Week 5

Main construction: GFP antibody

AIM:

To construct 8 different plasmids that will be further used as a genetic material for liposomal encapsulation.

REAGENTS USED:

Tab. 1 List of reagents used in the experiment

Name of reagent
LB medium with Ampicillin (100 mg/L)
GeneJet Miniprep plasmid extraction kit (Thermofisher, Lithuania)
Phusion polymerase Master Mix (Thermofisher, Lithuania)
PCR purification kit (Thermofisher, Lithuania)
NdeI, BcuI, NheI, EcoRI, HindIII, XbaI, PstI FastDigest restriction endonucleases (Thermofisher, Lithuania)
GeneJet Gel extraction kit (Thermofisher, Lithuania)
T4 DNA Ligase (Thermofisher, Lithuania)
DH5α and TG1 competent cells
Previously constructed pRSET F plasmid in DH5α strain
Previously ordered igA, Lpp-OmpA, GFP antibody and Mistic genes DNA sequences

DNA electrophoresis components: Agarose, TAE buffer, EtBr.
DreamTaq polymerase (Thermofisher, Lithuania)

Suppl. 1. List of order primers used in the experiment.

igA constructs

1. To fuse igA with FLAG-tag (pRSET F) – through EcoRI and HindIII (FLAG – EcoRI – igA – HindIII)

Tm=63.7 PGR - 1

Beta F EcoRI - gaaagaattcAAAGCCAGAGAGTTGGCAAG

Beta R HindIII – tgtaaagcTTAGAAACGAATCTGTATTTTAATTTGTCC

2. To fuse igA with FLAG-tag (pRSET F) + Mstx (C terminus) (FLAG – EcoRI – igA – SpeI – Mstx – HindIII)

igA- Tm=63.7 PGR - 2

Beta F EcoRI-
gaaagaattcAAAGCCAGAGAGTTGGCAAG

Beta R –STOP NheI –
TCGTGCTAGCTAAGAAACGAATCTG

Mstx- Tm=64.5 PGR - 3

PB+ F - CGGGCAATGTAGCGAGTACG

Mstx R – HindIII – tctcaAGCTTCTTTTTCGCCTTCTTCG

3. To fuse GFP antibody (N terminus) with igA (NdeI-GFP antibody-SpeI-igA-PstI)

Camelid- Tm=66.6 PGR - 4

Camelid F – NdeI – atctcATATGCAGGTTCAACTTGTAGAAAGCG

BB R – CTGCAGCGGCCGCTACTAGTA

igA- Tm=58 PGR - 5

PB F – GCAATGTAGCGAGTACG

PB R – TCCTGTAATGCTAGTCTGC

4. To fuse igA with GFP antibody (N terminus) and Mstx (C terminus) (NdeI- GFP antibody – SpeI - igA-NheI-Mstx-HindIII)

Camelid- Tm=66.6 PGR - 6

Camelid F – NdeI – atctcATATGCAGGTTCAACTTGTAGAAAGCG

BB R – CTGCAGCGGCCGCTACTAGTA

iga (-STOP)- Tm=65.6 PGR - 7

PB+ F – CGGGCAATGTAGCGAGTACG

beta R –STOP NheI – TCGTGCTAGCTAAGAAACGAATCTG

Mstx-Tm=64.5 PGR - 8

PB+ F - CGGGCAATGTAGCGAGTACG

Mstx R – HindIII – tctcaagcTTCTTTTTCGCCTTCTTCG

Lpp-ompA (LO) constructs

5. To fuse LO with FLAG (C terminus) (NdeI – LO – FLAG – HindIII)

Tm=62.4 PGR - 9

LO F NdeI – atgtcaTATGTCTAGCAATGCCAAAATCG

LO R FLAG HindIII – ttagaagcttaCTTATCGTCATCGTCTTTGTAGTCggatccacgtgtggc

6. To fuse LO with FLAG (C terminus) and Mstx (N terminus) (NdeI – Mstx – SpeI – LO – FLAG – HindIII)

Mstx- Tm=55.3 PGR - 10

Mstx F NdeI – tcaccaTATGTATTGCACCTTTTTTG

PB R – TCCTGTAATGCTAGTCTGC

LO- Tm=63 PGR - 11

PB+ F – CGGGCAATGTAGCGAGTACG

LO R FLAG HindIII – ttagaagcttaCTTATCGTCATCGTCTTTGTAGTCggatccacgtgtggc

7. To fuse LO with GFP antibody (C terminus) (NdeI – LO – SpeI – GFP antibody – HindIII)

LO- Tm=62.4 PGR - 12

LO F NdeI – atgtcaTATGTCTAGCAATGCCAAAATCG

PB+ R – GGGTCCTGTAATGCTAGTCTGC

Camelid- Tm=64.6 PGR - 13 BB F – GAATTCGCGGCCGCTTCTAG

Camelid R – HindIII – atacaagcttaGGAGGAGACGGTCACTTGG

8. To fuse LO with Mstx (N terminus) and GFP antibody (C terminus) (NdeI – Mstx – SpeI – LO – NheI – GFP Antibody – HindIII)

