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Optimization of DNA Extraction for Low-Biomass Microbiome Studies in Tissue from patients with Colorectal Cancer

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Abstract

Colorectal cancer (CRC) is the second most common cause of cancer-related deaths worldwide with an unknown cause. (1) CRC is the second most common type of cancer in Norway and the occurrence of CRC has tripled during the past 60 years. As a result of late detection, around 40 % will die from the disease within 5 years of diagnosis. (2)

To understand the impact tumor bacteria can have on various cancer hallmarks, characterization of the tumor microbiome can be an essential step. (3) In this study, we focused on optimizing the extraction efficiency for tissue samples with low bacterial biomass to mitigate bias arising from discrepancies in various extraction procedures. We tested several parameters, including the duration and intensity of bead beating, to enhance extraction efficiency. To estimate extraction efficiency, we developed an assay for the absolute quantification of bacterial load using qPCR and SYBR green dye. The extraction was a success, and the amplification of the tissue samples gave good results for both intensity of bead beating. The development of an assay and qPCR was successfully executed and gave a reliable and reproduceable absolute quantification based on standard curve, which was used to calculate the bacterial load for our samples. The next step in the process would be Next-Generation Sequencing, which is coming as a future work.

Content

Abbreviations

CRC	Colorectal Cancer
C-section	Caesarean section
mCRC	metastatic CRC
rRNA	Ribosomal RNA
dsDNA	Double stranded DNA
gDNA	Genomic DNA
ZymoSTD	ZymoBIOMICS Microbial Community Standards
PCR	Polymerase chain reaction
qPCR	Quantitative polymerase chain reaction
TAE- buffer	Tris, acetic acid, EDTA- buffer
NTC	No template control
bp	Base pair
SNP	Single nucleotide polymorphism
DNA STD	ZymoBIOMICS Microbial Community DNA Standard
NGS	Next generation sequencing
N/A	Not available

Introduction

1.1 Colorectal cancer

Colorectal cancer (CRC) is the second most common cause of cancer-related deaths worldwide and is the third most diagnosed cancer worldwide.(1) The colon can be divided into two sections; the right colon, including cecum, ascending colon, and right 2/3 transverse colon, and the left colon, which includes left 1/3 transverse colon, descending colon, and sigmoid colon. The primary role of the right colon involves the absorption of water and certain nutrients, whereas the primary role of the left colon is to store and excrete feces. (1)

It is suggested that bacteria may interfere with the molecular mechanisms underlying CRC, due to a present of an imbalance of the gut microbiome (4). There are three ways colon cancer can be present; sporadic, familial clustering and inherited syndromes. The sporadic colon cancer has an average age diagnosis older than 50 years old and is most likely linked to environmental factors. This differentiates from patients with a true inherited pattern that carries a higher risk at a younger age (younger than 50 years). The last one, familial clustering is the absence of identifiable inherited syndrome. (5)

Most CRC cases occur in individuals over the age of 55. The disease originates from small growths known as polyps, in the intestines. Polyps are prevalent, with approximately 1 in 4 55-years old having them. Most polyps are benign and will never be dangerous, but a few of them have potential to grow and develop into cancer. (2) The cause of CRC remains unclear, but it is suggested that the occurrence of CRC is strongly related to environmental factors, lifestyle, and diet. This is because of the correlation between migration, religious factors, and the CRC.(1) CRC is a disease influenced by multiple facts. The epithelial cells within the colorectal mucosa may experience hyperplasia, atypical hyperplasia (mild, moderate, severe) and the formation of adenomas, which can progress to carcinoma over time. Typically, this progression is triggered by carcinogenic factors that induce structural alternations in DNA, ultimately resulting in the malignant transformation of cells and the development of cancer.(1)

The leading cause of death in patients with CRC is metastasis. The most common metastatic site is the liver, followed by the lungs, lymph nodes and peritoneum. At initial diagnosis approximately 25% of CRC patients have distant metastases and during the disease almost 50% will develop metastases. The primary strategy to treat metastatic CRC (mCRC) includes immunotherapy, chemotherapy, targeted therapy, and their combinations. The effectiveness of these therapies is still compromised by drug resistance as the primary culprit. (6)

In Norway, CRC is the second most common type of cancer, and the occurrence of CRC has tripled during the past 60 years (2). Every year more than 4000 new cases of CRC are discovered in Norway. Worldwide, Norwegian women are at the top in the number of new cases of CRC while Norwegian men are in 9th place. The treatment of CRC is becoming increasingly better but since it is often detected late, about 40% will die from the disease within 5 years of diagnosis. However, over 95% of patients who are diagnosed with the disease in the earliest phase are alive after 5 years. (2)

1.2 Microbiome

The term gut microbiota refers to the microorganisms that settles in the intestinal tract and lives in symbioses with the host. The various interactions between the human body and the microbiome may be commensalistic, mutualistic or pathogenic. When it comes to microbes there are trillions involved. It is mostly bacteria but also viruses and fungi. The microbiota is established already at birth, as well as later by being exposed to microbes in the environment. (7, 8)

As mentioned above, the microbiota begins to develop as early as birth and the way a baby is born can influence how it develops. Both vaginal delivery and caesarean section (C-section) gives exposure to microbes but with different types. During the vaginal birth delivery, the newborn is exposed to maternal vaginal bacteria and their microbiota composition tend to reflect this region, while the C-section birth is influenced by the maternal skin flora and tend to have less diverse microbiota. Generally, newborns delivered vaginally show a higher level of similarity in gut microbiota with their mothers, compared to newborns delivered by C-section. The difference in gut microbiota can be that the newborns delivered by C-section has a higher risk of developing infections or allergies. (9)

The interaction between the body and microbiota plays a crucial role in sustaining overall health and well-being, seen as the microbes' influence metabolism and immune regulation, which affects the mental function. Age, nutrition, lifestyle, inherited genes, hormonal changes, and underlying diseases are factors that influence the human microbiome at any specific moment. Dysbiosis or an impaired biota can cause an inflammation in the brain that can lead to psychopathology, such as anxiety or depression. Other diseases that have been linked to dysbiosis of the human microbiota are cancer, cardiovascular disease, bowel inflammatory disease and difficult-to treat bacterial infections. (7, 8)

One of the pathogenic factors affecting the CRC metastasis is gut microbiota as certain pathogenic bacteria, such as *Streptococcus bovis* (*S. bovis*) and *Enterococcus faecalis* (*E. faecalis*), trigger inflammation and therefore contribute to the development of CRC. The specific function of the gut microbiota in tumor metastasis remains under investigated but there is still an increasing concern regarding the significant contribution of the gut microbiota to the development of mCRC. The concern is because bacteria such as *Fusobacterium nucleatum* (*F. nucleatum*), *Bacteriodes fragilis* (*B. fragilis*), and *Escherichia coli* (*E. coli*) can engage with the CRC cells, thereby promoting tumor invasiveness, by invading the tumor microenvironment.(6)

To get a better understanding of CRC and what causes the disease, getting more information about the microbiota and what kind of microorganisms that live there will help finding information about how the microorganisms in the microbiome contribute to the development of CRC and how one can treat or avoid the disease.

1.3 Intratumoral microbiome

In several kinds of tumor tissues there have been found different microbial components which are strongly associated with the cancer initiation and development. According to studies the gut microbiota is important for regulation of host immune responses, however the intratumoral microbiota could also play a role in influence local immune responses within the tumor microenvironment, and further affect tumor progression. The antitumor immunity can be influence by the intratumoral microbiota, either by enhancing or decreasing antitumor immune responses and inducing different immunotherapy efficacies and outcomes. (10)

Seen as intratumor bacteria have been identified in numerous cancer types but these bacteria are still lacking a comprehensive characterization, characterization of the tumor microbiome can be an essential step to understand the impact that tumor bacteria can have on various cancer hallmarks. The gut microbiome has appeared to have diverse effects on tumor biology, influencing processes like transformation, tumor progression and the response to anticancer therapies which includes immunotherapy. (3)

The potential sources of intratumoral microorganisms can be classified into three categories: through mucosal barrier sources, from adjacent normal tissue or through hematogenous spread. The mucosal barrier source includes pancreatic cancer, colorectal cancer, and other digestive tract tumors. The organs involved have a cavity that is exposed externally.

Microorganisms living on the mucous membrane may invade the tumor because the mucosal barrier is damaged during the formation of the tumor. The source from adjacent normal tissue stems from a study revealing the presence of bacteria in organs previously believed to lack them, with bacterial patterns in tumor tissues closely mirroring those in nearby healthy tissues. Additionally, the immune system suppression and lack of oxygen in tumors encourage bacterial growth. However, the origins of microorganisms in healthy tissues remain uncertain, and it may spread from the tumor site. Because of this, further research is required for confirmation. Through hematogenous spread, microorganisms from the mouth and intestines may travel through the bloodstream to reach the tumor site, infiltrating the tumor via compromised blood vessels. (10)

To study the intratumoral microbiota there are different studies using different types of methods to find the best way to get more information. Our study wants to compare and optimize our method to get unbiased results and have control over possible contamination.

Aim

The main aim is to optimize the protocol for the extraction of low-biomass microbiome in tumor tissue. To achieve this, our first objective is to evaluate how the intensity and duration of bead beating affect the efficiency of bacterial extraction. Next, we wanted to assess the bacterial load in our tissue samples, for which we have another object to establish an assay for the absolute quantification of bacterial load. These steps will be useful for further studies and downstream applications, including next-generation sequencing.

Methods

Table 1:Primer names of primers used in this project and their 5'-3' sequence.

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 (11)
16S Amplicon PCR Forward Primer	TCGTCGGCAGCGTCAGATGTGTATAA GAGACAGCCTACGGGNGGCWGCAG (12)
16S Amplicon PCR Reverse Primer	GTCTCGTGGGCTCGGAGATGTGTATAA GAGACAGGACTACHVGGGTATCTAATCC (12)

3.1 Tissue samples

Individuals were enrolled from an ongoing perspective, clinical-molecular biomarker outcomes study, the ACROBATICC project (13). The bachelor project involving human participants performed in compliance with national regulations and approved by the Norwegian Regional Ethics Committee (REK Helse Vest, #2012/742). All activities involving human samples performed in accordance with the ethical standards of the Stavanger University Hospital and/or national committee and with the 1964 Helsinki Declaration and its later subsequent updates or comparable ethical standards. (13)

3.2 DNA Extraction

The efficiency of the extraction process from tumor tissue was assessed by utilizing the Zymbiomics Microbial Community Standard (Later mentioned as ZymoSTD, Zymo Research, Lot. 13683). This standard is a mock microbial community that includes three easy-to-lyse Gram-negative bacteria, five tough-to-lyse Gram-positive bacteria and two tough-to-lyse yeasts. (14)

DNA was extracted using Zymbiomics DNA Miniprep kit (D4300T, Zymo Research) according to manufacture instructions. The ZymoSTD was extracted alongside the tumor tissue samples, including samples of tumor tissue that were spiked with 10% of the standard.

All samples underwent the same extraction process to ensure consistency. All steps were performed in sterile conditions at the Laminar Flow Hood. Samples with ZymoSTD were prepared with 75 µl ZymoSTD, 95 µl Solid tissue buffer blue (Zymo Research, Lot. 221990), 20 µl ultrapure distilled water (Invitrogen, lot. 2436576) and 10 µl of 20 mg/µl Proteinase K (Stock concentration, Zymo Research, lot. 196556 and Omega Bio-tek, lot. 27845EM253) in the RNase- and DNase free microcentrifuge tubes.

Tissue samples were prepared using about 15 mg of tissue sample of colorectal tumor. Tissue samples were cut, transferred in the tubes, and mixed, with 95 µl of solid tissue buffer blue, 95 µl of ultrapure distilled water and 10 µl of proteinase K.

Spike-in samples were prepared with the same amount of tissue, around 15 mg, and mixed with 95 µl solid tissue buffer blue, 87.5 µl ultrapure distilled water, 7.5 µl ZymoSTD and 10 µl proteinase K.

All tubes were vortexed, spined, and placed on heating block. The samples incubated at 55 °C with vigorous shaking overnight (o/n). The incubation allowed the tissue to fully break down and helps the proteinase K to break down proteins and enzymes.

3.3 Bead Beating and DNA extraction.

The samples were taken of the heat and transferred to 2 ml ZR BashingBead lysis tube (Zymo research, lot. 960009) and mixed with 750 µl ZymoBIOMICS Lysis solution (Zymo research, Lot. 227803). In this experiment, the different duration and intensity were used to test for what the best optimal results on quality and quantity of DNA extraction. The homogenizer, FastPrep®-24 is used to ensure that the microbial extraction is bias free. The tubes were placed in a MP FastPrep-24 homogenizer, and bead beated for 5, 6 and 7 minutes. When bead beating the samples, both intensity of 4 m/s and 6 m/s were used.

Afterwards, the BashingBead lysis tubes were centrifuged at 10.000 x g for 1 minute, and then 400 µl of the supernatant was transferred to the Zymo-spin III-F Filter (Zymo research, lot 430539) in a collection tube, and centrifuged again at 8.000 x g for 1 minute. The III-F filter was discarded.

To the remaining supernatant 1200 µl ZymoBIOMICS DNA binding buffer (Zymo research, lot.22487) was added and mixed well. Next, 800 µl of the mixture was transferred to a Zymo-spin IICR column (Zymo research, lot. 817622) in a collection tube and centrifuged at 10.000

x g for 1 minute. The flow through was discarded from the collection tube and the step was repeated. After that, 400 µl of ZymoBIOMICS DNA Wash Buffer 1 (Zymo research, lot. 228076) was added to the IICR column in a new collection tube and centrifuged at 10.000 x g for 1 minute, the flow-through was discarded. 700 µl of ZymoBIOMICS DNA Wash buffer 2 (Zymo research, lot. 228956) was added to the IICR column in a collection tube. The tube was centrifuged at 10.000 x g for 1 minute and the flow-through was discarded. An addition, 200 µl of DNA Wash buffer 2 was added to the IICR column and centrifuged at 10.000 x g for 1 minute.

The IICR columns were transferred to clean 1.5 ml microcentrifuge tubes and between 50-100 µl of Ultrapure distilled water was added directly to the column matrix. The tubes incubated for 1 minute before they were centrifuged at 10.000 x g for 1 minute to elute the DNA. The eluted DNA is now suitable for PCR and other downstream applications.

3.4 Increasing and purification of DNA concentration.

To get the best result possible and increasing of DNA concentration and purification was done on samples with bead beating intensity of 4 m/s. Increasing of DNA concentration was done by adding 15 µl of sample, 5 µl of 10 mM in water $AcNH_4$ and 40 µl of 100% ethanol, giving a total volume of 60 µl, in microcentrifuge tubes. 8 tubes were made in total, one for each sample. The mixture was vortexed and spined to mix it well. After it was stored in -20 °C, overnight.

To purify the DNA the samples centrifuged at max speed for 30 minutes. The supernatant was carefully removed, and the pellet was carefully washed with 200 µl of cold 75% ethanol. The samples were centrifuged again at max speed for 15 minutes. The liquid was removed, and the tubes were placed on ice to dry, for 10 minutes. Lastly, the samples were resuspended in 10 µl of 40 mM Tris, 20 mM Acetate and 1 mM EDTA (TAE buffer) or ultra-pure water.

3.5 DNA concentration measurement

Qubit 4 Fluorometer is a device used to accurately measure the quality of DNA, RNA, or protein. The fluorescent dyes bind selectively to DNA, RNA, or protein and only when the optimized dyes are bound to the target, a fluorescent signal will emit. The Qubit measure the intensity of the signal from fluorescent dyes bound to specific biological molecules. (15)

Using Qubit is a practical way to check the DNA concentration before next-generation sequencing (NGS) as it measures intact dsDNA. (16) In this experiment Qubit ds DNA Broad range assay (Invitrogen) was used for measurement of DNA after DNA extraction from ZymoSTD and cancer tissue sample. 190 µl of 1x ds DNA BR Working solution (Invitrogen, lot. 2610295) was added to 0.5 ml Qubit assay tubes (Invitrogen, lot. Q32865) with 10 µl Qubit 1x dsDNA BR Assay standard 1 and 2 (Invitrogen, lot. 2525748 and lot. 2525748). For the samples, 198 µl 1x ds DNA BR Working solution and 2 µl of the sample were added to the 0.5 ml Qubit assay tubes. The tubes were vortexed, spined, placed in the dark for 2 minutes, before they were measured.

In addition to Qubit, NanoDrop was also used to measurement of DNA concentration. The NanoDrop spectrophotometer is one of the most useful methods to estimate DNA concentration and purity through absorbance measures.(17) Measurement of both bead beating for 4 m/s and 6 m/s was taken. 2 µl of the samples were transferred to PCR tubes, then measured.

3.6 PCR

The genomic DNA purified from human tissue was assessed on the presence of bacterial DNA using primers amplifying either V3V4 region or full gene or bacterial 16S rRNA.

Both V3V4 and 16S rRNA gene PCR were made with primers suitable for them. For 16S rRNA gene PCR, 10 µM 27F and 10 µM 1492R were used. While for V3V4 16S Amplicon PCR Forward Primer and 16S Amplicon PCR Reverse Primer were used.

