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Comparative study of commercial master mixes in bacterial quantification

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Preface

This thesis was performed in association with Alimetrics Ltd. I would like to thank Dr. Marko Lauraeus for offering me the chance to take a leap into the world of research and life sciences. Thank you for Kalle-Juhani Riihinen and all my coworkers at Alimetrics for all the guidance, continuous support and good spirit.
My gratitude to my instructor Tiina Soininen for all the advice she gave me and Minna Paananen-Porkka for checking the language.
Special thanks to my family, to my mother (you are the best!) and my beloved late father for always believing in me and never questioning my choices.
And last but not least, a warm thank you to Antti who is always there for me with a smile on his face.

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Abstract

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This thesis project was done in association with Alimetrics Ltd Espoo. The main goals of this project was to find out whether commercial ready-to-use polymerase chain reaction (PCR) reagents (master mix) fulfill the quality criteria required and whether they can be easily implemented for different instruments and matrixes.

PCR is an extensively applied, sensitive and versatile tool used to amplify small amounts of DNA to a large number of copies. With the quantitative real-time polymerase chain reaction (qPCR) the specific amount of bacteria in the sample matrix can be quantified. However, possible reagent derived contaminations are considered to hamper the quantification and limit the utility of this otherwise excellent method when analyzing samples with a low number of DNA. Furthermore, the change of the both reagent and the instrument requires laborious optimization and validation steps that lead to extra costs. Therefore, for the end-user it is relevant to discover the reagent that contains as few reagent-derived contaminants as possible and can easily be adapted between different instruments if necessary.

In this project four commercial reagents based on SYBR Green chemistry were compared with three instruments. As samples, nine DNA samples from different origins and three standard DNAs were used. No template controls were used to determine the purity of reagents. Each reagent with replicates was tested with all three instruments.

According to the results, none of compared reagents were completely pure, each containing a foreign reagent-derived DNA that was detected in no template controls. Therefore, these reagents were not suitable for matrixes that were known to contain only small amounts of DNA. In addition, there were shown differences between instruments when tested with same reagent. Hence, none of the compared reagents fulfilled the quality requirements; nor can the reagents be implemented for different instruments and matrixes.

Keywords	master mix, qPCR, SYBR Green, S16 rDNA

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Tiivistelmä

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Suuntautumisvaihtoehto	Prosessitekniikka
Ohjaajat	Kehitysjohtaja Kalle-Juhani Riihinen Lehtori Tiina Soininen

Opinnäytetyö tehtiin Alimetrics Oy:lle Espoossa. Työn päätavoitteina oli selvittää täyttävätkö polymeraasiketjureaktiossa (PCR) käytettävät kaupalliset reagenssit (*master mixit*) niille asetettuja laatuvaatimuksia ja ovatko kyseiset reagenssit helposti implementoitavissa eri laitteistoille ja matriiseille.

PCR on yleisesti käytetty erittäin tarkka ja monipuolinen DNA –tutkimusmenetelmä, jolla voidaan monistaa pieni määrä DNA:ta moninkertaiseksi. Kvantitatiivisella polymeraasiketjureaktiolla (qPCR) voidaan määrittää tarkasti näytteessä olevien bakteerien määrä, mutta reagenssien sisältämien epäpuhtauksien on havaittu häiritsevän määritystä sekä estävän hyvin pieniä bakteerimääriä sisältävien näytteiden luotettavan analysoinnin. Lisäksi sekä reagenssin että laitteiston vaihto vaatii työläitä ja lisäkustannuksia tuovia optimointeja. Kuluttajan kannalta olisi olennaista löytää reagenssi, joka sisältäisi mahdollisimman vähän epäpuhtauksia ja olisi helposti toteutettavissa eri laitteistoilla tarpeen niin vaatiessa.

Työssä verrattiin neljää SYBR Green –kemiaan perustuvaa kaupallista reagenssia sekä kolmea PCR –laitetta. Vertailussa näytteinä olivat yhdeksän eri DNA-näytettä, kolme standardi –DNA:ta sekä kontrollit ilman DNA:ta reagenssien puhtauden määrittämiseksi. Jokainen reagenssi rinnakkaisnäytteineen testattiin jokaisella laitteella.

Tulosten mukaan mikään verratuista reagensseista ei ollut täysin puhdas, vaan sisälsivät reagenssien mukana tulevaa vierasta DNA:ta, joka voitiin havaita DNA:n monistumisena kontrollinäytteissä. Tämän vuoksi testatut reagenssit eivät myöskään olleet ideaalisia matriiseille joiden tiedettiin sisältävän vain vähän DNA:ta. Lisäksi myös laitteistolla todettiin olevan eroja samalla reagenssilla testattuna. Näin ollen mikään verratuista tuotteista ei täytä toivottuja puhtauskriteerejä eivätkä reagenssit ole suoraan implementoitavissa eri laitteiden kesken.

Avainsanat	*master mix*, qPCR, SYBR Green, S16 rDNA

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1 Introduction

Polymerase chain reaction (PCR) is an extensively applied tool that is used to amplify a small amount of DNA to a large number of copies. PCR is a reaction that requires a large number of reagents and optimal conditions. However, this complex method offers an exact and versatile tool for DNA analysis. [1, p. 1.]

Quantitative real-time polymerase chain reaction (qPCR) has become an extensively applied tool in molecular microbiology. It is a remarkable research tool that can provide valuable and reliable information in analyzing microbiological communities. However, there are some downsides in this otherwise excellent method. Especially reagent-derived DNA contamination problems limit the utility of qPCR when a low detection limit is required. [2.]

Therefore, it is relevant to discover the most suitable ready-to-use reagent mix, the master mix, for the end-user's purposes at the most reasonable price. Flexible transition from one master mix to another could also provide major cost savings in routine analytics. The purpose of this project was to discover whether the different master mixes can be transferred and adapted to a new qPCR instrument platform without laborious assay optimization and validation steps. Moreover, another essential rationale was to find out whether some of the existing master mixes meet the required quality criteria in terms of minimal background DNA contamination. This would shed light on the applicability of these commercial reagents in the analysis of samples with low amounts of endogenous bacteria.

Several approaches to reduce the amount of endogenous bacteria DNA in real-time PCR have been described in background literature. None of these methods, however, proved very effective and reproducible, especially when low copy numbers of bacterial ribosomal DNA (rDNA) have to be discriminated from contaminating endogenous DNA derived from the PCR reagents. Even if these procedures slightly decrease contamination levels, they induce redundant stages and costs to the application

protocol. These contamination risks are required to be considered among master mix manufacturers. [2; 3; 4; 5; 6.]

This project was performed in association with Alimetrics Ltd. During the past decade, Alimetrics team has actively developed and applied qPCR technique to assess the analysis of microorganisms in biological or industrial sample matrices. As they perform over 100,000 qPCR reactions annually, the intention is to further improve qPCR analytics comprising the science with reduced costs. [7.]

2 Review of the Literature

2.1 Polymerase Chain Reaction (PCR)

In 1984 Kary Mullis developed the PCR method that involves the replication of DNA. The discovery was awarded the Nobel Prize in chemistry in 1993. The innovation to apply the *Taq* polymerase enzyme in PCR revolutionized the implementation of the PCR method. Nowadays there are number of techniques implemented in this widely accepted method among research and it can be routinely used in DNA amplification *in vitro*. [1, p. 3; 8, p. 149.]

2.2 Principle of PCR

The PCR analysis consists of three steps. First, the sample solution that contains the template DNA is heated (>90 °C). Heat breaks the hydrogen bonds between the base pairs and double stranded DNA (dsDNA) is denaturized as two single strands. Second, the solution is cooled down (50 -75 °C). Two single stranded oliconucleotides, i.e. primers, excessively present in solution, bind to opposite strands of the template single stranded DNA (ssDNA). The annealing temperature is primer-specific and primers are required to be complementary with the target strand. The third phase is the primer extension. The sample solution is heated again (72 -78 °C). At an optimal temperature a heat-stable polymerase initiates the DNA synthesis, polymerization, by adding

deoxyribonucleotide triphosphates (dNTPs), and a DNA strand identical to the template is synthesized. The synthesis initiates from the 3' end and continues only at a raised temperature wherein the interaction between primers and template is highly specific. The principle of PCR is illustrated in Figure 1. [8, p. 149–150; 9, p. 385–387; 10, p. 123–125; 11.]

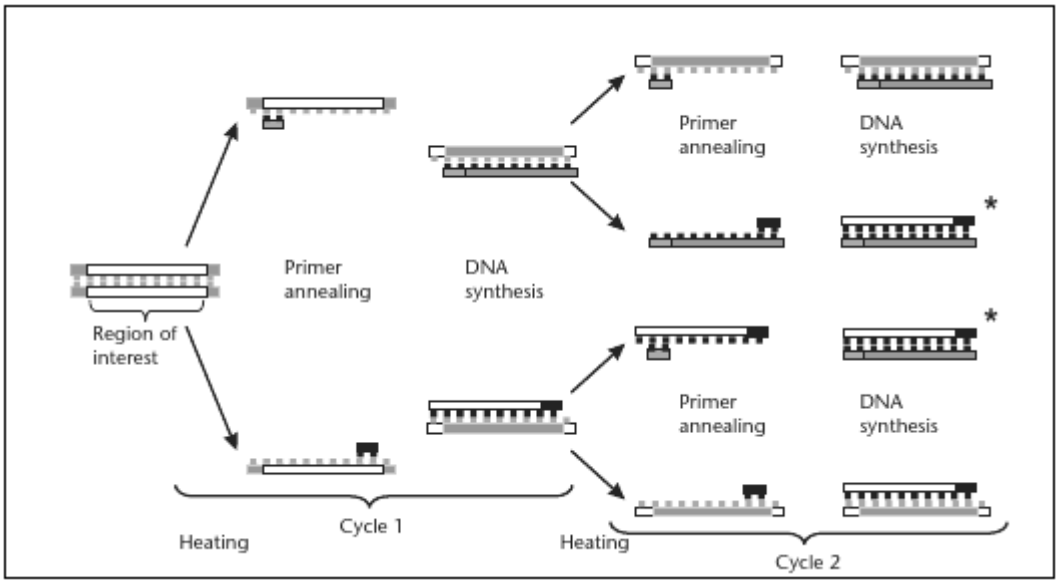

Figure 1. Polymerase chain reaction. [12, p. 12.]

The three stages of thermal cycle (Figure 2), melting, annealing and extension are repeated in several *n* amounts of cycles. The optimal number of cycles is dependent on the initial concentration of template DNA. A typical PCR procedure consists of 15 to 40 cycles and a PCR with 30 cycles is completed in 1–2 hours. The order of magnitude for the amplification coefficient is 10^6. Thus, one molecule of the template DNA can be polymerized to a million new identical DNA molecules in a short period of time. [9, p. 385–387; 13, p. 108.]

