Lab Note Book of Sweet Spirulina

TU Dresden iGEM team 2019

22. May - Arnau and Sebastian

We followed the High Efficiency Transformation Protocol changing SOC by LB to insert pCAMBIA1305 in *E. coli*.

- For C2987H: Thaw a tube of NEB 5-alpha Competent *E. coli* cells on ice for 10 minutes.
- Add 5 µl of the solution with the plasmid DNA to the cell mixture. Carefully flick the tube 4-5 times to mix cells and DNA. **Do not vortex.**
- Place the mixture on ice for 30 minutes. Do not mix.
- Heat shock at exactly 42°C for exactly 30 seconds. Do not mix.
- Place on ice for 5 minutes. Do not mix.
- Pipette 950 µl of room temperature LB into the mixture.
- Place at 37°C for 60 minutes. Shake vigorously (250 rpm) or rotate.
- Warm selection plates to 37°C.
- Mix the cells thoroughly by flicking the tube and inverting, then perform several 10-fold serial dilutions in LB.
- Spread 50-100 µl of each dilution onto a selection plate and incubate overnight at 37°C.

The colonies were plated with Kanamycin and stored at 37ºC

08. June - Behbood and Victoria

We made a mini-prep in order to extract the plasmid from the cultures set the last day

Miniprep:

Note: *Keep buffer P1 and P3 on ice, but not P2 (contains SDS, will precipitate!)*

- Spin down the cells at 11 000 rpm for 1 minute;
- Discard the supernatant (remove as much as possible)
- Add 200 µL of P1 buffer
- Re-dissolve the pellet by vortexing or use a pipette, make sure you have homogenous suspension!
- Add 200 µL of P2 buffer
- Mix the tubes by turning the tube upside down 5 times, do not mix by vortexing or with a pipette

Note: *To much force can cause physical shearing of genomic DNA, which then will contaminate the plasmid DNA (pDNA) preparation*

- Continue immediately, the solution should not stand for more than 5 minutes
- Add 200 µL of P3
- Mix by shaking the tube
- Let the solution stand for 5 minutes on ice
- Spin the tubes at 13 200 rpm for 5 minutes
- During incubation and centrifugation prepare new 1.5 mL reaction tubes, label them accordingly and add 450 µL of 2-propanol to each

- After centrifugation take out the supernatant with a pipette and transfer it into the corresponding tube with 2-propanol; avoid carry over of any white stuff
- Mix by shaking the tube
- Spin the tubes at 13 200 rpm for 10 minutes
- Discard the supernatant completely
- Carefully add 400 µL of 70 % ethanol and centrifuge again at 13 200 rpm for 3 min
- You can either decant or pipette out the 70 % EtOH with the large pipette, then short spin and then use a pipette with a yellow tip to remove the remaining 70 % EtOH
- Dry the pellet for about 5 min at 50 ºC with lids open in a thermomixer
- Solve the pellet in 30 µL of TE buffer

Note: *You can solve the pDNA also in 10 mM Tris-Cl pH 8.5 or water, but because of DNA integrity TE buffer is strongly recommended for long-term storage of DNA*

Buffer composition:

- **P1:** 50 mM Tris/HCl, pH 8.0, 10 mM EDTA, 200 µg/mL RNAse A
- **P2:** 200 mM NaOH, 1% SDS
- **P3:** 3.0 M KAc, pH 5.5 (potassium acetate)

From this we get 48.2 ng/µL of DNA measured with Nanodrop. The sample was diluted to get a final concentration of 5 ng/µL before make a PCR.

The PCR was done in order to extract the parts from the pCAMBIA1304 that we planned to make biobricks of and include them in our final construct. This parts were the promoter CaMV, the Hygromycin resistance gene and the NOS terminator.

PCR:

- Final volume = 50 µL
  - Master mix = 25 µL
  - Primers = 2,5 µL of each primer 10 µM
  - Template (pCAMBIA1304: 1 µg/mL) = 5 µL
  - H_2O = 15 µL

Four reactions were set and performed, one for amplify the CaMV promoter (CaMV), one for the gene of the Hygromycin resistance (HyR), one for the NOS terminator (NOS) and one with water as control. For this PCR we used primers including the Prefix and Suffix from the Biobrick standard assembly.

30 cycles	Annealing	65ºC	30 sec
	Extension	72ºC	1:15 min
	Denaturation	94ºC	1 min
	Final Extension	72ºC	3 min

Then an agarose gel was prepared for test the results of the PCR

Agarose gel preparation 0.7%:

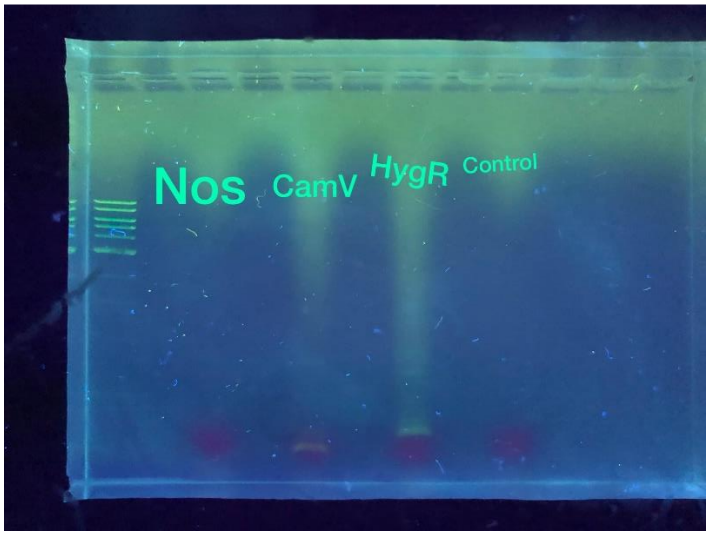
- Solve 0.42 g of agarose in 60 mL of 1 x TBE buffer
- Melt the agarose in the microwave until boiling
- Take out the solution and mix it by shaking the bottle
- Put the solution back into the microwave and let it boil again
- Repeat mixing and boiling until the agarose is completely melted

- Set up an agarose gel box with spacers and a comb
- Take out some solution with a blue pipette tip and put it along the inside of the spacers in the gel box and let it solidify
- Add 2 µL of RotiR-GelStain to the remaining solution and mix by shaking the bottle
- Pour the rest of the solution into the gel box
- Let it stand for at least 30 minutes until the gel becomes solid
- Fill the gel box up with 1 x TBE buffer so that the gel is just covered

An agarose gel was loaded in the following way:

The ladder used was the 1kb plus ladder of NEB lab

Ladder 3.5 µL	NOS	CaMV	HygR



13. June - Behbood

As the day of before we didn't achieved the amplification of our parts of interest we have to repeat the PCR. Before we made a mini-prep, this time using the mini-prep kit of Jenna Bioscience, the DNA (pCAMBIA1304) extraction was kept as a stock.

Mini-prep Kit from Jenna Bioscience (PP-204XS):

Buffer	**PP-204XS**
Lysis Buffer	3,2 mL
RNAaseA	0,8 mg
Neutralization Buffer	3,2 mL, add 0,8 mg RNaseA
Activation Buffer	1,2 mL
Washing Buffer	Add 12 mL Ethanol (final volume 15mL)
Elution Buffer	1 mL

1. **Cell Harvest and Lysis:**
   - Harvest the bacterial cell culture (1-3 ml) by centrifugation [1 min 10.000 rpm)
   - Re-suspend pelleted bacterial cells in 300 µl Lysis Buffer by pipetting or vortexing for 1 min.

