The crucial combination of DNA polymerase, qPCR assay and background matrix

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Submission: 20181123

Abstract
Quantitative PCR (qPCR) is a commonly used method today in the fields of molecular diagnostics, forensic and food analysis. The real-time measurements of amplification and quantification of specific DNA molecules is one of many reasons why qPCR is the method of choice. However, presence of inhibitory compounds limit this method for example by interacting with either the DNA polymerase or the DNA template, increasing the errors and impairing the detection limit. This is why pre-PCR processing, where the effect of inhibitory compounds are handled prior to qPCR amplification, is of importance. A well-designed robust qPCR assay is essential to obtain reliable results. However, the choice of DNA polymerase also plays a significant role since different DNA polymerases are more or less tolerant to different types of inhibitors. The aims of this project were to study the amplification efficiency and detection limit when combining DNA polymerases with different qPCR assays. Moreover, the performance of the DNA polymerases with qPCR assays was also studied when more or less inhibitory matrices were added. Lastly, due to several parameters affecting the qPCR, a method with the purpose of determining the extension rate assay of the DNA polymerase was set up in the lab. The results showed that the performance of DNA polymerases varied depending on both qPCR assay and matrix. Moreover, the prominence of a robust qPCR assay was clearly observed. The results from the enzymatic rate assay indicated that performance of one DNA polymerase was decreased when inhibitory matrices were added, while the performance of another DNA polymerase on the contrary increased. However, further investigations are needed to elucidate the mechanisms behind this.

Keywords: qPCR · DNA polymerase · qPCR assay · PCR inhibitors · Rate assay

1. Introduction
Quantitative PCR (qPCR) is a method widely used today in various scientific fields, for example in forensic analysis, food analysis and molecular diagnostics [1, 2]. qPCR is preferable due to the real-time measurements of amplification and quantification of specific DNA molecules. Compared to conventional PCR, where the results are obtained in the end of the analysis, qPCR has a higher sensitivity and specificity [3-5]. Moreover, by using qPCR instead of conventional PCR, the analysis time is shortened and less manual input is required since the need for gel electrophoresis is removed. This also reduces the risk of cross-contamination [3, 6]. Fluorescent-labeled dyes, such as EVAGreen, or hydrolysis probes interact with DNA in qPCR experiments, which make it possible to follow amplification in real-time. However, even though qPCR usually is the choice for DNA quantification, it has shown to have its limitations [3, 7, 8]. These limitations are for example that the presence of inhibitory compounds in the analyzed samples often leads to increased errors, impaired detection limits or even false results [7-11]. The inhibitors affecting the amplification

efficiency act either by interacting directly with the DNA polymerase or by binding to the DNA template, leading to that the primers and DNA polymerase can no longer bind to the DNA template [2, 7, 12].

In order to reduce or remove the inhibitory compounds from samples, pre-PCR processing can be applied, a concept where all steps of the analysis chain are taken into consideration [11, 12]. These steps are sampling, sample treatment and DNA amplification (qPCR) [6, 11, 12]. The first step, sampling, refers to the importance of handling the sample in a correct way, so the uptake of the sample is maximized, with a minimal risk of uptake of inhibitory compounds [6, 12]. Sample treatment, is the second step in which the nucleic acid is isolated with as high efficiency as possible while the effect of inhibitory compounds is removed or reduced. However, there is a trade-off between yield and purity, because increased purification often leads to loss of DNA [6, 11, 12]. It is important to choose the most appropriate buffers, reagents and DNA polymerase in the third step, DNA amplification, to further support the amplification of the sample [11, 12].

High yield of a specific product with as few cycles as possible without errors are to aim for when performing qPCR, where the amplification efficiency determines the product yield [5]. If the amplification efficiency is close to 100 %, the amount of PCR products is doubled with every PCR cycle and detected by fluorescent dyes or hydrolysis probes. The cycle where the fluorescence generated from the PCR products can be separated from the fluorescent background noise is called the cycle of quantification (Cq). This is thus the crossing point of the amplification curve and the so called baseline of background noise, and can be calculated based on different mathematical models such as linear regression or second derivative maximum method. The Cq value depends on the amount of template DNA in the starting material, and is used to calculate the concentration of starting template DNA from a standard curve with known concentrations. However, the Cq value also depends on the amplification efficiency and can vary with all parameters in a qPCR (qPCR assay, DNA polymerase, magnesium concentration, annealing temperature etc.). Thus a standard curve and an individual Cq value are valid only for an exact and defined set of qPCR parameters.

In order to have a well-designed qPCR assay, the importance of carefully planning the design, optimizing and validating the qPCR assay, can never be highlighted enough [10, 13]. There are several vital parameters affecting the qPCR results that are important to consider for an optimized assay [10, 14-17]. Data analysis of the primers and amplicons are important in order to study potential secondary structures formed [10, 17]. These secondary structures can lead to a less efficient DNA polymerization giving higher Cq values, reflecting that less copies of the target are made compared to low Cq values, where more copy numbers of the target are produced. Further, it is vital to optimize the annealing temperature of the primers using a temperature gradient analysis and evaluate the primer concentration to focus on primer characteristics [10]. In short, the results of an optimized and well-designed qPCR assay include high amplification efficiency (95-105 %), a linear standard curve ($R2 > 0.980$), no or low formation of primer-dimers and comparable results between reproduced experiments [10]. If these parameters/conditions are not considered beforehand, a non-robust qPCR assay may be obtained. However, also with a well-optimized and robust qPCR assay, there are limitations of the qPCR analysis under specific conditions, such as when inhibitors are present in the samples.

It is known that the efficiency of DNA polymerases differ greatly depending on the inhibitor type in the background matrix [18, 19]. However, that the performance of DNA polymerases can differ depending on the qPCR assays that are used, is not well described in literature [20]. Thus, the aim of this project was to study the efficiency and detection limit when combining DNA polymerases with different qPCR assays. Moreover, the performance of the DNA polymerases with different qPCR assays was studied when more or less inhibitory background matrices were added. Finally, a new method, focusing solely on the DNA polymerase activity and more precisely on the rate of nucleotide incorporation were set up and optimized in the lab [21, 22]. This enzymatic rate assay can be used to compare the rate of nucleotide incorporation for different DNA polymerases, with and without inhibitory compounds present in matrices.

2. Materials and Methods

2.1 Preparation of DNA samples

To prepare DNA from bacteria, *Salmonella typhimurium* (CCUG-31969) was enriched in lysogeny broth (LB) medium, while *Listeria monocytogenes* (12MOB052LM) and *Bacillus cereus* (F2085) were enriched in brain heart infusion (BHI) medium. LB medium and BHI medium both contain important ingredients promoting the bacteria to grow. From these cultures of *S. typhimurium*, *L. monocytogenes* and *B. cereus*, and from human blood sample, DNA was extracted and purified with Thermo Scientific GeneJET Genomic DNA Purification Kit (Thermo Fisher Scientific, Waltham, Massachusetts, USA, product number K0722), which is a spin column extraction kit. The first steps of the protocol differ depending on organism. In this protocol, mammalian DNA is extracted with lysis solution, proteinase K and ethanol (96-100 %), while gram-negative bacteria (*S. typhimurium*) is extracted with digestion solution, proteinase K, RNase A solution, lysis solution and ethanol (96-100 %). Extraction of DNA from gram-positive bacteria (*L. monocytogenes* and *B. cereus*) include a prepared lysis buffer (20 mM Tris-HCl, pH 8.0, 2 mM EDTA, 1.2 % Triton X-100 and lysozyme to 20 mg/mL), lysis solution, proteinase K, RNase A solution and ethanol (96-100 %). After extraction and purification of the DNA from the four organisms, DNA concentration was measured with both BioDrop (BioDrop, Cambridge, UK) and Qubit 3.0 Fluorometer (Thermo Fisher Scientific). BioDrop is a spectrophotometer, measuring absorption of at 260 nm, the wavelength where DNA absorbs light. Further, the ratio of absorptions at 260 nm versus 280 nm is commonly used to assess contamination of the DNA sample, since protein and especially aromatic compounds, absorb light at 280 nm. The preferred ratio for DNA, where the product is considered pure, is 1.8. Qubit, however, is a fluorometer measuring intercalating dyes binding to dsDNA in a sample. Qubit dsDNA BR Assay Kit protocol was used with 5 µL sample (Thermo Fisher Scientific, product number Q32851). To analyze the intactness of the DNA, an agarose gel electrophoresis was performed (0.9 % agarose gel running for 45 minutes on 100 V with 0.01 % GelRed from Biotium) and pictures were taken with BioOne Quantity (BioRad, Hercules, California, USA).

2.2 qPCR assays

Five validated and published qPCR assays (i.e primer sets) were used in order to evaluate the different DNA polymerases (Table 1). For human DNA, RB1 assay with two primer combinations 80F/235R and 157F/325R was chosen. For *S. typhimurium*, InvA assay was chosen, for *L. monocytogenes*, HlyA assay was chosen, and for *B. cereus*, A_bac assay was chosen.

Table 1. This table indicates the organisms used in this project with corresponding target gene/assay name, amplicon length, primer names and primer sequence. Forward primer is indicated with F, and reverse primer is indicated with R. The names of the primers in A_bac assay are shortened to F0, R0, F1, R1, F2 and R2, where F1, R1, F2 and R2 are modified versions of F0 and R0 (the red letters and underscore indicate changes in the original sequence).