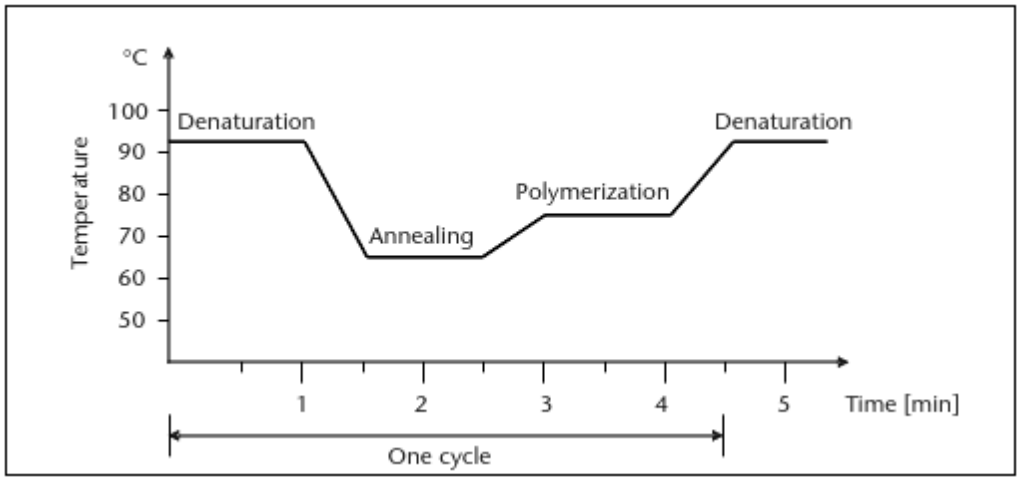

Figure 2. Temperature profile of thermal cycle. [12, p. 41.]

After completed, the last cycle final extension is performed. The samples are incubated at a higher temperature (72 °C or more) from 5 to 15 minutes. In the final extension the projecting ends of synthesized dsDNA are filled and the *Taq* polymerase enzyme adds extra A deoxyribonucleotide triphosphates (dATPs) according the base pairing rules (A:T and G:C). The polymerase enzyme catalyzes the synthesis of dsDNA from the 5' end to the 3' end. [1, p. 34; 14.]

In this thesis only the quantitative real-time polymerase chain reaction (qPCR) is discussed more closely in chapter 2.4 as it was the technique applied in the experiment.

2.3 Master Mix

Other PCR reagents besides the template DNA and paired primers are available as ready-to-use PCR premixes, master mixes. The master mix contains a polymerase enzyme (*Taq* polymerase in most cases), dNTPs and a buffer in optimal concentration for the reaction. [1, p. 26; 14.]

Commercial master mixes are available in two different kind of assays based either on a dsDNA binding dye or on a probe. The mostly applied dsDNA binding dye is SYBR Green I. [15.]

Master mixes are usually provided as 10X concentrations. In most implementations 1X or 2X concentrations are required; thus, the buffer is diluted with sterile laboratory water. The final reaction volume is obtained by adding the template DNA and complementary primers. [14.]

Nowadays most manufacturers specialized in biochemical solution have a wide selection of master mixes optimized for different applications. They are convenient in use and enable reproducibility. [16.]

2.3.1 Polymerase enzyme

The polymerase enzyme initiates the primer extension on ssDNA. The polymerase extends the annealed primer by adding complementary dNTPs on the template sequence (Figure 3). [13, p. 107–108.]

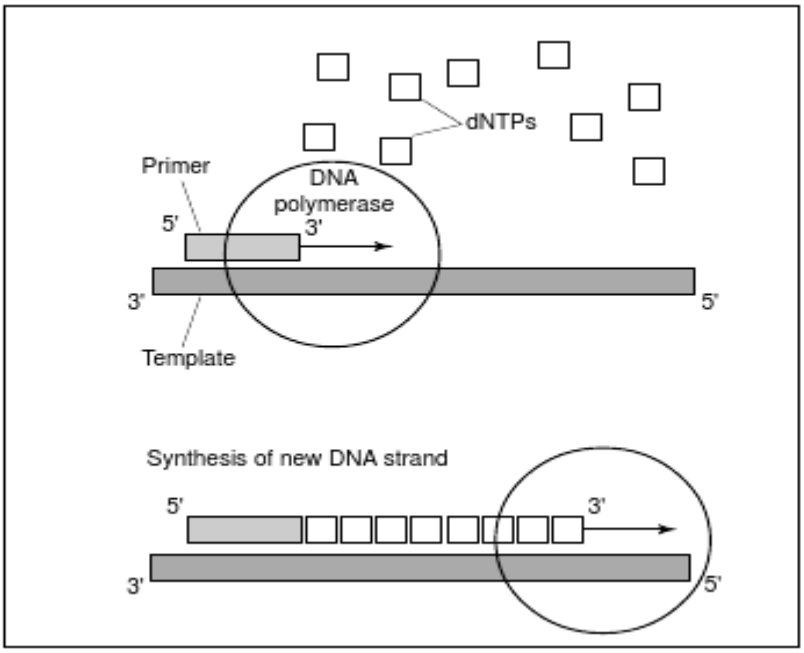

Figure 3. Primer extension and dsDNA polymerization by a polymerase enzyme. [1, p. 5.]

The first polymerase enzymes used in PCR were heat labile; thus, it was required that a fresh enzyme be added on each cycle. Later the discovery of a heat-stable enzyme facilitated the PCR protocol. [1, p. 35.]

An enzyme originated from bacterium *Thermus aquaticus*, *Taq* is the most applied polymerase enzyme in PCR. The *Taq* polymerase enzyme was first isolated by Thomas D. Brock. It is highly heat-stable; therefore, it can tolerate the high temperatures of thermal cycling without getting denaturized. However, this convenient heat stable enzyme has a relatively low replication fidelity as its downside. Hence new heat stable polymerase enzymes are isolated in order to improve the proofreading activity. [17, p. 245–246.]

2.3.2 Deoxyribonucleotide triphosphates (dNTPs)

The presence of all four deoxyribonucleotide triphosphates (dATP, dCTP, dGTP and dTTP) in reaction is essential for new DNA strand formation so that the DNA synthesis and amplification can take place. In general concentrations between 50 –200 µM are applied. It is also important to have all four dNTPs present in equimolar concentration. As dNTPs interact with Mg^{2+} ions, their concentration level must exceed the Mg^{2+} concentration. [1, p. 25; 8.]

Too low concentrations of dNTPs in reaction may decrease the efficiency, while too high concentrations can hamper the fidelity of PCR. [1, p. 25.]

2.3.3 Magnesium

The magnesium concentration affects the reaction specificity and efficiency. The presence of Mg^{2+} in the reaction is crucial for the polymerase action of the *Taq* polymerase enzyme as a soluble complex is formed between the Mg^{2+} ions, the polymerase and the DNA. In general commercial buffers contain 1.5 mM of Mg^{2+} in 1X concentration. However, Mg^{2+} has an equimolar binding with dNTPs and, therefore, the dNTP concentration in the buffer affects the free Mg^{2+} concentration. [1, p. 24; 14.]

A low Mg^{2+} concentration may help in eliminating non-specific priming,although too low concentration of free Mg^{2+} ions in reaction may induce a low yield of the desired product. In high Mg^{2+} concentrations (excess Mg^{2+} ions in reaction) the *Taq*

polymerase enzyme has higher probability to make errors. Excess Mg^{2+} ions can also hamper the denaturation during amplification. [1, p. 24; 15.]

DNA polymerases are relatively sensitive to the fluctuation of the Mg^{2+} ion concentration; thus, it should be optimized individually for each template-primer combination. [13, p. 110.]

In master mixes, magnesium is present as Magnesium chloride ($MgCl^2$). Some buffers are supplied with a separate magnesium stock solution. This allows free optimization of the Mg^{2+} concentration for the end user. Thus, it is always important to make sure whether magnesium is added in the commercial master mix used or not. [1, p. 25; 15.]

2.3.4 Primers

The primer, a short nucleotide complementary to the target sequence of the template DNA acts as a starting point for new dsDNA synthesis. In DNA amplification there are always two kinds of primer used, forward and reverse. In general, primers are from 10 to 30 base pairs (bp) long. The length of a primer is important since it is required to find and match the desired sequence in the template. The length of the primer increases the specificity as, on the other hand, a shorter primer has more options for binding and, thus, a higher chance to make mistakes. [1, p. 26; 12, p. 12.]

Two important points must be considered when new primers are designed. First, the primers should not be complementary with each other or with itself. Thus, the possibility of primers to join together (self-priming) is avoided and the formation of primer dimers is hindered. As primer dimers take a conformation as a dsDNA (Figure 4), it results in giving false positive signals when the SYBR Green I chemistry is applied in quantification. [1, p. 27; 12, p.12; 13, p. 110.]

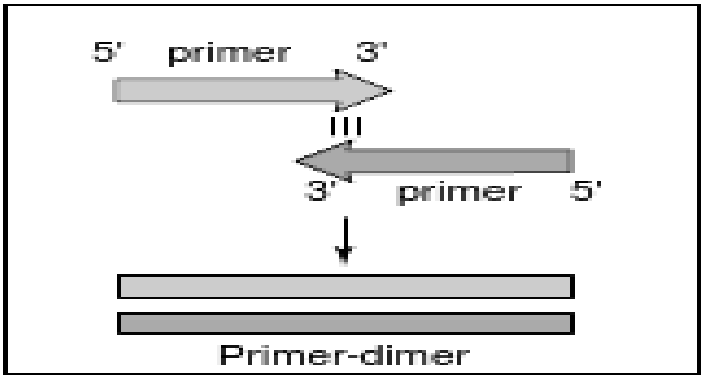

Figure 4. Primer dimer formation due to self-priming resulting in a false positive signal. [1, p. 26.]

Another issue that needs to be taken into account is A:T and G:C pairing. Melting temperatures (T_m) between the forward and the reverse primers should not vary more than 5 °C as primers are required to melt and anneal at nearly equal temperatures. T_m is the temperature where the primer is half adhered to the template DNA and half loose in the reaction solution. Since the primer with a higher content of G:C pairing requires an increased temperature to melt, the number of pairings in both primers should be nearly equal. T_m can be calculated according Equation 1. For primer design there are generally used commercial computer programs. [1, p. 27–28; 12, p. 12; 13, p. 110.]

$$T_m = 2(A + T) + 4(G + C) \quad (1)$$

Where

(A+T) is the number of A and T bp in primer sequence
(G+C) is the number of G and C bp in primer sequence

However, calculated T_m is usually too high for annealing. Therefore annealing temperature T_a is generally calculated from T_m according equation 2. [13, p. 110.]

$$T_a = T_m - 5\,℃ \quad (2)$$

In some cases even a lower T_a is required; therefore, it is specified experimentally. [13, p. 110.]

2.3.5 Buffer

Tris-HCl is a bipolar buffer that has a pH range from 6.8 to 8.3. The function of Tris-HCl in buffer is to keep the pH at an optimal level for polymerase activity. The optimal pH and ionic strength of the buffer may vary by the polymerase used in the reaction. [1, p. 24.]

The presence of salt ions in the solution is known to lower the melting temperature (T_m). Potassium Chloride (KCl) is also considered to contribute to primer and polymerase annealing. However, too high salt concentrations may result in aberrant products through primer mismatching. [1, p. 24.]

