Lysostaphin

Week 7

Summarized below are the experiments conducted this week in chronological order. Click on the experiment name to view it. To go back to this summary, click **Summary** in the footer.

Summary

At this point we have confirmed that the construct we have been working with is in actual fact 3A Assembled Esp Basic. Thus, to avoid further cross-contamination of samples, we decided to work with lysostaphin from the beginning, that being the BioBrick present in the plasmid. Hence, the aim of this week was to obtain 3A assembled lysostaphin constructs of pSB1C3-T7-Lys.

1 Digestion of Lysostaphin from plasmid Biobrick

Responsible

Reskandi Rudjito and Bethel Tesfai Embaie

Protocols used

- Digestion and Ligation
- Gel purification

Modifications and comments to protocols

Digestion - Reduced the reaction volume to 10 μl

Experimental Set Up

Table 1: Concentrations of the lysostaphin plasmid sample used for digestion

Sample	Concentration [ng/μl]
Lysostaphin 1	829.0

Table 2: Digestion reaction of Lysostaphin plasmid

Reaction	Volume [μl]
DNA	0.5
Buffer SH 10 X	1.0
Pst1	0.5
Xba1	0.5
Sterile water	7.5

Sample Calculation

1 μl of restriction enzyme is used for every 1 μg/μl of DNA.
Since the lysostaphin plasmid is just under 1μg/μl, thus the amount of enzyme used in the reaction is kept in accordance to the protocol.

Results and Conclusions

The gel electrophoresis result of the double digestion is shown in Figure 1. Based on this figure it can intrepreted that double digestion was a success. The two bands observed on well 2-4 represent represent the linearized pSB1C3 backbone and the digested Lys part, respectively. The expected sizes of each DNA fragment are shown in Table 3. The smeared bands around 700 bp was later cut with a clean scapel and gel purified to obtain pure digested Lys fragments.

Table 3: Expected DNA fragment sizes of digested lysostaphin

Sample	Fragment size [bp]
Digested Lys	765
Linearized pSB1C3	2068
Undigested Lys plasmid	2809

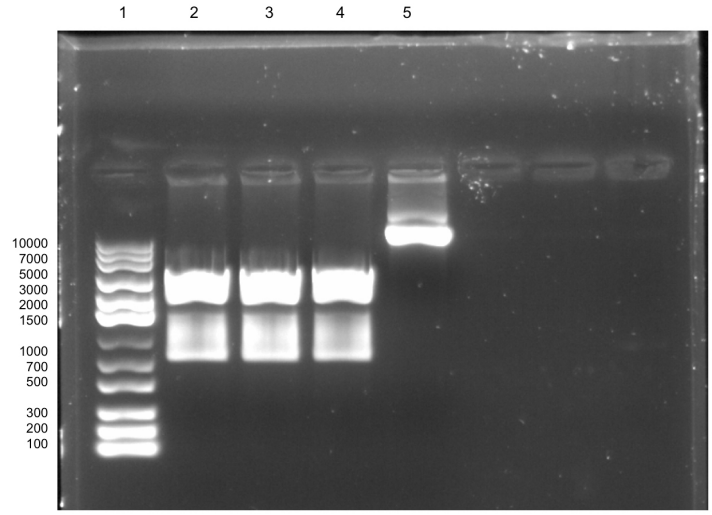

Figure 1: Double digestion of lysostaphin plasmid with EcoR1 and PstI. (1) DNA Ladder (2) Double digested Lys 1 (3) Double digested Lys 2 (4) Double digested Lys 3 (5) Undigested Lys.

Discussion and Troubleshooting

The smeared bands observed on the gel is most likely due to the overload of template used in the gel electrophoresis. An excess of template DNA was loaded into the gels to minimize loss during gel purification.

2 3A Assembly and Transformation of pSB1A3-T7-Lys

Responsible

Bethel Tesfai Embaie and Sigrun Stulz

Protocols used

- 3A Assembly
- Transformation

Modifications to protocols

Transformation was done on both TOP10 and BL21(DE3) cells.

