

PROLUNG

SENSING

LAB BOOK 2

iGEM
Stockholm

Cloning of OmpR responsive promoter into pSB4A5 backbone

Digestion 1

Aim

In order to express as much OmpR as possible, the gene requires a low copy plasmid backbone. Therefore the OmpR+YFP gene has to be cloned from the high copy number plasmid pSB1C3 to pSB4A5.

The following reactions will be done to perform this:

- Digest OmpR promoter + YFP gene from pSB1C3
- Digest Constitutive promoter + RFP from low copy number plasmid backbone pSB4A5
- Ligate OmpR + YFP in pSB4A5

Procedure

Digestion

1. Prepare the following Master Mixes

Table 1: Master Mixes for 20 μ l

Enzyme Master Mix for Plasmid Backbone (20 μ l total, for 5 runs)
2 μ l Tango Buffer
1.1 μ l EcoRI
1.1 μ l PstI
15.8 μ l dH ₂ O

1. Digest Plasmid Backbone

- Add 4 μ l linearized plasmid backbone (25 ng/ μ l for 100 ng total)

- Add 4 μ l of Enzyme Master Mix
2. Digest G-block 1
- Add 5 μ l G-block 1 (20 ng/ μ l for 100 ng total)
 - Add 5 μ l of Enzyme Master Mix

see below for specific addition of DNA and H₂O

Table 2. Added substances

Sample	DNA	DNA amount (ng)	H ₂ O
M30011	1.76	100	3.24
pSB4A5	0.76	100	3.24
K592101	2.68	100	2.32

Digest all three reactions at 37 °C for 60 min, heat kill 80 °C at 20 min

Results

After digestion, we realised we didn't need K592101 for this experiment, but instead BBa_I6211. The gel clearly shows that pSB4A5 was digested, so this sample was purified from the gel for ligating M30011.

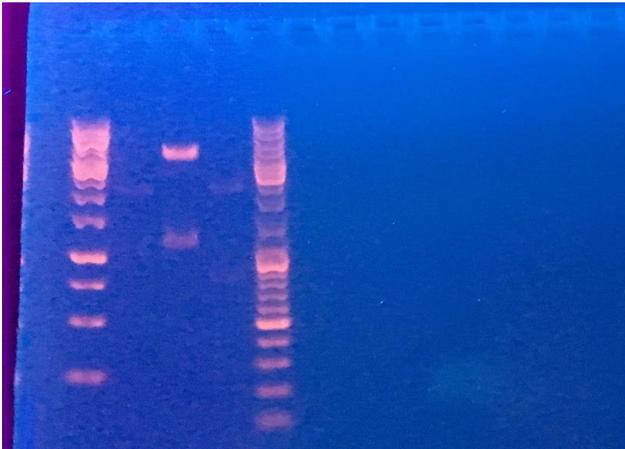


Figure 1. Gel of digestion of BBa_M30011, pSB4A5 and K592101

1. M30011 - VERY weak bands. wasnot further used
2. pSB4A5 - strong bands, backbone was purified from the gel
3. K592101 - weak bands, the "insert" was purified from the gel

After digestion, the parts of the gel containing the backbone of pSB4A5 and the insert of K592101 were purified for ligation

Digestion 2

Aim

We had previously used the wrong biobrick for OmpR+YFP, so the digestion had to be remade with the correct biobrick BBa_I6211. The previous digestion of BBa_M30011 failed, so we decided to redo it.

