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Optimalization of DNA Extraction for Low-Biomass Microbiome Studies in Tissue from Patients with Colorectal Cancer

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Abstract

Colorectal cancer is one of the most common tumors in the world, resulting in around 10% of tumor related deaths annually. Numbers of patients under 50 years old diagnosed with CRC are still rising due to unclear yet reason.

The primary objective for this study is to establish an efficient method for extracting low microbiota from tissue resected from patients with colorectal cancer, suitable for downstream application and next- generation sequencing. Initially, we aim to analyze various homogenization and bead beating conditions for the extraction of intratumoral bacterial DNA from tissue samples.
Subsequently, our focus shifts to assessing the bacterial load in the samples as the second objective. This involves establishing and validating a method for the quantification of bacterial load, with a particular emphasis on developing a standard curve for accurate quantification.

The tests performed in this thesis helped us understand the importance with establishing and optimalisation of methods used for extracting of intratumoral bacterial DNA from tissue samples from patients with colorectal cancer.

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Abbreviations

bp	Base pair
CRC	Colorectal Cancer
dsDNA	Double stranded DNA
gDNA	Genomic DNA
NTC	No template control
PCR	Polymerase chain reaction
qPCR	Quantitative polymerase chain reaction
RFU	Relative Fluorescens unit
rRNA	Ribosomal RNA
SNP	Single nucleotide polymorphisms
StdZymo	ZymoBIOMICS Microbial Community Standards
TAE- buffer	Tris, acetic acid, EDTA- buffer

1. Introduction

1.1 Colorectal cancer

Colorectal cancer is one of the most common tumors worldwide, resulting in around 10% of all tumors related death annually. Highest precent of deaths due to CRC as for 2023 occurred in Asia with 54,2% followed by Europe with 26,9%. Regardless of age and ethnicity, studies shows that men are 1,5 times more likely to develop CRC that woman. (1)

Approximately 41% of all colorectal cancers occur in the proximal colon, with around 22% involving distal colon. Approximately 28% involves rectum area. However, it is known that there can occur potential differences in the site of origin depending on the age and gender. (3)

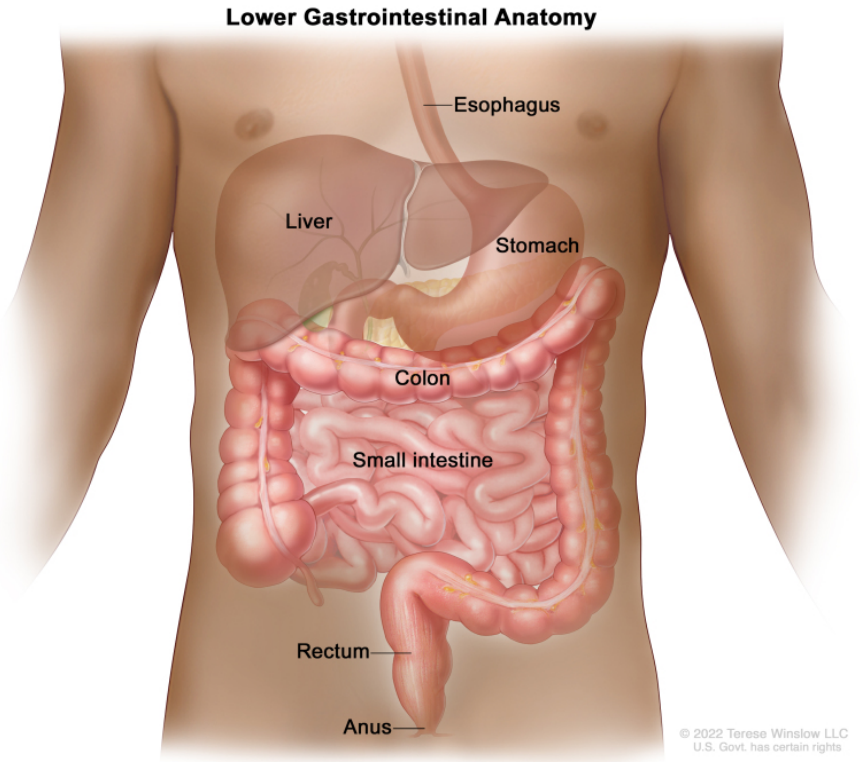

Figure 1:Anatomy of lower gastrointestinal tract. From: NIH, National Cancer Institute, 2024. (2)

Main reason why more and more people suffer from colorectal cancer are factors such as ageing and dietary habits, mostly in high- income countries. Risk factors such as obesity, lack of physical exercise and smoking are known to increase the risk of colorectal cancer. (4) Main group suffering from colorectal cancer are older people over 50 years old, but numbers of early- onset young patients below 50 are still increasing due to unclear yet reasons. (3)

Other known reason for occurrence of colorectal cancer are genetic variations. Hereditary colorectal cancer syndromes include Lynch syndrome (Hereditary nonpolyposis colorectal cancer), Familial adenomatous polyposis (FAP) and MUTYH- associated polyposis (MAP). Out of those syndromes, it is known that Lynch syndrome and Familial adenomatous polyposis causes majority of hereditary colorectal cancer, which still accounts for only about 5% of all colorectal cancers. (3)
The presence of family history, especially in first degree relatives, even without any known hereditary colon cancer syndromes, increases the risk of development of colorectal cancer in about 20% of cases. (3)

Out of patients with diagnosed colorectal cancer with metastasizes to liver, the ones that underwent surgery for removal of part or whole liver 71% lives longer that 5 years after surgery. (5)
There are few treatments for colorectal cancer, but adjuvant chemotherapy is usually only recommended for patients with lymph node metastases. Other form of treatment is surgery, which is only curative therapy for localized colorectal cancer. (6)
Other form for treatment of colorectal cancer can be systematic therapy, consisting of cytotoxin chemotherapy, biologic therapy such as antibodies to cellular growth factors, immunotherapy, and their combinations. (7)

The best treatment for patients with metastasizes is surgery, with adjuvant treatment of chemotherapy. The best duration time of the treatment is not easy to define, but most of the time optimal duration is set to be 6 months. Drug that is used in chemotherapy for CRC is 5-fluorouracil (5FU). (8)

There are known some factors that have been associated with a decrease in the incidence of colorectal cancer. Those factors include regular physical activity, diet rich in fruits and vegetables, high fiber diet, folate rich diet, calcium intake, dairy products, Vitamin D, Vitamin B6, magnesium intake, fish consumption and non- steroidal Anti- inflammatory drugs (NSAIDS) (3).
The overall survival for patients with CRC is good but there are still some aggressive forms of colorectal cancer and therefore it´s important to learn more about biology of development of cancer, and there is much more that needs to be learn.

1.2 Microbiome

The microbiota in humans is composed of communities of bacteria, viruses and fungi that are known to have greater complexity that the human genome itself. Projects such as European Metagenomics of the Human Intestinal Tract and the Human Microbiome Project have reported that microbiome in human has up to 3.3 million different unique protein- encoding genes, which means it is much bigger than the whole human genome which has only up to 23 000 genes. Whole human microbiota has extensive functions such as development of immunity, defense against pathogens, host nutrition including production of short- chain fatty acids which are important in host energy metabolism, fat storage, synthesis of vitamins as well as an influence on human behavior. (9)

The gastrointestinal tract has very comprehensive microbiome. It is known to consist of components such as diverse consortium of bacteria, archaea, fungi, protozoa, and viruses that inhabit gut of all mammals. (10)
Studies in humans, but also other mammals have shown that gut microbiome is very important in a range of different physiological processes that are extremely important for host health. Processes that gut microbiome is responsible for are for example energy homeostasis, gut epithelial health, metabolism, and neurobehavioral development.
Recent studies shows that gastrointestinal microbiome and changes in it are associated with diseases in both humans and animals. Diseases that are associated with gut microbiome are for example inflammatory bowel syndrome, asthma, obesity, metabolic syndrome, cardiovascular disease, immune- mediated conditions and neurodevelopmental conditions such as autism spectrum disorder. (10)

Most dramatic changes in microbiome happens in infancy and early childhood. The main reason to that is the intestinal microbiome of an infant is affected by gestational age, meaning if they were born in full term or premature, mode of delivery- vaginal birth or caesarean section and type of feed- breast milk or formula.
The complicity of microbiome in infants in this early life development stage is believed to be important in maintaining homeostasis with the host´s immune system and has a big impact on health later in life. (9)

1.3 Intratumoral microbiome and CRC

Different analyses and studies have shown that state of pathological microbial imbalance or dysbiosis is prevalent in the gut microbiome of patients with colorectal cancer. The studies show that different types of bacteria are found inside the gut microbiota of patients with colorectal cancer. Some of those bacteria are known to interact with human cancer cells *in vitro* and trigger disease pathways. Bacteria can affect colorectal cancer development by directly or indirectly affect host cells and/ or their environment by processes such as bacterial metabolism and its secreted molecular complements, attachment, invasion and translocation or host defense modulation. Studies also shown that chemotherapy efficiency can be affected by microbiome composition. There are 11 identified bacterial strains, that shows resistance against pathogenic infections, and are able to improve the therapeutic efficiency of immune checkpoint inhibitors. It's been also suggested that those bacteria interfere with the molecular mechanisms underlying colorectal cancer (11)

It has been shown that several types of bacteria are associated with colorectal cancer. One of them is *Fusobacterium nucleatum,* today considered a potential colorectal cancer biomarker. Higher content of *Fusobacterium* is observed in stool and colon tissues in CRC patients. Fusobacterium has been shown to expand myelin- derived immune cells, while also inhibiting T- cell responses and also was associated with macrophage activation following an upregulation of certain microRNAs. Therefore, *Fusobacterium* is considered to be one of the biomarkers for CRC. (11)

Another bacteria associated with colorectal cancer is *Peptostreptococcus ssp.* Studies shows that patients with this bacteria present have increased risk of developing colorectal cancer. *P. stomatis* potentially can contribute to the acidic and hypoxic tumor microenvironment, which is known to promote bacterial colonization.
Other type of *Peptostreptococcus, Peptostreptococcus anaerobius* has been found in higher amount in stool and colon tissue in CRC patients. The protumorigenic effect of this bacteria is shown to work via TLR2 and TLR4 which later leads to reactive oxygen species accumulation, which supports cholesterol synthesis and cellular proliferation. (11)

Also, *Prevotella intermedia* has been reported to be associated with a higher risk of developing CRC, especially in African- American cohorts, but while studying 526

metagenomics CRC fecal samples, *P. intermedia* has been also identified in multinational multicohort. Role of this bacteria in case of colorectal cancer has been speculated to be either modulation of the innate immune response via neutrophil suppression, or by causing p53 mutations and has also been shown in pancreatic cancer.

Parvimonas micra, is a bacteria believed to give rise to a protumorigenic and inflammatory environment. It happens because *P. micra* has been shown to interfere with the normal functioning of the NOD2 signaling pathway in periodontitis. (11)

The best ways to study microbiome and bacteria in colorectal cancer are analyses such as 16S rRNA next- generation sequencing, and furthermore use of different databases or computational pipelines for the analysis.
Analysis and studies on content of different bacteria in samples from colorectal cancer patient are great way to make credible microbiome composition which can later influence chemotherapy effects (11)
In this study we would like to further focus on expanding our understanding on the role of microbiome in CRC biology.

The aim for this study is to establish the most effective methods for extracting low microbiota from tissue resected from patients with colorectal cancer, evaluate best time and speed for bead beating process and establish the most effective method for bacterial load in tissue samples.

2. Methods

In this thesis we used different primers that are summarized in the table 1.

Table 1: table over all primers used in the paper.

Primer name	sequence 5'-3'
MA_pf-16S-F	GCGAATCGACGGGAGCTT
1492R	TACGGCTACCTTGTTACGACTT
MRT-125	CGACGTTGTAAAACGACGGCC
MRT-156-F	CCATTCGCCATTCAGGCTGC
MRT-261-F	CTGATTCTGTGGATAACCGTATTACCGC
V6-1	CNACGCGAAGAACCTTANC
V6-2	ATACGCGARGAACCTTACC
V6-3	CTAACCGANGAACCTYACC
V6-4	CAACGCGMARAACCTTACC
V6_Fp-R1	CGACRRCCATGCANCACCT
27F	AGAGTTTGATCCTGGCTCAG (13)
16S Amplicon PCR forward primer	TCGTCGGCAGCGTCAGATTGTTATAAGAGACAGCC TACGGGNGGCWGCAG (12)
16S Amplicon PCR reverse primer	GTCTCGTGGGCTCGGAGATGTGTATA AGAGACAGGACTACHVGGGTATCTAATCC (12)

2.1 Sample preparation

Participants were enrolled in the ongoing ACROBATICC project, a clinical- molecular biomarker outcomes study (14). The bachelor project involving human participants was conducted in compliance with national regulations and approved by the Norwegian regional Ethics Committee (REK Helse Vest, #2012/742). All activities involving human samples were performed according to the ethical standards of Stavanger University Hospital and/or national committee, adhering to the 1964 Helsinki Declaration and its subsequent updates or comparable ethical standards.

Extraction of DNA from colorectal cancer samples was carried out over the period of two days, both days using ZymoBIOMICS Mini prep kit (Zymo Research). Work on the samples was performed under sterile conditions to avoid contamination.

During the first day of sample preparation, we have digested tissue in the lysis buffer with proteinase K in order to release the DNA from the lysed cell and degrade proteins.