2. **Neutralization:**
    - Add 300 µl of Neutralization Buffer (containing RNase A) to sample and mix gently by inverting the tube 4-6 times (do not vortex!).
    - Centrifuge at 10,000 g (11070 rpm) for 5 min at room temperature in a microcentrifuge.
    - The color of the binding mixture should change to bright yellow indicating a pH of 7.5 required for optimal DNA binding. An orange or violet color shows a pH >7.5 and indicates an inefficient DNA adsorption. In this case, it is recommended to adjust the pH of the mixture by addition of a small volume of 3 M sodium acetate, pH 5.0 before proceeding.
3. **Column Activation:**
    - Place a Binding Column into a 2 ml collection tube.
    - Add 100 µl of Activation Buffer into the Binding Column.
    - Centrifuge at 10,000 g for 30 sec in a micro-centrifuge.
4. **Column Loading:**
    - Apply the supernatant from step 2 into the activated Binding column by decanting or pipetting.
    - Centrifuge at 10,000 g for 30 sec.
5. **Column Washing:**
    - Place the DNA loaded Binding Colum into the used 2 ml tube.
    - Apply 500 µl of Washing Buffer (containing Ethanol) to the Binding Column.
    - Centrifuge at 10,000 g for 30 sec and discard the flow-through. Optional Secondary Washing: Recommended only for DNA >200 bp, if highly purified DNA (for DNA sequencing, transfection etc.) is required.
    - Add 700 µl of Washing Buffer to the Binding Column.
    - Centrifuge at 10,000 g for 30 sec and discard the flow-through.
    - Centrifuge again for 2 min to remove residual Washing Buffer.
6. **Elution:**
    - Place the Binding Column into a clean 1.5 ml microtube (not provided in the kit).
    - Add 30-50 µl Elution Buffer or dd-water to the center of the column membrane.
    - Incubate for 1 min at room temperature.
    - Centrifuge at 10,000 g for 1 min to elute DNA. Ultra-pure plasmid DNA is now ready to use

The final amount of DNA extract measured with Nanodrop was 233ng/ µL

<u>24. June - Arnau and Behbood</u>

Make PCR of HyR gene and NOS from pCAMBIA1304 with primers, this time the primers didn't include the Prefix and Suffix because we believe that as the ones with the Prefix and Suffix were to big the reaction wasn't working. So we planned to used first ones without and then make a second PCR with the primers that include the Prefix and Suffix in order to make the biobricks. Also we realized that the primers for the CaMV promoter had another homology region in the pCAMBIA1304 and then we decided to not amplify it from this plasmid and use a different promoter.

We set 6 reactions, 2 controls, 2 for the HyR and 2 for the NOS terminator:

- Final volume = 50 µL
  - Master mix = 25 µL
  - Primers = 2,5 µL of each primer 10 µM
  - Template (pCAMBIA1304: 1 µg/mL) = 5 µL
  - H_2O = 15 µL

Then we start the PCR with the following standard:

30 cycles	Anealing	65ºC	30 sec
	Extension	72ºC	1:15 min
	Denaturation	94ºC	1 min
	Final Extension	72ºC	3 min

Store at 4ºC overnight

25. June - Arnau and Victoria

We prepared a gel for running the PCR product of the last day

Agarose gel preparation 0.7%:

7. Solve 0.42 g of agarose in 60 mL of 1 x TBE buffer
8. Melt the agarose in the microwave until boiling
9. Take out the solution and mix it by shaking the bottle
10. Put the solution back into the microwave and let it boil again
11. Repeat mixing and boiling until the agarose is completely melted
12. Set up an agarose gel box with spacers and a comb
13. Take out some solution with a blue pipette tip and put it along the inside of the spacers in the gel box and let it solidify
14. Add 2 µL of RotiR-GelStain to the remaining solution and mix by shaking the bottle
15. Pour the rest of the solution into the gel box
16. Let it stand for at least 30 minutes until the gel becomes solid
17. Fill the gel box up with 1 x TBE buffer so that the gel is just covered

The ladder used was the 1kb plus ladder of NEB lab

Load scheme:

Ladder	Control	Control	HyR	HyR	Nos	Nos

We don’t get any result in the gel

Repeat the PCR:

- Volume per reaction = 50 µL
  - Master mix = 25 µL
  - Primers = 2,5 µL of each primer 10 µM
  - Template (pCAMBIA1304: 1 µg/mL) = 5 µL
  - H_2O = 15 µL

We set 6 reactions, 2 controls and 2 reactions for each amplification target.

We change the annealing temperature from 65ºC to 64ºC

30 cycles	Annealing	64ºC	30 sec
	Extension	72ºC	1:15 min
	Denaturation	94ºC	1 min
	Final Extension	72ºC	3 min

We prepared another agarose gel but this time 1%

Agarose gel preparation 1%:

1. Solve 0.6 g of agarose in 60 mL of 1 x TBE buffer
2. Melt the agarose in the microwave until boiling
3. Take out the solution and mix it by shaking the bottle
4. Put the solution back into the microwave and let it boil again
5. Repeat mixing and boiling until the agarose is completely melted
6. Set up an agarose gel box with spacers and a comb
7. Take out some solution with a blue pipette tip and put it along the inside of the spacers in the gel box and let it solidify
8. Add 2 µL of RotiR-GelStain to the remaining solution and mix by shaking the bottle
9. Pour the rest of the solution into the gel box
10. Let it stand for at least 30 minutes until the gel becomes solid
11. Fill the gel box up with 1 x TBE buffer so that the gel is just covered

The ladder used was the 1kb plus ladder of NEB lab

Ladder	Negative control NOS	Negative control HyR	HyR w/o Prefix and Suffix	HyR w/o Prefix and Suffix	NOS w/o Prefix and Suffix	NOS w/o Prefix and Suffix

(This time we get an amplification of HyR without prefix and suffix, but we didn't take a picture of the gel)

25. June – Sophie

We repeat the PCR for the NOS using the primers without the Prefix and Suffix. The annealing temperature was set at 70ºC. Also we made a gel extraction of the PCR product for the HyR, and then we made a second PCR with the primers with Prefix and Suffix.

We set three reactions, 2 for NOS terminator and 1 negative control

1) Volume per reaction = 50 µL
   - **a.** Master mix = 25 µL
   - **b.** Primers = 2,5 µL of each primer 10 µM
   - **c.** Template (pCAMBIA1304: 1 µg/mL) = 5 µL
   - **d.** H_2O = 15 µL

30 cycles	Annealing	70,6ºC	30 sec
	Extension	72ºC	40 sec
	Denaturation	94ºC	1 min
	Final Extension	72ºC	3 min

2) Gel Extraction

Epi 1: 250 mg

Epi 2: 230 mg

Protocol for gel extraction:

- Add 750 ml and 690 ml Extraction buffer (3x volume compared to weight)
- Add 250 mL of isopropanol to both
- Add 100 µL of Activation buffer
- Load 700 µL wash buffer two times
- 40 µL Elution buffer

The concentrations measured with Nanodrop: 12 ng/µL and 9,5 ng/µL

We set three reactions, 2 for HyR gene and 1 negative control

1) Volume per reaction = 50 µL
   - **a.** Master mix = 25 µL
   - **b.** Primers = 2,5 µL of each primer 10 µM
   - **c.** Template (pCAMBIA1304: 1 µg/mL) = 5 µL

d. H_2O = 15 µL

30 cycles	Annealing	70,6ºC	30 sec
	Extension	72ºC	40 sec
	Denaturation	94ºC	1 min
	Final Extension	72ºC	3 min

28. June – Victoria, Sophie and Behbood

We run a gel of the PCR products from the last day.