Procedure

Digestion

2. Prepare following Master Mixes

Table 3: Master Mixes for 20 μ l

Enzyme Master Mix for Plasmid Backbone (20 μ l total, for 5 runs)
2 μ l Tango Buffer
1.1 μ l EcoRI
1.1 μ l PstI
15.8 μ l dH ₂ O

3. Digest Plasmid Backbone
 - Add 4 μ l linearized plasmid backbone (25 ng/ μ l for 100 ng total)
 - Add 4 μ l of Enzyme Master Mix
4. Digest G-block 1
 - Add 5 μ l G-block 1 (20 ng/ μ l for 100 ng total)
 - Add 5 μ l of Enzyme Master Mix

see below for specific addition of DNA and H₂O

Table 4. Added substances for digestion

Sample	DNA (μ l)	DNA amount (ng)	H ₂ O (μ l)
M30011	3.52	200	1.48
pSB4A5	0.76	100	3.24
I6211	0.96	100	4.04

Results

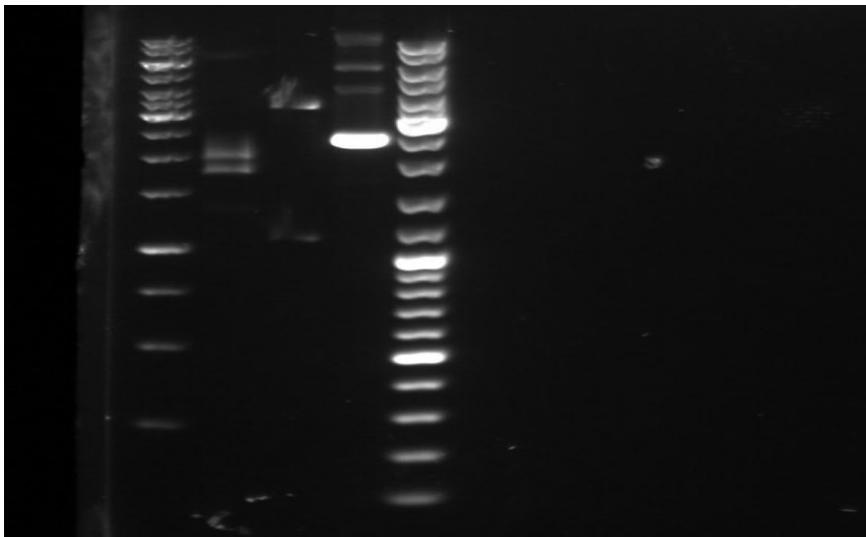


Figure 2. Gel of digestion of BBa_I6211, pSB4A5 and pSB1C3 (control).

1. I6211 - visible bands
2. pSB4A5 digested - visible bands
3. pSB1C3 plasmid (control) - strong bands

Gel purification

Aim

Purify the vector from its original insert part for ligation.

Procedure

The gel purification protocol was used with no modifications

Mistakes

1/3 of required QG was used due to a calculation error

Results

After consulting our advisors about the low yield after gel extraction, we decided to not proceed with these samples and instead ligate the parts without purification.

Table 5. DNA concentration after gel extraction

Sample	DNA concentration (ng/ μ l)
M30011	2
pSB4A5	4

Ligation

Aim

Ligate BBa_I6211 into pSB4A5

Background

The original idea was to purify the pSB4A5 backbone and the BBa_I6211 from the agarose gel and thereafter ligate these pieces. However, after consulting Max we decided to directly mix the digested parts in the ligation reaction, with 1:3 molar excess of the I6211 to pSB4A5 to increase chances of correct ligation of I6211 instead of the original RFP "insert" in pSB4A5.

The correct ligation should result in white colonies after transformation and re-ligation of the RFP gene will result in red colonies

Procedure

The amount of insert DNA was calculated using ligation calculator

1. Prepare the following ligation mixture (20 μ l)

Component	Amount (μ l)
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T4 ligase buffer	2
T4 ligase	1
Digested pSB4A5 (vector)	6
Digested l6211 (insert)	6.9
ddH ₂ O	4.1
Total volume	20