The reaction master mix contained 1x HotStarTaq Master mix (Quagen, lot. 172023740), 0.2 µM of forward primer, 0.2 µM of reverse primer, template of 30-60 ng and ultra-pure water. The total volume was 30 µl. The mix was dispensed into PCR tubes then placed in the thermal cycler and programmed according to the manufacturer's instructions.

1% agarose gel was prepared by mixing 3 gr agarose (Bioline, lot. ES520-B072820), 300 ml 1x TAE buffer and 30 µl Gelred 10,000 x in water (Biotium, lot. 9G0529).

3 µl of Generuler 1 Kb DNA Ladder (Thermo scientific, lot. 2791887) was added to one of the first wells as a ladder. To the samples 10 µl of 6x TriTrack DNA loading dye (Thermo Scientific, lot. 2822347) was added, then 20 µl of the samples were added to the agarose gel wells.

3.7 Development Of standard curve with plasmid containing16S rRNA gene

We wanted to establish a qPCR method for the quantification of bacterial DNA in genomic DNA purified from human tissue. To do that, we have designed a plasmid containing the 16S rRNA gene from *Peribacillus frigoritolerans,* a bacterium that has only one copy of the 16S rRNA gene. For quantification of bacterial load in this experiment, qPCR was used. The set of primers for amplifying the V6 region was employed as previously described (3). Preparation of plasmid included the amplification of gene of interest from *Peribacillus frigoritolerans,* gel purification of amplified PCR fragment, assembly of PCR fragment with the plasmid, transformation of assembled plasmid to the chemically competent cell, colony PCR, plasmid prep, plasmid verification by PCR and plasmid verification by sequencing.

3.7.1 Amplification of gene of interest from gDNA from Peribacillus frigoritolerans

The first step of the preparation of plasmid is amplification of 16S rRNA gene from Peribacillus frigoritolerans. This was done by using primers that was designed from the gDNA of the *Peribacillus frigoritolerans* bacteria. Initially, the PF-16-S and 1492R primers were prepared by diluting them to a final concentration of 100 µM. The forward primer, P1-16S-F was diluted with 263 µl of water while the reverse primer, 1492R, was diluted with 295 µl of water. Additionally, DNA from 8801 *Peribacillus frigoritolerans* was diluted by adding 12.5 µl ultra-pure water to 5µl bacteria. The diluted bacteria had a concentration of 4 ng/µl.

3.7.2 Gel extraction of PCR product

A reaction mix were prepared containing 1x Kapa Hifi HotStart ready mix (Roche, Cat. KK2602), 0.3 µM Forward primer, 0.3 µM reverse primer, 3% v/v DMSO, 1 µl template, and Ultra-pure water. The total volume was 25 µl. The forward primer used was PF-16-S, and the reverse primer used was 1492R. The reaction was made with kapa polymerase. 25 µl of the reaction mix were transferred to PCR tubes, then the tubes were vortexed and spined down. The PCR protocol was performed with the following cycling protocol:

Table 2: PCR cycling protocol for gel extraction of PCR product.

Step	Time	Temperature
Initial denaturation	3 min	95 °C
Denaturation	20 sec	98 °C
Annealing	15 sec	55-70 °C
Extension	1 min	72 °C
Number of cycles	25	
Final extension	10 min	72 °C

3 µl of Generuler 1 Kb DNA Ladder was added to one of the first well as a ladder. 5 µl of 6x TriTrack DNA loading dye was added to the samples before 9 µl of the samples were added to the 1% agarose gel in TAE buffer wells. The electrophoresis was performed for 30 minutes at 140V.

The agarose gel was put under a UV illuminator to highlight the band and then the gel was cut around the band by a sterile scalpel and transferred to the microcentrifuge tube to measure the weight. The equal volume of binding buffer XP2 (Omega Bio-Tek, lot.BB081216QG2365) was added to the cleaved agarose band. Each tube was then incubated for 7 minutes on 60 °C, the tubes were vortexed for 2-3 minutes each.

A HiBind DNA Mini column (Omega, lot. BVT081016R2271031356) was placed in a 2 ml collection tube and 700 µl of the solution containing dissolved agarose with PCR product were added to the column and centrifuged at 10.000 x g for 1 minute at room temperature. The filtrate was discarded. The steps were repeated until all samples has been transferred to the column. 300 µl Binding buffer XP2 (Omega, lot. BB081216QG2365), centrifuged a 13.000 x g for 1 minute and the filtrate was discarded. 700 µl SPW wash buffer (Omega, lot. SPW071416JC2353) was added, centrifuged at 13.000 x g for 1 minute and the filtrate was discarded. The empty HiBind DNA mini column was centrifuged at 13.000 x g for 2 minutes to dry the column matrix. The column was transferred to a clean 1.5 ml microcentrifuge tube and 50 µl Ultra-pure water was added directly to the center of the column membrane. The tubes sat at room temperature for 2 minutes before centrifuged at 13.000 x g for 1 minute.

3.7.3 NEBuilder® HiFi DNA assembly of 16S rRNA gene DNA from Peribacillus frigooritolerans and transformation of assembly to the 10-Beta bacteria cells

After gel purification the PCR product with highest DNA concentration was chosen for assembly with the plasmid.

The ssOB1 and ssOB2 bridges were diluted to 100 µM with ultra-pure water, vortexed and spined. The bridges were further diluted to 1µM with NEBuffer 2 (New England Biolabs, lot. 10162785) by mixing of 1 µl of MA_ssOB1, 1 µl of MA_ssOB2 and 98 µl NEBuffer 2.

A reaction mixture of assembly was prepared by adding 1.49 µl of pBS_VBB, 1.84 µl of PCR fragment, 0.25 µl of the bridge mixture and 3.58 µl of NEBuilder HiFi DNA Assembly Master Mix (New England Biolabs, Cat. E2621). The reaction mixture was incubated for 1 hour at 50 °C.

When the incubation was done 2 µl of the mixture made was added to the thawed-on ice 10-beta bacteria (50-100 µl) (New England Biolabs, lot. C3019H) in a new tube. The new mixture sat for 20 minutes on ice before it was placed on 42 °C heat for 30 seconds. The tube was placed on ice for 2 minutes then 1 ml of prewarmed outgrowth medium (New England BioLabs, Lot. 10186900) was added. It was then incubated at 37 °C, 10.000 rpm shake for 1 hour. When the incubation was done, samples of 75 µl, 100 µl and 150 µl was plated on LB agar plates with 100 µg/ml ampicillin.

The tube with the rest of the mixture was spined down and 600 µl of media was discarded before a new plate with 150 µl sample was plated onto a new LB agar plate with ampicillin. The four plates were incubated overnight at 37 °C.

3.7.4 Colony PCR with the target plasmid

A reaction master mix was prepared by adding 1x HotStarTaq Master mix, 0.2 µM of forward primer, 0.2 µM of reverse primer, 3% v/v DMSO and ultra-pure water. The total volume was 15 µl. One colony from the template was added to the tubes. 11 tubes were prepared, 10 with template and 1 negative control. Before added to the reaction master mix, the primers MRT-125 and V6_Fp_R1 was diluted with water to 10 µM.

The reaction mix was transferred to PCR tubes, vortexed and spined down. The PCR was performed with the following cycling protocol:

Table 3: PCR cycling protocol for colony PCR with the target plasmid.

Step	Time	Temperature
Initial denaturation	15 min	95 °C
Denaturation	30 sec	95 °C
Annealing	30 sec	55 °C
Extension	1 min, 10 sec	72 °C
Number of cycles	27	
Final extension	10 min	72 °C

To the tubes 3 µl of loading dye was added. 3 µl of DNA ladder and 18 µl of sample were added to the 1% agarose gel wells, then electrophoresis was performed for 30 minutes at 140V.

3.7.5 Plasmid mini prep

Four overnight cultures were prepared by adding 10 ml LB media with 100 µl/ml of ampicillin to four sterile glass tubes, then one culture was added to each tube. The cultures that were picked were three colonies from the 75 µl template LB agar plate, one colony from the 100 µl template LB agar plate and one colony from the 150 µl template LB agar plate. The tubes were incubated at 37 °C with 250 rpm shake over-night.

Purification of the DNA was done using the QIAprep spin miniprep kit (QIAGEN, ref:27104).

5 ml of the overnight culture were transferred to a tube and centrifuged at 4000 x g for 5 minutes, the fluid was discarded. 350 µl of solution I (Omega, lot. 47830FM98), mixed with RNase A, were added to the tube and vortexed. 350 µl of solution II (Omega, lot. 47458FO105) was added and the tube was turned to mix it slightly. The tube incubated in room temperature for 2-3 minutes before 450 µl of solution III (Omega, lot. 44640FP90) was added and centrifuged at 13.000 x g for 10 minutes. In a QIAprep 2.0 spin column (QIAGEN, lot. 154028393) the fluid from last step was added and centrifuged at 13.000 x g for 1 minute and the filtrate was discarded. This step was repeated one more time. 500 µl of HBC buffer diluted with 100% isopropanol (Omega, lot. 44822CB163) was added and centrifuged at 13.000 g x for 1 minute, the filtrate was discarded, and the step was repeated. 700 µl DNA Wash buffer (Omega, lot. 45741AM198) was added to the column and centrifuged at 13.000 x g for 30 seconds. After, the empty column was centrifuged at 13.000 x g for 2 minutes before

it was transferred to a new 1,5 ml microcentrifuge tube. 50 µl of Elution buffer (Omega, lot. 49043BD458) was added and the tubes sat in room temperature for 1 minute, then centrifuged at 13.000 x g for 1 minute.

3.7.6 Verification of Plasmid by PCR

The plasmid was diluted to 4 ng/µl by using 0.5 µl of the plasmid and dilute it with the correct volume of ultra-pure water.

A reaction mix was prepared by adding 1x HotStarTaq master mix, 0.2 µM MRT-156 primer (Invitrogen), 0.2 µM 1492R primer (Invitrogen), 3% v/v DMSO (Thermo Scientific, lot. P24I065), 1 µl template and ultra-pure water. The total volume was 20 µl. A total of 6 PCR tubes were made, vortexed and spined down. The PCR was performed using the following cycling program:

Table 4: PCR cycling protocol for verification of plasmid.

Step	Time	Temperature
Initial denaturation	15 min	95 °C
Denaturation	30 sec	94 °C
Annealing	30 sec	55 °C
Extension	1 min, 40 sec	72 °C
Cycles	25	
Final extension	10 min	72 °C

4 µl of loading dye was added to the tubes. 3 µl of DNA ladder and 24 µl of the template mixture were added to the agarose gel wells, then electrophoresis was performed for 30 minutes at 140V.

3.7.7 Verification of plasmid by sequencing

Before the plasmid could be sent to be sequenced, some preparation had to be done. The plasmid was diluted to 80 ng/µl by adding the correct volume of ultra-pure water. 12 µl of the new diluted plasmid was transferred to a tube and 3 µl of 20 µM of the primers were added. The primers used were V6_Fp_R1, 156 forward primer and 261 reverse primer. The plasmid solution was then sent to sequencing.

3.8 qPCR for plasmid containing 16S rRNA gene.

Quantitative PCR (qPCR) is a PCR-based technique that combines the amplification of a target DNA sequence with the quantification of its concentration in the reaction. This technique is a commonly used analytical method for evaluation DNA copy number, viral load, single nucleotide polymorphism (SNP) detection and allelic discrimination. This is because the technique allows for the determination of the initial template concentration. (18)

The preparation of reaction mix for qPCR was done in sterile conditions using a sterile bench. The reaction mix included 1x SsoAdvanced Universal SYBR Green Supermix (later mentioned as SYBR Green Supermix, BIO-RAD, cat.1725271), variable volume and concentration of both forward and reverse primer, 2-5 µl of template and Ultra-pure water. The total volume of the reaction mix was 20 µl.

The different concentrations of primer used ranged from 0.1-0.3 µM, and the primers used were V6-Fp forward primer and V6-Fp-R reverse primer. For the template, 2 µl with plasmid and 5 µl of DNA standards were added.

The DNA STD used was ZymoBIOMICS Microbial Community DNA standard (Zymo Research, Cat. D6305). The template was diluted using ten-fold dilution.

The qPCR was performed using the following cycling program:

Table 5: qPCR cycling protocol for plasmid containing 16S rRNA gene.

Step	Time	Temperature
Initial denaturation	3 min	98 °C
Denaturation	10 sec	98 °C
Annealing	15 sec	64 °C
Cycles	40	
Melting curve		50- 95 °C

3.8.1 optimizing qPCR plasmid 16S rRNA gene.

After watching the first qPCR an optimizing of qPCR plasmid 16S rRNA gene was made. The changes that were made were regarding the temperature, gradient and primers. The primer ration used was now 0.2 µM of both forward and reverse primer. This time the qPCR had a gradient with 70-54 °C in range, but the rest of the cycling program remained the same.

3.8.2 Primer matrix

In one of the qPCR that was tested, the results showed possible primer dimers so a primer matrix was made to check if the primer dimers would continue appearing in some concentrations.

Four different reaction mixes were made with different primer concentrations. The reaction mix was prepared by adding 1x SYBR green super mix, V6-FP forward primer, V6-PF-R reverse primer and ultra-pure water, with the total volume of 20 µl. The primers of the first reaction mix had a concentration of 0.1 µM of both forward and reverse primer. The second reaction mix had primer concentration of 0.1 µM of forward primer and 0.2 µM of reverse primer. The third reaction mix had primer concentration of 0.2 µM of forward primer and 0.1 µM of reverse primer. The fourth reaction mix had primer concentration of 0.15 µM of both forward and reverse primer.

The reaction mix was transferred to the qPCR wells, and each reaction mix had three parallels. The cycling program was the same as Table 5.

Results

4.1 Measurement of DNA concentration using Qubit fluorometer

After extracting the DNA, Qubit fluorometer was used to measure the DNA concentration, with the intensity of 4 m/s. Samples 1-3 were ZymoSTD, samples 4-6 was tissue samples and samples 7-9 was a spike-in including both tissue sample and ZymoSTD. The ZymoSTD were included to have a known concentration of the DNA, while the spike-in were includes to show some results, even if the tissue samples did not.

Samples 1,4,7 had a bead beating duration of 5 minutes, samples 2,5,8 had a bead beating duration of 6 minutes and samples 3,6,9 had a bead beating duration of 7 minutes. A negative control was also included to check for contamination.

The results of DNA concentration in samples with bead beating intensity of 4 m/s are shown in Table 6. The table shows that the ZymoSTD has a DNA concentration range of 485-600 ng, the tissue samples ranged from 1625-1795 ng and the spike-in ranged from 580-2005. The negative control showed a result that was too low, indicating no contamination.

Table 6 Results of Qubit measurements of 10 samples with bead beating intensity 4 m/s with different minutes.

Sample number	**Sample type and their bead beating time**	**Weight of tissue (in mg)**	**Results (ng/µl) with 50 µl**	**Results ng**
1	ZymoSTD 5	-	10.8	540
2	ZymoSTD 6	-	12	600
3	ZymoSTD 7	-	9.7	485
4	Tissue 5	15	34.7	1735
5	Tissue 6	15	35.9	1795
6	Tissue 7	17	32.5	1625
7	Spike-in 5	17	40.1	2005
8	Spike-in 6	16	29.3	1465
9	Spike-in 7	14	11.6	580
10	Negative control	-	Low	Low

After the measurement of samples with bead beating intensity of 4 m/s, a Qubit measurement of samples with a bead beating intensity of 6 m/s shown in Table 7 was done to verify if there would be any difference in results from the intensity of 4 m/s. In this part, only 50 µl of water was used to elute the samples, before the concentration was measured. The DNA concentration for the ZymoSTD samples ranged from 535-915 ng, the tissue samples ranged from 1670-2445 ng, and the spike-in samples ranged from 1775-3100 ng. The concentration for the ZymoSTD was deficient and were then eluted again with an additional 50 µl of water to check if the DNA concentration would increase.

Table 7 Results of Qubit measurements of DNA concentration of 10 samples with bead beating intensity 6 m/s with different minutes.

Sample number	Sample type and their bead beating time	Weight of tissue (in mg)	Results (ng/µl) with 50 µl water	Results ng	Results (ng/µl) with 100 µl water	Results ng
1	ZymoSTD 5	-	18.3	915	14.7	1470
2	ZymoSTD 6	-	10.7	535	6.26	626
3	ZymoSTD 7	-	12.0	600	8.32	832
4	Tissue 5	25	33.4	1670	-	-
5	Tissue 6	20	48.9	2445	-	-
6	Tissue 7	19	35.2	1760	-	-
7	Spike-in 5	19	62.0	3100	-	-
8	Spike-in 6	22	35.5	1775	-	-
9	Spike-in 7	19	37.3	1865	-	-
10	Negative control	-	Low	Low	-	-

Table 8 shows the results of a second round of samples with bead beating intensity of 6 m/s that were diluted with 100 µl water and had a bead beading intensity of 6 m/s and measured with Qubit. The second round was performed since the first round (Table 7) gave some low results. When the samples were taken of the heat the samples were not fully digested, which could affect the result. Table 8 shows that the DNA concentration for the ZymoSTD samples

ranged from 1940-2150 ng, the tissue samples ranged from 4120-4770 ng and the spike-in samples ranged from 3890-6160 ng. The negative control gave a results of "low", indicating no contamination.