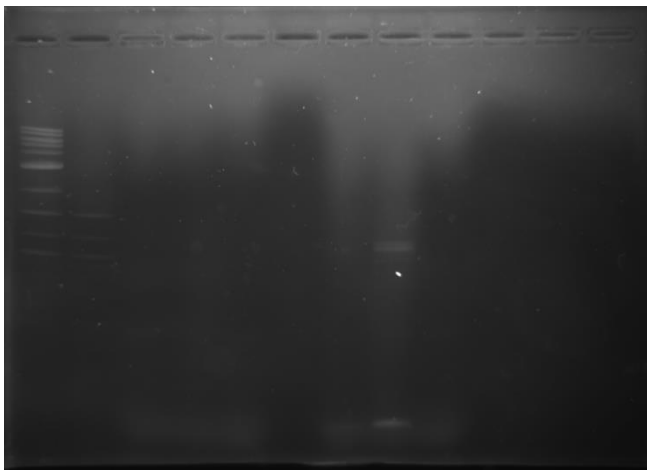
Agarose gel preparation 0.7%:

1. Solve 0.42 g of agarose in 60 mL of 1 x TBE buffer
2. Melt the agarose in the microwave until boiling
3. Take out the solution and mix it by shaking the bottle
4. Put the solution back into the microwave and let it boil again
5. Repeat mixing and boiling until the agarose is completely melted
6. Set up an agarose gel box with spacers and a comb
7. Take out some solution with a blue pipette tip and put it along the inside of the spacers in the gel box and let it solidify
8. Add 2 µL of RotiR-GelStain to the remaining solution and mix by shaking the bottle
9. Pour the rest of the solution into the gel box
10. Let it stand for at least 30 minutes until the gel becomes solid
11. Fill the gel box up with 1 x TBE buffer so that the gel is just covered

The ladder used was the 1kb plus ladder of NEB lab

Loading scheme

1 kb	100 b	NOS C	NOS 1	NOS 2	HyR C	HyR + P&S 1	HyR + P&S 2



We achieved the amplification of HyR with Prefix and Suffix (second PCR). Then we proceed with the gel extraction.

Protocol for gel extraction:

- Add 750 ml and 690 ml Ex buffer
- Add 250 mL of isopropanol to both
- Add 100 µL of activation buffer
- Load 700 µL wash buffer two times
- 40 µL Elution buffer

Concentrations measured with Nanodrop: 74 ng/µL

Then we made a PCR of NOS with Prefix and Suffix, this time we set a two-step PCR which work better for primers with a high annealing temperature, and also a reaction with primers without Prefix and Suffix

- Volume per reaction = 50 µL
  - Master mix = 25 µL
  - Primers = 2,5 µL of each primer 10 µM
  - Template (pCAMBIA1304: 1 µg/mL) = 5 µL
  - H_2O = 15 µL

30 cycles	Annealing	70,6ºC	30 sec
	Extension	72ºC	40 sec
	Denaturation	94ºC	1 min
	Final Extension	72ºC	3 min

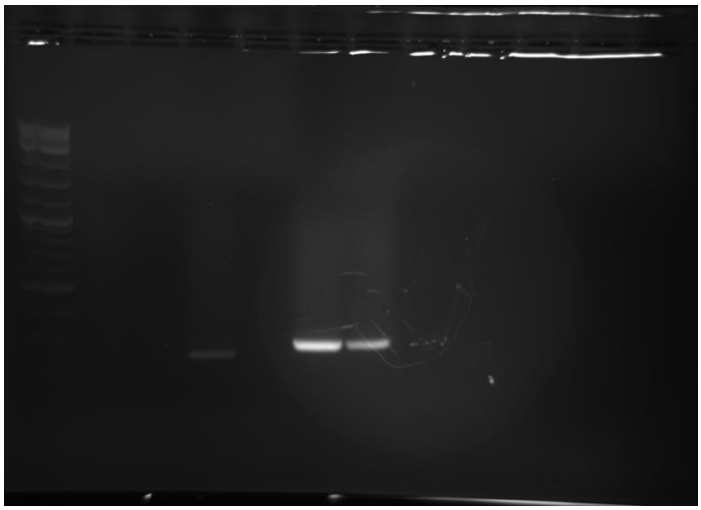
Agarose gel preparation 0.7%:

1. Solve 0.42 g of agarose in 60 mL of 1 x TBE buffer
2. Melt the agarose in the microwave until boiling
3. Take out the solution and mix it by shaking the bottle
4. Put the solution back into the microwave and let it boil again
5. Repeat mixing and boiling until the agarose is completely melted
6. Set up an agarose gel box with spacers and a comb
7. Take out some solution with a blue pipette tip and put it along the inside of the spacers in the gel box and let it solidify
8. Add 2 µL of RotiR-GelStain to the remaining solution and mix by shaking the bottle
9. Pour the rest of the solution into the gel box
10. Let it stand for at least 30 minutes until the gel becomes solid
11. Fill the gel box up with 1 x TBE buffer so that the gel is just covered

The ladder used was the 1kb plus ladder of NEB lab

Loading scheme

Ladder	NOS w/o P+S Negative Control	NOS w/o P+S	NOS w/o P+S	NOS with P+S Negative Control	NOS with P+S	NOS with P+S



We achieved the amplification of the NOS terminator without prefix and suffix and with prefix and suffix

30. June – Behbood and Arnau

We proceed with the gel extraction of the NOS terminator biobrick, the amplification of the NOS including prefix and suffix. The other one was discarded.

1) Agarose gel extraction for NOS biobrick

Weight 1: 160 mg

Weight 2: 240 mg

Protocol for gel extraction (protocol Jenna Bioscience DNA-gel extraction kit)

- Add 480 ml and 720 ml Ex buffer (volume x3)
- Add 160/240 mL of isopropanol to both (volume 1:1)
- Add 100 µL of activation buffer
- Load 700 µL wash buffer two times
- 40 µL Elution buffer

The measurements with Nanodrop indicate a DNA concentration in the first sample of 12.4 ng/µL and 22.4 ng/µL in the second.

03. July - Sophie

We set the cultures of Spirulina (*Arthrospira platensis*) in different media cultures to compare the growing:

- Kröger's Artificial Sea Salt
- NEPC (North East Pacific Collection)
- DM (fresh water media)
- Red Sea Salt

The liquid cultures were set in the incubator with light at 26 ºC

05. July - Victoria

We tried to plate Spirulina in LB plates in order to pick one colony and start a monoclonal culture.

For this we made differ dilutions of the liquid culture 1:20 -> 1:100 -> 1:10 x2

The plates were stored at 37ºC, unfortunately nothing grown.

08. July - Arnau

This time we tried to prepare our own plates with Zarrouk media.

100mL of Zarrouk media were mixed with 1.5 g of agar. Then we autoclave them and after that the media was set in plates. Unfortunately due to the temperature of the autoclave the Zarrouk media precipitate and acquire a dark colour, and nothing grown in the plates.

11. July - Arnau

This time we tried to set liquid cultures of Spirulina at higher pHs as we found reports that Spirulina can grow at up to 11 pH. Two cultures at pH 9.6 and pH 10.1 in Red Sea Salt media were prepared. However it was observed that the increase in the pH cause a precipitation of the media and nothing grown there.

26. July - Arnau and Sophie

This time we tried to integrate the biobricks already done into pBluescript plasmid in order to send them to sequence to check that the PCRs haven't affected the sequence of them.

The protocol for the digestion, ligation and transformation was the following:

DIGESTION:

[NOS] -> 22,4 ng/µL -> 22,3 µL

[HyR] -> 74 ng/µL -> 6,8 µL

[pBlueScript] 3,3 µg/µL -> 1:100 dilution -> 33ng/µL -> 15 µL

Double digestion (NEB lab protocol)

	NOS	HyR	pBS
Part/Plasmid 500 ng	22,3 µL	6,8 µL	15 µL
EcoRI-HF	1 µL	1 µL	1 µL
PstI	1 µL	1 µL	1 µL
10x NEB buffer 2.1	5 µL	5 µL	5 µL
H_2O	20,7 µL	36,2 µL	28 µL

Incubate at 37 ºC for 10 minutes

Incubate at 80ºC for 20 minutes

LIGATION:

	NOS	HyR
Digest product	2 µL	2 µL
pBS digested	2 µL	2 µL
10x T4 ligase buffer	2 µL	2 µL
T4 ligase	1 µL	1 µL
H_2O	13 µL	13 µL

Incubate at Room Temperature 10 minutes

Incubate at 80ºC 20 minutes

For the transformation 2 µL of ligation product were added to 50 µL of competent E. coli NEB 5 alpha cells and pBS not modified as control (3 transformations)

Protocol for transformation with heat shock of the competent cells

- Put on ice 30 minutes
- Put at 42ºC 30 seconds
- Put on ice 5 minutes
- Add 950 µL LB
- Incubate 37ºC one hour

Plate them in plates with ampicillin (for control) and X-Gal and IPTG (also control)

Incubate at 37ºC 16 hours

(The plates were prepared adding 10 µL of 100mM IPTG and 20 µL of 2% X-Gal)

However nothing grown in the plates from the digestion-ligation, only in the ones of the control.

