construction:
Today we checked the colonies from yesterday's C3-BB1+BB3 ligation. The transformation was successful. We observed red colonies for the plates with cells transformed with ligate mixtures, as well as a lot of red colonies on the positive control, colonies on the ligation negative control and no colonies on the transformation negative control. Tomorrow we will do colony PCR screening to check if any of the colonies contain vector with the BB3 insert. I will also include Rebecca's colonies for screening (AraC3-BB1.2 ligate transformants).

Biobrick amplification:
I also attempted to amplify biobricks BB1, BB1.2 and BB3.

IMG_0769.jpg



From left to right: BB1 #1 and #2, BB1.2 #1 and #2, BB3 #1 and #2, negative control. We can see that negative control also has a band for the same size as the biobricks should be. After discussing, we have come to the conclusion that the water, which I used for the master mix, might be contaminated. Tomorrow I will do the amplification of the biobricks again, using new water. I will also do it in the blue PCR machine instead of the one downstairs.

Stella:::
Transformation AraC + 1,2
also ligation AraC + 1

DONDERDAG 25-7-2019

ayleen:::
transformation of BB1 Arac3 into top 10 cells by using 8µl DNA and 2 µl KCM.
plated on chl, arabinose plates due to lack of simple chl plates.
incubated overnigh 37 degrees.

Sanchari + Stella
Phusion Colony PCR - AraC & 1,2
Phusion Colony PCR - AraC combined with 1,2 - Unsuccessful

Phusion Colony PCR - AraC-BB1.2 on orange

	A	B	C
1	STEP	TEMP	TIME
2	Initial Denaturation	98°C	30 seconds
3	25 Cycles	98°C 63°C 72°C	10 seconds 30 seconds 3 minute
4	Final Extension	72°C	10 minutes
5	Hold	4°C	

Table27

	A	B	C
1	STEP	TEMP	TIME
2	Initial Denaturation	98°C	30 seconds
3	25 Cycles	98°C 63°C 72°C	10 seconds 30 seconds 1 minute
4	Final Extension	72°C	7 minutes
5	Hold	4°C	

Digestion C3 BB3

Autoclaved water : enough for total volume of 20µl ()
Buffer H: 2µl
Acetyl BSA: 0,2µl
DNA(1µg/µl): 1µl
Add Restriction enyzyme 1: 0,5µl EcoR1: 0,42µl
Add restriction enzyme 2: 0,5 µl Pst1: 0,5µl
Final volume 20µl

Mix gently by pipetting up and down and then spin down the sample.
Incubate at 37°C for 1- to 4 hours (4 hours)
After incubation heat inactivation at 65°C to inactivate the restriction enzymes, or Gel purification, or PCR purification Kit

Plasmid A characterization

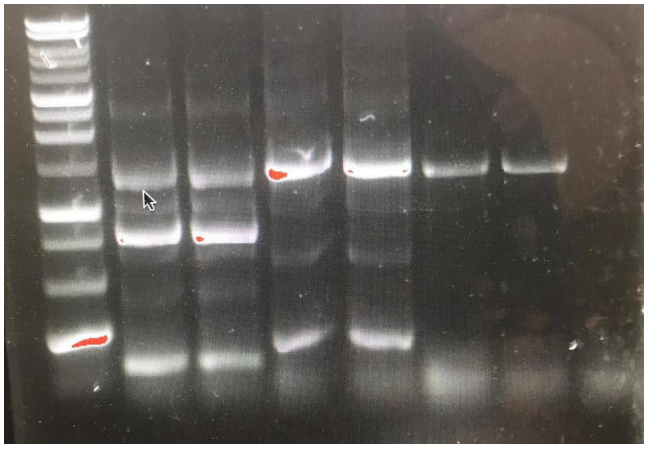
Today we transformed top 10 with the AraC3_BB1 vector
We digested BB2 and ligated it into AraC3. Incubation at 16°C overnight, they will be transformed into top10 at friday the 26th.

Milda:::

Biobrick amplification:

Today I repeated biobrick amplification, this time using pure water. The PCR was successful:

WhatsApp Image 2019-07-25 at 20.55.13.jpeg



From left to right: BB1 #1 and #2, BB1.2 #1 and #2, BB3 #1 and #2, negative control. The negative control does not have the band any more, so the water must indeed have been contaminated. Tomorrow I will do PCR purification of the amplified biobricks and perform digestion (using >20 ng/ul of the template). Appearantly such amplification works even with the set up that I used:

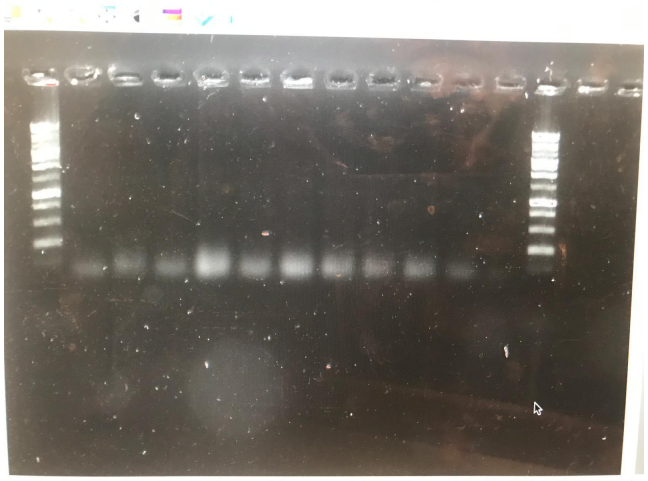
STEP	TEMP	TIME
Initial	98 degrees	30 seconds
25 cycles	98 degrees	10 seconds
	66 degrees	30 seconds
	72 degrees	1 minute
Final extension	72 degrees	2 minutes
Hold	4 degrees	

However, there is another setup which is even better, using 30 cycles, and more than 5 minutes of final extension. Tobi is going to attempt it tomorrow for comparison.

Plasmid A construction:

As for the plasmid A, today we did colony PCR with C3 positve control, C3-BB1 ligation negative control and C3-BB1+BB transformants (four colonies from two plates each). Colony PCR did not work (as seen below). The reason might be that this time we used Pfu polymerase which uses a different buffer and in this buffer the annealing temperature of the primers might be different. We used annealing temperature of 63 degrees, but we will attempt 60 degrees tommorrow when we repeat the experiment.

WhatsApp Image 2019-07-25 at 18.49.19.jpeg



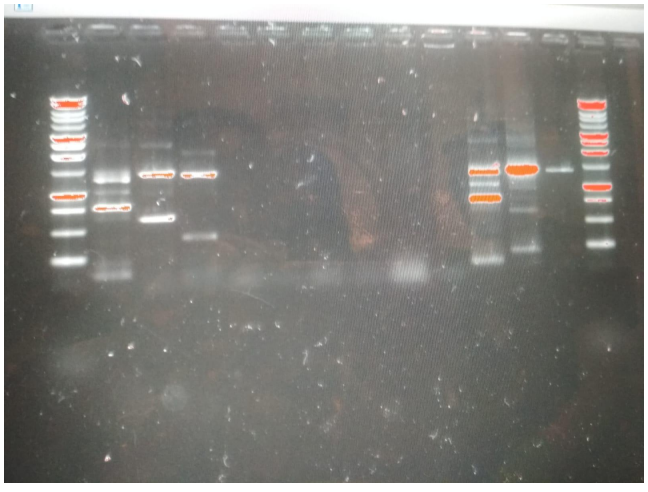
VRIJDAG 26-7-2019

Milda:::

Biobrick amplification:
Today I PCR purified the amplified biobricks.
Nanodrop measurements:
BB1 - 143.4;
BB1.2 - 147.8; '
BB3 - 11.3.
Simultaneously, Aneli amplified the biobricks using an alternative PCR machine setup, but the results are not vastly different than before. She then PCR purified them.

Plasmid A construction:
We did colony PCR with 5 minutes of inital denaturation and reduced annealing temperature of 60 degrees. It did not work. The major problem is probably the lysis step. It should be done separately after suspending the colony in 20 ul of water, by heating it in 95 degrees for 10 minutes, before doing PCR reaction.

WhatsApp Image 2019-07-26 at 15.57.58.jpeg



From left to right: biobricks BB1, BB1.2 and BB3 (new setup); colony PCR (did not work); biobricks BB1, BB1.2 and BB3 (PCR purified, previous amplification setup).
On Monday we will digest the biobricks and repeat the colony PCR with improved protocol.

