Week 11

Colony PCR of pCDFDuet-BamC, pET28b-SurA and pRSET-MstX-igA-GFP nano

AIM:

To conduct a colony PCR analysis of pCDFDuet-BamC, pET28b-SurA and pRSET-MstX-igA-GFP nano to ascertain whether the plasmids were constructed properly.

REAGENTS USED:

Tab. 1 List of reagents used in the experiment

Name of reagent	**Lot number**
DreamTaq Green PCR MasterMix (2x)	00563791
ddH2O	-
T7 Promoter primer	356109
T9 Terminator primer	363827

Tab. 2 List of primers used in the experiment

Name of primer pair	**Forward sequence**	**Reverse sequence**

T7 Promoter,forward primer	TAATACGACTCACTATAGGG	
T7 Terminator, reverse primer		GCTAGTTATTGCTCAGCGG

EXPERIMENT DESCRIPTION:

Colony PCR is a high-throughput method for determining the presence or absence of insert DNA in desired plasmid constructs.

EXPERIMENT PROTOCOL:

The following ThermoFisher protocol was used:

1. Gently vortex and briefly centrifuge DreamTaq Green PCR Master Mix (2X) after thawing.
   Place a thin-walled PCR tube on ice and add the following components for each 50 µL reaction:
   DreamTaq Green PCR Master Mix (2X) 25 µL
   Forward primer 0.1-1.0 µM
   Reverse primer 0.1-1.0 µM
   Water, nuclease-free to 50 µL
   Template DNA (take some cells from a desired colony using a tip of a pipette and put the tip into the PCR tube containing Master Mix + Primers+Water)
   Total volume can be up to 50 µL
2. Gently vortex the samples and spin down.
3. When using a thermal cycler that does not contain a heated lid, overlay the reaction mixture with 25 µL of mineral oil.

PCR was performed (the exact programme is described below)

Agarose gel analysis was performed

REACTION SET:

Reagent	Amount
DreamTaq Green PCR MasterMix (2x)	5 µl
T7 forward primer	0.5 µl
T7 reverse primer	0.5 µl
ddH2O	4 µl

PCR program:

1 cycle	95°C	3 minutes
30 cycles	95°C	30 seconds
	56°C	30 seconds
	72°C	2 minutes
1 cycle	72°C	5 minutes
1 cycle	4°C	indefinite period

RESULTS:

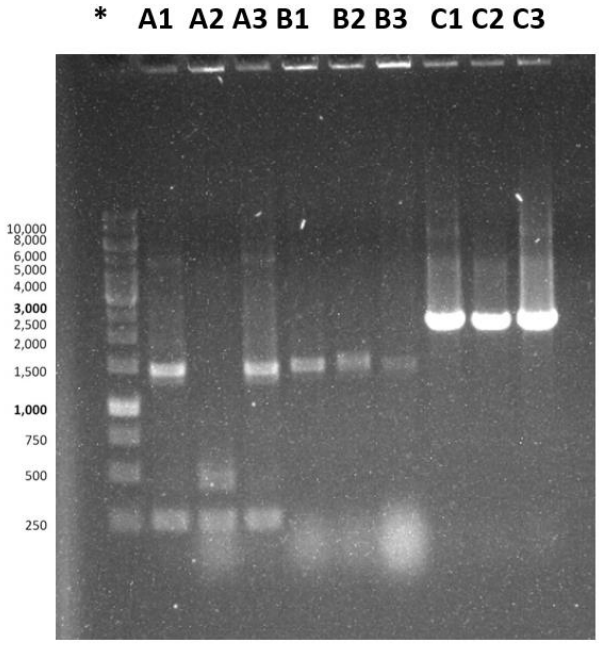


Fig. 1 Electrophoresis 1% agarose gel of colony PCR results. *-Generuler 1 kb; A1-pCDFDuet-BamC 1;A2-pCDFDuet-BamC 9; A3-pCDFDuet-BamC 10; B1-pET28b-SurA 60; B2-pET28b-SurA 65; B3 -pET28b-SurA 71;C1-pRSET-MstX-igA-GFP nano 22; C2-pRSET-MstX-igA-GFP nano 34;C3-pRSET-MstX-igA-GFP nano 59.