Mstx- Tm=55.3 PGR - 14

Mstx F NdeI – tcaccaTATGTATTGCACCTTTTTTG

PB R – TCCTGTAATGCTAGTCTGC

LO-Tm=58 PGR - 15

PB F – GCAATGTAGCGAGTACG

PB R – TCCTGTAATGCTAGTCTGC

Camelid- Tm=64.6 PGR - 16

BB F – GAATTCGCGGCCGCTTCTAG

Camelid R – HindIII – atacaagcttaGGAGGAGACGGTCACTTGG

EXPERIMENT DESCRIPTION:

All construction elements were amplified via PCR by using ordered primers. PCR products were cleaned with PCR clean-up kit. PCR products and pRSET F were cut with appropriate restriction endonucleases. Cut PCR products and pRSET F were excised from agarose gel and purified with gel extraction kit. 8 different ligation mixes were prepared and transformed into DH5α and TG1 competent cells. Successful clones were selected by performing colony PCR. Potential plasmids were sent for sequencing.

EXPERIMENT PROTOCOL:

1. LB medium with ampicillin containing pRSET F/DH5α cells was grown for 16 hours at 37°C temperature in a shaking water bath.
2. Cells were centrifuged at 5000 g for 10 min. and the pRSET F plasmid was extracted by using GeneJet Miniprep plasmid extraction kit (Thermofisher, Lithuania) according to the instructions.
3. 16 different inserts (underlined number in **Supp.** Nr.1 represents one insert with their amplification primers and their Tm's) were amplified via PCR.

PCR mix (V=100 µl):

Reagent	Amount	Final concentration
2x Phusion master mix	50 µl	1x
Template (~ 200 ng/µl)	1 µl	~ 2 ng/µl
Forward primer	2 µl	0.5 µM
Reverse primer	2 µl	0.5 µM
H_2O (nuclease-free)	45 µl	

PCR program:

1 cycle	98°C	30 seconds
35 cycles	98°C	10 seconds
	X°C (according to the primer Tm)	30 seconds
	72°C	60 seconds
1 cycle	72°C	5 minutes
1 cycle	4°C	indefinite period

4. Obtained amplified inserts were then purified by using PCR purification kit (Thermofisher, Lithuania) according to the instructions.

5. 16 different inserts were cut with appropriate FD restriction endonucleases to obtain sticky DNA ends for correct ligation. 16 nearly similar restriction reaction mixes were prepared that differed only in the used amplified inserts and FD restriction endonucleases.

Basic restriction mix (V=20 µl):

Reagent	Amount	Final concentration
PCR product Nr. X (purified)	Y µl (1 µg DNA)	50 ng/µl
10X FD Green buffer	2 µl	1X
FD restriction endonucleases	each 1 µl	1 reaction
H_2O (nuclease-free)	up to 20 µl	

Different inserts were cut with appropriate restriction endonucleases.

PCR product Number	First FD restriction endonuclease	Second FD restriction endonuclease
PCR product Nr. 1 (purified)	EcoRI	HindIII
PCR product Nr. 2 (purified)	EcoRI	NheI
PCR product Nr. 3 (purified)	SpeI	HindIII
PCR product Nr. 4 (purified)	NdeI	SpeI
PCR product Nr. 5 (purified)	SpeI	PstI
PCR product Nr. 6 (purified)	NdeI	SpeI
PCR product Nr. 7 (purified)	SpeI	NheI
PCR product Nr. 8 (purified)	SpeI	HindIII
PCR product Nr. 9 (purified)	NdeI	HindIII
PCR product Nr. 10 (purified)	NdeI	NheI
PCR product Nr. 11 (purified)	SpeI	HindIII
PCR product Nr. 12 (purified)	NdeI	NheI
PCR product Nr. 13 (purified)	XbaI	HindIII
PCR product Nr. 14 (purified)	NdeI	NheI
PCR product Nr. 15 (purified)	SpeI	NheI

PCR product Nr. 16 (purified)	XbaI	HindIII

pRSET F was also cut with different FD restriction endonucleases to obtain correct sticky ends that will be used for ligation. Altogether 3 cut pRSET F vectors that differed in sticky ends were produced. They have been marked with letters (A, B and C).

pRSET F vector	**First FD restriction endonuclease**	**Second FD restriction endonuclease**
A	EcoRI	HindIII
B	NdeI	PstI
C	NdeI	HindIII

Reaction mixes were incubated for 1 hour at 37°C temperature.

1. Cut inserts and pRSET F vectors were excised from 1% agarose gel and purified by using GeneJet Gel extraction kit (Thermofisher, Lithuania) according to the instructions.
2. 8 ligation mixes were prepared. All PCR products that were used as inserts in one ligation reaction are displayed in Supp. 1 (Red number stands for one ligation mix).

