Lysostaphin

Week 8

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.

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1 Digestion of Lysostaphin, pSB1A3 and pSB1C3

Responsible

Reskandi Rudjito and Bethel Tesfai Embaie

Protocols used

- Digestion and Ligation
- PCR Purification

Modifications and comments to protocols

No modification to the protocol was done

Experimental Set Up

The digestion of lysostaphin plasmid, pSB1A3 and pSB1C3 was done in parallel in reaction volumes of 10-20 μl. Since the backbone pSB1A3 and pSB1C3 were relatively low in concentration, a larger volume of the backbone was added to the digestion reaction to obtain a final concentration of 200 ngμl. The initial concentrations and digestion reactions of each sample are stated below.

Table 1: Concentrations of the lysostaphin plasmid, pSB1A3 and pSB1C3 used for digestion

Sample	Concentration [ng/μl]
Lysostaphin 1	829.0
pSB1A3	49.0
pSB1C3	41.0

Table 2: Digestion reaction of Lysostaphin plasmid

Reaction	Volume [μl]
Lysostaphin plasmid	1.5
Buffer SH 10 X	2.0
Pst1	1.0
Xba1	1.0
Sterile water	14.5

Table 3: Digestion reaction of pSB1A3

Reaction	Volume [μl]
pSB1A3	4.0
Buffer SH 10 X	1.0
Pst1	0.2
EcoR1	0.2
Sterile water	4.6

Table 4: Digestion reaction of pSB1C3

Reaction	Volume [μl]
pSB1C3	5.0
Buffer SH 10 X	1.0
Pst1	0.2
EcoR1	0.2
Sterile water	3.6

Sample Calculation

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

Results and Conclusions

The atomic weight of magnesium is concluded to be $24\,\mathrm{g\,mol^{-1}}$, as determined by the stoichiometry of its chemical combination with oxygen. This result is in agreement with the accepted value.

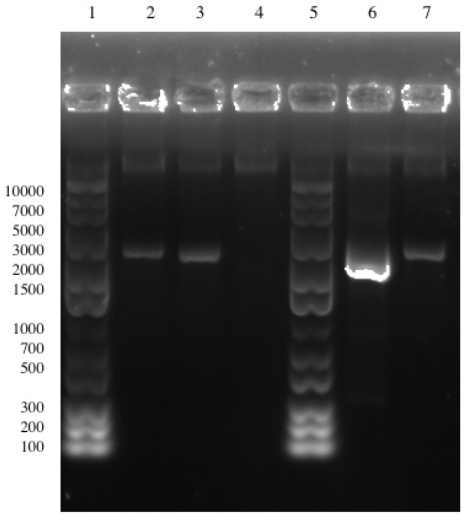

Figure 1: Digested Backbone. (1) DNA Ladder (2) Digested pSB1C3 Sample 1 (3) Digested pSB1C3 Sample 2 (3) Empty well (4) DNA Ladder (5) Undigested pSB1A3 (6) Digested pSB1C3

Discussion and Troubleshooting

Gel electrophoresis was only performed on the digested backbones since a gel confirming the presence of digested Lys had already been done in week 7. Based on the gel electrophoresis result (Figure 1), it can be concluded that the DNA had not run smoothly. This is clearly shown by the poor separation of the ladder. However, bands in the range of 2000-3000 bp are shown and these bands correspond to the expected size of the digested backbones. In well 6 the stronger

band which correspond to undigested pSB1A3 seems to have migrated much further down than the digested pSB1A3 which was loaded in well 7. This anomaly was most likely caused by the difference in the loading dye used when loading the samples.

2 3A Assembly and Transformation of pSB1C3-T7-Lys

Responsible

Reskandi Rudjito, Bethel Tesfai Embaie, Aman Mebrahtu and Shuangjia Xue

Protocols used

- 3A Assembly
- Transformation

Modifications to protocols

The backbone used has been changed to pSB1C3 instead of pSB1A3. Ligation was performed overnight at a temperature of 16 oC instead of 10 minutes at room temperature.

Experimental Set Up

To perform the 3A assembly, all three DNA fragments consisting of the T7 promoter, lysostaphin and pSB1C3 backbone are digested and purified prior to ligation. The concentrations of each DNA fragment are stated in Table 5.