Organism	Target gene/Assay name	Amplicon length (bp)	Primer name	Primer sequence (5' → 3')	References
Homo sapiens	Human retinoblastoma 1 (RB1) Chromosome 13	156 (RB1_80F – RB1_235R) 168 (RB1_157F – RB1_325R)	RB1_80F RB1_157F RB1_235R RB1_325R	AGG TTG CTA ACT ATG AAA CAC TGG C CCA GAA AAT AAA TCA GAT GGT ATG TAA CA TGG TTT AGG AGG GTT GCT TCC CCA TCT CAG CTA CTG GAA AAC ATT C	Niederstätter et al, 2007 [23]
Salmonella typhimurium (CCUG-31969)	Invasion protein A (*InvA*)	88	InvA_F InvA_R	CGT GTC CTT TGG TAT TAA TCC AAC AAT C CCG GAG TTT CTC CCC CTC TTC	Richmond et al, 2008 [3] and Sidstedt et al, 2018 [7]
Listeria monocytogenes (12MOB052LM)	Thiol-activated cytolysin (HlyA)	64	HlyA_F HlyA_R	CAT GGC ACC ACC AGC ATC T ATC CGC GTG TTT CTT TTC GA	Rodriguez-Lazaro et al, 2004 [24]
Bacillus cereus (F2085)	DNA-directed RNA polymerase β subunit, A_bac_rpoB (A_bac)	80	A_bac_F0 A_bac_R0 A_bac_F1 A_bac_R1 A_bac_F2 A_bac_R2	ACC TCT TCT TAT CAG TGG CCC GTA AAG TCT TCA ATC ACC TCT TCT TAT CAG TG_ CCC GTA AAG TCT TCA AT_ AAA CCT CTT CTT ATC AGT G_ ACC CGT AAA GTC TTC AAT_	Ehrs et al, 2009 [25]

2.3 DNA polymerases

TaKaRa Ex *Taq* HS DNA polymerase (Ex *Taq*), Tempase Hot Start DNA polymerase (Tempase), Immolase DNA polymerase (Immolase), KAPA3G Plant DNA polymerase (KAPA3G), Tth DNA polymerase (Tth), *Taq* DNA polymerase (*Taq*), AccuStart II *Taq* DNA polymerase (AccuStart II) and PerfeCTa ToughMix (ToughMix) (the DNA polymerase in ToughMix is AccuStart II) were assessed in this project (Table 2). Due to its previously observed robustness for various sample types and qPCR assays, Ex *Taq* was considered a reference DNA polymerase when optimizing assays.

Table 2. The different DNA polymerases used in this project, their manufacturer, product number, which organism they derive from and activation is presented in this table.

DNA polymerase	Manufacturer/Product number	Organism	Hot start
TaKaRa Ex *Taq* HS DNA polymerase	TaKaRa Bio, Shiga, Japan/RR006A	Modified version of *Taq* DNA polymerase	Yes
Tempase Hot Start DNA polymerase	Ampliqon Liveline Ltd, Brighton, UK/A221103	Modified version of *Taq* DNA polymerase	Yes
Immolase DNA polymerase	Bioline, London, UK/BIO-21046	Novel unspecified organism	Yes
KAPA3G Plant DNA polymerase	KAPA Biosystems Inc, Wilmington, Massachusetts, USA/KK7251	Plant derived DNA	Yes
Tth DNA polymerase	Roche Diagnostics, Risch, Switzerland/11 480 022 001	*Thermus thermophilus*, recombinant (*E.coli*)	Yes
Taq DNA polymerase	TaKaRa Bio, Shiga, Japan/R007A	*Thermus aquaticus*	Yes
PerfeCTa ToughMix	Quanta BioSciences, Beverly, Massachusetts, USA/95112-250	Modified version of *Taq* DNA polymerase	Yes
AccuStart II *Taq* DNA polymerase	Quanta BioSciences, Beverly, Massachusetts, USA/95141-250	Modified version of *Taq* DNA polymerase	Yes

2.4 Screening of DNA polymerases in combination with different qPCR assays

To investigate the efficiency between the polymerases in combination with different qPCR assays, each polymerase was tested with all qPCR assays (Figure 1). Triplicates of each sample were tested. First, the qPCR assays were optimized using Ex *Taq*, regarding primer concentration and annealing temperature. As many parameters as possible were standardized between qPCR assays, thus EVAGreen was used instead of probe (Biotium, Hayward, California, USA, product number 31000). EVAGreen is a fluorescent dye that increases its fluorescence by 1000-fold when bound to dsDNA, making it possible to detect the generation of PCR products after each PCR cycle, and to subsequently obtain a melt curve analysis. The melt curve analysis is advantageous in these experiments to observe primer-dimer formations, in contrast to using a probe where primer-dimer formations pass undetected. Moreover, probes are specific to each qPCR assay, and would thus add to the difference in parameters between the qPCR assays. Furthermore, the five qPCR assays were used due to similar annealing temperature. The qPCR experiments were performed with BioRad CFX96 (BioRad) with a reaction volume of 20 µL. If nothing else is stated, triplicates of all samples were analyzed. Except otherwise indicated, final reaction concentrations were 2.5 mM $MgCl_2$ (Roche Diagnostics), 0.3 µM primer, 1X of the buffer received with each polymerase, 0.2 µM deoxyribonucleotide triphosphate (dNTP) (Roche Diagnostics, product number 11814362001), 1X EVAGreen, 1 U DNA polymerase and template concentrations of 10 ng/µL to 0.001 ng/µL for both RB1 assays, and 1000 pg/µL to 0.001 pg/µL for HlyA, InvA and A_bac assays. However, for the A_bac assay, a primer concentration of 0.5 µM instead of 0.3 µM was used, since optimization experiments showed that this primer concentration was more optimal. The buffers received with each DNA polymerase were used because they are produced and optimized for each DNA polymerase. There are likely several facilitators

present in ToughMix, but it is not known which ones. No other facilitators were added to the reaction. The reaction was initiated by activating the DNA polymerases at 95 °C for 5 minutes (except for Tempase and Immolase that require 15 minutes of activation), following denaturation at 95 °C for 10 seconds, annealing at 60 °C for 20 seconds, and extension at 72 °C for 30 seconds. After 40 cycles, a melt curve analysis was performed to determine the melting temperature of the produced product. An agarose gel electrophoresis (1 % agarose gel running for 60 minutes on 100 V with 0.01 % GelRed) was performed for a selection of samples to confirm the melt curve results from the qPCR BioRad instrument.

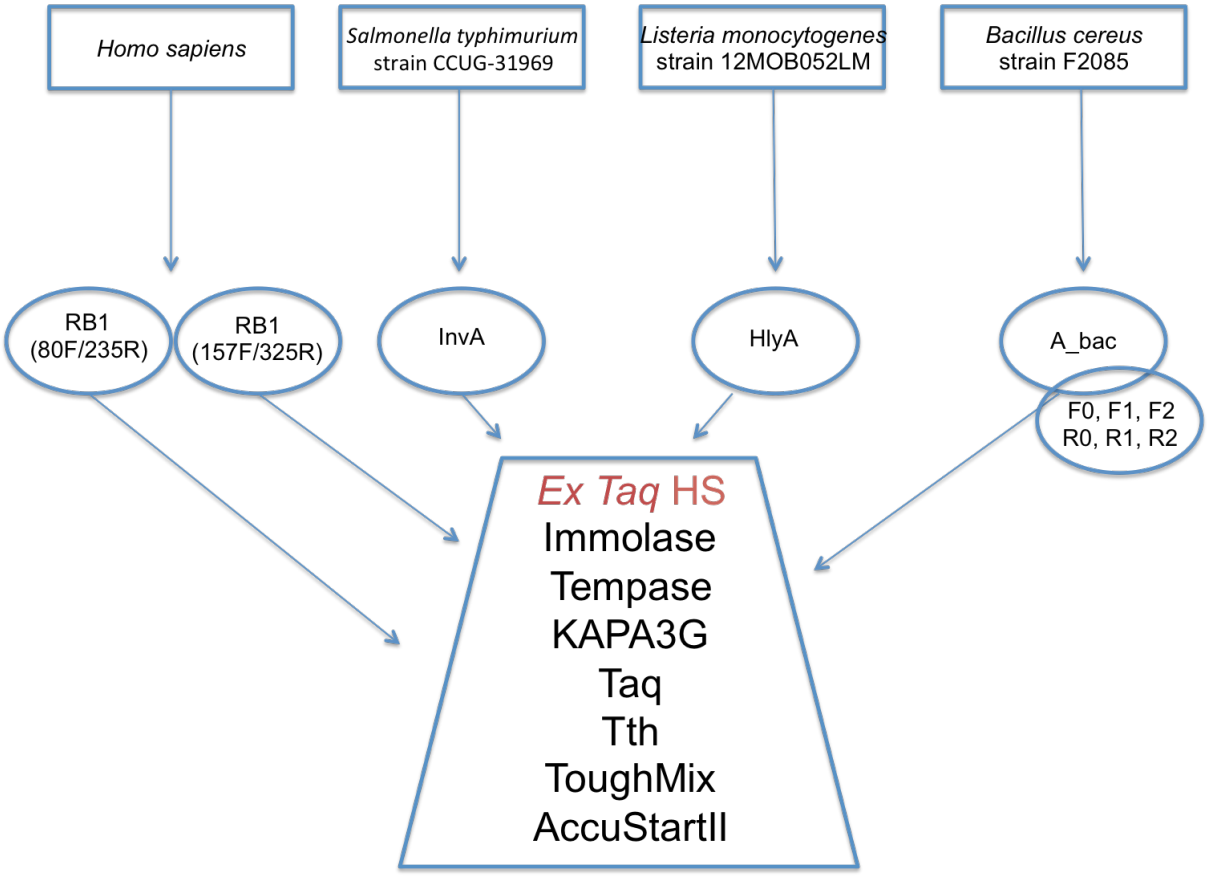


Figure 1. An overview of how the screening of the different DNA polymerases was performed. At the top, organism names are indicated, following respective qPCR assay. RB1 assay was performed with two primer pairs (80F/235R and 157F/325R), and different primers and primer pairs were used for A_bac assay as well. The DNA polymerases shown in the figure were then analyzed in combination with each assay. Ex *Taq*, which is indicated with a red color, was considered a reference DNA polymerase when optimizing qPCR assays.

2.4.1 Identification of primer-dimer product (A_bac assay)

Primer-dimer formations were observed in the A_bac assay, and this was investigated further. To confirm and identify the primer-dimers obtained with the A_bac assay, Sanger sequencing was performed. Moreover, new primer pairs were ordered (F1, R1, F2, R2), but with an altered sequence by adding and/or deleting nucleotides (Table 1). Furthermore, another batch of A_bac primers (F0 and R0) was reordered from Thermo Fisher Scientific and also from a different company (Integrated DNA Technologies, Skokie, Illinois, USA). This was done to either confirm or rebut the primer-dimer formation that was observed with the first batch of A_bac primers.