Table 8: Results of Qubit measurement of DNA concentration for 10 samples with bead beating intensity 6 m/s with different minutes.

Sample number	Sample type and their bead beating time	Weight of tissue (in mg)	Results (ng/µl) With 100 µl water	Results ng
1	ZymoSTD 5	-	19.8	1980
2	ZymoSTD 6	-	19.4	1940
3	ZymoSTD 7	-	21.5	2150
4	Tissue 5	15	47.1	4710
5	Tissue 6	14	47.7	4770
6	Tissue 7	13	41.2	4120
7	Spike-in 5	12	61.6	6160
8	Spike-in 6	14	55.3	5530
9	Spike-in 7	11	38.9	3890
10	Negative control	-	Low	Low

The samples from bead beating with 4 m/s intensity went through an increase and purification to see if the DNA concentration would be improved. The concentration was measured with Qubit, shown in Table 9. The table shows that the DNA concentration of ZymoSTD ranged from 9.48-60.6 ng, the tissue samples ranged from 234-445 ng and the spike-in samples ranged from 318-338 ng. When comparing the results in Table 9 with Table 6, most of the samples gave a lower DNA concentration after the increasing and purification of DNA. The three exceptions are the tissue sample with a bead beating duration of 6 minutes and the spike-in samples with a bead beating duration of 6 and 7 minutes.

Table 9: Results of Qubit measurement of increasing and purification of DNA concentration for 10 samples with bead beating intensity 4 m/s with different minutes.

Sample number	Sample type and their bead beating time	Results (ng/µl) in 10 µl	Results ng
1	ZymoSTD 5	6.06	60.6
2	ZymoSTD 6	0.95	9.48
3	ZymoSTD 7	4.94	49.4
4	Tissue 5	25.2	252
5	Tissue 6	44.5	445
6	Tissue 7	23.4	234
7	Spike-in 5	33.8	338
8	Spike-in 6	31.8	318
9	Spike-in 7	31.8	318
10	Negative control	Low	Low

4.2 Measurement of DNA concentration using NanoDrop

In addition to Qubit measurement of the samples, NanoDrop measurement was also used to see how similar the results would be when comparing to the Qubit measurement results. Table 10 shows the results of NanoDrop measurement for 10 samples of both bead beating with 4 m/s intensity and bead beating with 6 m/s intensity. The table shows the results when measuring ng/µl as well as the absorbance at 260/280 and 260/230.

Table 10: measurements using NanoDrop for 10 samples of bb 4 m/s and 10 samples of bb 6 m/s.

Sample number	Sample type and their bead beating time	Bead beating 4 m/s			Bead beating 6/s		
		ng/ µl	260/280	260/230	ng/ µl	260/280	260/230
1	ZymoSTD 5	14.6	1.98	0.05	28.0	1.76	0.4
2	ZymoSTD 6	16.8	1.74	0.57	32.5	1.79	0.15
3	ZymoSTD 7	11.8	1.77	0.09	29.6	1.84	0.42
4	Tissue 5	46.7	1.78	1.34	60.3	1.83	0.46
5	Tissue 6	45.2	1.76	1.58	66.6	1.84	0.40

6	Tissue 7	41.1	1.82	1.89	47.4	1.82	1.99
7	Spike-in 5	53.2	1.82	0.46	71.7	1.87	0.23
8	Spike-in 6	36.1	1.78	0.60	74.1	1.85	1.30
9	Spike-in 7	13.6	1.77	0.70	59.3	1.87	1.05
10	Negative control	5.7	1.46	0.78	0.3	-1.18	0.09

4.2 PCR for bacterial 16S rRNA gene

PCR was performed for ZymoSTD samples with bead beating intensity of 4 m/s. The PCR was done with both full length 16S rRNA gene primers (Figure 1) and variable regions V3V4 of 16S rRNA gene primers (Figure 2), later referred as 16S rRNA and V3V4 primer sets In both PCRs. Well 1 contains a sample that have 5 minutes of bead beating, well 2 contains a sample that have 6 minutes of bead beating and well 3 contains a sample that have 7 minutes of bead beating. Figure 1 Shows that PCR for ZymoSTD with 16S rRNA gene primers gives a result at level of 1500 bp, while Figure 2 shows that PCR for ZymoSTD with V3V4 primers gives a result at level of 500 bp. Both figures have strong bands, but the bands are slightly stronger in Figure 2.

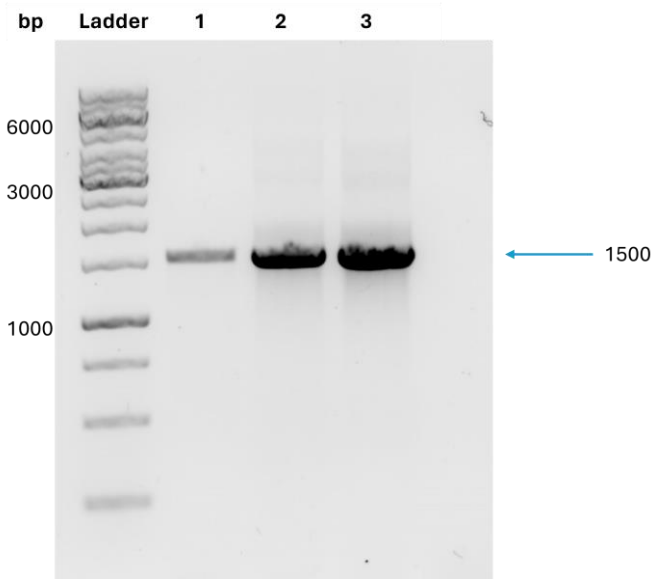

Figure 1: PCR for ZymoSTD with 4m/s bead beating using 16S rRNA gene primers. Well 1 is 5 minutes of bead beating, well 2 is 6 minutes of bead beating and well 3 is 7 minutes of bead beating.

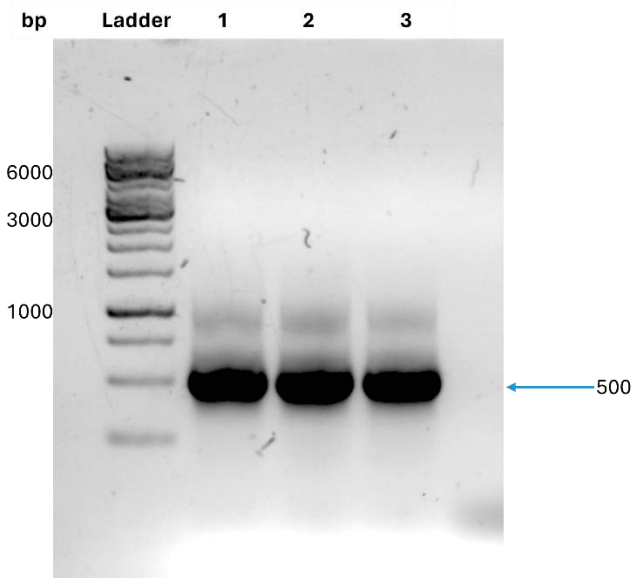

Figure 2: PCR for ZymoSTD with 4m/s bead beating using V3V4 primers. Well 1 is 5 minutes of bead beating, well 2 is 6 minutes of bead beating and well 3 is 7 minutes of bead beating.

After the PCR for ZymoSTD gave reliable results, another PCR was performed for the tissue samples and spike-in, both with bead beating intensity at 4 m/s. Well 1-3 contains tissue samples, well 4-6 contains spike-in samples and well 7-8 are negative controls. PCR for the samples using 16S rRNA gene primers (Figure 3) gave a result at the level of 1500 bp, and PCR for the samples using V3V4 primers (Figure 4) gave a result at the level of 500 bp. Well 7-8 does not show any results in either of the figures meaning that there was no contamination. In both PCR results the bands in well 1-3 are fait but could still be detected in the gel. The bands in well 4-6 are stronger but that is expected since it is a spike-in that includes bacterial DNA.

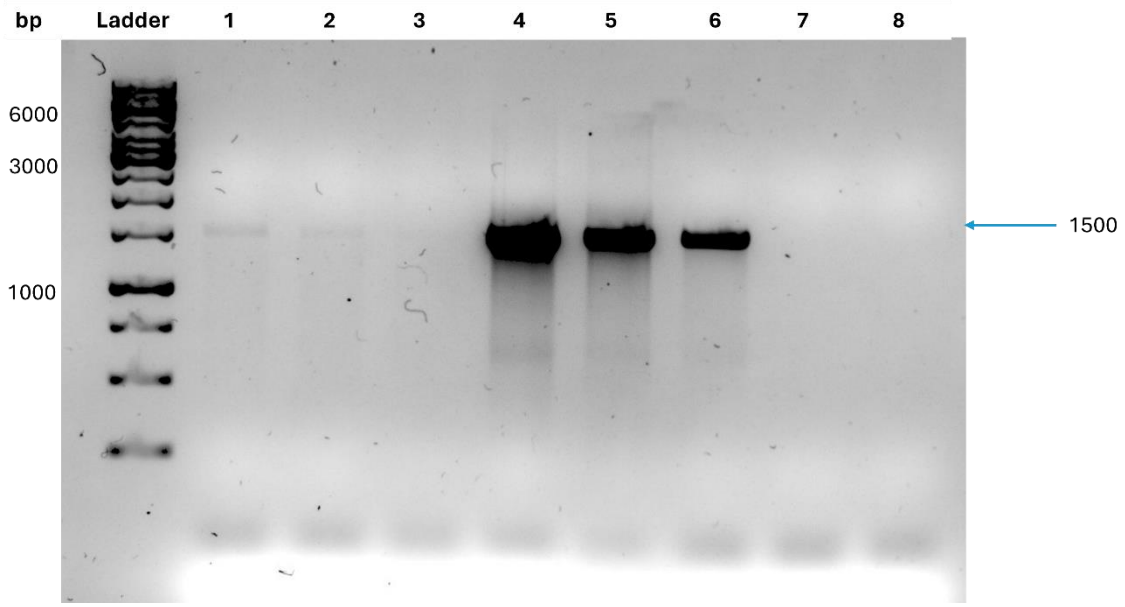

Figure 3 PCR for tissue samples and spike-in with 4m/s bead beating using 16S rRNA gene primers. Well 1-3 are tissue samples with the bead beating time of 5,6 and 7 minutes. well 4-6 are spike-ins with bead beating time of 5,6,7 minutes. Well 7 is a negative control for tissues extraction, while well 8 is negative control for PCR.

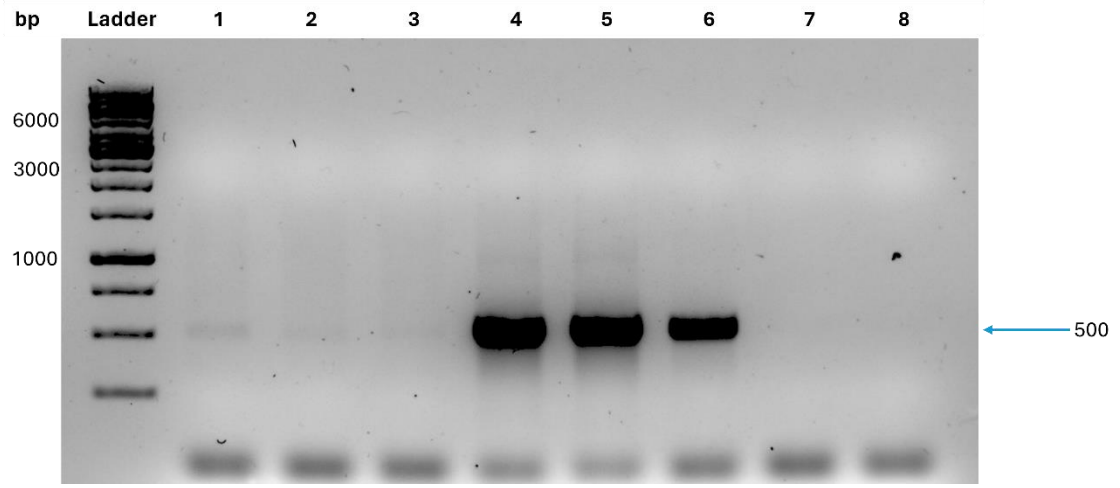

Figure 4 PCR for tissue samples and spike-in with 4m/s bead beating using V3V4 primers. Well 1-3 are tissue samples with the bead beating time of 5,6 and 7 minutes. well 4-6 are spike-ins with bead beating time of 5,6,7 minutes. Well 7 is a negative control for tissues, while well 8 is negative control for PCR.

Seeing that the PCR for samples with bead beating intensity of 4 m/s gave reliable results, the same test was performed with samples with bead beating intensity of 6 m/s. Wells 1-3 contains ZymoSTD, wells 4-6 contains tissue, 7-9 contains spike-in and 10-11 are negative controls. Figure 5 shows that the PCR with 16S rRNA gene primers had a result at level of 1500 bp, while Figure 6 shows that PCR with V3V4 primers had a result at level of 500 bp.

The negative controls had no results which indicates no contamination. As expected, the bands in well 1-3 and 7-9 quite strong, this is because it contains bacteria. In Figure 5, the bands in well 4-6 are a bit weak. The bands in well 4-6 in Figure 6 are a bit stronger, especially the band in well 5.

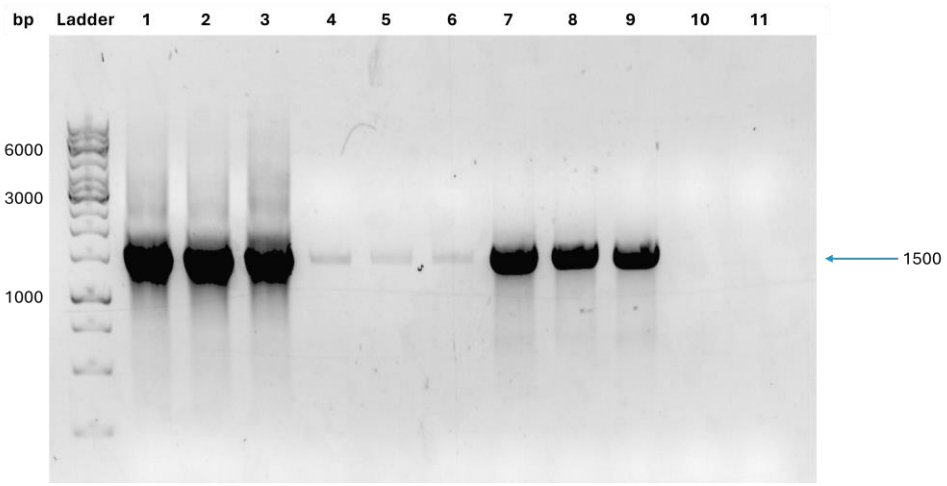

Figure 5: PCR for bead beating 6 m/s with 16S rRNA gene primers. well 1-3 are ZymoSTD with bead beading time of 5,6 and 7 minutes. well 4-6 are tissue samples with bead beating time of 5,6 and 7 minutes. well 7-9 are spike-in with bead beating time of 5,6 and 7 minutes. well 10 is negative control for samples, well 11 is negative control for PCR.

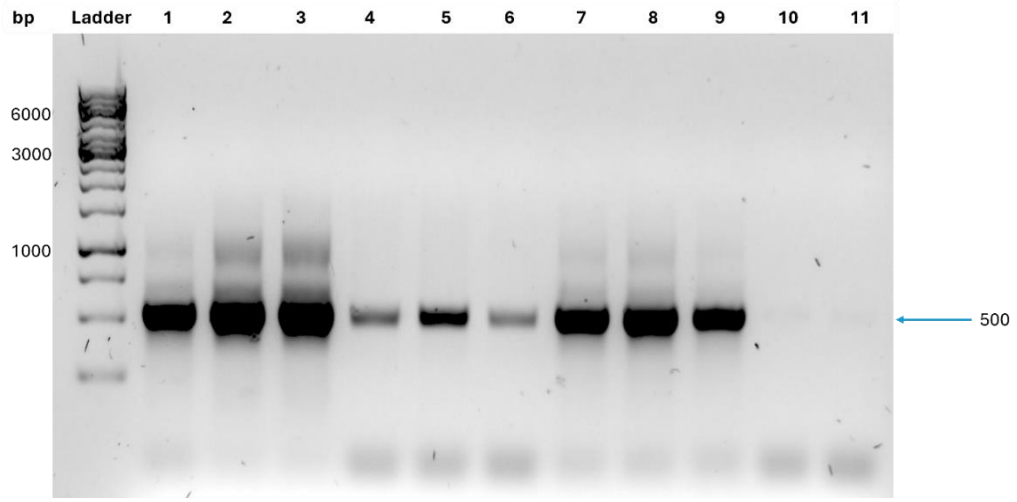

Figure 6: PCR for bead beating 6 m/s with V3V4 primers. well 1-3 are ZymoSTD with bead beading time of 5,6 and 7 minutes. well 4-6 are tissue samples with bead beating time of 5,6 and 7 minutes. well 7-9 are spike-in with bead beating time of 5,6 and 7 minutes. well 10 is negative control for samples, well 11 is negative control for PCR.

