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Rapid detection of *Streptococcus agalactiae* 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 the detection of *Streptococcus agalactiae* by using the isothermal SIBA®-method. *S. agalactiae,* i.e. group B streptococcus (GBS), is the leading cause of severe neonatal infections. In addition, it causes infections for pregnant women, the elderly and people, who have some chronic disease.

The experimental part of this thesis was executed at Orion Diagnostica's Research and Development laboratory. The thesis was started by conducting oligoscreening to find the most suitable primer combinations. Along with the screening, GBS was grown on blood agar plate and LB broth. The genomic DNA was extracted from LB broth and quantified with qPCR. Primer combinations that passed the oligoscreening were tested with the genomic DNA. Suitable assays were optimized, the sensitivity and specificity of the assays were tested, and the best assay was freeze-dried. In addition, the effect of different lytic enzymes to SIBA® reaction and GBS cells was tested. Lastly, the developed SIBA GBS assay was tested with clinical samples by using freeze-dried reagents.

A rapid method for the detection of *Streptococcus agalactiae* was successfully developed during this thesis. With freeze-dried reagents, the assay detected GBS in 5.7 minutes at its best, when genomic DNA was used as a template. Clinical samples were tested with both qPCR and freeze-dried SIBA®-reagents, and in both tests 1/3 GBS positive samples was detected. Samples that were not detected could have been low positive samples, or the sample preparation method may not have been optimal.

On the basis of the results of this thesis, the assay could be further developed by optimizing the reaction conditions of freeze-drying. In addition, sample preparation methods should be developed further. Also, testing the sensitivity of the qPCR method and possible optimization of it should be done, since there was not enough time to do these during this thesis.

Keywords	*Streptococcus agalactiae*, SIBA, qPCR, diagnostics

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Tämän insinöörityön tavoitteena oli kehittää uusi ja nopea tapa *Streptococcus agalacitaen* tunnistamiseksi isotermaalista SIBA®- teknologiaa hyödyntäen. *S. agalactiae* eli B-ryhmän streptokokki (GBS) on beetahemolyytinen bakteeri, joka on merkittävä vastasyntyneiden infektioiden aiheuttaja. Lisäksi se voi aiheuttaa infektioita raskaana oleville naisille, vanhuksille sekä henkilöille, joilla on jokin perussairaus.

Tämän insinöörityön kokeellinen osuus suoritettiin Orion Diagnostica Oy:n tuotekehityslaboratoriossa. Työ aloitettiin oligoseulonnalla sopivan alukeparin löytämiseksi. Seulonnan ohella *S.agalactiaea* kasvatettiin verimaljalla ja LB-liemessä sekä pystytettiin kirjallisuuteen perustuva qPCR-menetelmä GBS:n tunnistamiseksi. LB-liemestä eristettiin genomista DNA:ta, joka kvantitoitiin qPCR:n avulla. Seulonnasta läpi päässeet alukeparit testattiin genomisella DNA:lla, ja jatkoon päässeet menetelmät optimoitiin. Optimoitujen menetelmien herkkyys ja spesifisyys testattiin sekä toimiva menetelmä kylmäkuivattiin. Lisäksi testattiin erilaisten lyysaysentsyymien vaikutusta SIBA®-reaktioon sekä entsyymien lyysaystehoa GBS-soluihin. Lopuksi menetelmää testattiin kliinisillä näytteillä kylmäkuivattuja reagenssejа käyttäen.

Insinöörityön aikana onnistuttiin kehittämään nopea menetelmä *S.agalactiaen* tunnistamiseksi. Kylmäkuivatuilla reagensseilla menetelmä tunnisti GBS:n nopeimmillaan 5,7 minuutissa, kun templaattina käytettiin genomista DNA:ta. Kliiniset näytteet testattiin sekä qPCR:llä että kylmäkuivatuilla SIBA®-reagensseilla, ja molemmissa testeissä positiivisista näytteistä 1/3 detektoitui. Näytteet, jotka eivät detektoituneet, saattoivat olla heikkoja positiivisia tai käytetty näytteenkäsittelymenetelmä ei soveltunut testiasetelmaan.