To get the sequence of the primer-dimers, Sanger sequencing was performed. Due to the short product length of approximately 30-50 base pairs, the amplicons had to be inserted in a vector to enable sequencing. This vector was then transformed into competent cells. First, a conventional PCR was performed on Applied Biosystems GeneAmp PCR System 9700 to amplify the DNA with a reaction volume of 50 µL (Thermo Fisher Scientific). Final concentrations were 2 mM $MgCl_2$, 1 µM primers, 1X of PCR buffer II, 0.2 µM dNTP and 1 U AccuStart II DNA polymerase. No template DNA was used in this experiment because the product studied was the primer-dimer formation. The reaction was initiated by activating AccuStart II DNA polymerase at 94 °C for 5 minutes, following denaturation at 94 °C for 30 seconds, annealing at 60 °C for 30 seconds, and extension at 72 °C for 30 seconds. The cycling continued for 35 cycles. After conventional PCR, an agarose gel electrophoresis (0.8 % agarose gel running for 55 minutes on 100 V with 0.01 % GelRed) was performed to obtain bands of primer-dimer formations. The bands were visualized using UV light and the band corresponding to the primer-dimer product was excised from the gel and purified according to QiaQuick gel extraction kit and GeneJet PCR purification kit, which are based on spin columns (Qiagen, product number 28706 and Thermo Fisher Scientific, product number K0702). To confirm that extraction and purification succeeded, the DNA concentration of the samples was measured on Qubit where Qubit dsDNA HS Assay Kit protocol was used with 5 µL sample (Thermo Fisher Scientific, product number Q32851). Moreover, an agarose gel electrophoresis (2 % agarose gel running for 100 minutes on 100 V with 0.01 % GelRed) was performed to verify the presence of correct primer-dimer product.

After extraction and purification of the primer-dimer product, a ligation reaction and subsequent transformation was performed (pGEM-T and pGEM-T Easy Vector Systems, Promega, Madison, Wisconsin, USA, product number A1380). The reaction components of a standard ligation reaction were 10X Rapid Ligation Buffer, T4 DNA ligase (1 µL), pGEM-T Easy Vector 50 ng (1 µL), PCR product (2 µL), T4 DNA Ligase 3 Weiss units/µL (1 µL) and 5 µL of SQ water. The final ligation reaction volume was 10 µL. Samples were incubated overnight at 4 °C. After incubation, transformation of the vector (containing the primer-dimer product) to JM109 high-efficiency competent cells was then performed. LB plates with ampicillin (150 µg/µL), IPTG (100 mM) and X-Gal (50 mg/mL) were prepared, and the transformation cultures were then plated. Moreover, the plates were incubated for 24 hours at 37 °C. After incubation, the plates were placed in a fridge for 2 hours. White colonies on each plate were then selected and placed into liquid LB medium containing ampicillin (100 µg/µL) for incubation at 37 °C, 200 rpm for 20 hours. Then, 10 clones were picked and the plasmid from each was purified based on spin columns (Thermo Scientific GeneJET Plasmid Miniprep Kit). The plasmid concentration was then measured on Qubit (BR, 2 µL sample). Lastly, before sequencing, a restriction enzyme (EcoRI) was used to cleave the primer-dimer product and the plasmid (Thermo Scientific FastDigest EcoRI). A gel electrophoresis (2 % agarose gel running for 80 minutes on 100 V with 0.01 % GelRed) was then used to visualize the cleaved plasmid and primer-dimer product. Finally, samples were sent for sequencing with T7 as primer (Eurofins Scientific, Brussels). The result files from sequencing were then analyzed with Unipro Ugene [26].

2.4.2 Preparation of background matrices

After the systematic screening of qPCR assays with the different DNA polymerases, the effect of adding matrices representing more or less inhibitory samples was investigated. To a total reaction volume of 20 µL, 5 µL of matrix was added to investigate how the DNA polymerases were affected in the presence of possibly disturbing compounds from the matrices. The less inhibitory matrices were eluates generated from two commonly used extraction methods: 1.

EZ1 DNA Tissue kit (Qiagen, Hilden, Germany, product number 953034) using EZ1 Biorobot and 2. a Chelex-based protocol [27]. To produce the EZ1 eluate, 200 µL of Super Q (SQ) water was added to a 2 mL sample tube and extraction was performed according to the manufacturer's instructions. To prepare the Chelex eluate, a Chelex solution of 20 % was prepared in a glass bottle. 1 mL of this solution was then transferred to an Eppendorf tube, where 10 µL of proteinase K was added. Then, the sample was incubated at 56 °C, for 60 minutes, at 1500 rpm on BioShake iQ (QInstruments, Jena, Germany). The sample was then incubated for another 20 minutes at 100 °C. Lastly, the sample was centrifuged for 1 minute at 11000 rcf. Background matrices with a more pronounced and well-known inhibitory effect were also investigated (see enzymatic rate assay in results): human blood and humic acid (HA) (Sigma-Aldrich, Taufkirchen, Germany, product number 53680) [7, 28]. Human blood was used in two different concentrations; 0.4 % and 0.04 % [7, 28]. Likewise, HA was also prepared in two different concentrations; 40 ng/µL and 10 ng/µL [7, 28]. Both human blood and HA were diluted in TE buffer to obtain the correct concentrations. The EZ1 and Chelex matrices were used for the screening experiments, while all of the matrices (EZ1, Chelex, blood and HA) were used to investigate how the extension rates of two different DNA polymerases were affected.

2.5 Enzymatic rate assay

In order to analyze the efficiency of DNA polymerases more directly, a method was adapted from Montgomery and coworkers [21, 22]. The extension rates of the DNA polymerases (how fast the DNA polymerase incorporates nucleotides per cycle) were studied and the DNA polymerases investigated in this experiment were Ex *Taq* and Tempase.

2.5.1 Quantification of DNA polymerase

To determine the molarity of the DNA polymerase stock solution, quantification of DNA polymerases was performed. In order to quantify DNA polymerases, sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed on 15-well Mini-PROTEAN TGX gels on BioRad (Mini-PROTEAN Tetra Cell, 4-Gel System). Components in running buffer for 10X SDS-PAGE were 250 mM Tris, 1.92 M glycine and 1 % SDS. For 1X, dilute 10X stock with 900 mL SQ water. 10 µL of sample buffer was prepared and loaded to the wells with 5 µL sample, Laemmli sample buffer 2X µL and β–Mercaptoethanol as components. BSA (Thermo Scientific, Albumin Standard, product number 23209) was used as standard with duplicates of each concentration (50, 100, 200 and 300 ng). Electrophoresis was then performed for 40 minutes at 200 V. After electrophoresis, the gels were stained in 50 mL Oriole Fluorescent Gel Stain for 90 minutes covered with aluminum foil on a shaker. Then, pictures were acquired with BioOne Quantity, where a standard curve was made based on the pixel intensity of the BSA bands. By receiving molecular weight and concentration of the DNA polymerases, the molarity of stock DNA polymerase concentration was calculated. The concentration of Tempase DNA polymerase was difficult to determine due to a smear on the gel, thus only *Ex Taq* was quantified using SDS-PAGE. For Tempase, the activity (U) was used instead, meaning that the protein concentration of Ex *Taq* was used for Tempase as well because both DNA polymerases had the same concentration (1 U). So the same amount of Tempase was added as for Ex *Taq*.

2.5.2 DNA polymerase extension template

The extension template used in this experiment is a self-complementary oligonucleotide, which forms a hairpin with a free 3' end and a 14 base pair stem [21, 22]. Moreover, for extension, a 25 base pair overhang is present. The sequence of the self-complementary oligonucleotide is

tagcgaaggatgtgaacctaatcccTGCTCCCGCGGCCGatctgcCGGCCGCGGGAGCA,
where capital letters indicate self-complementary sequences [21, 22].

2.5.3 DNA polymerase extension assay

The experiments were performed with BioRad instrument with a reaction volume of 20 µL. Triplicates of each sample were tested. Final concentrations were 2.5 mM $MgCl_2$, 0.1 µM oligonucleotide template, 1X of the buffer received with each polymerase, 0.2 µM deoxyribonucleotide triphosphate (dNTP), 1X EVAGreen, 1 U polymerase. Before starting the reaction, the DNA polymerases were diluted in 50 mmol/L Tris, 300 µg/mL BSA (Roche Diagnostics) and 0.03 % Tween 20 [21, 22]. The reaction was initiated by activating the Ex *Taq* at 95 °C for 5 minutes and Tempase at 95 °C for 15 minutes, following extension at 72 °C for 1 second. After 360 cycles, an extension curve analysis was performed to evaluate the extension rates.

2.5.4 DNA polymerase activity and extension rates

In theory, the extension rate (nucleotides incorporated per polymerase per cycle), can be calculated given by [21, 22]:

$$[Template]\, x\, \frac{L}{(F(max) - F(min))}\, x\, (F(t) - F(\min))$$

where [Template] is the template concentration in nanomoles/liter, L is the length of the extension template, F(max) is the maximum fluorescence value, F(min) is the minimum fluorescence value and F(t) is the fluorescence value at a given point.

By dividing this equation by the concentration of DNA polymerase, average nucleotides per polymerase per cycle can be calculated. These results can then be incorporated as extension rate curves, where the linearity in the curves specifies the rate of the DNA polymerase per nucleotide per cycle.

2.6 qPCR data analysis

Cq values are a measurement of the amount of target copies produced, where low Cq values reflects that more copies of the target are made, compared to high Cq values, where fewer copies are produced. The resulting Cq values of the DNA polymerases were used to compare the DNA polymerases and the qPCR assays. However, a DNA polymerase may result in high Cq values, but still be reliable due to high amplification efficiency (95-105 %) and a linear standard curve (R2 > 0.980), so the amplification efficiency, detection limit and a linear standard curve of each DNA polymerase were also taken into account when comparing the DNA polymerases and the qPCR assays.

3. Results

3.1 Preparation of template genomic DNA

The quality of the DNA extracted from bacteria or human blood was assessed using agarose gel electrophoresis. The results showed that the DNA from all organisms was intact (Figure 2). DNA concentration measurements using Qubit showed 10.3 ng/µL of human, 18.2 ng/µL of *S. typhimurium*, 46.8 ng/µL of *L. monocytogenes* and 11.7 ng/µL of *B. cereus*. The 260/280 ratios of absorption on BioDrop of each sample were approximately 1.8 for all samples, indicating pure products.