4.3 Assembly of plasmid

A plasmid containing the full length of 16S rRNA gene from *Peribacillus frigoritolerans* was designed. After the plasmid was designed, a PCR with the target plasmid was performed. 8 parallels containing samples with the designed plasmid with 16S rRNA gene primer was used. Figure 7 shows that the PCR gave a result at the level of 1500 bp for the plasmids and all of them had a strong band.

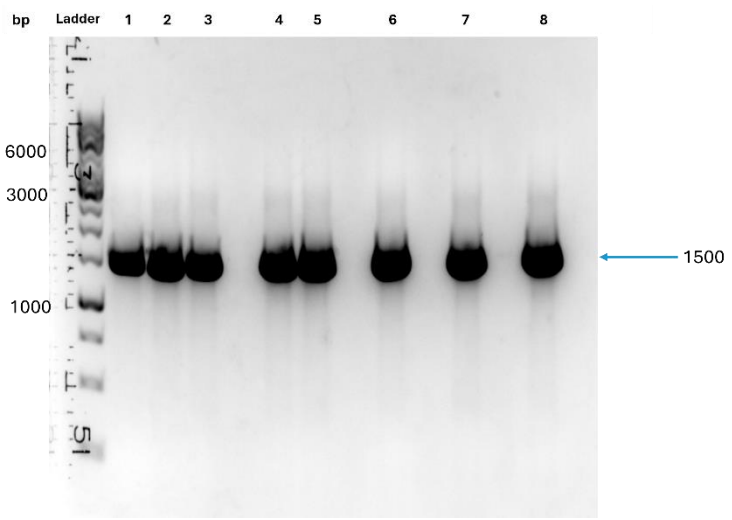

Figure 7: PCR for plasmids with 16S rRNA gene primer. Well 1-8 contains parallels with plasmid.

After the PCR for 8 parallels with the target plasmid gave reliable results, gel extraction was performed and purified. The four bands in well 4-7 (Figure 7) were chosen and cut out by a sterile scalpel and transferred to a microcentrifuge tube. After adding the equal volume of binding buffer XP2 to the cleaved agarose gel and the microcentrifuge tube was incubated, the samples of the PCR product were measured using NanoDrop to find out the DNA concentration. The results are shown in Table 11.

Table 11: Measurement using NanoDrop for 4 sample of gel extractions.

Sample	ng/µL	260/280	260/230
4	19.3	1.75	0.14
5	48.7	1.74	0.69
6	17.1	1.88	0.42
7	40.3	1.78	1.16

The PCR results of the gel extraction were high enough and showed that the test could continue the assembly of insert and then transformation to the 10-Beta bacteria cells. The assembly was done with NEBuilder HiFi DNA.

A PCR was taken for 10 different bacteria colonies from LB agarose plates with ampicillin, containing the 10-Beta bacterial cells. Well 1-3 are samples from 75 µl sample LB agar plate, well 4-6 are samples from 100 µl sample LB agar plate and well 7-9 are samples from 150 µl sample LB agar plate. A negative control was also made. Shown in Figure 8, the bacteria colonies gave a result at the level of 1000 bp, except well 7. The reason well 7 does not have any results may be because too much material was used, and it got stuck in the well.

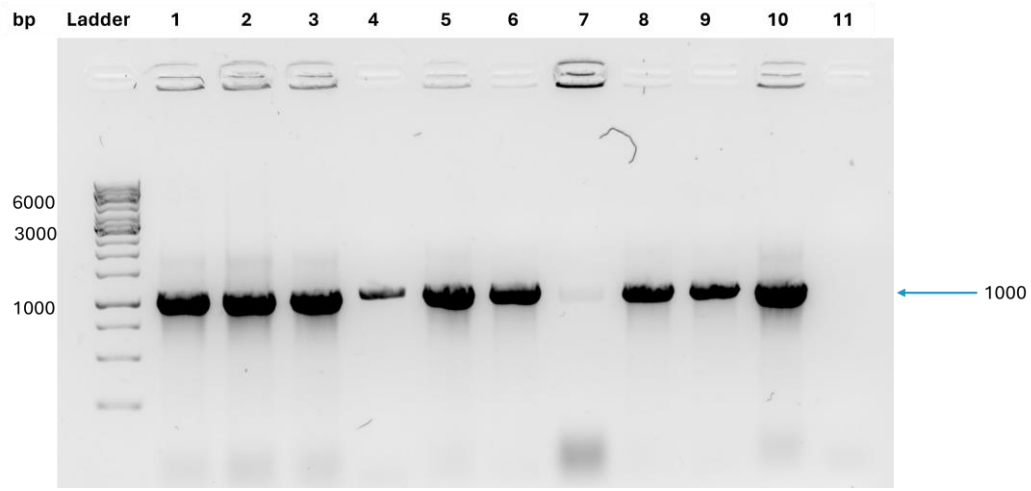

Figure 8: PCR for 10 different bacterial colonies using 10-Beta bacteria cells. Well 11 is a negative control for PCR.

Table 12 shows the five samples that were picked from overnight bacterial cultures for miniprep to amplify and purify the plasmid with insert of interest. The samples used from the overnight bacterial culture were picked by looking at the PCR, Figure 8, and choosing the strongest bands. 3 of the samples were taken from the same plate with 75 µl sample LB agar plate, same colonies as well 1-3 in Figure 8. Sample 4 was taken from the plate with 100 µl sample LB agar plate, the colony used in well 5 in Figure 8. The last sample, sample 5, was taken from 150 µl sample LB agar plate, same colony as used in well 10 in Figure 8. The DNA concentration of the chosen plasmid was measured using NanoDrop.

Table 12: Measurement using NanoDrop for overnight culture samples with Peribacillus frigoritolerans.

Colony number	description	ng/µl	260/280	260/230
1	Taken from 75 µl sample LB agar plate	181.7	1.85	2.18
2	Taken from 75 µl sample LB agar plate	353.4	1.86	2.18
3	Taken from 75 µl sample LB agar plate	235.5	1.85	2.23
5	Taken from 100 µl sample LB agar plate	178.0	1.85	2.16
10	Taken from 150 µl sample LB agar plate	274.2	1.86	2.23

DNA samples were diluted to 4 ng/µl. Figure 9 shows the PCR results of the diluted overnight samples. Well 6 is a negative control. The samples showed a bp of about the level of 1500 bp. No result in well 6 indicated absence of contamination. PCR with the plasmid verified correct insert, and the results were as expected. Plasmid purified from colony #2, 3 and 10 were sent for sequencing at Microsynth facility to verify the correct insert. The result shows the successful incorporation of insert (data is not shown). Plasmid from colony #2 was used as a standard for absolute quantification of bacterial load by qPCR.

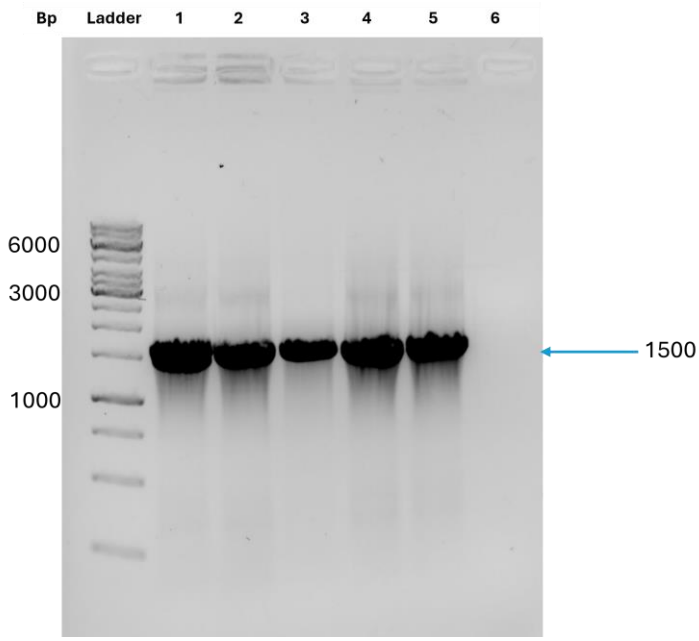

Figure 9: PCR for 5 overnight culture containing the Peribacillus frigoritolerans bacteria. *Well 1 contains colony 1, well 2 contains colony 2, well 3 contains colony 3, well 4 contains colony 5 and well 5 contains colony 10.* Well 6 is a negative control for PCR.

4.4 Quantification of bacterial load by qPCR

A qPCR was performed with a plasmid extracted from earlier in the project. The plasmid chosen was plasmid 2, from Table 12 which had a concentration of 353.4 ng/µl. The plasmid was diluted 1:100 to 3,53 ng/µl and further diluted using the 10-fold method 7 times. The sample dilution ranged from $3.5 - 3.5 \times 10^{-7}$ ng/µl. Eight samples of the diluted plasmid were used for the first trial shown in Table 13, as well as ZymoBIOMICS Microbial Community DNA standard (DNA STD). The DNA STD was used as a positive control. The primer concentration was 0.3 µM for both forward and reverse primer. The Table 13 includes the sample name, the Cq average and the standard deviation of the parallels.

Table 13: qPCR results of plasmid from earlier in the project, and ZymoBIOMICS Microbial Community DNA standard. The primer concentration was 0.3 µM for both forward and reverse primer.

Sample name	Template	Cq mean	Cq SD	Cq mean ± SD
Plasmid Dilution 1	Plasmid	8.88	0.21	8.88 ± 0.21
Plasmid Dilution 2	Plasmid	11.73	0.98	11.73 ± 0.98
Plasmid Dilution 3	Plasmid	12.78	0.21	12.78 ± 0.21
Plasmid Dilution 4	Plasmid	15.56	0.05	15.56 ± 0.05
Plasmid Dilution 5	Plasmid	19.24	0.16	19.24 ± 0.16
Plasmid Dilution 6	Plasmid	23.63	0.39	23.63 ± 0.39
Plasmid Dilution 7	Plasmid	26.95	0.25	26.95 ± 0.25
Plasmid Dilution 8	Plasmid	28.93	0.59	28.93 ± 0.59
DNA STD	DNA STD	19.77	0.08	19.77 ± 0.08
NTC	Water	30.10	0.91	30.10 ± 0.91

4.4.1 Optimalization of qPCR

After the first trial some changes were made to optimize the qPCR to find out what annealing, temperature and primer concentration gave the best Cq result for our samples. Some of the changes included the temperature, gradient and primer concentration. The first dilution used in the first trial were dropped because the concentration was too high and provided lowering of noise to signal ratios.

Table 14 shows the qPCR of plasmid with three different primer mixes. Primer mix 1 had a primer concentration of 0.3 µM of both forward and reverse primer. Primer mix 2 had a primer concentration of 0.2 µM of both primers. Primer mix 3 had a mix of primer with a concentration of 0.3 µM for both primers. Primer mix 1 and 2 had the plasmid 2 as template. The plasmid was first diluted 1:100, to 0.35 ng/µl, and then further diluted to 2 ng/µl. The plasmid was even further diluted to 0.02 ng/µl using the 10-fold method. It was only the

plasmid sample with concentration of 0.02 ng/µl that was used in this qPCR. Primer mix 3 had the DNA STD as template. 5 µl of each template was added the primer mixes. Three corresponding NTC were included to the qPCR. The gradient for the qPCR was 54-70 °C.

Table 14: qPCR results of plasmid with two different primer mix with different primer concentration and ZymoBIOMICS Microbial Community DNA standard. Primer mix 1 has a forward/reverse primer concentration of 0.3/0.3 µM. primer mix 2 has a forward/reverse primer concentration of 0.2/0.2 µM. DNA STD has a primer mix with a concentration of 0.3 µM. NTC to the corresponding primer mixes. The qPCR had a 54-70 ° C gradient.

Sample name	Template	Temperature	Cq mean	Cq SD	Cq mean ± SD
Primer mix 1	Plasmid	70 °C	26.93	9.06	26.93 ± 9.06
Primer mix 2	Plasmid	70 °C	26.10	1.47	26.10 ± 1.47
Primer mix 3	DNA STD	70 °C	29.05	0.15	29.05 ± 0.15
NTC primer mix 1	Water	70 °C	37.68	N/A	37.68 ± N/A
NTC primer mix 2	Water	70 °C	N/A	N/A	N/A ± N/A
NTC primer mix 3	Water	70 °C	N/A	N/A	N/A ± N/A
Primer mix 1	Plasmid	69 °C	17.90	1.70	17.90 ± 1.70
Primer mix 2	Plasmid	69 °C	19.35	0.44	19.35 ± 0.44
Primer mix 3	DNA STD	69 °C	24.46	0.04	24.46 ± 0.04
NTC primer mix 1	Water	69 °C	31.41	N/A	31.41 ± N/A
NTC primer mix 2	Water	69 °C	38.23	N/A	38.23 ± N/A
NTC primer mix 3	Water	69 °C	35.42	N/A	35.42 ± N/A
Primer mix 1	Plasmid	67 °C	14.31	0.06	14.31 ± 0.06
Primer mix 2	Plasmid	67 °C	15.22	0.10	15.22 ± 0.10
Primer mix 3	DNA STD	67 °C	21.53	0.07	21.53 ± 0.07
NTC primer mix 1	Water	67 °C	27.66	N/A	27.66 ± N/A
NTC primer mix 2	Water	67 °C	N/A	N/A	N/A ± N/A
NTC primer mix 3	Water	67 °C	30.51	N/A	30.51 ± N/A
Primer mix 1	Plasmid	63,9 °C	13.55	0.08	13.55 ± 0.08
Primer mix 2	Plasmid	63,9 °C	14.10	0.08	14.10 ± 0.08
Primer mix 3	DNA STD	63,9 °C	20.31	0.05	20.31 ± 0.05
NTC primer mix 1	Water	63,9 °C	26.77	N/A	26.77 ± N/A
NTC primer mix 2	Water	63,9 °C	28.54	N/A	28.54 ± N/A
NTC primer mix 3	Water	63,9 °C	27.62	N/A	27.62 ± N/A

Primer mix 1	Plasmid	60,2 °C	13.12	0.04	13.12 ± 0.04
Primer mix 2	Plasmid	60,2 °C	13.57	0.12	13.57 ± 0.12
Primer mix 3	DNA STD	60,2 °C	19.91	0.04	19.91 ± 0.04
NTC primer mix 1	Water	60,2 °C	26.07	N/A	26.07 ± N/A
NTC primer mix 2	Water	60,2 °C	28.31	N/A	28.31 ± N/A
NTC primer mix 3	Water	60,2 °C	27.76	N/A	27.76 ± N/A
Primer mix 1	Plasmid	57,1 °C	13.14	0.03	13.14 ± 0.03
Primer mix 2	Plasmid	57,1 °C	13.81	0.12	13.81 ± 0.12
Primer mix 3	DNA STD	57,1 °C	20.00	0.04	20.00 ± 0.04
NTC primer mix 1	Water	57,1 °C	25.39	N/A	25.39 ± N/A
NTC primer mix 2	Water	57,1 °C	28.75	N/A	28.75 ± N/A
NTC primer mix 3	Water	57,1 °C	27.90	N/A	27.90 ± N/A
Primer mix 1	Plasmid	55,1 °C	13.21	0.00	13.21 ± 0.00
Primer mix 2	Plasmid	55,1 °C	13.25	0.70	13.25 ± 0.70
Primer mix 3	DNA STD	55,1 °C	20.06	0.03	20.06 ± 0.03
NTC primer mix 1	Water	55,1 °C	26.22	N/A	26.22 ± N/A
NTC primer mix 2	Water	55,1 °C	28.80	N/A	28.80 ± N/A
NTC primer mix 3	Water	55,1 °C	28.86	N/A	28.86 ± N/A
Primer mix 1	Plasmid	54 °C	13.38	0.05	13.38 ± N/A
Primer mix 2	Plasmid	54 °C	14.04	0.03	14.04 ± 0.03
Primer mix 3	DNA STD	54 °C	20.20	0.04	20.20 ± 0.04
NTC primer mix 1	Water	54 °C	28.69	N/A	28.69 ± N/A
NTC primer mix 2	Water	54 °C	29.01	N/A	29.01 ± N/A
NTC primer mix 3	Water	54 °C	28.20	N/A	28.20 ± N/A

After the two first qPCR was completed, a primer matrix was done to check for primer dimers, and to find out the best temperature for the samples. The primer matrix consisted of four different primer mixes that had different forward/reverse primer concentrations. Primer mix 1 had a primer concentration of 0.1/0.1 µM, primer mix 2 had a primer concentration of 0.1/0.2 µM, primer mix 3 had a primer concentration of 0.2/0.1 and primer mix 4 had a

concentration of 0.15/0.15 µM. In this qPCR the template used was just water. The primer matrix also had a 54-70 °C gradient. The results are shown in Table 15.

Table 15: qPCR results for four primer mixes with different primer concentration. Primer mix 1 had a forward/reverse primer concentration of 0.1/0.1 µM. Primer mix 2 had a forward/reverse primer concentration of 0.1/0.2 µM. Primer mix 3 had a forward/reverse primer concentration of 0.2/0.1 µM. Primer mix 4 had a forward/reverse primer concentration of 0.15/0.15 µM.