1. pCDFDuet-BamC 1: gel results show that the plasmid was constructed properly (simulation's length 1437bp – the band of approximate 1400 bp is visible)
2. pCDFDuet-BamC 9: gel results show that the plasmid was constructed improperly (simulation's length 1400 bp – the band of approximate 400 bp is visible)
3. A3 pCDFDuet-BamC 10: gel results show that the plasmid was constructed properly (simulation's length 1437bp – the band of approximate 1400 bp is visible
4. B1 pET28b-SurA 60 : gel results show that the plasmid was constructed properly (simulation's length 1500 bp – the band of approximate 1500 bp is visible)
5. B2 pET28b-SurA 65 : gel results show that the plasmid was constructed properly (simulation's length 1500 bp – the band of approximate 1500 bp is visible)
6. B3 pET28b-SurA 71 : gel results show that the plasmid was constructed properly (simulation's length 1500 bp – the band of approximate 1500 bp is visible)
7. C1 pRSET-MstX-igA-GFP nano 22: gel results show that the plasmid was constructed properly (simulation's length 2300 bp – the band of approximate 2300 bp is visible)

8. C2 pRSET-MstX-igA-GFP nano 34: gel results show that the plasmid was constructed properly (simulation's length 2300 bp – the band of approximate 2300 bp is visible)

C3 pRSET-MstX-igA-GFP nano 59: gel results show that the plasmid was constructed properly (simulation's length 2300 bp – the band of approximate 2300 bp is visible)

CONCLUSIONS:

Most of the constructs were constructed successfully. Bands of the needed lengths were received. However, pCDFDuet-BamC 9 was constructed improperly and the experimental band size differed from the theoretical band size. Thus, pCDFDuet-BamC 9 cannot be used for the further work.

Restriction analysis of pCDFDuet-BamC, pET28b-SurA, pRSET-MstX-igA-GFP nano, pCDFDuet

AIM:

To conduct a restriction analysis pCDFDuet-BamC, pET28b-SurA, pRSET-MstX-igA-GFP nano, pCDFDuet to ascertain whether plasmids were constructed properly.

REAGENTS USED:

Tab. 1 List of reagents used in the experiment

Name of reagent	Lot number
FastDigest PstI	00627842
FastDigest NotI	00616216
FastDigest HindIII	00620168
FastDigest BcuI	00636621
FastDigest NheI	00634416
FastDigest XbaI	00631594
10x FastDigest Green Buffer	00638694
GeneRuler 1 kb DNA Ladder	00643884

EXPERIMENT DESCRIPTION:

Restriction analysis is performed in order to ascertain whether the desired plasmids were constructed properly. The restriction mixture is prepared individually for each plasmid considering their sequences and concentrations. Then, after an incubation for a needed for restriction to take place time, an electrophoresis is performed to visualize the results and later analyse them using various techniques. After the whole set of procedures is made it is possible to conclude how well-prepared desired plasmids are.

EXPERIMENT PROTOCOL:

1. Select restriction enzymes and the reaction buffer to digest the plasmid.
2. In a 1.5 mL tube combine the following:

   DNA

   Restriction Enzyme(-s)

   Buffer

ddH_2O up to 20 µl

3. Mix by pipetting.
4. Incubate tube(-s) at a temperature of 37 °C for 15 minutes.
5. To visualize the results of the digest, conduct gel electrophoresis.