2.4 Quantitative Real-Time Polymerase Chain Reaction (qPCR)

qPCR has become an extensively applied tool in molecular microbiology that allows a quantitative measurement of the desired DNA in a homogenous assay. It is considered to be the most advanced method for DNA detection. In qPCR a small amount of the specific fragment of the DNA can be rapidly amplified and the product measured as it emerges (Figure 5). The detection is enabled by several chemistries that generate a fluorescent signal. qPCR has many advantages when compared to traditional PCR methods. It enables fast results with high sensitivity, specificity, throughput and absolute quantification. Furthermore, the specificity can be adjusted from individual bacterial strains to the phylum level or for the quantification of functional genes. [7; 18; 19; 20;]

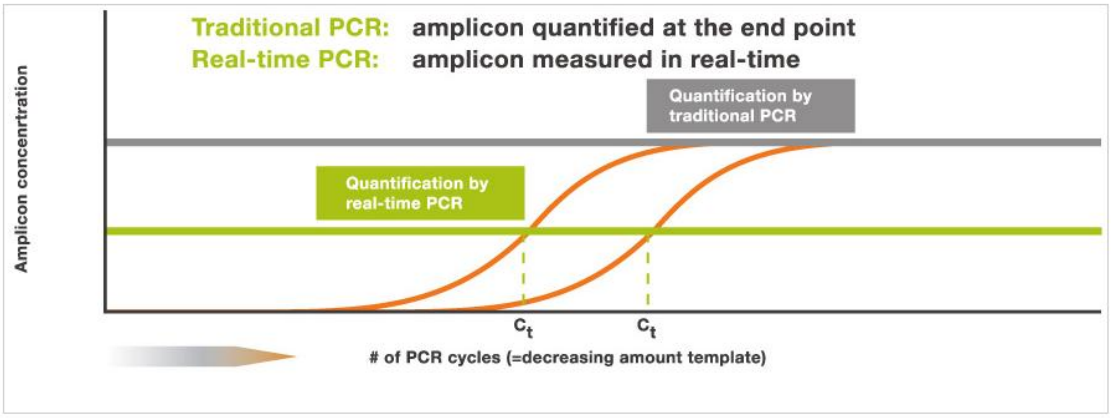

Figure 5. Amplicon measurements in traditional and real-time PCR. [7].

qPCR analytics comprises a number of applications used in various fields of diagnostics, e.g. medicine, forensic, molecular science and testing. It is used routinely in laboratories for example for pathogen detection, viral quantification, quality controlling and assay validation. [8, p. 151; 9; 15; 18.]

2.4.1 DNA amplification in qPCR

When DNA is amplified, the run has three different phases: exponential, linear and plateau (Figure 6). In exponential phase, if a 100% reaction efficiency is assumed, the product is doubled in each cycle repeated. In qPCR data is measured in this phase as it provides the most reliable data for quantification. The exponential amplification of PCR can be described according Equation 3. [18; 21, p. A-8.]

$$X_n = X_m(1 + E_X)^{n-m} \tag{3}$$

where

X_n = number of target molecules cycle n (in order n ≥ m)
X_m = number of target molecules cycle m (in order m ~ n)
E_X = target amplification efficiency (from 0 to 1)
$n - m$ = number of cycles run between cycle m and cycle n

In linear phase amplification the process has already consumed some of the reagents. Hence, the reaction starts to slow down and growth does not occur in exponential scale anymore. The plateau phase, also known as the end point phase, is the last phase in amplification where the reaction reaches its end and no new dsDNA is synthesized. [18.]

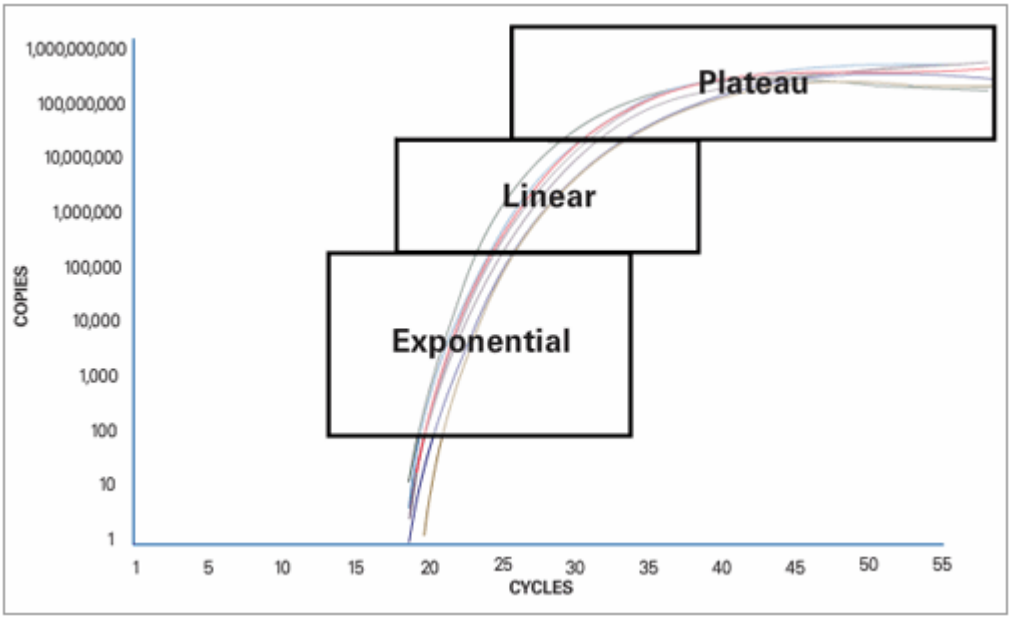

Figure 6. Three phases in DNA amplification. [18.]

As quantification data is collected in exponential phase of amplification, it is necessary to determine the level of detection, the Threshold line. The Threshold line is the level in which the fluorescent intensity is greater than the background. The Cycle Threshold (Ct) is the amplification cycle where the sample reaches the Threshold line. The Threshold line and Ct are illustrated in Figure 7. [18.]

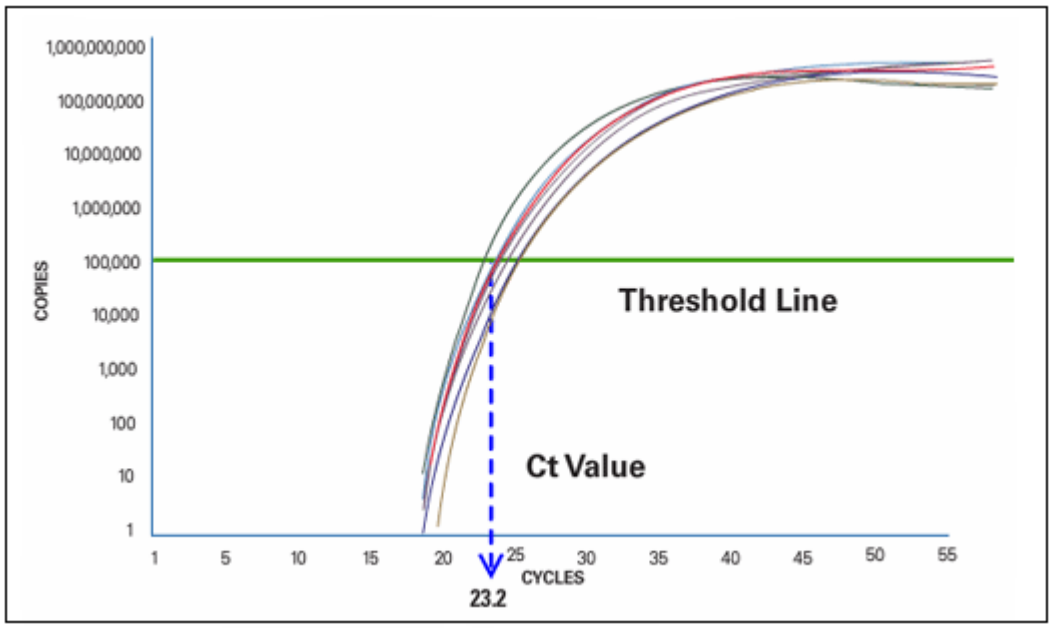

Figure 7. The Threshold line and Cycle Threshold in amplification plot. [18.]

The Ct values of samples with unknown concentration are compared to known standards series (Figure 8). Thus, the exact concentration of samples can be determined. [18.]

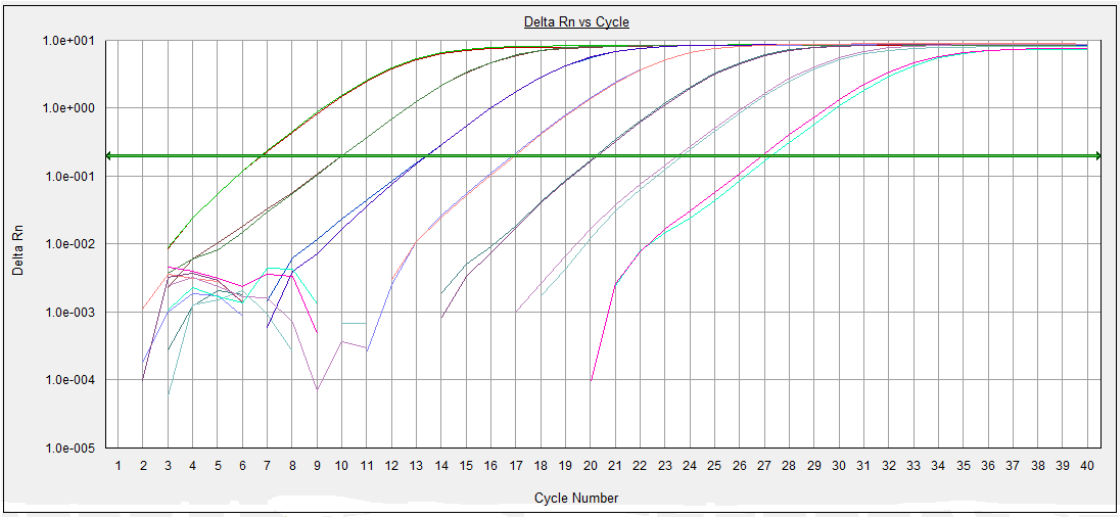

Figure 8. Amplification plot of Clostridium leptum standard series. The curves with Ct value of 7 contains 1·10 ^{8} copies, Ct value 10 1·10 ^{7} copies, Ct value between 13 and 14 1·10 ^{6} copies, Ct value 17 1·10 ^{5} copies, Ct value between 20 and 21 1·10 ^{4} copies, Ct value between 23 and 24 1·10 ^{3} copies and Ct value between 27 and 28 1·10 ^{2} copies. (Copyright Alimetrirics Ltd.)

2.4.2 SYBR Green I

SYBR Green I, (2-[*N*-(3-dimethylaminopropyl)-*N*-propylamino]-4-[2, 3-dihydro-3-methyl (benzo-1, 3-thiazol-2-yl) methylidene]-1-phenylquinolinium), is a green-emitting cyanine dye. It binds non-specifically to dsDNA and emits a fluorescent signal. Therefore the intensity of fluorescence is proportional to the amount of dsDNA in amplification (Figure 9). [22; 23; 24.]