Sample name	Template	Temperature	Cq mean	Cq SD	Cq mean ± SD
Primer mix 1	Water	70 °C	N/A	N/A	N/A
Primer mix 2	Water	70 °C	N/A	N/A	N/A
Primer mix 3	Water	70 °C	N/A	N/A	N/A
Primer mix 4	Water	70 °C	N/A	N/A	N/A
Primer mix 1	Water	69 °C	N/A	N/A	N/A
Primer mix 2	Water	69 °C	N/A	N/A	N/A
Primer mix 3	Water	69 °C	39.25	0.19	39.25 ± 0.19
Primer mix 4	Water	69 °C	N/A	N/A	N/A
Primer mix 1	Water	67 °C	39.25	0.19	39.25 ± 0.19
Primer mix 2	Water	67 °C	N/A	N/A	N/A
Primer mix 3	Water	67 °C	31.48	0.11	31.48 ± 0.11
Primer mix 4	Water	67 °C	33.69	0.14	33.69 ± 0.14
Primer mix 1	Water	63,9 °C	29.12	0.12	29.12 ± 0.12
Primer mix 2	Water	63,9 °C	34.54	0.57	34.54 ± 0.57
Primer mix 3	Water	63,9 °C	29.12	0.12	29.12 ± 0.12
Primer mix 4	Water	63,9 °C	30.86	0.43	30.86 ± 0.43
Primer mix 1	Water	60,2 °C	29.49	0.21	29.49 ± 0.21
Primer mix 2	Water	60,2 °C	33.51	1.06	33.51 ± 1.06
Primer mix 3	Water	60,2 °C	29.51	0.14	29.51 ± 0.14

Primer mix 4	Water	60,2 °C	30.04	0.49	30.04 ± 0.49
Primer mix 1	Water	57,1 °C	29.51	0.14	29.51 ± 0.14
Primer mix 2	Water	57,1 °C	33.87	0.28	33.87 ± 0.28
Primer mix 3	Water	57,1 °C	28.86	0.20	28.86 ± 0.20
Primer mix 4	Water	57,1 °C	30.44	0.56	30.44 ± 0.56
Primer mix 1	Water	55,1 °C	29.86	0.52	29.86 ± 0.52
Primer mix 2	Water	55,1 °C	33.77	0.63	33.77 ± 0.63
Primer mix 3	Water	55,1 °C	29.76	0.94	29.76 ± 0.94
Primer mix 4	Water	55,1 °C	29.21	2.51	29.21 ± 2.51
Primer mix 1	Water	54 °C	34.74	6.70	34.74 ± 6.70
Primer mix 2	Water	54 °C	34.42	0.13	34.42 ± 0.13
Primer mix 3	Water	54 °C	27.71	1.64	27,71 ± 1.64
Primer mix 4	Water	54 °C	30.17	0.91	30.17 ± 0.91

Table 15 shows that 70 °C was too high of a temperature for the qPCR since it gives no signal. We concluded that the best annealing temperature to use is between 57.1-63.9 °C.

After doing a qPCR to check for primer dimers, and to find out the best temperature, a new qPCR was done with six primer mixes with different forward/reverse primer concentrations, results shown in Table 16. This qPCR was done to find out the best primer concentration for our designed plasmid. Primer mix 1 had a 0.1/0.1 µM concentration, primer mix 2 had a 0.1/0.2 µM concentration, primer mix 3 had a 0.2/0.2 µM concentration, primer mix 4 had a concentration of 0.15/0.15 µM concentration, primer mix 5 had a concentration of 0.2/0.2 µM and primer mix 6 had a concentration of 0.3/0.3 µM. In this qPCR test the forward primer was a primer mix that consisted of 4 types of forward primers, where 10 µl of each primer were mixed. There was only one reverse primer. The gradient for the qPCR was 57,1-63,9 °C.

Table 16: qPCR results for 6 primer mixes with different primer concentration. Primer mix 1 had a forward/reverse primer concentration of 0.1/0.1 µM. Primer mix 2 had a forward/reverse primer concentration of 0.1/0.2 µM. Primer mix 3 had a forward/reverse primer concentration of 0.2/0.1 µM. Primer mix 4 had a forward/reverse primer concentration of 0.15/0.15 µM. Primer mix 5 had a forward/reverse primer concentration of 0.2/0.2 µM. Primer mix 6 had a forward/reverse primer concentration of 0.3/0.3 µM.

Sample name	Template	Temperature	Cq mean	Cq SD	Cq Mean ± SD
Primer mix 1	Water	63,9 °C	N/A	N/A	N/A
Primer mix 2	Water	63,9 °C	38.78	0.50	38.78 ± 0.50
Primer mix 3	Water	63,9 °C	39.99	N/A	39.99 ± N/A
Primer mix 4	Water	63,9 °C	37.19	0.05	37.19 ± 0.05
Primer mix 5	Water	63,9 °C	35.42	0.83	35.42 ± 0.83
Primer mix 6	Water	63,9 °C	32.46	0.39	32.46 ± 0.39
Primer mix 1	Water	60,2 °C	38.34	0.57	38.34 ± 0.57
Primer mix 2	Water	60,2 °C	33.63	1.68	33.63 ± 1.68
Primer mix 3	Water	60,2 °C	36.05	0.33	36.05 ± 0.33
Primer mix 4	Water	60,2 °C	34.15	0.06	34.15 ± 0.06
Primer mix 5	Water	60,2 °C	33.52	0.32	33.52 ± 0.32
Primer mix 6	Water	60,2 °C	31.53	0.72	31.53 ± 0.72
Primer mix 1	Water	57,1 °C	36.77	0.24	36.77 ± 0.24
Primer mix 2	Water	57,1 °C	34.89	0.17	34.89 ± 0.17
Primer mix 3	Water	57,1 °C	35.47	0.14	35,47 ± 0.14
Primer mix 4	Water	57,1 °C	34.18	0.69	34.18 ± 0.69
Primer mix 5	Water	57,1 °C	32.95	0.13	32.95 ± 0.13
Primer mix 6	Water	57,1 °C	31.69	0.04	31.69 ± 0.04

Some of the results in Table 16 shows no results, this is most likely because of a pipetting mistake. Looking at the table, it looks like primer mix 1, which had a primer concentration of 0.1 µM gave the best result, and therefor may be the most optimal primer concentration to use.

After testing for primer dimers and trying to figure out the best primer concentration, another qPCR was done with plasmid. Two primer mixes were used with different concentration of primers. Primer mix 1 had a forward/reverse primer concentration of 0.1/0.2 µM while primer mix 2 had a forward/reverse primer concentration of 0.15/0.15 µM. The corresponding NTC was also included. The plasmid was diluted to 2 ng/µl and was furthered diluted using the 10-fold method 7 times, giving 8 samples of diluted plasmid in total. The concentration in the diluted plasmid samples ranged from $2 - 2 \times 10^{-7}$ ng/µl. In this qPCR the annealing was changed to 20 seconds instead of 15 seconds. The results are shown in Table 17.

Table 17:qPCR results of plasmid from earlier in the project, with two different primer mix with different primer concentrations. Primer mix 1 had a forward/reverse primer concentration of 0.1/0.2 µM. Primer mix 2 had a forward/reverse primer concentration of 0.15/0.15 µM.

Sample name	Template	Cq mean	Cq SD	Cq mean ± SD
Primer mix 1. Dilution 1	Plasmid	1.52	0.53	1.52 ± 0.53
Primer mix 1 Dilution 2	Plasmid	18.52	0.14	18.52 ± 0.14
Primer mix 1 Dilution 3	Plasmid	22.18	0.17	22.18 ± 0.17
Primer mix 1 Dilution 4	Plasmid	25.84	0.10	25.84 ± 0.10
Primer mix 1 Dilution 5	Plasmid	29.44	0.13	29.44 ± 0.13
Primer mix 1 Dilution 6	Plasmid	33.12	1.04	33.12 ± 1.04
Primer mix 1 Dilution 7	Plasmid	33.94	0.31	33.94 ± 0.31
Primer mix 1 Dilution 8	Plasmid	34.55	0.35	34.55 ± 0.35
NTC primer mix 1	Water	35.24	1.44	35.24 ± 1.44
Primer mix 2 Dilution 1	Plasmid	13.95	0.14	13.94 ± 0.14
Primer mix 2 Dilution 2	Plasmid	17.37	0.11	17.37 ± 0.11
Primer mix 2 Dilution 3	Plasmid	21.14	0.06	21.14 ± 0.06
Primer mix 2 Dilution 4	Plasmid	24.90	0.14	24.90 ± 0.14
Primer mix 2 Dilution 5	Plasmid	28.35	0.18	28.35 ± 0.18
Primer mix 2 Dilution 6	Plasmid	31.57	0.59	31.57 ± 0.59
Primer mix 2 Dilution 7	Plasmid	33.39	0.16	33.39 ± 0.16
Primer mix 2 Dilution 8	Plasmid	33.78	0.20	33.78 ± 0.20
NTC primer mix 2	water	33.49	0.31	33.49 ± 0.31

The previous qPCR with annealing for 20 seconds did not result in better results, and we concluded that the most optimal annealing time was 15 seconds. A qPCR with the plasmid and the extracted DNA with bead beating intensity of 4 m/s was then done, Table 18. Two different primer mixes were made since the template volume for the samples were different from each other. 5 µl of each plasmid dilution were added, while only 2 µl of the samples with bead beating 4 m/s were added. Both primer mixes had a forward/reverse primer concentration of 0.1/0.1 µM. The plasmid was diluted using the 10-fold method, giving a total of seven plasmid samples. The concentration of the plasmid dilutions ranged from $2 - 2 \times 10^{-6}$. For the samples with bead beating with intensity of 4 m/s only the tissue samples and spike-in was used.

A standard curve was made from the results in Table 18, and are shown in Figure 10. The standard curve gave a $y = -4{,}1415x + 38{,}305$ and $R^2 = 0{,}9957$.

Table 18: qPCR results with plasmid and samples from bead beating 4 m/s. The plasmid was diluted using the 10-fold method. The primer concentration was 0.1 µM of both forward and reverse primer.

Sample name	Template	Cq mean	Cq SD	Cq mean ± SD
plasmid Dilution 1	Plasmid	10.03	0.16	10.03 ± 0.16
Plasmid Dilution 2	Plasmid	12.22	0.39	12.22 ± 0.39
plasmid Dilution 3	Plasmid	17.55	0.36	17.55 ± 0.36
Plasmid Dilution 4	Plasmid	21.64	0.37	21.64 ± 0.37
Plasmid Dilution 5	Plasmid	26.06	0.19	26.06 ± 0.19
Plasmid Dilution 6	Plasmid	30.04	0.24	30.04 ± 0.24
Plasmid Dilution 7	Plasmid	33.97	0.08	33.97 ± 0.08
Tissue 1	Tissue	33.74	1.29	33.74 ± 1.19
Tissue 2	Tissue	34.09	N/A	34.09 ± N/A
Tissue 3	Tissue	35.23	0.93	35.23 ± 0.93
Spike-in 1	Tissue + ZymoSTD	24.65	1.17	24.65 ± 1.17
Spike-in 2	Tissue + ZymoSTD	29.93	5.49	29.93 ± 5.49
Spike-in 3	Tissue + ZymoSTD	26.83	2.43	26.83 ± 2.43
Neg. Control	Water	37.17	2.50	37.17 ± 2.50
NTC	Water	N/A	N/A	N/A

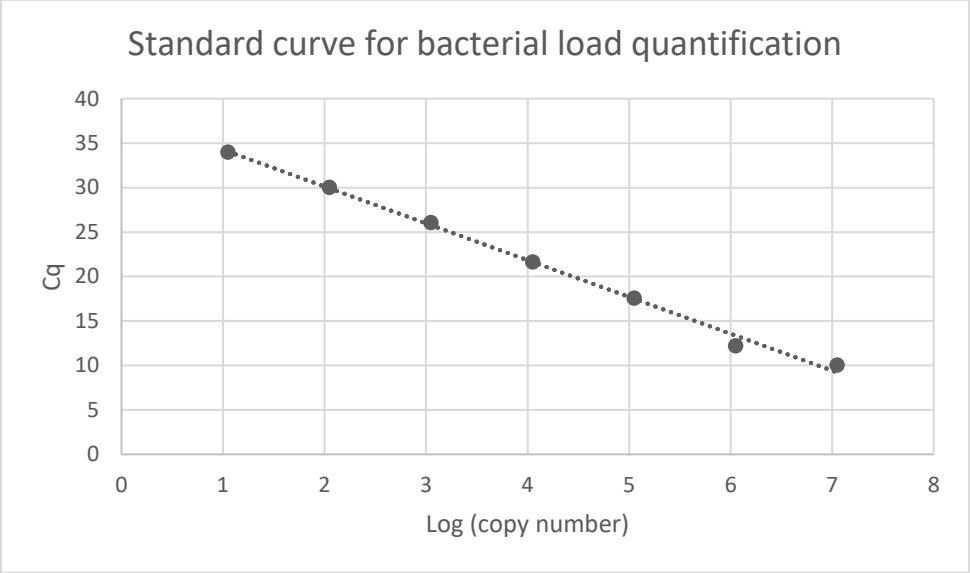

Figure 10: Standard curve made from qPCR with designed plasmid. The plasmid was diluted 7 times using the 10-fold method. The start concentration was 0.2 ng/µl.

Since the previous qPCR with plasmid and the extracted DNA with bead beating intensity of 4 m/s gave reliable results another one was performed, this time with the ZymoSTD included. In this qPCR a new plasmid concentration was used, and the plasmid had a concentration of 486 ng/µl. The plasmid was diluted to 2 ng/µl, this dilution was not used in the qPCR. The diluted plasmid was further diluted six times, using the 10-fold method, giving six diluted samples that was used in the qPCR. The concentration of the 6 diluted plasmid samples ranged from $0.2 - 2 \times 10^{-5}$. Two primer mixes were made since the added volume for the samples of the plasmid and the bead beating 4 m/s samples was different from each other. Both primer mixes had a forward/reverse primer concentration of 0.1/0.1 µM. The ZymoSTD samples were diluted 1:100. A NTC was also included. The results are shown in Table 19.

Table 19: qPCR for plasmid and samples from 4m/s intensity bead beating. The plasmid was diluted using the 10-fold method. The primer concentration was 0.1 µM for both forward and reverse primer.

Sample name	Template	Cq mean	Cq SD	Cq mean ± SD
Plasmid Dilution 1	Plasmid	16.60	0.42	16.60 ± 0.42
Plasmid Dilution 2	Plasmid	19.63	0.17	19.63 ± 0.17
Plasmid Dilution 3	Plasmid	24.34	0.31	24.34 ± 0.31
Plasmid Dilution 4	Plasmid	29.09	0.23	29.09 ± 0.23

Plasmid Dilution 5	Plasmid	33.85	0.29	33.85 ± 0.29
Plasmid Dilution 6	Plasmid	38.65	0.35	28.65 ± 0.35
ZymoSTD 1	ZymoSTD	23.93	0.35	23.93 ± 0.35
ZymoSTD 2	ZymoSTD	24.05	0.03	24.05 ± 0.03
ZymoSTD 3	ZymoSTD	24. 82	0.04	24.82 ± 0.04
Tissue 1	Tissue	33.07	0.08	33.07 ± 0.08
Tissue 2	Tissue	34.41	0.23	34.41 ± 0.23
Tissue 3	Tissue	35.46	0.10	35.46 ± 0.10
Spike-in 1	Tissue + ZymoSTD	22.68	0.20	22.68 ± 0.20
Spike-in 2	Tissue + ZymoSTD	22.94	0.02	22.94 ± 0.02
Spike-in 3	Tissue + ZymoSTD	24.66	0.12	24.66 ± 0.12
Neg. Control	Water	36.24	0.01	36.24 ± 0.01
NTC	Water	N/A	N/A	N/A

Lastly, a qPCR was done with plasmid and extracted DNA with bead beating intensity of 6 m/s. Just as the last qPCR, two primer mixes were made, both with forward/reverse primer concentration of 0.1/0.1 µM. The plasmid was diluted to 2 ng/ µl, that was not used in the test, and further diluted six times using the 10-fold method, giving 6 diluted samples. The concentration of the diluted plasmid samples ranged from $0.2 - 2 \times 10^{-6}$. The ZymoSTD was also diluted 1:100. 5 µl of the plasmid samples were used while only 2 µl of the extracted DNA samples were used.

Table 20: qPCR results of plasmid and samples from 6 m/s intensity bead beating. The plasmid was diluted using the 10-fold method. The primer concentration was 0.1 µM for both forward and reverse primer.

