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Rapid Detection of *Streptococcus pyogenes* Using Strand Invasion Based Amplification (SIBA®) Method

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The aim of this Bachelor's thesis was to develop a new and rapid method for detecting *Streptococcus pyogenes* by using the isothermal nucleic acid amplification method, SIBA®, which is owned by Orion Diagnostica Oy. *S. pyogenes* is a human pathogen causing hundreds of millions pharyngitis infections globally every year. In addition, *S. pyogenes* causes severe infections such as necrotizing fasciitis and streptococcal toxic syndrome. These infections require rapid diagnosis and treatment. Normally, *S. pyogenes* i.e. group A *Streptococcus* (GAS) infections are detected from clinical specimens by culturing bacteria on blood agar plates or with PCR.

This thesis was conducted for Orion Diagnostica Oy and the experimental part of the thesis was performed in Orion Diagnostica's research and development laboratory. The experimental part was started by culturing different Streptococcal strains on blood agar. The genomic DNA of each strain was extracted and quantified by qPCR. Primers and invasion oligonucleotide were already designed and tested for possible interactions. The assay development started by screening potential LNA probes for the existing assay. The best performing primer combinations and LNA probe were tested with extracted GAS gDNA. The assay was optimized by titrating oligonucleotide and enzyme concentrations and the sensitivity was also tested. The assay specificity was tested by using other Streptococcal gDNAs. Lastly, the most suitable assays were lyophilized and the effect of drying was examined with template tests.

A rapid and sensitive method for detecting GAS was developed during this thesis. The assay reached the sensitivity of even one molecule of target DNA. When the assay performance was tested with lyophilized SIBA-reagents, GAS was detected in 8.5 minutes. Due to the schedule, the assay was only tested with gDNA and not with clinical specimens.

Some unspecific amplification was seen during the assay development, which might have an effect for the assay sensitivity. The assay development should be continued by screening more potential primers and IOs for the assay. After finding oligonucleotide combination, which causes less unspecific amplification, the assay reagents should be optimized and the performance should be tested with clinical specimens. Clinical specimens contain the target DNA, but also other bacteria and human-DNA, which may have an inhibitory effect on the assay performance.

Keywords	*Streptococcus pyogenes*, diagnostics, SIBA, qPCR

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Tämän insinöörityön tavoitteena oli kehittää uusi ja nopea menetelmä *Streptococcus pyogenesin* tunnistamiseksi hyödyntäen Orion Diagnostican omistamaa isotermaalista nukleiinihappojen monistusmenetelmää SIBAa. *S. pyogenes* on ihmisen patogeeni, joka aiheuttaa satoja miljoonia nielutulehduksia vuosittain ympäri maailmaa. Lisäksi *S. pyogenes* aiheuttaa vakavia infektioita, kuten lihansyöjäbakteeri-infektiona tunnettua nekrotisoivaa faskiittia ja toksista sokkioireyhtymää. Nämä sairaudet vaativat nopeaa diagnosointia ja hoidon aloittamista mahdollisimman pian. *S. pyogenes* eli A-ryhmän streptokokki tunnistetaan yleensä viljelemällä bakteereita veriagarilla tai käyttämällä PCR-tekniikkaa.

Tämän insinöörityön toimeksiantajana oli Orion Diagnostica Oy ja työn kokeellinen osuus suoritettiin Orion Diagnostican tutkimus- ja tuotekehityslaboratoriossa. Kokeellinen osuus aloitettiin viljelemällä eri kantojen streptokokkeja veriagarilla. Kasvatettujen bakteerien genomiset DNA:t eristettiin ja kvantitoitiin hyödyntäen qPCR-tekniikkaa. Työssä käytetyt alukkeet ja keskioligo oli valmiiksi suunniteltu ja niiden yhteisvaikutuksia oli alustavasti tutkittu ennen tässä suoritetun osuuden aloitusta. Testimenetelmän kehitys aloitettiin seulomalla potentiaalisia LNA-koettimia. Tehokkaimmin toimivat alukeparit testattiin genomisella DNA:lla käyttäen myös potentiaalisinta LNA-koetinta. Kehitettyä testimenetelmää optimoitiin mm. titraamalla reaktiokomponentteja. Testimenetelmän herkkyys määritettiin ja lisäksi spesifisyys varmistettiin käyttämällä muita streptokokki-kantoja templaattina. Viimeiseksi tutkittiin kylmäkuivauksen vaikutusta kehitetyn testin toimintaan.

Insinöörityön aikana kehitettiin nopea metodi A-ryhmän streptokokin tunnistamiseksi. Testi osoittautui hyvin sensitiiviseksi ja jopa yksi molekyyli kohde-DNA:ta detektoitui. Kylmäkuivatuilla reagensseilla tehdyissä kokeissa kehitetty testi tunnisti A-ryhmän streptokokin 8,5 minuutissa. Aikataulusta johtuen testimenetelmän toimivuutta kokeiltiin vain genomisella DNA:lla eikä lainkaan kliinisillä näytteillä, kuten alun perin oli suunniteltu.

Työn aikana testimenetelmässä havaittiin epäspesifistä monistumista, ja tämä saattaa heikentää testin herkkyyttä. Testimenetelmän kehitystä tulisi jatkaa seulomalla lisää alukepareja ja keskioligoita, jolloin tavoitteena olisi löytää yhdistelmiä, joista aiheutuu vähemmän epäspesifistä monistumista. Toimivien yhdistelmien löydyttyä testi tulisi optimoida ja selvittää kuivauksen vaikutukset. Lisäksi testin toimivuus tulisi testata käyttäen kliinisiä näytteitä. Kliiniset näytteet sisältävät kohde-DNA:n lisäksi myös muita bakteereita ja ihmisen DNA:ta, ja nämä saattavat vaikuttaa testin toimintaan inhiboivasti.

Avainsanat	*Streptococcus pyogenes*, diagnostiikka, SIBA, qPCR

Contents

Abbreviations

ATCC	American Type Culture Collection
CDC	Centers for Disease Control and Prevention
CO_2	Carbon dioxide
COH	Columbia agar plate with 5 % horse blood
Ct-value	Cycle threshold value
DNA	Deoxyribonucleic acid
dsDNA	Double-stranded DNA
FD	Freeze-dried
F primer	Forward primer
GAS	Group A *Streptococcus*
gDNA	Genomic DNA
IO	Invasion oligonucleotide
MgAc	Magnesium acetate
PBS	Phosphate-Buffered Saline
PCR	Polymerase chain reaction
qPCR	Quantitative polymerase chain reaction
R primer	Reverse primer
SIBA	Strand Invasion Based Amplification

TSH	Trypcase soy agar plate with 5 % horse blood

1 Introduction

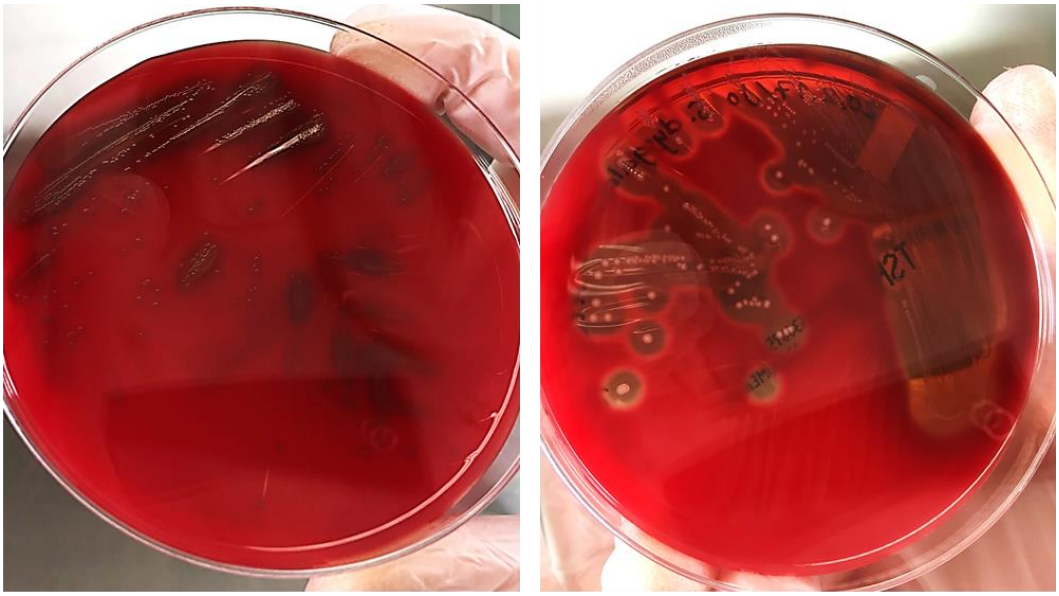
Streptococcus pyogenes, also known as group A *Streptococcus* (GAS), is a human pathogen causing every year over 600 million pharyngitis infections around the world [1]. Also, the number of infections of necrotizing fasciitis and streptococcal toxic syndrome are increasing. Culturing clinical specimens is typically used for diagnosing GAS infections. Also, polymerase chain reaction (PCR) is a reliable method for detecting GAS in clinical specimens. [2.]

The experimental part of this thesis took ten weeks and it was performed in Orion Diagnostica's R&D laboratory in Espoo. The aim of this thesis was to develop and optimize rapid assay for the detection of *S. pyogenes* using Strand Invasion Based Amplification (SIBA®) technology. SIBA is an isothermal DNA amplification technology, which is owned by Orion Diagnostica Oy. After optimization, reagents are freeze-dried and the performance is tested with DNA and clinical specimens.

2 Streptococci

Streptococci are part of the normal flora. Especially they occur in mouth and intestinal tract and also on the skin and in the upper respiratory and genital tracts. Streptococci are gram-positive, non-spore forming, non-motile cocci that occur in chains or pairs. Most of streptococci are facultative anaerobes and can tolerate both microaerobic and anaerobic environments. Some of them are obligate anaerobes and can live only in the total absence of oxygen. [3.]

Streptococci are divided into groups by the type of hemolysis on blood agar. Hemolysis means breaking down red blood cells and there are three types of hemolysis: alpha-hemolysis (α-hemolysis), beta-hemolysis (β-hemolysis) and gamma-hemolysis (γ-hemolysis). In α-hemolysis the red blood cells are partly lysed and that can be seen as green circle around bacterial colonies. In β-hemolysis, the red blood cells are completely lysed, and a clear zone can be seen around colonies. When there is no red blood cell lysis seen, it is called the γ-hemolysis. α- and β-hemolysis are presented in Figure 1.



Alpha hemolysis of *Streptococcus pneumoniae* (ATCC 6305)

Beta hemolysis of *Streptococcus dysgalactiae* (ATCC 12394)

Figure 1. Alpha and beta hemolysis seen on blood agar.

Streptococci can also be classified based on their biochemical reactions and differences in their cell wall, including antigenic differences. [3, 4.]

2.1 Streptococcus pyogenes

S. pyogenes, commonly known as GAS is a β-hemolytic gram-positive cocci. *S. pyogenes* is a common bacterial pathogen and also part of the human normal flora. Normally, it is not causing harm on healthy individuals, but for people with weaker defense system, it may lead to rapidly spreading illnesses. [3, 5.]