1. pCDFDuet-BamC digest (two colonies)

Name of the reagent	**Amount**
0.5 µg DNR pCDFDuet-BamC	3 µl and 2.5 µl
FD PstI	0.2 µl
FD NotI	0.2 µl
FD Green Buffer	2 µl
ddH2O	14.6 µl and 15.1 µl

2. pET28b-SurA digest (two colonies)

Reagent	**Amount**
0.25 µg DNR pET28b-SurA	4.5 µl and 5.5 µl
FD PstI	0.2 µl
FD NotI	0.2 µl
FD Green Buffer	2 µl
ddH2O	13.1 µl and 12.1 µl

3. pRSET-MstX-igA-GFP nano digest (two colonies)

Reagent	**Amount**
0.5 µg pRSET-MstX-igA-GFP nano	1.5 µl and 1.5 µl
FD PstI	0.2 µl

FD NotI	0.2 µl
FD Green Buffer	2 µl
ddH2O	16.4 µl and 16.4 µl

4. pCDFDuet digest (two colonies)

Reagent	Amount
0.25 µg DNR pCDFDuet	7 µl and 5.5 µl
FD PstI	0.2 µl
FD NotI	0.2 µl
FD Green Buffer	2 µl
ddH2O	10.6 µl and 12.1 µl

5. Using Benchling the digest of each desired plasmid with appropriate enzymes was simulated. After the real digestion electrophoresis was conducted and the results were compared.

Simulations:

pCDFDuet-BamC digest

Start	End	Length	Left Cutter	Left Overhang	Right Cutter	Right Overhang
448	990	543	PstI	3'	PstI	3'
991	1110	120	PstI	3'	NotI	5'
1111	447	4078	NotI	5'	PstI	3'

pET28b-SurA digest

Start	End	Length	Left Cutter	Left Overhang	Right Cutter	Right Overhang

167	1218	1052	NotI	5'	PstI	3'
1219	166	5480	PstI	3'	NotI	5'

pRSET-MstX-igA-GFP nano digest

Start	End	Length	Left Cutter	Left Overhang	Right Cutter	Right Overhang
452	2189	1738	SpeI	5'	HindIII	5'
2190	451	3105	HindIII	5'	SpeI	5'

pCDFDuet digest

Start	End	Length	Left Cutter	Left Overhang	Right Cutter	Right Overhang
1567	2366	800	NheI	5'	XbaI	5'
2367	1566	2981	XbaI	5'	NheI	5'

RESULTS:

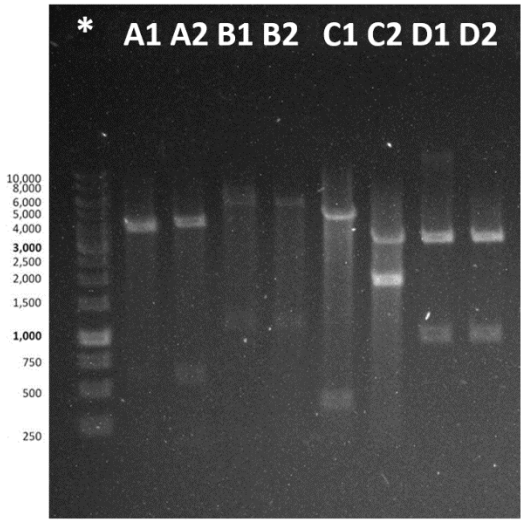


Fig. 1 Electrophoresis of the restriction analysis in 1% agarose gel. *-Generuler 1 kb prestained; A1-pCDFDuet-BamC 12; A2- pCDFDuet-BamC 31; B1-pET28b-SurA 71; B2-pET28b-SurA 72; C1-pRSET-MstX-igA-GFP nano 34; C2-pRSET-MstX-igA-GFP nano 50; D1-pCDFDuet 1; D2-pCDFDuet 2.