Experimental Set Up

In order to perform the 3A assembly, all three DNA fragments consisting of the T7 promoter, lysostaphin and pSB1A3 backbone are digested and purified prior to ligation. The concentrations of each DNA fragment are stated in Table 4.

Table 4: Concentrations DNA fragments for 3A Assembly

Sample	Concentration [ng/μl]	Size [bp]
Digested T7 promoter (E and S)	6.6	50
Digested Lys (X and P)	6.2	765
Digested pSB1A3	2.6	2200

Sample Calculation

In this 3A assembly, we have decided to go forward with an insert:vector molar ratio of 3:1. The calculations shown on the tables below are adjusted so that each reaction contains 50 ng of backbone and the inserts are adjusted according to the desired molar ratio. Since the concentrations of the purified DNA fragments were relatively low, this resulted in an overload of reaction volume which is indicated by a minus sign for sterile water.

Table 5: 3A Assembly Reaction

Component	Volume
T7 promoter	0.52
Lys	8.41
pSB1A3	19.23
T4 DNA Ligase	0.5
T4 DNA Ligase Buffer 10X	3
Sterile water	-1.66

Results and Conclusions

The ligated product was transformed into Top 10 and BL21(DE3) cells, however after 16 hours of incubation there was no sign of colonies. We incubated the plates for an additional few hours but there were still no colonies observed. The ligation and transformation of 3A assembled lys was unsuccessful.

Discussion and Troubleshooting

As mentioned above, the concentration of the DNA fragments that we had worked with was very low. This could have affected the efficiency of the ligation process. Also, in this particular ligation procedure the incubation period with T4 DNA ligase was performed at room temperature for 10 minutes. We later realized that this may have been too short, and future ligations should be done at a lower temperature but for a longer period of time.
Another possible troubleshoot could have been the concentration of ampicillin used in the agar plates. In this transformation, we used plates with an ampicillin concentration of 100 μM/ml. Thus, to test whether this was too high, we decided to lower the ampicillin concentration to 50 μM/ml (Addgene, 2016).
Addgene. 2016. Making LB Agar Plates. https://www.addgene.org/plasmid-protocols/bacterial-plates/. Accessed in August 2016

3 Re-Transform pSA1C3-T7-Lys

Responsible

Reskandi Rudjito and Bethel Tesfai Embaie

Protocols used

- Transformation

Modifications to protocols

LB plates were made with 50 μM/ml of ampicillin instead of 100 μM/ml

Experimental Set Up

The transformation was done on both Top10 and BL21 (DE3) cells and the set up is written in the table below.

Table 6: Re-Transformation of pSB1C3-T7-Lys

Sample	Comment
pSB1A3-T7-Lys Top10	Transformed into Top10 cells
pSB1A3-T7-Lys BL21 (DE3)	Transformed into BL21 (DE3) cells
positive control Top 10	No antibiotics
positive control BL21	No antibiotics

Sample Calculation

Amount of ampicillin stock 100 mM/ml = 50 μL of antibiotic for every 100 ml of LB

Results and Conclusions

Only the positive control showed growth of colonies. Plates with ampicillin had no colonies indicating either ligation, transformation or both procedures were unsuccessful.

Discussion and Troubleshooting

Hence, based on this re-transformation procedure, the decrease of ampicillin concentration had not improved the result. At this point we have been getting inefficient ligation and transformation results and it is difficult to pin point the exact problem. To ensure that the problem was not solely on human error, we redid the ligation and transformation, which will be described in the next section.

4 3A Assembly and Transformation of pSB1A3-T7-Lys

Responsible

Reskandi Rudjito and Bethel Tesfai Embaie

Protocols used

- 3A Assembly
- Digestion and Ligation
- PCR purification
- Transformation

Modifications to protocols

LB plates were made with 50 μM/ml of ampicillin instead of 100 μM/ml.

Experimental Set Up

As mentioned in section 2, to perform the 3A assembly, all three DNA fragments consisting of the T7 promoter, lysostaphin and pSB1A3 backbone are digested and purified prior to ligation. The concentrations of each DNA fragment are stated in Table 4.