2.1.1 Symptoms and types of infection

Normally, GAS illnesses are harmless and classical symptoms could include common throat pain or skin rash and sometimes there are not any symptoms. GAS can also cause common infections such as scarlet fever, impetigo and erysipelas [3, 6]. GAS infections are also the most common cause of acute pharyngitis, which often occur among children between ages 5 and 15. If these infections caused by GAS are left untreated, they might

spread and result in more severe illnesses, for example, rheumatic fever, glomerulonephritis or reactive arthritis. For example, glomerulonephritis is found to be a post-infection resulting from previous GAS infection in throat or on skin [7]. In rare cases, GAS infections might lead to quickly spreading necrotizing fasciitis, which is also known as flesh-eating disease [8]. These sequelae occur one to three weeks after the acute GAS illness [3, 6].

2.1.2 Spread of infection and risk factors

S. pyogenes can cause illnesses in healthy people of any age who do not have immunity against that specific GAS serotype. However, the prevalence of GAS infection is higher in children and elderly because of their lower immunity. Normally, human defense mechanisms prevent organisms from invading the body. Infections caused by GAS commonly happen when the bacteria enters to the body, for example, from the upper respiratory tract or wounds on the skin. GAS mainly spreads through person-to-person transmission by fomites, respiratory secretions and skin contact. Also, contaminated surfaces and objects and dust particles play an important role in transmitting GAS. [3, 9.]

2.2 Diagnosis and GAS detection

2.2.1 Culturing

When a patient is suspected of *S. pyogenes* infection, a throat swab culture can be done to detect the bacteria. According to literature, culturing bacteria from clinical specimens is the most common method for diagnosing group A streptococcal infections [10, 11]. *S. pyogenes* is normally cultured on blood agar and an even more optimal choice would be a selective media for culturing gram-positive bacteria. Culturing bacteria on blood agar is an effective way to identify if the colonies are β-hemolytic. The colony morphology of *S. pyogenes* is typically round with moist or smooth surface. The colonies appear in white-grayish color and β-hemolytic zone around the colonies is clearly seen (Figure 2).

Figure 2. *S. pyogenes* cultured on blood agar. Beta hemolytic zone around colonies is clearly seen.

For most of the Streptococcal strains, optimal incubation conditions are when temperature is between 35 °C and 37 °C and carbon dioxide (CO_2) is 5 % and incubated overnight. [11, 12.]

Typically, *S. pyogenes* colonies appear after a 24-hour incubation in optimal conditions. Sometimes, only few typical GAS colonies appear during the incubation, and then it is more presumable that the patient is only a streptococcal carrier and not infected by GAS. It should also be considered that non-optimal conditions for incubation and imperfect specimen collection might lead to misinterpretations when examining the results. [11.]

2.2.2 Lancefield antigen test

When diagnosing GAS from clinical specimens the Lancefield antigen test should always be performed. With Lancefield antigen test, the β-hemolytic streptococci can be divided into different groups. The grouping is based on the presence of antigens on surface of streptococci. The presence of antigens can be tested using commercial kits, which are based on antigen extraction from the bacterial cell wall and then the antigen is identified based on agglutination. The group A antigen is found from *S. pyogenes*, but also from

other Streptococcal species, for example *Streptococcus anginosus*. Consequently, more identification tests must be performed for reliable species detection, for example, PYR test and bacitracin test. [11.]

2.2.3 DNA-based analysis methods

Identifying pathogens or other bacteria with DNA-based analysis methods require some sample preparation before starting the amplification protocol. Sample preparation could include, for example, DNA extraction. Extracted DNA can then be used as a template in the amplification.

DNA can be extracted using different commercial kits such as EZ1 DNA Tissue Kit (Qiagen), which is designed to be used with EZ1 Advanced XL -extraction robot (Qiagen). EZ1 DNA Tissue Kit is optimal for molecular biology applications and for purification of gDNA from tissue and other samples, for instance bacterial cultures. The kit contains all reagents for extraction and purification of the DNA from bacterial cultures. The purification procedure is based on magnetic-particle technology which ensures that the DNA is high-quality and can be directly used in other applications, for example in SIBA and PCR. Extraction is based on using silica-coated magnetic-particles to which the DNA binds. The cells are lysed with lysis buffer and silica-coated magnetic particles are added to reaction. Lysis buffer contains chaotropic salts, which are helping to create optimal conditions for the DNA to bind with silica. Chaotropic salts are destabilizing molecule bonds, which leads also to destabilization of nucleases. Nucleases bind with silica because chaotropic salts inhibit their association with water molecules. At the end of purification procedure, magnetic-particles are separated from DNA by using a magnet and the DNA is washed and eluted in elution buffer. [13: 4,7; 14.]

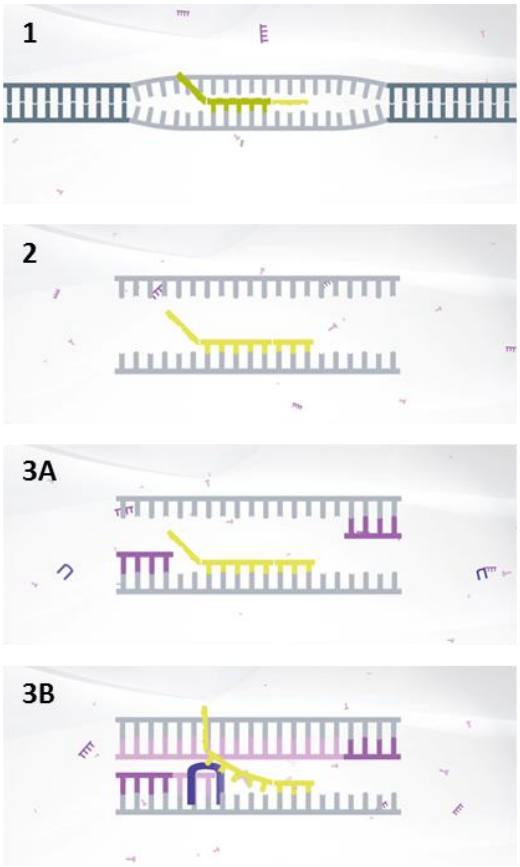
2.2.3.1 *PCR*

PCR is commonly used method for identifying pathogenic bacteria. It has been addressed that PCR targeting transcriptional regulator genes provide rapid and reliable results for detecting bacteria. Transcriptional regulators are DNA binding proteins that play an important role in adaptation and survival of bacteria in different conditions. For detecting *S. pyogenes*, a putative transcriptional regulator gene spy1258 can be used as a target because it is uniquely present in *S. pyogenes* genome. [15.]

2.2.3.2 *Strand Invasion Based Amplification (SIBA®)*

SIBA can also be used detecting different pathogens, for example *S. pyogenes*. SIBA technology is highly sensitive and can detect even single molecule of the target DNA. SIBA does not require target-specific probes. SIBA is an isothermal nucleic acid amplification method owned by Orion Diagnostica Oy and it was first described by Hoser M. et al. [16, 17.]

In isothermal processes the temperature remains constant. The SIBA technology relies on two primers and an invasion oligonucleotide (IO) and requires the presence of recombinase enzyme. In SIBA reaction single-stranded IO recognizes the complementary region of a target DNA and invades into the double-stranded DNA with the help of recombinase enzyme. As a result of this event, the double-stranded DNA dissociates into single-stranded DNAs and target specific amplification primers can bind into the single-stranded DNA. Then with DNA polymerase enzyme, two double-stranded DNAs are synthetized. At next, IO is attached to next target sequence and the cycle is repeated. As a result, the target DNA is exponentially amplified. This real-time progress of the SIBA reaction can be followed by using qPCR instruments and fluorescent dyes. Amplification cycle is presented in Figure 3. [17.]



1. Invasion.
Invasion oligonucleotide (yellow) penetrates double-stranded DNA target with the help of a recombinase enzyme.

2. Dissociation.
The invasion process dissociates the double-stranded DNA into single-stranded DNAs.

3. Amplification.
Specific amplification primers anneal single-stranded DNA (3A).
DNA polymerase synthesizes double-stranded DNAs (3B).

Figure 3. Steps of SIBA reaction. [18.]

In SIBA reaction the primers are not substrates for the recombinase, and for that reason, they are not able to extend the target DNA without the presence of the IO. Also, 2'-O-methyl RNA is included to the IO, which ensures that it cannot take part in the target DNA extension. Therefore, SIBA is resistant to unspecific amplification and all artifacts produced during the SIBA reaction, are not amplified that effective than in the target-dependent reactions such as PCR. [16, 17.]

The SIBA reaction includes recombinase and its cofactors, polymerase, nucleic acid components and also a system for producing ATP. As a recombinase, UvsX is used due to its fast turnover of ATP. Also, inorganic phosphate is produced by UvsX, and to avoid its inhibitory effect for the SIBA reaction, sucrose and sucrose phosphorylase are added too. The optimal concentrations of the recombinase and other proteins and components vary depending on the target analyte. [17.]

SIBA and other isothermal nucleic acid amplification methods offer great advantages over PCR. Firstly, isothermal amplification methods do not require thermal cycling and

the amplification can be done without large laboratory equipment. Secondly, SIBA is also a more rapid method for detecting pathogens because it takes approximately two hours to get results in PCR when in SIBA it takes less than an hour [10, 17]. In addition, SIBA can be used in multiplexed assays. There are at least two target-analytes which are tested simultaneously in the same reaction tube. Target-analytes are detected with specific probes that are dual-labeled with a reporter dye at 5' end and a quencher at the 3' end [19]. Using multiplexed tests reduces the sample processing time and costs. [16, 17.]

Freeze-drying

Lyophilization, also known as freeze-drying, is a method used for preserving biological materials such as proteins, microbes, vaccines and also diagnostic tests. Also commercial SIBA reaction kit includes freeze-dried (FD) reagents. Drying diagnostic tests using vacuum- or freeze-drying makes the products more stable in storage and also easier to use. Freeze-drying enables drying the product without heating in high temperatures. In the process water is removed from a product by first freezing it and then decreasing the surrounding pressure. In this primary drying the ice changes directly from solid to vapor (sublimation). The boundary water molecules are removed from the product in the next phase of freeze-drying which is known as secondary drying. In secondary drying, the product temperature is increased in order to help water desorption from the product. [20, 21.]

3 Materials and methods

3.1 Bacterial strains and cultivation

American Type Culture Collection's (ATCC) strains were used in this study. Strains are shown in Table 1.

Table 1. ATCC strains used in this study.