CONCLUSIONS:

1. pCDFDuet-BamC plasmids from two separate colonies are both constructed improperly because three bands of different lengths must be visible in a gel, but only two of them are present. However, the analysis should be conducted one more time considering the fact that the bottom part of the gel is slightly less demonstrative. Moreover, the problem might be with the map/sequence of this construct in benchlings therefore the analysis with different 'single-cutters' restriction enzymes may be conducted later if needed.
2. Both of the B1-pET28b-SurA are constructed properly. Two bands of an approximately 5000 bp and 1000 bp are visible that meets the simulation's patterns/results.
3. pRSET-MstX-igA-GFP nano. Considering the colony 34, the bands are quite different from the needed ones,5000 bp and 400 bp differs from 3100 bp and 1740 bp from stimulation. Therefore the plasmid from the colony 34 cannot be used in following experiments. However, the bands of proper lengths are visible in the gel pattern of the plasmid from the colony 50.

4. Purified pCDFDuet vectors both show the needed pattern, therefore they can be used later to conduct desired experiments.

BamA mRNA purification

AIM:

Outer membrane protein assembly complex BAM is involved in the assembly and insertion of beta-barrel proteins into the outer membrane. BamA is the only protein of the BAM complex that inserts directly into the membrane. We hypothesized that by adding mRNA into our system we would ensure that BamA folds and inserts into liposome membrane correctly. Thus, it was decided to purify BamA mRNA and add it to reaction mixture as a template instead of DNA.

REAGENTS USED:

Tab. 1 List of reagents used in the experiment

Name of reagent	**Cat. No.**	**Manufacturer**
AanI Restriction Enzyme	ER2061	Thermo Fisher Scientific, Lithuania
FastDigest (FD) Green Buffer	B72	Thermo Fisher Scientific, Lithuania
UltraPure DNase/RNase-Free Distilled Water	10977023	Thermo Fisher Scientific, Netherlands
Phenol/ Tris-saturated pH 7-8/ chloroform mixture	A156.2	Carl Roth GmbH + Co. KG, Germany
Trichlormethan/Chloroform	7331.1	Carl Roth GmbH + Co. KG, Germany

96 % ethanol	64-17-5	Lach-Ner, Czech Republic
10x Reaction Buffer with $MgCl_2$	B43	Thermo Fisher Scientific, Lithuania
TranscriptAid T7 High Yield Transcription Kit: TranscriptAid Enzyme Mix 5X TranscriptAid Reaction Buffer DNase I, RNase-free, 1U/µL ATP ,Tris buffered 100 mM CTP, Tris buffered 100 mM GTP, Tris buffered 100 mM UTP, Tris buffered 100 mM 3M Sodium Acetate Solution, pH 5.2 1 mL DEPC-treated Water 0.5 M EDTA, pH 8.0	K0441	Thermo Fisher Scientific
GeneJet RNA purification kit: Lysis buffer **without β-mercaptoethanol** Wash Buffer 1 Wash Buffer 2 Water, nuclease-free	K0731	Thermo Fisher Scientific, Lithuania

EXPERIMENT DESCRIPTION:

Firstly, plasmid DNA is linearized by restriction digestion (downstream of the insert to be transcribed). We need to make circular DNA linear in order to ensure efficient synthesis of BamA transcript. Therefore, restriction was done with AanI (PsiI) restriction enzyme that cuts downstream the terminator. Then *in vitro* transcription (IVT) reaction was ran to make BamA mRNA. After that, the RNA was purified using two distinct methods: standard phenol (pH 4.7): chloroform method and purification using GeneJET RNA purification columns. BamA transcript concentration and purity were examined using NanoDrop 2000 spectrophotometer (Thermo Scientific, United States) as well as Agilent Bioanalyzer 2100 (Agilent Technologies, United States).

EXPERIMENT PROTOCOL

Restriction

Mix all the components listed in the **Table 2** thoroughly, vortex and incubate for ≥30 min at 37 °C. After incubation inactivate the restriction enzyme by incubating for 5 min at 65 °C.