Table 7: Concentrations DNA fragments for 3A Assembly

Sample	Concentration [ng/μl]	Size [bp]
Digested T7 promoter (E and S)	6.6	50
Digested Lys (X and P)	6.2	765
Digested pSB1A3	2.7	2200

As a repetition of the previous transformation procedure, both TOP10 and Bl21 cells were used. Positive controls plated onto LB plates without antibiotics were done to ensure that the cells were viable.

Table 8: Re-Transformation of pSB1C3-T7-Lys

Sample	Comment
pSB1A3-T7-Lys Top10	Transformed into Top10 cells
pSB1A3-T7-Lys BL21 (DE3)	Transformed into BL21 (DE3) cells
positive control Top 10	No antibiotics
positive control BL21	No antibiotics

Sample Calculation

To repeat the same experiment as mentioned in section 2, we have kept the insert:vector molar ratio of 3:1. The calculations shown on the tables below are adjusted so that each reaction contains 50 ng of backbone and the inserts are adjusted according to the desired molar ratio. Since the concentrations of the purified DNA fragments were relatively low, this resulted in an overload of reaction volume which is indicated by a minus sign for sterile water.

Table 9: 3A Assembly Reaction

Component	Volume [μl]
T7 promoter	0.26
Lys	4.21
pSB1A3	9.26
T4 DNA Ligase	0.25
T4 DNA Ligase Buffer 10X	1.5
Sterile water	-0.47

Results and Conclusions

Unfortunately no colonies were observed from the ligation and transformation procedure.

Discussion and Troubleshooting

To ensure that ligation was a success, we decided to perform a PCR on the ligated plasmid with the verification primers VF2 and VR. Since these primers anneal to the plasmid backbone, theoretically a band of the ligated inserts should be visible after a PCR reaction. This experiment is explained in the next section.

5 PCR of Ligated pSB1A3-T7-Lys

Responsible

Reskandi Rudjito and Bethel Tesfai Embaie

Protocols used

- PCR with Q5 polymerase

Modifications to protocols

The reaction volume of PCR was reduced to 12.5 μl to preserve the limited amount of DNA sample available.

Experimental Set Up

The PCR reaction was done on one ligated product of pSB1A3-T7-Lys. The reaction composition and condition are described in the tables below.

Table 10: PCR Reaction of ligated pSB1A3-T7-Lys with Q5

Component	Volume μl Reaction	Final Concentration
Q5 High-Fidelity 2X Master Mix	6.25	1X
10 M Forward Primer (VF2)	0.625	0.5 M
10 M Reverse Primer (VR)	0.625	0.5 M
Template DNA	0.5	<1,000 ng
Nuclease-Free Water	4.5	-

Table 11: PCR conditions of Q5 Polymerase (30 cycles)

Step	Temperature [°C]	Time [seconds]
Initial Denaturation	98	30
Denaturation	98	10
Annealing	52	30
Extension	72	20
Final Extension	72	120
Hold	4	-

Sample Calculation

No further calculations were done.

Results and Conclusions

No bands were observed on the gel.

Discussion and Troubleshooting

It was likely that ligation did not succeed, therefore no or very few plasmids were present in the reaction leading to an inefficient PCR.

6 Digestion of pSB1A3

Responsible

Reskandi Rudjito and Bethel Tesfai Embaie

Protocols used

- Digestion and Ligation

Modifications to protocols

Digestion: since the original pSB1A3 sample is low in concentration, 4 μl of sample was used to obtain a total concentration of 100 ng/μl in the digestion reaction

Experimental Set Up

The digestion reaction used to digest the linearized pSB1A3 plasmid is written in the table below. The amount of of enzyme used was adjusted to the amount of plasmid present in the reaction.

Table 12: Double digestion of pSB1A3 with EcoR1 and Pst

Reaction	Volume [μl]
pSB1A3 (25 ng/μl)	4
Buffer SH 10X	2
EcoRI	0.1
Pst	0.1
Sterile water	3.8

Sample Calculation

1 μl of restriction enzyme is used for every 1 μg/μl of DNA.