Species	Strain
S. pyogenes	Rosenbach, ATCC 19615
S. dysgalactiae	(Diemhofer) Garvie et al., ATCC 9926
S. dysgalactiae subsp. *equisimilis*	Vandamme et al. emend. Vieira et al., ATCC 12388
S. dysgalactiae subsp. *equisimilis*	Vandamme et al. emend. Vieira et al., ATCC 12394
S. agalactiae	Lehmann and Neumann, ATCC 13813
S. agalactiae	Lehmann and Neumann, ATCC 12386
S. agalactiae	Lehmann and Neumann, ATCC 27956
S. intermedius	Prevot emend. Whiley and Beighton, ATCC 27335
S. mutans	Clarke, ATCC 31377
S. pneumoniae	(Klein) Chester, ATCC 6305

Strains were cultured from frozen stocks on blood agar plates (Biomerieux) using sterile inoculating loops. *S. pyogenes* (ATCC 19615) was cultured on Columbia agar plate with 5 % horse blood (COH) and other Streptococci strains were cultured on trypcase soy agar plates with 5 % horse blood (TSH). The plates were incubated overnight (o/n) at 37 °C until isolated colonies appeared.

After cultivations, one bacterial colony from every plate was suspended into liquid microbial growth medium Luria broth (Sigma-Aldrich). Parts from the same bacterial colonies were also subcultured on new blood agar plates aiming to grow pure cultures. Plates and suspensions were incubated at 37 °C o/n and stored at 4 °C for later use. Subculturing of isolated bacterial colonies will make sure that all bacteria on new culture are genetically identical.

3.2 DNA templates and genome extraction

Genomic DNA (gDNA) of cultured streptococci was extracted by using EZ1 Advanced XL -extraction robot (Qiagen) with EZ1 Advanced Bacteria Card (Qiagen) and DNA Tissue Kit (Qiagen). The gDNA was extracted from broth cultures according to the manufacturer's protocol [13: 39]. Before extraction, overnight bacterial culture suspensions were centrifuged at 1000 x g for 4 minutes and supernatants were discarded. Bacterial pellets were washed with calcium and magnesium free phosphate-buffered saline (PBS). PBS and supernatants were discarded. Pellets were suspended to 5 ml of PBS. Before purification of the DNA, bacterial suspensions were prepared according manufacturer's instructions for gram-positive bacteria [13:40].

3.3 qPCR quantification

PCR method was set up aiming to quantify, i.e. to determine the copy number of extracted *S. pyogenes* gDNA. The PCR program was designed on the basis of work by Kodani M. et al. [22] and the program is presented in Figure 4.

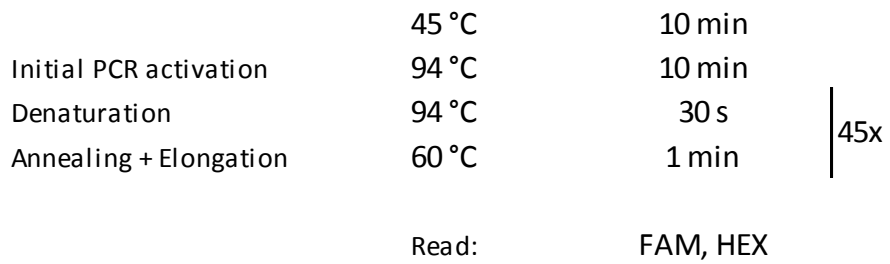


Figure 4. qPCR program used for quantification. The duration of each step and temperatures used are described here.

Primers and probes (Eurofins) used in this PCR are shown in Table 2. They are targeting *spy1258* gene, which encodes transcriptional regulator of *S. pyogenes* [6].

Table 2. Primers and probe used in qPCR quantification.

Oligonucleotide	**Sequence 5' → 3'**
CDC Strep forward	GCACTCGCTACTATTTCTTACCTCAA
CDC Strep reverse	GTCACAATGTCTTGGAAACCAGTAAT
CDC Strep probe	FAM-CCGCAACZCATCAAGGATTTCTGTTACCA

PCR quantification was done with two different Master Mixes: Express One-Step Superscript qRT-PCR Universal kit (Thermo Fisher Scientific) and iTaq Universal Probes Supermix (Bio-Rad Laboratories). PCR reaction was done in total volume of 20 µl, which contained 10 µl of kit's ready-to-use Master Mix and concentration for both CDC Strep primers was 0.4 µM per reaction. The CDC Strep probe concentration was 0.2 µM per reaction, and 2 µl of DNA template was used per reaction. To get the reaction volume to 20 µl, correct amount of nuclease-free water was added. qPCR quantification was performed by using CFX96 Real-Time PCR detection instrument (Bio-Rad Laboratories).

Synthetic double stranded GAS CDC DNA (dsDNA, Centers for disease control and prevention) was used for quantification of extracted *S. pyogenes* gDNA. Synthetic DNA was

ordered from Thermo Fisher Scientific, and the quantity and the sequence length were known. The DNA copy number was calculated using Formula 1.

$$\text{number of copies} = \frac{quantity \times 6{,}022{*}10^{23}}{length \times 10^{9} \times 650} \quad (1)$$

In qPCR quantifications the synthetic GAS DNA was used as a standard and the used copy numbers per microliter (cp/µl) were 10^6, 10^5, 10^4, 10^3, 10^2, 10 and 1. Two parallel reactions of every standard concentration were made. The extracted DNA of *S. pyogenes* was diluted with nuclease-free water 1:10, 1:100, 1:1000, $1{:}10^4$, $1{:}10^5$, $1{:}10^6$ and $1{:}10^7$. Also extracted DNA stock was used. Four parallel reactions were made of each *S. pyogenes* DNA dilution.

3.4 SIBA reaction conditions

SIBA reactions were performed at 20 µl reaction volume. SIBA reaction is isothermal and the reaction temperature is remaining the same through the whole amplification, at 40 °C for at least 60 min. SIBA reactions were performed using commercial SIBA reagent kit (Orion Diagnostica Oy) and $1{:}10^5$ dilution Sybr Green I (Thermo Fisher Scientific). The enzymes used, unless otherwise mentioned, were 250 ng/µl UvsX and gp32 of each. The concentration of IO was 175 nM and concentration of primers was 250 nM in this study. The reactions were started by adding a correct amount of target DNA (prepared in 10 mM magnesium acetate (MgAc)) or only 10 mM MgAc. SIBA reactions were performed by using Agilent MX3005P qPCR instrument (Agilent Genomics) and Agilent AriaMX Real-Time PCR instrument (Agilent Genomics). SIBA amplification was detected with Sybr Green (FAM-channel) and with cyanine 5 (Cy5-channel).

3.4.1 Probe screening

The assay used in this thesis was earlier designed in Orion Diagnostica, and the aim was to develop the assay further. The assay's forward (F) and reverse (R) primers and IO have also been screened for self-priming and unspecific interactions between oligonucleotides in SIBA reaction conditions. All oligonucleotides in this assay were ordered from Integrated DNA Technologies (IDT) or Eurofins, and they are shown in Table 3.

Table 3. Assay's F and R primers and IO.

Primers and IO
F6
R2
F7
R5
IO

In this thesis, three different locked nucleic acid (LNA) probes were screened for the assay. LNA probes are dual-labeled DNA probes that are more sensitive and have better target specificity than DNA and RNA probes [23]. The aim was to find one LNA probe with good amplification speed and with clean and specific amplification signals. All three LNA probes (Table 4) are labeled with cyanine 5 (Cy5) fluorophore and with specific quencher.

Table 4. LNA probes tested in this thesis.

LNA probes
Cy5_1
Cy5_2
Cy5_3

All three LNA probes were tested with one F and R primer combination, F7/R5. Based on the results, the best performing LNA probe Cy5_3 was then tested also with another primer combination F6/R2 to determine if there are any differences in LNA probe performance with different primer combinations. Extracted *S. pyogenes* gDNA was used as template and experiments were done using 1000 copies and 100 copies of DNA per reaction. Also reactions without DNA template and reactions without LNA probe were tested. Four parallel reactions were done per each different reaction.

3.4.2 Oligonucleotide titration

The reason for oligonucleotide titration was to get the GAS assay faster and more specific, thus LNA probe and primer concentrations were titrated. In previous experiments used concentrations of R and F primers were 250 nM and concentration of LNA probe Cy5_3 was also 250 nM per reaction. IO concentration was 175 nM per reaction. In the

oligonucleotide titration, the concentrations of F primer and IO remained the same as earlier and only R primer and LNA probe concentrations were titrated. R primer concentrations were titrated between 100 nM and 400 nM and LNA probe concentrations between 100 nM and 250 nM. All combinations tested are presented in Table 5.

Table 5. Tested R primer and LNA probe concentrations (nM). IO concentration was 175 nM and F primer concentration was 250 nM in every reaction.

R primer	LNA probe	R primer	LNA probe
100	100	250	100
100	150	250	150
100	175	250	175
100	250	250	250
150	100	300	100
150	150	300	150
150	175	300	175
150	250	300	250
175	100	400	100
175	150	400	150
175	175	400	175
175	250	400	250

Oligonucleotide titrations were performed using only one F and R primer combination. The combination F7/R5 was chosen to be used in the oligonucleotide titration because of its good performance in previous experiments.

The used *S. pyogenes* gDNA copy number was 10 000 copies per reaction and four parallel reactions were done of each different R primer and LNA probe concentration. All R primer and LNA probe concentrations were also tested without DNA template, and template volume was replaced with correct amount of 10 mM MgAc per reaction.

3.4.3 Gp32 and UvsX titrations

Assay optimization was continued by titrating concentrations of gp32 and UvsX proteins. Concentrations for R primer and LNA probe were chosen based on previous experiment's results, and they are 100 nM for R primer and 250 nM for LNA probe. The IO and F primer concentrations remained the same as in the previous experiment. UvsX and

gp32 concentrations were titrated between 150 ng/µl and 400 ng/µl per reaction by changing both concentrations equally to the same direction. It was also tested to change both concentrations at the same time but in different directions. The latter one was done with concentrations 150 – 300 ng/µl of UvsX and 200 – 300 ng/µl of gp32. All combinations tested are shown in Table 6.

Table 6. UvsX and gp32 combinations tested. Concentrations are shown in ng/µl per reaction.

UvsX	Gp32	UvsX	Gp32	UvsX	Gp32
150	150	200	250	300	200
150	200	200	300	300	250
150	250	250	200	300	300
150	300	250	250	350	350
200	200	250	300	400	400

S. pyogenes gDNA was used as template in all gp32 and UvsX titrations. The used DNA copy number per reaction was 10 000. Four parallel reactions were done of each different reaction. All protein concentration combinations were also tested without DNA template and template volume was replaced with correct amount of 10 mM MgAc per reaction.

3.4.4 Specificity

Specificity of the assay was determined by using gDNAs of ten different *Streptococcus* strains that are presented in Table 1. Strains were cultured on blood agar plates and the gDNAs were extracted from cultures using EZ1 Advanced XL -extraction robot. The nucleic acid concentration of each eluate was measured with NanoDrop spectrophotometer (Thermo Scientific) by taking three parallel measurements per eluate and calculating average concentrations for each eluate.

Extracted DNA stocks from all used *Streptococcus* strains were diluted to equal concentrations, 5 ng/µl. Also, a GAS template with known copy number of 100 000 copies was used as a positive control. Two parallel reactions were done of each template with all different primer combinations.

The assay specificity was tested with three different R and F primer combinations which are presented in Table 7.

Table 7. F and R primer combinations used in specificity tests.