Samples with mock community were prepared using ZymoBIOMICS Microbial Community Standards (lot. 73683, Zymo Research, later mentioned as stdZymo), the stdZymo has known propositional content of bacteria and yeast. To prepare these samples 95µl of solid tissue blue buffer (lot. 221990, Zymo Research), 75µl of stdZymo, 20µl of ultra-pure distilled water (2436576, Invitrogen) and 10µl of 20 mg/µl proteinase K (stock concentration, lot. 196556, Zymo Research) were all added to a 2 ml RNase- and DNase- free microcentrifuge tube.

Three of samples were prepared using tissue with unknown content of bacteria and yest, collected from patient with colorectal cancer. To prepare those samples ~15mg of tissue was added to the 2ml collection tube. To the tissue sample 95µl of solid tissue blue buffer, 95µl ultra-pure distilled water and 10µl proteinase K were added.

The last samples were prepared using spike in method, which means they contain both standard from ZymoBIOMICS and tissue samples collected from patients with colorectal cancer. The reason for that is to control the extraction of the DNA from the sample with tissue.
To prepare those samples, both ~15mg of tissue sample and 7,5µl of stdZymo was added to 2ml microcentrifuge tube, together with 95 µl of solid tissue blue buffer, 87,5µl ultra-pure distilled water, and 10µl proteinase K.

All samples, together with one negative control sample made without standard nor tissue sample, were put to incubation at 55°C with vigorous shaking overnight.

2.2 DNA extraction

After overnight incubation the lysate was used for DNA extraction using the ZymoBIOMICS DNA Miniprep kit according to manufacture instruction.

Firstly all 200µl of lysate from previous step was transformed into a ZR BashingBead lysis tube 0.1&0.5mm (960009, ZymoBIOMICS), and 750µl of lysis solution (227803, Zymo Research) was added to each of the samples.
After that a homogenizer FastPrep24 (MP biomedical) was used on 4m/s or 6 m/s setting. Bead beating in this case was used to lyse, homogenize, and grind bacterial microbiota, which is hard to lyse.
One of each type of sample was then bead- beated for 5, 6 or 7 minutes. Bead beating was done at one minute at the time with 5 minutes break in between. Reason for that was to find out most optimal time of bead beating, which would result in better lysis of wide range of bacterial community and produce high yield of DNA.

After bead beating was done, tubes were centrifuged at 10000 x g at 1 minute, and 400µl of a sample was transferred to a Zymo- spin III-F filter (430539, Zymo Research) with a collection tube. Samples were then centrifuged on 8000 x g for 1 minute. In some cases, a small pellet from bashing beads was formed at the bottom of the tube. In those cases, whole supernatant from those samples was carefully transferred to new collection tube, to ensure no contamination from bashing beads.
To that 1,2ml of binding buffer (224872, Zymo Research) was added and supernatant from the previous step was vortex to make sure the solution is homogeneous. From that, 800µl was transferred to a Zymo- spin IICR column (817622, Zymo Research) in a new collection tube, and all samples were centrifuged again at 1000 x g for 1 minute. This step was repeated one more time.
After last steps all DNA was bind to the column and needed to be cleaned to make sure there is no contamination with the cell debris, RNA, and proteins. To do that first 400µl of DNA wash buffer 1 (228076, Zymo Research) was added to the IICR column in the new collection tube, and thereafter samples were centrifuged again at 10000 x g at 1 minute. After that DNA wash buffer 2 was used (228956, ZymoBIOMICS). First 700µl was added, samples were

centrifuged and following that 200µl of the same wash buffer was added. Samples were again centrifuged. Between each of washes, waste from collection tube was disposed.

After washing steps, IICR column was transferred to the new 1,5ml microcentrifuge tube, and either 50µl or 100µl of ultra-pure distilled water was added and incubated for 1 minute. Amount of water varies between different attempts described in results section.
Finally, samples were centrifuged at 10000 x g for 1 minute, and DNA collected at the bottom of collection tube was used further to measure concentration of the DNA, and downstream application.

2.3 DNA concentration measurements

After the extraction of the DNA, its concentration of both unknown samples, and known standards from ZymoBIOMICS was measured on fluorometer Qubit, using Qubit 4 from invitrogen. The reason why Qubit was used is to ensure the results will be as accurate as possible for dowsed application.

Qubit works by measuring the intensity of the signal from fluorescent dye bound to the specific molecules that needs to be studied. It uses specialized curve- fitting algorithm to make a calibration curve, with help from standard samples with known concentration. Then the unknown sample concentration can be calculated based on the curve, by comparing the relative fluorescence units in the sample to the RFUs in standards. (15)

To prepare the samples with standards 190µl of 1x dsDNA Broad Range working solution (2610295, Invitrogen) was added to the Qubit assay tubes (Q32856, Invitrogen), and for unknown samples, 198 µl of the same working solution was added to the Qubit assay tubes where samples will be.
Then to one of the tubes prepared for standard, 10 µl of Qubit 1x dsDNA BR standard #1 (2581660, Invitrogen) was added, and to the second tube Qubit 1x dsDNA BR standard #2 (2581660, Invitrogen) was added. To the rest of testing tubes 2 µl of unknown samples was added. Measurements was done on fluorometer Qubit, and results are shown in the result section.

2.4 NanoDrop DNA measurements

DNA was also measured using NanoDrop, control for quality of DNA. NanoDrop is a full-spectrum UV- spectrophotometer. It can be used for both high- and low concentration samples and requires only 2 µl of sample to be able to read results. Using NanoDrop it's possible to get results in ng/µl but also 260/ 280, and 260/230 values. (16)

To prepare samples for the NanoDrop, 2µl of every DNA sample was transferred to new tube in the sterile bench to avoid contamination, and the measurements was carried out on NanoDrop. NanoDrop requires to use blank before testing, and for that ultra-pure water was used. Results of NanoDrop measurements are shown in result section.

Up- concentration and purification of samples

DNA samples from the last step were up concentrated and then purified to check if DNA concentration will be higher. To do that 15µl of sample was transferred to new tube, and then 5 µl of $AcNH_4$ was added. Sample was thoroughly vortex, and then 40 µl of ice- cold 100% ethanol was added. Samples were incubated at -20°C overnight
Next day, samples were centrifuged on top speed for 30 minutes, and the supernatant was carefully removed and discharged. Pellet that formed on the bottom of tube was washed with 200µl of ice- cold 75% ethanol. Then samples were centrifuged again, at top speed for 30 minutes.
After centrifuging, supernatant was again carefully removed and discharged, and sample was incubated on dry ice for 10 minutes to ensure complete evaporation of residual ethanol. After 10 minutes, samples were resuspended in 10µl of water and DNA concentration was measured on fluorometer Qubit, using Qubit 4 from Invitrogen.

Preparing of agarose gel

To prepare agarose gel used for PCR, 3 grams of Agarose, Molecular Grade (lot. ES520-B072820, Bioline) was measured and mixed with 300ml of 1x TAE buffer, and the whole mixture was wormed up in microwave until agarose was dissolved.
At the end 30µl of GelRed 10,000 in water (lot. 9G0529, Biotium) was added. Mixture was stored in heating cabinet at 60°C for up to one week.

2.5 PCR

To verify whether extracted DNA from tissue contained bacterial DNA, the polymerase-chain reaction was performed, to amplify bacterial 16S rRNA gene. We have tested both amplification of variable regions V3V4 of bacterial 16S rRNA gene at 500bp and full 16S rRNA gene at about 1500bp. To do that firstly both primers, for V_3V_4 and full 16S rRNA were diluted 1:10 to 10µl in ultra-pure water. Then two reaction mixes were made. Each one by adding 25µl of HotStarTaq Master Mix (lot. 172023740, QIAGEN), 1 µl of 10µM forward primer 16S Amplicon PCR forward primer for V3V3 and for 16S rRNA: 27F primer, 1µl of 10µM reverse primer Amplicon PCR reverse primer for V3V4 and for 16S rRNA gene 1492R primer, 1µl template at varying concentrations and ultra- pure distilled water, making up to 50µl of total reaction mixture.

All samples were placed in the thermal cycler for 25 cycles following the program shown in *table 2.*

Table 2: thermal cycle condition used for V3V4 and 16S samples

	V_3V_4		16S	
step	time	temperature	time	temperature
Initial heat activation	10 minutes	95°C	10 minutes	95°C
Denaturation	30 seconds	94°C	30 second	94°C
Annealing	30 second	52°C	30 second	54°C
Extension	30 second	72°C	1min 30sec	72°C
Final extension	10 minutes	72°C	10 minutes	72°C

After thermal cycle was done, 10µl of 6X Tritrack DNA loading dye (lot. 2822347, Thermo Scientific) was added to each sample. All samples together with 3 µl of Gene Ruler 1kb DNA ladder (lot. 2791887, Thermo Scientific) were transformed to 1% agarose gel and electrophoresis was carried out at 140V for 30 minutes in 1X TAE buffer.
At the end picture of gel was taken.

2.6 development of standard curve for quantification of bacterial load using plasmid containing 16S rRNA gene.

Design of plasmid and primers

The design, the plasmid and primers for cloning used in this project was performed by Mitchellrey Magbanua Toleco, with the help of Benchling software. The sequence of 16S rRNA for *Peribactillus frigoritolerans* was downloaded in FASTA format on NCBI website. This 16S rRNA gene contains V6 region that will later on be used as a standard for quantification of bacterial load with qPCR

Amplification of target fragment of full gene 16S rRNA from *Peribactillus frigoritolerans*

In the first step of plasmid preparation, the target sequence of 16S rRNA gene from *Peribactillus frigoritolerans* was amplified using the designed primers (table 2). First, designed primers P1-16S-F and 1492R were first diluted so that their final concentration would be 100µM. Forward primer P1-16S-F was diluted with 263µl of water, and reverse primer 1492R was diluted with 295µl of water.
DNA from *8801 Peribactillus frigoritolerans* was also diluted to 4 ng/µl in ultra-pure water. To do that 12,5µl of water was added to 5µl of bacteria.

Then the reaction mix was made, using 12,5µl of HiFi HotStart Ready Mix (cat. KK2602, Roche), 0,3µl of forward primer (P1-16S-F), 0,3µl of reverse primer(1492R), 9,25µl of ultra-pure water and 0,75µl of 3% DMSO (P24I065, Thermo scientific). To each sample 1µl of 4ng/µl of genomic DNA from *Peribactillus frigoritolerans* was added.

Samples were put into thermal cycler for 25 cycles. Conditions that were used are shown in *table 3.*

Table 3: thermal cycle conditions used for PCR with 16S plasmid

step	time	temperature
Initial denaturation	3 minutes	95°C
Denaturation	20 seconds	98°C
Annealing	15 seconds	55°C -70°C gradient
Extension	1minute	72°C
Final extension	1 minute	72°C

After thermal cycle was done, PCR reaction was mixed with loading buffer, and gel electrophoresis was done in 1% agarose gel. Picture of gel was taken using ChemiDoc system (Bio-Rad)

Gel extraction

After the size of amplified gene interest was confirmed by the gel electrophoresis in 1% agarose gel and 1kb ladder, the band with the PCR product was extracted from the gel. For gel extraction, corresponding band containing DNA was cut out using sterile scalpel and UV illuminator in the darkness.

Then, the pieces of gel were weighed, and volume of binding buffer XP2 (lot. BB081216QG2365, Omega Bio-Tek) equivalent to weight of gel was added (if gel weigh is 0.3g, 0.3ml of buffer was added).

Samples were then incubated for 7 minutes on 60°C, tubes were vortex each 2-3 minutes. HiBend DNA mini column (BVT081016R2271031356, Omega Bio-Tek) was inserted into 2ml collection tube, and up to 700µl of agarose/ DNA mixture was transformed into mini

column. Samples were then centrifuged at 10,000 x g for 1 minute. Filtrate was discarded and steps were repeated until all of sample was transformed to the column.

After all sample was transformed, and filtrate was discarded, 300µl of binding buffer was added to the same mini column, and samples were centrifuged at top speed at 10,000 x g for 1 minute.
Filtrate was once again discarded, and 700µl of SPW Wash Buffer (SPW071416JC2353, Omega Bio-Tek) was added to the samples. Samples were than centrifuged at top speed for 1 minute. Filtrate was discarded, and empty mini column was centrifuged for 2 minutes at maximum speed to dry the column matrix. Mini column was then transferred to the clean 1,5ml microcentrifuge tube, and 50µl of Elution Buffer (EB032316JC2013, Omega Bio- Tek) was added. Samples were then centrifuged at maximum speed for 1 minute, and DNA was measured on NanoDrop.

One step assembly of PCR product and vector

After gel extraction, the concentration of PCR products was high enough to allow continuation of the assembly of plasmid and transformation of assembly to the 10-beta bacteria cells were done.
To do that, first both bridges, MA- SSOB1 and MA-SSOB2 were diluted in water to 100µM in ultra-pure water. Then they were further diluted in NEBuffer 2 (lot. 10162785, New England BioLabs) to 1µM, and mixed by adding 1µl of bridge 1, 1µl of bridge 2 and 98µl of buffer.