Insinöörityön tulosten pohjalta menetelmän kehittämistä voi jatkaa kylmäkuivatun SIBA®-reaktion olosuhteiden optimoinnilla. Lisäksi näytteenkäsittelymenetelmiä tulee kehittää. Myös qPCR-menetelmän herkkyyden testaus ja mahdollinen optimointi olisi hyvä tehdä, sillä niitä ei ehditty tämän insinöörityön aikana suorittaa.

Avainsanat	*Streptococcus agalactiae*, SIBA, qPCR, diagnostiikka

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Appendix 1. Raw data from qPCR

Abbreviations

ATCC	American Type Culture Collection
ATP	Adenosine triphosphate
CDC	Centers for Disease Control and prevention
cp	Copies
CPS	Capsular polysaccharide
Ct- value	Cycle threshold value
DNA	Deoxyribonucleic acid
dsDNA	Double stranded DNA
ELISA	Enzyme-linked immunosorbent assay
EOD	Early-onset disease
F	Forward primer
FD	Freeze-dried
gDNA	genomic DNA
GBS	Group B Streptococcus
IAP	Intrapartum antibiotic prophylaxis
IO	Invasion oligonucleotide
LAMP	Loop-mediated isothermal amplification
LOD	Late onset disease

MgAc	Magnesium Acetate
NFW	Nuclease free water
NTC	No template control
OGR	Orion Genread
PEG	Polyethylene glycol
R	Reverse primer
rRNA	Ribosomal RNA
R&D	Research and development
SIBA	Strand Invasion Based Amplification
qPCR	Quantitative polymerase chain reaction

1 Introduction

Streptococcus agalactiae, i.e. group B streptococcus (GBS), is a leading cause of serious neonatal infections, which may lead to death [2]. There is no vaccine available for GBS [23]. GBS is usually transferred to a newborn from the GBS colonized mother during the labor. However, it is possible to try to prevent the GBS disease in newborns by giving intrapartum antibiotic prophylaxis (IAP) to the colonized mother during the labor. Current methods for GBS diagnosis include for example culturing and PCR. Pregnant women can be screened for GBS during pregnancy weeks 35- 37 with cultivation. However, GBS colonization is a dynamic state, which may come and go, therefore, the result of screening might not be accurate at the time of the labor. [8, 11, 12]

The experimental part of this thesis lasted nine weeks and was executed at Orion Diagnostica's research and development laboratory. The aim of this thesis was to develop a rapid and accurate method for the detection of GBS using strand based invasion amplification (SIBA®) technology owned by Orion Diagnostica. SIBA® is an isothermal nucleic acid amplification method that can be performed on relatively small and low cost devices [22].

2 Theory

2.1 Streptococci

Streptococci are gram-positive, catalase-negative, spherical bacteria that often occur as chain or pairs. Some of the streptococci species are facultative anaerobic and some obligate anaerobic. They usually belong to a normal flora of humans and different animal species. Streptococci appear, for example, on the skin, in the mouth, in intestines, and in the respiratory tract. Most of the streptococci cause opportunistic infections, but some streptococci species are virulent, which means they can cause serious infections regardless of the defense mechanism of the host organism. [1, 2]

Streptococci can be divided into alpha-, beta- and gamma-hemolytic groups based on their hemolytic activity, i.e. red blood cell lysing activity (Figure 1). Alpha-hemolytic streptococci lyse red blood cells and hemoglobin partially. Alpha hemolysis can be seen on

the blood agar plate as a greenish area under and around the colonies. [1, 2] Greenish color is caused by the oxidation of hemoglobin to methemoglobin [28]. Beta-hemolytic streptococci lyse red blood cells and hemoglobin completely, which can be seen on the blood agar plate as lightened or transparent area under and around the colonies. Gamma-hemolytic, i.e. non-hemolytic streptococci, do not cause hemolysis. [1, 2]