Sample name	Template	Cq mean	Cq SD	Cq mean ± SD
Plasmid Dilution 1	Plasmid	14.46	0.16	14.46 ± 0.16
Plasmid Dilution 2	Plasmid	18.09	0.09	18.09 ± 0.09
Plasmid Dilution 3	Plasmid	22.63	0.32	22.63 ± 0.32
Plasmid Dilution 4	Plasmid	27.37	0.22	27.37 ± 0.22
Plasmid Dilution 5	Plasmid	31.61	0.31	31.61 ± 0.31
Plasmid Dilution 6	Plasmid	36.03	0.27	36.03 ± 0.27

ZymoSTD 1	ZymoSTD	23.07	0.01	23.07 ± 0.01
ZymoSTD 2	ZymoSTD	24.12	0.34	24,12 ± 0.34
ZymoSTD 3	ZymoSTD	23.61	0.00	23.61 ± 0.00
Tissue 1	Tissue	28.52	0.14	28.52 ± 0.14
Tissue 2	Tissue	26.17	0.04	26.17 ± 0.04
Tissue 3	Tissue	30.79	0.12	30.79 ± 0.12
Spike-in 1	Tissue + ZymoSTD	21.97	0.33	21.97 ± 0.33
Spike-in 2	Tissue + ZymoSTD	21.74	0.51	21.74 ± 0.51
Spike-in 3	Tissue + ZymoSTD	22.85	0.09	22.85 ± 0.09
Neg. Control	Water	38.63	0.72	38.63 ± 0.72
NTC	Water	N/A	N/A	N/A

After the last 2 qPCR were performed, with samples with bead beating intensity of 4 m/s and 6 m/s, two standard curves were made for each of them. Appendix figure 17 shows the standard curve for the samples with bead beating intensity of 4 m/s, while Appendix figure 18 shows the standard curve for the samples with bead beating intensity of 6 m/s. The standard curve, Appendix figure 17, was used to calculate the bacteria count in the unknown samples with a bead beating intensity of 4 m/s in the sample stock. The bacteria count was calculated in ZymoSTD samples, tissue samples, spike-in samples, and the negative control. Table 21 shows that tissue sample with bead beating duration of 5 minutes had the highest bacteria count of 16113.24 bacteria in sample, while tissue sample with bead beating duration of 7 minutes had the lowest bacteria count of 4737.78 bacteria in sample.

Appendix figure 18 was the standard curve used to calculate the bacteria load for unknown samples with bead beating intensity of 6 m/s. Also here were the bacteria count in sample stock calculated in ZymoSTD samples, tissue samples, spike-in samples, and the negative control. Table 21 shows that for the tissue samples, the highest bacteria count was in tissue sample with a bead beating duration of 6 minutes and intensity of 6 m/s, counted to 19360.75 bacteria in sample. The lowest bacteria count in tissue samples with bead beating intensity of 6 m/s were found in the tissue sample with bead beating duration of 7 minutes, counted to 17073.08 bacteria in sample.

Table 21: Bacteria count for unknown samples including ZymoSTD samples, tissue samples, spike-in samples that contain both ZymoSTD and tissue, and negative control. The unknown samples had both bead beating intensity of 4 m/s and 6 m/s. The bacteria count was Calculated with use of standard curves made from the plasmid results.

Sample	Copy number per µl in sample
ZymoSTD bb 5 min 4 m/s	344333728
ZymoSTD bb 6 min 4 m/s	323023300
Zymo STD bb 7 min 4 m/s	217931615
Tissue bb 5 min 4 m/s	16113.24
Tissue bb 6 min 4 m/s	8102.87
Tissue bb 7 min 4 m/s	4737.78
Spike-in bb 5 min 4 m/s	3253096.16
Spike-in bb 6 min 4 m/s	2855588.61
Spike-in bb 7 min 4 m/s	1182509.83
Negative ctrl bb 7 min 4 m/s	3180.11
ZymoSTD bb 5 min 6 m/s	197854284.47
ZymoSTD bb 6 min 6 m/s	113869073.47
Zymo STD bb 7 min 6 m/s	148918246.38
Tissue bb 5 min 6 m/s	56223.04
Tissue bb 6 min 6 m/s	193607.49
Tissue bb 7 min 6 m/s	17073.08
Spike-in bb 5 min 6 m/s	1764740.43
Spike-in bb 6 min 6 m/s	1991769.37
Spike-in bb 7 min 6 m/s	1113604.83
Negative ctrl bb 7 min 6 m/s	275.19

Discussion

5.1 Qubit versus NanoDrop

NanoDrop spectrophotometer is currently the most useful way to determine DNA concentration and purity as it measures absorbance of samples' microvolumes. The DNA purity is determined by the absorbance ratios 260/280 and 260/230. The absorbance rations also determine the presence of contaminants. (17)

The purity in both nucleic acid and protein extraction have been measured by the ratio of absorbance at wavelengths of 260 and 280 nm. For DNA, the ratio that is accepted as "pure" is ~1.8. The ratio of A_{260}/A_{230} is frequently calculated as the absorbance at 230 nm is accepted as being the results of other contamination. To detect potential issues with the sample purity, analyzing sample spectra can be valuable. A low 260/230 ratio can be a result of contaminant absorbed at 230 nm or less. A low 260/280 ratio can be due to a contaminant absorbing at 280 nm or less. The wavelength of the through in samples spectrum should ideally be around ~230 nm. If there's absorbance by a contaminant at a lower wavelength, it usually shifts the through wavelength. Similarly, the wavelength of the peak in the sample spectrum should be around ~260 nm. The peak absorbance wavelength may shift by the absorbance by a contaminant.(19)

Even though NanoDrop is widely used, there are some challenges with this method, such as lack of selectivity for distinguishing between DNA, RNA, or protein. Another problem is that the absolute values from the measurement exhibit wide variability due to the presence of contaminants and differences in base compositions. Lastly, the spectrophotometers precision tends to be deficient at low concentrations of DNA and RNA. As a result of this, Qubit, which is a fluorescence-based quantitation method, has become more used. (20)

For the nucleic acid of interest, Qubit is often more specific and more sensitive. The Qubit fluorometer has a system that is simple, fast, and easy to use. The Qubit measure the nucleic acids by identifying fluorescent dyes in various Qubit Assays that uniquely bind to their target. Even at low concentrations, the dyes releases fluorescence when bound to their target, and within minutes the dyes are absorbed and ready to be read by Qubit fluorometers. (21) The instrument only needs a small sample volume of 1-20 µl, leaving more sample for analysis. Unlike the NanoDrop, The Qubit can measure both DNA and RNA accurately. (22)

In a comparison done by Invitrogen, where the Invitrogen™ Qubit™ Fluorometer and the Thermo Scientific™ NanoDrop™ ND-1000 Spectrophotometer was compared, it was

concluded that the Qubit fluorometric quantitation offers a more accurate method for quantitating nucleic acid that is more selective and sensitive compared with the NanoDrop ND.100 Spectrophotometer. (22)
In this project, both Nanodrop and Qubit were used to determine the DNA concentrations. Using both instruments can be helpful as the Qubit shows accurate quantitation and the Nanodrop can show if contaminant is present.

The DNA concentration for both samples with bead beating intensity at 4 m/s and 6 m/s were higher when measured with NanoDrop (Table 10) than when measure with Qubit (Table 6 and Table 7). As mentioned above, the NanoDrop is less accurate and the measurement can vary because of other substances mixed in, which can be why the DNA concentrations were higher when measured with NanoDrop.

5.2 Bead beating

It would be interesting to see if not only different bead beating duration would give different results, but also if the bead beating intensity would have an impact. In this project, three different durations were evaluated, along with two different bead beating intensities.

The durations used when bead beating was 5, 6 and 7 minutes. The different durations were tested to check if there would be an impact in the DNA concentration. Looking at both Table 6 and Table 8, it shows that the highest DNA concentration, measured with Qubit, was in the tissue samples with bead beating duration of 6 minutes. The figures also show that the DNA concentration decreases at the duration of 7 minutes, showing that 7 minutes is not the most ideal duration.

The bead beating intensities evaluated were intensities of 4 m/s and 6 m/s. Looking at the Qubit measurement of bead beating 4 m/s, Table 6, and bead beating 6 m/s, Table 8, the DNA concentration measured with Qubit are slightly higher in the tissue samples with bead beating intensity of 6 m/s. The DNA concentration from tissue samples with bead beating intensity of 6 m/s is ranging from 4120-4770 ng while the tissue samples with bead beating intensity of 4 m/s is ranging from 1625-1795 ng. Even if the tissue samples had a higher DNA concentration at bead beating intensity of 6 m/s, both bead beating intensities gave reliable results.

Looking at the qPCR results of bead beating intensity of 4 m/s, Table 19, and bead beating intensity of 6 m/s, Table 20, the Cq average does not have a big difference between the tissue samples with different bead beating intensities. The Cq average from tissue samples with bead beating intensity 4 m/s ranged from 33.07-35.46, while the tissue samples with bead beating intensity of 6 m/s ranged from 26.17-30.79.

Since both bead beating intensities gave reliable results, it indicates that the tissue samples can be amplified the same way.

5.3 qPCR with SYBR green

qPCR is a technique used to amplify specific fragments of DNA, like PCR, but qPCR also allows to quantify the amount of target DNA in the samples. qPCR is a real-time method, meaning that the amplification of the target DNA can be monitored in real-time. During qPCR, fluorescent dyes (SYBR green in case of this project) or probes are added to binds to the target DNA as it amplifies. When the probes are stimulated by a laser, they will emit light at different wavelengths. The amount of target DNA present in the sample before amplification can be determined by measuring the light emitted at each wavelength. qPCR is a useful technique as it can detect varying levels of expression within the same well, giving it the benefit of high throughput and a wider dynamic range. It can also be a more practical option due to its ability to multiplex, amplifying multiple targets in one experiment, along with its rapid results. (23)

Some of the qPCR tests done had a high Cq for the NTCs. A few more tests were done to check if the high NTC Cq could be because of primer dimers, and the NTC were also moved on the plate to check if it were contamination from other wells. After a getting a few qPCR tests with NTC that had a high Cq value, a suspicion that it could be on account of contamination in the water occurred. A new water was ordered and used in the newer qPCR tests. When comparing the NTC from the first qPCR shown in Table 13 and the qPCR shown in Table 18, it is shown that the Cq went from 30.1 to N/A. From Table 18, is where the new water was used, which could strengthen the suspicion that the high NTC Cq were indeed from a contamination in the water.

5.4 Quantification of bacteria load

The bacteria load was calculated in unknown samples with different bead beating intensity. The unknown samples consisted of ZymoSTD samples, tissue samples and spike-in samples that consisted of both ZymoSTD and tissue. The intensity of the bead beating used were 4 m/s and 6 m/s. At Table 21, it is showed that the bacteria load is higher in the tissue samples that were bead beated with a higher intensity, 6 m/s, than in the tissue samples with bead beating intensity of 4 m/s.

The bacteria load between the tissue samples and spike-in samples with bead beating intensity of 4 m/s were calculated. The spike-in sample with bead beating of 5 minutes were 201-fold higher in bacteria load than the tissue sample with same bead beating duration. The bacteria load in the spike-in sample with bead beating duration of 6 minutes were 352-fold higher than the tissue sample with same bead beating duration. The bacteria load in the spike-in sample with bead beating duration of 7 minutes were 249-fold higher than the tissue sample with the same duration.

The bacteria load between the tissue samples and spike-in samples were also calculated in samples with bead beat intensity of 6 m/s. The bacteria load in spike-in sample with bead beating of 5 minutes were 31-fold higher than the bacteria load in tissue sample with same bead beating duration. The bacteria load in the spike-in sample with bead beating duration of 6 minutes were 10-fold higher than the tissue sample with same bead beating duration. The bacteria load in the spike-in sample with bead beating duration of 7 minutes were 65-fold higher than the tissue sample with the same duration.

The bacteria load between the tissue samples with different intensities were also calculated. The difference between the tissue samples with bead beating of 5 minutes showed that the tissue sample with bead beating intensity of 6 m/s was 3-fold higher than the tissue sample with bead beating intensity of 4 m/s. In the samples with bead beating duration of 6 minutes, the tissue sample with bead beating intensity of 6 m/s were 23-fold higher than the tissue sample with intensity of 4 m/s. Lastly, in the tissue samples with duration of 7 minutes, the tissue sample with intensity of 6 m/s were 3-fold higher than the tissue sample with intensity of 4 m/s.

Looking at the bacteria load for both tissue samples with different intensity and the difference between the tissue samples, it shows that the tissue samples with a bead beating intensity of 6 m/s gave a bigger bacteria load. The difference in bacteria load between the tissue samples

and spike-in samples with bead beating intensity of 4 m/s were significantly higher than the difference between the tissue samples and spike-in samples with bead beating intensity of 6 m/s.

A study was done by Jolanda Kool and her colleagues where they compared several approaches to investigate and identify potential biases in microbiome research. In the study they used mock communities and multiple fecal samples. (24) The study found that a higher number of cycles during PCR would lead to an increase in contaminants detected in the negative controls. The study suggests a recommendation of 25 PCR cycles as optimal parameters, at least for human fecal samples, to reduce the effect of contamination. (24) In our study, 25 cycles were used in PCR, it gave a positive outcome for the tissue sample, and there was no imply for contaminants. This could indicate that this number of cycles were the most optimal parameter for human tissue samples.

In the study done by Jolanda Kool and her colleagues, it was noticed how the immediately frozen samples gave different results than the samples stored in room temperature, even when the room temperatures samples were preserved in stabilization buffers. (24) The samples stored in room temperature for 3-5 days had a higher amount of *Enterobacteriaceae* compared to the same sample when directly frozen. This was prevented using stabilization buffer. This indicated the importance of immediate frozen storage is a critical aspect in fecal microbiota, although storing samples at room temperature with stabilization buffer can be an suitable compromise when freezing them immediately is not possible.(24) Even though the study is done on human fecal samples, whereas our study used human tissue samples, the storage of samples may have the same importance. If the tissue used in our study had been stored in room temperature instead of frozen there might have given a different result of DNA and showed different types of bacteria than the tissue samples stored frozen.

Conclusion

In this study, an assay for the absolute quantification of bacterial load was successfully developed, using qPCR and SYBR green dye. A plasmid containing the 16S rRNA gene from *Peribacillus frigoritolerans* was designed to use in a qPCR method for the quantification of bacterial DNA in genomic DNA purified from human tissue. DNA was extracted from human tissue with use of bead beating with different duration and intensity. The duration used was 5-7 minutes and the intensity was 4 m/s and 6 m/s. The different intensity and duration did not affect the yield. The standard curves were then used to calculate the bacteria load in the unknown samples.

When measuring the DNA concentration using NanoDrop and Qubit, the differences were quite small between the samples with two different bead beat intensity, but the calculated bacteria load the difference were significant. The tissue samples with bead beating intensity of 6 m/s had a higher bacteria load, with between 3- and 23-fold higher bacteria load than the tissue samples with bead beating intensity of 4 m/s. This indicates that a bead beating intensity of 6 m/s is the most optimal intensity to get the best bacteria load. The results showed that the bacteria load could be quantified and there were not a significant differences between the samples that had a longer bead beating duration.

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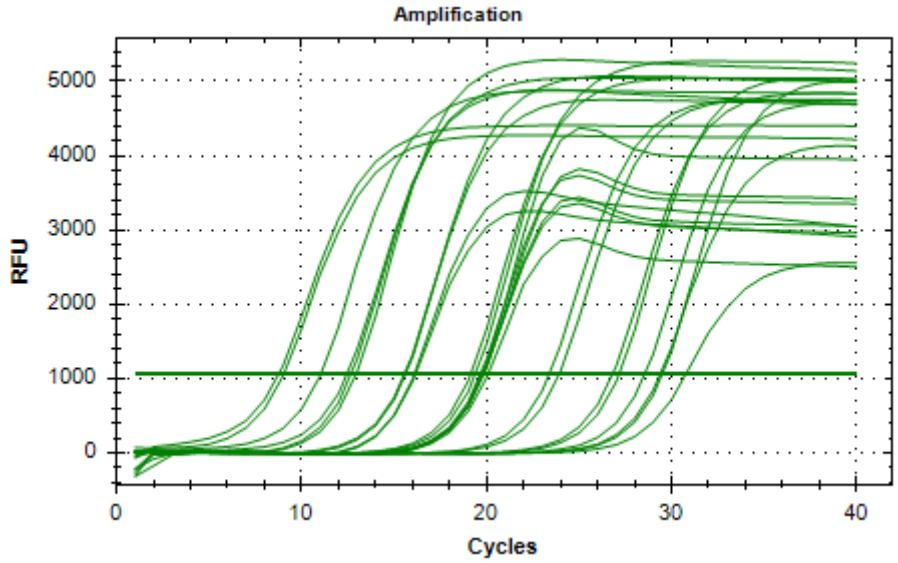
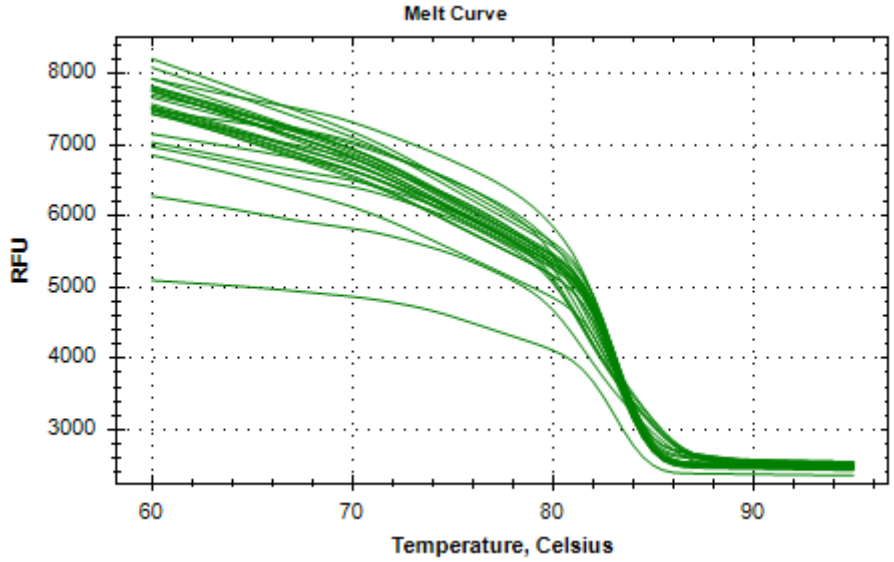
Appendix

Appendix figure 1**Feil! Fant ikke referansekilden.** shows the qPCR results from Table 13 in a curve, while Appendix figure 2 shows the melting curve for the same qPCR test. The Curve is for plasmid samples and DNASTD. The plasmid samples had a concentration range of $3.5 - 3.5 \times 10^{-7}$ ng/µl.