2. Mix gently
3. Let stand 1h room temperature
4. Heat inactivate enzyme 10 min 65 C
5. Put on ice for transformation

Transformation

Aim

Transform the ligated product l6211 + pSB4A5

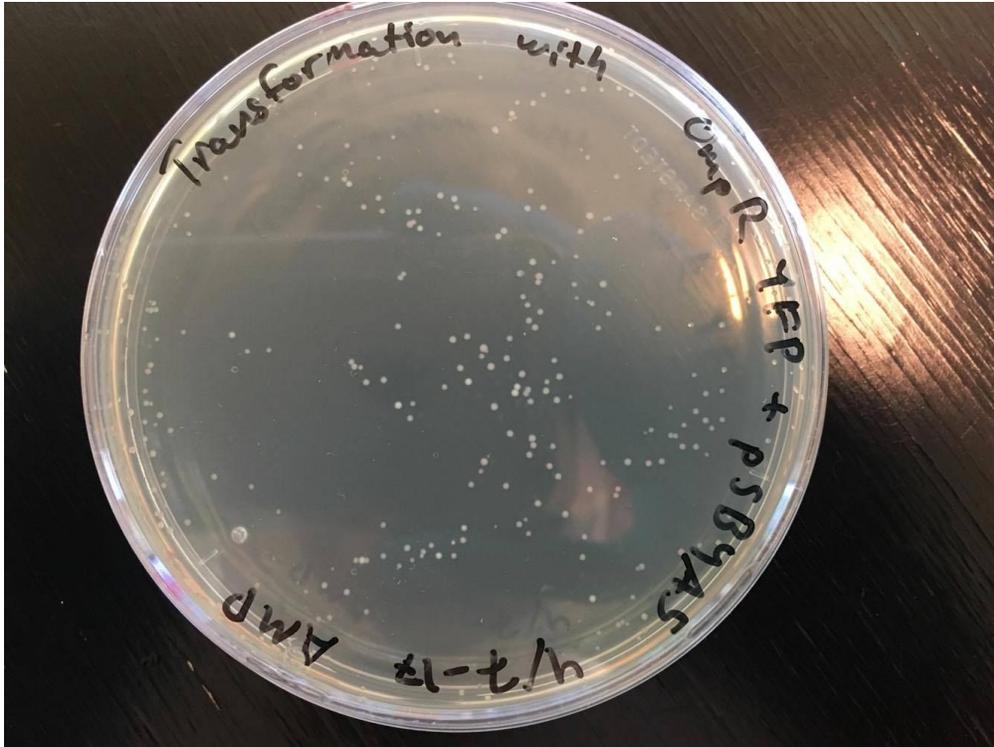
Procedure

The transformation protocol was used with no modifications

Results

Expected: white colonies indicate successful ligation and transformation. Red colonies indicate re-ligation of digested product

Obtained results:



Several white colonies, few red colonies

Conclusion

The ligation and transformation were successful as mainly white colonies were visible

Flasks

Aim

Ensuring that the white colonies were "truly white" and did not turn red

Procedure

1. Pick a colony with a pipette tip and put in an E-flask containing:
10 ml nutrient broth
10 μ l ampicillin
2. Incubate the flask (37°C, 200 rpm) for 8h

Note: 2 flasks with white colonies were made

Results

The colonies did not turn red, ie they were truly white.

Testing of OmpR promoter

1. Preparations of negative control
- cultivation

Aim

Prepare cells that will be used for negative control in osmolarity test

Procedure

Top 10 cells were streaked out on agar plates without antibiotics and incubated overnight (14h 30 min) in 37°C

Results

Many visible colonies after incubation

Osmolarity test 1

Aim

Demonstrate that our system can sense osmotic pressure changes and activate transcription of a gene when osmotic pressure is increased. Furthermore, characterization of OmpR promoter biobrick and demonstrate that it is truly dependent on osmotic pressure changes and at which OD's the promoter is mainly active.