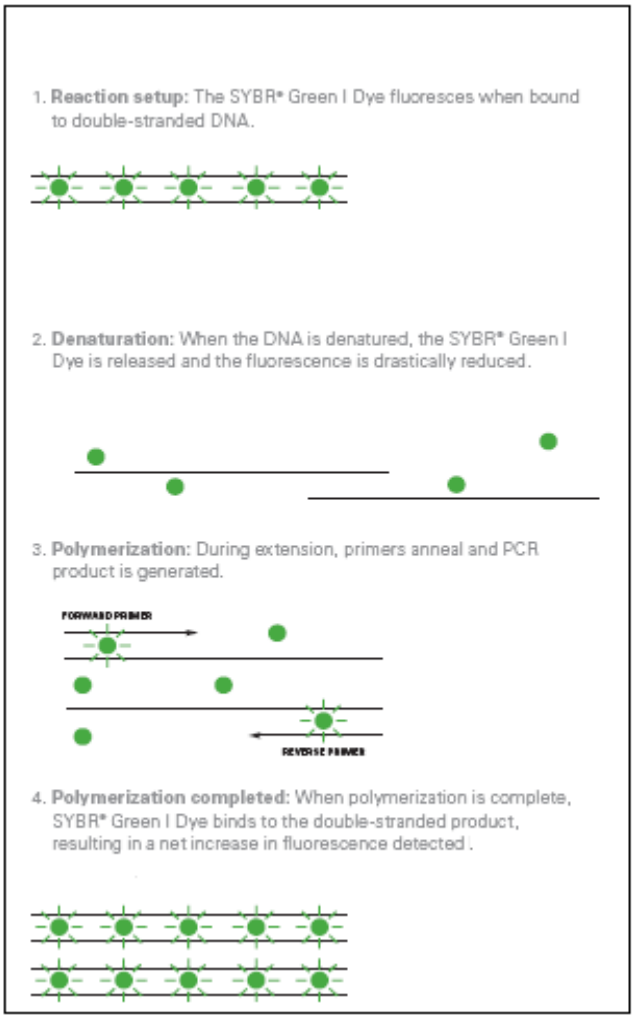

Figure 9. SYBR green I dye chemistry. [18.]

SYBR Green I is a simple and economical solution as qPCR chemistry since there is no probe required. It is sensitive, flexible and safe to use and it does not inhibit PCR. However, the use of SYBR Green I may cause false positives if primer dimers or other artifacts are synthesized during amplification. This may prevent the use of SYBR Green I chemistry in some situations. [11; 19; 23; 25;]

2.4.3 qPCR instrumentation

As the implementation of the qPCR analysis is promptly increasing in the field of research, instrumentation and techniques are getting more advanced. Higher throughput time for samples, simplicity and fast analyzing time are due to a high degree of automation. As PCR analysis gets more automated, the hands-on-time of

samples decreases. Therefore, the possibilities of contaminations due to handling are reduced. [12, p. 312–313; 19.]

The qPCR analysis is most commonly performed with a 96-well formatted instrument. The 48- and 384-well formats with a reaction volume from 5 to 100 µl are also implemented. Instruments consist of a thermal block cycler (Peltier element block or heated air), an excitation source (a tungsten halogen, a LED, a high intensity xenon lamp or an argon ion laser) and a detection unit (a CCD camera, a PMT, emission filters or photodetectiondiodes). Excitation spectrums can be selected with various filters with a wavelength range from 350 nm to 750 nm. The data is collected and analyzed by separate a desktop the data station. [16; 26; 27.]

Various manufacturers, such as Applied Biosystems, Hybaid, Eppendorf, Stratagene and Roche, supply PCR instruments. Figure 10 illustrates a Roche LightCycler® 480 instrument that was applied in this project. [1, p. 57–58.]

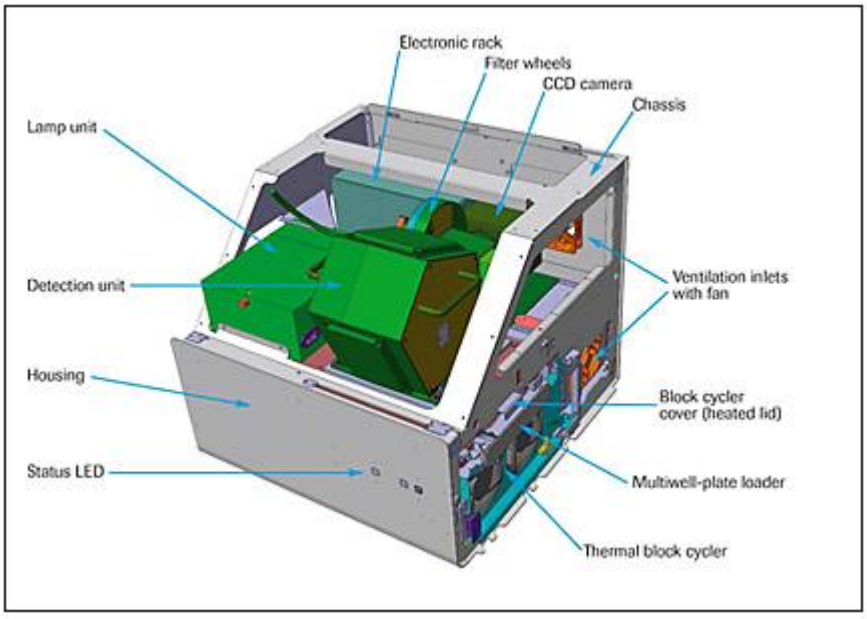

Figure 10. Roche LightCycler® 480 Instrument.[16.]

2.5 16S ribosomal RNA gene

The living cells can be classified in three different families, domains: the Bacteria, the Eucaryota and the Archaea (Figure 11). Bacteria are further classified as prokaryotes and all the animal cells and fungi as eukaryotes. [28, p. 1.]

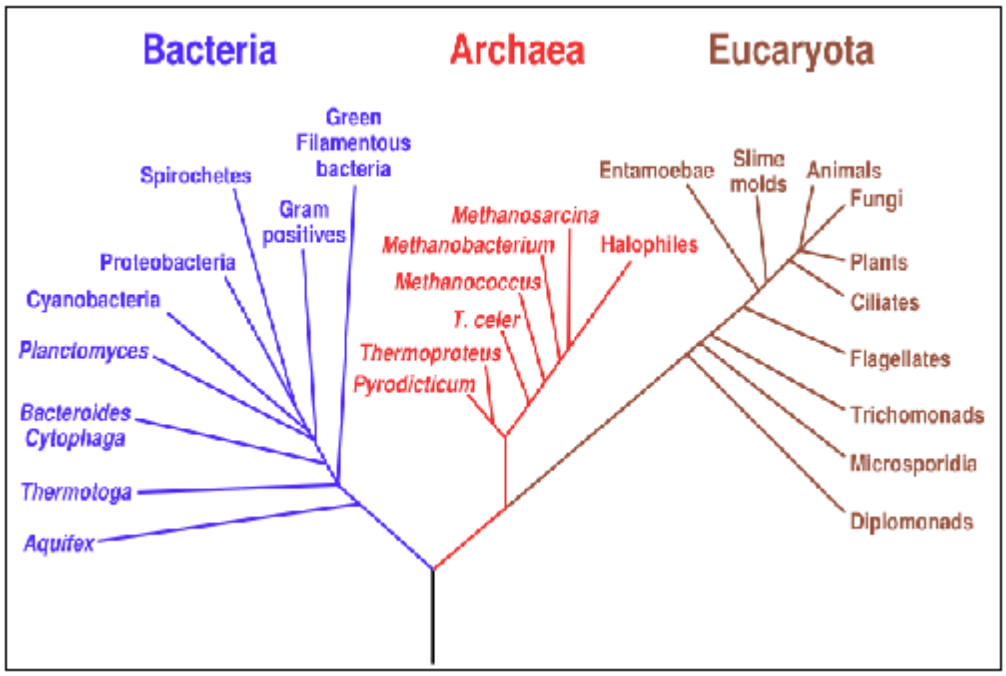

Figure 11. Phylogenetic tree of families. [29.]

The cell structure of prokaryotes differs from eukaryote by several features. The prokaryotic cell is not surrounded by the nuclear membrane in contrast to eukaryotic cell. The formation of chromosomes also varies as they are circular in prokaryotes and linear in eukaryotes. The cytoplasm of eukaryotes contains cell organelles such as mitochondria, Golgi apparatus and peroxisomes. In prokaryotes the cytoplasm is highly undifferentiated. Prokaryotic cells, thus bacteria also, are surrounded by a cell wall which is more substantial compared to the plasma membrane surrounding eukaryotic cells. [28, p. 2.]

2.5.1 Ribosome and ribosomal RNA in prokaryotes

The ribosome is the part in the cell that where the protein synthesis occurs. The messenger RNA (mRNA) delivers the genetic information to ribosomes. The ribosome

attaches itself on the mRNA and starts to read the information of base sequences that the mRNA contains. [13, p. 20; 30.]

The ribosomal RNA (rRNA) is the component in ribosomes that catalyzes the peptide-bond formation. [8, p. 25.]

The formation of a prokaryotic ribosome consists of two subunits, a large subunit (50S) and a small subunit (30S). The 16S rRNA molecule is located in small 30S subunit with 21 different proteins. The 16S gene is one of the most conserved genes. The 16S gene marks evolutionary distances and relatedness of different organisms, even though the absolute rate of change in the 16S gene between species is unknown. Therefore, the 16S gene is an excellent tool for bacterial identification at genus and species level. [8, p. 823; 30.]

2.5.2 Universal primers for 16S rRNA gene

In 1980s it was evidenced that a comparison of the stable part of the genetic code can determine phylogenetic relationships of all life forms. Since then, the 16S ribosomal RNA gene has been found to be most commonly used part of the gene for this purpose. [30.]

Bacterial detection by PCR is extensively applied by using the 16S ribosomal RNA (16S rRNA) gene as a target sequence. In prokaryotes, the 16S rDNA occurs in at least one copy in a genome and contains several conserved regions, which have remained constant throughout the evolution nearly in all bacterial species. Thus, the universality of these gene fragments makes the 16S rDNA an ideal target in the qPCR when the intention is to analyze the total bacterial load in the samples of interest. The length of the 16S rRNA gene is approximately 1,550 bp and between the conserved regions there are located variable regions that enable group- or species-specific targeting (Figure 12). [2; 3; 4; 7.]

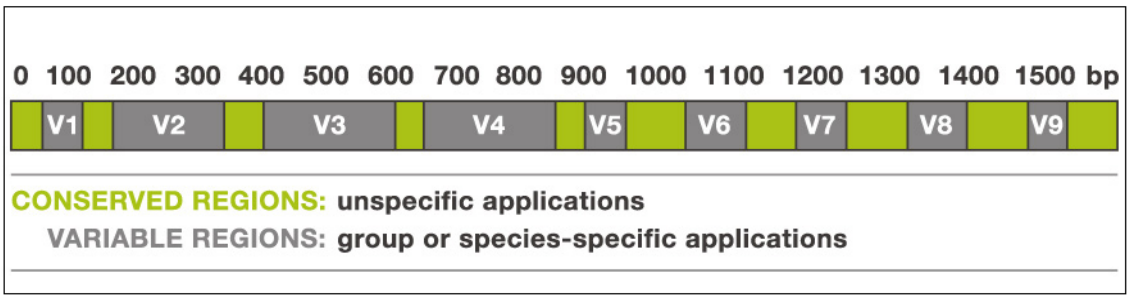

Figure 12. Conserved and variable regions in 16S rRNAgene. [7.]

However, the performance of this broad-range rDNA PCR approach, especially when applied to samples with low bacterial counts, can be crucially affected by the amplification of the contaminant DNA present in the PCR reagents. In a number of occasions cross-reaction with the unwanted DNA hampers the applicability of the qPCR technique by reducing the assay sensitivity as well as producing false-positive results. [2; 3; 4.]