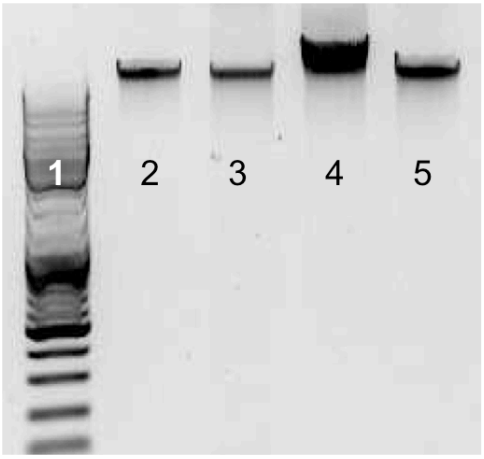


Figure 2. Agarose gel electrophoresis was performed in order to confirm the quality of the extracted and purified DNA samples. Lane 1 indicates ladder (GeneRuler DNA Ladder Mix, 0.5 µg/µL, Thermo Fischer Scientific), lane 2 indicates human DNA, lane 3 indicates *Bacillus cereus*, lane 4 indicates *Salmonella typhimurium* and lane 5 indicates *Listeria monocytogenes*.

3.2 Screening of DNA polymerases in combination with different qPCR assays

Different efficiencies between the DNA polymerases within each qPCR assay and between the qPCR assays were observed (Figure 3). Comparing DNA polymerases within each assay, the Cq values resulting from the different DNA polymerases indicated a difference for InvA assay, where Ex *Taq*, Tth and AccuStart II gave lower Cq values (28.67 ± 0.10, 27.79 ± 0.48 and 28.05 ± 0.05 at a DNA concentration of 1 pg/µL) than the other DNA polymerases (Figure 3A). *Taq* reached the detection limit of InvA already at a DNA concentration of 0.1 pg/µL, thus no detection occurred at the concentration of 0.01 pg/µL (indicated with no bar being present). The results for the HlyA assay showed that Ex *Taq* and Tth, again, gave the lowest Cq values (25.87 ± 0.12, 25.49 ± 0.11 at a DNA concentration of 1 pg/µL) while Immolase gave the highest Cq values (28.20 ± 0.10) (Figure 3B). However, even though Tth contributed to a lower Cq value at 10 and 1 pg/µL, its detection limit was reached at 1 pg/µL. KAPA3G, Tth and *Taq* resulted in the lowest Cq values (25.76 ± 0.06, 25.19 ± 0.04, 25.98 ± 0.16 at a DNA concentration of 1 ng/µL) for RB1 80F/235R assay, and Tempase with the highest Cq values at all DNA concentrations (27.78 ± 0.06) (Figure 3C). For RB1 157F/325R assay, Ex *Taq* together with KAPA3G and AccuStart II gave lower Cq values (27.59 ± 0.02, 27.82 ± 0.08 and 28.19 ± 0.03 at a DNA concentration of 1 ng/µL) compared to the other DNA polymerases (Figure 3D). Immolase and Tempase resulted in higher Cq values (28.51 ± 0.08 and 29.70 ± 0.04 at a DNA concentration of 1 ng/µL). Lastly, Ex *Taq* and AccuStartII were the DNA polymerases representing the lowest Cq values (26.89 ± 0.08 and 27.33 ± 0.09 at a DNA concentration of 100 pg/µL) for the A_bac assay (Figure 3E). Even at the highest concentration, 100 pg/µL, Tempase gave a high Cq-value (28.20 ± 0.03) compared to the other DNA polymerases. At 0.1 pg/µL, none of the DNA polymerases indicate reliable Cq values due to primer-dimer formations with the A_bac assay. Comparing the qPCR assays, InvA, HlyA and RB1 80F/235R seem to be more robust than RB1 157F/325R and especially A_bac assay, which means that these assays are more stable at lower DNA concentrations and result in higher precision. Even at the highest concentration (100 pg/µL), the results of the melt curve of A_bac assay indicated primer-dimer formation. To summarize, some qPCR assays are more robust, such as InvA, HlyA and RB1 80F/235R assays, and result in a superior detection limit with almost no primer-dimer formation. Less robust qPCR assays such as A_bac show primer-dimer formations at higher DNA concentrations. Moreover, some DNA polymerases seem to perform poorly through all qPCR assays, such as Tempase. Other

DNA polymerases, such as Immolase seem to vary in their performance depending on qPCR assay.

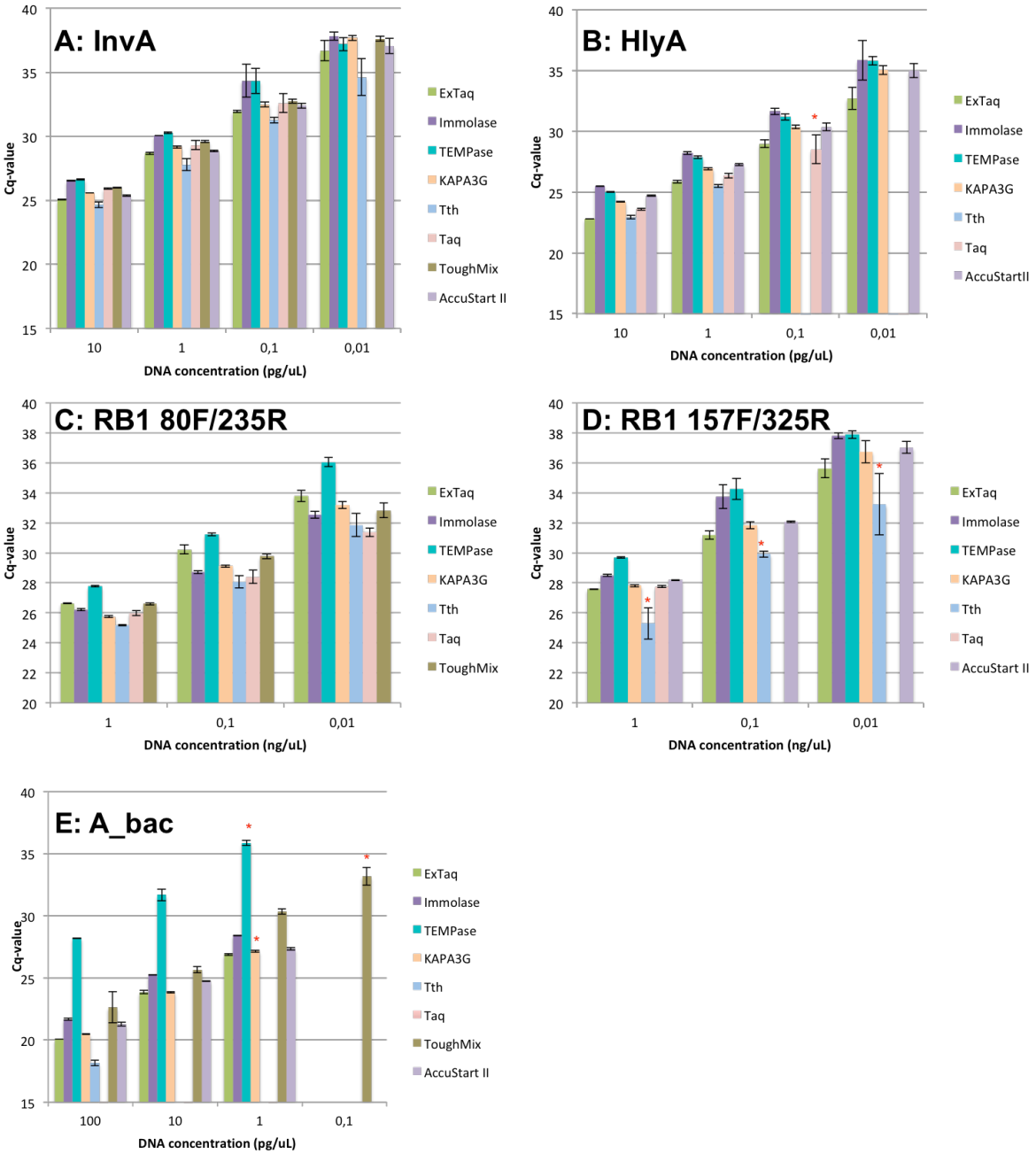


Figure 3. Five qPCR assays were used to investigate efficiencies of DNA polymerases, both within and between each assay. All of the samples were tested in triplicates. The red asterisks above some of the DNA polymerases indicate that approximately 50 % of the amplified qPCR products were the correct product and 50 % were primer-dimer products, as seen in the melt curve. These Cq values are thus generated from amplification of a high amount of incorrect qPCR products and are thereby not reliable. A: InvA assay (*Salmonella typhimurium*) is a robust qPCR assay, where the Cq values given by the DNA polymerases differed only slightly depending on DNA polymerase. B: Similar to InvA, HlyA assay (*Listeria monocytogenes*) is a robust assay. C: RB1 80F/235R assay (human) is also an assay with a low detection limit. D: RB1 157F/325R assay (human) produces higher Cq values than the RB1 80F/235R assay. E: A_bac assay (*Bacillus cereus*) gave high Cq values already at a higher concentration, with primer-dimer formations for Tth at 10 pg/ µL.

To confirm the results seen on the melt curves of each qPCR assay, an agarose gel electrophoresis was performed (Figure 4). The results of no primer-dimer formation observed in the melt curve (Figure 4A) were confirmed with the gel results (Figure 4B). Likewise, the primer-dimer formations seen on the melt curves were also seen on the gel (Figure 4C-D).