Basic ligation mix (V=20 µl):

Reagent	**Amount**	**Final concentration**
Cut pRSET F	X µl (10 - 100 ng DNA)	0.5 - 5 ng/µl
Cut insert(s)	X µl	3:1 (insert:vector) molar ratio
10x T4 DNA Ligase buffer	2 µl	1X
T4 DNA Ligase	0.2 µl for 1 insert 0.4 µl for 2 inserts 0.6 µl for 3 inserts	0.1 U/µl (for one insert) 0.2 U/µl (for two inserts) 0.3 U/µl (for three inserts)
H_2O (nuclease-free)	Up to 20 µl	

Cut pRSET F vector and inserts that were mixed in one reaction are listed below:

Ligation mix number:	pRSET F vector:	PCR product number(s):
1	A	1
2	A	2, 3
3	B	4, 5
4	C	6, 7, 8
5	C	9
6	C	10, 11
7	C	12, 13
8	C	14, 15, 16

Ligation mixes were incubated at 22°C temperature for 1 hour.

5. Each 10 µl of ligation mix was used for chemical transformation to DH5α and TG1 competent cells.

5.1. 0.1 mL of pre-warmed (on ice) competent cells were gently mixed with 10 µl of ligation mix.

5.2. Cells were 15 minutes incubated on ice.

5.3. 1 minute heat-shock at 42°C temperature.

5.4. 2 minute heat-shock on ice.

5.5. 1 mL of LB medium were added to transformed cells. Cells were further incubated for 1.5 hour in a shaking water bath at 37°C temperature.

5.6. Transformed cells were streaked on LB plate with ampicillin (100 mg/L) and incubated for 16 hours at 37°C temperature.

6. Successful clones were checked via colony PCR by using IDT Readymade T7 promoter and terminator primers and DreamTaq polymerase.

7. Selected successful clones were grown in 5 ml of LB medium with ampicillin (100 mg/L) for 16 hours in a shaking water bath at 37°C temperature. 500 µl of grown cell culture was then mixed with

glycerol (1:1) and put in a -70°C for stocking. Potential constructs were then extracted from the remaining 4.5 ml of grown cell culture by using Gene Jet Plasmid Miniprep kit and were sent for sequencing.

RESULTS:

5 µl of PCR product was analysed on 1% agarose gel after the insert amplification using the ordered primers to ensure that the amplification is correct. All PCR products were correct size as anticipated (Fig. 1).

Fig. 1 All insert amplicons were correct length. Each lane number matches to the underlined PCR product numbers in Supp. 1.