The 16A rRNA gene-targeted universal primers are applied to estimate the total bacterial load in complex communities. Several studies have reported the use of more than a single set of primers when bacteria were detected. However, conserved regions of the 16S rDNA offer a tool for bacterial detecting with just one primer set as well. The variation in the number of copies of the 16S rRNA operons between species limits the absolute determination of bacteria in a sample matrix by the qPCR. The qPCR is, nevertheless, still considered to be the most precise and sensitive method applied when detecting bacteria from a difficult matrix with a multi-species population and possible impurities. Bergey's Manual of Systematic Bacteriology offers great information on universal 16S priming since it covers references on bacterial taxonomy and uses the 16SrRNA gene sequence analysis as the cornerstone. [6; 30.]

2.6 Reagent derived contamination

Difficulties in reaching high sensitivity and clear negative controls when ready-to-use PCR reagents are applied in qPCR analytics have been reported. Contamination can be potentially introduced from several sources in the reagent manufacturing process, *e.g.* water, plastic ware and inappropriate process conditions. [2; 3; 6.]

The contamination of the master mix with an unwanted DNA is commonly derived from the manufacturing process of enzymes. More closely it has been indicated that there are contaminations with a bacterial DNA in *Taq* polymerase enzymes, originated from its bacterial production host. As mentioned previously, there have been several approaches to reduce the unwanted DNA in PCR reagents, none of which are reported with outstanding results. These include the use of a long-wave UV light, ultra filtration, incubation with different restriction enzymes and the use of DNA cross-linking dyes such as propidiummonoazide. Currently only two different methods, DNAse and ethidiummonoazide treatments, have shown encouraging results in *Taq* polymerase decontamination. It should also be taken account that the degree of reagent-derived contamination may be batch-dependent. Thus optimization in decontamination procedures may also be required since unnecessary excessive consumption increases pre-PCR expenses. [2; 3; 4; 5; 31.]

Since the PCR procedure is delicately disturbed by possible contaminants in the reaction, the correct working methods should be taken into account. Negative templates or negative primer controls are an efficient way to observe the possible contamination of reagents. [13, p. 109.]

3 Aims of the Study

The use of commercial master mixes is widely implemented in PCR analytics. However, there are several reports considering the contamination problems in commercial PCR reagents. Even though reagent manufacturers inform that their reagents fulfill the quality criteria set for the PCR analysis, the contaminations are still noticed in numerous occasions. Other problems occurred applying the PCR method are the lack of universality of master mixes in different PCR instruments and the suitability of master mix components for the amplification of bacterial DNA deriving from different matrices.

When changing from one master mix to another with same instrumentation or from an instrument to another with same master mix, there are laborious optimizations required. After all, the idea in the implementation of ready-to-use reagents and kits should be their simplicity and adaptability.

The general goals of this study were i) to find out whether the quality criteria promised by manufacturers were fulfilled and ii) to test if the master mixes were easily implemented to different instrumentations without laborious optimizations.

4 Materials and Methods

4.1 Study design

A total of four commercial PCR master mixes based on the SYBR Green I chemistry from different suppliers and referred as 1, 2, 3 and 4 were used. Two replicate plates with three different instruments were compared. Each plate contained 27 negative template controls (NTCs), 16 standards and 45 DNA extracts of different matrix types.

All reactions were performed in a 2X Master mix concentration in a total volume of 15µl.

NTCs (Sigma-Aldrich mol.g. water) were used to control the background contamination.

4.2 Samples

All the DNA samples used in this study were provided by Alimetrics Ltd. The template DNAs were extracted from complex communities of DNA originated from different environments (Table 1). Each reaction contained 5µl of template.

Table 1. DNA sample origin and numbering.

Sample	**DNA origin**
Matrix 1	Chicken caecum bacterial DNA
Matrix 2	Chicken ileum bacterial DNA
Matrix 3	Bacterial DNA from rye doe
Matrix 4	Bacterial DNA from end product of card board process a
Matrix 5	Bacterial DNA from end product of card board process b
Matrix 6	Bacterial DNA from end product of card board process c
Matrix 7	Fish digesta bacterial DNA
Matrix 8	Human fecal bacterial DNA
Matrix 9	Pig ileum bacterial DNA
Matrix 10	No template control
Matrix 11	Standard 1, *Clostridium leptum*
Matrix 12	Standard 2, *Bacillus subtilis*

Each matrix was tested in various dilutions (Table 2).

Table 2. Dilution numbering and coefficients.

Dilution number	Dilution coefficient
1	10
2	100
3	1000
4	10000
5	100000
6	1000000
7	10000000
8	100000000
9	16
10	No dilution

The plate configuration is illustrated in Figure 13. The standards are highlighted with the green color and the NTCs with the yellow color.

	1	2	3	4	5	6	7	8	9	10	11	12
A	matrix 11 dil 1	matrix 4 dil 9	matrix 5 dil 9	matrix 6 dil 9	matrix 9 dil 2		matrix 1 dil 2	matrix 2 dil 2	matrix 8 dil 2	matrix 7 dil 1	matrix 3 dil 1	matrix 12 dil 1
B	matrix 11 dil 2	matrix 4 dil 9	matrix 5 dil 9	matrix 6 dil 9	matrix 9 dil 3		matrix 1 dil 3	matrix 2 dil 3	matrix 8 dil 3	matrix 7 dil 2	matrix 3 dil 2	matrix 12 dil 2
C	matrix 11 dil 3	matrix 4 dil 9	matrix 5 dil 9	matrix 6 dil 9	matrix 9 dil 4		matrix 1 dil 4	matrix 2 dil 4	matrix 8 dil 4	matrix 7 dil 3	matrix 3 dil 3	matrix 12 dil 3
D	matrix 11 dil 4	matrix 4 dil 9	matrix 5 dil 9	matrix 6 dil 9	matrix 9 dil 5		matrix 1 dil 5	matrix 2 dil 5	matrix 8 dil 5	matrix 7 dil 4	matrix 3 dil 4	matrix 12 dil 4
E	matrix 11 dil 5	matrix 4 dil 9	matrix 5 dil 9	matrix 6 dil 9	matrix 9 dil 6		matrix 1 dil 6	matrix 2 dil 6	matrix 8 dil 6	matrix 7 dil 5	matrix 3 dil 5	matrix 12 dil 5
F	matrix 11 dil 6	matrix 10 dil 10	matrix 10 dil 10	matrix 10 dil 10	matrix 10 dil 10		matrix 10 dil 10	matrix 10 dil 10	matrix 10 dil 10	matrix 10 dil 10	matrix 10 dil 10	matrix 12 dil 6
G	matrix 11 dil 7	matrix 10 dil 10	matrix 10 dil 10	matrix 10 dil 10	matrix 10 dil 10		matrix 10 dil 10	matrix 10 dil 10	matrix 10 dil 10	matrix 10 dil 10	matrix 10 dil 10	matrix 12 dil 7
H	matrix 11 dil 8	matrix 10 dil 10	matrix 10 dil 10	matrix 10 dil 10	matrix 10 dil 10		matrix 10 dil 10	matrix 10 dil 10	matrix 10 dil 10	matrix 10 dil 10	matrix 10 dil 10	matrix 12 dil 8

Figure 13. Plate set-up of the qPCR analysis.

DNA extraction and purification

As pretreatment, the sample was pipetted in an Eppendorf tube together with a washing buffer and ethylenediaminetetraacetic acid (EDTA). The solution was vortexed and centrifuged. The liquid phase was pipetted away and the pellet was stored for DNA extraction.

In DNA extraction the pellet from the previous step was first suspended in a Tris-HCl buffer with EDTA and the Proteinase K enzyme. The mix was vortexed and incubated. After incubation the cells were dispersed and the DNA was extracted with a phenol-chloroform-isoamyl alcohol mixture. The extraction phase was repeated to ensure the purity and yield of the product. Next, the water phase was collected and the DNA was precipitated with natrium chloride (NaCl) and isopropanol. The precipitate was centrifuged to the bottom of the Eppendorf tube, the liquid phase was tossed away and the precipitated DNA was washed twice with ice cold ethanol. The DNA pellet was dried and suspended in a TE buffer (Tris-Hcl with EDTA).

4.3 Primers

The 16S rRNA-targeted universal forward and reverse primers, Ali 613 and Ali 614, were used. Sequences of primers are illustrated in Table 2. The end product with these primers is 466 bp long. In each reaction primer, the concentrations were 20 pmol/µl.

Table 2. Ali 613 and Ali 61416S rRNA universal primer sequences.

Primer	**Sequence**
ALI 613 Forward	TCCTACGGGAGGCAGCAGT
ALI 614 Reverse	GGACTACCAGGGTATCTAATCCTGTT

The Ali 613 and Ali 614 primers

Ali 613 and Ali 614 primers are designed to attach flanking the variable regions V3 and V4. These regions are highlighted with the red color in Figure 14.

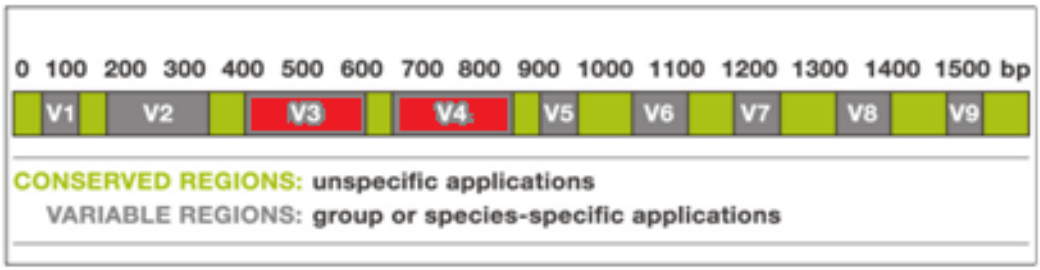

Figure 14. Binding sites of the universal primers applied in this study.

4.4 Standards

As controlled standards, a 10-fold dilution series of 16S rRNA genes from three individual bacterial species, *Bacillus subtilis*, *Clostridium leptum* and *Lactobacillus crispatus*, were applied (Table 3).

Table 3. Number of copies in standard dilution series

Dilute	**Number of copies**
1	$1 \cdot 10^{8}$
2	$1 \cdot 10^{7}$
3	$1 \cdot 10^{6}$
4	$1 \cdot 10^{5}$
5	$1 \cdot 10^{4}$
6	$1 \cdot 10^{3}$
7	$1 \cdot 10^{2}$
8	$1 \cdot 10^{1}$

Each reaction contained 5µl of standard.

4.5 Instrumentation

Quantitative Real-Time PCR amplification reactions were performed on 96-well plates (Thermo Scientific AB gene PCR Plate) with three different instruments: the Applied Biosystems ABI PRISM 7000 Sequence Detection System, the Eppendorf Mastercycler® ep realplex ^2 and the Roche LightCycler®480. Each plate was sealed.

Table 4. Instruments and numbering.

Instrument number	**Instrument**
1	Applied Biosystems ABI PRISM 7000 Sequence Detection System
2	Eppendorf Mastercycler® ep realplex ^2
3	Roche LightCycler®480

The amplification comprised an initial hold step at 50 °C for 2 minutes, initial denaturation at 95 °C for 10 minutes, annealing and synthesis at 58°C for 1 minute. The denaturation between cycles was performed at 95 °C for 15 seconds. Thermal cycles were run for 40 times. The melting curve analysis was not performed.