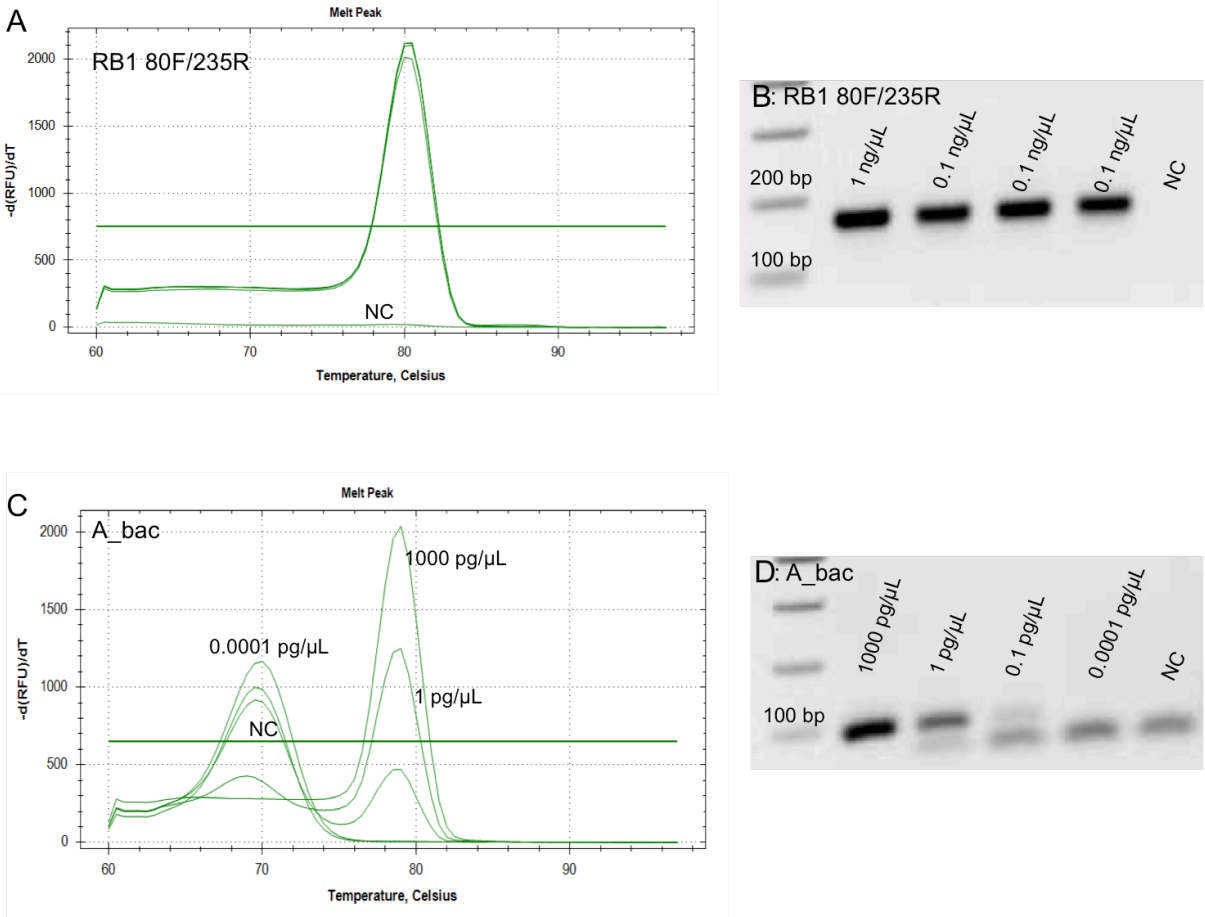


Figure 4. Melting peaks and gel electrophoresis results of the PCR products formed by two different qPCR assays, one more robust than the other. The applied DNA polymerase was Ex *Taq*. A: Melt curve of RB1 80F/235R assay. The DNA concentrations of the samples were 1 ng/µL, triplicates of 0.1 ng/µL and negative control (NC) without template DNA. B: Agarose gel electrophoresis of RB1 80F/235R assay. The samples on the gel were the same as in A. C: Melt curve of A_bac assay. The DNA concentrations of the samples are 1000 pg/µL, 1 pg/µL, 0.1 pg/µL, 0.0001 pg/µL and NC without template DNA. A formation of primer-dimers can be seen. D: Agarose gel electrophoresis of A_bac assay. The samples on the gel were the same as in C, confirming the primer-dimer formation seen in the melt curve.

3.2.1 Identification of primer-dimer product (A_bac assay)

The prominent primer-dimer product formed with the A_bac assay was further investigated by Sanger sequencing. The results from 10 different clones transformed with the primer-dimer product specified the same sequence, represented by the sequence of forward and reverse primers overlapping with a single base pair (Table 1 and Figure 5A-B). A new batch of A_bac primers, including primers with altered sequence either by adding or deleting nucleotides, were purchased and applied to explore if the primer-dimer formation could be removed applying the altered primers. The results varied depending on A_bac primer combination. Two primer combinations, F1R0 and F2R0, showed no formation of primer-dimers for DNA concentrations 1000 to 1 pg/µL (Figure 5C). Only low amounts of primer-dimers were formed at DNA concentrations of 0.1 pg/µL. For combinations including primer F0 however, primer-dimer formation still occurred. Surprisingly, the primer-dimer formation

seen with the first batch of primers was no longer observed with the new batch of A_bac F0R0 primers ordered from Integrated DNA Technologies (Figure 5D). Lastly, a new batch of A_bac F0 and R0 primers were ordered from the first company (Thermo Fisher Scientific), to rule out the possibility of incorrectly manufactured primers in the first primer batch and the results with the new batch of A_bac primers showed no primer-dimer formation. This suggests that the A_bac primers, and more specifically the F0 primer, in the first batch from Thermo Fisher Scientific were incorrectly manufactured.

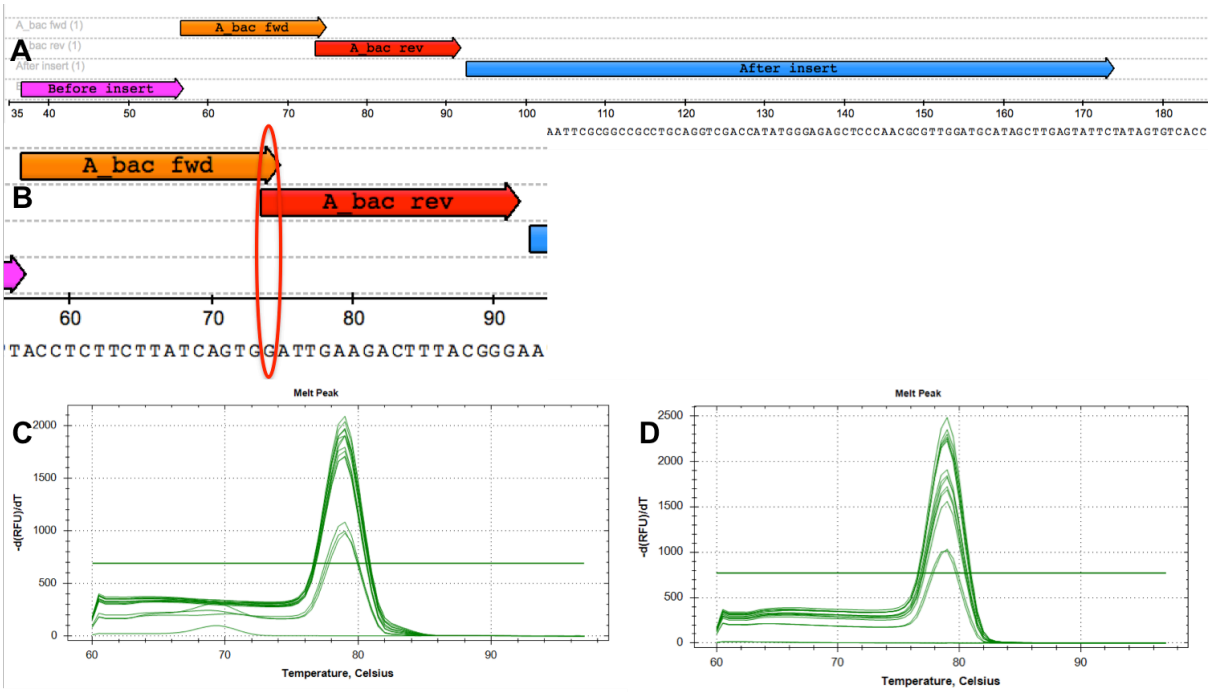


Figure 5. A: The primer-dimer product of the A_bac assay. Binding of forward (fwd) primer with reverse (rev) primer with only one base pair can be seen in the figure. B: Zoom of A. C: The DNA polymerase Ex *Taq* in combination with primer pairs F2R0 indicated no primer-dimer formation for the DNA concentrations 1000-1 pg/µL. At 0.1 pg/µL, low amounts of primer-dimers were formed. D: The DNA polymerase Ex *Taq* in combination with primer pairs F0R0 from a new batch. No unspecific product was formed for the DNA concentrations 100-0.1 pg/µL.

3.2.2 Addition of background matrices

The effect of adding EZ1 matrix to the reactions depended both on qPCR assay and DNA polymerase. Applying EZ1 matrix to the invA assay, no Cq shifts were observed regardless of the DNA polymerase used, indicating a robustness of the assay (Figure 6A-B). Applying some DNA polymerases, such as AccuStart II, amplification with A_bac assay was also barely affected by the added EZ1 matrix (Figure 6C-D). In contrast, for other DNA polymerases with the A_bac assay, there was a large shift in Cq values compared to positive control (PC). At a DNA concentration of 10 pg/µL, Ex *Taq* and Immolase resulted in less pronounced Cq shifts with added EZ1 matrix (Ex *Taq*: PC 23.79, EZ1 25.29 ± 0.05, Immolase: PC 25.22, EZ1 26.46 ± 0.18), whereas Tempase resulted in higher Cq values when EZ1 was added (PC 26.26, EZ1 36.07 ± 0.49). Moreover, no amplification at all was observed with ToughMix at this DNA concentration, as EZ1 matrix was added. However, the amplification with AccuStart II (the DNA polymerase of the ToughMix) was barely affected by the addition of EZ1 matrix (PC 25.00, EZ1 25.36 ± 0.06 at a DNA concentration of 10 pg/µL, PC 28.06, EZ1 28.50 ± 0.22 at a DNA concentration of 1 pg/µL) (Figure 6D). Ex *Taq*, Immolase and Tempase resulted in higher Cq values with addition of EZ1 matrix and 1 pg DNA/µL (Ex *Taq*: PC 26.51, EZ1 29.34 ± 0.46, Immolase: PC 27.95, EZ1 29.26 ± 0.25,

Tempase: PC 29.52, EZ1 37.73 ± 0.42). Similar to 10 pg/µL, ToughMix gave no Cq values with EZ1 matrix added at 1 pg DNA/µL. Lastly, applying different DNA concentrations with different DNA-polymerases and EZ1 matrix, almost no observable changes of Cq values were seen with the InvA assay, while a critical inhibition of ToughMix and Tempase were observed with the A_bac assay (Figure 6E-F).