The reaction mixture was made by adding 1,49µl of pBS-VBB, 1,84µl of PCR fragment, 0,25µl of bridge mixture and 3,58µl of NEB HiFi Assembly Master Mix (#E2621, New England Biolabs).
Samples were then incubated for 1 hour, at 50°C

Transformation of assembly to 10- beta bacteria cells

After incubations were done, 2µl of PCR mixture from previous step was used for transformation into NEB® 10-beta Competent E. coli (C3019H, New England Biolabs). First, the competent bacteria were thawed on ice for 20 minutes, the 2µl of assembly were added to bacteria and incubated for 20 minutes on ice. Thereafter bacterial cells were heat- shocked on warm block for 30 second at 42°C and then transferred to ice and incubated for 2 minutes. After that, 1ml of pre- warmed outgrowth media (lot. 10186900, New England BioLabs) was added to the samples, and they were again put for incubation for 1 hour at 37°C with vigorous shaking.
After incubation, 75µl, 100µl and 150µl of sample was plated onto LB agar with 100µg/ml Ampecilin. Rest of the sample was thoroughly mixed and plated onto new LB agar.
All plates were incubated overnight at 37°C

Colony PCR with the target plasmid

After LB agar plates incubated overnight, the colony PCR was done. To do that firstly, primers were diluted 100µM in water. The 90,75µl of HotStarTaq Master Mix, 3,63µl of forward primer (P1-16S-F), 3,63µl of reverse primer (1492R), 5,44µl DMSO and 78,04µl ultra-pure distilled water was mixed. 15µl of mixture was added to PCR tube, and one colony was that added to each tube.
Samples were then put to thermal cycler for 27 cycles. Conditions that were used are shown in *table 4.*

Table 4: thermal cycle conditions used for PCR with bacteria with 16S plasmid

step	time	temperature
Initial denaturation	15 minutes	95°C
Denaturation	30 seconds	95°C
Annealing	30 seconds	55°C
Extension	1min 10sec	72°C
Final extension	10 minutes	72°C

After that, samples together with ladder were put into 1% agarose gel, and electrophoresis was carried out
Picture of the gel was taken.

Based on the results from PCR, 5 colonies were picked out and put for overnight culture preparation using LB media with 100µg/ml ampicillin. Samples were put at 37°C with 250rpm shaking overnight.

Plasmid mini prep

After overnight incubation, plasmid was purified using QIAprep spin Mini Prep Kit (ref. 27104, QIAgen).
From the overnight culture, 5ml was transformed in to 15ml collection tube, and centrifuged at 4000 x g for 5 minutes. Supernatant was carefully removed and 350µl of solution I (lot. 47830FM98, Omega Bio-Tek) was added, followed by addition of 350µl of solution II (47458F0105, Omega Bio- Tek).
Samples were gently mixed ensure a proper mixing of reagents.
After that 450µl of solution III (44640FP90, Omega Bio-Tek) was added, and samples were centrifuged at maximum speed for 10 minutes.
Next, QIAprep 2.0 spin columns (154028393, QIAgen) were transferred into the collection tube. 600µl of sample was transferred into the spin column, and sample was centrifuged at maximum speed for 1 minute. Step was repeated that all samples would be applied to the column. After columns were centrifuged, filtrate was discarded and 500µl of HBC buffer (lot. 44822CB163, Omega Bio-Tek) was added and column were centrifuged at maximum speed for 1 minute. Step was repeated.
Filtrate was again discarded, and 700µl of DNA Wash Buffer (lot. 45741AM198, Omega Bio-Tek) was added. Samples were centrifuged at maximum speed for 1 minute, and step was repeated.
After column were centrifuged, filtrate was discarded, and empty spin columns were centrifuged at maximum speed for 2 minutes, to dry the filter.

Spin columns were then placed in new 1,5ml microcentrifuge tube, and 50µl of elution buffer (49043BD458, Omega Bio-Tek) was added. After incubation at room temperature, for 1 minute, samples were centrifuged at maximum speed for 1 minute.
DNA concentrations were measured by NanoDrop.

Verification of plasmid by PCR

To verify the plasmid, we have performed the PCR reacting.
To do that 0,5 µl of each sample with plasmid was diluted to 4ng/ml in water.
Reaction mix was then made, using 10µl of HotStarTaq Master Mix, 0,4µl of MRT-156 of 200µM forward primer, 0,4µl of 1492 of 200µM reverse primer, 0,6µl of 3% DMSO, 1µl of template and 7,6µl of water.
Samples were then put to thermal cycler for 25 cycles. Conditions that were used are shown in *table 5.*

Table 5: thermal cycle conditions used for PCR for verification of plasmid

step	time	temperature
Initial denaturation	15 minutes	95°C
denaturation	30 seconds	95°C
Annealing	30 seconds	55°C
Extension	1min 40sec	72°C
Final extension	10 minutes	72°C

After that, samples together with ladder were added to the 1% agarose gel, and electrophoresis was carried out.
Picture of the gel was taken.

Verification of plasmid by sequencing

To prepare samples for sequencing, plasmid was diluted to 80 ng/µl in water.
To that, 3µl of 20mM primes was added. Primers that were used are V6PFR, 156FR and 261 RV. Samples were then send to Microsynth Seqlab (Germany) for sequencing.

qPCR

Quantitative PCR (qPCR), also called for real- time PCR is a technique that amplifies the target DNA sequence together with quantification of the concentration of the species DNA in the reaction. qPCR is mostly used to evaluate DNA copy number in samples, SNP detection and viral load. (17)

To prepare for qPCR all needed calculation for both dilution of the sample and reaction mixes was made. Then sample were diluted to 2 ng/µl and thereafter the 10- fold dilution was used 6 to 8 times.
Reaction mix used in qPCR was made with 10µl Sso Advanced Universal SYBR Green Supermix (Cat. #1725271, BIO-RAD), primers which concentration varies from 100 to 300, and ultra- pure water added in the amount that makes the total volume of the samples 18µl. To that 2µl of template was added

Both reaction mix, and each of dilutions of the sample were carefully pipetted into the 96- well plate and sealed. Plates was then placed into the Real- Time PCR system for 40 cycles at the conditions shown in table 6.

Table 6: Real- Time PCR conditions used for qPCR testing

step	time	temperature
Initial denaturation	3 minutes	98°C
denaturation	10 seconds	98°C
Annealing	15 seconds	60°C
Extension	5 seconds	60°C
Final extension	5 seconds	95°C

Optimalisation of qPCR

Based on the results from first qPCR run, test needed optimalisation to get best possible results next times.
To do that dilution was calculated again and decision was made to used different dilutions that would give better results. The amount of template used was changed from 2µl to 5µl for samples with unknown content.
Both temperature gradient, and primer gradient was done to determine in which conditions samples would show the best results.
Based on the results from first run, NTC had too high concentrations and therefore both water and primers were replaced with new ones to make sure no contamination will take place again.
All the conditions were tested with both NTC samples and ZymoBIOMICS Microbial Community DNA standard (D6305, Zymo Research) to ensure that the readings are correct.

After couple of testes with both primer matrix and temperature gradient, best conditions were chosen and the original test with samples was again carried out.

qPCR with the extracted DNA and designed plasmid

After the process for qPCR was optimized and right conditions was determined, qPCR with the designed plasmid samples and extracted DNA samples was carried out.
To do that, first plasmid with concentration 486 ng/µl was diluted 1:10, and then 5µl of that was added to 116,5µl of ultra-pure water, making concentration of sample 2ng/µl. Then sample was diluted 6 times using 10-fold method.
Samples with ZymoStd were diluted 1:100, and tissue samples were used undiluted.
To reaction mixes were made, using 10µl of Sso Advanced Universal SYBR Green Supermix, 0,2µl of 100µM of forward primer (V6-FP-F), 0,2µl of 100µM of reverse primer (V6-FP-R), 2µl of template for ZymoStd or 5µl of template for plasmid and tissue samples, and topped with ultra-pure water so that the total volume of the sample was 20µl.
Samples were then carefully placed in Real- Time PCR system for 40 cycles at the conditions shown in table 7.

Table 7: Real- Time PCR conditions used for qPCR testing

step	time	temperature
Initial denaturation	3 minutes	98°C
denaturation	10 seconds	98°C
Annealing	15 seconds	60°C
Extension	5 seconds	60°C
Final extension	5 seconds	95°C

3. Results

3.1 DNA extraction

Qubit fluorometer was used to determine the concentration of DNA from purified samples. DNA was extracted using ZymoBIOMICS Mini prep kit, from samples containing ZymoBIOMICS Microbial Community Standards, tissue samples and tissue samples with spike of ZymoStd. Beat beating used in this test was 4 m/s. Results are shown in table 8.

Table 8: results of DNA measurements of samples with ZymoStd tissue and tissue with spike. Beat beating 4m/s, DNA is measured by Qubit.

Sample number	Content of the sample	Weight of tissue sample	Result
1	Microbial Community Standards	No tissue	10.8 ng/µl
2	Microbial Community Standards	No tissue	12 ng/µl
3	Microbial Community Standards	No tissue	9.17 ng/µl
4	Tissue sample	15 mg	34.7 ng/µl
5	Tissue sample	15 mg	35.9 ng/µl
6	Tissue sample	17 mg	32.5 ng/µl
7	Spike and tissue	15 mg	40.1 ng/µl
8	Spike and tissue	16 mg	29.3 ng/µl
9	Spike and tissue	14 mg	11.6 ng/µl
10	Negative control	No tissue	Low

The same test was performed using beat beating on higher speed as 6 m/s to test if amount and quality of DNA in samples would be different from 4 m/s. Test was also done with ZymoBIOMICS Microbial Community Standards, tissue samples and samples of tissue with spike in of ZymoStd. This time samples were also eluted with only 50µl of water, and after the measurement samples with Std was eluted one more time, with 50µl more water. Results are shown in table 9.

Table 9: Results of DNA measurements of samples with ZymoStd, tissue, and tissue with spike- in with bead beating 6 m/s. DNA concentration measurements is performed on Qubit

Sample number	Content of the sample	Weight of tissue sample	Result with 50µl of water	Results with 100µl of water
1	Microbial Community Standards	No tissue	18.3 ng/µl	14.7 ng/µl
2	Microbial Community Standards	No tissue	10.7 ng/µl	6.26 ng/µl
3	Microbial Community Standards	No tissue	12 ng/µl	8.32 ng/µl
4	Tissue sample	25 mg	33.4 ng/µl	
5	Tissue sample	20 mg	48.9 ng/µl	
6	Tissue sample	19 mg	35.2 ng/µl	
7	Spike and tissue	19 mg	62 ng/µl	
8	Spike and tissue	22 mg	35.5 ng/µl	
9	Spike and tissue	19 mg	37.3 ng/µl	
10	Negative control	No tissue	Low	

Test with bead beating 6 m/s was done one more time, this time all samples were eluted with 100µl of water. After overnight incubation with shaking, one of the samples didn't fully digest this time, and were in gel form. Test was also done with ZymoBIOMICS Microbial Community Standards, unknown tissue samples and samples made using spike in method. Results are shown in table 10.

Table 10: Results of DNA measurements of samples with ZymoStd, tissue, and tissue with spike- in with bead beating 6 m/s. DNA concentration measurements is performed on Qubit

Sample number	Content of the sample	Weight of tissue sample	Result
1	Microbial Community Standards	No tissue	19.8 ng/µl
2	Microbial Community Standards	No tissue	19.4 ng/µl
3	Microbial Community Standards	No tissue	21.5 ng/µl
4	Tissue sample	15 mg	47.1 ng/µl
5	Tissue sample	14 mg	47.7 ng/µl
6	Tissue sample	13 mg	41.2 ng/µl
7	Spike and tissue	12 mg	61.6 ng/µl
8	Spike and tissue	14 mg	55.3 ng/µl
9	Spike and tissue	11 mg	38.9 ng/µl
10	Negative control	No tissue	Low

After testing on Qubit, samples bead beated on 4 m/s were tested on NanoDrop to check the quality and amount of DNA in samples. Results are shown in table 11.

Table 11: Results of DNA measurements of samples with ZymoStd, tissue, and tissue with spike- in with bead beating 4m/s. DNA concentration measurements is performed on NanoDrop

Sample content	ng/µl	260/280	260/230
Microbial Community Standard	14.6	1.98	0.05
Microbial Community Standard	16.8	1.74	0.57
Microbial Community Standard	11.8	1.77	0.09
Tissue sample	46.7	1.78	1.34
Tissue sample	45.2	1.76	1.58
Tissue sample	41.1	1.82	1.89
Spike and tissue	53.2	1.82	0.46
Spike and tissue	34.1	1.78	0.60
Spike and tissue	13.6	1.77	0.70
Negative control	5.7	1.48	0.78

From NanoDrop results can we see that 260/280 ratio was around 1,8 which indicates pure DNA of good quality, 260/230 ratio was very low which can indicate some form of contamination in samples. Contamination is also supported by results from sample number 10 which should be negative but gave results in both 260/280 and 260/230 tests.
After that samples that were bead beated on 6m/s were also tested on NanoDrop to check for quality and amount on DNA in samples. Results are shown in table 12.

Table 12: Results of DNA measurements of ZymoStd, tissue, and tissue with spike- in with bead beating 6m/s. DNA measurements is performed on NanoDrop

Sample content	ng/µl	260/280	260/230
Microbial Community Standard	28	1,76	0.40
Microbial Community Standard	32.5	1.79	0.15
Microbial Community Standard	29.6	1.84	0.42
Tissue sample	60.3	1.83	1.46
Tissue sample	66.6	1.84	0.49
Tissue sample	47.4	1.82	1.99
Spike and tissue	71.7	1.87	0.23
Spike and tissue	74.1	1.85	1.30
Spike and tissue	59.3	1.87	1.05
Negative control	0.3	-1.18	0.09

From NanoDrop results can we see that 260/280 ratio was around 1,8 which shows that DNA in samples was pure and had good quality, 260/230 ratios were low which can mean that some form of contamination was present in the samples.