Background

iGEM Stockholm 2015 have previously demonstrated that OmpR is dependent on osmotic pressure changes using sucrose gradient. iGEM UCL 2015 have previously demonstrated that OmpR is dependent on osmotic pressure changes using NaCl gradient and measured it's activity over increasing OD. We have decided to base

our test on a combination of the work of the previous iGEM teams mentioned and will test both a sucrose gradient (5%, 10% and 15%) as well as a NaCl gradient (0.05%, 0.1%, 0.2% and 0.4%). The gradients used are the same as the previous teams have used. The protocol used comes from iGEM Stockholm 2015 and is modified according to their experience.

The fluorescent protein used in this experiment is YFP.

Material

Preparation

- Sterile filter for sucrose
- Falcon tubes
- 300 g sucrose
- ddH₂O
- Sterile bottles
- 1 g NaCl
- 30 E- flasks
- 672 ml nutrient broth
- 400 µl ampicillin
- 150 µl chloroamphenicol
- 136 µl kanamycin

OD measurement

- Cuvettes
- OD spectrophotometer

Fluorescence measurement

- Thermofisher 96 well black plates
- Fluorostar omega
- 1X PBS

Procedure

1. Prepare the solutions in appendix 1
2. Choose sample with similar fluorescent protein as a positive control and sample

without

fluorescent protein as a negative control.

3. Use only 0%, 15% sucrose and 0.4% NaCl nutrient broth for both the positive and the negative controls.

4. Prepare overnight culture of the bacteria in 10 mL osmo media in E-flasks. Inoculate one fresh colony in each flask and incubate overnight at 37°C.

5. Do not forget to include negative and positive controls. Check the OD next morning and note it down.

6. Dilute the overnight culture using 1:20 dilutions (2 ml of overnight cultured bacteria into 38 mL of respective osmo media in Erlenmeyer flask) and let them grow in the incubator at 37°C until they reach OD 0.3.

7. Add Kanamycin 340 µg to 1 mL culture to inhibit protein synthesis and immediately put the sample in refrigerator. When all the samples are done, centrifuge all the samples and remove the media. Suspend the cell pellets in 1 mL of PBS and perform fluorescence analysis directly using plate reader. Replace Kanamycin with other antibiotics if your cells contain Kanamycin resistance gene.

8. Use excitation and emission length accordingly (depending on the fluorescent protein).

An example that we used is the excitation length 485-12 nm ± 10 and emission length 520 nm ± 30 to measure YFP. The same will go with the controls.

9. Add 100 µl of sample to a 96 well plate and measure in Fluorostar. For each sample, 5 replicated were made and the average values of each sample were used.

10. Do not forget to include negative and positive control during measurement. Use only media as a blank.

DATA ANALYSIS:

Measure the fluorescence and compare with the controls and blank. Plot it as a

graph to observe relative change with different osmole concentrations.

Results

1.1 Growth curve

All cells grew after incubation in their respective media (figure 4) and reached OD 0.3.