30. July - Arnau

This time we tried to construct the biobricks for the homology arms (first the right one) with the genome of spirulina. The DNA extraction of spirulina was done using the DipGene protocol and then a two-step PCR of the homology regions was done.

DNA extraction procedure of 2 mL of spirulina from the liquid culture of NPEC:

- Spin 11.000 rpm 1 minute
- Add 200 µL of P1 and vortex
- Add 200 µL of P2 and incubate at 55ºC adding 20 µL of proteinase K for 5 minutes
- Dip the paper during 2 minutes
- Wash with 500 µL wash buffer 30 seconds
- Put it in the PCR mix

Buffer composition:

- **P1:** 50 mM Tris/HCl, pH 8.0, 10 mM EDTA
- **P2:** 200 mM NaOH, 1% SDS
- **Wash buffer:** 10 mM Tris [pH 8.0], 0.1% Tween-20

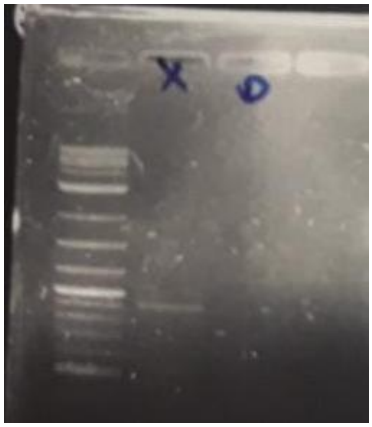
Prepare Master mix Q5 High-Fidelity 2x Master Mix (two reactions, one with primers with Prefix and Suffix and one without, and one negative control)

- Volume per reaction 50 µL
    - 2,5 µL each primer
    - 25 µL Q5 master mix
    - DNA paper
    - 22,5 µL water

Agarose gel preparation 0.7%:

1. Solve 0.42 g of agarose in 60 mL of 1 x TBE buffer
2. Melt the agarose in the microwave until boiling
3. Take out the solution and mix it by shaking the bottle
4. Put the solution back into the microwave and let it boil again
5. Repeat mixing and boiling until the agarose is completely melted
6. Set up an agarose gel box with spacers and a comb
7. Take out some solution with a blue pipette tip and put it along the inside of the spacers in the gel box and let it solidify
8. Add 2 µL of RotiR-GelStain to the remaining solution and mix by shaking the bottle
9. Pour the rest of the solution into the gel box
10. Let it stand for at least 30 minutes until the gel becomes solid
11. Fill the gel box up with 1 x TBE buffer so that the gel is just covered

The ladder used was the 1kb plus ladder of NEB lab



The x in the image correspond to the PCR product using the primers without Prefix and suffix

<u>01. August – Arnau, Victoria and Behbood</u>

We made an electro competent cells stock. We also made a new DNA extraction of Spirulina and PCR of the left homology arm without Prefix and Suffix, another PCR of the left homology arm with the primers with Prefix and Suffix and second PCR of the product of the first PCR of the right homology arm (two-step PCR)

Protocol for making electro competent cells:

1. Cultivate them until the OD550 = 0.72
2. Keep on ice during all the process
3. Spin at 5000 rpm at 4ºC 10 minutes
4. Discard the supernatant
5. Re-suspend in cold water
6. Spin at 5000 rpm at 4ºC 10 minutes
7. Repeat the wash
8. Spin again
9. Re-suspend in 45 mL of cold water
10. Transfer to a falcon tube and spin
11. Discard the supernatant
12. Re-suspend in 800 µL of 10% glycerol
13. Aliquot each 35 µL in Eppendorf and put in liquid nitrogen
14. Store a -70ºC

DNA extraction procedure (with 2 mL of spirulina culture NPEC)

- Spin 11.000 rpm 1 minute
- Add 200 µL of P1 and vortex
- Add 200 µL of P2 and incubate at 55ºC adding 20 µL of proteinase K for 5 minutes
- Dip the paper during 2 minutes
- Wash with 500 µL wash buffer 30 seconds
- Put it in the PCR mix

Buffer composition:

- **P1:** 50 mM Tris/HCl, pH 8.0, 10 mM EDTA
- **P2:** 200 mM NaOH, 1% SDS
- **Wash buffer:** 10 mM Tris [pH 8.0], 0.1% Tween-20

Prepare Master mix Q5 High-Fidelity 2x Master Mix (for each reaction and control)

- Volume per reaction 50 µL
  - 2,5 µL each primer
  - 25 µL Q5 master mix
  - DNA paper
  - 22,5 µL water

30 cycles	Annealing	64ºC	30 sec
	Extension	72ºC	1:15 min
	Denaturation	94ºC	1 min
	Final Extension	72ºC	3 min

Two step PCR

30 cycles	Annealing	70,6ºC	30 sec
	Extension	72ºC	40 sec
	Denaturation	94ºC	1 min
	Final Extension	72ºC	3 min

02. August - Sophie and Arnau

We run a gel with the PCR products of the last day.

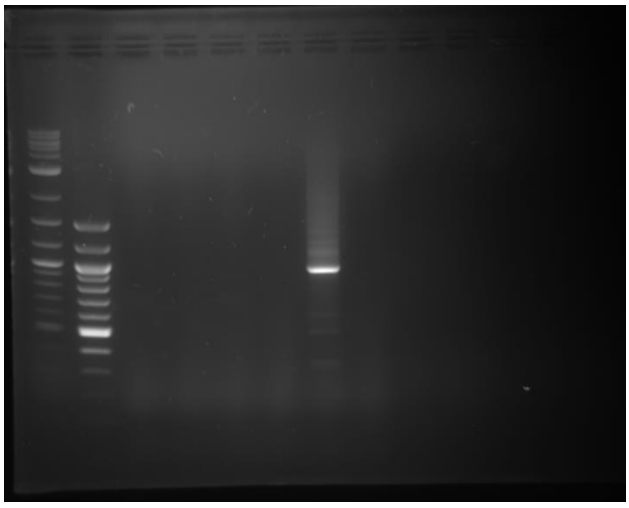
Agarose gel preparation 0.7%:

1. Solve 0.42 g of agarose in 60 mL of 1 x TBE buffer
2. Melt the agarose in the microwave until boiling
3. Take out the solution and mix it by shaking the bottle
4. Put the solution back into the microwave and let it boil again
5. Repeat mixing and boiling until the agarose is completely melted
6. Set up an agarose gel box with spacers and a comb
7. Take out some solution with a blue pipette tip and put it along the inside of the spacers in the gel box and let it solidify
8. Add 2 µL of RotiR-GelStain to the remaining solution and mix by shaking the bottle
9. Pour the rest of the solution into the gel box
10. Let it stand for at least 30 minutes until the gel becomes solid
11. Fill the gel box up with 1 x TBE buffer so that the gel is just covered

The ladder used was the 1kb plus ladder of NEB lab

Loading scheme:

1 kb ladder	100 b	Left Homology arm (LH)	LH control	LH + Prefix & Suffix	LH + Prefix & Suffix	Right Homology Arm (RH) + Prefix & Suffix	RH + P&S	RH control



The amplification of the second PCR of the right homology arm with Prefix and Suffix can be seen in the gel image.