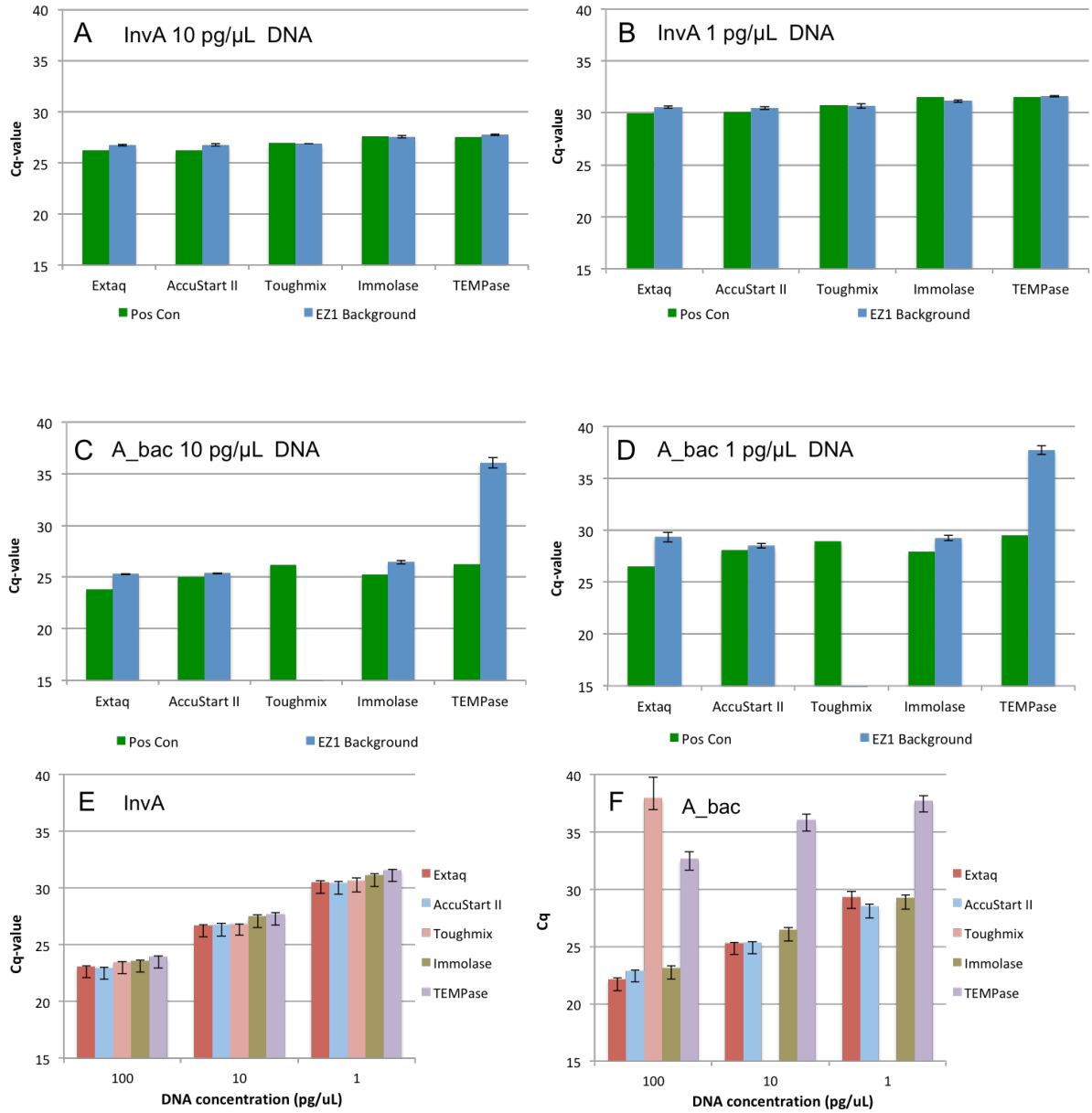


Figure 6. The resulting Cq values of the different DNA polymerases in the absence and presence of EZ1 matrix were compared. All of the matrix samples were analyzed in triplicates, while the positive controls were analyzed with one replicate. The results of positive controls (A-D) are shown as green staples, while the results of EZ1 matrices are shown as blue staples. The different DNA polymerases tested were Ex *Taq*, AccuStart II, ToughMix, Immolase and Tempase. A: InvA assay with a template DNA concentration of 10 pg/µL. B: InvA assay with a template DNA concentration of 1 pg/µL. C: A_bac assay with a template DNA concentration of 10 pg/µL. D: A_bac assay with a template DNA concentration of 1 pg/µL. E: A comparison of the resulting Cq values with the addition of EZ1 matrix given by the different DNA polymerases for InvA assay. F: A comparison of the resulting Cq values with the addition of EZ1 given by the different DNA polymerases for A_bac assay.

Similar to the results of adding EZ1 matrix, addition of Chelex matrix resulted in no or small changes in Cq values when different DNA polymerases were applied with the InvA assay, again indicating a very robust assay (Figure 7 A-B). Ex *Taq*, however, gave higher Cq values when Chelex matrix was added to the reaction (PC 25.34, Chelex 26.77 ± 0.24 at a DNA concentration of 10 pg/µL). In contrast to the EZ1 matrix, addition of Chelex matrix caused no or slight changes in Cq values with the A_bac assay. ToughMix in combination with Chelex matrix gave a slightly higher Cq value compared to control (PC 29.08, Chelex 30.6 ± 0.42 at a DNA concentration of 1 pg/µL) (Figure 7C-D). Lastly, applying different DNA concentrations with different DNA polymerases and Chelex matrix resulted in similar Cq values with the InvA assay, except for with Ex *Taq* (Figure 7E). However, with the A_bac assay, Chelex matrix in combination with ToughMix and Tempase resulted in higher Cq values (Figure 7F).

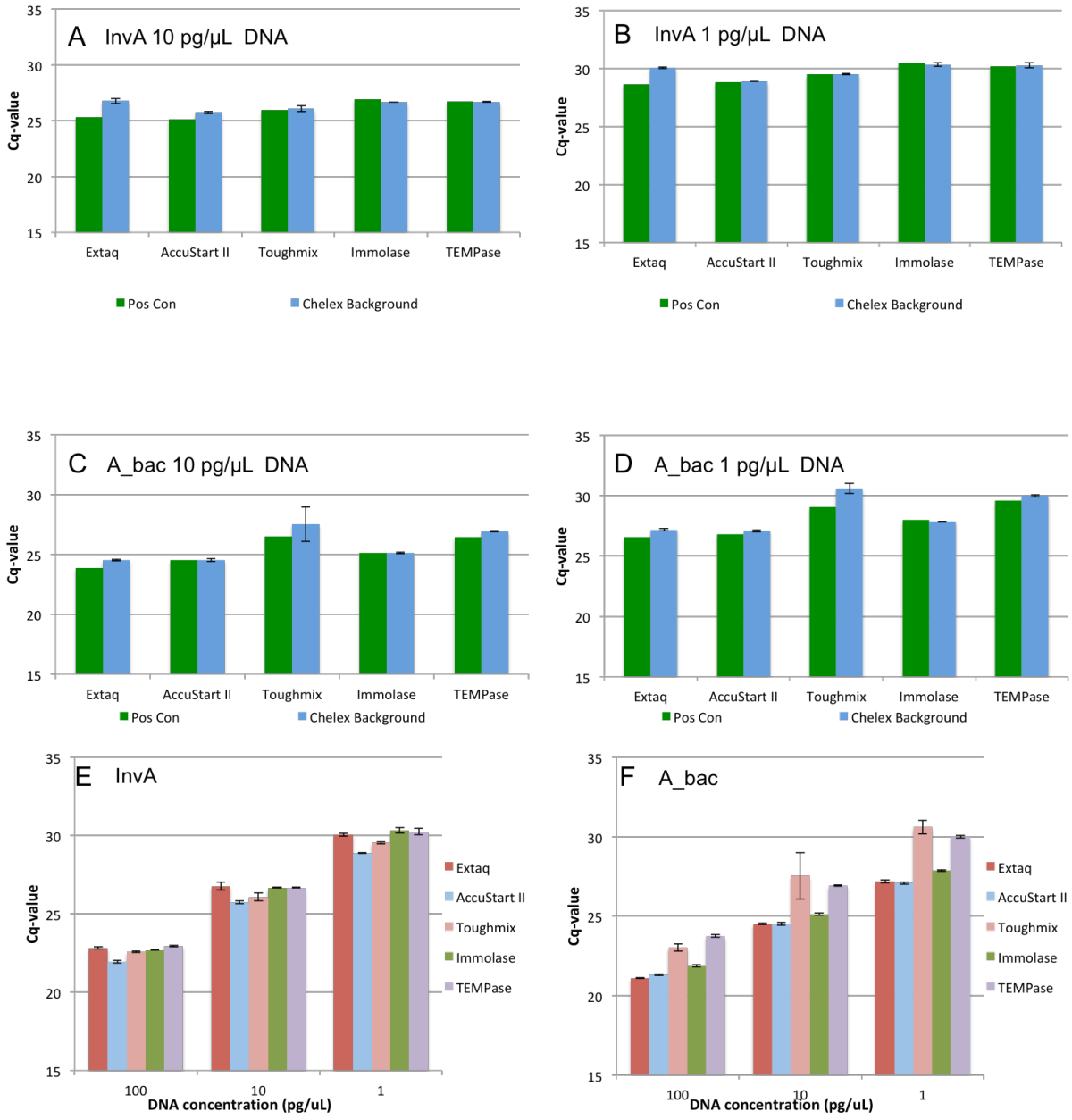
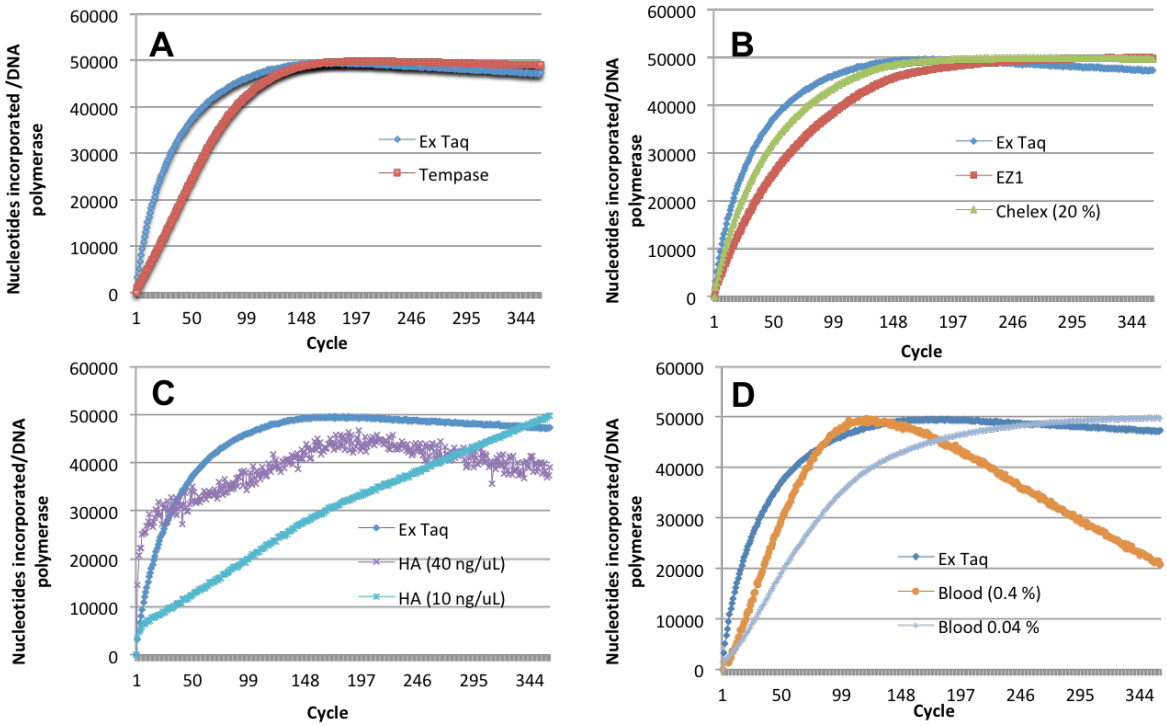