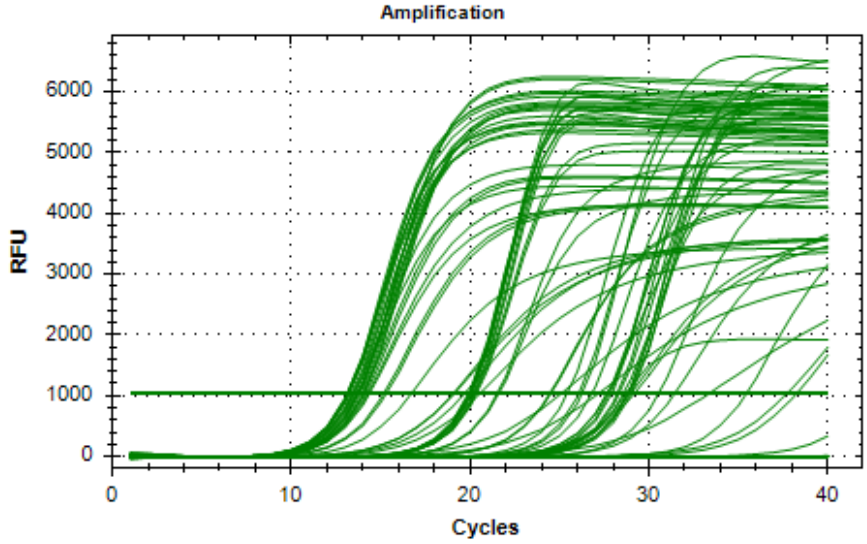


Appendix figure 1: Results for qPCR standard and Plasmid 2. The primer concentration was 0.3 µM for both forward and reverse primer. The plasmid was diluted using a 10-fold.

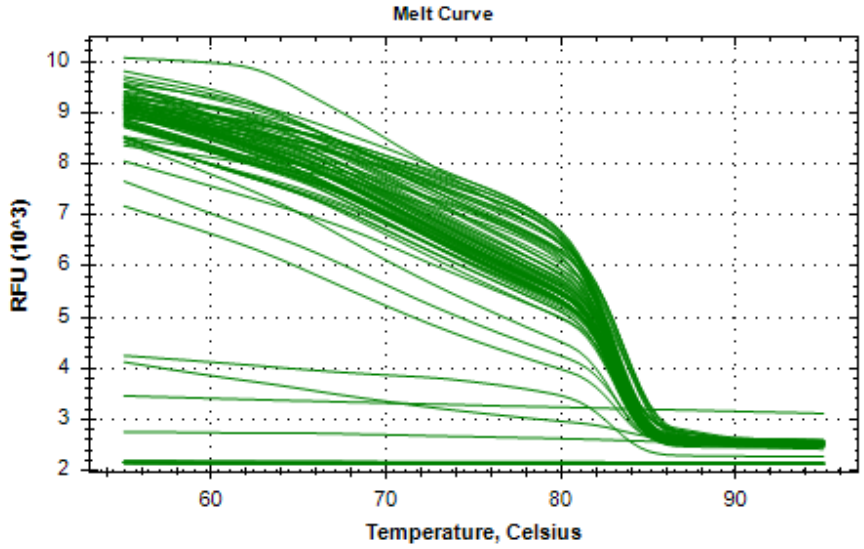


Appendix figure 2: Melting curve for qPCR standard and Plasmid 2. The primer concentration was 0.3 µM for both forward and reverse primer. The plasmid was diluted using a 10-fold.

Appendix figure 3 shows the results from Table 14 in a curve. Appendix figure 4 shows the melting curve for the same qPCR test. The curve has samples from plasmid that were diluted. plasmid samples had a concentration of 0.02 ng/µl.

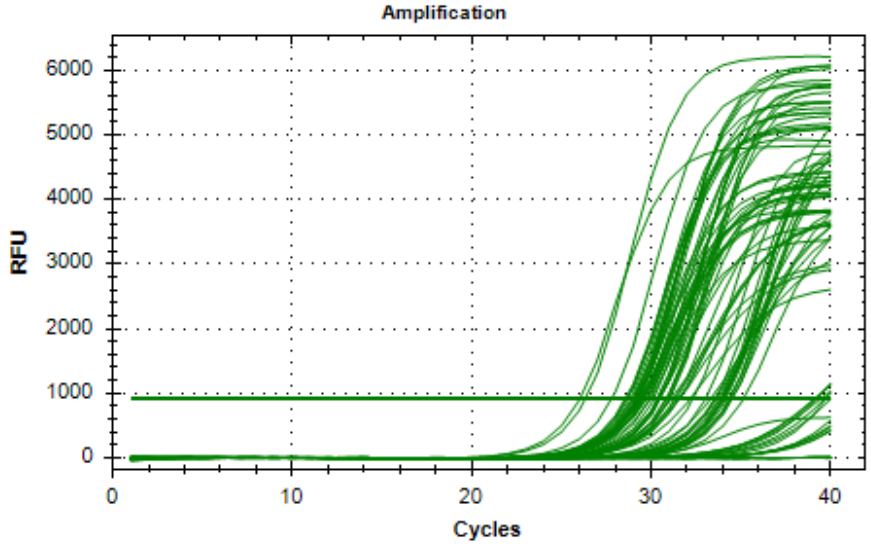


Appendix figure 3:qPCR for optimalization of the plasmid with different forward/reverse primer concentrations, the concentrations used were 0.2/0.2 µM, 0.3/0.3 µM and a primer mix with 0.3 µM concentration. Had a 54-70 °C gradient.

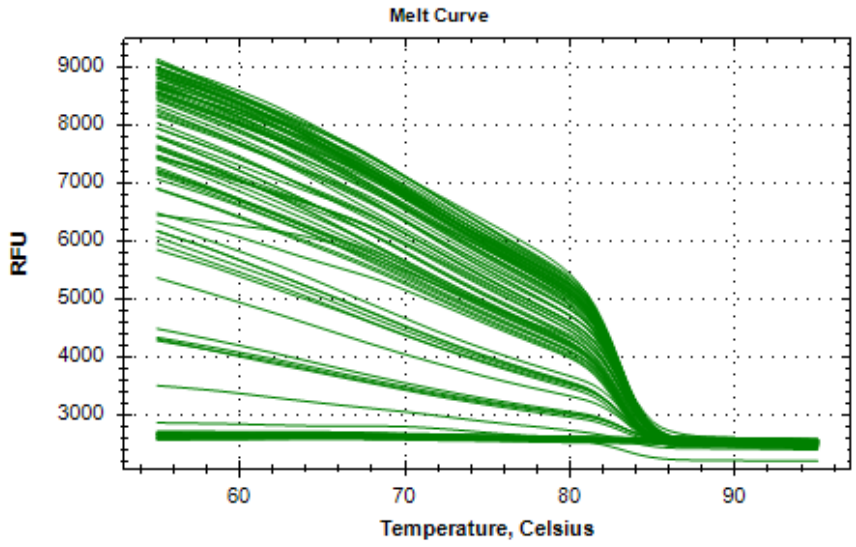


Appendix figure 4: Melting curve for qPCR for optimalization of the plasmid with different forward/reverse primer concentrations, the concentrations used were 0.2/0.2 µM, 0.3/0.3 µM and a primer mix with 0.3 µM concentration. Had a 54-70 °C gradient.

Appendix figure 5 shows the results from Table 15 in a curve. Appendix figure 6 shows the melting curve for the same qPCR. The curve is of 4 primer mixes, which all had water as template.

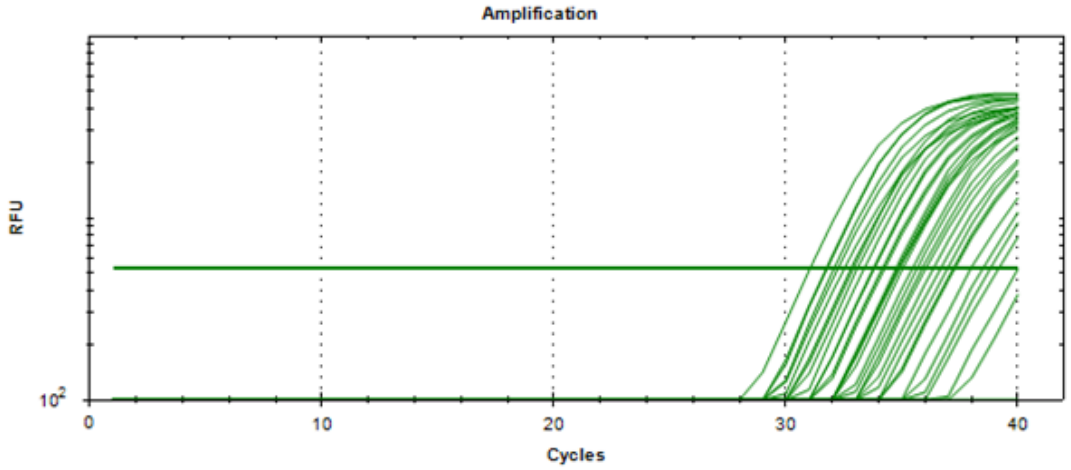


Appendix figure 5: qPCR for Primer matrix. Four primer mix with different forward/reverse primer concentration, primer mix 1 had 0.1/0.1 µM concentration, primer mix 2 had 0.1/0.2 µM concentration, primer mix 3 had 0.2/0.1 µM concentration and primer mix 4 had 0.15/0.15 µM concentration. Had a 54-70 °C gradient.

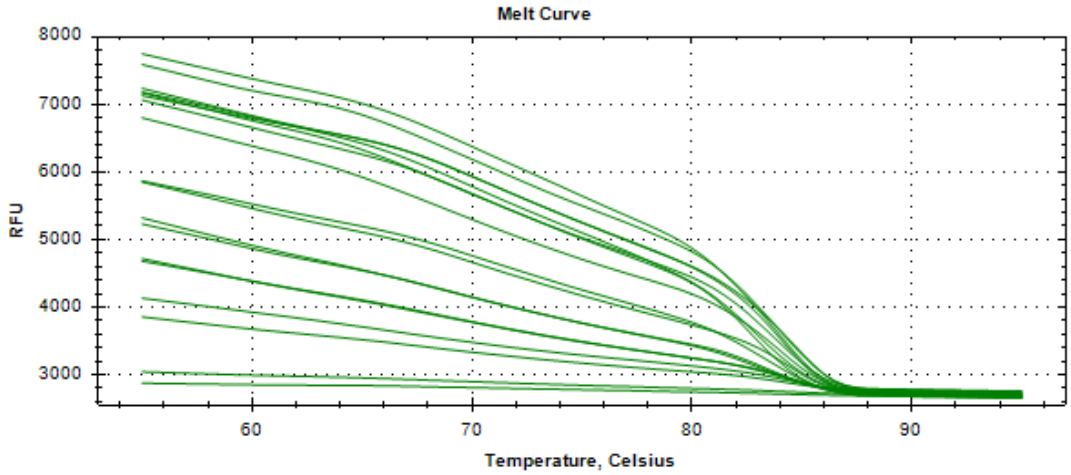


Appendix figure 6: Melting curve for qPCR for Primer matrix. Four primer mix with different forward/reverse primer concentration, primer mix 1 had 0.1/0.1 µM concentration, primer mix 2 had 0.1/0.2 µM concentration, primer mix 3 had 0.2/0.1 µM concentration and primer mix 4 had 0.15/0.15 µM.

Appendix figure 7 shows the results from Table 16 in a curve. Appendix figure 8 shows the melting curve from the same qPCR. The curve is for 6 primer mixes which all had water as template.

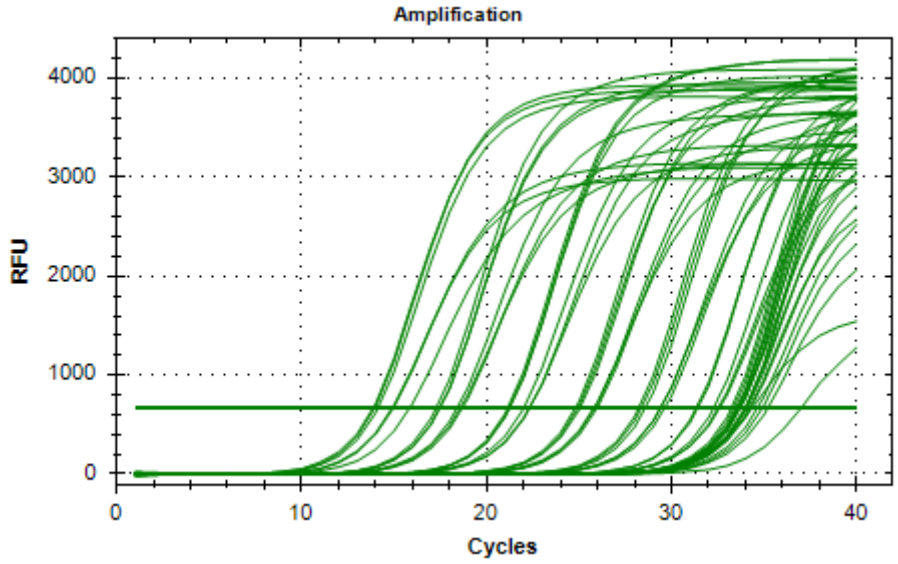


Appendix figure 7: qPCR of 6 different primer mixes with different forward/reverse primer concentrations. Primer mix 1 had 0.1/0.1 µM concentration, primer mix 2 had 0.1/0.2 µM concentration, primer mix 3 had 0.2/0.1 µM concentration, primer mix 4 had 0.15/0.15 µM concentration, primer mix 5 had 0.2/0.2 µM concentration and primer mix 6 had 0.3/0.3 µM concentration. Had a 54-70 °C gradient.

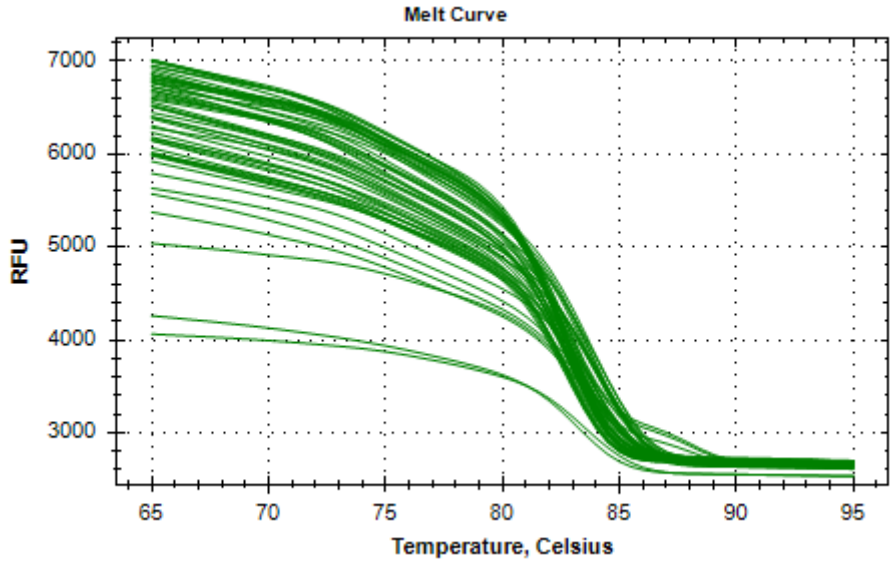


Appendix figure 8: Melting curve for qPCR of 6 different primer mixes with different forward/reverse primer concentrations. Primer mix 1 had 0.1/0.1 µM concentration, primer mix 2 had 0.1/0.2 µM concentration, primer mix 3 had 0.2/0.1 µM concentration, primer mix 4 had 0.15/0.15 µM concentration. Had a 54-70 °C gradient.