MAANDAG 29-7-2019

Figuring out colony pcr

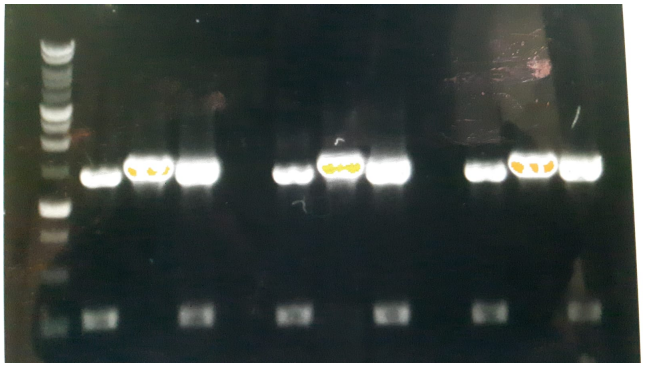
first test different temperature sets for AraC3 C3 and AraC3/C3 mix

Colony PCR 200µM Primer 2mM Magnesium

	A	B	C	D	E	F	G
1	STEP	TEMP	TIME	Component	25 µl reaction	50 µl reaction	Final Concentration
2	Initial Denaturation	95°C	2min	5X Standard Taq Reaction Buffer	5 µl	10 µl	1X
3	25 Cycles	95°C 59.3°C 72°C	30 seconds 30 seconds 2min	10 mM PCR neucleotide mix	0.5 µl	1 µl	200 µM
4	Final Extension	72°C	2 min	10 µM Forward Primer	1.25 µl	2.5 µl	0.5 µM (0.05–1 µM)
5	Hold	4°C		10 µM Reverse Primer	1.25 µl	2.5 µl	0.5 µM (0.05–1 µM)
6				Bacterial lysate	1µl	1µl	<1,000 ng
7				Taq DNA Polymerase	0.125 µl	0.25 µl	1.25 units/50 µl PCR
8				Nuclease-free water	15.9ul	to 50 µl	

touch colony slightly with pipet tip and resuspend it into 20µl of water. Lyse the cells at 95°C for 10 minuts, use 1µl of that lysate as template.
For testing i need Purified AraC3 and C3 aswell as glycerol stock/colonies AraC3 and C3

grafik.png



C3/AraC3/C3+AraC3/negative at 57,3/58,3/59,3 °C

Milda:::

Biobrick amplification:
We measured on Nanodrop Anali's biobricks:
BB1 - 49.8 ng/ul
BB1.2 - 25 ng/ul
BB3 - 20.7 ng/ul

Using biobricks amplified by me and Anali, we have now done biobrick digestion:
340 ng of BB3 with XbaI and PstI
340 ng of BB3 with EcoRI and PstI
400 ng of BB1 with XbaI and PstI
400 ng of BB1.2 with XbaI and PstII
We incubate the digestion mixtures for 3 hours at 37 degrees. We then inactivated the reaction for 10 minutes at 65 degrees.

Characterization of C3-BB1:
I prepared overnight cultures of Top10 cells, C3 SD negative control, C3 positive control and C3-BB1 plasmid containing cells.
Tomorrow I will (after measuring OD) set up the FLUOstar, as well as do growth curves of these cel.

DINSDAG 30-7-2019

Digestion of BB1 and BB2

Table28

	A	B	C	D	E
1		BB 1	negative	BB2	negative
2	Buffer H	2		2	
3	Water	11,22		13,2	
4	BSA	0.2		0,2	
5	DNA	800ng=5,58		800ng=3,6	
6	Pst	0,5		0,5	
7	Xbal	0,5		0,5	

Ligation
Ligation: araC3+BB1

Table29

	A	B	C
1		BB 1 (40ng/µl)	Negative
2	T4 buffer	4	4
3	T4 Ligase	1	1
4	Vector	0.5	0.5
5	Insert	2,5	water
6	H2O	12	6,7

5:1 BB1(97,94ng=2,5µl):Vector(50ng=0,5µl)

Ligation: araC3+BB2

Table30

	A	B	C
1		BB 2 (40ng/µl)	Negative
2	T4 buffer	4	4
3	T4 Ligase	1	1
4	Vector	0.5	0.5
5	Insert	3,9	water
6	H2O	10,6	6,7

5:1 BB1(155,8ng=3,9µl):Vector(50ng=0,5µl)

Milda:::

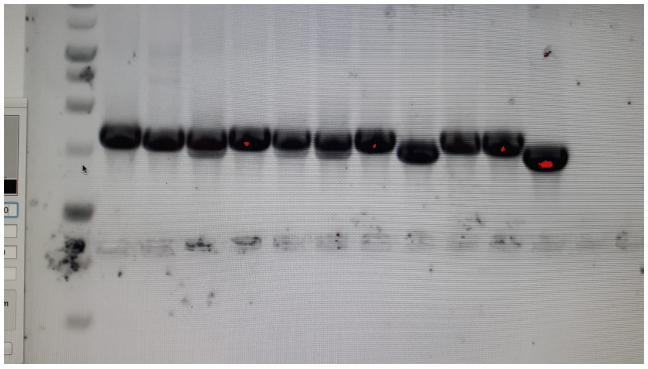
Characterization of C3-BB1:

In the morning I first set up FLUOstar for C3-BB1 characterization, including four samples for both 1:10 and 1:20 dilutions (all cells diluted to 0.3 OD), as well as Top10 cells, SD and C3-positive as controls in the same dilutions.

I then did growth curves for the same cells for 5 hours, first seeding them from the night cultures to 0.05 OD and then growing for one hour before continuing taking time series samples. I collected samples for Western Blot at 180 min, 240 min, 300 min time points, pelleting the cells, removing the supernatant and freezing them.

Tobi:
Result of colony PCR:
AraC3_BB1 shows no deviation from the AraC3 positive controll
AraC3_BB2 shows some bands with longher sizes where group one and 4 show the highest bands

grafik.png



Today we digested 800ng of BB1 and BB2 andstarted the ligation into AraC3 with a 5:1 ration over night

WOENSDAG 31-7-2019

For Lisa:
We will transform in the afternoon around 2PM, leave the ligation in the +16 until then.
Two transformations per ligation and we will plate 50µl of each. Do we have enough plates?
Also we will do Colony PCR, but first please run the colony PCR again, at the picture you can see that some of them have higher bands, and also very faint higher bands which i havent noticed. So first run those 10 samples again and then depending on those results run single colony PCRs.

Update of Lisa
Take single colonies from group 5 and 6 and resuspend them in 20µL of water
Work under sterile conditions. With a pipette of 20-200µL touch the colony.
Of those 20µL, take 5µL and do lysis (95°C for 10min)
The 15µL save them! Write date + name on tube

With the lysed bacteria , do colony PCR.
The program for that is saved in the machine under colony PCR => see Benchling

Milda:::

Today we are beginning construction of one construct: AraC-BB1.2 + BB3. We will first construct AraC-BB1.2. For that, we have AraC DD with SpeI and PstI, and we have BB1.2 DD with XbaI and PstI. We follow an updated protocol, in which we first dephosphorylate the vector, in order to avoid vector self-ligation (protocol for dephosphorylation can be found here: https://international.neb.com/protocols/0001/01/01/vector-dephosphorylation-protocol). Antarctic phosphatase can be borrowed from another lab.

Vector dephosphorylation:
Vector DNA 200 ng -> 20 ul
Antarctic phosphatase buffer (10x) 2 ul
Antarctic phosphatase (5000 U/ml) 1 ul
H20 up to 20 ul
Incubate at 37 degrees for 30 minutes. Inactivate at 80 degrees for 2 minutes.

We purify the dephosphorylated vector using PCR purification kit. The concentration is 3.6 ng/ul.

We use 40 ng (11.1 ul) for ligation in a 1:5 ligation reaction (with 68.39 ng of BB1.2 insert). Incubate overnight in 16 degrees.
Tomorrow Sanchari will do the transformation, and on Friday, if it worked - colony PCR.