4.6 Factual number of copies

The detected Ct values of the samples were deduced to factual copy numbers from the Ct values of the standards. The intercept and slope of standard dilutions series were calculated and the number of copies were calculated according to Equation 4.

$$y = a + bx \tag{4}$$

Where

a is the intercept of standard Ct values and known number of copies
b is the slope of standard Ct values and known number of copies

x is the detected Ct value of sample

As the Ct values detected were from samples with various dilutions, the dilution coefficients were taken into account to achieve the factual number of copies for each sample according equation 5.

$$y_{factual} = 10^{a+bx} \cdot z \quad (5)$$

Where

a is the intercept of standard Ct values and known number of copies
b is the slope of standard Ct values and known number of copies
x is the detected Ct value of sample
z is the dilution coefficient

Thus, all results are comparable.

4.7 Statistical analysis

One way ANOVA (analysis of variance) was used to find out whether differences in master mixes and instruments are significant. Differences were considered significant when $P < 0.05$. Statistical evaluations were calculated with data analysis and statistical software Stata.

ANOVA is one of the most applied statistical analysis method and it is suitable for various kinds of studies. [32, p. 1.]

> ANOVA places no restriction on the number of groups or conditions that may be compared, while factorial ANOVA allows examination of the influence of two or more independent variables or factors on a dependent variable. [32, p. 1.]

5 Results and discussion

Firs, ANOVA was executed t for all samples to find out whether there are significant differences in master mixes and in instrumentation at a general level. Table 5 displays the results (p-values) and those results that indicate significance in either master mix or instrument are highlighted with the yellow color

Table 5. P-values for master mixes and instruments by matrix.

Matrix	p-value master mix	p-value instrument
1	0,0767	0,1568
2	0,0261	0,1367
3	0,0026	0,3009
4	0,0016	0,0000
5	0,0000	0,0016
6	0,0000	0,0001
7	0,5360	0,9487
8	0,0894	0,2355
9	0,151	0,0057
11	0,0410	0,2864
13	0,0741	0,4154

The results in Table 5 indicate that there are significant differences in the master mix in numerous matrixes. For matrixes 2, 3, 4, 5, 6, 11, and 12 the master mix showed significance. As matrixes 4, 5 and 6 (Bacterial DNA samples from end product of card board process), significance was expected as they would be the matrixes with the lowest number of copies detected (when NTCs were not taken into account). The smaller the number of copies in the sample, the higher is the variance in results; thus, different master mixes gives different levels of results for such samples. The significance of master mix can also result from differences between matrixes. Since DNA samples in the experiment were derived from various origins, some of them may contain impurities in spite of properly executed extraction protocol. Impurities can act as inhibitors in the reaction, inhibiting the activity of polymerase enzyme or primers. Particularly ileum samples are challenging since they can contain plenty of possible disruptive components (Matrix 2). However, this presumption cannot be proven by this experiment.

Note that NTCs (Matrix 10) are missing from Table 5 since the observations that are based on them and their significance is discussed later.

In instrumentation, significance was found for matrixes 4, 5, 6 and 9. Again matrixes 4, 5 and 6 were expected to produce such results because of their low number of copies. This results in a conclusion that different instruments have different levels of sensitivity

and without optimizations that result in insufficient detecting when a low detection limit is required.

5.1 Differences of master mixes between instrumentation

Different master mixes were compared in all matrix types with each instrument individually to find out whether the master mixes show differences between instruments. Tables 6, 7, and 8 present the results for all matrixes with each master mix and each instrument.

Table 6. Variations between master mixes by matrix in Instrument 1.

Matrix	Mastermix	Mean	Std. Dev.	Freq.
1	1	8.450e+09	2.750e+09	10
	2	1.218e+10	5.473e+09	10
	3	1.527e+10	1.759e+10	10
	4	9.920e+09	5.754e+09	10
	Total	1.146e+10	9.719e+09	40
	p-value			**0,4367**
2	1	1.233e+08	53506074	10
	2	2.422e+08	2.336e+08	10
	3	1.233e+08	82310388	9
	4	3.317e+08	4.086e+08	10
	Total	2.072e+08	2.500e+08	39
	p-value			**0,1864**
3	1	2.630e+09	1.095e+09	10
	2	3.610e+09	1.307e+09	10
	3	4.730e+09	1.310e+09	10
	4	2.039e+09	9.375e+08	10
	Total	3.252e+09	1.529e+09	40
	p-value			**0,0001**
4	1	17960	5104.9431	10
	2	3284	3922.4828	10
	3	14180	5438.5047	10
	4	17600	2366.4319	10
	Total	13256	7342.4267	40
	p-value			**0,0000**
5	1	2876	1459.9787	10
	2	10050	3857.8203	10
	3	3136	2708.2245	5
	4	22100	7978.4432	10
	Total	10455.429	9323.1503	35
	p-value			**0,0000**
6	1	2890	993.8142	10
	2	10060	4140.102	10
	3	2838	2314.6749	5
	4	18700	4667.857	10
	Total	9448.2857	7453.6973	35
	p-value			**0,0000**
7	1	1.103e+09	2.348e+09	10
	2	2.411e+09	5.054e+09	10
	3	1.677e+09	3.466e+09	8
	4	1.193e+09	2.514e+09	10
	Total	1.592e+09	3.414e+09	38
	p-value			**0,8325**
8	1	3.590e+09	1.627e+09	10
	2	5.560e+09	1.972e+09	10
	3	3.810e+09	2.695e+09	10
	4	4.330e+09	1.296e+09	10
	Total	4.323e+09	2.042e+09	40
	p-value			**0,1303**
9	1	2.102e+08	1.582e+08	10
	2	3.301e+08	2.014e+08	10
	3	1.750e+08	1.186e+08	8
	4	4.350e+08	3.943e+08	10
	Total	2.935e+08	2.591e+08	38
	p-value			**0,1127**
11	1	4.713e+08	3.369e+08	16
	2	4.324e+09	8.799e+09	16
	3	7.129e+09	1.608e+10	15
	4	1.751e+10	4.023e+10	16
	Total	7.362e+09	2.258e+10	63
	p-value			**0,1708**
13	1	1.526e+09	1.297e+09	16
	2	5.589e+09	1.024e+10	16
	3	2.969e+09	3.423e+09	14
	4	1.454e+10	3.291e+10	16
	Total	6.259e+09	1.793e+10	62
	p-value			**0,1701**

With Instrument 1 (Table 6) significant differences between master mixes occurred in matrixes 3, 4, 5 and 6. In Matrix 3, Master mix 3 produced a higher result in the number of copies compared to others. In Matrix 4, Master mix 2 produced a significantly lower result. In matrixes 5 and 6 Master mix 4 produced higher results than other master mixes.

Matrixes 4, 5, and 6 were known to be more difficult matrixes compared to other ones because of their relatively low number of copies.

However, in matrixes 5 and 6 the lower number of frequencies in comparison with other master mixes may hamper the reliability of these results.

Table 7. Variations between master mixes by matrix in Instrument 2.

Matrix	Mastermix	Mean	Std. Dev.	Freq.
1	1	9.430e+09	4.057e+09	10
	2	1.080e+10	4.109e+09	10
	3	6.840e+09	7.123e+09	5
	4	9.210e+09	6.732e+09	10
	Total	9.389e+09	5.324e+09	35
	p-value			**0,621**
2	1	1.242e+08	51341558	10
	2	2.594e+08	2.407e+08	10
	3	1.544e+08	94283084	5
	4	6.522e+08	1.140e+09	10
	Total	3.180e+08	6.404e+08	35
	p-value			**0,2622**
3	1	3.350e+09	2.205e+09	10
	2	3.940e+09	1.571e+09	10
	3	4.960e+09	1.284e+09	5
	4	1.715e+09	1.213e+09	10
	Total	3.281e+09	1.947e+09	35
	p-value			**0,0051**
4	1	17990	11775.346	10
	2	4748	4908.6789	10
	3	25200	18444.511	5
	4	45900	10503.439	10
	Total	23210.857	19302.229	35
	p-value			**0,0000**
5	1	2443	1532.4059	10
	2	12950	5801.5802	10
	3	4820	3106.7668	5
	4	52000	18299.97	10
	Total	19943.714	23230.448	35
	p-value			**0,0000**
6	1	2960	2070.534	10
	2	13580	3548.0198	10
	3	4696	3672.8844	5
	4	52400	14369.721	10
	Total	20368	22389.561	35
	p-value			**0,000**
7	1	1.452e+09	2.736e+09	8
	2	1.607e+09	3.343e+09	10
	3	1.963e+09	4.381e+09	5
	4	9.123e+08	1.789e+09	10
	Total	1.413e+09	2.867e+09	33
	p-value			**0,9192**
8	1	3.590e+09	1.971e+09	10
	2	6.590e+09	4.032e+09	10
	3	2.214e+09	8.891e+08	5
	4	4.790e+09	2.603e+09	10
	Total	4.593e+09	3.087e+09	35
	p-value			**0,0328**
9	1	2.549e+08	2.417e+08	10
	2	3.914e+08	2.277e+08	10
	3	1.844e+08	1.455e+08	5
	4	6.713e+08	9.335e+08	10
	Total	4.028e+08	5.450e+08	35
	p-value			**0,2712**
11	1	4.831e+08	3.889e+08	16
	2	5.535e+09	1.200e+10	16
	3	1.129e+10	2.465e+10	8
	4	5.272e+10	1.344e+11	16
	Total	1.840e+10	7.437e+10	56
	p-value			**0,1804**
13	1	1.607e+09	1.551e+09	16
	2	8.669e+09	1.759e+10	16
	3	3.814e+09	4.598e+09	8
	4	5.365e+10	1.389e+11	16
	Total	1.881e+10	7.650e+10	56
	p-value			**0,1947**

Instrument 2 (Table 7) gave quite similar results as Instrument 1. Again problems occurred with matrixes 3, 4, 5 and 6. In Matrix 3, Master mix 4 had lower results; in Matrix 4, Master mix 4 had a significantly higher result. The result with Master mix 2 , in contrast, was significantly lower when compared to those of other matrixes. The variation between master mixes 2 and 4 in Matrix 4 was quite remarkable.
In Matrix 5 there was again remarkable variance in results between master mixes 1 and 4, Master mix 4 producing the highest result. In matrixes 5 and 6 the results were similar to each other. Master mix 1 produced the lowest results and master mix 4 the highest. Again the variance between the lowest and highest results was significant.

With Instrument 2 significant variation in results occurred also in Matrix 8. In this matrix (human fecal bacterial DNA), there was difference in results between master mixes 2 and 3.

Again, it should be noticed that there were again a low number of frequencies in Master mix 3.

Table 8. Variations between master mixes by matrix in Instrument 3.