Figure 7. The resulting Cq values of the different DNA polymerases in the absence and presence of Chelex (20 %) were compared. All of the matrix samples were analyzed in triplicates, while the positive controls were analyzed in a single replicate. The results of positive controls (A-D) are shown as green staples, while the results of Chelex backgrounds are shown as blue staples. The applied DNA polymerases were Ex *Taq*,

AccuStart II, ToughMix, Immolase and Tempase. A: InvA assay with a template DNA concentration of 10 pg/µL. B: InvA assay with a template DNA concentration of 1 pg/µL. C: A_bac assay with a template DNA concentration of 10 pg/µL. D: A_bac assay with a template DNA concentration of 1 pg/µL. E: A comparison of the resulting Cq values with the addition of Chelex given by the different DNA polymerases for InvA assay. F: A comparison of the resulting Cq values with the addition of Chelex matrix given by the different DNA polymerases with the A_bac assay.

3.3 Enzymatic rate assay

A method to determine enzymatic rate, developed by Montgomery and coworkers [21, 22] was established and optimized for the qPCR instruments and DNA polymerases used in this master thesis project. The DNA polymerases assessed with the enzymatic rate assay were Ex *Taq* and Tempase. After quantification of Ex *Taq* using SDS-PAGE, the qPCR rate assay was performed and the results are shown as extension rate curves (Figure 8). Matrices (EZ1, Chelex 20 %, HA 40 ng/µL and 10 ng/µL, human blood 0.4 % and 0.04 %) were then added to the reaction to investigate how the rate of the DNA polymerases was affected. Studying the extension curves for Ex *Taq* and Tempase (Figure 8A), it is clear that the slope for Ex *Taq* is steeper, indicating a higher rate of extension compared to Tempase. Seemingly, the matrices Chelex and EZ1 also cause a slight decrease in the slope for Ex *Taq*, indicating that these matrices slow down the DNA polymerase (Figure 8B). With the more prominent PCR inhibitors, HA and blood, the slope was clearly lowered and DNA polymerase extension rate decreased. In contrast, with Tempase, the results indicated that the DNA polymerase extension rate was increased with the different matrices, although further replicates of the data is needed to make any conclusions. Due to the time limit of the project, the DNA polymerase rate has not yet been calculated for these experiments, thus the results are shown as comparative curves and not as determined extension rates. To calculate the rate, more work with data analysis is needed, where the linearity in the curves is used to specify the rate of the DNA polymerase per nucleotide per cycle.



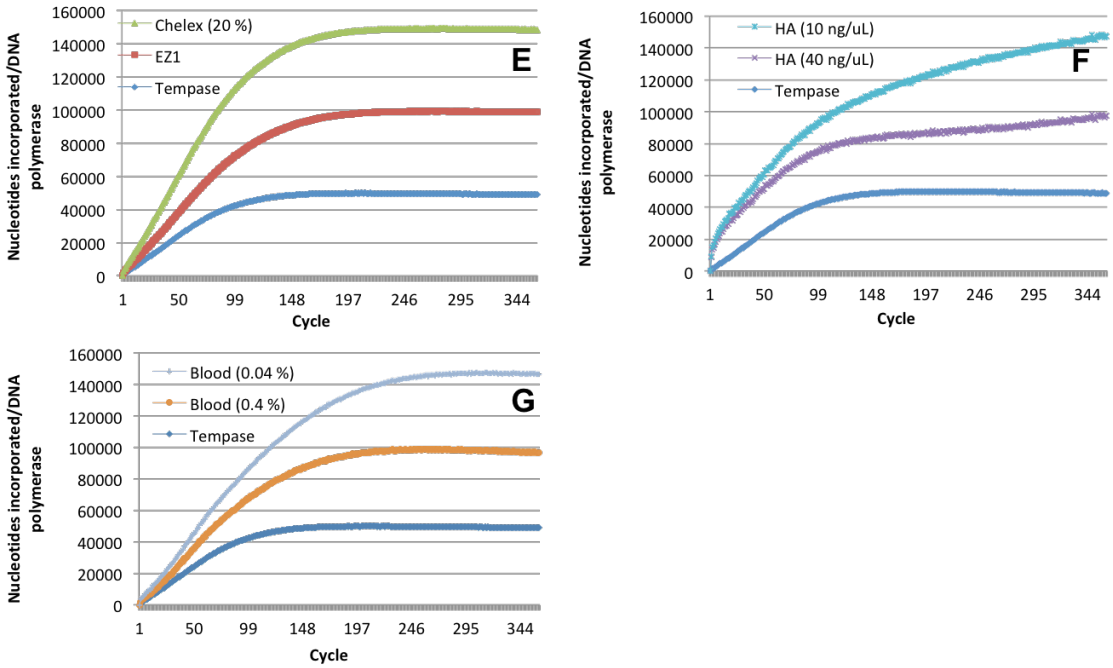


Figure 8. Extension rate curves of the DNA polymerases Ex *Taq* and Tempase. A: Comparison of Ex *Taq* and Tempase, B: Ex *Taq* with EZ1 matrix and Chelex matrix, C: Ex *Taq* with PCR inhibitor humic acid (HA) in two concentrations (40 ng/µL and 10 ng/µL), D: Ex *Taq* with PCR inhibitor blood in two concentrations (0.4 % and 0.04 %), E: Tempase with EZ1 matrix and Chelex matrix, F: Tempase with PCR inhibitor HA in two concentrations (40 ng/µL and 10 ng/µL), G: Tempase with PCR inhibitor blood in two concentrations (0.4 % and 0.04 %).

4. Discussion

It is well known that Cq values can vary depending on the DNA polymerase used with a certain qPCR assay, even though the amplification efficiency and the detection limit are similar. It has also previously been shown that the performance between different DNA polymerases varies depending on the type of inhibitors in the background matrix [18, 19]. One study have reported that different DNA polymerases might be biased in their priming efficiency, depending on the sequence of the primer-template junction [20], however this phenomenon is not further acknowledged in the literature. Moreover, that the combination of different DNA polymerases and specific qPCR assays are more or less sensitive to common background matrices such as eluates from DNA extraction methods, is to our knowledge not previously described at all. In this project, the performance of different DNA polymerases in combination with five validated and published qPCR assays is evaluated, and moreover, the effect of adding more or less inhibitory matrices explored. The advantage of studying qPCR assays from different organisms is that their genomes differ from each other, both regarding size and composition. To eliminate differences originating from the DNA extraction, each organism was extracted with the same method.

4.1 Screening of DNA polymerases in combination with different qPCR assays

The first part involved screening of several DNA polymerases with five qPCR assays, where one of the qPCR assays, A_bac, was shown to be less robust than the other assays (InvA, HlyA and RB1). A robust qPCR assay is defined to provide reliable results even though conditions are not absolutely optimal or when low amounts of qPCR inhibitors are present in the sample [10]. On the contrary, a non-robust qPCR assay is more sensitive to small changes in the conditions, for example an altered annealing temperature or the presence of qPCR inhibitors [10]. It is important to remember that even though most DNA polymerases in

combination with the robust qPCR assays resulted in similar Cq values, there were small differences in Cq values. This is expected, as the efficiency of DNA polymerases differ, both between each other and of course between different qPCR assays. A consistently higher Cq value given by a specific DNA polymerase and qPCR assay is often seen and does not necessarily suggest an ill-performing DNA polymerase. As long as the efficiency is high, the standard curve within the dynamic range is linear and the detection limit is low, the individual Cq values given by the DNA polymerase is less important. In this study, we saw that some of the DNA polymerases that gave lower Cq values at the higher DNA concentrations resulted in an impaired detection limit and thus had a more narrow dynamic range. Thus it is important to study more aspects than the Cq values when comparing the capacity and performance of the DNA polymerases.

