Cloning protocol

1. LB medium (with certain antibiotic) with cells containing chosen cloning vector plasmid was grown for 16 hours at 37°C temperature in a shaking water bath.
2. Cells were centrifugated at 5000g for 10min. and the cloning plasmid was extracted by using GeneJet Miniprep plasmid extraction kit (Thermofisher, Lithuania) according to the instructions.
3. Cloning inserts were amplified via PCR with Phusion of SuperFi polymerase.

PCR mix (V=100µl):

Reagent	**Amount**	**Final concentration**
2x Phusion/SuperFi master mix	50 µl	1x
Template (~ 200ng/µl)	1 µl	~ 2ng/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	15-30 seconds/1kb
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. Amplified inserts were cut with appropriate FD restriction endonucleases to obtain sticky DNA ends for correct ligation. Cloning vector was also cut with appropriate FD restriction endonucleases to obtain correct sticky ends that will be needed for ligation.
   Reaction mixes were incubated for 1 hour at 37°C temperature.

   Basic restriction mix (V=20µl):

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

6. Cut insert and cloning vector were excised from 1% agarose gel and purified by using GeneJet Gel extraction kit (Thermofisher, Lithuania) according to the instructions.

7. Extracted insert and cloning vector were put into a ligation mix and incubated for 1h at 22°C.
   Basic ligation mix (V=20 µl):

Reagent	**Amount**	**Final concentration**
Cut vector	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	

8. Each 10 µl of ligation mix was used for chemotransformation to DH5α or TG1 competent cells.

1) 0.1 mL of pre-warmed (on ice) competent cells were gently mixed with 10 µl of ligation mix.
2) Cells were 15 minutes incubated on ice.
3) 1 minute heat-shock at 42°C temperature.
4) 2 minute heat-shock on ice.
5) 1mL of LB medium were added to transformed cells. Cells were furtherly incubated for 1,5 hour in a shaking water bath at 37°C temperature.
6) Transformants were streaked on LB plate with appropriate antibiotic and incubated for 16 hours at 37°C temperature.

9. Successful clones were checked via colony PCR by using primers covering the MCS and DreamTaq polymerase.