DONDERDAG 1-8-2019

Ayleen:::
transformation of Arac3-bb1 and arac3-bb2 and AraC3-bb1.2
plated colonies of Arac3-bb2 (5,1 - 5,3 - 5,4 -6,2 - 6,3 - 6,4)

overlapextentions of BB1 (two sets of primers) and BB2 (one set of primers)

MAANDAG 5-8-2019

Rebecca: Biobrick amplification - Only BB3 was purfied and Nanodroped -
Transformation of Sancharis samples -AraC3 BB1.2

DINSDAG 6-8-2019

Sanchari :
Today we digested 1000ng of AraC3 in three tubes(total= 3000ng) using plp 1.1 with a concentration of 139.39 ng/ul.

Digest 3 ug = 1ug + 1ug, 1ug=3000ng
Digested AraC3 conc. 1000ng/20ul = 50ng/ul
5 hours in 37 degrees

Table1

	A	B	C
1		All three tubes	Neg
2	Buffer B	4	
3	BSA	0.4	
4	PstI	1	
5	SpeI	1	
6	DNA	7.18	0
7	H2O	6.42	13.6
8	20 ul		

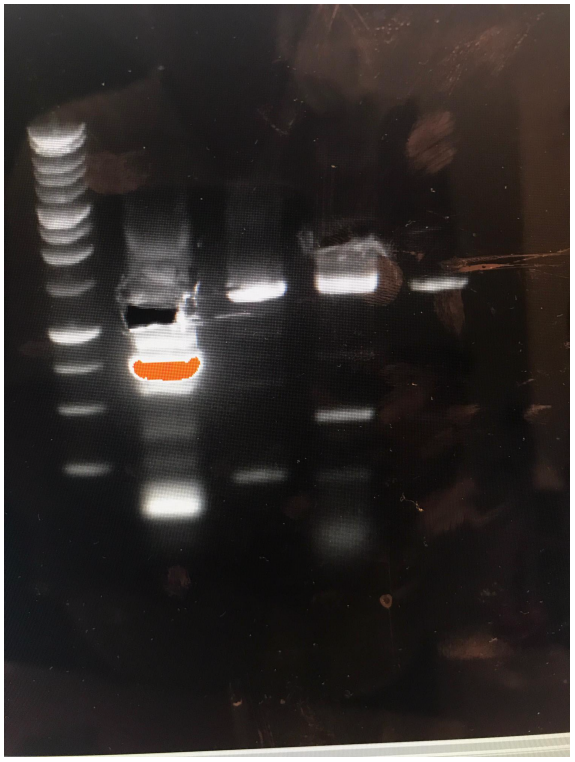
Sequencing:
Volumens: 12µl per reaction
concentrations: 1,5ng/100bp
Brick1: 19,27
Brick1.2: 20,7
Brick2: 30,67
Brick3: 20,025

Milda:::

Today I am amplifying biobricks BB1, BB1.2, BB2 and BB3. The amplification setup on the blue machine is "Amp." with the following cycles:

Initial denaturation	98 degrees	30 secs
30 cycles	98 degrees	10 secs
	66 degrees	30 secs
	72 degrees	1 min 10 secs
Final extension	72 degrees	15 mins
Hold	4 degrees	

IMG_0876.jpg



BB1 and BB3 were gel purified (see above the cut bands), concentrations were 12.1 and 5.8 ng/ul respectively; BB1.2 and BB2 were PCR purified, concentrations were 3.6 and 1.2 ng/ul respectively.

WOENSDAG 7-8-2019

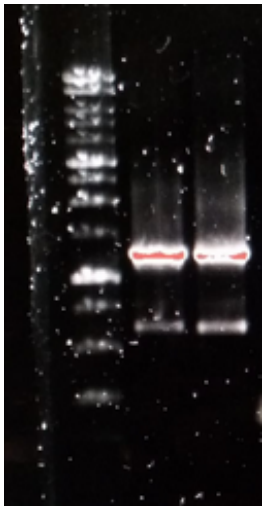
Tobi
9 to 12 PCR first round Brick 1/1.2/2/3 with OE primers using Q5

OE_1_1+2_F_0008 :_: CGCCAGGTTGAATGAATTCGC 64.5 2:10min
OE_1_1+2_R_0009 :CAAATAATCAATGTTGGCCGGCTTGACGG 65.0 2:10min
OE_2_1+2_F_00010 : GTCAAGCCGGCCAACATTGATTATTTGCACGGCGTCAC 65 2:10 2:10min
OE_2_1+2_R_00011 GTATTTGGAGGTACTGCAGCGG 65 2:10min
OE_1_1+3_F_00012 CGCCAGGTTGAATGAATTCGC 64.5 2:10min
OE_1_1+3_R_00013 CGAGAAGATAGTCTTGACGGCTAGCTCAGTCCT 65.6 2:10min

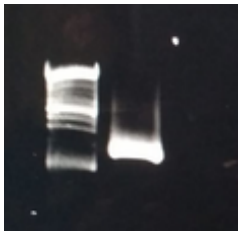
OE_3_1+3_F_00014 AGCTAGCCGTCAAGACTATCTTCTCGCTTATCGTGTT 63.6 1:30
OE_3_1+3_R_00015 GTATTTGGAGGTACTGCAGCGG 65 1:30

We made BB1(1+2)´BB1(1+3)´BB1.2(1+3)´BB2(1+2)´and BB3(1+3)

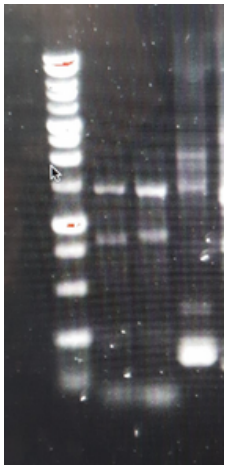
BB1(1+2)/BB1(1+3) png



BB3.png



BB1.2(1+3)/BB 2(1+2).png



We need to gel purifiy them

Milda:::

Today Stella plasmid purified the plasmids form the successful transformation colonies (AraC3-BB1.2). I did double digestion of 1 ug this plasmid (concentration 150 ng/ul) with PstI and SpeI for 3 hours at 37 degrees. Then I did dephosphorylation of this digested vector, as well as of AraC3 vector.

Stella:::

NanoDrop PLP AraC3 BB1.2

	A	B	C
1	155.6	144.5	150.05
2	187.0	198.2	195.6
3	158.7	164.4	161.55
4	161.6	184.0	172.8
5	159.2	149.2	154.2
6	178.5	179.4	178.95

Also beginning on working with Overlap Extension. Tomorrow step one and two will begin.
BB1(2) - 68°C Annealing temperature for Q5
BB1.2(1) - 68°C
BB2(1) - 69°C
BB3(1) - 66°C
All primers are on primers at Benchling where I have also written their Q5 Tm

Q5 Overlap Extension from neb

	A	B	C	D
1	Component	25 µl Reaction	50 µl Reaction	Final Concentration
2	Q5 High-Fidelity 2X Master Mix	12.5 µl	25 µl	1X
3	10 µM Forward Primer	1.25 µl	2.5 µl	0.5 µM
4	10 µM Reverse Primer	1.25 µl	2.5 µl	0.5 µM
5	Template DNA	1 µl	variable	< 1,000 ng
6	Nuclease-Free Water	to 25 µl	to 50 µl	
7	Notes: PCR machine without a heated lid. Thermocycling Conditions for a PCR:			
8	Initial Denaturation	98°C	30 seconds	
9	30 Cycles	98°C	10 seconds	
10		Annealing Tm	30 seconds	
11		72°C	30 seconds/kb	
12	Final Extension	72°C	2 minutes	
13	Hold	4–10°C		

DONDERDAG 8-8-2019

Sanchari :::

Ligation of AraC3 +BB1 [1:5 ratio]
Ligation of AraC31.2 + BB3 [1:3 ratio]

Successful ligation.

Transformation was done for both on 09/08. Positive colonies observed

MAANDAG 12-8-2019

Colony PCR was performed for AraC3+BB1 and AraC3 1.2+BB3.
Positive colonies for both were observed . These positive colonies were further grown overnight at 37 °C in liquid cultures.
9th colony from 2nd plate of AraC31.2+BB3. 5th colony from 2nd plate of AraC3+BB1.