Results and Conclusions

It would be difficult to observe whether digestion had worked if we had not perform a ligation and transformation right after. Nonetheless, we decided to run a gel on all the digested products to ensure that the right DNA fragment was present in each sample. The gel we ran here is shown in the figure below. The digested pSB1A3 was later purified and measured with nanodrop to obtain a concentration of 6.8 [ng/μl].

Discussion and Troubleshooting

Based on Figure 2, well 2 and 4 show GS and EB which are irrelevant to this lab book. The digested pSB1A3 was loaded into well 3, however no bands could be observed. This was likely due to a human error whilst pipetting the sample into the gel. Well 5 and 6 show T7 and Lys

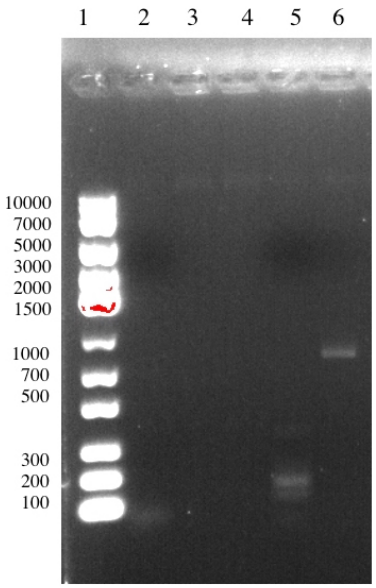

Figure 2: Double digestion of lysostaphin plasmid with EcoR1 and PstI. (1) DNA Ladder (2) Digested GS (3) Digested pSB1A3 (4) Digested ESP Basic (5) Diegsted T7 (6) Digested Lysostaphin

which are fragments needed in the 3A Assembly of pSB1A3-T7-Lys. The T7 sample show bands at around 100-200 bp. These bands are the remains of the double digestion from a PCR product with the verification primers. The T7 itself which has a size of 50 bp is undetectable in this gel. Well 6 show the digested Lys at 765 bp.

7 3A Assembly and Transformation of pSB1A3-T7-Lys

Responsible

Reskandi Rudjito and Bethel Tesfai Embaie

Protocols used

- 3A Assembly
- Digestion and Ligation
- PCR purification
- Transformation

Modifications to protocols

LB plates were made with 50 μM/ml of ampicillin instead of 100 μM/ml

Experimental Set Up

As mentioned in section 2 and 4, to perform the 3A assembly, all three DNA fragments consisting of the T7 promoter, lysostaphin and pSB1A3 backbone are digested and purified prior to ligation. The concentrations of each DNA fragment are stated in Table 13.

Table 13: Concentrations DNA fragments for 3A Assembly

Sample	Concentration [ng/μl]	Size [bp]
Digested T7 promoter (E and S)	6.6	50
Digested Lys (X and P)	6.2	765
Digested pSB1A3	6.8	2200

As a repetition of the previous transformation procedure, both TOP10 and Bl21 cells were used. Positive controls plated onto LB plates without antibiotics were done to ensure that the cells were viable.

Table 14: Transformation of pSB1A3-T7-Lys

Sample	Comment
pSB1A3-T7-Lys Top10	Transformed into Top10 cells
pSB1A3-T7-Lys BL21 (DE3)	Transformed into BL21 (DE3) cells
positive control Top 10	No antibiotics
positive control BL21	No antibiotics

Sample Calculation

As mentioned in section 2 and 4, we have kept the insert:vector molar ratio of 3:1. The calculations shown on the tables below are adjusted so that each reaction contains 50 ng of backbone and the inserts are adjusted according to the desired molar ratio.

Table 15: 3A Assembly Reaction

Component	Volume [μl]
T7 promoter	0.52
Lys	8.41
pSB1A3	7.35
T4 DNA Ligase	0.5
T4 DNA Ligase Buffer 10X	2.0
Sterile water	1.22

Results and Conclusions

Again, no colonies were observed from the ligation and transformation procedure.

Discussion and Troubleshooting

To improve ligation transformation efficiency, we looked into increasing the insert to vector ratio and increasing it into 10:1. In addition, we decided to run the ligation overnight at a temperature of 16 °C instead of 10 minutes at room temperature.