Tab. 2 Reagents for restriction reaction:

Reagent	**Amount (1x)**
AanI Restriction Enzyme	0.5 µL
FastDigest (FD) Green Buffer	2 µL
Ultrapure DNase/RNase-Free Distilled Water	to 20 µL
Template DNA	500 ng

DNA extraction

1. Add equal volume of phenol/ Tris-saturated pH=7-8/ chloroform mixture to DNA sample after restriction and mix for 2-5 min inverting the tube by hand.
2. Centrifuge at 14000 rpm for 10 min. Transfer the supernatant to new 1.5 ml Eppendorf tube. Repeat this procedure twice with equal amount of chloroform.
3. Precipitate the DNA by adding 1/10th volume of 3M sodium acetate pH=5.2 and two volumes of 96 % ethanol and vortex thoroughly.
4. Incubate at -20 °C for at least 30 min. Collect the pellet by centrifugation at 13500 rpm for 25 min. Remove the supernatant and rinse the pellet with 500 µL of 70 % ethanol.
5. Centrifuge at 13 500 rpm for 25 min. Remove the supernatant and dry the pellet leaving the sample at 37 °C for 10 min.
6. Resuspend the pellet in 7 µL DEPC-treated water. Store DNA at -20 °C or -70 °C until use.

High yield *in vitro* transcription (IVTT)

- Thaw all the components, mix and centrifuge briefly to collect all drops.
- Keep Transcript Aid Enzyme Mix and nucleotides on ice.
- Combine the following reaction components listed in Table 3 at room temperature.
- Incubate at 37 °C for 2 h.

Tab. 3 Reagents for High Yield *in vitro* Transcription:

Reagent	**Amount (1x)**
DEPC-treated water	to 20 µL
5x Transcript Aid Reaction Buffer	4 µL
Transcript Aid Enzyme Mix	2 µL
NTP mix	8 µL
Template DNA	1 µg

mRNA purification

a) Using purification columns

1. To remove all genomic DNA add components listed in **Table 4** to an RNAse-free tube, mix thoroughly and incubate for 30 min at 37 °C. To inactivate the DNAse I add 1 µL 50 mM EDTA and incubate at 65 °C for 10 min.

Tab. 4 Reagents for genomic DNA removal:

Reagent	Amount (1x)
DEPC-treated water	to 10 µL
10x Reaction Buffer with $MgCl_2$	1 µL
DNAse I, RNAse-free	1 µL
RNA	1 µg

Adjust the volume of the reaction mixture to 100 µL with water, nuclease free.

2. Add 300 µL of Lysis buffer without β-mercaptoethanol, mix thoroughly by vortexing or pipetting.
3. Add 180 µL of 96 % ethanol and mix by pipetting.
4. Transfer the mixture to the GeneJET RNA purification column inserted into the collection tube.
5. Centrifuge the column for 1 min at 12 000 x g.
6. Discard the collection tube containing the flow-through solution. Place the column into a new 2 mL collection tube (included).
7. Add 700 µL of Wash Buffer 1 and centrifuge for 1 min at 12 000 x g.
8. Discard the flow-through and place the purification column back into the collection tube.
9. Add 600 µL of Wash Buffer 2 and centrifuge for 1 min at 12 000 x g.
10. Discard the flow-through and place the purification column back into the collection tube.
11. Add 250 µL of Wash Buffer 2 and centrifuge for 2 min at 12 000 x g.
12. Empty the collection tube and re-spin the purification column for 1 min at max speed.
13. Transfer the column to a sterile 1.5 mL RNAse-free microcentrifuge tube.
14. Add 30 µL of Water, nuclease-free to centre of column membrane.
15. Centrifuge for 1 min at 12000 x g.
16. Discard the purification column. Store RNA at -20 °C or -70 °C until use.