Some of the DNA polymerases generally resulted in lower Cq values and better detection limits regardless of which qPCR assay that was applied, for example Ex *Taq* and AccuStart II. Other DNA polymerases seemed to work well in combination with one qPCR assay, but not at all for another qPCR assay, for example Tth, Taq, Immolase, ToughMix and KAPA3G. One DNA polymerase, Tempase, consistently resulted in high Cq values or no amplification at all with all the qPCR assays. In previous studies however, Tempase proved to be one of the best performing DNA polymerases in combination with a qPCR assay targeting Francisella DNA and high amounts of HA [29]. Apparently, Tempase is not a good choice for the qPCR assays assessed in the current study, but it could be that it works better in presence of inhibitors. Although Accustart II is the DNA polymerase used in ToughMix, AccuStart II resulted in a more efficient amplification compared to ToughMix. This is probably due to the unknown buffer components in ToughMix, which are delivered as a proprietary premixed master mix. ToughMix is produced to manage samples containing a lot of inhibitory background, so it is likely that some of the buffer components are different facilitators.

The reason why some DNA polymerases performed well in combination with one qPCR assay, but not another may be due to that some DNA polymerases are less efficient at amplifying for example GC-rich regions, or more easily detach from the template-primer duplex due to heavier secondary structures [16, 17, 30-32]. It might also be due to that some DNA polymerases are biased in their priming efficiency to certain primer sequences, as previously described by Pan and coworkers [20]. Future studies including primers that have been slightly shifted a few base pairs could be of interest to elucidate this phenomenon further. Also, different batches of the same DNA polymerase was not compared, so if there is a difference in the performance of the same DNA polymerase between different batches may also be a future experiment. Hopefully, more knowledge regarding the robustness of different combinations of qPCR assays and DNA polymerases would improve the detection limit and precision of diagnostic qPCR.

4.1.1 Identification of primer-dimer product (A_bac assay)

The primer-dimer formations seen in A_bac assay were further studied by sequencing the product. The results of Sanger sequencing showed a primer-dimer formation, where only one base pair held the two primers together (GC binding). This is a primer-dimer very unlikely to occur because the binding of only one base pair is not that strong. In an attempt to eliminate the primer-dimer formation, the primers were redesigned, with an altered sequence either adding and/or deleting nucleotides. Different combinations of these primers were tested, where two combinations, F1R0 and F2R0, showed no or very low formation of primer-dimers. In contrast, any combination including F0 primers from the first batch consistently led to primer-dimer formations. However, the fact that no primer-dimer products were seen with

the reordered F0 primers suggests that the first batch of F0 primers were defected. Exactly how these primers could lead to formation of the sequenced primer-dimer product is unknown, but it might be due to additional nucleotides that were complementary to bases of the reverse primer, leading to a hybridization involving more than 1 base pair. Bustin and Hugget indeed describe the importance of selecting primers that are robust and selective for a qPCR assay but also acknowledge that primer combinations sometimes fail to work without predictable reasons [10]. Nevertheless, in our study the impaired amplification of the A_bac assay were due to a defective primer batch, highlighting the importance of having a reliable manufacturer.

4.1.2 Addition of background matrices

It is already known that different DNA polymerases work differently for different inhibitory matrices, for example blood and soil [7, 28]. Moreover, many studies involving qPCR inhibitors have been published [6, 8]. When inhibitors are present, they may disturb the amplification of DNA by interacting with the DNA polymerase, or by binding directly to the DNA [2, 8, 33]. If the DNA concentration is low, it is even more important with a robust qPCR assay and an efficient DNA polymerase [8, 19]. That commonly used extraction methods may affect the performance of different DNA polymerases depending on qPCR assay is not well described [8]. Therefore, the second part of the project was to study the performance of DNA polymerases in InvA and A_bac assays when matrices with compounds from the extraction were added. In this study, a difference between a robust qPCR assay (InvA) and a non-robust qPCR assay (A_bac) was clearly observed. Moreover, that the performance of the different DNA polymerases differed depending on qPCR assay was also seen. The resulting Cq values of the different DNA polymerases are more similar in a robust qPCR assay, while in a non-robust qPCR assay the Cq values are more spread out depending on the DNA polymerase. This might be due to the capacity of the different DNA polymerases. With EZ1 matrix and InvA assay, only slight differences were observed comparing the positive control with addition of EZ1 matrix. The efficiency of the DNA polymerases was similar as well. As mentioned, this may be due to the robust InvA assay. On the other hand, with EZ1 matrix and A_bac assay, observable changes in Cq values resulting from the different DNA polymerases comparing positive control with addition of EZ1 matrix were seen. Furthermore, ToughMix and Tempase showed less or no amplification at all, where, as mentioned, no amplification of ToughMix probably is a result of the reaction mix delivered with the DNA polymerase. It is most likely due to the presence of unknown component of the buffer, and not the DNA polymerase itself, since AccuStart II has shown to be one of the more efficient DNA polymerases throughout this study. Apparently, although the effect of the matrices depends greatly on the robustness of the qPCR assay [10], it is also clear that different DNA polymerases is more or less compatible with different qPCR assays, particular in the presence of specific matrices.

With Chelex matrix and InvA assay, the results were similar to the effects of the DNA polymerases when adding EZ1 to the samples in InvA assay. The robustness of the InvA assay was apparent as almost no change was observed comparing positive control with addition of Chelex of each DNA polymerase, except for Ex *Taq*, where the resulting Cq values were higher with the presence of Chelex indicating lowered DNA polymerase capacity. Despite the robust InvA assay, Ex *Taq* still gave higher Cq values when chelex, but not EZ1 matrix, was added, supporting that different combinations of DNA polymerases and qPCR assays are affected differently by background matrices.

It is important to remember that the A_bac assay investigated in this study included a defective primer batch, which resulted in a prominent formation of primer-dimer product. Therefore, results from A_bac assay were used as a model of a non-robust qPCR assay and the results should be interpreted with caution. The results would probably have been different if the new A_bac primers were used instead. To repeat the experiments with the correct A_bac primers would be an interesting future study to perform, including the addition of EZ1 and Chelex matrices. It would also be interesting to study the addition of other qPCR inhibitors, like blood or HA, to compare the differences in efficiency of the DNA polymerases with the different qPCR assays. Further, experiments combining 2 or more polymerases and buffer systems with different assays would have been interesting to continue with, since such polymerase blending previously has shown to improve the detection [19].

4.2 Enzymatic rate assay

To compare the different DNA polymerases more directly and not only the outcome of a complete qPCR reaction, an attempt to isolate the DNA polymerase extension step was done by setting up a method estimating the extension rate of the DNA polymerase. In order to compare the extension rate between different DNA polymerases, it is necessary to know the polymerase concentration, which is only provided by the manufacturers as unit/µL. The first part in setting up this novel method was thus to quantify the DNA polymerase with SDS-PAGE and the second part to optimize a rate qPCR assay using the BioRad qPCR instrument. As mentioned, this enzymatic rate assay was adapted from Montgomery and coworkers [21, 22]. Ex *Taq* resulted in lower extension rates when matrices were added, while Tempase on the contrary showed an increased extension rate with addition of matrices. However, this partly agrees with the previous study including Tempase, which proved to be one of the best performing DNA polymerases when HA was added [29]. The extension rate assay has not been extensively validated within this project, but has great potential for future studies of how DNA polymerases are affected under different conditions. The first challenge is to find the linearity in each curve to calculate the extension rate of the DNA polymerase. Another challenge would be to quantify other DNA polymerases than Ex *Taq* with SDS-PAGE and subsequently continue the rate evaluation with and without added matrix samples. What can be learnt with experiments like these is how the rate that the DNA polymerase incorporates nucleotides is affected by adding specific components or inhibitors. There were limitations in this project, for example that Tempase was not quantified with SDS-PAGE. However, the experiments done within this study show that Ex *Taq* works at a slower rate when blood and HA are present in the samples, in line with results of previous studies [7, 28, 33]. It has been shown that mutated forms of *Taq* DNA polymerase manage to amplify DNA in the presence of PCR inhibitors in soil and blood samples [33]. Thus, other interesting future experiments would be to study the extension rate of other types of DNA polymerases when inhibitors such as blood or HA is added to the samples.

5. Conclusion

In conclusion, this study has shown that the performance of a DNA polymerase depends to a high degree on the robustness of the qPCR assay. However, we have also observed that other factors might play a role, and that is the specific combination of DNA polymerase, qPCR assay and background matrix. The results suggest that some DNA polymerases are less tolerant and thus more sensitive to certain types of background matrices, and that this depends on the assessed qPCR assay. Interestingly, the background matrices analyzed within this study has not previously been described to affect qPCR amplification negatively; on the contrary these matrices are eluates from DNA extraction methods commonly used prior to diagnostic

qPCR. This study shows the importance of optimizing and evaluating any qPCR assay in combination with at least two different DNA polymerases, especially when introducing a new background matrix. A DNA polymerase performing very well for some matrices with one qPCR assay can seemingly fail to work with other qPCR assays as different background matrices are added. However, more studies focusing on the cause of why some DNA polymerase performs better for one qPCR assay, and not another, with or without the presence of matrices, is required. This could hopefully be done using the enzymatic rate assay that was set up and optimized within this study, indeed showing promising initial results. Yet another lesson learned from this study is to use a reliable manufacturer for reagents supplies, and if the qPCR fail to work it might be a good idea to analyze any new primers for disturbing primer-dimer formation by using intercalating dyes instead of hydrolysis probes. It might be as simple as ordering new primers to solve the problem.

Acknowledgements

I would like to thank Johannes Hedman and Peter Rådström for letting me take part in this interesting project. To my supervisors Linda Jansson and Maja Sidstedt who always have had their doors open for me, I am really thankful for all your guidance during this project. You have challenged me to think in new perspectives. This would not have been possible without you. I would also like to thank Yasmine Akel and Lovisa Kling for keeping me company in the lab and for all of your help. Thanks to the rest of the group at TMB for your assistance, I have learned a lot from all of you. Nick Relaji, thank you for your support with the enzymatic rate assay.

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