Appendix figure 9 shows the results from Table 17 in a curve. Appendix figure 10 shows the melting curve from the same qPCR. The curve is for two primer mixes that had plasmid as template. The diluted plasmid had a concentration range of $2 - 2 \times 10^{-7}$ ng/µl

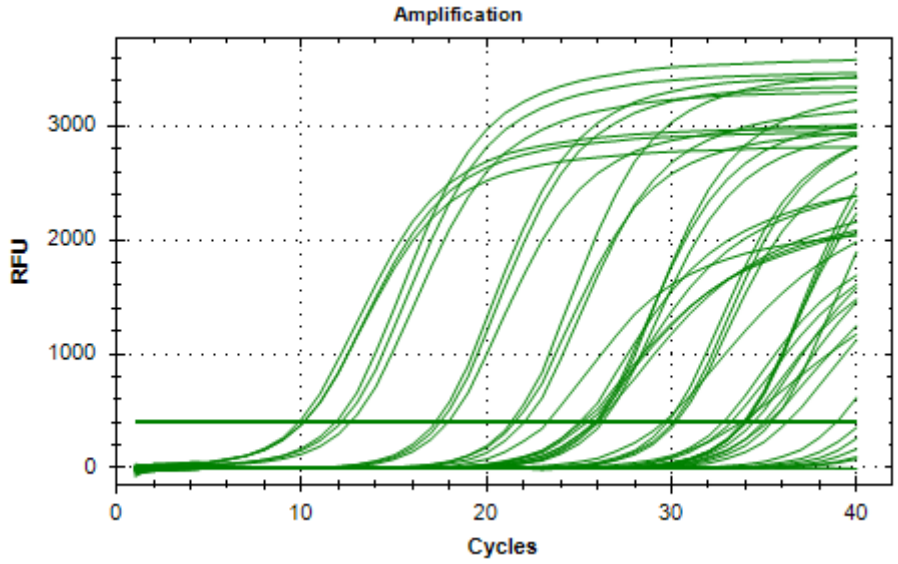


Appendix figure 9: qPCR for samples with DNA standard with two different primer mixes. The DNA standard was diluted using the 10-fold method. Primer mix 1 was had a 0.1/0.2 µM concentration of forward/reverse primers, primer mix 2 had a 0.15/0.15 µM concentration of forward/reverse primer.

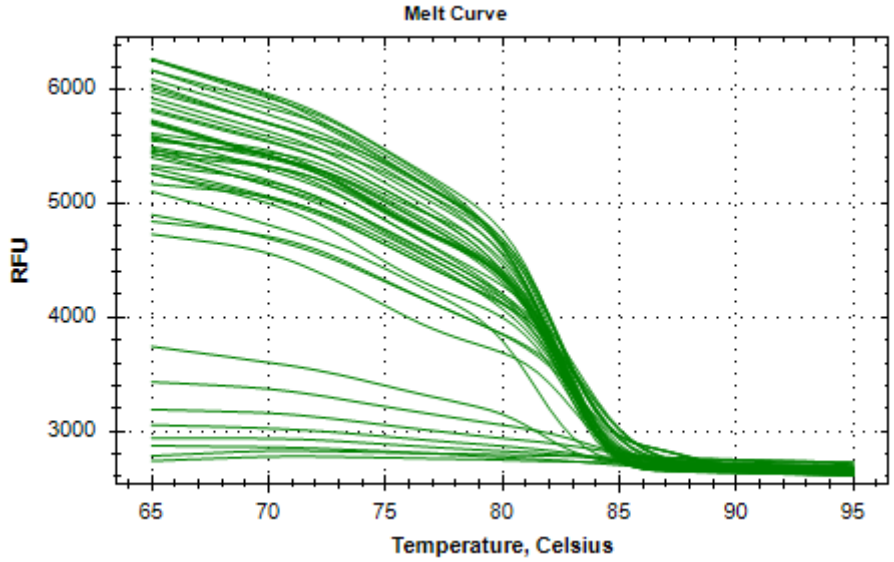


Appendix figure 10: Melting curve for qPCR for samples with DNA standard with two different primer mixes. The DNA standard was diluted using the 10-fold method. Primer mix 1 was had a 0.1/0.2 µM concentration of forward/reverse primers, primer mix 2 had a 0.15/0.15 µM concentration of forward/reverse primer. The qPCR was taken 08.03.2024.

Appendix figure 11 shows the results from Table 18 in a curve. Appendix figure 12 shows the melting curve from the same qPCR. The curve is for plasmid samples and samples with bead beating intensity of 4 m/s. The diluted plasmid had a concentration range of $2 - 2 \times 10^{-6}$ ng/µl.

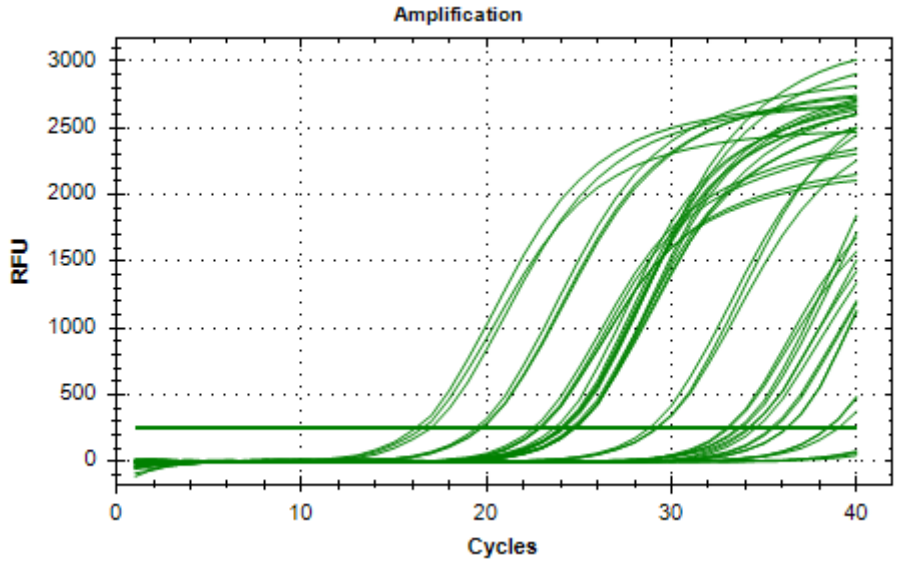


Appendix figure 11: qPCR for DNA standard and samples with 4 m/s bead beating, 3 parallels for each. The DNA standard was diluted using the 10-fol method. Both primer mixes had a 0.1/0.1 µM concentration of forward/reverse primer.

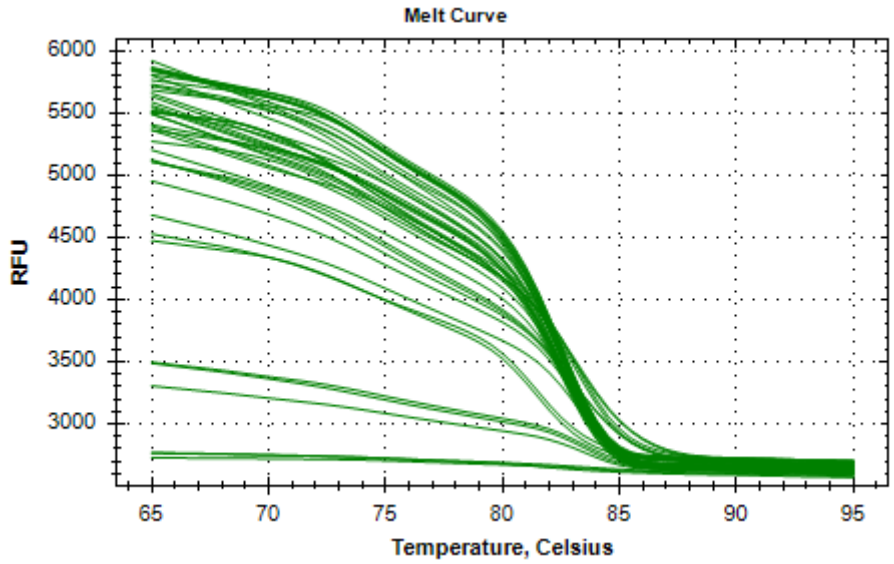


Appendix figure 12: Melting curve for qPCR for DNA standard and samples with 4 m/s bead beating, 3 parallels for each. The DNA standard was diluted using the 10-fol method. Both primer mixes had a 0.1/0.1 µM concentration of forward/reverse primer. The qPCR was taken 11.03.2024.

Appendix figure 13 shows the results from Table 19 in a curve. Appendix figure 14 shows the melting curve from the same qPCR. The curve is for plasmid samples and samples with bead beating with 4 m/s. The diluted plasmid had a concentration range of $0.2 - 2 \times 10^{-5}$ ng/µl. The ZymoSTD samples were diluted 1:100.

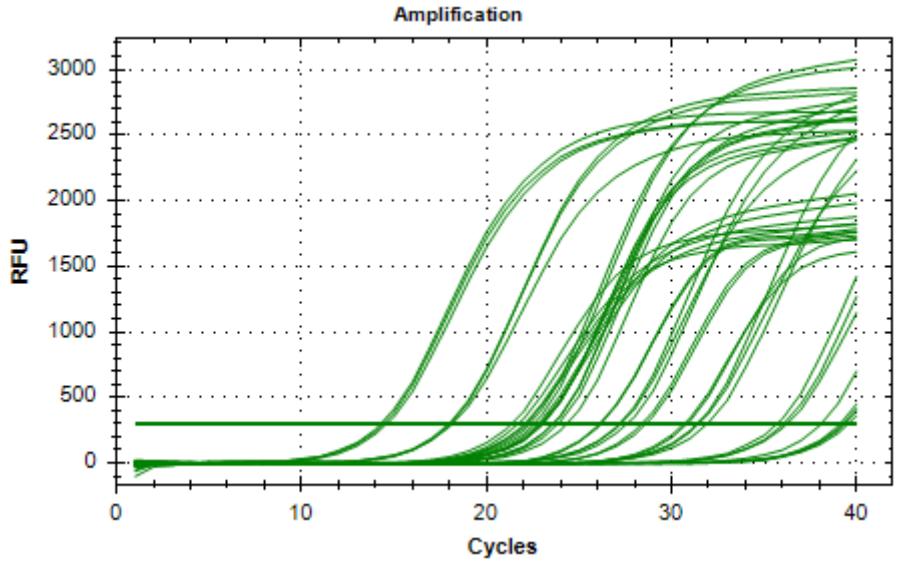


Appendix figure 13: qPCR for plasmid and samples with 4 m/s bead beating. The plasmid was diluted using the 10-fold method. Both primer mixes had a 0.1/0.1 µM concentration of forward/reverse primers. The qPCR was taken 14.03.2024.

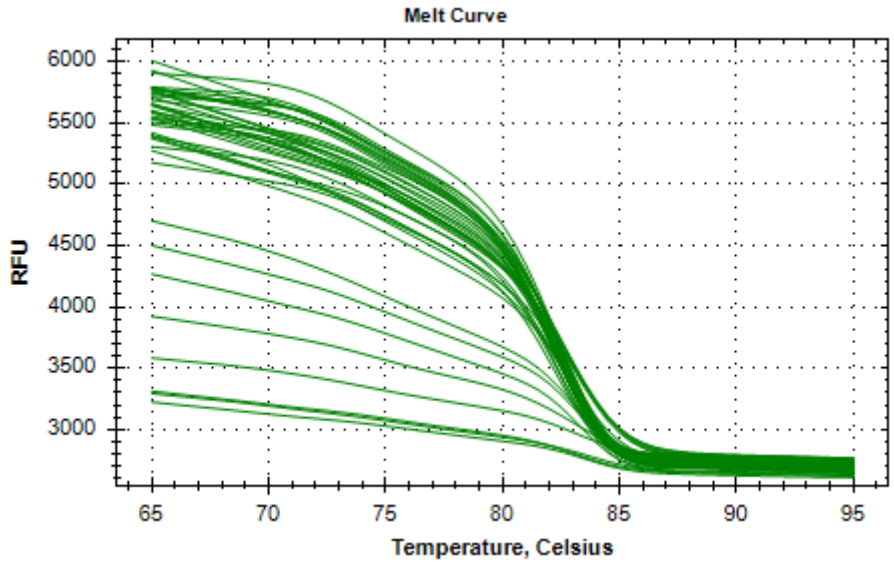


Appendix figure 14: Melting curve for qPCR for plasmid and samples with 4 m/s bead beating. The plasmid was diluted using the 10-fold method. Both primer mixes had a 0.1/0.1 µM concentration of forward/reverse primers. The qPCR was taken 14.03.2024.

Appendix figure 15 shows the results from Table 20 in a curve. Appendix figure 16 the melting curve form the same qPCR. The curve is for plasmid samples and samples with bead beating with 6 m/s. The diluted plasmid had a concentration range of $0.2 - 2 \times 10^{-5}$ ng/µl. The ZymoSTD samples were diluted 1:100.

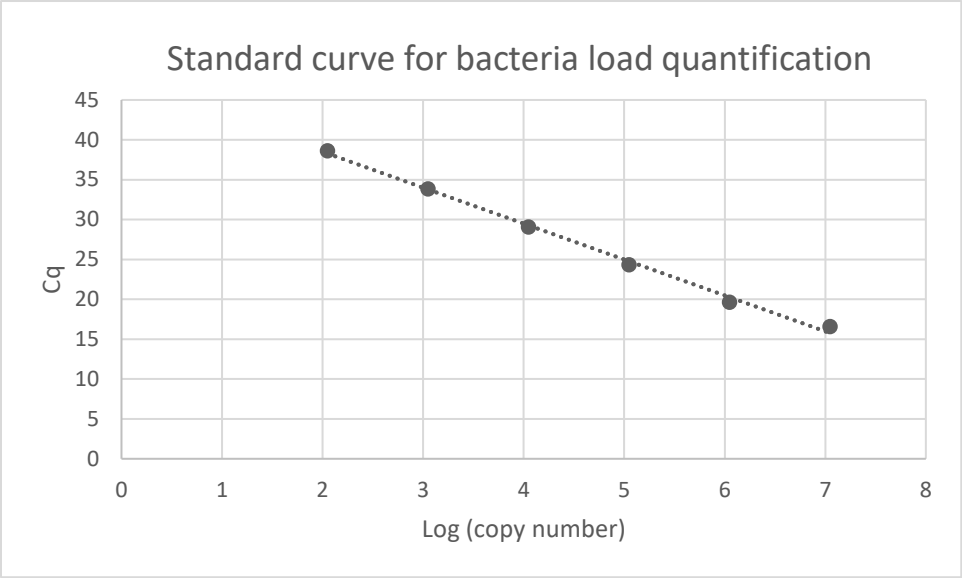


Appendix figure 15: qPCR for plasmid and samples with 6 m/s bead beating. The plasmid was diluted using the 10-fold method. Both primer mixes had a 0.1/0.1 µM concentration of forward/reverse primers.



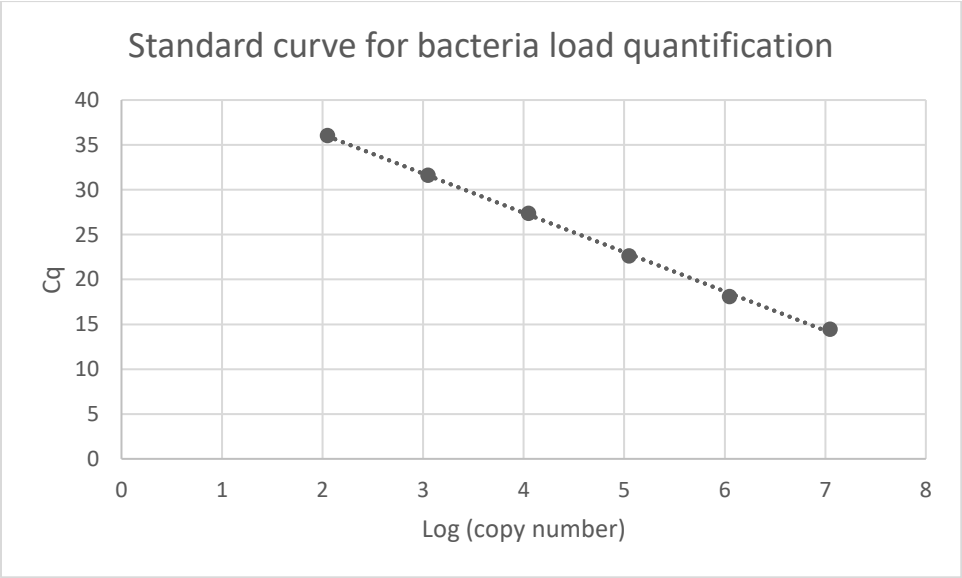
Appendix figure 16: Melting curve for qPCR for plasmid and samples with 6 m/s bead beating. The plasmid was diluted using the 10-fold method. Both primer mixes had a 0.1/0.1 µM concentration of forward/reverse primers. The qPCR was taken 15.03.2024.

A standard curve was made from the results from Table 19 and Appendix figure 13. The curve is from designed plasmid that was diluted 6 times using the 10-fold dilution method. The first plasmid had a concentration of 0.2 ng/µl. The standard curve gave a $y = -4{,}5052x + 47{,}514$ and $R^2 = 0{,}6464$.



Appendix figure 17: Standard curve made from qPCR with designed plasmid. The plasmid is diluted 6 times using the 10-fold dilution. The first plasmid had a concentration of 0.2 ng/µl

Another standard curve was made from the results in Table 20 and Appendix figure 15. The curve is made from designed plasmid samples that were diluted six times using the 10-fold dilution method. The standard curve gave a $y = -4{,}3761x + 44{,}93$ and $R^2 = 0{,}999$,



Appendix figure 18: Standard curve made from qPCR with designed plasmid. The plasmid is diluted 6 times using the 10-fold dilution. The first plasmid had a concentration of 0.2 ng/µl.