3.2 Up- concentration and purification of samples

DNA samples that were bead beated on 4m/s were up- concentrated and purified to check if the quality and concentration of DNA would be improved for downstream applications. Measurement was done on Qubit and results are shown in table 12.

Table13: results of DNA measurements of samples with ZymoStd, tissue, and tissue with spike- in with beat beating 4 m/s after up concentration and purification. DNA concentration measurements is performed on Qubit.

Sample number	Content of the sample	Weight of tissue sample	Result
1	Microbial Community Standards	No tissue	6.06 ng/µl
2	Microbial Community Standards	No tissue	0.948 ng/µl
3	Microbial Community Standards	No tissue	4.94 ng/µl
4	Tissue sample	15 mg	25.2 ng/µl
5	Tissue sample	15 mg	44.4 ng/µl
6	Tissue sample	17 mg	23.4 ng/µl
7	Spike in sample	15 mg	33.8 ng/µl
8	Spike in sample	16 mg	31.8 ng/µl
9	Spike in sample	14 mg	31.8 ng/µl
10	Negative control	No tissue	Low

Results from samples shows that up- concentration didn't help in getting better quality and higher amount of DNA in samples.

3.3 PCR

When performing PCR first test was done with ZymoBIOMICS Microbial Community Standards with both V3V4 primer (figure 2) and full length 16S rRNA primer (figure 3). PCR was done in 1% agarose gel.

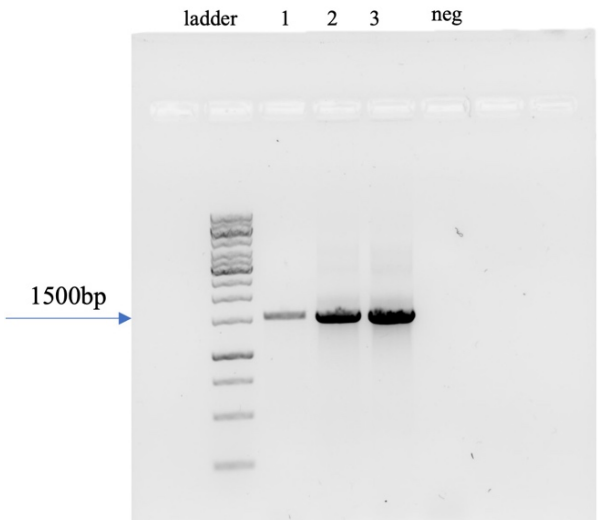

Figure 2: PCR with ZymoBIOMICS std with V3V4 primer, bead beating 4m/s. (1) bb time 4min, (2) bb time 5min, (3) bb time 6min, (neg) negative control

Results from PCR with ZymoBIOMICS std with V3V4 primer shows strong band in sample nr 2 and 3, and lighter one in sample 1. All band are showing at 1500bp.

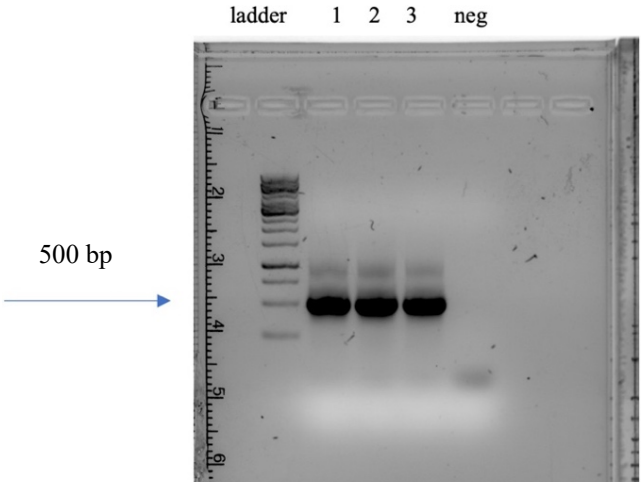

Figure 3: PCR with ZymoBIOMICS std with 16S primer, bead beating 4m/s. (1) bb time 4min, (2) bb time 5min, (3) bb time 6min, (neg) negative control

Results from PCR with ZymoBIOMICS std with 16S primer shows all three band being very strong and showing at 500bp.

After PCR with just Std samples were successful, PCR with unknown samples and spike in were done. All samples were tested using both V3V4 primer (figure 4) and 16S primer (figure 5). PCR was done in 1% agarose gel.

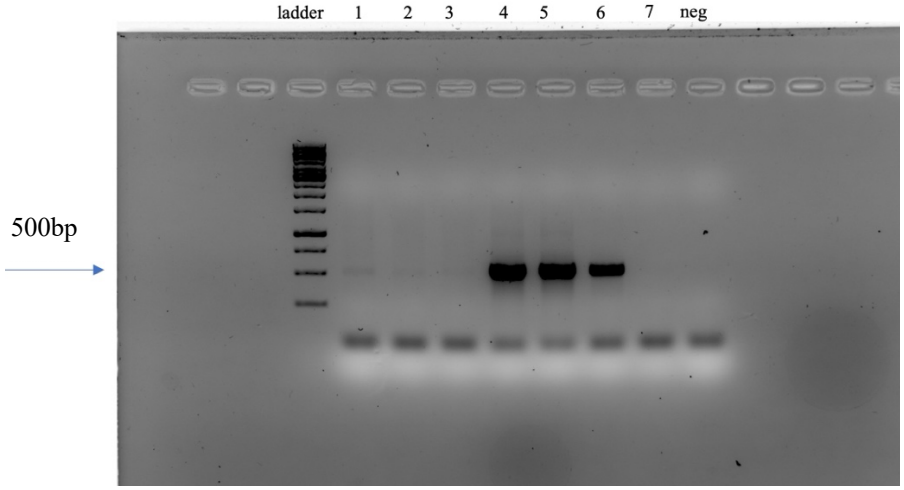

*Figure 4: PCR with tissue samples and spike in samples with V3V4 primer, bb 4m/s. (**1**) tissue sample bb time 4min, (**2**) tissue sample bb time 5min, (**3**) tissue sample bb time 6min, (**4**) spike- in bb time 4min, (**5**) spike- in bb time 5min, (**6**) spike- in bb time 6min, (7) negative control for extraction of DNA, (**neg**) negative control for PCR*

Results from PCR with V3V4 primer shows strong band at 500bp in samples 4,5, and 6 which are samples with spike- in, much lighter bands at 500bp show in samples 1,2, and 3 which are samples with only tissue. Samples 7 and negative don't show any bands which indicates that no contamination was present.

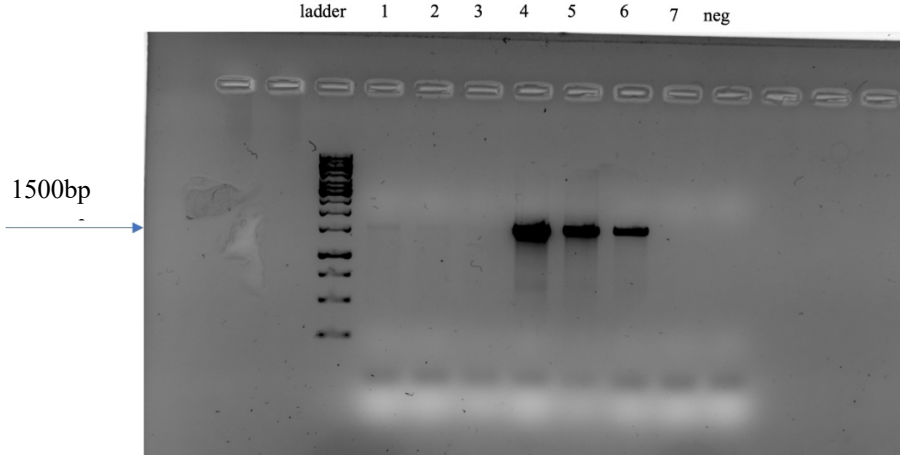

*Figure 5: PCR with tissue samples and spike in samples with 16S primer, bb 4m/s. (**1**) tissue sample bb time 4min, (**2**) tissue sample bb time 5min, (**3**) tissue sample bb time 6min, (**4**) spike- in bb time 4min, (**5**) spike- in bb time 5min, (**6**) spike- in bb time 6min, , (7) negative control for extraction of DNA, (**neg**) negative control for PCR*

Results from PCR with 16S primer shows strong bands at 1500bp in samples 4,5, and 6 which are samples with spike- in, lighter bands at 1500bp in samples 1,2, and 3 which were samples with tissue input. No bands in sample 7 and negative which indicates that no contamination was present.

Lastly PCR with all samples were done, including std, unknown sample and spike- in. All samples were tested with both V3V4 primer (figure 6) and 16S primer (figure 7). PCR was done in 1% agarose gel.

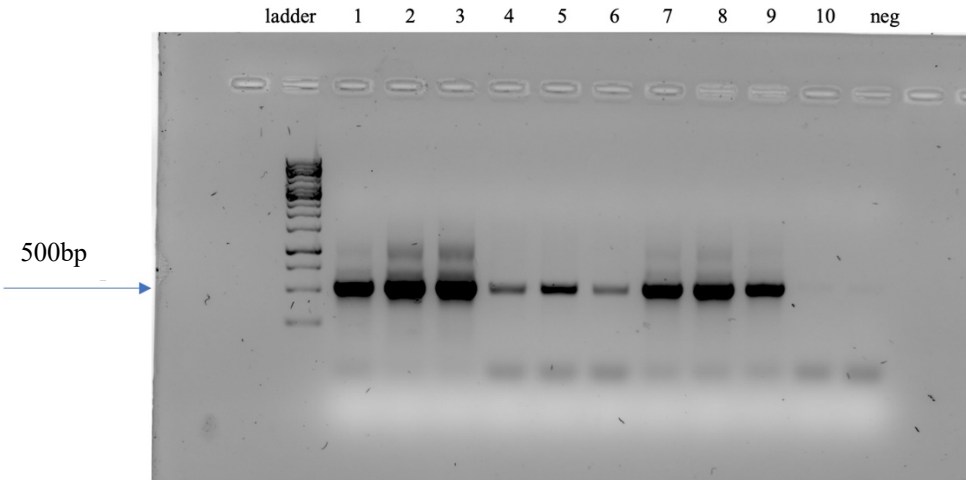

*Figure 6: PCR with std, tissue samples and spike in with V3V4 primer, bb 4m/s. (**1**) std bb time 4min, (**2**) std bb time 5min, (**3**) std bb time 6min, (**4**) tissue sample bb time 4min, (**5**) tissue sample bb time 5min, (**6**) tissue sample bb time 6min, (**7**) spike- in bb time 4min, (**8**) spike- in bb time 5min, (**9**) spike- in bb time 6min, (**10**) negative control for extraction of DNA, (**neg**) negative control for PCR.*

Results from PCR with V3V4 primer shows very strong band at 500bp in samples 1,2 and 3 which were samples with ZymoBIOMICS std, much lighter bands at 500bp are visible in samples 4,5 and 6 which were the ones with only tissue input, stronger bands at 500bp are visible in samples 7,8 and 9 were spike- in samples were. There are no band visible in samples 10 and negative which indicates that there was no contamination.

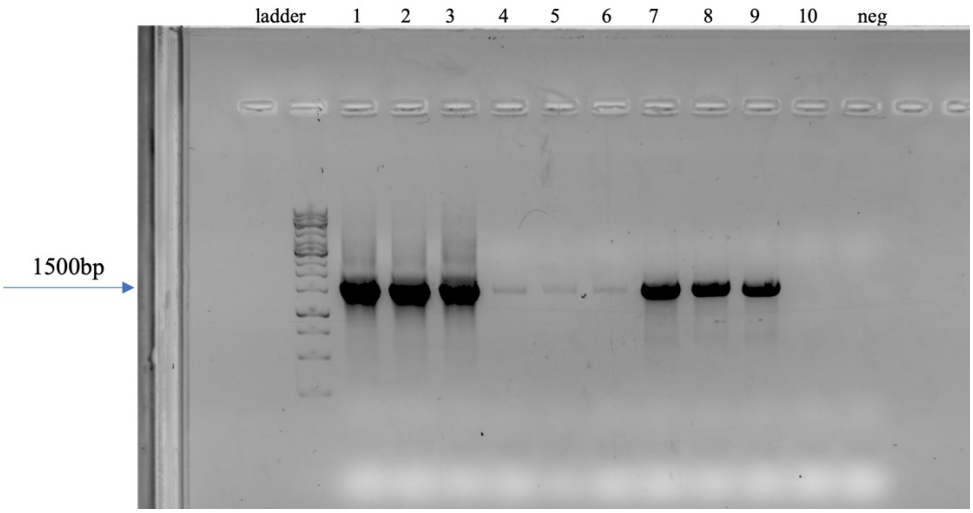

*Figure 7: PCR with std, tissue samples and spike in with 16S primer, bb 4m/s. **(1)** std bb time 4min, **(2)** std bb time 5min, **(3)** std bb time 6min, **(4)** tissue sample bb time 4min, **(5)** tissue sample bb time 5min, **(6)** tissue sample bb time 6min, (7) spike- in bb t time 4min, **(8)** spike- in bb time 5min, **(9)** spike- in bb time 6min, **(10)** negative control for extraction of DNA, (**neg**) negative control for PCR.*

Results from PCR with 16S primer shows very strong band at 1500bp in samples 1,2 and 3 which were samples with ZymoBIOMICS std, very faint bands at 1500bp are visible in samples 4,5 and 6 which were the ones with only tissue input, stronger bands at 1500bp are visible in samples 7,8 and 9 were spike- in samples were. There are no band visible in samples 10 and negative which indicates that there was no contamination.