Screen Shot 2019-08-13 at 13.58.00.png

DINSDAG 13-8-2019

Sanchari and Milda

Plasmid Preps of AraC3+ BB1.
Digestion of AraC3+BB1 with Spe1 and Pst1.
Dephosphorylation of AraC3BB1 vector
Ligation with double digested BB2 overnight
We also prepared one plate of each liquid culture and glycerol stocks for both the cultures

Nerea & Stella

We took liquid colony from Milda and Sanchari (Model plasmid, AraC+ BB1.2 + BB3)
We started a growth curve to characterize this plasmid (no arabinose was added).
We also took samples at 1h, 3h and 5h to keep aside for the Wester Blot
The OD were as follows:

Table36

	Time	OD	WB sample	D
1	0	0.059	-	
2	1 (30min)	0.072	-	
3	2(1h)	0.120	1mL	
4	3(1h30)	0.243	-	
5	4(2h)		-	
6	5(2h30)		-	
7	6(3h)			
8	7(3h30)		-	
9	8(4h)		-	
10	9(4h30)		-	
11	10(5h)			

For the WB, 10^8 cells/mL were needed, so calculations where done using this calculator:
https://www.chem.agilent.com/store/biocalculators/calcODBacterial.jsp?_requestid=57942

WB samples were spin down for 1 min at max speed and the super natent was dispossed. The pellet was kept at the fridge until the collection of the last tube was done. After this, lysis was done.

WOENSDAG 14-8-2019

Transformation of AraC3 BB1 + BB2 ligation mixtures

DONDERDAG 15-8-2019

Ayleen:::::
Digestion of BB1+3 from overlapextention, see renewed protocol in protoocl digestion.

DIGESTION OF BIOBRICKS 1 and 3 ligated with overlap extension (AraC3 VECTOR) with edited protocol from NEBcloner/Aman

Digest 200 ng

Incubate at 37°C for 5-15 minutes as both enzymes are Time-Saver qualified (NEBcloner).
After incubation heat inactivation at 65°C 10 min to inactivate the restriction enzymes, or Gel purification, or PCR purification Kit

Table37

	A	B	C
1	COMPONENT	50 µl REACTION	neg
2	DNA	1 µg	0µl
3	10X NEBuffer 3.1	5 µl (1X)	5µl
4	XbaI	1.0 µl (or 10 units)	1µl
5	PstI	1.0 µl (or 10 units)	1µl
6	Nuclease-free Water	to 50 µl	43 µl

Amount BB1+3 digested--> 260ng--> 25 uL taken from a concetrstion of 10,4 ng/ul
Final digestion concntration of BB1+3 --> 260ng/50uL--> 5,2 ng/uL

https://www.neb.com/protocols/2014/05/07/double-digest-protocol-with-standard-restriction-enzymes
https://nebcloner.neb.com/#!/protocol/re/double/PstI,XbaI
https://www.ecosia.org/search?q=ligation+protocol+50+uL+reaction+
http://nebiocalculator.neb.com/#!/ligation

However the enzymes are from promega and not NEB.

Overlap Extension Overview part 1 (Stella)

Thursday 08/08 - Starting Step 1, Amplifying all BioBricks with primers with overhangs. (**Tobi + Stella**)
Friday 09/08 - Gel electrophoresis and purification. All successful, except BB1.2. But low yield. (**Tobi + Stella**)
Monday 12/08 - Trying out Step 2 and 3 - with Q5 protocol. Step 2 was unsuccessful at 70°C, but successful at 65°C. This has later become optimized with a Tm of **67°C in Step 2**.for BB1+3. The first time we forgot to add both DNA samples in one tub**e. (Stella + Ayleen**)
Tuesday 13/08 - Helping out with growth curve for transformed cells w. BB1.2+3, while the DNA was sent to sequencing. (**Stella + Nerea**)
Wednesday 14/08 - Step 2 and Step 3, performed and gel purified. BB1+3 successfully created - with a max concentration of 7.9 ng/ul in 29 ul.
Thursday 15/08 -
Ayleen completed digestion of BB1+3 and heat inactivation. However, the vector is in too low concentration to use for ligation. Also, it is faulty. Also, the vector might be faulty due to the wrong protocol for digestion. **Stella** amplified BB1+3 to have for digestion/ligation/transformation. And started Step 1 for BB2, since we were out of it. But this was unsuccessful.
Nerea purified the BB1+3 samples and nanodropped them.
Friday 16/08 - Amplify BB2 and purify with gel purification (**Sanchari + Milda**). If sample with good concentration - Step 2 and Step 3 can be performed.

Step 1: Overlap Extension (25 ul)

	Reagent	Amount
1	Q5	12.5
2	F-primer	1.25
3	R-primer	1.25
4	DNA	1
5	H20	4+5

Amplify BB2 and gel purify. Depending on concentration - tells amount of DNA for step 2. Also, skip negative control because it is taking a lot of Q5 Master Mix.

Step 2: Overlap Extension (50ul) - O...

	Reagent	Amount
1	Q5	12.5
2	DNA	3.3 ul + 4.76 ul
3	H20	17.7

Step 3: Overlap Extension (50ul)

	Reagent	Amount
1	Q5	25
2	F-primer	5
3	R-primer	5
4	DNA	10 from step 2
5	H20	10

Q5 NORMAL PCR MIX & Thermocycling

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	1.25µl		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	1,25 µl	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			

VRIJDAG 16-8-2019

Milda:::

This week we have been following the plasmid construction to-do list as can be seen on: https://docs.google.com/document/d/17xAOX3-x2ADCxuzcKzVY6iWo0SBCL9wjMI7LaRc0C7s/edit

We have successfully reached the transformation stage until we received sequencing results. These results showed that instead of biobricks we had been inserting shorter pseudo-bricks into our vector. After long discussion, we came to the conclussion that these short fragments were created because of longer than necessary digestion of the amplified biobricks. Instead of the recommended 15 minutes, we had been digesting our biobricks for as long as three hours. This resulted in enzymes exhibiting the so-called star activity and unspecific cleavage of biobricks, as illustrated by a gel image below.

WhatsApp Image 2019-08-15 at 17.27.56.jpeg

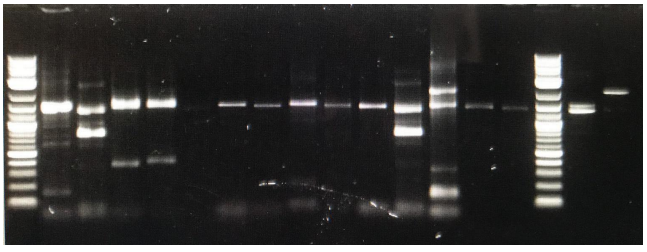


Figure above. Star activity as illustrated by short bands below the actual length of the biobricks. Last two wells - two control bricks. For our latest experiments, we have been using the biobricks seen in the first four wells, in which short fragments are clearly present. Note: some short fragments can still be present even if they are not seen on the gel! This was just to illustrate the point.

Because of these mistakes, we now have to repeat a large part of our work. We firstt have to amplify our biobricks, because we do not have enough left, double digest them properly (as well as te vector), and then ligate them.

Biobrick amplification:

Protocol found here: https://international.neb.com/protocols/2012/12/07/protocol-for-q5-high-fidelity-2x-master-mix-m0492
Annealing temperature calculator: http://tmcalculator.neb.com/#!/main

Biobrick amplification

	A	B	C	D	E	F
1		**BB1**	**BB1.2**	**BB2**	**BB3**	**Negative control**
2	Q5 High Fidelity 2x MM	12.5	12.5	12.5	12.5	12.5
3	10 uM FP	1.25	1.25	1.25	1.25	1.25
4	10 uM RP	1.25	1.25	1.25	1.25	1.25
5	Template DNA	1	1	1	1	0
6	Water	9	9	9	9	10
7	**TOTAL**	**25**	**25**	**25**	**25**	**25**

Primer sequences:
GACGCCAGGTTGAATGAATTCGCGGCCGCTT
CCGTATTTGGAGGTACTGCAGCGGCCGCTA

#1 PCR set-up (based on experience):

Initial denaturation	98 degrees	30 secs
30 cycles	98 degrees	10 secs
	66 degrees	30 secs
	72 degrees	1 minute
Final extension	72 degrees	2 minutes
Hold	4 degrees	

#2 PCR set-up (based on current calculations):
Current calculations showed 72 degrees as the optimal annealing temperature for these primers. Aman suggested going for 70 degrees instead to try out. But to match with Stella, I made it 69 degrees.