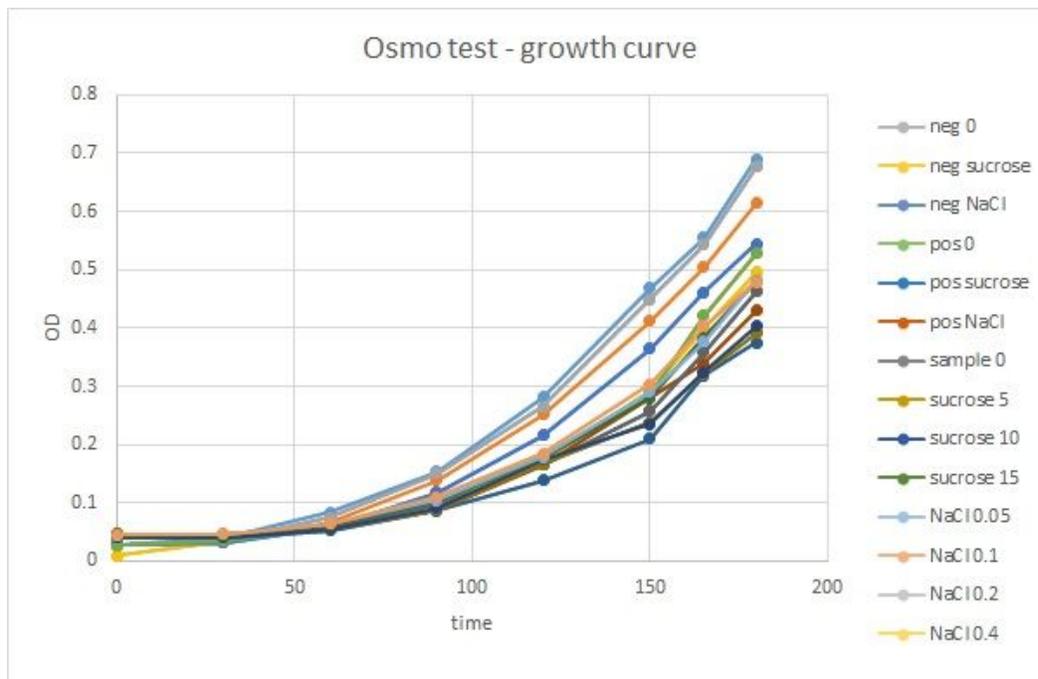


Figure 4. Growth curve

1.2 Fluorescence

Three fluorescence measurements were made. The graphs show no trend in increasing fluorescence when the sucrose or salt concentration is increased (figure 5).