1) A competence assay for the competent cells was performed showing that the transformation efficiency is of 466,4 UFC/pg of DNA)

Also the first PCR of the left homology arms was repeated.

DNA extraction procedure (with 2 mL of spirulina culture NPEC)

- Spin 11.000 rpm 1 minute
- Add 200 µL of P1 and vortex
- Add 200 µL of P2 and incubate at 55ºC adding 20 µL of proteinase K for 5 minutes
- Dip the paper during 2 minutes
- Wash with 500 µL wash buffer 30 seconds
- Put it in the PCR mix

Buffer composition:

- **P1:** 50 mM Tris/HCl, pH 8.0, 10 mM EDTA
- **P2:** 200 mM NaOH, 1% SDS
- **Wash buffer:** 10 mM Tris [pH 8.0], 0.1% Tween-20

Prepare Master mix Q5 High-Fidelity 2x Master Mix (for each reaction and control)

- Volume per reaction 50 µL
    - 2,5 µL each primer
    - 25 µL Q5 master mix
    - DNA paper
    - 22,5 µL water

30 cycles	Annealing	64ºC	30 sec
	Extension	72ºC	1:15 min
	Denaturation	94ºC	1 min
	Final Extension	72ºC	3 min

Then we extract the DNA extraction of the biobrick for the right homology arm.

Protocol for gel extraction of Jenna Bioscience DNA-gel extraction kit:

Weight of the cut from the gel: 160 mg

- Add 480 µL Ex buffer (volume x3 respect the weight)
- Add 160/240 mL of isopropanol to both (volume 1:1)
- Add 100 µL of activation buffer
- Load 700 µL wash buffer two times
- 40 µL Elution buffer

When measuring with nanodrop the values and the plot were strange, looks like the protocol of extraction failed, therefore the sample was discarded, the PCR of the right homology arm had to be repeated.

<u>05. August - Behbood</u>

We run the gel of the first PCR of the left homology arm (no prefix and suffix)

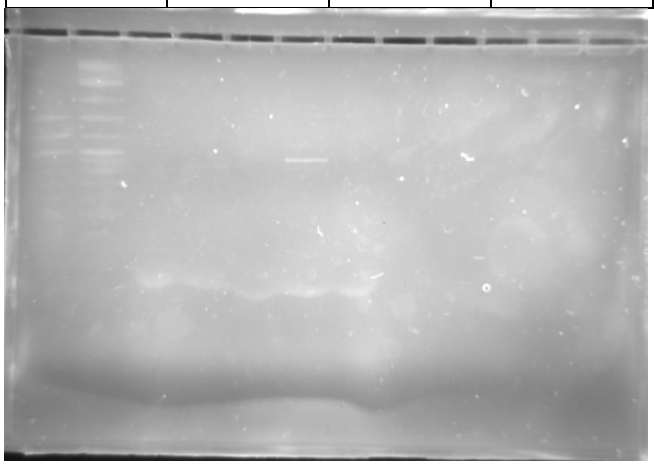
Agarose gel preparation 0.7%:

1. Solve 0.42 g of agarose in 60 mL of 1 x TBE buffer
2. Melt the agarose in the microwave until boiling
3. Take out the solution and mix it by shaking the bottle

4. Put the solution back into the microwave and let it boil again
5. Repeat mixing and boiling until the agarose is completely melted
6. Set up an agarose gel box with spacers and a comb
7. Take out some solution with a blue pipette tip and put it along the inside of the spacers in the gel box and let it solidify
8. Add 2 µL of RotiR-GelStain to the remaining solution and mix by shaking the bottle
9. Pour the rest of the solution into the gel box
10. Let it stand for at least 30 minutes until the gel becomes solid
11. Fill the gel box up with 1 x TBE buffer so that the gel is just covered

The ladder used was the 1kb plus ladder of NEB lab

100 kb ladder	1 kb ladder	Left Homology arm	Left Homology arm	Negative control	Right homology arm without Prefix and suffix 30.07.2019



09. August - Arnau and Behbood

We repeat the DNA extraction of Spirulina, followed by the first PCR of both homology arms with the primers without prefix and suffix and also a two-step PCR with the primers with Prefix and Suffix.

DNA extraction procedure (with 2 mL of spirulina culture NPEC)

- Spin 11.000 rpm 1 minute
- Add 200 µL of P1 and vortex
- Add 200 µL of P2 and incubate at 55ºC adding 20 µL of proteinase K for 5 minutes
- Dip the paper during 2 minutes
- Wash with 500 µL wash buffer 30 seconds
- Put it in the PCR mix

Buffer composition:

- **P1:** 50 mM Tris/HCl, pH 8.0, 10 mM EDTA
- **P2:** 200 mM NaOH, 1% SDS
- **Wash buffer:** 10 mM Tris [pH 8.0], 0.1% Tween-20

Prepare Master mix Q5 High-Fidelity 2x Master Mix (for each reaction and control)

- Volume per reaction 50 µL
  - 2,5 µL each primer
  - 25 µL Q5 master mix
  - DNA paper
  - 22,5 µL water

First PCR cycles:

30 cycles	Annealing	64ºC	30 sec
	Extension	72ºC	1:15 min
	Denaturation	94ºC	1 min
	Final Extension	72ºC	3 min

Two step PCR:

30 cycles	Annealing	70,6ºC	30 sec
	Extension	72ºC	40 sec
	Denaturation	94ºC	1 min
	Final Extension	72ºC	3 min

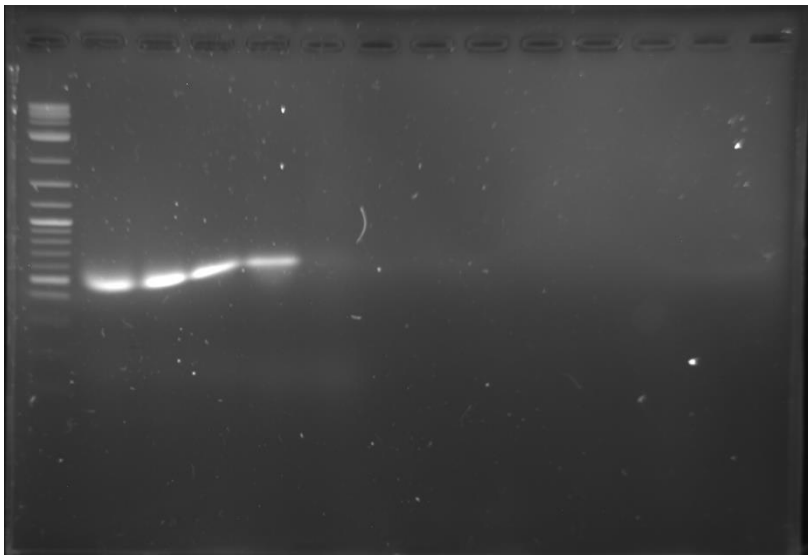
Agarose gel preparation 0.7%:

1. Solve 0.42 g of agarose in 60 mL of 1 x TBE buffer
2. Melt the agarose in the microwave until boiling
3. Take out the solution and mix it by shaking the bottle
4. Put the solution back into the microwave and let it boil again
5. Repeat mixing and boiling until the agarose is completely melted
6. Set up an agarose gel box with spacers and a comb
7. Take out some solution with a blue pipette tip and put it along the inside of the spacers in the gel box and let it solidify
8. Add 2µL of RotiR-GelStain to the remaining solution and mix by shaking the bottle
9. Pour the rest of the solution into the gel box
10. Let it stand for at least 30 minutes until the gel becomes solid
11. Fill the gel box up with 1 x TBE buffer so that the gel is just covered

The ladder used was the 1kb plus ladder of NEB lab

Loading scheme in the gel:

Ladder	Left arm without P&S	Left arm with P&S	Right arm without P&S	Left arm with P&S	Negative controls



All PCRs were successful

10. August - Arnau and Behbood

We made a gel extraction of homology biobricks, followed by digestion and ligation action, and transformation with the product.