Table 5: Concentrations DNA fragments for 3A Assembly

Sample	Concentration [ng/μl]	Size [bp]
Digested T7 promoter (E and S)	2.2	55
Digested Lys (X and P)	7.6	765
Digested pSB1C3	8.9	2029

To increase the chances of a positive transformation, 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 6: 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

Since we have been acquiring negative ligation and transformation results, we decided to increase the insert:vector molar ratio to 10:1. 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. The final reaction volume was 20 μl.

Table 7: 3A Assembly Reaction

Component	Volume [μl]
T7 promoter	2.59
Lys	11.44
pSB1A3	2.81
T4 DNA Ligase	0.5
T4 DNA Ligase Buffer 10X	2.0
Sterile water	0.67

Results and Conclusions

Two colonies were observed from TOP10 cells transformed with pSB1C3-T7-Lys.

Discussion and Troubleshooting

By increasing the insert:vector molar ratio to 10:1 as well as prolonging the ligation incubation period, the transformation had worked. The emergence of only two colonies is sufficient but indicates that the transformation had been inefficient. One solution to increase transformation efficiency would be to purify the ligation product prior to transformation. This would remove undesired DNA fragments as well as salts and enzymes that may interfere with the transformation process.

3 Colony picking and PCR of pSB1C3-T7-Lys

Responsible

Aman Mebrahtu and Reskandi Rudjito

Protocols used

- Colony picking
- Colony PCR

Modifications to protocols

Colony PCR: Two PCR master mixes were made since many colonies containing other combat proteins were picked in parallel with Lys

Experimental Set Up

The transformed colonies were picked were first dipped into a PCR mix and later cultivated in LB containing chloramphenicol 20 μM/ml. The colony PCR was done with verification primers (VF2 and VR). Plasmids from the overnight cultures were then extracted using a Mini prep and sent for sequencing. Table 8 shows the composition used for the colony PCR and Table 9 shows the PCR conditions used in the thermocycler.

Table 8: Colony PCR Reaction

Master Mix	Component	Volume [μl]
1	PCR Grade Nucleotide mix	2
	R_Primer (VR)	10
	F_primer (VF2)	10
	Template DNA	-
	Sterile water	28
	Total Volume	50
2	PCR Reaction buffer 10 x	10
	Taq DNA Polymerase	2.5
	Sterile water	37.5
	Total Volume	50

Table 9: Colony PCR condition using Taq polymerase

Step	Cycles	Temperature [°C]	Time
Initial Denaturation	1	94	2 min
Denaturation	30	94	30 secs
Annealing		52	1 min
Extension		72	1 min
Final Extension	1	72	7 min
Hold	indefinitely	4	-

Sample Calculation

No further calculations were done.

Results and Conclusions

The result of the colony PCR is shown in the figure below.

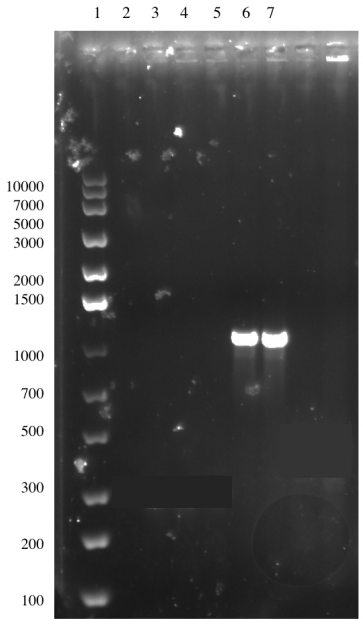

Figure 2: Colony PCR of Transformed Top10 cells containing pSB1C3-T7-Lys. (1) DNA Ladder (6) Lys Colony 1 (7) Lys Colony 2

Discussion and Troubleshooting

The result of the colony PCR on colonies containing pSB1C3-T7-Lys indicate that the 3A assembly as well as the transformation was successful. The two bands shown on Figure 2 are located just above the 1000 bp mark on the ladder. This correspond to the PCR product of pSB1C3-T7-Lys when it is amplified using the verification primers. The exact size of the amplified construct is 1093 bp.

4 Glycerol Stocks and Plasmid Purification of pSB1C3-T7-Lys

Responsible

Aman Mebrahtu and Reskandi Rudjito

Protocols used

- Glycerol Stock
- Plasmid Purification

Modifications to protocols

Plasmid purification: the DNA binding step was repeated to optimize the amount of DNA bound onto the column

Experimental Set Up

Table 10: Glycerol stock of Top10 cells containing pSB1C3-T7-Lys

Sample	Description
Lys colony 1	Small colony
Lys colony 2	Large colony

Sample Calculation

No calculations were done in this experiment.