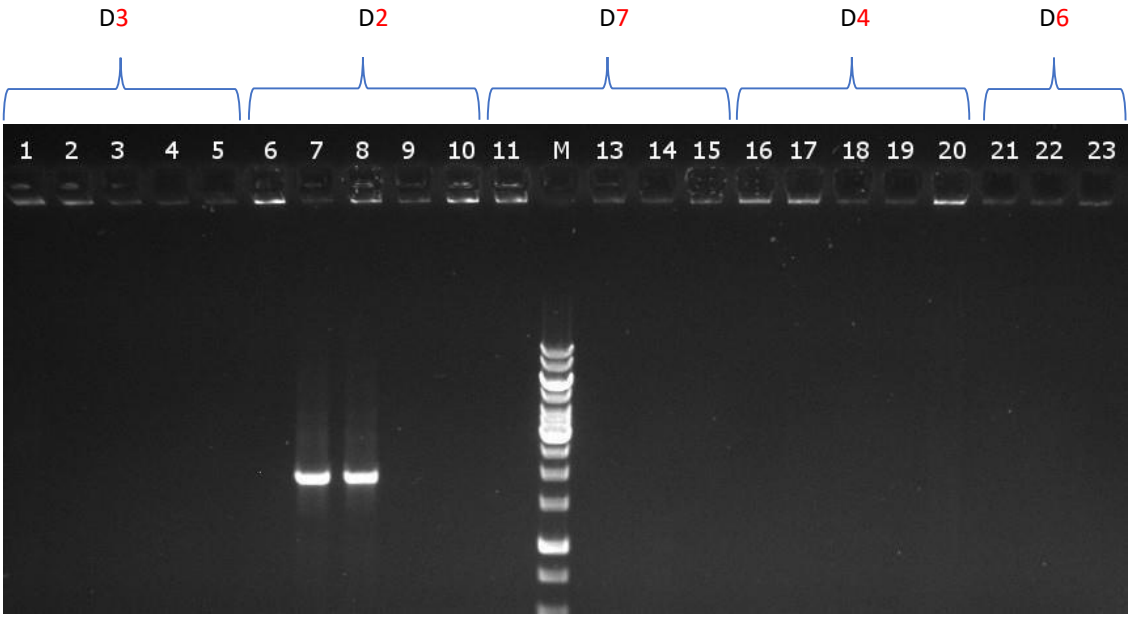
Colony PCR

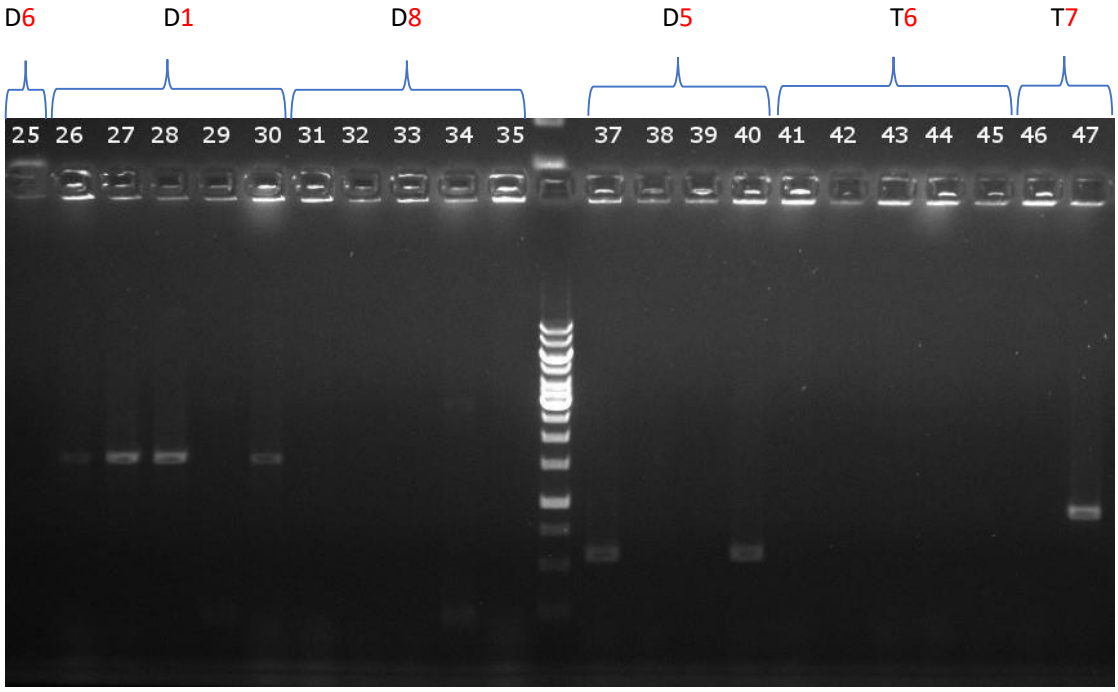
After the transformation successful clones were recognized by the length of PCR product amplified with T7 promoter and terminator primers. 8 constructs amplicons lengths are listed below:

Construct number (Ligation mix number):	**Amplicon length (bp):**

1	1556
2	1892
3	1946
4	2243
5	560
6	896
7	893
8	1229

Only 4 constructs were found during the first colony PCR that had the correct amplicon length – constructs' number 1 (colonies nr. 26, 27, 28, 30, 76, 79, 80), 2 (colonies nr. 7,8, 73, 75), 5 (colonies nr. 37, 40, 56, 59, 60), 7 (colony nr. 47) (Pic. 2).





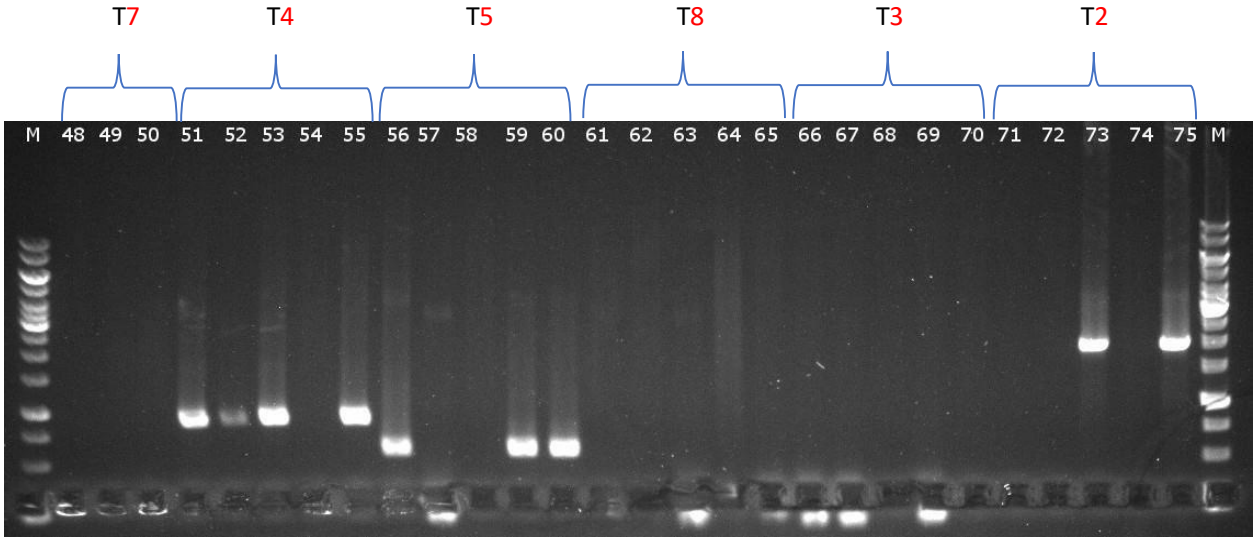


Fig. 2 Only 4 constructs out of 8 were found during the first colony PCR screening. T-TG1 strain, D-Dh5α strain colonies. Red number represents the number of the construct. White numbers represent the number of the colony. M-marker.