Weight of the cuts from the gel:

- Right Homology + P&S: 0,09 g
- Left Homology + P&S: 0,11 g
- Right Homology: 0,1 g
- Left Homology: 0,11 g

DNA extraction with the Zymoclean Gel DNA recovery kit D4007:

1. Excise the DNA fragment from the agarose gel using a razor blade, scalpel or other device and transfer it into a 1.5 ml microcentrifuge tube.
2. Add 3 volumes of ADB to each volume of agarose excised from the gel (e.g. for 100 µl (mg) of agarose gel slice add 300 µl of ADB).
3. Incubate at 37-55 °C for 5-10 minutes until the gel slice is completely dissolved. For DNA fragments > 8 kb, following the incubation step, add one additional volume (equal to that of the gel slice) of water to the mixture for better DNA recovery (e.g., 100 µl agarose, 300 µl ADB, and 100 µl water).
4. Transfer the melted agarose solution to a Zymo-Spin™ Column in a Collection Tube.
5. Centrifuge for 30-60 seconds. Discard the flow-through.
6. Add 200 µl of DNA Wash Buffer to the column and centrifuge for 30 seconds. Discard the flow-through. Repeat the wash step.
7. Add ≥ 6 µl DNA Elution Buffer or water directly to the column matrix. Place column into a 1.5 ml tube and centrifuge for 30-60 seconds to elute DNA.

The measurements with Nanodrop showed the following concentration of DNA:

- Right Homology arm + P&S: 2 ng/µL
- Left Homology arm + P&S: 3,4 ng/µL
- Right Homology arm: 2,2 ng/µL
- Left Homology: 2,3 ng/µL

Because of the low yield when measuring in the Nanodrop and also an strange curve of absorption with a lot of background noice we decided to repeat the PCR this time only with the primers with the Prefix and Suffix using the two-step program

Prepare Master mix Q5 High-Fidelity 2x Master Mix (for each reaction and control)

- Volume per reaction 50 µL
    - 2,5 µL each primer
    - 25 µL Q5 master mix
    - DNA paper
    - 22,5 µL water

Two-step PCR:

30 cycles	Annealing	70,6ºC	30 sec
	Extension	72ºC	40 sec
	Denaturation	94ºC	1 min
	Final Extension	72ºC	3 min

12. August - Arnau

We run a gel of the PCR products from the last day but no amplification were observed. So we made a new PCR and a new gel for run it.

Prepare Master mix Q5 High-Fidelity 2x Master Mix (for each reaction and control)

- Volume per reaction 50 µL
    - 2,5 µL each primer
    - 25 µL Q5 master mix
    - DNA paper
    - 22,5 µL water

Two-step PCR:

30 cycles	Annealing	70,6ºC	30 sec
	Extension	72ºC	40 sec
	Denaturation	94ºC	1 min
	Final Extension	72ºC	3 min

Agarose gel preparation 0.7%:

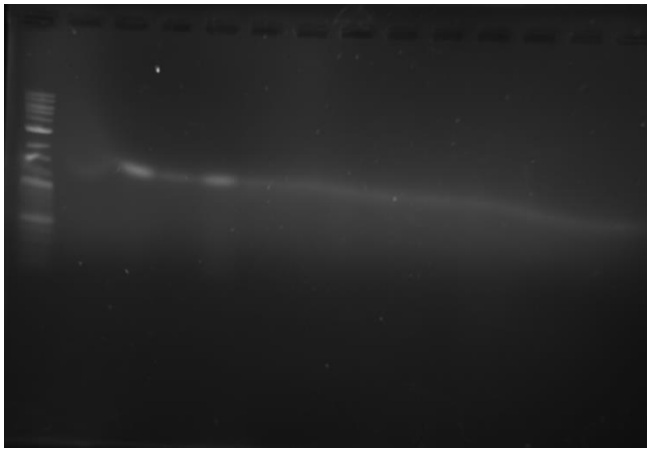
1. Solve 0.42 g of agarose in 60 mL of 1 x TBE buffer
2. Melt the agarose in the microwave until boiling
3. Take out the solution and mix it by shaking the bottle

4. Put the solution back into the microwave and let it boil again
5. Repeat mixing and boiling until the agarose is completely melted
6. Set up an agarose gel box with spacers and a comb
7. Take out some solution with a blue pipette tip and put it along the inside of the spacers in the gel box and let it solidify
8. Add 2 µL of RotiR-GelStain to the remaining solution and mix by shaking the bottle
9. Pour the rest of the solution into the gel box
10. Let it stand for at least 30 minutes until the gel becomes solid
11. Fill the gel box up with 1 x TBE buffer so that the gel is just covered

The ladder used was the 1kb plus ladder of NEB lab

Loading scheme:

Ladder	Left Homology arm of 10.08.2019	Right Homology arm of 10.08.2019	Left Homology arm of today	Right Homology arm of today	Negative control



DNA extraction with the Zymoclean Gel DNA recovery kit D4007:

8. Excise the DNA fragment from the agarose gel using a razor blade, scalpel or other device and transfer it into a 1.5 ml microcentrifuge tube.
9. Add 3 volumes of ADB to each volume of agarose excised from the gel (e.g. for 100 µl (mg) of agarose gel slice add 300 µl of ADB).
10. Incubate at 37-55 °C for 5-10 minutes until the gel slice is completely dissolved. For DNA fragments > 8 kb, following the incubation step, add one additional volume (equal to that of the gel slice) of water to the mixture for better DNA recovery (e.g., 100 µl agarose, 300 µl ADB, and 100 µl water).
11. Transfer the melted agarose solution to a Zymo-Spin™ Column in a Collection Tube.
12. Centrifuge for 30-60 seconds. Discard the flow-through.
13. Add 200 µl of DNA Wash Buffer to the column and centrifuge for 30 seconds. Discard the flow-through. Repeat the wash step.
14. Add ≥ 6 µl DNA Elution Buffer or water directly to the column matrix. Place column into a 1.5 ml tube and centrifuge for 30-60 seconds to elute DNA.

The results from the gel extraction measured with Nanodrop show a final yield for the left homology arm of 10.1 ng/µL and 17.3 ng/µL for the right homology arm

13. August - Arnau and Behbood

We tried the double digestion, ligation and transformation of the Biobricks of HyR, NOS, LH and RH with pBS in order to send them to sequence.

DNA extraction procedure with 2 mL of spirulina culture in NPEC media:

- Spin 11.000 rpm 1 minute
- Add 200 µL of P1 and vortex
- Add 200 µL of P2 and incubate at 55ºC adding 20 µL of proteinase K for 5 minutes
- Dip the paper during 2 minutes
- Wash with 500 µL wash buffer 30 seconds
- Put it in the PCR mix

Buffer composition:

- **P1:** 50 mM Tris/HCl, pH 8.0, 10 mM EDTA
- **P2:** 200 mM NaOH, 1% SDS
- **Wash buffer:** 10 mM Tris [pH 8.0], 0.1% Tween-20

Prepare Master mix Q5 High-Fidelity 2x Master Mix

- Volume per reaction 50 µL
  - 2,5 µL each primer
  - 25 µL Q5 master mix
  - DNA paper
  - 22,5 µL water

Second PCR cycles (2 step PCR):

30 cycles	Annealing	70,6ºC	30 sec
	Extension	72ºC	40 sec
	Denaturation	94ºC	1 min
	Final Extension	72ºC	3 min

Double digestion:

	NOS	HyR	pBS	RH	LH
Plasmid/DNA 500 ng	26,7 µL	8,1 µL	6 µL	20 µL	20 µL
Eco RI-HF	1 µL	1 µL	1 µL	1 µL	1 µL
Pst I	1 µL	1 µL	1 µL	1 µL	1 µL
10xNEB buffer	5 µL	5 µL	5 µL	5 µL	5 µL
H_2O	16, 3 µL	34,4 µL	37 µL	25 µL	25 µL

Incubate 37ºC 20 minutes (decided to increase from 10 to 20 minutes)

Incubate 80ºC 20 minutes

LIGATION:

Digest product	2 µL
pBS digested	2 µL
10x T4 ligase buffer	2 µL
T4 ligase	1 µL
H_2O	13 µL

Incubate at Room Temperature 20 minutes (increased from 10 to 20 minutes)

Incubate at 80ºC 20 minutes

Then the electro competent cells were transformed:

- 2 µL ligation product +35 µL electro competent cells
- Electroporate 1350 V
- Add 1mL LB cultivate 1 hour 37ºC 950 rpm
- Plate 100 µL in agar plates with Amp (for the pBS selection) and with Gal4 and IPTG (for insertion detection)

16. August – Victoria and Behbood

Liquid cultures of the colonies from the transformation were set.