Results and Conclusions

Table 11: Concentration of plasmid after purification

Sample	Concentration [ng/μl]
Lys colony 1	137
Lys colony 2	131

Discussion and Troubleshooting

To confirm that the sequence is correct, we first purified the plasmids from the liquid culture which we then sent for sequencing. Once we have confirmed that the sequence is correct, we can express the Lys in BL21 (DE3) cells and perform a functional test on the construct. We can also perform an overhang PCR which serves to add a BamHI restriction site right after the biobrick part. This BamHI sequence would then be used for fuse the biobrick with pre-designed

linker tags that also contain corresponding restriction site. The linker tags would later be used to fuse the protein onto the spider silk with the help of sortase.

5 Overhang PCR of pSB1C3-T7-Lys (3A Assembled Lys)

Responsible

Reskandi Rudjito

Protocols used

- PCR using Taq polymerase

Modifications to protocols

PCR reaction volume was adjusted to 25 μl

Experimental Set Up

The aim of this PCR is to add a BamHI restriction site on the 3'end of the lysostaphin gene. This is done by performing an overhang PCR using VF2 as a forward primer and Lys-Rev as the reverse primer. The Lys-Rev primer has also been designed to remove stop codons present on the original biobrick part. The characteristics of the Lys-Rev primer are written below.

Table 12: Lysostaphin Reverse Primer

Parameter	Description
Sequence	5' CGCGGATCCCTTTATAGTTCCCCAAAGAACACCTAAAG 3'
Tm []oC]	57
Restriction site	BamHI

Since we are using the VF2 primer in complement to the new Lys-rev primer, we have kept the PCR reaction composition and condition the same as to previous PCR reactions. The PCR is done on both 3A assembled Lys samples denoted 'Lys 1' and 'Lys 2'.

Table 13: Overhang PCR Reaction

Master Mix	Component	Volume [μl]
1	PCR Grade Nucleotide mix	1
	R_Primer (VR)	5
	F_primer (Lys Rev)	5
	Template DNA	2
	Sterile water	12
	Total Volume	25
2	PCR Reaction buffer 10 x	5
	Taq DNA Polymerase	0.5
	Sterile water	19.5
	Total Volume	25

Sample Calculation

Calculations for the PCR reaction are shown in Table 13.

Table 14: Overhang PCR condition using Taq polymerase

Step	Cycles	Temperature [°C]	Time
Initial Denaturation	1	94	2 min
Denaturation	30	94	30 secs
Annealing		52	1 min
Extension		72	1 min
Final Extension	1	72	7 min
Hold	indefinitely	4	-

Results and Conclusions

The result of the overhang PCR of Lys is shown as a gel in the figure below. The PCR product from sample Lys 1 was purified to obtain a final concentration of 23.5 ng/μl

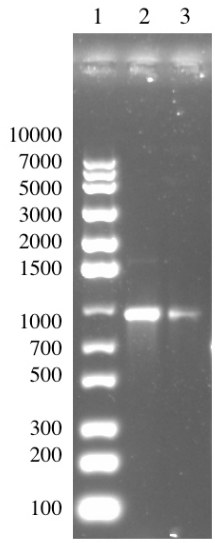

Figure 3: Overhang PCR of Lys. (1) DNA Ladder (2) Lys 1 (3) Lys 2

Discussion and Troubleshooting

The product size of the overhang PCR of pSB1C3-T7-Lys is 922 bp. Based on Figure 3, the DNA bands shown in well 2 and 3 correspond to the desired size of the overhang product. The DNA band in well 2 seemed to be stronger compared to the band in well 3. This is most likely due to the lack of homogeneity when mixing the two PCR master mixes together.

6 Digestion of Overhang Lys

Responsible

Reskandi Rudjito and Bethel Tesfai Embaie

Protocols used

- Digestion and Ligation
- PCR purification

Modifications to protocols

The amount of restriction enzymes added to the reaction was adjusted to the concentration of DNA being digested. The final concentration of the overhang purified PCR product used for digestion was 199.75 ng/μl.

Experimental Set Up

Similar to that of the 3A Assembly, 2 fragments would be directly cloned into a backbone. Here, the overhang Lys is digested with EcoR1 and BamHI and the linker tags (LT) would then be digested with BamHI and Pst1. The backbone is linearized with EcoR1 and Pst1. The three fragments would then be ligated together and transformed into competent cells.