3.4 Preparation of standard for bacterial load quantification

When preparing standard for bacterial load quantification, first PCR was performed to amplify full gene 16S rRNA gene from *Peribactillus frigoritolerans* bacteria, assembling the gene in the plasmid before transformation of assembly to the 10- beta bacteria cells (figure 8). PCR was done in 1% agarose gel.

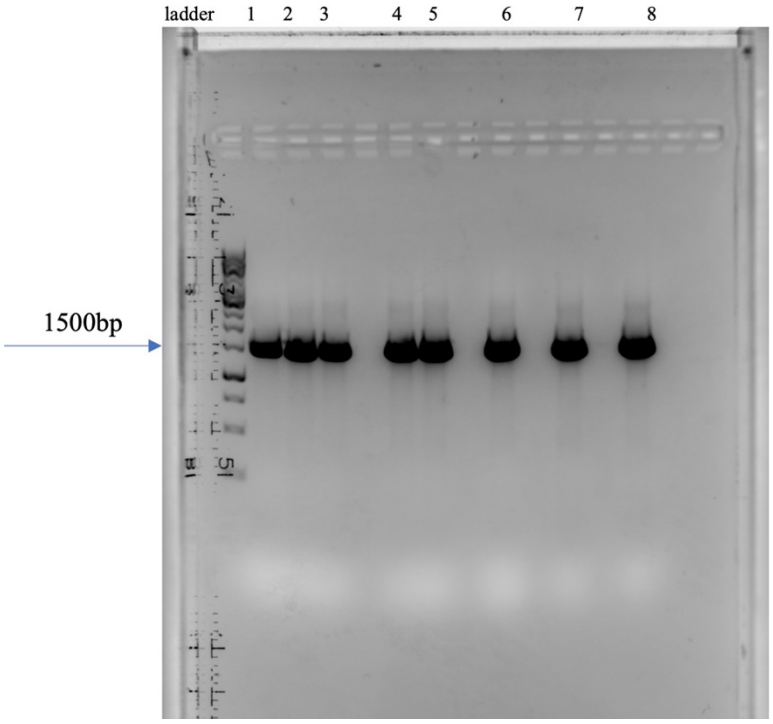

*Figure 8: PCR with full gene 16S rRNA from Peribactillus frigoritolerans bacteria. (**1-8**): 8 parallels of the gene.*

After PCR with the designed plasmid was done, PCR product was cut out using sterile scalpel and UV illuminator in the darkness, and the extraction of DNA from PCR product was done. Then the extracted DNA was measured on NanoDrop to check for amount and quality of DNA in samples and results are shown in table 14.

Table 14: Results of DNA measurements of the PCR product with the design plasmid performed on NanoDrop.

Sample number	Ng/µl	260/280	260/230
1	19,3	1,75	0,19
2	48,7	1,74	0,69
3	17,1	1,88	0,42
4	40,3	1,78	1,16

From NanoDrop results can we see that the 260/280 ratio is around 1,8 which indicates that quality of DNA is pure and high, but 260/230 ratio is low which can indicate some form of contamination.

3.5 One step assembly of PCR product and vector

After the PCR product was tested, and quality of DNA was good enough to continue, transformation of assembly to the 10- beta bacteria cells was done, and plasmid was placed in the bacteria and samples were incubated overnight. Later samples were collected, and colony PCR was done (figure 9). PCR reaction was mixed with loading buffer and gel electrophoresis was done in 1% agarose gel.

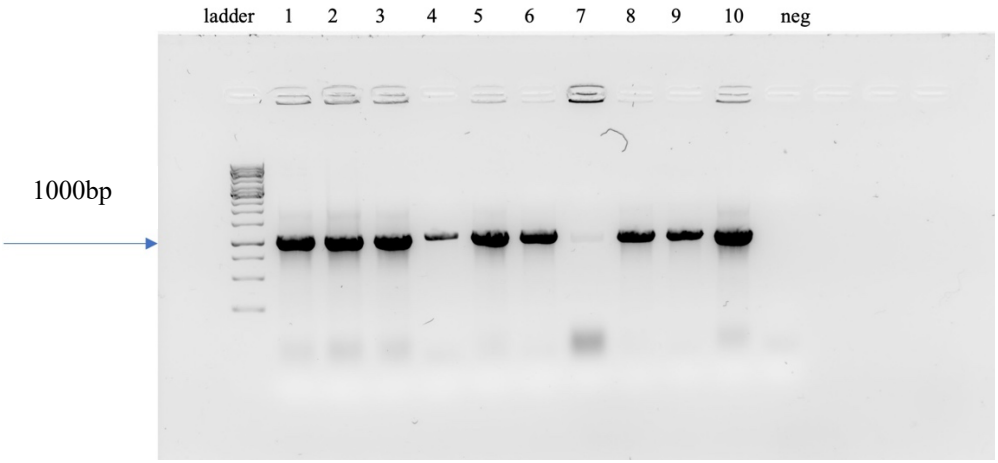

*Figure 9: colony PCR. (**1-3**) colonies from 75µl plate, (**4-6**) colonies from 100µl plate, (**7-10**) colonies from 150µl plate, (**neg**) negative control for PCR.*

After colony PCR, 5 best colonies were used for miniprep to amplify the plasmid in bacterial cells. DNA concentration after miniprep was measured on NanoDrop to check for DNA amount and quality and results are shown in table 15.

Table 15: Results of DNA measurements of the 10- beta bacteria with design plasmid performed on NanoDrop.

Sample number	Content of the sample	Ng/µl	260/280	260/230
1	Sample from 75µl plate	181,7	1,85	2,18
2	Sample from 75µl plate	353,4	1,86	2,18
3	Sample from 75µl plate	235,5	1,85	2,23
5	Sample from 100µl plate	178	1,85	2,16
10	Sample from 150µl plate	274,2	1,86	2,23

From the NanoDrop results can we see that 260/280 ratio is around 1,8 which shows that DNA is pure and of good quality, and 260/230 ratio is around 2 which shows that there is no contamination present in the samples.

Lastly verification of the plasmid by PCR was done to assure that the insert was cloned in correctly. Verification was done with 5 best samples from colony PCR. Samples that were choose are 1, 2, 3,5 and 10. PCR picture from verification of the plasmid is shown in figure 10. Gel electrophoresis was done in 1% agarose gel.

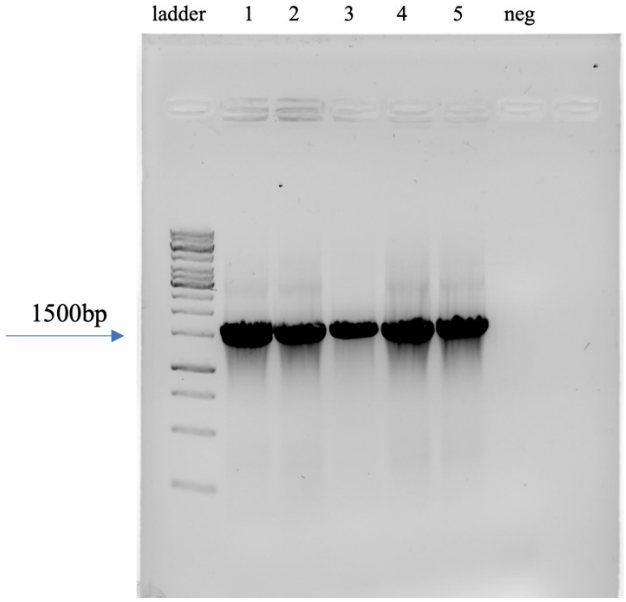

*Figure 10: verification of the plasmid. (**1**) sample nr 1 from colony PCR, (**2**) sample nr 2 from colony PCR, (**3**) sample nr 3 from colony PCR, (**4**) sample nr 5 from colony PCR, (**5**) sample nr 10 from colony PCR, (**neg**) negative control for PCR.*

Plasmid 2,3 and 10 was sent for sequencing to Mycrosynth to verify the correct insert. The results of sequencing confirmed the correct insert (data is not shown).

3.5 Absolute quantification of bacterial load using qPCR

To determine the target DNA sequence together with quantification of the concentration of the species DNA in the sample, quantitative PCR (qPCR) was performed.
For the first trial plasmid samples were first diluted 1:100 to 3,5 ng/µl and then diluted using 10- fold method 7 times. All 8 dilutions were used in qPCR. Results from first trial are shown in table 16.

Table 16: Results of qPCR testing of designed plasmid and ZymoBIOMICS Microbial Community DNA standard (DNA std) (D6305, Zymo Research)

Sample name	Cq mean ± SD
plasmid dilution 1	8,875± 0,20
plasmid dilution 2	11,725± 0,98
plasmid dilution 3	12,775± 0,20
plasmid dilution 4	15,555± 0,05
plasmid dilution 5	19,235± 0,16
plasmid dilution 6	23,625± 0,39
plasmid dilution 7	26,95± 0,25
plasmid dilution 8	28,93± 0,59
DNA std 1	19,91± 0,21
DNA std 2	19,7±0,99
DNA std 3	19,765±0,78
DNA std 4	16,11±0,42
NTC	30,1±0,90

Optimalization of qPCR

After the result from first time, prosses on optimalization of qPCR was done. The reason for that is too high Cq of NTC in the first trail, and to low Cq with the first dilution meaning the concentration of the first dilution should be reduced to achieve optimal noise- to- signal level. Optimalization was needed to check whether the Cq around 35 are caused by contamination in the reagents and primers or primer dimers.

First step was to perform temperature gradient with primer concentration 0,3mM and 0,2mM. Test was performed on both plasmid samples and samples with ZymoBIOMICS microbial community DNA standard (D6305, Zymo Research). Results are shown in table 17.

Table 17: results of qPCR optimalization using temperature gradient, with plasmid samples and ZymoBIOMICS Microbial Community DNA standard. (**Primer mix 1**): primer concentration 0,3mM, (**Primer mix 2**): primer concentration 0,2mM, (**DNA std**): ZymoBIOMICS Microbial Community DNA standard.

Sample name	Input	Temperature	Cq mean± SD
Plasmid primer mix 1	Plasmid	70°C	26,925± 9,05
plasmid primer mix 2	plasmid	70°C	26,1± 1,47
DNA std	DNA standard	70°C	29,045± 0,15
NTC 1	Water	70°C	37,68
NTC 2	Water	70°C	N/A
NTC 3	water	70°C	N/A
Plasmid primer mix 1	Plasmid	69°C	17,895± 1,70
Plasmid primer mix 2	Plasmid	69°C	19,35± 0,44
DNA std	DNA standard	69°C	24,46± 0,42
NTC 1	Water	69°C	31,41
NTC 2	Water	69°C	38,23
NTC 3	Water	69°C	35,42
Plasmid primer mix 1	Plasmid	67°C	14,305± 0,63
Plasmid primer mix 2	Plasmid	67°C	15,22± 0,99
DNA std	DNA standard	67°C	21,53± 0,07
NTC 1	Water	67°C	27,66
NTC 2	Water	67°C	N/A
NTC 3	water	67°C	30,51
Plasmid primer mix 1	Plasmid	63,9°C	13,55± 0,08
Plasmid primer mix 2	Plasmid	63,9°C	14,1± 0,08
DNA std	DNA standard	63,9°C	20,305± 0
NTC 1	Water	63,9°C	26,77
NTC 2	Water	63,9°C	28,54
NTC 3	Water	63,9°C	27,62
Plasmid primer mix 1	Plasmid	60,2°C	13,115± 0,03
Plasmid primer mix 2	Plasmid	60,2°C	13,565± 0,12
DNA std	DNA standard	60,2°C	19,905± 0,03
NTC 1	Water	60,2°C	26,07
NTC 2	Water	60,2°C	28,31
NTC 3	Water	60,2°C	27,76
Plasmid primer mix 1	Plasmid	57,1°C	13,14± 0,03
Plasmid primer mix 2	Plasmid	57,1°C	13,805± 0,12

DNA std	DNA standard	57,1°C	20± 0,04
NTC 1	Water	57,1°C	25,39
NTC 2	Water	57,1°C	28,75
NTC 3	Water	57,1°C	27,9
Plasmid primer mix 1	Plasmid	55,1°C	13,21± 0
Plasmid primer mix 2	Plasmid	55,1°C	13,745± 0,01
DNA std	DNA standard	55,1°C	20,06± 0,03
NTC 1	Water	55,1°C	26,22
NTC 2	Water	55,1°C	28,80
NTC 3	Water	55,1°C	28,86
Plasmid primer mix 1	Plasmid	54°C	13,375± 0,05
Plasmid primer mix 2	Plasmid	54°C	14,04± 0,03
DNA std	DNA standard	54°C	20,2± 0,04
NTC 1	Water	54°C	28,69
NTC 2	Water	54°C	29,01
NTC 3	Water	54°C	28,2

After temperature gradient, primer matrix together with temperature gradient was performed to check at what concentration and temperature primer dimers will disappear and at which temperature primers are most effective. No samples were used in this test, to ensure that primer result wouldn't be interrupted. Concentrations of primers used in this qPCR testing were 0,1mM, 0,2Mm and 0,15mM. Results are shown in table 18.