20. August - Arnau

Miniprep of the liquid cultures using the ZymoPure plasmid miniprep kit D4211

1. Centrifuge 0.5-5 ml1 of bacterial culture in a clear 1.5 ml tube at full speed for 15- 20 seconds in a microcentrifuge. Discard supernatant.
2. Add 250 µl of ZymoPURE™ P1 (Red) to the bacterial cell pellet and resuspend completely by vortexing or pipetting.
3. Add 250 µl of ZymoPURE™ P2 (Green) and immediately mix by gently inverting the tube 6-8 times. Do not vortex! Let sit at room temperature for 2-3 minutes. Cells are completely lysed when the solution appears clear, purple, and viscous.
4. Add 250 µl of ice cold ZymoPURE™ P3 (Yellow) and mix thoroughly by inversion. Do not vortex! Invert the tube an additional 3-4 times after the sample turns completely yellow. The sample will turn yellow when the neutralization is complete and a yellowish precipitate will form.
5. Incubate the neutralized lysate on ice for 5 minutes.
6. Centrifuge the neutralized lysate for 5 minutes at 16,000 x g.

7. Transfer 600 µl of supernatant from step 6 into a clean 1.5 ml microcentrifuge tube. Be careful not to disturb the yellow pellet and avoid transferring any cellular debris to the new tube.
8. Add 275 µl of ZymoPURE™ Binding Buffer to the cleared lysate from step 7 and mix thoroughly by inverting the capped tube 8 times.
9. Place a Zymo-Spin™ II-P Column in a Collection Tube and transfer the entire mixture from step 8 into the Zymo-Spin™ II-P Column.
10. Incubate the Zymo-Spin™ II-P/Collection Tube assembly at room temperature for 2 minutes and then centrifuge at 5,000 x g for 1 min. Discard the flow through.
11. Add 800 µl of ZymoPURE™ Wash 1 to the Zymo-Spin™ II-P Column and centrifuge at 5,000 x g for 1 min. Discard the flow through.
12. Add 800 µl of ZymoPURE™ Wash 2 to the Zymo-Spin™ II-P Column and centrifuge at 5,000 x g for 1 min. Discard the flow through.
13. Add 200 µl of ZymoPURE™ Wash 2 to the Zymo-Spin™ II-P Column and centrifuge at 5,000 x g for 1 min. Discard the flow through.
14. Centrifuge the Zymo-Spin™ II-P Column at ≥ 10,000 x g for 1 minute in order to remove any residual wash buffer.
15. Transfer the Zymo-Spin™ II-P Column into a clean 1.5 ml tube and add 25 µl of ZymoPURE™ Elution Buffer2,3 directly to the column matrix. Incubate at room temperature for 2 minutes, and then centrifuge at ≥ 10,000 x g for 1 minute in a microcentrifuge. Store the eluted plasmid DNA at ≤ -20°C.

21. August - Sophie

The Nanodrop measurments of the miniprep of yesterday show that the values were correct then we decided to perform a digestion assay to check if our biobricks were really inserted in the plasmid.

Double digestion:

	NOS	HyR	pBS	RH	LH
Plasmid/DNA 500 ng	26,7 µL	8,1 µL	6 µL	20 µL	20 µL
Eco RI-HF	1 µL	1 µL	1 µL	1 µL	1 µL
Pst I	1 µL	1 µL	1 µL	1 µL	1 µL
10xNEB buffer	5 µL	5 µL	5 µL	5 µL	5 µL
H_2O	16, 3 µL	34,4 µL	37 µL	25 µL	25 µL

Incubate 37ºC 20 minutes (decided to increase from 10 to 20 minutes)

Incubate 80ºC 20 minutes

Agarose gel preparation 0.7%:

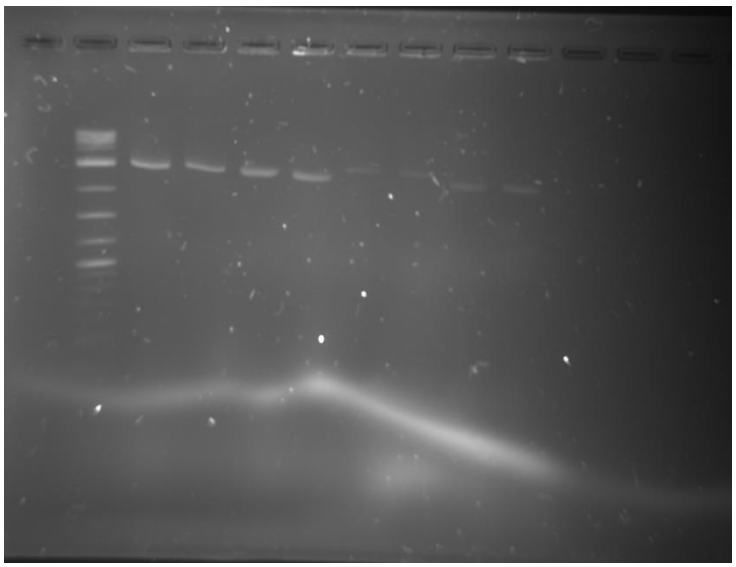
1. Solve 0.42 g of agarose in 60 mL of 1 x TBE buffer
2. Melt the agarose in the microwave until boiling

3. Take out the solution and mix it by shaking the bottle
4. Put the solution back into the microwave and let it boil again
5. Repeat mixing and boiling until the agarose is completely melted
6. Set up an agarose gel box with spacers and a comb
7. Take out some solution with a blue pipette tip and put it along the inside of the spacers in the gel box and let it solidify
8. Add 2 µL of RotiR-GelStain to the remaining solution and mix by shaking the bottle
9. Pour the rest of the solution into the gel box
10. Let it stand for at least 30 minutes until the gel becomes solid
11. Fill the gel box up with 1 x TBE buffer so that the gel is just covered

The ladder used was the 1kb plus ladder of NEB lab

Loading scheme of the gel:

1 kb ladder	HR	HR	HL	HL	NOS	NOS	HyR	HyR	Negative control



As only one band was visible at the high of the backbone we assume that the insertion didn't worked

22. August - Arnau and Sophie

We repeat the digestion. This time we decided to not use ladder because we were running out of materials in the lab.