Initial denaturation	98 degrees	30 secs
30 cycles	98 degrees	10 secs
	69 degrees	30 secs
	72 degrees	1 minute 10 secs
Final extension	72 degrees	2 minutes
Hold	4 degrees	

PCR purification:

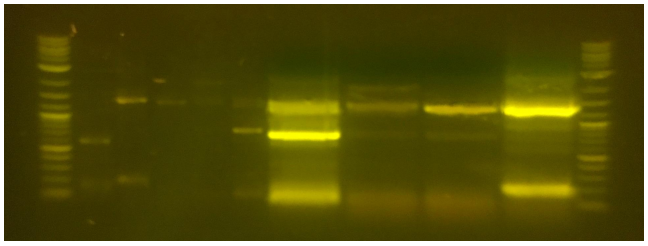
I PCR purified biobricks BB1.2 and BB3, and gel purified BB1 and BB2.

Concentrations:

BB1 #1 5.9
BB1.2 #1 47.9
BB2 #1 6.7

BB3 #1 14.7
BB1 #2 2.5
BB1.2 #2 46.1
BB2 #2 5.8
BB3 #2 48.2

IMG_0939.jpg



From the gel image it seems that the #1 conditions are preferrable for the biobricks, except for BB3, for which annealing temperature of 69 degrees is better. On Monday we will need to perform double digestion of the biobricks and ligate them into the vector.

MAANDAG 19-8-2019

Milda:::

Biobrick and vector digestion:

The enzymes being used are compatible with rapid digestion. Protocol for rapid digestion was found on: https://worldwide.promega.com/-/media/files/resources/protocols/technical-manuals/101/restriction-enzymes-protocol.pdf?la=en

Biobrick digestion

	A	B	C	D	E	F
1		BB1,x2	BB1.2	BB2,x2	BB3	
2	Buffer H	2	2	2	2	
3	BSA	0.2	0.2	0.2	0.2	
4	PstI	1	1	1	1	
5	XbaI	1	1	1	1	
6	DNA	15.8 (93ng) or 15.8 (70.1)	8.4(400ng)	15.8(106ng or 100)	8.3(400ng)	
7	Water	0	7.4	0	7.5	

Vector AraC3 digestion

	A	B
1		AraC3
2	Buffer B	2
3	BSA	0.2
4	PstI	1
5	SpeI	1
6	DNA	10.9 (1 ug)
7	H20	5.9

The tubes were incubated for 15 minutes at 37 degrees and inactivated at 65 degrees for 20 minutes.

Vector AraC3 dephosphorylation:

Protocol found on: https://international.neb.com/protocols/0001/01/01/vector-dephosphorylation-protocol

Vector AraC3 dephosphorylation

	A	B
1		AraC3 (3280 bp)
2	Vector DNA	10 ul of DD (500 ng)
3	Antarctic phosphatase buffer, 10x	2
4	Antarctic phosphatase	0.5
5	H20	7.5

The tube was incubated at 37 degrees for 30 minutes and inactivated at 80 degrees for 2 minutes. The product was PCR purified. Concentration was 10.4 ng / uL

Ligation of AraC3 + BB1 and AraC3 + BB1.2:

In AraC3+BB1 ligation, we are using 30 ng of AraC3 and 35.26 ng BB1.
In AraC3+BB1.2 ligation, we are using 30 ng of AraC3 and 37.87 ng BB1.2

Protocol found on: https://international.neb.com/Protocols/0001/01/01/dna-ligation-with-t4-dna-ligase-m0202
AraC3+BB1 ligation:
2.9 ul AraC DD dephosph. purified
7.6 ul BB1 DD
2 ul ligase buffer

1 ul T4 ligase
6.5 water

AraC3+BB1.2 ligation:
2.9 ul AraC DD dephosph. purified
1.9 ul BB1.2 DD
2 ul ligase buffer
1 ul T4 ligase
12.2 water

The tubes are incubated overnight at 16 degrees. Inactivated at 65 degrees for 10 minutes.

DINSDAG 20-8-2019

Ayleen:::
Ligation of Arac3 and BB1+3 from overlap extention. Protocol from: https://international.neb.com/Protocols/0001/01/01/dna-ligation-with-t4-dna-ligase-m0202

30 ng of Arac3 vector (conc 10,4 ng/ul)--> 2,9 uL
71,89 ng of insert BB1+3 (conc 5,2 ng/uL) to get a 3:1 ratio --> 13,8 uL
ligase buffer --> 2 uL
T4 ligase --> 1 uL
water --> 0,3 uL

neg control ; inster--> water 14,1 ng/uL

The tubes are incubated overnight at 16 degrees. Inactivated at 65 degrees for 10 minutes.

Milda:::

Did transformation today of AraC3+BB1 and AraC3+BB1.2 ligates.

WOENSDAG 21-8-2019

Milda:::

Transformation was successful. Many colonies observed on AraC3+BB1.2 plate but not as many on AraC3+BB1 plate (less than on self-ligate AraC3 plate, ligation negative control). Doing colony PCR today.

Colony PCR:

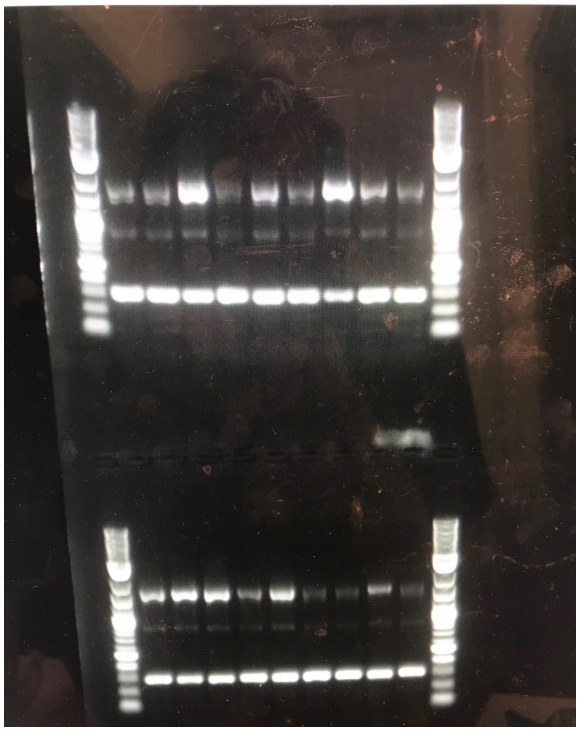
Colony PCR was done following Pfu Promega protocol, which can be found here:
https://worldwide.promega.com/-/media/files/resources/protocols/product-information-sheets/g/pfu-dna-polymerase-protocol.pdf?la=en

Pfu colony PCR set up

	A	B	C	D	E	F	G
1		**Volume**	**Master mix, 19x**	**Step**	**Temperature**	**Time**	
2	Pfu DNA Polymerase 10X Buffer with MgSO4	5	95	Initial denaturation	95	1.5 min	
3	dNTP mix, 10 mM each	1	19	Denaturation	95	45 secs	30 cycles
4	10 uM forward primer, VF2	2.5	47.5	Annealing	60	30 secs	
5	10 uM reverse primer, VR	2.5	47.5	Extension	73	3 mins	
6	DNA template	1	-	Final extension	73	5 mins	
7	Pfu DNA Polymerase (2–3u/µl)	0.5	9.5	Hold	4	Forever	
8	Water	37.5	712.5				

The picked colonies were resuspended in 20 ul of water and 5 ul of that was used for lysis at 95 degrees for 10 minutes. 1 ul of this mixture was then added to the colony PCR reaction mix.

IMG_0953.jpg



In the colony PCR gel we cannot see any constructs of the right size. It might be either of two cases: 1) I did not pick the right colony, so I should check more to see if any of them contain our construct; 2) Something went wrong in the construction process: either 15 minute digestion is not enough, dephosphorylation was inefficient, or the ligation itself did not work. At this point, I am genuinely hoping it is the first case. Tomorrow I will run the ligation mixtures on the gel to check if I see a construct there, and if I do, I will check more colonies on colony PCR.

Ayleen::::
Transformation of Arac3+bb1+3 into top10 cells.
Ratio
2 uL kcm
5 uL DNA
3 uL water

"positive control" was the religated vector - i.e. the negative control from ligation - meaning ligation of vector with no insert . Negative control was no DNA at all.