Primer combination	Primers
1	F7 / R5
2	F6 / R9
3	F6 / R10

On the basis of the results from probe screening, LNA probe Cy5_3 was used in all specificity tests and the concentration was 250 nM.

3.4.5 Sensitivity

The assay sensitivity was tested using three different F and R primer combinations to see which combination is performing best and has the highest sensitivity of detecting *S. pyogenes*. Primer combinations are the same as used in specificity tests, and they are presented in Table 7. LNA probe Cy5_3 was used in the assay's sensitivity tests and the concentration was 250 nM per reaction. The extracted *S. pyogenes* DNA was used as a template in this experiment. The template was diluted with nuclease-free water and the dilutions were based on the results from qPCR quantification. In this experiment, the template copy numbers used were 100 000, 10 000, 1000, 100, 10 and 1 copies per reaction. Two parallel reactions of every DNA concentration were done and with all of the primer combinations.

3.4.6 Freeze-drying

Effects of freeze-drying were tested on the reagents of the developed assay. Freeze-drying was performed using Julabo-dryer and Lyovac GT2 freeze-dryer (Steris). Oligo-nucleotides and their concentrations used in the freeze-drying are presented in Table 8.

Table 8. Five different Master Mixes were lyophilized. Oligonucleotide concentrations of each Master Mix are presented in nM per reaction.

	Concentration			
	F	**R**	**IO**	**LNA probe**
Master mix 1 F7/R5	250	100	175	250
Master mix 2 F7/R5	150	150	175	250
Master mix 3 F7/R5	200	200	175	250
Master mix 4 F6/R2	200	200	175	250
Master mix 5 F6/R2	250	250	175	250

Before lyophilization, the Master Mix was prepared and pipetted on 96-well plate, 70 µl per well. Plate was then centrifuged until all air bubbles were removed. Then, the plate was placed in pre-cooled Julabo-dryer. After two hours of pre-cooling the plate, the drying program was started.

After lyophilization FD reagents were tested with *S. pyogenes* extracted gDNA. The Master Mix 1 FD reagents were tested using only one reaction buffer (later Buffer 1). FD reagents with Master Mixes from 2 to 5 were tested using two different reaction buffers (later Buffer 1 and Buffer 2). The reaction volume with lyophilized SIBA reagents was 41 µl which contains 40 µl of reaction buffer and 1 µl of DNA template. FD reagents were also tested without DNA template and the template volume was replaced with correct amount of nuclease-free water. The used DNA copy number was 100 000 cp and 10 000 cp with Master Mix 1 FD reagents and 100 000 cp with FD reagents with Master Mixes from 2 to 5. Three replicates were done of each reaction with template and two replicates of reactions without template.

4 Results

In results analysis for all SIBA reactions, the detection times and signal levels were checked with both Cy5 and Sybr green in reactions with and without DNA template. Sybr Green I detection was measured in FAM-channel. On the basis of the signal levels and detection times, it was determined which reaction conditions were the most optimal and chosen further to next experiments.

4.1 qPCR quantification

All raw data from qPCR quantifications is presented in Appendices 1 and 2. The abbreviation *RFU* in qPCR amplification figures means *Relative fluorescence unit*.

4.1.1 qPCR quantification using Thermo Fisher Scientific's Master Mix

Synthetic *S. pyogenes* DNA was used as a standard. Since the copy number of that synthetic double stranded GAS CDC template DNA was known, the standard curve calculation was based on that. Sensitivity of this PCR was 200 cp per reaction because replicates with less copies of standard DNA did not amplify. All replicates of extracted *S. pyogenes* DNA stock and dilutions 1:10, 1:100 and 1:1000 amplified. Three out of four replicates of dilutions $1:10^4$ and $1:10^5$ amplified also. None of the more diluted templates amplified. Amplification of standards and extracted *S. pyogenes* DNA are presented in Figure 5.

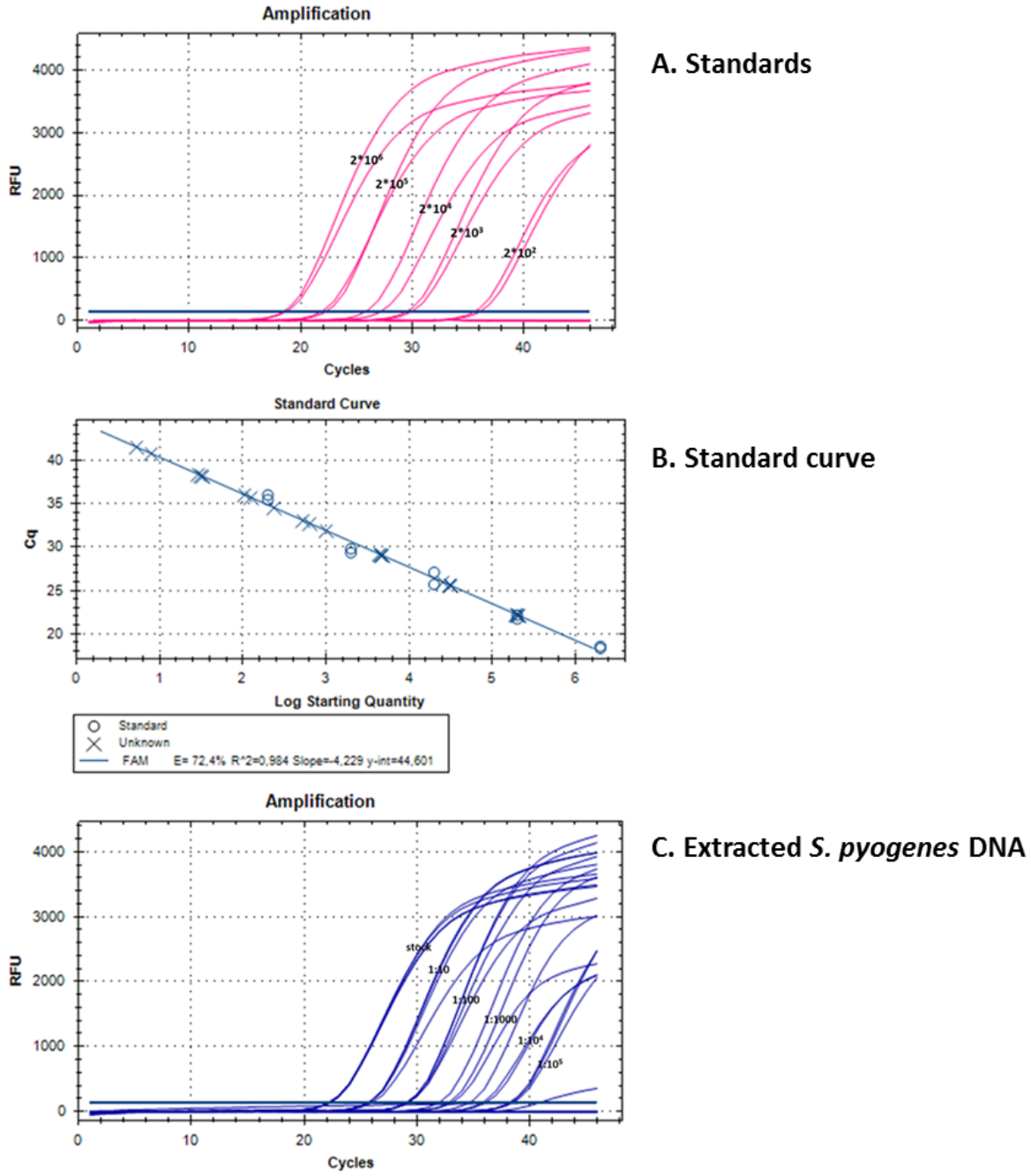


Figure 5. A. Amplification of standards (GAS CDC template). Two replicates of each concentration. B. Standard curve. C. Amplification of extracted *S. pyogenes* DNA. Non-diluted DNA is marked as *stock* in figure.

The copy number of extracted DNA was $3.92*10^5$ cp/µl. Concentration was calculated based on samples from stock to dilution 1:1000 since all their replicates amplified. Standard curve of this quantification is shown in Figure 5. None of the reactions without DNA template amplified.

4.1.2 qPCR quantification using Bio-Rad Laboratories' Master Mix

As it was in the other quantification, GAS CDC template DNA was used as a standard also in this experiment. The protocol in this quantification was similar with the first one. The quantification was done with Bio-Rad Laboratories' kit to determine if there are any differences on results.

This PCR was not as sensitive as the other one, since standards amplified only from $2*10^6$ to 2000 copies per reaction. All replicates of extracted *S. pyogenes* DNA stock and dilutions 1:10, 1:100 and 1:1000 amplified. Three replicates of dilution $1:10^4$ and two replicates of dilution $1:10^5$ amplified also. None of the reactions without DNA amplified. Amplification of standards and extracted DNA is presented in Figure 6.