Double digestion:

	NOS	HyR	pBS	RH	LH
Plasmid/DNA 500 ng	26,7 µL	8,1 µL	6 µL	20 µL	20 µL
Eco RI-HF	1 µL	1 µL	1 µL	1 µL	1 µL
Pst I	1 µL	1 µL	1 µL	1 µL	1 µL
10xNEB buffer	5 µL	5 µL	5 µL	5 µL	5 µL

H_2O	16, 3 µL	34,4 µL	37 µL	25 µL	25 µL

Incubate 37ºC 20 minutes (decided to increase from 10 to 20)

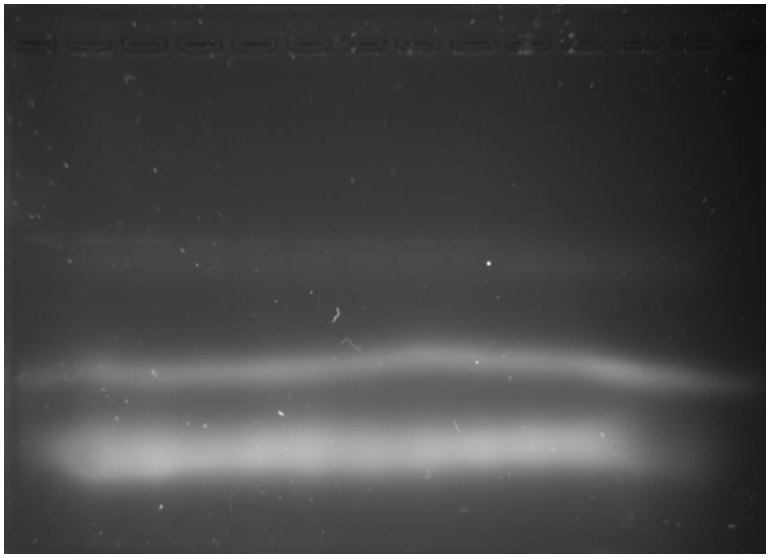
Incubate 80ºC 20 minutes

Agarose gel preparation 0.7%:

1. Solve 0.42 g of agarose in 60 mL of 1 x TBE buffer
2. Melt the agarose in the microwave until boiling
3. Take out the solution and mix it by shaking the bottle
4. Put the solution back into the microwave and let it boil again
5. Repeat mixing and boiling until the agarose is completely melted
6. Set up an agarose gel box with spacers and a comb
7. Take out some solution with a blue pipette tip and put it along the inside of the spacers in the gel box and let it solidify
8. Add 2 µL of RotiR-GelStain to the remaining solution and mix by shaking the bottle
9. Pour the rest of the solution into the gel box
10. Let it stand for at least 30 minutes until the gel becomes solid
11. Fill the gel box up with 1 x TBE buffer so that the gel is just covered

Loading scheme (two times each):

pBS digestion 1	pBS digestion 2	LH	HyR	NOS	RH



<u>23. August - Arnau</u>

This time we decide to try to make a gel running and a gel extraction of the digestion product of pBS in order to reduce the chances of this to re-ligate and increase the efficiency of the ligation.

Double digestion:

	pBS
Plasmid 500 ng	6 µL
Eco RI-HF	1 µL
Pst I	1 µL
10xNEB buffer	5 µL
H_2O	37 µL

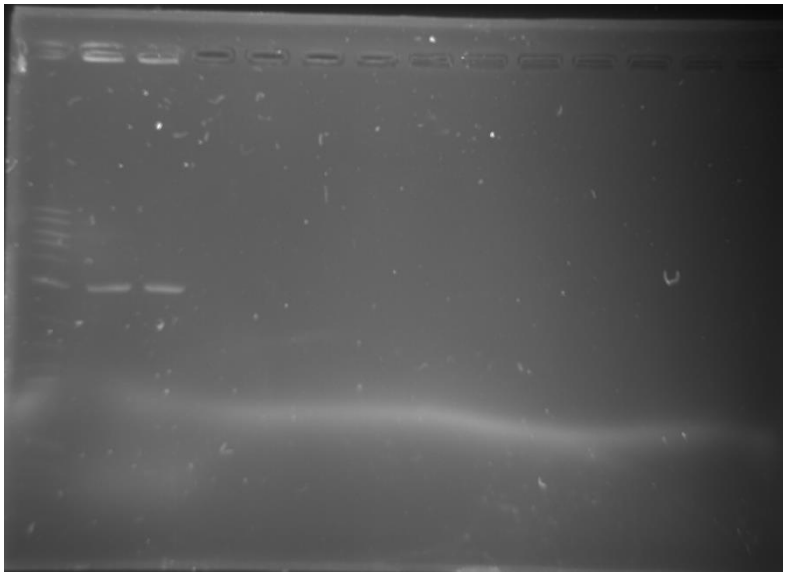
Agarose gel preparation 0.7%:

1. Solve 0.42 g of agarose in 60 mL of 1 x TBE buffer
2. Melt the agarose in the microwave until boiling
3. Take out the solution and mix it by shaking the bottle
4. Put the solution back into the microwave and let it boil again
5. Repeat mixing and boiling until the agarose is completely melted
6. Set up an agarose gel box with spacers and a comb
7. Take out some solution with a blue pipette tip and put it along the inside of the spacers in the gel box and let it solidify
8. Add 2 µL of RotiR-GelStain to the remaining solution and mix by shaking the bottle
9. Pour the rest of the solution into the gel box
10. Let it stand for at least 30 minutes until the gel becomes solid
11. Fill the gel box up with 1 x TBE buffer so that the gel is just covered

The ladder used was the 1kb plus ladder of NEB lab

Loading scheme:

1 kb ladder	pBS digestion product	pBS digestion product	Negative control



DNA extraction with the Zymoclean Gel DNA recovery kit D4007:

1. Excise the DNA fragment from the agarose gel using a razor blade, scalpel or other device and transfer it into a 1.5 ml microcentrifuge tube.
2. Add 3 volumes of ADB to each volume of agarose excised from the gel (e.g. for 100 µl (mg) of agarose gel slice add 300 µl of ADB).
3. Incubate at 37-55 °C for 5-10 minutes until the gel slice is completely dissolved. For DNA fragments > 8 kb, following the incubation step, add one additional volume (equal to that of the gel slice) of water to the mixture for better DNA recovery (e.g., 100 µl agarose, 300 µl ADB, and 100 µl water).
4. Transfer the melted agarose solution to a Zymo-Spin™ Column in a Collection Tube.
5. Centrifuge for 30-60 seconds. Discard the flow-through.
6. Add 200 µl of DNA Wash Buffer to the column and centrifuge for 30 seconds. Discard the flow-through. Repeat the wash step.
7. Add ≥ 6 µl DNA Elution Buffer or water directly to the column matrix. Place column into a 1.5 ml tube and centrifuge for 30-60 seconds to elute DNA.

The concentration was measured with Nanodrop with a result of 93 ng/µL. Then we proceed with the digestion of the biobricks.

Digestion of biobricks

	NOS	HyR	RH	LH
Plasmid/DNA 500 ng	26,7 µL	8,1 µL	20 µL	20 µL
Eco RI-HF	1 µL	1 µL	1 µL	1 µL
Pst I	1 µL	1 µL	1 µL	1 µL
10xNEB buffer	5 µL	5 µL	5 µL	5 µL
H_2O	16, 3 µL	34,4 µL	25 µL	25 µL

Incubate 37ºC 20 minutes (decided to increase from 10 to 20 minutes)

Incubate 80ºC 20 minutes

Ligation:

Digest product	2 µL
pBS digested	2 µL
10x T4 ligase buffer	2 µL
T4 ligase	1 µL
H_2O	13 µL

Incubate at Room Temperature 20 minutes (increased from 10 to 20 minutes)

Incubate at 80ºC 20 minutes

Transformation (electroporation):

- 2 µL ligation product +35 µL electro competent cells
- Electroporate 1350 V
- Add 1mL LB cultivate 1 hour 37ºC 950 rpm
- Plate 100 µL in agar plates with Amp (for the pBS selection) and with Gal4 and IPTG (for insertion detection)

The transformation wasn't successful, no colonies were detected in the plates

27.08.2019

At this point as we were lacking of resource and manpower to carry on with both projects and we had to decide to focus full time on only one of them. The project to be continued was chosen to be DipGene.