Matrix	Mastermix	Mean	Std. Dev.	Freq.
1	1	6.940e+09	2.195e+09	5
	2	5.440e+09	3.196e+09	5
	3	3.633e+10	3.174e+10	4
	4	1.910e+10	1.830e+10	5
	Total	1.593e+10	1.985e+10	19
	p-value			**0,0629**
2	1	2.860e+08	3.109e+08	4
	2	5.060e+08	1.401e+08	5
	3	2.600e+08	3.305e+08	3
	4	9.294e+08	1.397e+09	5
	Total	5.354e+08	7.765e+08	17
	p-value			**0,5974**
3	1	4.730e+09	7.061e+09	5
	2	3.478e+09	3.771e+09	5
	3	8.040e+09	1.039e+10	5
	4	2.066e+09	2.020e+09	5
	Total	4.579e+09	6.497e+09	20
	p-value			**0,554**
4	1	37400	24825.39	5
	2	46250	57629.203	2
	3	135333.33	42253.205	3
	4	4066.6667	1724.3356	3
	Total	53669.231	56409.801	13
	p-value			**0,0041**
5	1	15175	9358.1961	4
	2	7625	5415.0254	4
	4	17920	12182.446	5
	Total	13907.692	9954.6858	13
	p-value			**0,3158**
6	1	9725	6593.6207	4
	2	17200	16687.72	2
	4	8150	2648.8991	4
	Total	10590	7775.6672	10
	p-value			**0,439**
7	1	1.250e+09	2.767e+09	5
	2	8.616e+08	1.867e+09	5
	3	7.004e+09	9.894e+09	2
	4	8.258e+08	1.830e+09	5
	Total	1.688e+09	3.712e+09	17
	p-value			**0,1975**
8	1	9.750e+09	1.072e+10	5
	2	6.922e+09	7.679e+09	5
	3	3.600e+09	2.899e+09	4
	4	3.620e+09	2.722e+09	5
	Total	6.098e+09	6.975e+09	19
	p-value			**0,4938**
9	1	2.114e+08	2.263e+08	5
	2	1.225e+09	1.980e+09	5
	3	1.645e+09	1.775e+09	2
	4	1.640e+09	2.589e+09	4
	Total	1.065e+09	1.730e+09	16
	p-value			**0,6336**
11	1	9.980e+08	1.067e+09	8
	2	4.204e+09	9.615e+09	7
	3	7.380e+08	4.748e+08	5
	4	4.537e+09	8.409e+09	8
	Total	2.764e+09	6.508e+09	28
	p-value			**0,5946**
13	1	1.468e+09	2.442e+09	8
	2	2.132e+10	4.104e+10	8
	3	9.361e+09	1.369e+10	6
	4	8.048e+09	1.514e+10	8
	Total	1.009e+10	2.350e+10	30
	p-value			**0,4135**

With Instrument 3 (Table 8) Matrix 4 was the only one that showed significance in the variation of results as master mixes 3 and 4 differed from each other.

In its entirety, the results for Instrument 3 are considered to be unreliable as there were no replicate test plates run

5.2 Differences of instruments between master mixes

Different instruments were compared in all matrix types with each master mix individually to find out whether instruments show differences between master mixes. Tables 9, 10, 11 and 12 present the results for all matrixes with each instrument and each master mix.

Table 9. Variations between instruments by matrix in Master mix 1.

Matrix	Instrument	Mean	Std. Dev.	Freq.
1	1	8.450e+09	2.750e+09	10
	2	9.430e+09	4.057e+09	10
	3	6.940e+09	2.195e+09	5
	Total	8.540e+09	3.267e+09	25
	p-value			**0,3940**
2	1	1.233e+08	53506074	10
	2	1.242e+08	51341558	10
	3	2.860e+08	3.109e+08	4
	Total	1.508e+08	1.363e+08	24
	p-value			**0,0894**
3	1	2.630e+09	1.095e+09	10
	2	3.350e+09	2.205e+09	10
	3	4.730e+09	7.061e+09	5
	Total	3.338e+09	3.346e+09	25
	p-value			**0,5385**
4	1	17960	5104.9431	10
	2	17990	11775.346	10
	3	37400	24825.39	5
	Total	21860	15078.959	25
	p-value			**0,0284**
5	1	2876	1459.9787	10
	2	2443	1532.4059	10
	3	15175	9358.1961	4
	Total	4745.4167	5993.1446	24
	p-value			**0,0000**
6	1	2890	993.8142	10
	2	2960	2070.534	10
	3	9725	6593.6207	4
	Total	4058.3333	3799.6472	24
	p-value			**0,0014**
7	1	1.103e+09	2.348e+09	10
	2	1.452e+09	2.736e+09	8
	3	1.250e+09	2.767e+09	5
	Total	1.256e+09	2.461e+09	23
	p-value			**0,9599**
8	1	3.590e+09	1.627e+09	10
	2	3.590e+09	1.971e+09	10
	3	9.750e+09	1.072e+10	5
	Total	4.822e+09	5.283e+09	25
	p-value			**0,0592**
9	1	2.102e+08	1.582e+08	10
	2	2.549e+08	2.417e+08	10
	3	2.114e+08	2.263e+08	5
	Total	2.283e+08	2.008e+08	25
	p-value			**0,8743**
11	1	4.713e+08	3.369e+08	16
	2	4.831e+08	3.889e+08	16
	3	9.980e+08	1.067e+09	8
	Total	5.814e+08	5.921e+08	40
	p-value			**0,0810**
13	1	1.526e+09	1.297e+09	16
	2	1.607e+09	1.551e+09	16
	3	1.468e+09	2.442e+09	8
	Total	1.547e+09	1.626e+09	40
	p-value			**0,9795**

In Master mix 1 (Table 9) it was found that matrixes 4, 5 and 6 showed significance for instrumentation. In each of these matrixes Instrument 3 produced considerably higher results than instruments 1 and 2. This same phenomenon was also discovered with no template controls thus, conclusions are discussed later.

Table 10. Variations between instruments by matrix in Master mix 2.

Matrix 1

Instrument	Mean	Std. Dev.	Freq.
1	1.218e+10	5.473e+09	10
2	1.080e+10	4.109e+09	10
3	5.440e+09	3.196e+09	5
Total	1.028e+10	5.076e+09	25

p-value **0,0409**

Matrix 2

Instrument	Mean	Std. Dev.	Freq.
1	2.422e+08	2.336e+08	10
2	2.594e+08	2.407e+08	10
3	5.060e+08	1.401e+08	5
Total	3.018e+08	2.375e+08	25

p-value **0,0938**

Matrix 3

Instrument	Mean	Std. Dev.	Freq.
1	3.610e+09	1.307e+09	10
2	3.940e+09	1.571e+09	10
3	3.478e+09	3.771e+09	5
Total	3.716e+09	1.993e+09	25

p-value **0,9012**

Matrix 4

Instrument	Mean	Std. Dev.	Freq.
1	3284	3922.4828	10
2	4748	4908.6789	10
3	46250	57629.203	2
Total	7855.4545	18166.27	22

p-value **0,0024**

Matrix 5

Instrument	Mean	Std. Dev.	Freq.
1	10050	3857.8203	10
2	12950	5801.5802	10
3	7625	5415.0254	4
Total	10854.167	5179.137	24

p-value **0,1831**

Matrix 6

Instrument	Mean	Std. Dev.	Freq.
1	10060	4140.102	10
2	13580	3548.0198	10
3	17200	16687.72	2
Total	12309.091	5608.7439	22

p-value **0,1637**

Matrix 7

Instrument	Mean	Std. Dev.	Freq.
1	2.411e+09	5.054e+09	10
2	1.607e+09	3.343e+09	10
3	8.616e+08	1.867e+09	5
Total	1.779e+09	3.835e+09	25

p-value **0,7649**

Matrix 8

Instrument	Mean	Std. Dev.	Freq.
1	5.560e+09	1.972e+09	10
2	6.590e+09	4.032e+09	10
3	6.922e+09	7.679e+09	5
Total	6.244e+09	4.210e+09	25

p-value **0,8078**

Matrix 9

Instrument	Mean	Std. Dev.	Freq.
1	3.301e+08	2.014e+08	10
2	3.914e+08	2.277e+08	10
3	1.225e+09	1.980e+09	5
Total	5.336e+08	9.018e+08	25

p-value **0,1587**

Matrix 11

Instrument	Mean	Std. Dev.	Freq.
1	4.324e+09	8.799e+09	16
2	5.535e+09	1.200e+10	16
3	4.204e+09	9.615e+09	7
Total	4.799e+09	1.012e+10	39

p-value **0,9339**

Matrix 13

Instrument	Mean	Std. Dev.	Freq.
1	5.589e+09	1.024e+10	16
2	8.669e+09	1.759e+10	16
3	2.132e+10	4.104e+10	8
Total	9.967e+09	2.228e+10	40

p-value **0,2589**

In Master mix 2 (Table 10) there was a significance for instrumentation in matrixes 1 and 4. Again the differences were found in the results of Instrument 3.

Table 11. Variations between instruments by matrix in Master mix 3

Matrix	Instrument	Mean	Std. Dev.	Freq.
1	1	1.527e+10	1.759e+10	10
	2	6.840e+09	7.123e+09	5
	3	3.633e+10	3.174e+10	4
	Total	1.748e+10	2.114e+10	19
	p-value			**0,0969**
2	1	1.233e+08	82310388	9
	2	1.544e+08	94283084	5
	3	2.600e+08	3.305e+08	3
	Total	1.566e+08	1.479e+08	17
	p-value			**0,4084**
3	1	4.730e+09	1.310e+09	10
	2	4.960e+09	1.284e+09	5
	3	8.040e+09	1.039e+10	5
	Total	5.615e+09	5.095e+09	20
	p-value			**0,4930**
4	1	14180	5438.5047	10
	2	25200	18444.511	5
	3	135333.33	42253.205	3
	Total	37433.333	48571.56	18
	p-value			**0,0000**
5	1	3136	2708.2245	5
	2	4820	3106.7668	5
	Total	3978	2887.4356	10
	p-value			**0,3876**
6	1	2838	2314.6749	5
	2	4696	3672.8844	5
	Total	3767	3055.4434	10
	p-value			**0,3666**
7	1	1.677e+09	3.466e+09	8
	2	1.963e+09	4.381e+09	5
	3	7.004e+09	9.894e+09	2
	Total	2.483e+09	4.677e+09	15
	p-value			**0,3643**
8	1	3.810e+09	2.695e+09	10
	2	2.214e+09	8.891e+08	5
	3	3.600e+09	2.899e+09	4
	Total	3.346e+09	2.387e+09	19
	p-value			**0,4872**
9	1	1.750e+08	1.186e+08	8
	2	1.844e+08	1.455e+08	5
	3	1.645e+09	1.775e+09	2
	Total	3.741e+08	7.102e+08	15
	p-value			**0,0111**
11	1	7.129e+09	1.608e+10	15
	2	1.129e+10	2.465e+10	8
	3	7.380e+08	4.748e+08	5
	Total	7.176e+09	1.744e+10	28
	p-value			**0,5873**
13	1	2.969e+09	3.423e+09	14
	2	3.814e+09	4.598e+09	8
	3	9.361e+09	1.369e+10	6
	Total	4.580e+09	7.239e+09	28
	p-value			**0,1860**

In Master mix 3 (Table 11) there was a significance for instrumentation in matrixes 4 and 9, Instrument 3 resulting in higher results. Note that in this master mix there are no results for instrument 3 in matrixes 5 and 6 as the instrument was not able to detect any amplification in these matrixes. Therefore, it can be concluded that Master mix 3 is not suitable reagent to be implemented with Instrument 3.