DONDERDAG 22-8-2019

Milda:::

Today I am doing colony PCR to check as many colonies as possible from the plates. I will also run the ligation mixtures on the gel to see if there is a band of the construct there. As we are out of Pfu polymerase, I will do GoTaq colony PCR (protocol can be found: https://worldwide.promega.com/-/media/files/resources/protocols/product-information-sheets/g/gotaq-g2-dna-polymerase-protocol.pdf?la=en)

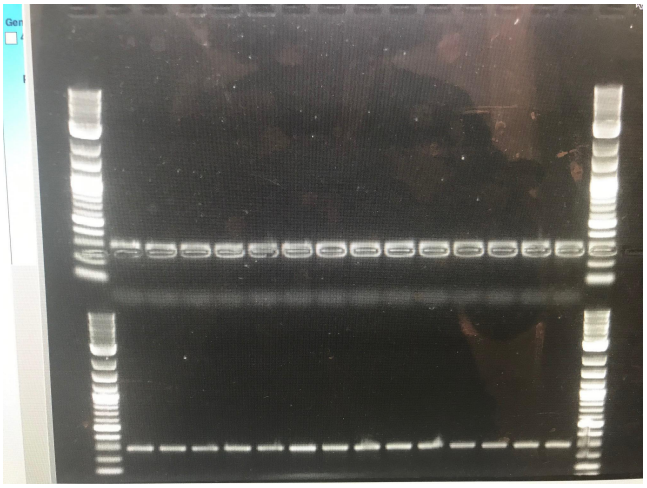
Colonies were picked and resuspended in 20 ul of water. 5 ul of that was lysed for 10 minutes in 95 degrees. The PCR set up was:

GoTaq colony PCR

	A	B	C	D	E	F
1		Volume	Master Mix, 27x	Initial denaturation	95°C	4 min
2	5X Green GoTaq® Reaction Buffer	10	270	denaturation	95°C	30 sec
3	PCR Nucleotide Mix, 10mM each	1	27	annealing	59.3°C	30sec
4	10 uM forward primer, VF2	2.5	67.5	extension	72°C	2 min
5	10 uM reverse primer, VR	2.5	67.5	final extension	72°C	2 min
6	GoTaq® G2 DNA Polymerase (5u/µl)	0.25	6.75	Hold	4°C	
7	template DNA	1	-			
8	Nuclease-Free Water	32.75	884.25			

Colony PCR did not work. You can see below:

IMG_0962.jpg



The problem is that these primers with these particular conditions produce unspecific product, which overwhelms the real one. Max's suggestion was to increase the annealing temperature, which is what Ayleen did (see below). However, even if colony PCR did work, there is a very low probability that we would see a colony with our construct because on the ligation negative controlo plate we saw a bunch of colonies, meaning the background is very high. We have discussed this issue with Max, and we have come to the conclusion that there might be two possible problems. Either dephosphorylation did not work (which is strange, as the conditions were identical as before), or the digestion did not work. However, Max said that he routinely does digestion for only 15 minutes, and it works fine. The problem might be with the enzymes themselves. Tomorrow, I will run a gel to see how the vector DD looks like, as well as the biobrick DD, and also try digesting the vector with each of the enzymes separately to see if they work at all (should produce a linear product).

Ayleen:::

Today I am doing colony PCR to check as many colonies as possible from the plates. checked the transformation of Arac3+BB1+3 and positve control.

Colonies were picked and resuspended in 20 ul of water. 5 ul of that was lysed for 10 minutes in 95 degrees. The PCR set up was:

Table38

	A	B	C	D	E	F
1		Volume	Master Mix, 13x	Initial denaturation	95°C	2min
2	5X Green GoTaq® Reaction Buffer	10	130	denaturation	95°C	30 sec
3	PCR Nucleotide Mix, 10mM each	1	13	annealing	62°C	30sec
4	10 uM forward primer, VF2	2.5	32,5	extension	72°C	2 min
5	10 uM reverse primer, VR	2.5	32,5	final extension	72°C	4 min
6	GoTaq® G2 DNA Polymerase (5u/µl)	0.25	3,25	Hold	4°C	
7	template DNA	1	-			
8	Nuclease-Free Water	32.75	884.25			

OBS:: Matsermix was made without GoTaq and added 49 uL and 1 uL DNA. And then 0,25 uL was added to each PCR tube. Meaning that the toatl volume was 50,25 instead of 50.

VRIJDAG 23-8-2019

Milda:::

First, I will check how the digestions have gone. I run a gel with vector DD (15 min) and biobrick DD (15 min) and vector DD (1 hour, from Joost).

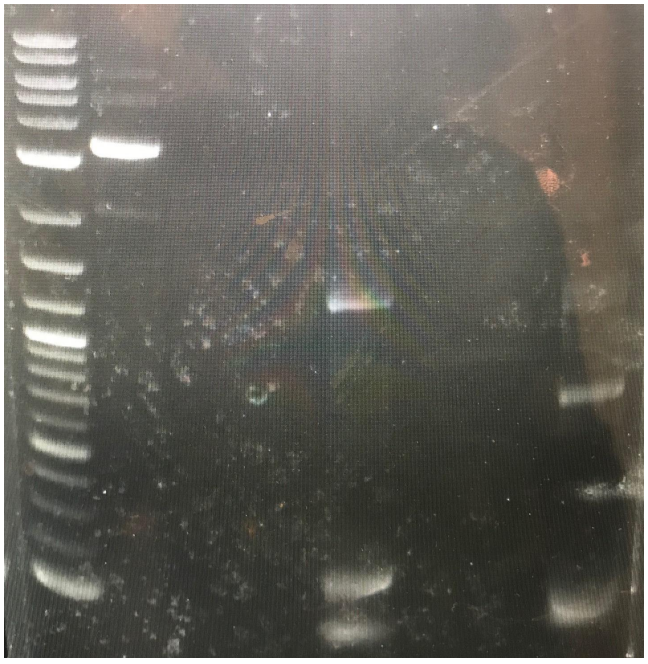
Then I will try out vector digestion with PstI only, with SpeI only, with both for 15 minutes, 30 minutes and 1 hour, as well as one biobrick digestion for the same time points (400 ng of every). In total, that is 12 digestions, and I will also run undigested vector and biobrick on gel (14 wells in total).

Digestions

	A	B	C	D	E	F
1		BB1.2	MM,4x		AraC3	MM, 10x
2	Buffer H	2	8	Buffer B	2	20
3	BSA	0.2	0.8	BSA	0.2	2
4	PstI	0.5	-	PstI	0.5	-
5	XbaI	0.5	-	SpeI	0.5	-
6	DNA	8.4(400ng)	-	DNA	4.6 (400 ng); 10.3	-
7	Water	8.4	-	H20	11.2 (or 12.2 for SD); 5.5/6.5	-

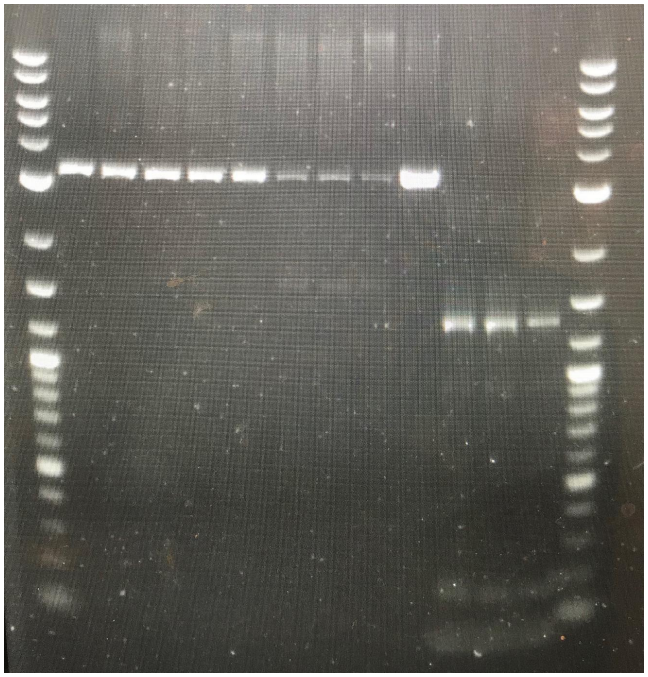
All reactions were inactivated at 65 degrees for 10 minutes.