Table 15: Digestion reaction of Overhang Lysostaphin

Reaction	Volume [μl]
Overhang Lys (23.5 ng/μl)	8.5
Buffer SB 10 X	2.0
EcoR1	0.2
BamH1	0.2
Sterile water	9.1

Sample Calculation

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

Results and Conclusions

The result of the digested overhang Lys compared to the undigested sample is shown in the figure below. The purified digested product had a final concentration of 7 ng/μl.

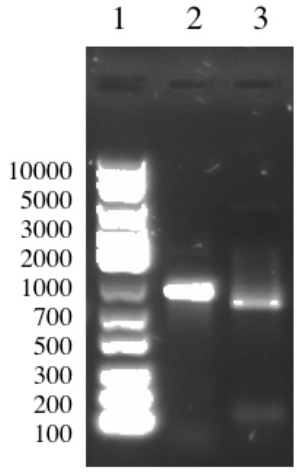

Figure 4: Digested Overhang Lys. (1) DNA Ladder (2) Undigested Overhang Lys (3) Digested Overhang Lys

Discussion and Troubleshooting

The digested overhang lys is 795 bp in size. This is more than 100 bp shorter than the undigested fragment which has a size of 922 bp. Thus, the difference between the digested and undigested fragments should be noticeable on a gel. Bearing this in mind, Figure 4 shows that there is a slight difference in size between the undigested and digested overhang Lys. The gel electrophoresis could have ran for a little longer for better separation of the bands, however based on this result we concluded that the digestion had worked.

7 Ligation and Transformation of pSB1C3-T7-Lys-LT

Responsible

Reskandi Rudjito, Bethel Tesfai Embaie and Sigrun Stulz

Protocols used

- 3A Assembly
- Transformation

Modifications to protocols

Experimental Set Up

To perform ligation procedure, all three DNA fragments consisting of the overhang Lys, linkertag and pSB1C3 backbone are digested and purified prior to ligation. The concentrations of each DNA fragment are stated in Table 16.

Table 16: Concentrations of DNA fragments for Ligation

Sample	Concentration [ng/μl]	Size [bp]
Digested Overhang Lys promoter (E and B)	7.0	795
Digested Linker Tags (B and P)	6.1	88
Digested pSB1C3 (E and P)	6.0	2029

To increase the chances of a positive transformation, 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 17: Transformation of pSB1C3-T7-Lys-LT

Sample	Comment
pSB1C3-T7-Lys-LT Top10	Transformed into Top10 cells
pSB1C3-T7-Lys-LT BL21 (DE3)	Transformed into BL21 (DE3) cells
positive control Top 10	No antibiotics
positive control BL21	No antibiotics

Sample Calculation

The insert:vector molar ratio used in the ligation was 10:1. 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. The final reaction volume was 20 μl.

Results and Conclusions

Colonies from the TOP10 cells were observed the next day indicating that ligation and transformation were successful. These colonies were cultivated and plasmids containing pSBC13-T7-Lys-LT were directly transformed into BL21 (DE3) cells.

Table 18: Ligation Reaction of pSB1C3-T7-Lys-LT

Component	Volume [μl]
Overhang Lys	12.99
Linker Tags	1.64
pSB1C3	4.17
T4 DNA Ligase	0.5
T4 DNA Ligase Buffer 10X	2.0
Sterile water	-1.29

8 Expression of pSB1C3-T7-Lys in BL21 (DE3)

Responsible

Bethel Tesfai Embaie

Protocols used

- Transformation
- Protein Expression
- Sonication

Modifications to protocols

No modification was done towards the protocol

Experimental Set Up

The plasmid containing the pSB1C3-T7-Lys construct was first transformed into BL21 (DE3) cells. The BL21 (DE3) cells containing pSB1C3-T7-Lys was then cultivated in LB + anitibiotic and induced for protein expression.

Table 19: Expression of Lysostaphin from BL21 (DE3)

Sample	Treatment
Lys 1	Induced 1 mM IPTG
	Induced 0.5 mM IPTG
	Uninduced
Lys 2	Induced 1 mM IPTG
	Induced 0.5 mM IPTG
	Uninduced

Sample Calculation

No calculations were done.

Results and Conclusions

The OD600 of the culture was at 0.89 before each sample was induced with IPTG. The concentration of IPTG was varied to see if lower IPTG induction would have an efect on the amount of proteins expressed. As lysostaphin is a bacteriolytic enzyme, there is a possibility that it could disrupt the cells of production, in this case the E. coli BL21 (DE3) cells. The protein samples were later subjected to SDS Page which will be mentioned in lab book week 9.