b) Using standard phenol:chloroform method

1. To 8 µL RNA add 15 µL 3M sodium acetate pH=5.2 and DEPC-treated water to 150 µL.
2. Add equal volume of 1:1 phenol/ Tris-saturated pH=4.7/ chloroform mixture and vortex thoroughly.
3. Centrifuge at 4 °C 12000 x g for 15 min. Transfer the supernatant to new 1.5 ml Eppendorf tube. Repeat this procedure twice with equal amount of chloroform.
4. Precipitate the RNA by adding 300 µL of room temperature (RT) isopropanol to a new 1.5 mL tube.
5. Transfer RNA-containing upper aqueous phase (clean supernatant) into isopropanol.
6. Invert by hand 10-20 times to mix.
7. Let sit at RT for 10 min.
8. Centrifuge at 4 °C 12000 x g for 10 min to precipitate RNA.
9. Remove supernatant and discard.
10. Add 1 mL of 75 % EtOH (with nuclease-free water) to pellet.
11. Centrifuge at 4 °C 7500 x g for 5 min.
12. Remove supernatant and discard.
13. Repeat EtOH wash (steps 10-12) for 2 times.
14. Pulse spin samples at RT.
15. Carefully remove remaining supernatant with pipette without disturbing the RNA pellet.
16. Leave tubes open at RT for 3-5 min to evaporate EtOH (optional).
17. Heat tubes open at 65 °C for 2-5 min to evaporate any remaining EtOH.
18. Add 20 µL of nuclease-free water to RNA pellet.
19. Heat tubes at 65 °C for 2-5 min to solubilize RNA.
20. Vortex tubes 5-10 s, pulse spin, and place solubilized RNA on ice immediately.
21. Quantify RNA concentration and purity. Store RNA at -20 °C or -70 °C until use.

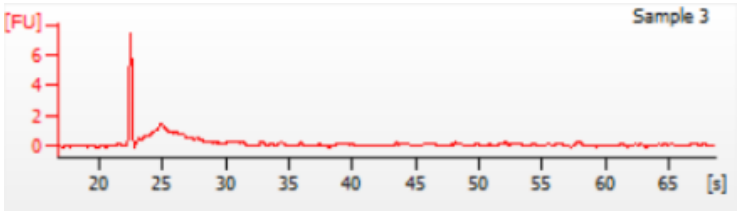
RESULTS:

The experiment was divided into 3 parts.

1st experiment consisted of: 3 – $mRNA_1$ of BamA (purified using columns), 4 – $mRNA_2$ of BamA (purified using columns), 6 – not purified RNA after *in vitro* transcription. We evaluated samples using Agilent Bioanalyzer 2100:

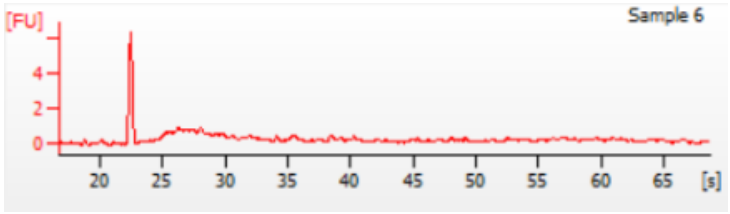
<u>1st experiment:</u>

- $mRNA_1$ (concentration 1.7 ng/µL)



- $mRNA_2$ (concentration 0.4 ng/µL)

- Not purified mRNA after IVT

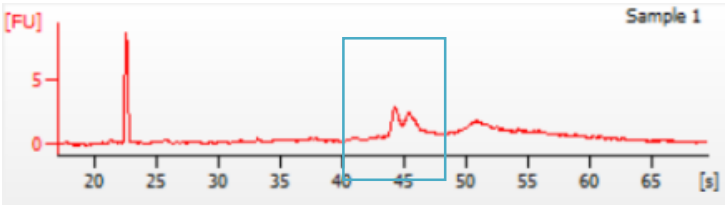


The highest peak is a ladder. According to the graphs, the purification was unsuccessful. We hypothesized that the buffer for genomic DNA removal from RNA preparations was not effective.