Table 18: Results of qPCR optimalization with temperature gradient and primer matrix without sample input. (**Primer mix 1**): 0,1mM forward/ 0,1mM reverse, (**Primer mix 2**): 0,1mM forward/ 0,2mM reverse, (**Primer mix 3**): 0,2mM forward/ 0,1mM reverse, (**Primer mix 4**): 0,15mM forward/ 0,15mM reverse.

Sample name	temperature	Cq mean± SD
Primer mix 1	70°C	N/A
primer mix 2	70°C	N/A
primer mix 3	70°C	N/A
primer mix 4	70°C	N/A
Primer mix 1	69°C	N/A
primer mix 2	69°C	N/A

primer mix 3	69°C	39,45± 0,32
primer mix 4	69°C	N/A
Primer mix 1	67°C	39,25± 0,18
primer mix 2	67°C	36,53± 4,55
primer mix 3	67°C	31,48± 0,11
primer mix 4	67°C	33,69± 0,14
Primer mix 1	63,9°C	31,63± 0,53
primer mix 2	63,9°C	34,54± 0,57
primer mix 3	63,9°C	29,12± 0,12
primer mix 4	63,9°C	30,86± 0,43
Primer mix 1	60,2°C	29,49± 0,21
primer mix 2	60,2°C	33,51± 1,06
primer mix 3	60,2°C	28,73± 0,25
primer mix 4	60,2°C	30,04± 0,49
Primer mix 1	57,1°C	29,51± 0,14
primer mix 2	57,1°C	33,87± 0,28
primer mix 3	57,1°C	28,86± 0,20
primer mix 4	57,1°C	30,44± 0,56
Primer mix 1	55,1°C	29,86± 0,52
primer mix 2	55,1°C	33,77± 0,63
primer mix 3	55,1°C	29,19± 0,52
primer mix 4	55,1°C	29,21± 2,51
Primer mix 1	54°C	29,74± 0,37
primer mix 2	54°C	34,42± 0,13
primer mix 3	54°C	27,71± 1,63
primer mix 4	54°C	30,17± 0,91

From the last test it was determined that best temperature for primers is between 64°C and 57°C. Test was performed one more time with temperature gradient between our preferable temperatures, and primer matrix. Test was again performed without template input. Primer concentrations used were 0,1mM, 0,2mM, 0,3mM and 0,15mM. Results are shown in table 19.

*Table 19: Results of qPCR optimalization with temperature gradient and primer matrix without sample input. (**Primer mix 1**): 0,1mM forward/0,1mM reverse, (**Primer mix 2**): 0,1mM forward/ 0,2mM reverse, (**Primer mix 3**): 0,2 forward/ 0,1 reverse, (**Primer mix 4**):0,15mM forward/ 0,15mM reverse, (**reaction mix 5**): 0,2mM forward/ 0,2mM reverse, (**Primer mix 6**): 0,3mM forward/ 0,3 mM reverse*

Sample name	temperature	*Cq mean* ± SD
primer mix 1	63,9°C	N/A
primer mix 2	63,9°C	38,775± 0,50
primer mix 3	63,9°C	39,99± 0
primer mix 4	63,9°C	37,185± 0,05
primer mix 5	63,9°C	35,415± 0,82
primer mix 6	63,9°C	32,455± 0,38
primer mix 1	60,2°C	38,34±0,56
primer mix 2	60,2°C	33,63± 1,68
primer mix 3	60,2°C	36,045± 0,33
primer mix 4	60,2°C	34,15± 0,05
primer mix 5	60,2°C	33,515± 0,31
primer mix 6	60,2°C	31,53± 0,72
primer mix 1	57,1°C	36,77± 0,24
primer mix 2	57,1°C	34,89± 0,17
primer mix 3	57,1°C	35,47± 1,14
primer mix 4	57,1°C	34,18± 0,69
primer mix 5	57,1°C	32,945± 0,13
primer mix 6	57,1°C	31,685± 0,03

After testing it was determined that temperature that works best is 64°C, and primer concentrations that are most preferable are 0,1mM forward/ 0,2mM reverse and 150mM forward/ 0,15mM reverse. One more test with those conditions was performed, this time with plasmid to check if results will still be correct. Input of sample was also increased to 5µl.

Plasmid samples were diluted to 2 ng/µl and then diluted 8 times using 10- fold dilution. 8 of dilutions from 10- fold was used for qPCR. Results are shown in table 20.

*Table 20: Results of qPCR optimalization using 8 10-fold dilutions of plasmid at 64 ℃. (**Reaction mix 1**): 0,1mM forward/ 0,2mM reverse, (**Reaction mix 2**): 0,15mM forward/ 0,15mM reverse.*

Sample name	Cq mean ± SD
reaction mix 1 dilution 1	15,2± 0,53
reaction mix 1 dilution 2	18,52± 0,14
reaction mix 1 dilution 3	22,18± 0,16
reaction mix 1 dilution 4	25,84± 0,10
reaction mix 1 dilution 5	29,44± 0,12
reaction mix 1 dilution 6	33,12± 1,04
reaction mix 1 dilution 7	33,94± 0,31
reaction mix 1 dilution 8	34,55± 0,35
reaction mix 2 dilution 1	13,95± 0,13
reaction mix 2 dilution 2	17,37± 0,11
reaction mix 2 dilution 3	21,14± 0,58
reaction mix 2 dilution 4	24,90± 0,13
reaction mix 2 dilution 5	28,35± 0,17
reaction mix 2 dilution 6	31,57± 0,59
reaction mix 2 dilution 7	33,39± 0,16
reaction mix 2 dilution 8	33,78± 0,19
NTC 1	35,24± 1,44
NTC 2	33,49± 0,31

After optimalization best concentration of primers, and temperature for qPCR was determine. It was also determined that there is a big possibility for contamination based on the results, so water and primers were changed to the new ones so no more contamination would occur.

qPCR with the extracted DNA and designed plasmid

After optimalization gave great results, samples with designed plasmid and extracted DNA could be tested to determine concentration of DNA in the sample.
In plasmid samples, first sample was diluted to 10ng/µl and later 10-fold method was used to dilute it 5 more times, all 6 dilutions were used in qPCR.
Samples with extracted DNA that were tested has been bead beated at 4m/s.
For the qPCR 2µl of sample was used. Temperature that was determined to work best was 64°C and primer concentration was 0,1mM forward/ 0,1mM reverse. Results are shown in table 21 and standard curve calculated from those results is shown in figure 11, where y= -4,5046x + 53,371 and R^2=0,9959.

*Table 21: Results of qPCR with designed plasmid and with extracted DNA. (**plasmid**): samples with designed plasmid, (**ZymoStd**): samples with ZymoStd, (**tissue**): samples with tissue DNA bb 4m/s, (**spike in**): samples spiked with ZymoStd.*

Sample name	Cq mean± SD
plasmid dilution 1	16,6± 0,41
plasmid dilution 2	19,63± 0,17
plasmid dilution 3	24,34± 0,31
plasmid dilution 4	29,09± 0,23
plasmid dilution 5	33,85± 0,28
plasmid dilution 6	38,65± 0,35
ZymoStd 1	23,93± 0,34
ZymoStd 2	24,05± 0,02
ZymoStd 3	24,82± 0,04
tissue 1	33,07± 0,07
tissue 2	34,41± 0,22
tissue 3	35,46± 0,09
spike in 1	22,68± 0,19
spike in 2	22,94± 0,02
spike in 3	24,66± 0,12
negative control	36,24± 0,01
NTC	N/A

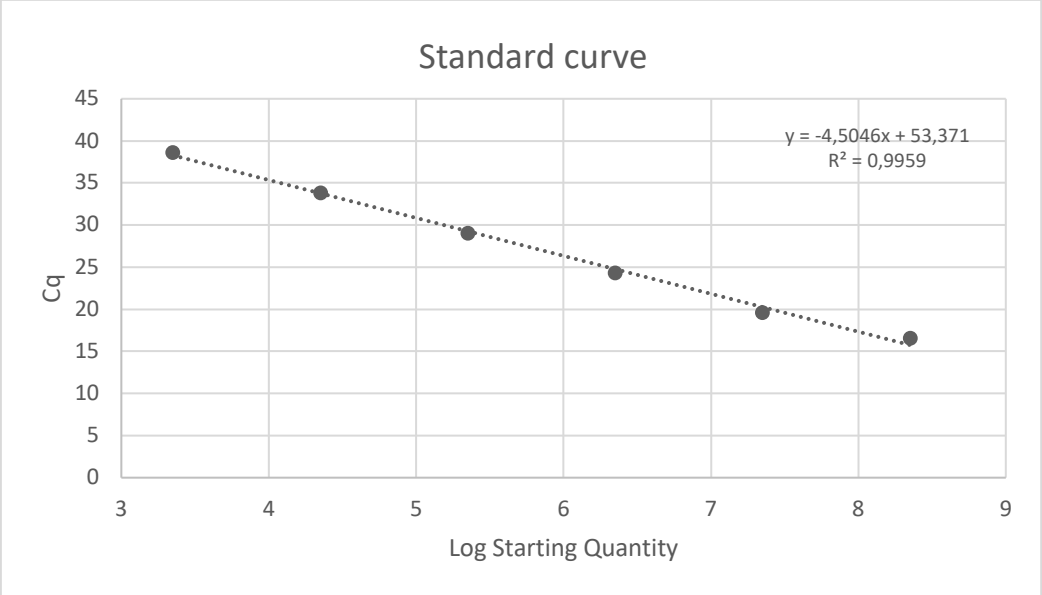

Figure 11: standard curve based on results from qPCR with designed plasmid. Plasmid is diluted 6 times using 10-fold method, start concentration is 0,2ng/µl.

Based on the standard curve, bacterial load in samples with tissue and spike in was calculated. Equation used for this is: $\frac{DNA_{conc}(g\ \mu l^{-1}) * 6{,}022*10^{23}}{DNA_{lenght}*660\ mol^{-1}}$. Results for bacterial load based on standard curve from figure 11 are shown in table 22.

Table 22: bacterial load in samples with tissue input and spike in, with bead beating 4m/s

	sample content	mean Cq	log	volume per	Copy /ul
ZymoStd 1	ZymoStd bb 4m/s 5min	23,93	6,53	2	342268143,20
ZymoStd 2	ZymoStd bb 4m/s 6min	24,05	6,51	2	322938631,10
ZymoStd 3	ZymoStd bb 4m/s 7min	24,82	6,33	2	217866271,0
tissue 1	tissue bb 4m/s 5min	33,07	4,50	2	321166,32
tissue 2	tissue bb 4m/s 6min	34,41	4,20	2	161901,17
tissue 3	tissue bb 4m/s 7min	35,46	3,97	2	94658,58
spike in 1	spike in bb 4m/s 5 min	22,68	6,81	2	65051902,14

spike in 2	spike in bb 4m/s 6 min	22,94	6,75	2	56956068,02
spike in 3	spike in bb 4m/s 7min	24,66	6,37	2	23643403,83

Same test was performed once again, this time with DNA samples with bead beating 6m/s. For samples with designed plasmid 5µl of template was used as input.
ZymoStd samples were first diluted 1:100 and then 2µl of sample was used as input.
Both tissue and spike in samples weren't diluted and 2µl of sample was used as input.
Primer concentration used this time was 0,1mM forward/ 0,1mM reverse, and reaction was put on 64°C. Results are shown in table 23.

*Table 23: Results of qPCR with designed plasmid and with extracted DNA. (**plasmid**): samples with designed plasmid, (**ZymoStd**): samples with ZymoStd, (**tissue**): samples with tissue DNA bb 4m/s, (**spike in**): samples spiked with ZymoStd.*

Sample name	Cq mean± SD
plasmid dilution 1	14,46± 0,15
plasmid dilution 2	18,09± 0,08
plasmid dilution 3	22,63± 0,32
plasmid dilution 4	27,37± 0,22
plasmid dilution 5	31,61± 0,30
plasmid dilution 6	36,03± 0,27
ZymoStd 1	23,07± 0,01
ZymoStd 2	24,12± 0,34
ZymoStd 3	23,61± 0
tissue 1	28,52± 0,14
tissue 2	26,17± 0,04
tissue 3	30,79± 0,12
spike in 1	21,97± 0,32
spike in 2	21,74± 0,50
spike in 3	22,85± 0,09
negative control	38,63± 0,72
NTC	N/A

Based on the results from table 23, bacterial load in samples bead beated on 6m/s was calculated one more time using the same equation. Results are shown in table 24. Standard curve for this run is shown in appendix (appendix, figure 10).

Table 24: bacterial load for samples with tissue input and spike in, with bead beating 6m/s.

	sample content	**mean Cq**	**log**	**volume per**	**copy number in stock**
ZymoStd 1	ZymoStd bb 6m/s 5min	23,07	6,29	2	197965722,8
ZymoStd 2	ZymoStd bb 6m/s 6min	24,12	6,05	2	113927875,8
ZymoStd 3	ZymoStd bb 6m/s 7min	23,61	6,17	2	148997849,4
tissue 1	tissue bb 6m/s 5min	28,52	5,05108	2	1124812,15
tissue 2	tissue bb 6m/s 6min	26,17	5,58813	2	3873735,82
tissue 3	tissue bb 6m/s 7min	30,79	4,5323	2	340643,41
spike in 1	spike in bb 6m/s 5 min	21,97	6,54798	2	35316690,55
spike in 2	spike in bb 6m/s 6 min	21,74	6,60054	2	39860248,33
spike in 3	spike in bb 6m/s 7min	22,85	6,34687	2	22226444,73

In summary, table 25 shows bacterial count in stock calculated for both runs with samples bead beated at 4m/s and 6m/s.