IMG_0967.jpg



The gel above, from left to right: AraC3 DD 15 minutes (1 ug), BB1 DD #1 (93 ng) and #2 (70.1 ng) 15 min, BB1.2 DD 15 min (400 ng), BB2 #1 (106 ng) and #2 (100 ng) DD 15 min, BB3 DD 15 min (400 ng). I loaded 2.5 ul which was obviously not to be visible on the gel for BB1.2 and BB2. For AraC3, it seems that 15 minutes was not enough for full digestion, because not all vector was linearized (above 3 kb), we can still see other bands. BB1.2 we can see as a band a little over 1.2 kb, the same for BB3, however, there are extra bands around 100 bp and less; and one more band around 700 bp for BB3. The parts which are cut out from the bricks are 15-30 bp whereas these bands look more around 100 bp. However, I did not load the amplified biobricks themselves on the gel, nor the biobricks before the amplification, so I cannot compare.

IMG_0970.jpg



The gel above: AraC3 +PstI (15 min), AraC3 +SpeI (15 min), AraC3 DD (15 min), AraC3 +PstI (30 min), AraC3 +SpeI (30 min), AraC3 DD (30 min), AraC3 +PstI (60 min), AraC3 +SpeI (60 min), AraC3 DD (60 min), BB1.2 DD (15 min), BB1.2 DD (30 min), BB1.2 DD (60 min). In this case, the 15 minutes seem to be enough for the vector, but it is because this time we used 400 ng (before - 1 ug). Here, AraC3 DD for 60 min was done on 500 ng. It seems that 1 hour on 400 ng produced star activity (extra band). BB1.2 double digestions produced the same results as the previous gel: there are two extra bands, but again there is nothing to compare it with.

What we need to do: run a gel with unamplified biobrick, amplified biobrick and digested biobrick. We need to understand where the extra bands in the digestion mixtures are coming from. It can be either from amplification, when the PCR purification does not remove the unspecific products, or the digestion as the enzymes could have degenerated over the summer and are now having star activity. We will order new enzymes which should remove the latter problem. However, the problem with amplification remains, because when the primers amplify unspecifically, they produce products with enzyme restriction sites, so these products get cut in double digestion, making it easy to clone them instead of full length bricks. For next steps, I think it would be important not only to use new enzymes but also do gel purification instead of PCR purification.

MAANDAG 26-8-2019

Tobi::: Soooo, i will amplify OE 1+3 at 68 °C because thats the temp we get suggested by the Q5 page using the primers:
OE 1_1+3_F CGC CAG GTT GAA TGA ATT CGC
OE 3_1+3_R GTA TTT GGA GGT ACT GCA GCG G

I will however use 3 different temps 66 68 and 70 °C

Amplification of OE1+3

	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	1,25 µl		25 Cycles	98°C 66/68/70°C 72°C	10 seconds 30 seconds 30 seconds/kb= 1 min 30sec
4	10 µM Reverse Primer	1.25 µl	1,25 µl	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			

Here the results we be written down togheter with a picture of the gel.

I will also addd overhangs to brick 2 (eg step 1 for BB2)
Here i will use the primers:
OE2_1+2F GTCAAGCCGGCC-AACATTGATTATTTGCACGGCGTCAC
OE2_1+2R GTATTTGGAGGTACTGCAGCGG

NEB recoments 69°C however i will try 67, 69 and 71

Rebecca and Sanchari:::
As recommended we ran the following samples on a gel: unamplified bricks (1ul), Amplified and PCR/gel purified bricks from the 16th of august. and digested biobricks (15 min digestion from the experiments performed by Milda on the 23rd of august. Will insert picture.

We noticed that for all unamplifed bricks only one band was present but in some cases the band did not seem clear or appears as a smear.
We also noticed that for biobrick 3 the amplified sequence is much shorter and shows up at 700bp. This is likely the fragemnt that was inserted and showed up in the sequencing result as only part of the tag and YFP protein. We tracked back previous amplificaion of the biobrick through the pictures saved in ImageJ and benchling to see when Biobrick 3 was last seen amplified at the correct size. It seems that the problem occured after the 5th/6th of august. Since the amplification produces a lot of unspecific bands of BB3 we need to reamplify this brick and check it before digestion.

Biobrick 1.2 - the cloning of biobrick 1.2 ino the AraC3 vector was confirmed successful by both colony PCR and sequencing before I left on the 9th of august. It sees there was 6 plasmidpreps of these cells prepared by Stella on the 9th of August. Only 2 remains and no glycerol stock. We need again to culture one of the positive colonies and save this. If we can amplify BB3 correctly and digest it with the 15 min protocol and thereby obtain the correct fragemnt we can clone it to the backbone and make the model plasmid. '

Biobrick 2 did not show up on the gel and for Biobrick one it seems to be amplified but the amount is dimished after gel purification of the band.

It also seems that after gel extraction, if the gel is not pooled in the extraction the concentration is very low. Until we can obtain substantially higher concentration of these biobricks after amplification there is little chance we will have enough for digestion and cloning. I suggest mutliple tubes and amplification of biobrick 2 - gel extraction and pooling from many tubes to obtain a higher conc.
Most likely we believe the problem at hand lies within the amplification still and that we are digesting many of the unspecific products that exist in the amplified and PCR purified samples before digestion.

As there is no more q5 we cannot proceed with amplification.

WOENSDAG 28-8-2019

Joost:::

Going to redo digestion and ligation of biobricks in AraC3 in the same way as I build the characterisation construct. Ive made a new notebook (sorry for that) because working in this book was almost impossible due to the lag.

BB1_1.2_3 Amplification

	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			30 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			

Amplification_2

	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			30 Cycles	98°C 72°C 72°C	10 seconds 30 seconds 30 seconds/kb= 1 min 30 sec
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			

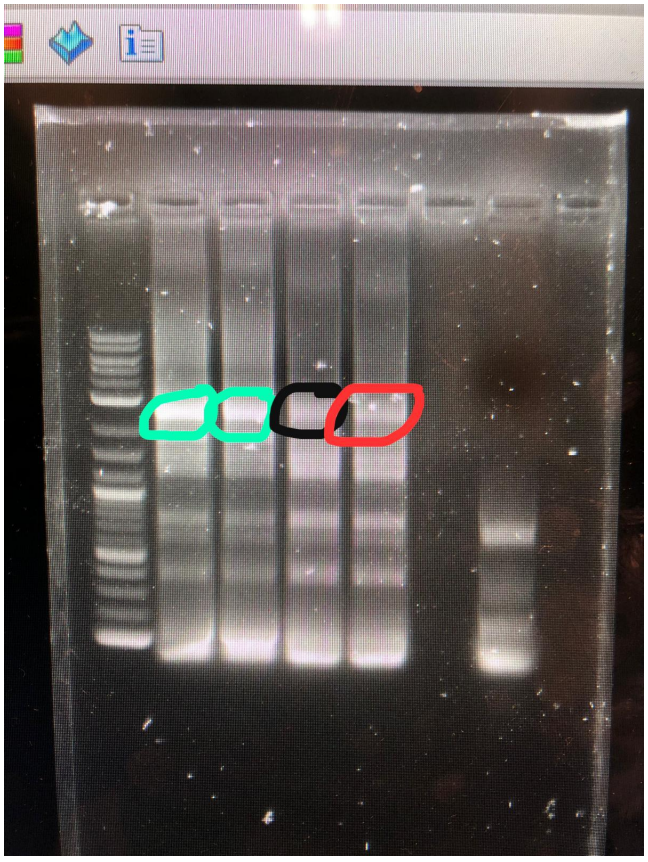
VRIJDAG 30-8-2019

Stella::::

Overlap Extension Overview part 2 (Stella)

previously BB1+3 was successfully created with Overlap Extension, here the overlap (Step 2) was made at 67°C(green) and 68°C(black&red).

IMG_3013.JPG



Then I tried to amplify BB2 at 69°C, 1:10 elongation time, like the first time it worked with Q5.
None of these tries were successful: 69°C, 69°C, 69°C, 64°C, 69°C, 67°C, 65°C.
During this time, I used several different sometimes old Master Mixes.

29/08 - I did six different PCR reactions for Overlap Extension for BB1, BB2 and BB1+3.
BB2: 69°C at 1:10 elongation time, 69°C at 1:10 elongation time with DMSO(1ul) and a touch-up between 69°C and 72°C where each cycle the annealing temperature increased with 0.5°C - in this reaction I also used Tobi's special longer amplification primer.
BB(1+3): I used some DNA from a previous step 2 from a week back and what I believed to be gel purified BB1+3 from the gel shown in the picture above. These gel purifications howeve turned out to not contain any DNA because someone forgot to add EtOH to the wash buffer. This might also explain why I never succeeded in amplifying already complete BB1+3 because I might have used the samples I thought contained BB1+3 but didn't. Luckily, I still have some BB1+3 left from the first successful Overlap Extension (65°C) with BB1+3. To make sure that I still had DNA after purification I ran all samples on a gel.
BB1: I amplified in one reaction BB1 with the OE primers for (1+2).