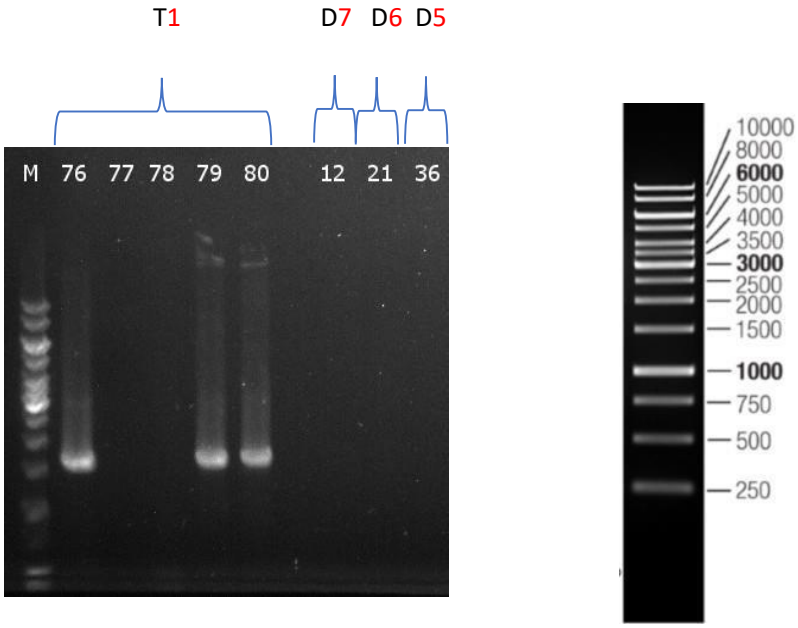
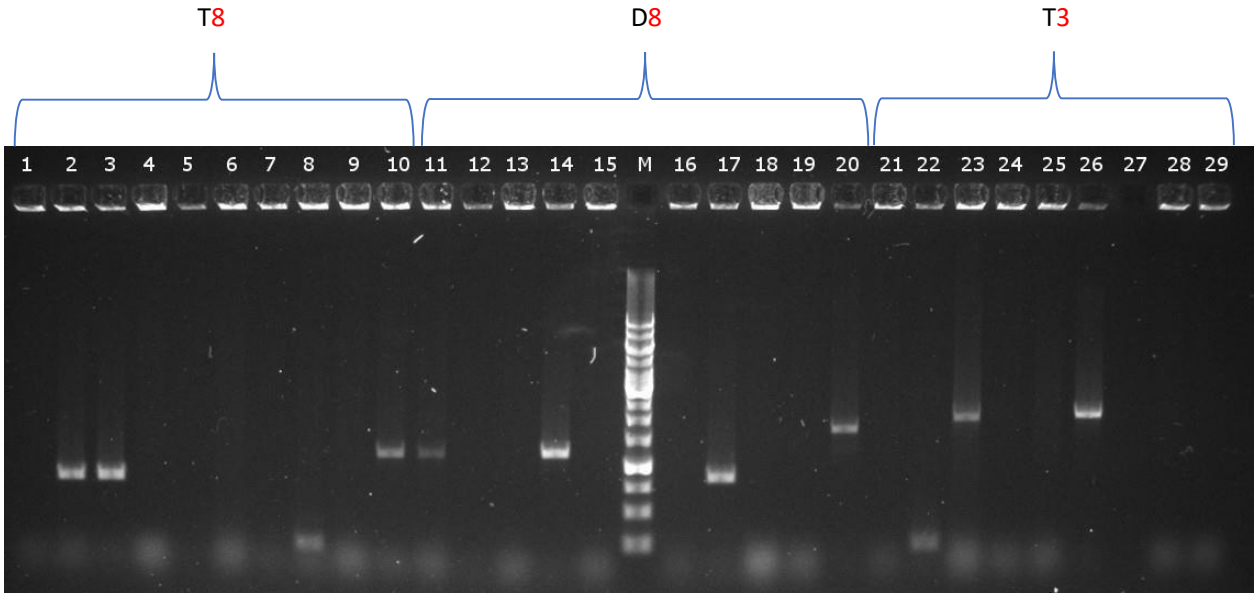