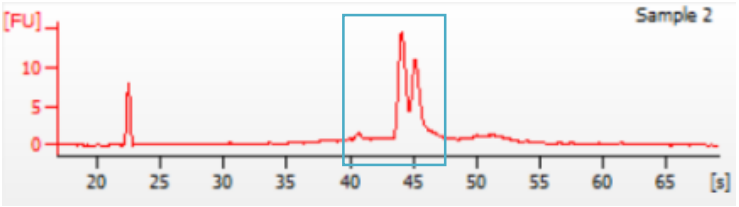
2nd experiment:

For the 2nd experiment we changed the buffer and purified mRNA after IVT by two distinct methods. Sample 1 – mRNA purified using columns, 2 – mRNA purified using standard phenol: chloroform method, 5 – not purified RNA after *in vitro* transcription.

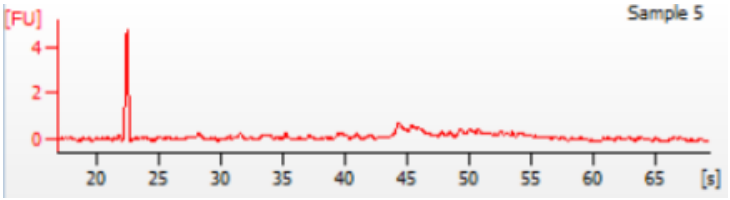
- mRNA purified using GeneJET RNA purification columns (concentration 1117 ng/µL)



- mRNA purified using standard phenol:chloroform method (concentration 3939 ng/µL)



- Not purified mRNA after *in vitro* transcription (concentration 259.8 ng/µL)

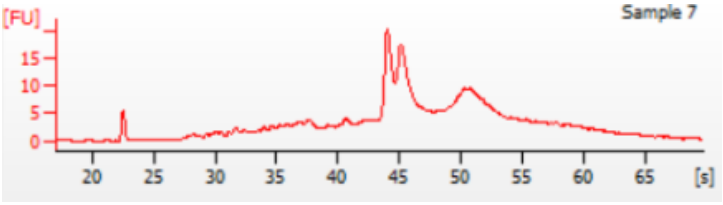


After changing the buffer BamA mRNA purification was successful. Two peaks (in the blue frames) can be seen after both purifications – using columns as well as standard method. The mRNA yield differed between both purification methods for about 3.5 times. According to this, for further experiments we used standard phenol: chloroform method instead of purification by columns.

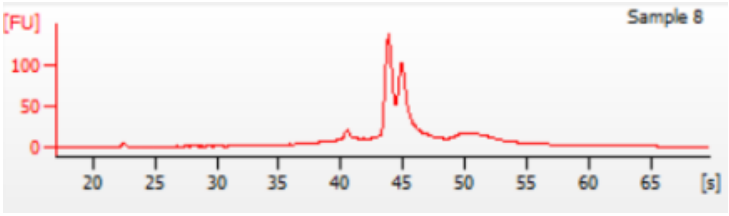
3rd experiment:

Also, we analysed mRNA repeatedly using 10x concentrated samples.