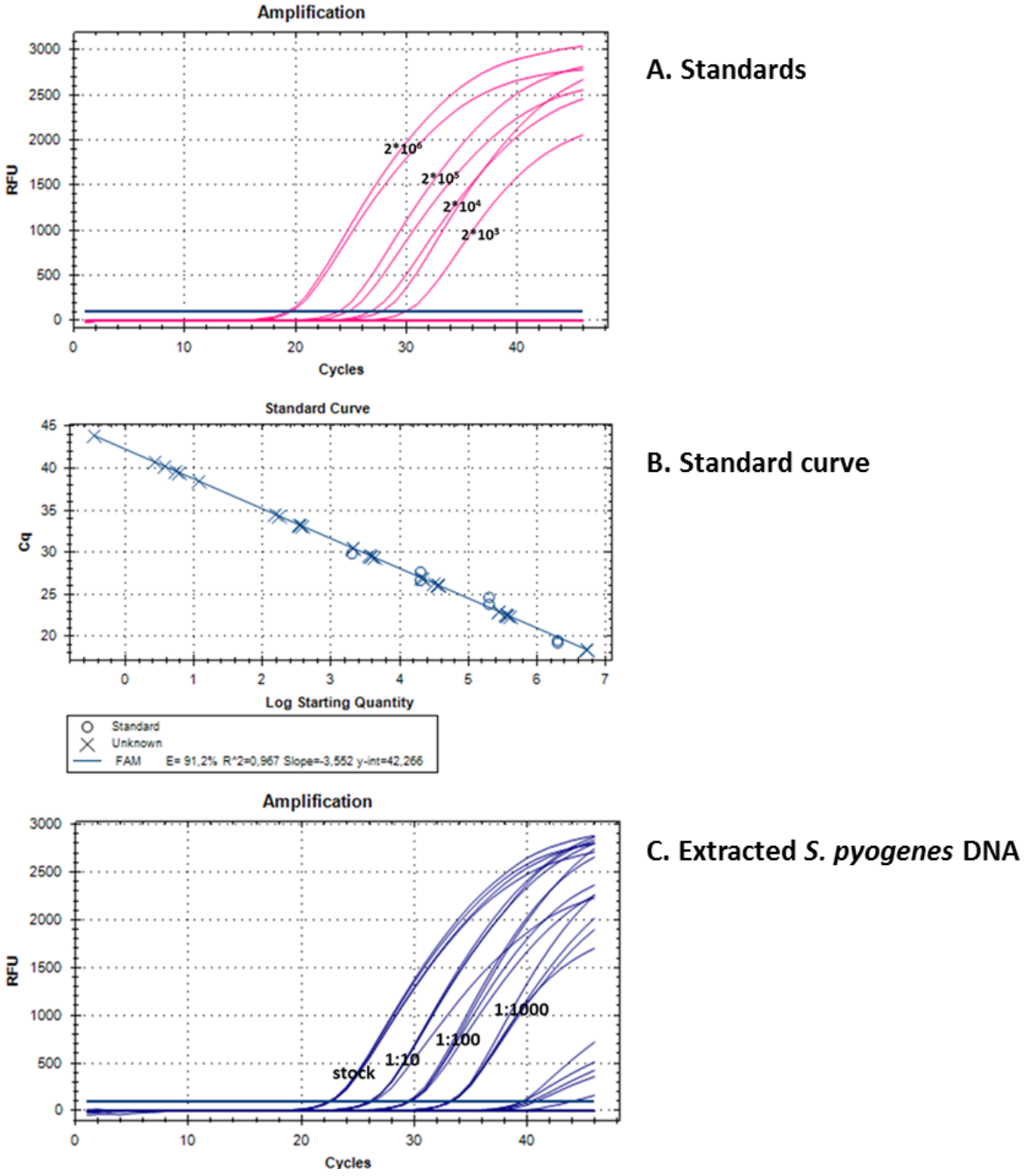


Figure 6. A. Amplification of standards (GAS CDC template). Two replicates of each concentration. B. Standard curve. C. Amplification of extracted *S. pyogenes* DNA. Non-diluted DNA is marked as *stock* in figure.

Based on this qPCR quantification, the copy number of extracted DNA was $3.72*10^5$ cp/µl which is almost the same as the other qPCR quantification revealed. Concentration was calculated based on samples from stock to dilution 1:1000 since all their replicates amplified. Standard curve of the quantification is shown in Figure 6.

Both PCR kits performed equally in quantification tests as the results in DNA copy numbers were almost the same, $4*10^5$ cp/µl. However, the quantification done with Thermo Fisher Scientific's kit was slightly more sensitive than the other one.

4.2 Probe screening

All probe screening experiments were performed using concentration 250 nM per reaction for F and R primers and for the screened LNA probes.

4.2.1 LNA probes with primer combination F7/R5

Three different LNA probes were tested with the primer combination F7/R5. Extracted *S. pyogenes* gDNA was used as a template in copy numbers of 1000 and 100 per reaction. With all three LNA probes, only 1000 cp/reaction amplified and was detected with fluorophore Cy5. Reactions with 100 copies of DNA were not detected with Cy5, except for one replicate with LNA probe Cy5_2. Some amplification was also detected with another fluorophore Sybr green in reactions with template and also without template. Experiment data including cycle threshold values (ct-values) is presented in Table 9.

Table 9. Raw data from the probe screening with primer combination F7/R5. Ct-values are presented in minutes. Extracted DNA was used as a template.

		Dye Cy5, Threshold 521.1		Dye FAM (Sybr), Threshold 393.6	
LNA probe	**DNA copy number**	**Ct (dR)**	**Average Ct**	**Ct (dR)**	**Average Ct**
Cy5_1	1000	No Ct	27.0	52.95	30.8
		15.45		13.86	
		29.37		24.88	
		36.26		31.37	
	100	No Ct	0.0	10.83	10.8
		No Ct		No Ct	
		No Ct		No Ct	
		No Ct		No Ct	
Cy5_2	1000	36.44	36.3	33.84	38.2
		36.63		35.62	
		No Ct		49.26	
		35.92		33.9	
	100	18.44	18.4	16.95	39.6
		No Ct		50.39	
		No Ct		35.99	
		No Ct		54.88	
Cy5_3	1000	19.66	18.2	17.33	16.5
		16.79		15.36	
		20.33		18.69	
		16.08		14.58	
	100	No Ct	0.0	20.47	19.0
		No Ct		20.21	
		No Ct		19.85	
		No Ct		15.43	

Amplification in reactions with LNA probe Cy5_1 were detected with Cy5, on average, in 27 minutes and reactions with LNA probe Cy5_2 were detected, on average, in 36 minutes. In the same reactions, Sybr green signal was detected, on average, in 31 minutes with LNA probe Cy5_1 and, on average, in 38 minutes with LNA probe Cy5_2. Amplification curves are shown in Figures 7 and 8.

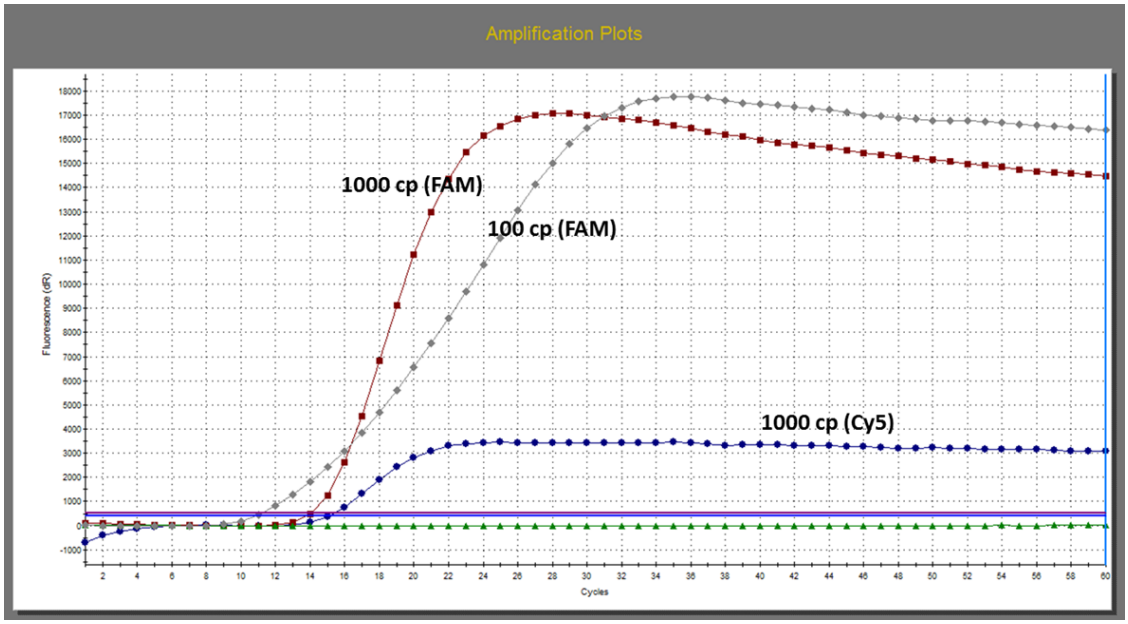


Figure 7. Amplification curves of single replicates of reactions with LNA probe Cy5_1.

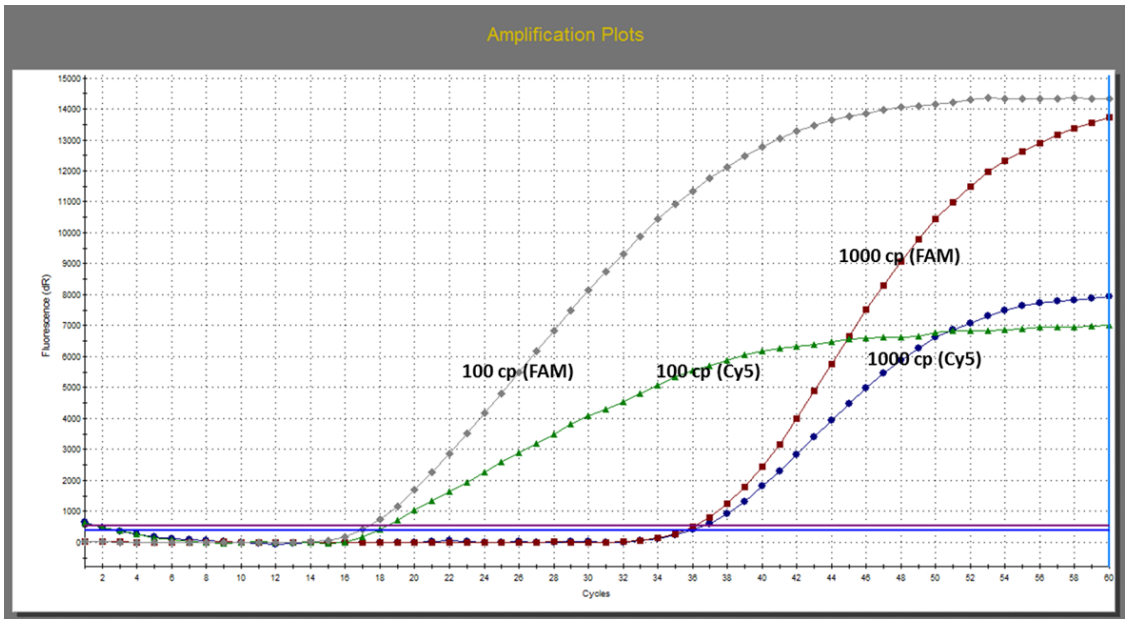


Figure 8. Amplification curves of single replicates of reactions with LNA probe Cy5_2.

Amplification in reactions with LNA probe Cy5_3 were detected with Cy5, on average, in 18 minutes and Sybr green signal was detected, on average, in 17 minutes. Thus, with LNA probe Cy5_3 the amplification was faster (with both Cy5 and Sybr green) when compared with other LNA probes. Cy5 and Sybr green signal levels are also slightly higher with LNA probe Cy5_3. Amplification of reactions with LNA probe Cy5_3 is shown in Figure 9.

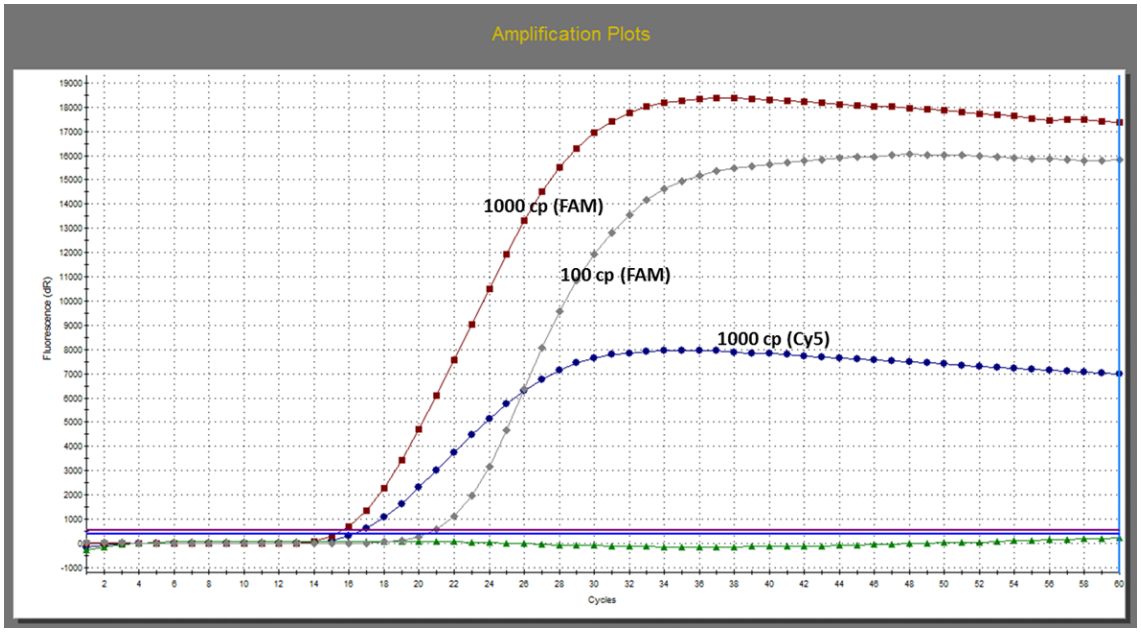


Figure 9. Amplification curves of single replicates of reactions with LNA probe Cy5_3.