Table 25: summary of all calculated bacterial count for different bead beating times and speeds.

Sample content	Bead beating time	Bead beating speed	Copy number in stock
ZymoStd	5min	4m/s	342268143,20
ZymoStd	6min	4m/s	322938631,10
ZymoStd	7min	4m/s	217866271
Tissue	5min	4m/s	321166,32
tissue	6min	4 m/s	161901,17
tissue	7min	4 m/s	94658,58
Tissue and spike	5min	4 m/s	65051902,14
Tissue and spike	6min	4 m/s	56956068,02
Tissue and spike	7min	4 m/s	23643403,83
ZymoStd	5min	6m/s	197965722,80
ZymoStd	6min	6m/s	113927875,80
ZymoStd	7min	6m/s	148997849,40
Tissue	5min	6 m/s	112412,15
Tissue	6min	6m/s	3873735,82
Tissue	7min	6 m/s	340643,41
Tissue and spike	5min	6 m/s	35316690,55
Tissue and spike	6min	6 m/s	39860248,33
Tissue and spike	7min	6 m/s	22226444,73

4. Discussion

4.1 DNA extraction

After DNA from all samples was extracted, measurements were performed, on both fluorometer Qubit, using Qubit 4 from Invitrogen and NanoDrop. The same samples were later also tested using quantitative real time PCR (qPCR).
Measured concentration from those instruments were similar, but with some variations. When using Qubit fluorometer we could only measure concentration of DNA ng/µl in tested sample, while when using NanoDrop as results we get concentration in ng/µl and in addition also evaluate ratio of 260/280 and 260/230 values which gives indication about the purity of our samples and provides us with more detailed results. (18) 260/280 is a ration of absorbance that is used to determine the purity of DNA and RNA in tested sample. 260/230 is a ration used to determine the presence of unwanted organic compounds in the samples (18).
So overall we can determine that out of Qubit fluorometer and NanoDrop, it is NanoDrop that provides better and more detailed results when it comes to quality of DNA in the samples. However, Qubit provide more precise measurements of DNA concentration.

Use of quantitative real time PCR (qPCR) allows us to see all of the DNA content of the sample, including DNA from alive organisms as well as DNA from dead organisms that are still present in the sample. qPCR is also most sensitive and is able to amplify all target DNA in the sample.
This is the best way to determine for example contamination, or other fails in the research, and also the best way to get most accurate results.

When comparing results measured on Qubit from samples bead beated on 4m/s (table 8) and 6m/s (table 10) we can see that samples bead beated on 6m/s have higher DNA concentration than samples bead beated on 4m/s. Measurements from NanoDrop also shows that DNA concentration in samples bead beated on 6m/s is higher than DNA concentration in samples bead beated on 4m/s.
Reason for that can be use of different tissue samples in both tests or contamination could be present. Other reason for that could be that bead beating samples on 6m/s lyse, homogenize, and grind bacterial microbiota better which results in higher DNA yield in the sample.

Similar research about comparing methods from sample collection to sequencing was performed in 2023 (19). Results from that paper shows that method used for cell disruption was a big contributor to variations in microbiota composition, and that the number of cycles during PCR can lead to an increase in contaminants detected in the negative control samples. This means that DNA extraction process have a huge impact on the microbial compositions in samples. (19) Optimal amount of PCR cycles from this research is set to be around 25 to avoid contamination, which matches our results.
Other result acquired from that research were that mock communities alone were not sufficient to control for quality in fecal microbiome samples. In our case mock community samples worked well and were big help, which shows that they work when testing tissue microbiome samples.

4.2 Testing the designed plasmid

When performing tests on the designed plasmid, first the PCR was done to amplify DNA and determine how much of the DNA is in the sample. After that was successful, the one step assembly of PCR product and vector was done to continue testing the plasmid in the 10- beta bacteria.
When doing that, quantitative real time PCR (qPCR) was used, and during the first trail, results didn't come out as expected and contamination was suspected. To determine whether that was the case, first the temperature gradient and then the primer matrix were done, results shown in tables 17, 18, 19 and 20.
Reason for testing the temperature gradient was to check if samples tested at different temperatures would give other results, which would exclude contamination and prove that the problem was the temperatures at which primers were tested.
Primer matrix was done by testing how different concentrations of the primers will act during qPCR at the gradient of annealing temperatures. Reason for that was to check for potential primer dimes that could be translated as contamination.
After those tests were performed and the most optimal temperature and primer concentration was determined the primers and ultra-pure water used was replaced due to still possible contamination. The test with designed plasmid was then performed once more, showing

wanted results. The conclusion to that is that the optimalisation of qPCR test we´ve done was successful and performed correctly.

From table 25 with summary of bacterial count in different samples, can we see that bead beating gives best results when put on 4m/s rather than 6m/s. From results can we also see that samples with ZymoStd have more copies than samples with only tissue or tissue with spike. From our results can we see that spike- in method works well and gives desirable results.

Using the equation: $\frac{bead\ beating\ 6m/s\ for\ 7min}{bead\ beating\ 5m/s\ for\ 5\ min} * 100\%$ can we calculate difference in copy/µl count on lowest/ shortest and highest/longest setting. This gives us 106% in difference. This shows us that the optimalisation of the process, and finding best conditions is crucial for the best results.

From table 25 can we also see that when comparing copy/µl count in tissue samples bead beated for 5 minutes at 4m/s and 6m/s the number of bacteria is much higher at 4m/s.

The longer time is used for bead beating process, the less copies is present in the samples, which shows that the most optimal duration for all types of samples used in this paper would be 4m/s at 5minutes.

5. Conclusion

The main aim for this study was to establish most efficient method for extraction of low microbiota from tissue from patients with colorectal cancer. The main expected result was to find the best time and speed for bead beating process, resulting in highest concentration of bacterial load in samples. All samples needed to be also suitable for downstream application and next- generation sequencing and that's why optimalization of processes was so important. Other big focus in this study was to establish and validate an effective method for quantification of bacterial load and developing a standard curve for accurate quantification of samples with unknown concentration of bacteria.

The results obtain in this study helps us understand more about how different speed and time of bead beating influence DNA concentration, quality of DNA and bacterial copy count in samples. We also learned how big impact contaminations have on samples, and how important it is to follow protocols to avoid contamination.

The overall survival for patients with CRC is good but there are still some aggressive forms of colorectal cancer and therefore it´s important to learn more about biology of development of cancer, and there is much more that needs to be learn.

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Appendix

When testing samples on qPCR relative fluorescence unit (RFU) is detected. Figure 1 shows in which of the cycles RFU was detected in different samples. Samples used in this test were the designed plasmid and ZymoBIOMICS Microbial Community DNA standard (D6305, Zymo Research)

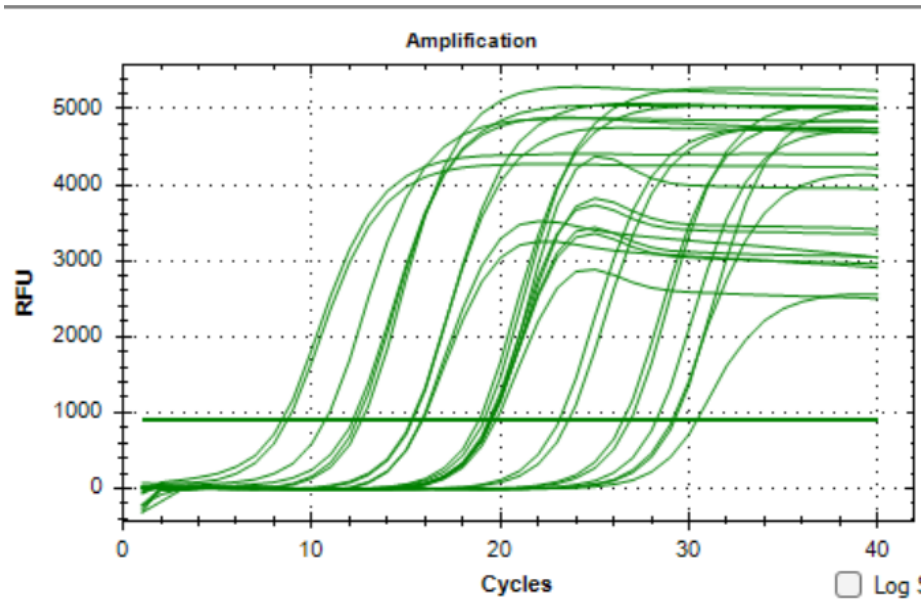

Figure 1: Results of qPCR testing of designed plasmid and ZymoBIOMICS Microbial Community DNA standard (DNA std).

Figure 2 shows in which of the cycles RFU was detected when performing temperature gradient. Samples used in this run were designed plasmid and ZymoStd.

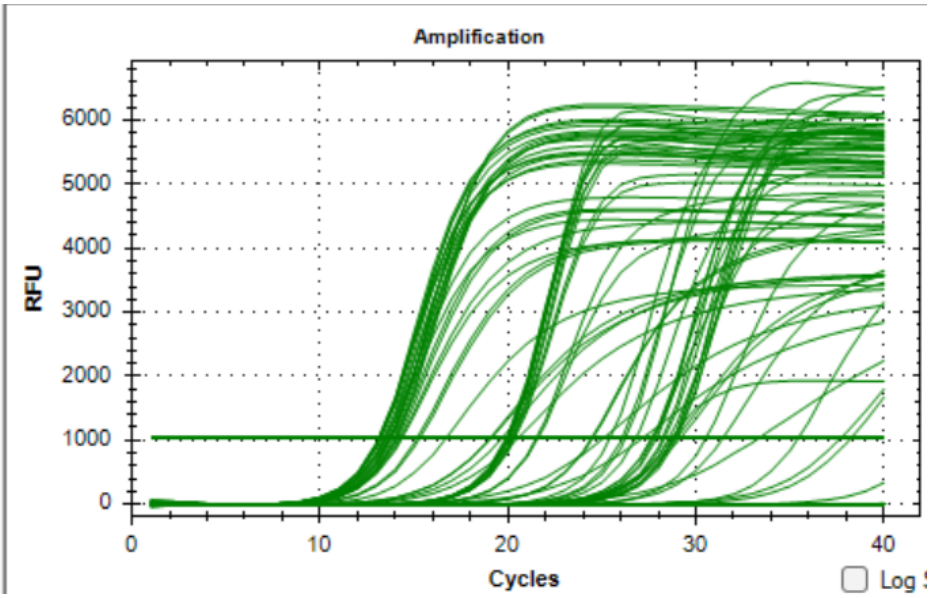

Figure 2: results of qPCR optimalization using temperature gradient, with plasmid samples and ZymoBIOMICS Microbial Community DNA standard.

Figure 3 shows in which cycle RFU was detected when performing qPCR optimalization with temperature gradient and primer matrix without sample input.

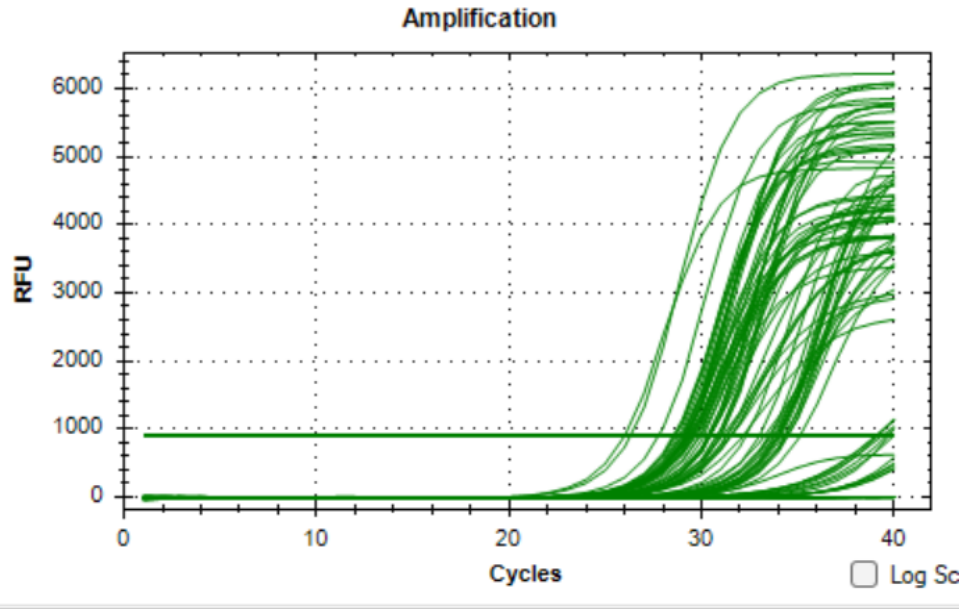

Figure 3: Results of qPCR optimalization with temperature gradient and primer matrix without sample input.

Figure 4 shows in which cycle RFU was detected when performing qPCR optimalization with temperature gradient and primer matrix without sample input.