- 10x concentrated Sample 1



- 10x concentrated Sample 2



- 10x concentrated Sample 5

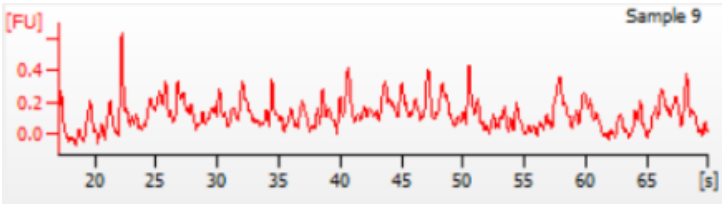
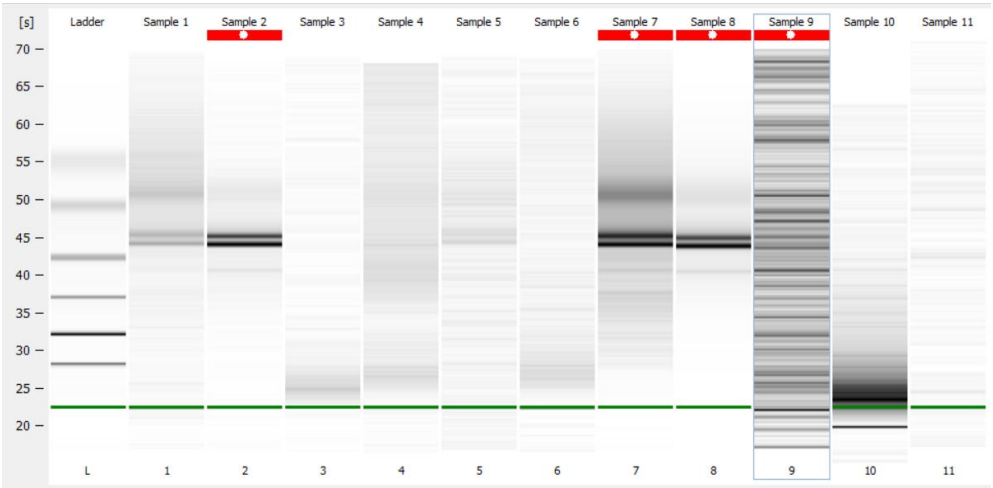


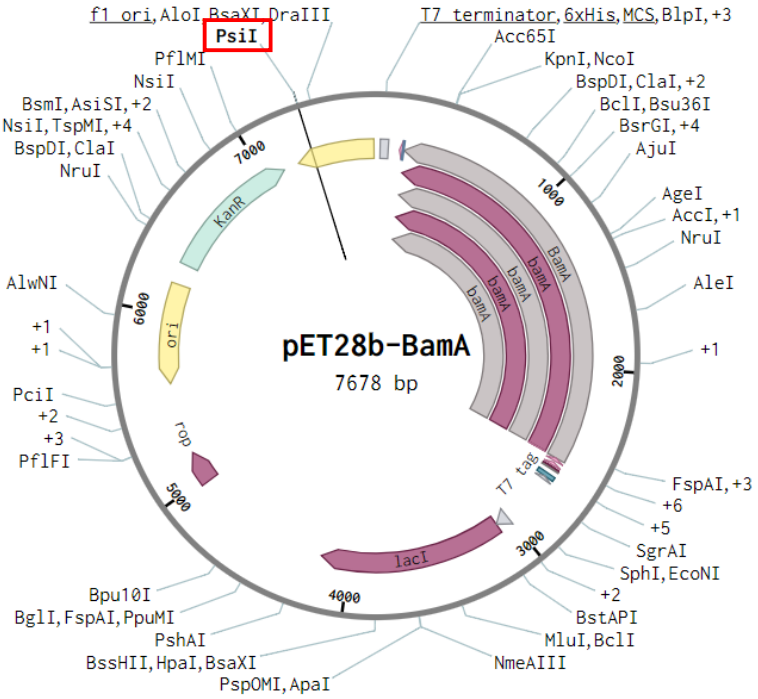
Fig. 1 Gel compiled using Bioanalyzer 2100 Expert Software



Samples 1 (same as Sample 7, but 10x diluted) and 2 (same as Sample 8, but 10x diluted) showed two separate strands of different length RNA fragments: 2385 nt and 2929 nt.

We figured out, that first fragment (2385 nt.) corresponds to correctly transcribed BamA gene. The longer fragment (2929 nt.) is transcript of plasmid starting from T7 promoter and ending at the restriction site (restriction enzyme PsiI (AanI)). Apparently, the RNA polymerase does not stop transcription at the T7 terminator and finishes transcription of the fragment all the way through to restriction site.

Fig. 2 pET28b plasmid with PsiI (AanI) restriction site (in a red frame)



CONCLUSIONS:

1. RNA purification was successful using both purification column and traditional phenol: chloroform method, however yield was 3.5 times higher using standard phenol: chloroform purification method.
2. There were two RNA transcripts because BamA transcription did not end at T7 terminator site.