On the basis of LNA probe screening results, LNA probe Cy5_3 was performing best and was taken further to next experiments done in this thesis. In reactions with other two LNA probes, Cy5_1 and Cy5_2, the Cy5 detection times were significantly slower and for that reason they were discarded.

4.2.2 LNA probe Cy5_3 with primer combinations F7/R5 and F6/R2

In previous experiments with LNA probe Cy5_3, the Cy5 detection times were slightly slow, and, for that reason, new batches of the F and R primers were also tested. In addition, it was also tested one more primer combination, F6/R2, to see if there are any differences in detection times.

Extracted DNA was used as a template in copy numbers of 1000 and 100 per reaction. With primers F7/R5, three of four replicates with 1000 cp DNA amplified and the average Cy5 detection time was 16 minutes. With the same primers and 100 cp DNA, all replicates were detected (Cy5), on average, in 38 minutes. Sybr green signal of these reactions was also detected, on average, in 17 minutes with 1000 cp DNA and in 38 minutes with 100 cp DNA. Amplification of reactions with primers F7/R5 are shown in Figure 10.

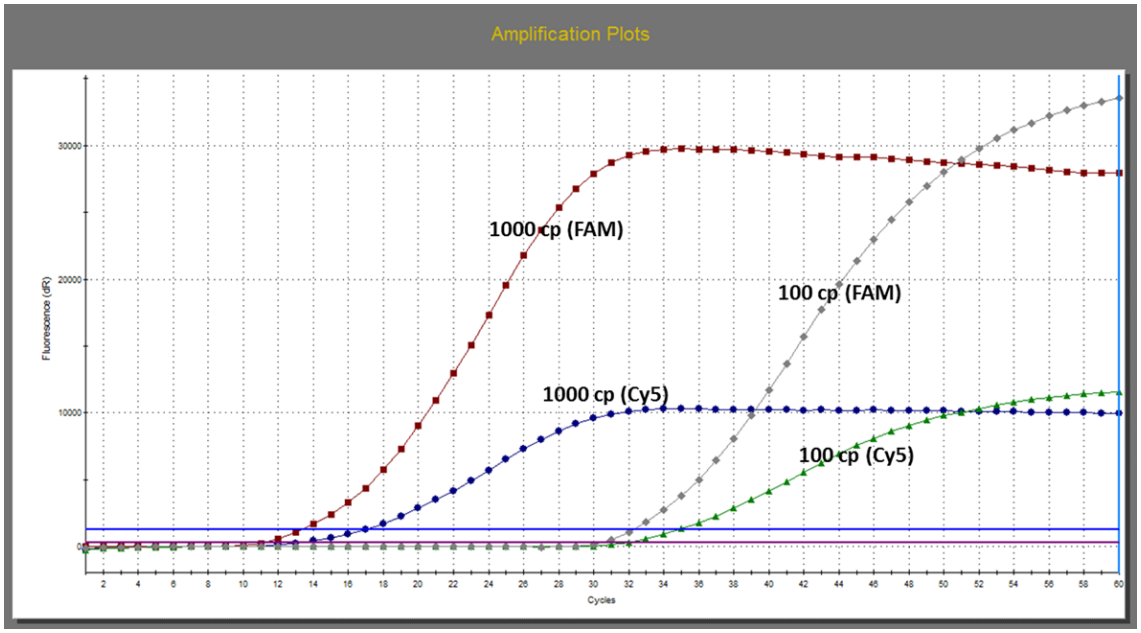


Figure 10. Amplification of single replicates of reactions with primer combination F7/R5 and LNA probe Cy5_3.

With primer combination F6/R2, all replicates with both DNA copy numbers were detected with Cy5. 1000 cp reactions were detected, on average, in 19 minutes and 100 cp reactions, on average, in 26 minutes. Sybr green detection occurs at the same time with Cy5 detection. Amplification of reactions with primers F6/R2 are presented in Figure 11.

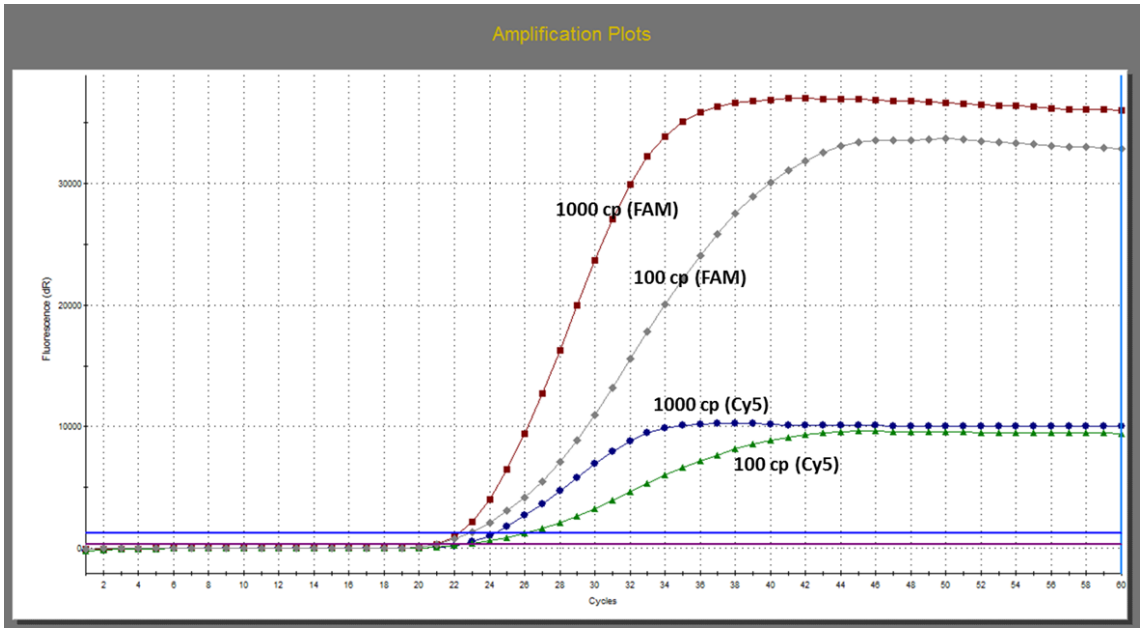


Figure 11. Amplification of single replicates of reactions with primer combination F6/R2 and LNA probe Cy5_3.

Ct-values and other data from this experiment are shown in Table 10.

Table 10. Raw data from the LNA probe Cy5_3 tests with primer combinations F7/R5 and F6/R2. Ct-values are presented in minutes. Extracted DNA was used as a template.

		Dye Cy5, Threshold 291.6		Dye FAM (Sybr), Threshold 1266.9	
Primer combination	**DNA copy number**	**Ct (dR)**	**Average Ct**	**Ct (dR)**	**Average Ct**
F7/R5	1000	13.96	16.3	14.66	16.7
		No Ct		No Ct	
		13.29		13.43	
		21.78		21.99	
	100	39.98	37.5	41.01	38.1
		40.49		40.83	
		37.64		38.17	
		31.9		32.37	
F6/R2	1000	22.4	19.3	22.38	19.3
		21.76		21.85	
		18.21		18.07	
		14.73		14.8	
	100	31.86	25.7	31.97	26.0
		35.14		35.5	
		22.5		22.88	
		13.2		13.49	

4.3 Titration of oligonucleotides

R primer and LNA probe concentrations were titrated to get the assay more specific and faster. There were 24 different concentration combinations that were tested and detection times were measured with both fluorophores, Cy5 and Sybr Green. The average detection times of four replicates are presented in Table 11.

Table 11. Average detection times in different R primer and LNA probe concentrations. Detection times are presented in minutes for both fluors.

R primer (nM)	LNA probe (nM)	Detection time (Cy5)	Detection time (FAM)
100	100	16.2	18.7
100	150	12.8	15.2
100	175	15.4	18.8
100	250	12.4	14.8
150	100	13.2	14.1
150	150	13.7	16.0
150	175	11.8	14.5
150	250	13.4	16.5
175	100	11.8	12.4
175	150	12.4	13.6
175	175	11.5	13.3
175	250	13.6	15.9
250	100	11.3	12.0
250	150	11.9	13.1
250	175	11.8	13.2
250	250	11.7	13.5
300	100	10.8	11.5
300	150	10.8	12.0
300	175	11.7	10.1
300	250	10.5	12.2
400	100	12.7	12.6
400	150	11.1	11.9
400	175	12.0	12.8
400	250	12.6	13.7

In all reactions the detection times with Cy5 were between 10.5 and 16.2 minutes. Sybr Green detection times were between 10.1 and 18.8 minutes. When checking the signal levels with both fluorophores, the combination of 100 nM R primer and 250 nM LNA probe was performing the best and was chosen further to be tested in next experiments.

4.4 Titration of enzymes

In the first enzyme titration, both enzymes were tested in same concentrations per reaction. On the basis of those results, the fastest Cy5 detection was in reactions where UvsX and gp32 concentrations were 200 ng/µl and 250 ng/µl. The detection time with the concentration of 200 ng/µl for both enzymes was, on average, 17.8 minutes, and with the concentration of 250 ng/µl for both enzymes was, on average, 17.7 minutes. Also, with the concentration of 150 ng/µl for both enzymes, it was seen that the Sybr green signal detection was the slowest, on average, 19 minutes.

In the second enzyme titration, enzymes in different concentrations were tested, a total of 12 different combinations. The fastest Cy5 detection times were reached with the concentrations of 200 ng/µl and 300 ng/µl of both enzymes per reaction. Those detection times were, on average, 15 minutes. In reactions without DNA template, it was also seen some amplification, but after 19 minutes. On the basis of signal levels and detection times, the combination of 300 ng/µl of both enzymes was chosen for the assay optimization.

4.5 Assay specificity and sensitivity

Assay specificity was tested with three different primer combinations. For the specificity tests, gDNAs were used as a template, and they were extracted from ten different *Streptococcus* strains and the strains are presented in Table 1. Average nucleic acid concentrations of extracted gDNAs are shown in Appendix 2. All primer combinations performed equally, and only *S. pyogenes* amplification was detected. Reactions with other Streptococcal DNA were not detected with Cy5, and no-template-control reactions were neither detected with Cy5. The best signal levels and fastest detection times were seen with F6/R9 with detection in 25 minutes and with F6/R10 with detection in 17 minutes.