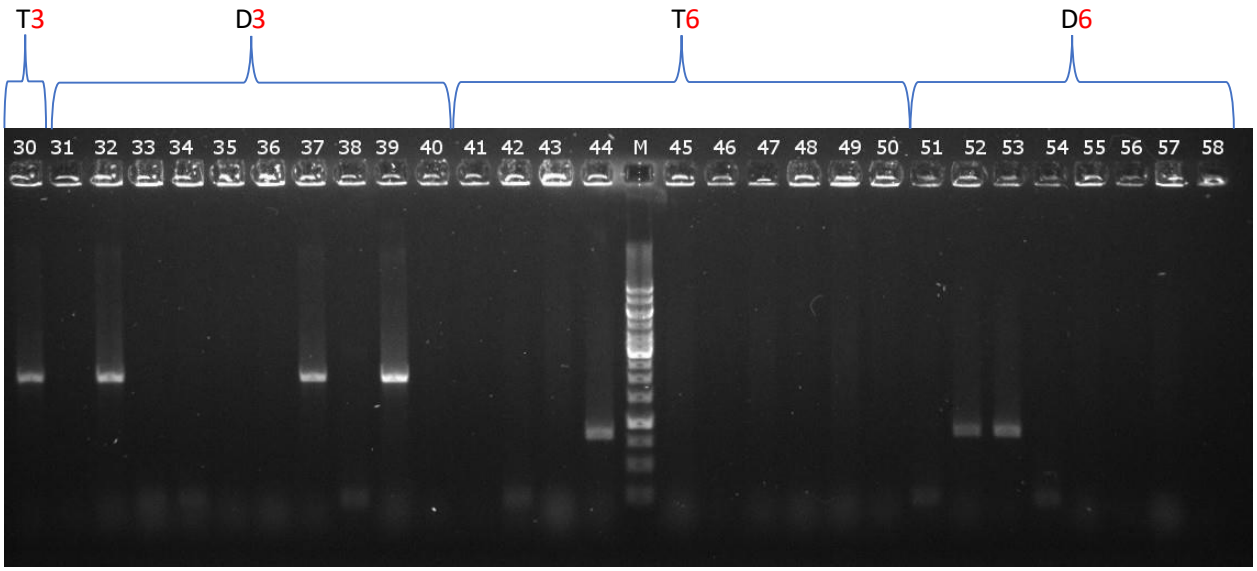
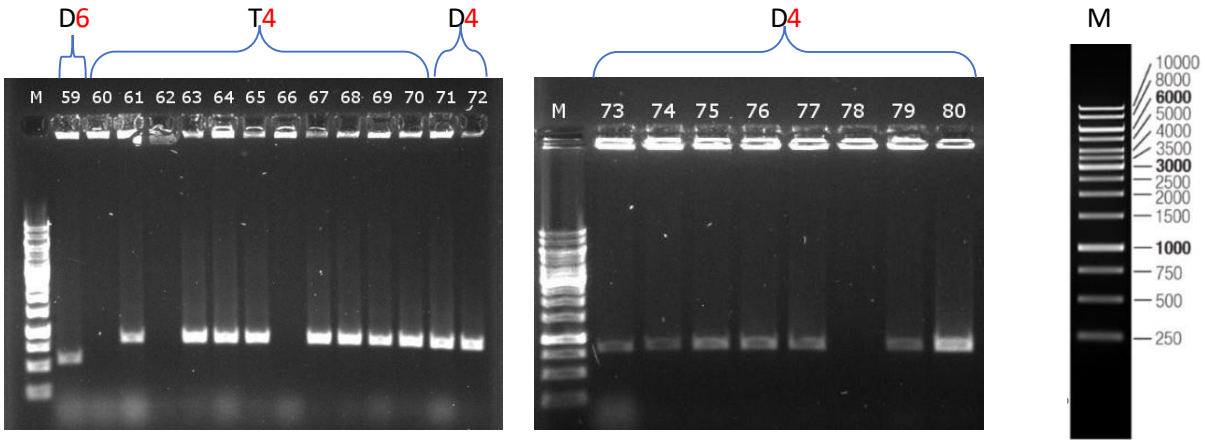