Results: This time the BB2 PCR reaction worked, as well as the BB1 reaction. However, the BB1+3 reaction was unsuccessful 1. because the purified BB1+3 didn't contain DNA and 2. because it has passed a week since that PCR was made. In step 3 of Overlap Extension, you take 10 ul from the PCR tube in step 2 - it is not purified. The BB2 and BB1 were gel extracted and purified.

30/08 - I continued to work on creating the switch model starting the Overlap Extension step 2, for this construct (BB1+2) at 66°C, 1:10 elongation time for 15 cycles. I took very little template DNA for this reaction so as not to waste product but will redo this with more DNA once the protocol works. In step 3, the protocol was 68°C, 1:40 elongation time for 30 cycles.
I also tried to amplify the overlapped construct BB1+3 from a vial I knew contained BB1+3, 69°C, 1:10 elongation time for 30 cycles.

Results: These were both unsuccessful. One lane showed unspecific binding BB1+2 and no overlap (with no band the right size) and the BB1+3 was a smear.

links:
http://www.geneinfinity.org/cc/cc_dnaconverter.html
https://www.unitconverters.net/weight-and-mass/microgram-to-nanogram.htm

amplification

	A	B	C
1	Initial Denaturation	98°C	30s
2	25 Cycles	98°C 66°C 72°C	10 seconds 30 seconds 30 seconds/kb= 1 min
3	Final Extension	72°C	2 minutes
4	Hold	4°C	

DINSDAG 3-9-2019

:::Stella:::
Today I PCR purified BB3 from yesterday with B2 binding buffer - got a good band in a gel.
Gel purified BB1.2 from overnight - got a low concentration - faint band in a gel.
The low concentration from 25 ul lead to the decision of trying 50 ul reaction -
Which I tried with BB1.2 and BB2 from which I need more DNA to be able to use in a PCR reaction after purification with the mastermix.

I also tried a first run of OE step 2 and 3 for 1.2 + 3.
Step 2: 67°C, 15 cycles, 0:45
Step 3: 68°C, 30 cycles, 1:45

In the check up gel I will look at the previously digested 1+3 using Promega enzymes and NEB buffer - just for checking.
I will also look at 1.2+3, BB1.2 and BB2.

:::Rebecca:::
Overnight culture
I found the plate which had the positive AraC3 BB1.2 colonies. I used colony 7 from this plate to make an overnight culture and one colony from the old AraC3 positive plate. These cultures were used to make 2 midipreps each.

Transformation
I transformed previously isolated AraC3 BB1.2 purified plasmid into top10 cells. My negative control was no DNA and my positive control was the B1C3 vector.
The transformation was very successful. The negative control clear and positive good and many greenish colonies on the two plates with AraC3 BB1.2 -> 5 colonies were selected from one of these plates for colony screening

WOENSDAG 4-9-2019

:::Stella:::
amplifying 1.2 three times and purifying 2 from yesterday.
Overlapping 1+2, the switch. At 66°C for step 2, elongation 1:10 & 15 cycles, and 68°C, elongation 1,45 for step 3, 30 cycles overnight.

:::Rebecca:::
Midiprep of AraC3 and AraC3 BB1.2 ovenight cultures.
20 ml (2x10ml) was used for midiprep and the rest was used to make 4 tubes each of glycerol stocks stored in the 2018 box

The elution step - there was no pellet and the samples had low DNA conc.

AraC3 and AraC3BB.12 midiprep

	A	B	C	D
1				Mean
2	1	80.2	67.3	73.75
3	2	82.3	79.1	80.7
4	3	25.0	22.4	23.7
5	4	46.8	50.4	48.6

DONDERDAG 5-9-2019

:::Rebecca:::
Colony PCR of 4 transformants from 3rd of September
Midiprep 1-4 was also run on the gel and so was the positive control plates: One AraC3 plate from 9/8 and one more recent AraC3 plate made by Milda 20/8.

The colony from AraC3 9/8 did not show on the gel, I will toss it.
Milda AraC3 20/8 colonies looked good.
The colony PCR from the transformants are worrying - 400 bp
The midiprep also look good, the plasmids are in supercoiled state but it was sufficient to elucidate the content of the 4 tubes.

ZATERDAG 7-9-2019

:::Rebecca:::
Overnight culture of
AraC3 BB1.2 from glycerol stock (from 4/9)
AraC3 BB1.2 random culture from transformation plate (3/9)

ZONDAG 8-9-2019

:::Rebecca::::
Overnight culture - 3 minipreps of each, see nanodrop results.

One each of these will be sent for sequencing.

Growth curve
AraC3 Neg control was cultured to OD0.7
AraC3BB1.2 was re-cultures to OD1.154
Cultures were rediluted to OD 0.05 and grown for 5 hours in different conditions.
Samples were taken at 180 min, 300 min and 1215 min

:::Jannah:::
Miniprep for 2 cultures AraC3 BB 1.2 T and AraC3 BB 1.2 GS

Table40

	T	GS	C	D
1	99.2	178.0		
2	89.0	200		
3	95.4	199.6		
4				

MAANDAG 9-9-2019

09/09 - Western Blotting Preparation
Growth curve
Time point 3 was taken, the OD was similar to time point 2.
Prepared a fluOstar plate and ran fluOstar for 5h - seems there is no RFP exp.
Westernblot sample prep
Sent to sequencing: Midipreps and miniprep:transformation of AraC3, AraC3 BB1.2

Cell Lysis - Western Blotting Preparation

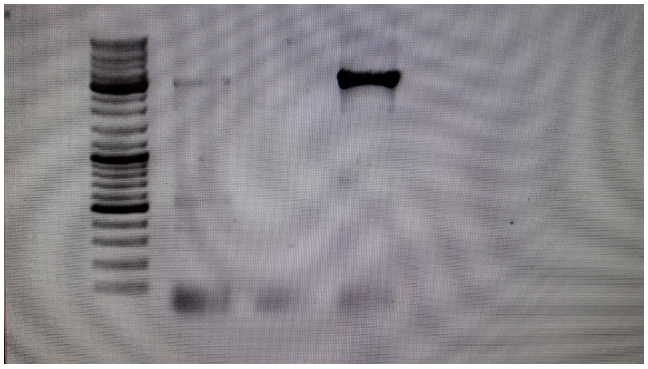
Cell Concentration

	Cell	t=1	volume (ul)	t=2	volume (ul)	t=3	volume (ul)
1	AraC3 -	9.12	109.78	16.1	62	28.9	35
2	AraC3 0.2%	8.55	117	15.1	66	25.3	39.5
3	AraC3 BB1.2 -	10.1	99	18.0	55.6	26.0	38.5
4	AraC3 BB1.2 0.2%	9.2	108.7	14.8	67.6	16.6	60
5	AraC3 BB1.2 0.4%	10.9	91.7	8.56	117	18.2	55
6	AraC3 BB1.2 0.6%	9.87	101.3	14.8	68	19.8	50.5

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

DINSDAG 24-9-2019

Rebecca
Ran a fluOstar - FLourecence measurement of the switch plasmid in diffferent arabinose concentrations and I also used the igem GFP measurement kit that uses Flourecin. We measured RFP with filter for Texas Red, Rhodamine, AlexaFlour and our own setting . We also let it grow overnight and still neither GFP nor RFP was detected in our samples. The measurement kit on the otherhand showed good results with intensity from 2500-25.

WOENSDAG 25-9-2019

Rebecca
Ran a new fluOstar - Measured absorbance of the switch plasmid - growth curve to use as results for the wiki.
Since we could not detect flourescence from our samples we decided to tranform the plasmid into an expression E.coli strain; Bcl21.
Transformation of SP18 (sequenced switch plasmid) into Bcl21 cells.

VRIJDAG 27-9-2019

Rebecca
Colony screeening of transformation plates - 20 colonies were screened. The same colonies were cultured in eppendorph tubes. Positive colonies will be used for flourescence experiments and westernblot prep.
Cells will be re-diluted and then grown overnight with arabinose.

DONDERDAG 3-10-2019

FRIDAY - 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