Also assay sensitivity was tested with three different primer combinations. Extracted *S. pyogenes* gDNA was used as a template. The template copy numbers used were 100 000, 10 000, 1000, 100, 10 and 1 copies per reaction. Primer combination F7/R5 was not working at all and none of the reactions with F7/R5 were detected. The second tested primer combination F6/R9 performed better and detection was seen with all replicates with 100 000 cp of DNA and with half of the replicates in reactions with 10 000 cp,

1000 cp and 100 cp of DNA. 100 000 cp was detected within 27 minutes and 100 cp was detected within 53 minutes.

The third primer combination was the most sensitive of all three. With this F6/R10 combination, all replicates with 100 000 cp, 10 000 cp, 1000 cp and 100 cp were detected. Also, one replicate with 1 cp DNA was detected. Compared to other two combinations, the detection times with F6/R10 were also faster with this primer combination. 100 000 cp DNA was detected with Cy5 within 17 minutes and 1 cp within 41 minutes.

In sensitivity tests, it was also seen amplification in reactions without DNA template with every primer combination. Detection times with Sybr green were 18 minutes with F7/R5, 20 minutes with F6/R9 and 17 minutes with F6/R10.

4.6 Template test with freeze-dried (FD) reagents

FD reagents with Master Mix 1 were tested with DNA template using one reaction buffer. 100 000 cp DNA was detected with Cy5 average in 10.5 minutes and with Sybr green, on average, in 10 minutes. 10 000 cp DNA was detected with Cy5 in 12.5 minutes and with Sybr green in 7.5 minutes. Also, in reactions without DNA template, the Sybr green detection times were about 7.5 minutes. Since the Sybr green detection times were that fast, the freeze-drying was done again using different Master Mixes.

FD reagents with Master Mixes from 2 to 5 were tested using two different reaction buffers and 100 000 cp of DNA. Cy5 detection with Master Mix 2 (F7/R5 150 nM) FD reagents was in 14.5 minutes with Buffer 1 and in 10.5 minutes with Buffer 2. Sybr green was detected in 14 minutes with Buffer 1 and in 10.5 minutes with Buffer 2. In reactions without template, Sybr green was detected in 18 minutes with both of the buffers.

With Master Mix 3 FD reagents (F7/R5 200 nM) the Cy5 detection was in 12.5 minutes with Buffer 1 and in 8.5 minutes with Buffer 2. Sybr green signal was detected in 11.5 minutes with Buffer 1 and in 8.5 minutes with Buffer 2. Amplification in no-template reactions was seen with Sybr green in 22 minutes with Buffer 1 and in 18 minutes with Buffer 2.

Master Mixes 4 and 5 contained primers F6/R2 in concentrations 200 nM and 250 nM per reaction. All replicates except for one were amplifying without DNA template in reaction and were also detected with both Cy5 and Sybr green fluorophores. Due to that unspecific amplification, these FD reagents with Master Mixes 4 and 5 were discarded.

5 Discussion and conclusions

This thesis aimed to optimize the existing, previously designed assay for detecting *S. pyogenes*. F and R primers and also IO were earlier screened for interactions between oligonucleotides and self-priming in SIBA reaction conditions. The assay was further optimized by titrating oligonucleotides and enzymes, testing different LNA probes and also sensitivity and specificity were tested. After optimizing the assay, the effects of freeze-drying were tested on the assay that was currently performing best.

In LNA probe screening, it was found that LNA probe Cy5_3 performs best. When testing that LNA probe with two primer combinations, F7/R5 and F6/R2, it was found that the LNA probe performs equally with both primer combinations. There was also some unspecific amplification seen with both primer combinations, which was detected with Sybr green signals. If these results are only analyzed by examining detection times, better primer combination would be F7/R5 since the detection times were slightly faster.

On the basis of the previous results, primer combination F7/R5 was also used in oligonucleotide and enzyme titrations. In oligonucleotide titrations, it was seen that there were many possible concentrations that were performing well. The best concentration, 100 nM R primer and 250 nM LNA probe, was chosen based on good Cy5 signal level and consistency between replicate results. Also, the results of reactions with 150 nM R primer and 250 nM LNA probe seemed fine.

In enzyme titration, it was found some properly performing concentrations of UvsX and gp32. The detection times were fastest with concentrations 200 ng/µl and 300 ng/µl and the latter was chosen to next experiments of this study. There was also seen some unspecific amplification detected with Sybr green in all reactions and obviously that unspecific amplification was not dependent of the enzyme concentration.

When specificity of the assay was tested, it was found that all three primer combinations were specific and the LNA probe signal (Cy5) was detected only in reactions with *S. pyogenes* DNA. However, the fastest detection times and best signal levels were seen with primer combination F6/R10. Nevertheless, all three primer combinations were used in sensitivity tests to determine possible differences between them. For some reason, one combination, F7/R5, was not working at all in sensitivity experiments. It is possible that the primer stocks used were frozen and melted too many times and for that reason they did not work properly. Two other primer combinations were almost equal in sensitivity testing. It was found that F6/R10 combination was slightly more sensitive and even 1 cp of DNA was detected in one replicate.

The GAS assay was lyophilized using five different Master Mixes including two primer combinations in variable concentrations. When the other primer combination, F6/R2, was tested with two different reaction buffers, it was noticed that there was unspecific amplification in almost all reactions without DNA template, and for that reason FD reagents containing these primers were discarded. The other primer combination, F7/R5, was performing better in FD reagent testing though there was some amplification detected with Sybr green too. Primer combination F7/R5 was performing well in FD reagents with both tested reaction buffers.

The priority purpose of this thesis was to develop and optimize an assay for detecting GAS. The original plan was to test the assay performance also with clinical specimens, but due to the schedule, that part was excluded from the experimental part of this thesis. The developed assay detected GAS properly when extracted DNA was used as a template. In addition, this particular assay was performing decently in sensitivity test and even one molecule was detected. Also, the best performing LNA probe is specific and binds only with its target DNA. However, some unspecific amplification was seen during the assay development. That unspecific amplification result in non-productive consumption of assay reagents and that way can reduce the sensitivity. The assay optimization should be continued with oligonucleotide screening because it could be possible to find proper oligonucleotide combination, which would cause less unspecific amplification. After finding proper combination of oligonucleotides, other reaction components could be optimized, and the assay could be even faster.

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Appendix 1. Data of qPCR quantifications

Sample	Well	Fluor	Content	Cq	Cq Mean	Cq Std. Dev	Starting Quantity (SQ)	Log Starting Quantity	SQ Mean	SQ Std. Dev
stock GAS	A01	FAM	Unkn-1	22,11	22,17	5,80E-02	2,08E+05	5,32E+00	2,02E+05	6,39E+03
	B01	FAM	Unkn-1	22,13	22,17	5,80E-02	2,06E+05	5,31E+00	2,02E+05	6,39E+03
	C01	FAM	Unkn-1	22,2	22,17	5,80E-02	1,98E+05	5,30E+00	2,02E+05	6,39E+03
	D01	FAM	Unkn-1	22,23	22,17	5,80E-02	1,95E+05	5,29E+00	2,02E+05	6,39E+03
1:10 GAS	A02	FAM	Unkn-2	25,95	25,68	1,85E-01	2,56E+04	4,41E+00	2,99E+04	2,87E+03
	B02	FAM	Unkn-2	25,61	25,68	1,85E-01	3,09E+04	4,49E+00	2,99E+04	2,87E+03
	C02	FAM	Unkn-2	25,56	25,68	1,85E-01	3,18E+04	4,50E+00	2,99E+04	2,87E+03
	D02	FAM	Unkn-2	25,59	25,68	1,85E-01	3,13E+04	4,50E+00	2,99E+04	2,87E+03
1:100 GAS	A03	FAM	Unkn-3	29,11	29,1	6,30E-02	4,59E+03	3,66E+00	4,63E+03	1,58E+02
	B03	FAM	Unkn-3	29,18	29,1	6,30E-02	4,42E+03	3,65E+00	4,63E+03	1,58E+02
	C03	FAM	Unkn-3	29,06	29,1	6,30E-02	4,73E+03	3,68E+00	4,63E+03	1,58E+02
	D03	FAM	Unkn-3	29,04	29,1	6,30E-02	4,77E+03	3,68E+00	4,63E+03	1,58E+02
1:1000 GAS	A04	FAM	Unkn-4	33,08	33,06	1,11E+00	5,30E+02	2,73E+00	6,05E+02	3,19E+02
	B04	FAM	Unkn-4	32,72	33,06	1,11E+00	6,43E+02	2,81E+00	6,05E+02	3,19E+02
	C04	FAM	Unkn-4	31,9	33,06	1,11E+00	1,01E+03	3,00E+00	6,05E+02	3,19E+02
	D04	FAM	Unkn-4	34,55	33,06	1,11E+00	2,38E+02	2,38E+00	6,05E+02	3,19E+02
1:10 000 GAS	E01	FAM	Unkn-5	N/A	0	0,00E+00	N/A	N/A	0,00E+00	0,00E+00
	F01	FAM	Unkn-5	36,04	37,52	2,84E+00	1,06E+02	2,02E+00	7,97E+01	6,30E+01
	G01	FAM	Unkn-5	35,72	37,52	2,84E+00	1,26E+02	2,10E+00	7,97E+01	6,30E+01
	H01	FAM	Unkn-5	40,8	37,52	2,84E+00	7,92E+00	8,98E-01	7,97E+01	6,30E+01
$1:10^5$ GAS	E02	FAM	Unkn-6	38,17	39,1	1,64E+00	3,31E+01	1,52E+00	2,48E+01	1,31E+01
	F02	FAM	Unkn-6	41,55	39,1	1,64E+00	5,27E+00	7,22E-01	2,48E+01	1,31E+01
	G02	FAM	Unkn-6	38,26	39,1	1,64E+00	3,15E+01	1,50E+00	2,48E+01	1,31E+01
	H02	FAM	Unkn-6	38,41	39,1	1,64E+00	2,91E+01	1,46E+00	2,48E+01	1,31E+01
$1:10^6$ GAS	E03	FAM	Unkn-7	N/A	0	0,00E+00	N/A	N/A	0,00E+00	0,00E+00
	F03	FAM	Unkn-7	N/A	0	0,00E+00	N/A	N/A	0,00E+00	0,00E+00
	G03	FAM	Unkn-7	N/A	0	0,00E+00	N/A	N/A	0,00E+00	0,00E+00
	H03	FAM	Unkn-7	N/A	0	0,00E+00	N/A	N/A	0,00E+00	0,00E+00
$1:10^7$ GAS	E04	FAM	Unkn-8	N/A	0	0,00E+00	N/A	N/A	0,00E+00	0,00E+00
	F04	FAM	Unkn-8	N/A	0	0,00E+00	N/A	N/A	0,00E+00	0,00E+00
	G04	FAM	Unkn-8	N/A	0	0,00E+00	N/A	N/A	0,00E+00	0,00E+00
	H04	FAM	Unkn-8	N/A	0	0,00E+00	N/A	N/A	0,00E+00	0,00E+00
Std $2*10^6$	A06	FAM	Std-1	18,53	18,42	1,57E-01	2,00E+06	6,30E+00	2,00E+06	0,00E+00
	B06	FAM	Std-1	1,83E+01	18,42	1,57E-01	2,00E+06	6,30E+00	2,00E+06	0,00E+00
Std $2*10^5$	A07	FAM	Std-2	21,71	21,92	3,05E-01	2,00E+05	5,30E+00	2,00E+05	0,00E+00
	B07	FAM	Std-2	2,21E+01	21,92	3,05E-01	2,00E+05	5,30E+00	2,00E+05	0,00E+00
Std $2*10^4$	A08	FAM	Std-3	27,08	26,38	9,90E-01	2,00E+04	4,30E+00	2,00E+04	0,00E+00
	B08	FAM	Std-3	2,57E+01	26,38	9,90E-01	2,00E+04	4,30E+00	2,00E+04	0,00E+00
Std $2*10^3$	A09	FAM	Std-4	29,81	29,58	3,25E-01	2,00E+03	3,30E+00	2,00E+03	0,00E+00
	B09	FAM	Std-4	2,94E+01	29,58	3,25E-01	2,00E+03	3,30E+00	2,00E+03	0,00E+00
Std $2*10^2$	A10	FAM	Std-5	36,02	35,74	3,90E-01	2,00E+02	2,30E+00	2,00E+02	0,00E+00
	B10	FAM	Std-5	3,55E+01	35,74	3,90E-01	2,00E+02	2,30E+00	2,00E+02	0,00E+00
Std 20 cp	A11	FAM	Std-6	N/A	0	0,00E+00	2,00E+01	1,30E+00	0,00E+00	0,00E+00
	B11	FAM	Std-6	N/A	0	0,00E+00	2,00E+01	1,30E+00	0,00E+00	0,00E+00
Std 2 cp	A12	FAM	Std-7	N/A	0	0,00E+00	2,00E+00	3,01E-01	0,00E+00	0,00E+00
	B12	FAM	Std-7	N/A	0	0,00E+00	2,00E+00	3,01E-01	0,00E+00	0,00E+00
NTC	E12	FAM	NTC	N/A	0	0,00E+00	N/A	N/A	0,00E+00	0,00E+00
	F12	FAM	NTC	N/A	0	0,00E+00	N/A	N/A	0,00E+00	0,00E+00
	G12	FAM	NTC	N/A	0	0,00E+00	N/A	N/A	0,00E+00	0,00E+00
	H12	FAM	NTC	N/A	0	0,00E+00	N/A	N/A	0,00E+00	0,00E+00