Fig. 3 All constructs except for 4 were found during the second colony PCR screening. T – TG1 strain colony, D – DH5α strain colony. Red number – construct number. White number – colony number. M – marker.

During the second colony PCR we managed to find colonies that had all of the remaining constructs except for number 4, which clones had the incorrect amplicon length (Pic. 3). 3 (colonies nr. 23, 26, 32, 37, 39), 6 (colonies nr. 44, 52, 53), 8 (colonies nr. 10, 11, 14) – all had the correct amplicon length, which was the indication of the correctly assembled constructs.







The third colony PCR was mainly focused on finding colony that had the correctly assembled construct number 4. We finally managed to find the last construct by screening 80 colonies out of the

construct nr. 4 transformation agar plate. 7 colonies had the correct amplicon length – colonies nr. 3, 22, 34, 50, 53, 54, 59 (Fig. 4).

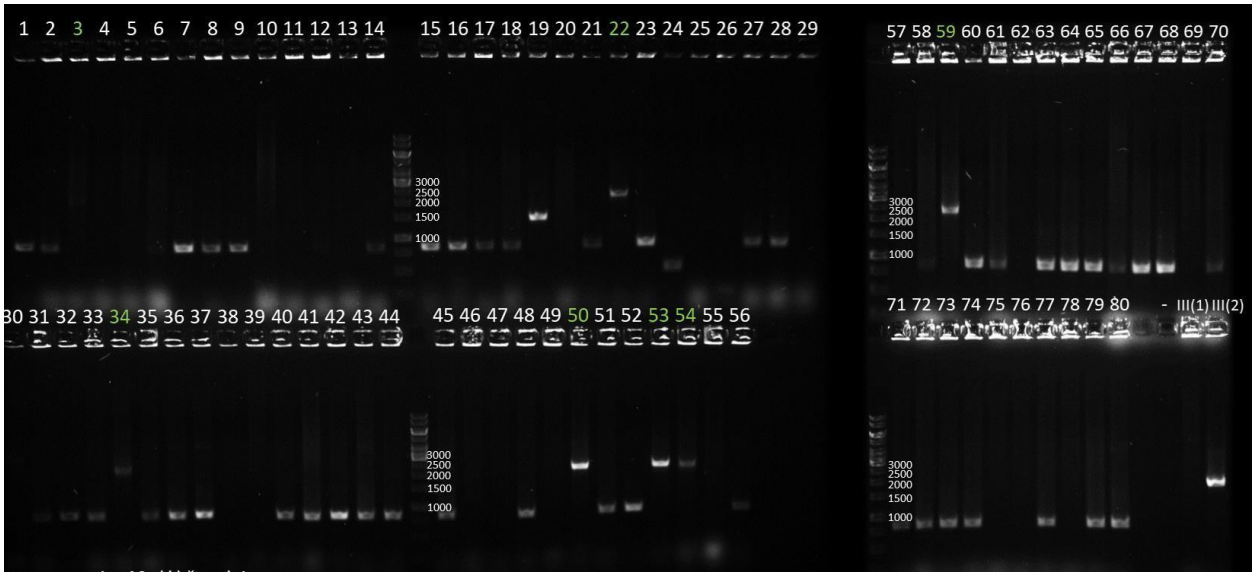


Fig. 4 Screening of 80 colonies in search of the 4 construct. White number – colony number, green number – colonies that had the correct amplicon length.

RESTRICTION ANALYSIS

2 constructs out of 8 could had the highest chance of being assembled incorrectly due to the homologous sticky ends in one of the inserts. They were constructs number 4 and 8. Colonies that had those constructs were analysed by cutting the extracted construct with appropriate restriction endonucleases. Correctly assembled constructed were differentiated from the rest by the length of restriction products on agarose gel (Fig. 5).4 (SpeI and HindIII) 8 (NheI and HindIII)

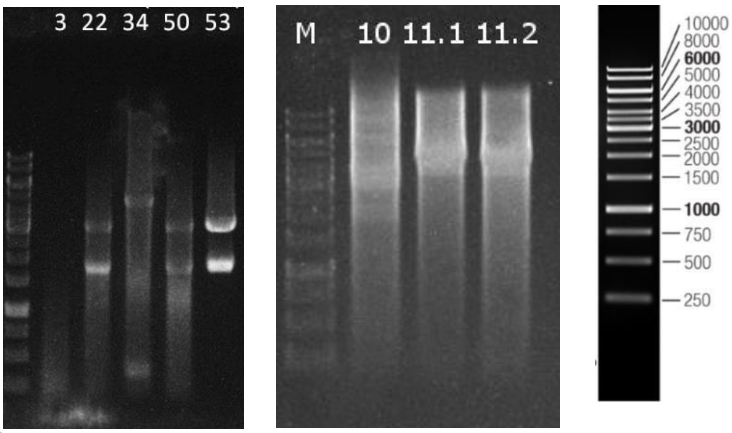


Fig. 5 Restriction analysis of constructs number 4 and 8. Used restriction endonucleases are stated in brackets. White number – colony number. M – marker.

Correctly assembled construct number 4 should have gave 2 bands (1738 and 3105 bp). That is visible in constructs from colonies nr. 22, 50 and 53.

Correctly assembled construct number 8 should have gave 1 band (3829 bp). That is visible in constructs from colonies nr. 11.1, 11.2.

SEQUENCING

Based on the results from colony PCR and restriction analysis, we have extracted 8 constructs from 22 different colonies for sequencing (Tab. below). The inserts will be sequenced from both sides by using IDT Premade T7 promoter and terminator primers.