Table 12. Variations between instruments by matrix in Master mix 4

Matrix		Matrix	
1	Instrument | Mean Std. Dev. Freq. 1 | 9.920e+09 5.754e+09 10 2 | 9.210e+09 6.732e+09 10 3 | 1.910e+10 1.830e+10 5 Total | 1.147e+10 1.002e+10 25 p-value **0,1634**	2	Instrument | Mean Std. Dev. Freq. 1 | 3.317e+08 4.086e+08 10 2 | 6.522e+08 1.140e+09 10 3 | 9.294e+08 1.397e+09 5 Total | 5.794e+08 9.636e+08 25 p-value **0,522**
3	Instrument | Mean Std. Dev. Freq. 1 | 2.039e+09 9.375e+08 10 2 | 1.715e+09 1.213e+09 10 3 | 2.066e+09 2.020e+09 5 Total | 1.915e+09 1.261e+09 25 p-value **0,8234**	4	Instrument | Mean Std. Dev. Freq. 1 | 17600 2366.4319 10 2 | 45900 10503.439 10 3 | 4066.6667 1724.3356 3 Total | 28139.13 17904.944 23 p-value **0,0000**
5	Instrument | Mean Std. Dev. Freq. 1 | 22100 7978.4432 10 2 | 52000 18299.97 10 3 | 17920 12182.446 5 Total | 33224 20528.867 25 p-value **0,0001**	6	Instrument | Mean Std. Dev. Freq. 1 | 18700 4667.857 10 2 | 52400 14369.721 10 3 | 8150 2648.8991 4 Total | 30983.333 21117.202 24 p-value **0,0000**
7	Instrument | Mean Std. Dev. Freq. 1 | 1.193e+09 2.514e+09 10 2 | 9.123e+08 1.789e+09 10 3 | 8.258e+08 1.830e+09 5 Total | 1.007e+09 2.038e+09 25 p-value **0,9357**	8	Instrument | Mean Std. Dev. Freq. 1 | 4.330e+09 1.296e+09 10 2 | 4.790e+09 2.603e+09 10 3 | 3.620e+09 2.722e+09 5 Total | 4.372e+09 2.144e+09 25 p-value **0,6264**
9	Instrument | Mean Std. Dev. Freq. 1 | 4.350e+08 3.943e+08 10 2 | 6.713e+08 9.335e+08 10 3 | 1.640e+09 2.589e+09 4 Total | 7.343e+08 1.208e+09 24 p-value **0,2442**	11	Instrument | Mean Std. Dev. Freq. 1 | 1.751e+10 4.023e+10 16 2 | 5.272e+10 1.344e+11 16 3 | 4.537e+09 8.409e+09 8 Total | 2.900e+10 8.937e+10 40 p-value **0,3792**
13	Instrument | Mean Std. Dev. Freq. 1 | 1.454e+10 3.291e+10 16 2 | 5.365e+10 1.389e+11 16 3 | 8.048e+09 1.514e+10 8 Total | 2.888e+10 9.113e+10 40 p-value **0,3784**		

In Master mix 4 (Table 12) the significance for instrumentation was found in matrixes 4, 5 and 6, Instrument 2 resulting in higher results in each of the three matrixes. The results for Master mix 4 differ from those of other master mixes. That in, this Master mix 4 implemented with Instrument 3 produced the lowest results. This indicates that master mix 4 is not suitable reagent to be implemented with instrument 3.

With Instrument 3, it was noticed that the detection in general takes place with significantly higher Ct values. This was considered as a technical feature of this instrument, since the factual number of copies was similar to that other of instruments in those results where problems did not occur.

When looking at these results, it should also be taken into account that the number of replications with Instrument 3 is not enough to produce reliable results.

5.3 Master mix implementation on changing instrumentation

All the p-values from tables 6 to 12 are collected in diagram (Figure 14). The values in the figure are in a minus logarithmic 10 -scale so that the ones that have a p-value below 0.05 are placed above the red line.

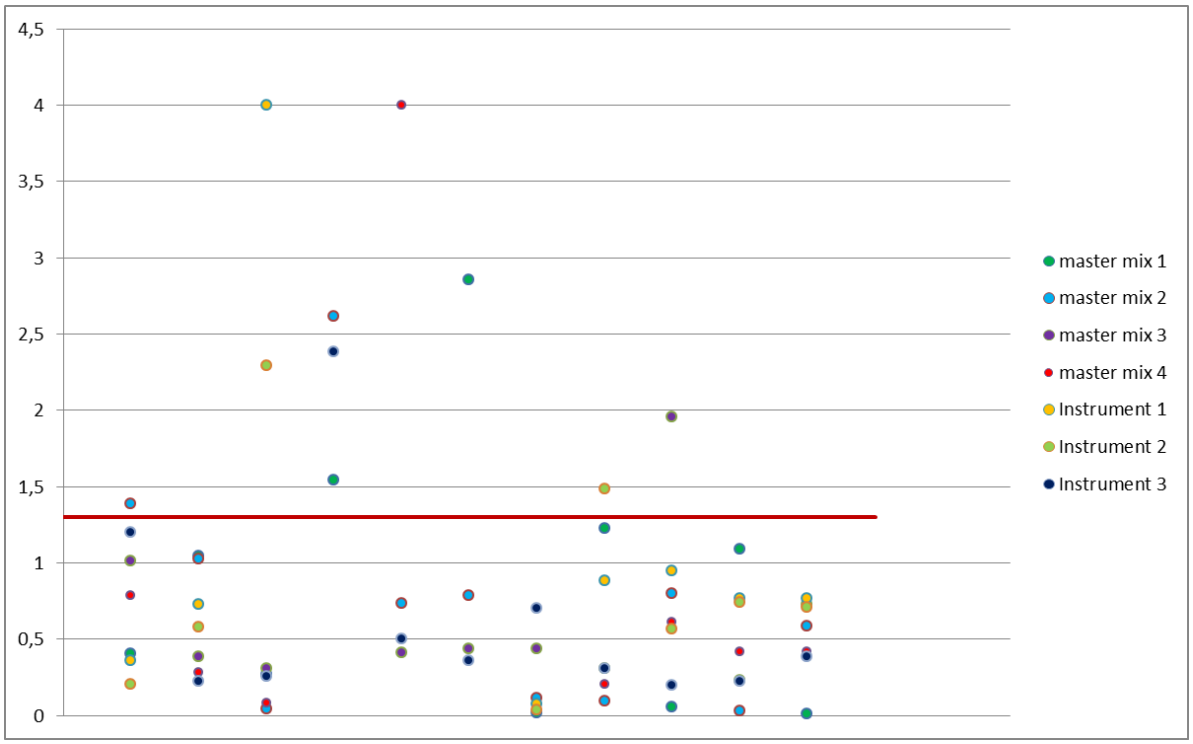

Figure 15. P-values of instruments between master mixes and master mixes between instruments. Number of copies of copies on y-axis.

In Figure 14 it can be observed that in each master mix and instrument there can be found results that indicate that there were significances in master mixes between instrumentation. Therefore, any of the master mixes cannot be directly implemented with the instruments tested in this study.

5.4 Levels of contaminations

The contamination level of master mixes was studied with no template controls (NTCs). Since the manufacturers promise that there are no reagent-derived contaminations in master mixes that may hamper the quantification, there should be a significantly low number of copies detected in NTC samples or, in an ideal case, no copies detected at all.

The average number of copies and the standard errors for each master mix and instrument were calculated. The results are illustrated in Figure 15.

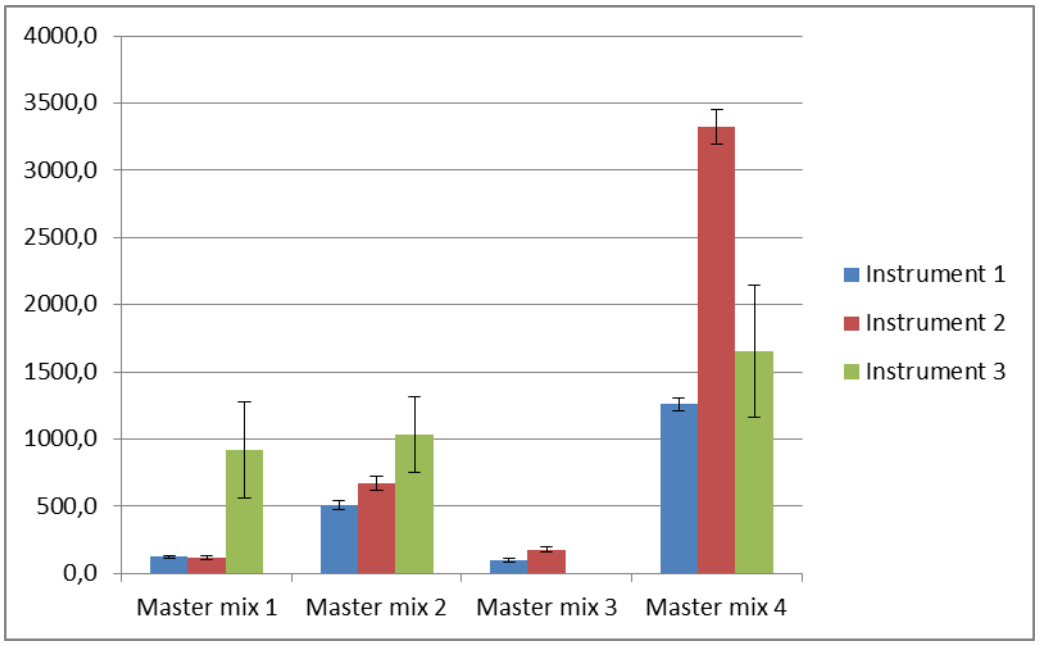

Figure 16. Number of copies detected.

The results show clearly that Master mix 4 had the highest number of copies detected in the NTCs. This suggests that Master mix 4 was highly contaminated. Master mixes 1 and 3 had the lowest level of contamination. The results for these two master mixes were significantly higher with Instrument 3, as were also many of the results for the other matrixes.

In Master mix 3 with Instrument 3 there was no detection at all. Since there was found similar problems with some matrixes that were detected successfully with other combinations of master mixes and instruments, and there are a relatively large number of copies detected with instruments 1 and 2 in Master mix 3; nevertheless, this result cannot be considered to prove the total purity of Master mix 3.

From the results of the NTCs it can be also observed that instruments 1 and 2 produce quite similar results when implemented with master mixes 1, 2 and 3. However, since the quantification needs to be precise, the minor differences in the results show that transfer and adaption between instruments and master mixes is not possible without optimizations.

Note that in these results it should be taken into account that the detected numbers of copies in NTCs are not on the range of the standards that were detected reliably. The last three dilutions of the standards were not on the linear range. In standards, $1 \cdot 10^4$ copies were the smallest number that could be quantified in linear range of standards. Hence, the numbers determined in NTCs are not absolute. However, the results still indicate the impurities present in reagents since there were some copies detected.

Note also that the abnormal result in Master mix 4 detected with Instrument 2 may also be due a human caused contamination.

6 Conclusions

In this project the general goals were to find out whether the master mixes contain reagent derived contaminants and if the master mixes were easily implemented to different instrumentations without laborious optimizations.

There were found differences in master mixes and instruments with several matrixes. In this project there were intentionally several types of matrixes examined so that it could be investigated how many restrictions and requirements the DNA sample origin set when specifying a suitable master mix and instrument.

Since some matrixes were proved to be more challenging, the next step could be a study with more replicates executed with complex matrixes. In this way there could be manufactured master mixes expressly for different matrix types, e.g. for ileum or blood samples.

The current situation requires that the end user performs laborious and expensive experiments to find the most suitable reagent and instrument for each matrix type. Once the proper reagent is found, it might still need more optimizations, e.g. in Mg^{2+} concentration and, therefore, the expenses increase.

None of the master mixes was found to be completely pure. Thus, more studies to find new possible ways to reduce the amount of endogenous bacteria in PCR reagents should be done. However, at this point, if neither the manufacturers nor the end user can suggest any ways to bring PCR reagents to the desired level of purity, the detection of a low number of copies in bacterial samples set quite a challenge. Therefore, it is important to execute an adequate number of NTCs in each PCR assay so that the background and thereby the level of possible contamination can be determined.

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