Table 1.1. Raw data from the first qPCR quantification.

Sample	Well	Fluor	Content	Cq	Cq Mean	Cq Std. Dev	Starting Quantity (SQ)	Log Starting Quantity	SQ Mean	SQ Std. Dev
stock GAS	A01	FAM	Unkn-01	22,56	2,25E+01	1,03E-01	3,53E+05	5,55E+00	3,79E+05	2,56E+04
	B01	FAM	Unkn-01	22,31	2,25E+01	1,03E-01	4,14E+05	5,62E+00	3,79E+05	2,56E+04
	C01	FAM	Unkn-01	22,44	2,25E+01	1,03E-01	3,81E+05	5,58E+00	3,79E+05	2,56E+04
	D01	FAM	Unkn-01	22,49	2,25E+01	1,03E-01	3,69E+05	5,57E+00	3,79E+05	2,56E+04
1:10 GAS	A02	FAM	Unkn-02	26,27	2,61E+01	1,08E-01	3,20E+04	4,50E+00	3,55E+04	2,40E+03
	B02	FAM	Unkn-02	26,04	2,61E+01	1,08E-01	3,69E+04	4,57E+00	3,55E+04	2,40E+03
	C02	FAM	Unkn-02	26,08	2,61E+01	1,08E-01	3,61E+04	4,56E+00	3,55E+04	2,40E+03
	D02	FAM	Unkn-02	26,04	2,61E+01	1,08E-01	3,70E+04	4,57E+00	3,55E+04	2,40E+03
1:100 GAS	A03	FAM	Unkn-03	29,62	2,95E+01	1,13E-01	3,64E+03	3,56E+00	3,94E+03	2,93E+02
	B03	FAM	Unkn-03	29,5	2,95E+01	1,13E-01	3,92E+03	3,59E+00	3,94E+03	2,93E+02
	C03	FAM	Unkn-03	29,34	2,95E+01	1,13E-01	4,34E+03	3,64E+00	3,94E+03	2,93E+02
	D03	FAM	Unkn-03	29,52	2,95E+01	1,13E-01	3,87E+03	3,59E+00	3,94E+03	2,93E+02
1:1000 GAS	A04	FAM	Unkn-04	33,21	3,32E+01	7,80E-02	3,53E+02	2,55E+00	3,59E+02	1,86E+01
	B04	FAM	Unkn-04	33,26	3,32E+01	7,80E-02	3,43E+02	2,54E+00	3,59E+02	1,86E+01
	C04	FAM	Unkn-04	33,08	3,32E+01	7,80E-02	3,86E+02	2,59E+00	3,59E+02	1,86E+01
	D04	FAM	Unkn-04	33,21	3,32E+01	7,80E-02	3,55E+02	2,55E+00	3,59E+02	1,86E+01
1:10 000 GAS	E01	FAM	Unkn-05	39,64	4,02E+01	5,47E-01	5,49E+00	7,40E-01	3,99E+00	1,41E+00
	F01	FAM	Unkn-05	40,21	4,02E+01	5,47E-01	3,79E+00	5,78E-01	3,99E+00	1,41E+00
	G01	FAM	Unkn-05	40,73	4,02E+01	5,47E-01	2,71E+00	4,32E-01	3,99E+00	1,41E+00
	H01	FAM	Unkn-05	N/A	0,00E+00	0,00E+00	N/A	N/A	0,00E+00	0,00E+00
$1:10^5$ GAS	E02	FAM	Unkn-06	N/A	0,00E+00	0,00E+00	N/A	N/A	0,00E+00	0,00E+00
	F02	FAM	Unkn-06	N/A	0,00E+00	0,00E+00	N/A	N/A	0,00E+00	0,00E+00
	G02	FAM	Unkn-06	39,46	4,17E+01	3,12E+00	6,15E+00	7,89E-01	3,25E+00	4,10E+00
	H02	FAM	Unkn-06	43,88	4,17E+01	3,12E+00	3,52E-01	-4,53E-01	3,25E+00	4,10E+00
$1:10^6$ GAS	E03	FAM	Unkn-07	N/A	0,00E+00	0,00E+00	N/A	N/A	0,00E+00	0,00E+00
	F03	FAM	Unkn-07	N/A	0,00E+00	0,00E+00	N/A	N/A	0,00E+00	0,00E+00
	G03	FAM	Unkn-07	N/A	0,00E+00	0,00E+00	N/A	N/A	0,00E+00	0,00E+00
	H03	FAM	Unkn-07	N/A	0,00E+00	0,00E+00	N/A	N/A	0,00E+00	0,00E+00
$1:10^7$ GAS	E04	FAM	Unkn-08	N/A	0,00E+00	0,00E+00	N/A	N/A	0,00E+00	0,00E+00
	F04	FAM	Unkn-08	N/A	0,00E+00	0,00E+00	N/A	N/A	0,00E+00	0,00E+00
	G04	FAM	Unkn-08	N/A	0,00E+00	0,00E+00	N/A	N/A	0,00E+00	0,00E+00
	H04	FAM	Unkn-08	N/A	0	0	N/A	N/A	0,00E+00	0,00E+00
Std $2*10^6$	A06	FAM	Std-01	19,47	1,93E+01	2,08E-01	2,00E+06	6,30E+00	2,00E+06	0,00E+00
	B06	FAM	Std-01	19,18	1,93E+01	2,08E-01	2,00E+06	6,30E+00	2,00E+06	0,00E+00
Std $2*10^5$	A07	FAM	Std-02	24,6	2,42E+01	5,70E-01	2,00E+05	5,30E+00	2,00E+05	0,00E+00
	B07	FAM	Std-02	23,8	2,42E+01	5,70E-01	2,00E+05	5,30E+00	2,00E+05	0,00E+00
Std $2*10^4$	A08	FAM	Std-03	26,64	2,71E+01	6,98E-01	2,00E+04	4,30E+00	2,00E+04	0,00E+00
	B08	FAM	Std-03	27,63	2,71E+01	6,98E-01	2,00E+04	4,30E+00	2,00E+04	0,00E+00
Std $2*10^3$	A09	FAM	Std-04	29,84	2,98E+01	0,00E+00	2,00E+03	3,30E+00	2,00E+03	0,00E+00
	B09	replicate was excluded from the analysis								
Std $2*10^2$	A10	FAM	Std-05	N/A	0,00E+00	0,00E+00	2,00E+02	2,30E+00	0,00E+00	0,00E+00
	B10	FAM	Std-05	N/A	0,00E+00	0,00E+00	2,00E+02	2,30E+00	0,00E+00	0,00E+00
Std 20 cp	A11	FAM	Std-06	N/A	0,00E+00	0,00E+00	2,00E+01	1,30E+00	0,00E+00	0,00E+00
	B11	FAM	Std-06	N/A	0,00E+00	0,00E+00	2,00E+01	1,30E+00	0,00E+00	0,00E+00
Std 2 cp	A12	FAM	Std-07	N/A	0,00E+00	0,00E+00	2,00E+00	3,01E-01	0,00E+00	0,00E+00
	B12	FAM	Std-07	N/A	0,00E+00	0,00E+00	2,00E+00	3,01E-01	0,00E+00	0,00E+00
NTC	G11	FAM	NTC	N/A	0,00E+00	0,00E+00	N/A	N/A	0,00E+00	0,00E+00
	H11	FAM	NTC	N/A	0	0	N/A	N/A	0,00E+00	0,00E+00
	G12	FAM	NTC	N/A	0,00E+00	0,00E+00	N/A	N/A	0,00E+00	0,00E+00
	H12	FAM	NTC	N/A	0	0	N/A	N/A	0,00E+00	0,00E+00

Table 2.2. Raw data from the second qPCR quantification.

Appendix 2. DNA concentrations of extracted gDNAs

Species	Strain	Concentration (ng/µl)
S. pyogenes	ATCC 19615	8.20
S. dysgalactiae	ATCC 9926	10.37
S. dysgalactiae	ATCC 12388	13.07
S. dysgalactiae	ATCC 12394	10.27
S. agalactiae	ATCC 13813	8.23
S. agalactiae	ATCC 12386	7.97
S. agalactiae	ATCC 27956	8.47
S. intermedius	ATCC 27335	14.23
S. mutans	ATCC 31377	8.87
S. pneumoniae	ATCC 6305	8.07

Table 2. Concentrations of extracted gDNAs.