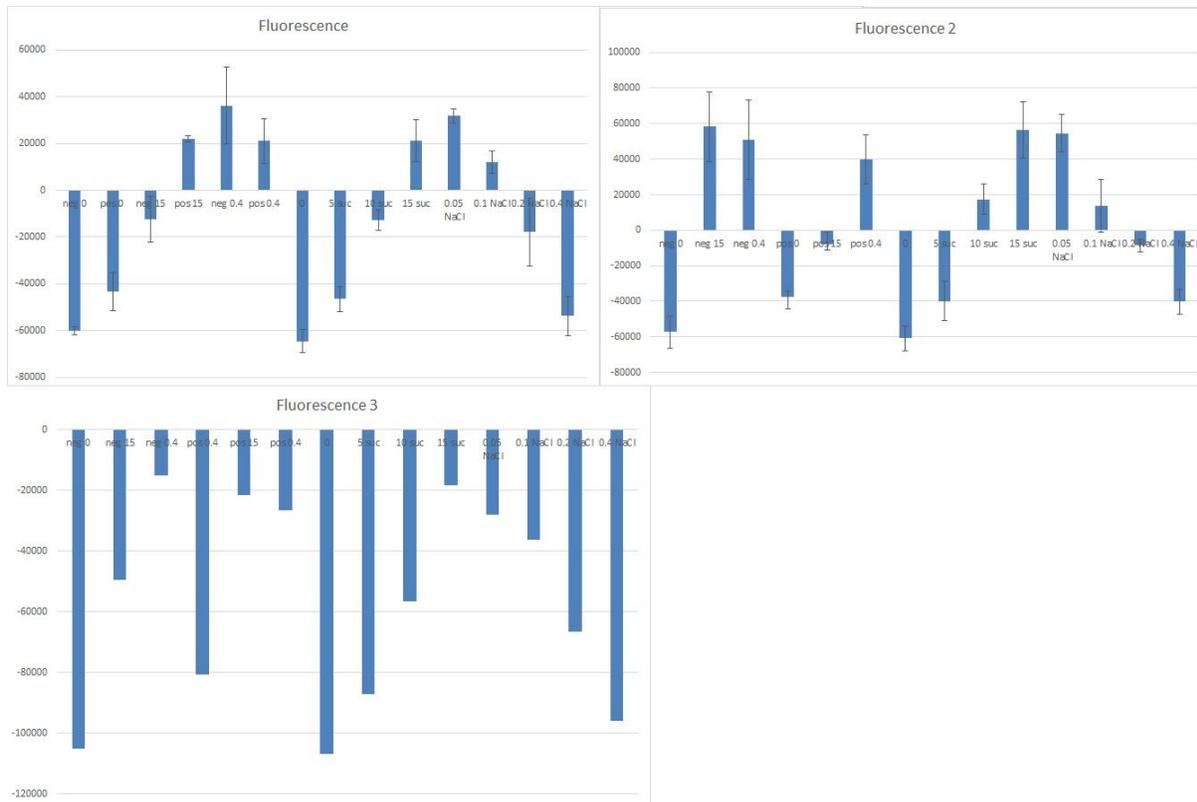


Figure 5. Fluorescence at OD 0.1, 0.2 and 0.3.

Discussion

The fluorescence measurements were very inconsistent and demonstrated several negative values, which was very unexpected. In the list below, some possible explanations for this are listed.

The salt gradient resulted in decreasing fluorescence with increasing NaCl concentration. A possible explanation for this is that the peak was already reached with lower salt concentrations. Therefore, in the next experiment, lower concentrations of NaCl will be tested

Troubleshoot

- Use the same gain for all the samples; do not adjust gain in the settings.
- Repeat the experiment at least three times to have statistical relevance.

- Forgot to spin down the cell culture and resuspend in PBS. The fluorescence was measured in respective osmo media which gave strange results.

- The excitation and emission wavelengths for YFP varies from the available filter in the Fluorostar, which could have made the results strange.

- When using YFP, there will be much background from the cells

- The cells were diluted in normal nutrient broth instead of their osmo media because of unclear protocol

Conclusion

There is no trend in increasing fluorescence when increasing the sucrose concentration, and when comparing to the rest of the results the values are very strange.

Appendix 1

Recipes for sucrose and NaCl gradients

0% sucrose

For 10 ml:

5 ml nutrient broth

5 ml ddH₂O

Required for osmo test: 30 ml (3 samples)

5% sucrose

For 10 ml:

5 ml nutrient broth

5 ml 10% sucrose solution

Required for osmo test: 10 ml (1 sample)

10% sucrose

For 10 ml:

5 ml nutrient broth

5 ml 20% sucrose solution

Required for osmo test: 10 ml (1 sample)

15% sucrose

For 10 ml:

5 ml nutrient broth

5 ml 30% sucrose solution

Required for osmo test: 30 ml (3 samples)

0.05% NaCl

For 10 ml:

5 ml nutrient broth

5 ml 0.1% NaCl solution

Required for osmo test: 10 ml (1 sample)

0.1% NaCl

For 10 ml:

5 ml nutrient broth

5 ml 0.2% NaCl solution

Required for osmo test: 10 ml (1 sample)

0.2% NaCl

For 10 ml:

5 ml nutrient broth

5 ml 0.4% NaCl solution

Required for osmo test: 10 ml (1 sample)

0.4% NaCl

For 10 ml:

5 ml nutrient broth

5 ml 0.8% NaCl solution

Required for osmo test: 30 ml (3 samples)

Recipes for sucrose solutions from 30% stock solution

10% sucrose

For 10 ml:

3.33 ml 30% sucrose solution

7.67 ml ddH₂O

20% sucrose

For 10 ml:

6.67 ml 30% sucrose solution

3.33 ml ddH₂O

Recipes for NaCl solution from 1% stock solution

0.1% NaCl

For 10 ml:

1 ml 1% NaCl solution

9 ml ddH₂O

0.2% NaCl

For 10 ml:

2 ml 1%NaCl solution

8 ml ddH₂O

0.4% NaCl

For 10 ml:

4 ml 1% NaCl

6 ml ddH₂O

0.8% NaCl

For 10 ml:

8 ml NaCl

2 ml H₂O

Required for osmo test: 30 ml (3 samples)

Kanamycin (340 µg/ml)

For 20 ml:

136 µl kanamycin (50 mg/ml)

19864 μ l ddH₂O