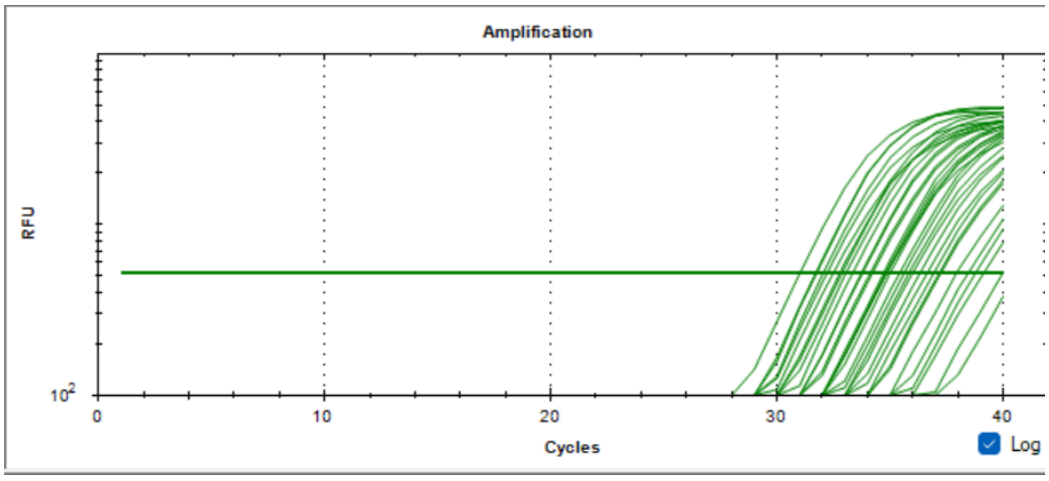

Figure 4: *Results of qPCR optimalization with temperature gradient and primer matrix without sample input.*

Figure 6 shows in which cycle RFU was detected when performing qPCR with 8 samples of 10- fold dilution of the designed plasmid. qPCR was performed at 64°C.

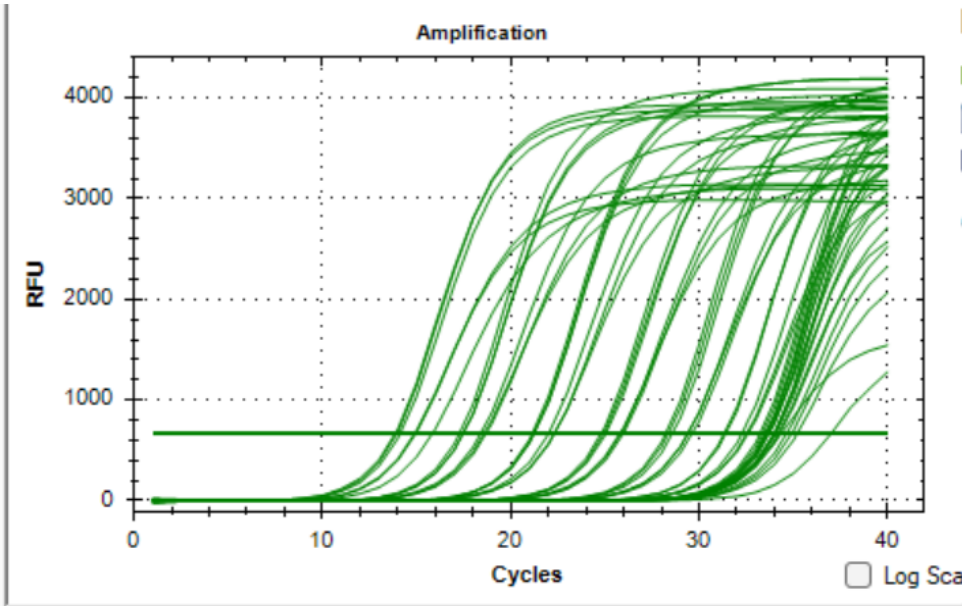

Figure 6: Results of qPCR optimalization using 8 10-fold dilutions of plasmid at 64 ℃.

Figure 7 shows at which cycle RFU was detected when performing qPCR with designed plasmid and extracted DNA that was bead beated at 4m/s. Samples in this run were put at 64°C and primer concentration used was 0,1M forward/ 0,1M reverse.

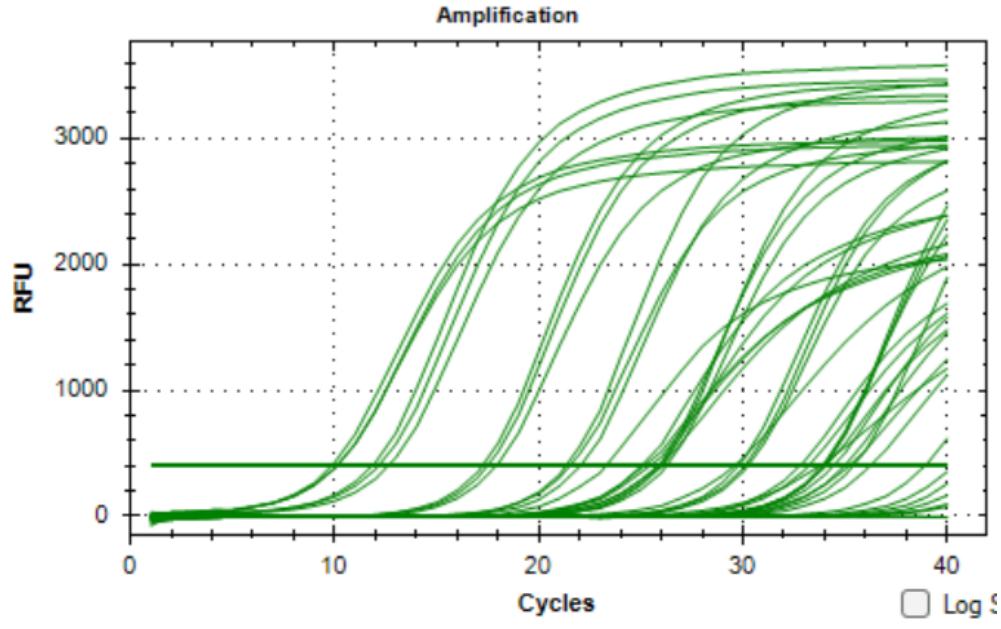

Figure 7: Results of qPCR with designed plasmid and with extracted DNA.

Figure 7 shows at which cycle RFU was detected when performing second qPCR with designed plasmid and extracted DNA that was bead beated at 4m/s. Samples in this run were put at 64°C and primer concentration used was 0,1M forward/ 0,1M reverse.

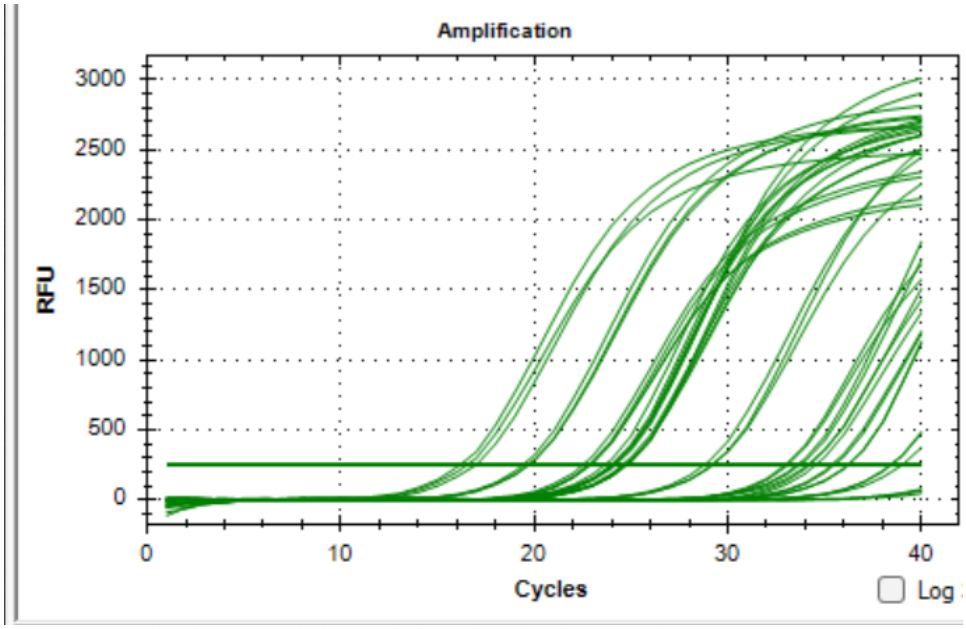

Figure 8: Results of qPCR with designed plasmid and with extracted DNA.

Figure 7 shows at which cycle RFU was detected when performing third qPCR with designed plasmid and extracted DNA that was bead beated at 6m/s. Samples in this run were put at 64°C and primer concentration used was 0,1M forward/ 0,1M reverse.

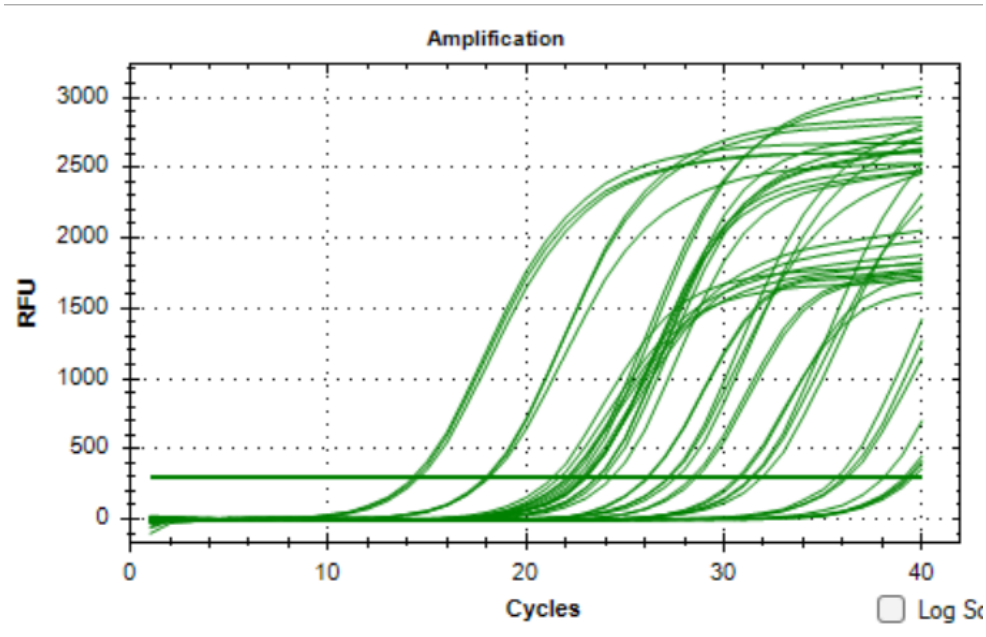

Figure 9: Results of qPCR with designed plasmid and with extracted DNA

Figure 10 shows standard curve calculated from plasmid samples from qPCR. Plasmid samples were diluted 6 times using 10- fold method, and first concentration used is 0,2ng/µl. Standard curve was later used to calculate bacterial load in samples with unknown concentration. Results of that are shown in table 24.

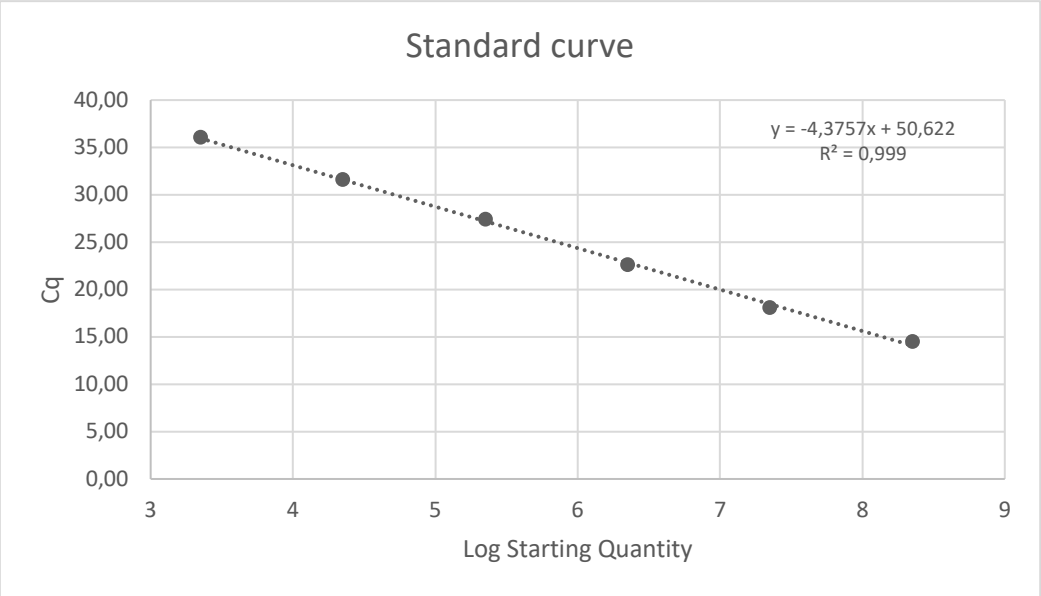

Figure 10: standard curve based on results from qPCR with designed plasmid. Plasmid is diluted 6 times using 10-fold method, start concentration is 0,2ng/µl.