Name of the construct	Construct size (kb)	DNA concentration (µg/ml)	Primer name	T_{melt}, °C	Primer sequence
TG1. 79.	4.2	0.455	T7 fw	51.8	TAA TAC GAC TCA CTA TAG GG
D1. 28.	4.2	0.183	T7 fw	51.8	TAA TAC GAC TCA CTA TAG GG
D1. 27.	4.2	0.270	T7 fw	51.8	TAA TAC GAC TCA CTA TAG GG
TG2. 73.	4.5	0.369	T7 fw	51.8	TAA TAC GAC TCA CTA TAG GG
D2. 7.	4.5	0.258	T7 fw	51.8	TAA TAC GAC TCA CTA TAG GG
D2. 8.	4.5	0.399	T7 fw	51.8	TAA TAC GAC TCA CTA TAG GG
TG3. 26.	4.5	0.418	T7 fw	51.8	TAA TAC GAC TCA CTA TAG GG
D3. 39	4.5	0.435	T7 fw	51.8	TAA TAC GAC TCA CTA TAG GG
TG4. 53.	4.8	0.397	T7 fw	51.8	TAA TAC GAC TCA CTA TAG GG
TG4. 50	4.8	0.407	T7 fw	51.8	TAA TAC GAC TCA CTA TAG GG
D4. 22.	4.8	0.353	T7 fw	51.8	TAA TAC GAC TCA CTA TAG GG
D5. 37.	3.2	0.281	T7 fw	51.8	TAA TAC GAC TCA CTA TAG GG
D5. 40.	3.2	0.209	T7 fw	51.8	TAA TAC GAC TCA CTA TAG GG

TG5. 56.	3.2	0.355	T7 fw	51.8	TAA TAC GAC TCA CTA TAG GG
TG6. 52.	3.5	0.202	T7 fw	51.8	TAA TAC GAC TCA CTA TAG GG

TG6. 53.	3.5	0.230	T7 fw	51.8	TAA TAC GAC TCA CTA TAG GG
TG6. 44.	3.5	0.270	T7 fw	51.8	TAA TAC GAC TCA CTA TAG GG
TG7. II. 1.	3.5	0.337	T7 fw	51.8	TAA TAC GAC TCA CTA TAG GG
TG7. II. 2.	3.5	0.363	T7 fw	51.8	TAA TAC GAC TCA CTA TAG GG
TG7. 47.	3.5	0.309	T7 fw	51.8	TAA TAC GAC TCA CTA TAG GG
D8. 11. 1	3.8	0.273	T7 fw	51.8	TAA TAC GAC TCA CTA TAG GG
D8. 11. 2	3.8	0.238	T7 fw	51.8	TAA TAC GAC TCA CTA TAG GG
TG1. 79.	4.2	0.455	T7 rev	57.6	GCTAGTTATTGCTCAGCG G
D1. 28.	4.2	0.183	T7 rev	57.6	GCTAGTTATTGCTCAGCG G
D1. 27.	4.2	0.270	T7 rev	57.6	GCTAGTTATTGCTCAGCG G
TG2. 73.	4.5	0.369	T7 rev	57.6	GCTAGTTATTGCTCAGCG G
D2. 7.	4.5	0.258	T7 rev	57.6	GCTAGTTATTGCTCAGCG G
D2. 8.	4.5	0.399	T7 rev	57.6	GCTAGTTATTGCTCAGCG G
TG3. 26.	4.5	0.418	T7 rev	57.6	GCTAGTTATTGCTCAGCG G
D3. 39	4.5	0.435	T7 rev	57.6	GCTAGTTATTGCTCAGCG G
TG4. 53.	4.8	0.397	T7 rev	57.6	GCTAGTTATTGCTCAGCG G
TG4. 50	4.8	0.407	T7 rev	57.6	GCTAGTTATTGCTCAGCG G
D4. 22.	4.8	0.353	T7 rev	57.6	GCTAGTTATTGCTCAGCG G

D5. 37.	3.2	0.281	T7 rev	57.6	GCTAGTTATTGCTCAGCG G
D5. 40.	3.2	0.209	T7 rev	57.6	GCTAGTTATTGCTCAGCG G
TG5. 56.	3.2	0.355	T7 rev	57.6	GCTAGTTATTGCTCAGCG G
TG6. 52.	3.5	0.202	T7 rev	57.6	GCTAGTTATTGCTCAGCG G
TG6. 53.	3.5	0.230	T7 rev	57.6	GCTAGTTATTGCTCAGCG G
TG6. 44.	3.5	0.270	T7 rev	57.6	GCTAGTTATTGCTCAGCG G
TG7. II. 1.	3.5	0.337	T7 rev	57.6	GCTAGTTATTGCTCAGCG G
TG7. II. 2.	3.5	0.363	T7 rev	57.6	GCTAGTTATTGCTCAGCG G
TG7. 47.	3.5	0.309	T7 rev	57.6	GCTAGTTATTGCTCAGCG G
D8. 11. 1	3.8	0.273	T7 rev	57.6	GCTAGTTATTGCTCAGCG G
D8. 11. 2	3.8	0.238	T7 rev	57.6	GCTAGTTATTGCTCAGCG G

CONCLUSIONS:

Based on the results from colony PCR and restriction analysis, we have successfully constructed 8 different constructs that will be further used in the project for liposomal encapsulation.