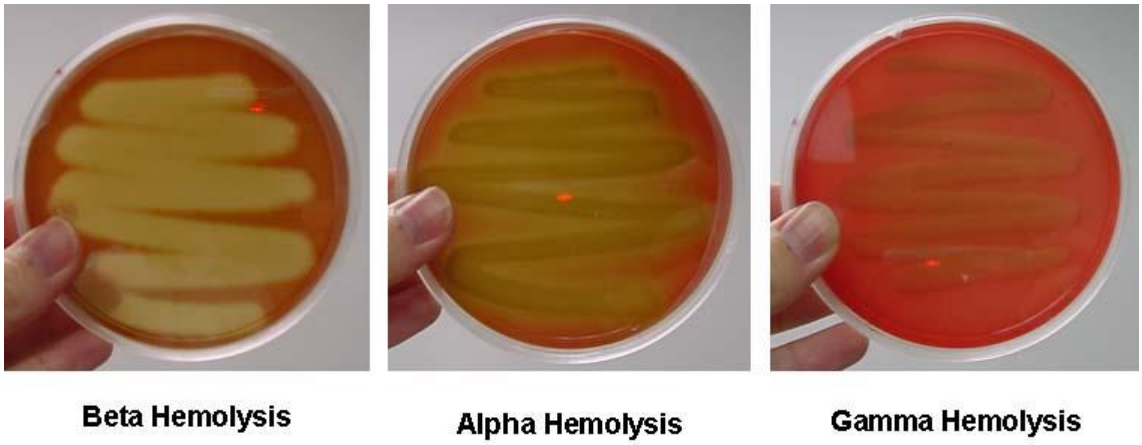

Figure 1. Beta, alpha and gamma hemolysis on blood agar plates [3].

Streptococci can also be classified serologically by using Lancefield grouping, in which bacteria are divided into groups from A to V, excluding I and J, based on the carbohydrate antigens on their cell wall [26]. *Streptococcus pneumoniae* and Viridans streptococci cannot be classified by Lancefield grouping since they lack the carbohydrate antigen [33].

On the basis of 16s rRNA sequences, streptococci can be divided into six groups: the *S. pyogenes* group, the *S. anginosus* group, the *S. bovis* group, the *S. mitis* group, the *S. mutans* group and the *S. salivarus* group [29].

2.2 *Streptococcus agalactiae*

Streptococcus agalactiae, also known as group B streptococcus or GBS, is a weak beta-hemolytic (Figure 2) and facultative anaerobic bacterium. It has the Lancefield group B antigen, hence the name group B streptococcus. [1, 2] GBS can be divided in ten differ-

ent serotypes Ia, Ib, and III to IX based on the capsular polysaccharide (CPS) components on their cell wall [26]. It has numerous virulence factors, such as the pore-forming toxin and sialic acid-rich capsular polysaccharide [27]. GBS was first found in the 1930s and discovered as a human pathogen in 1938 [16].

Figure 2. Streptoccus agalactiae (ATCC 12386) pure culture on a blood agar plate. Weak hemolysis is seen around the colonies.

GBS bacterium causes infections in both humans and animals. It is a significant veterinary pathogen since it causes bovine mastitis in dairy cows. GBS is the main cause of severe neonatal infections. [2] It also causes infections for pregnant women, the elderly, and people with some chronic disease. It is common to be an asymptotic carrier of GBS. [14] GBS-bacterium can be found on the skin, in the throat, in gastrointestinal tract, in bladder, and in vagina without it causing any symptoms. Around 10-30 % of pregnant women have GBS in their rectum or vagina. [9]

2.2.1 Group B Streptococcus in newborns

In newborns, GBS causes two types of infection; the early-onset disease (EOD) and the late onset disease (LOD). The early-onset disease appears at the latest within six days of birth, but mostly within the first 24 hours of birth. In the early-onset disease, the source

of bacteria is in most cases a GBS colonized mother. GBS is transferred from the mother to the baby in uterus or during the labor. The EOD causes, for example, sepsis, pneumonia, and meningitis. [11,12] The early-onset disease may also cause long-term problems, such as deafness or learning difficulties, especially in the case of meningitis. EOD may also lead to death. [11] Fever, difficulties in feeding, difficulties in breathing, irritability, lethargy, and blueish skin color are symptoms of the early-onset disease [9]. In 2015, 13 EOD cases were detected in Finland and 840 cases in the USA. [10,12] The amount of EOD cases in the UK in 2015 was 517 [35].

There are several risk factors for the early-onset disease. GBS colonized mother, preterm delivery and prolonged (>18 hours) rupture of membranes expose the infant to EOD. In addition, mother's GBS bacteriuria during pregnancy and fever during labor are risk factors. Demographic risk factors for EOD include mother's young age and African American race. However, over half of the EOD cases are with newborns, who do not have any risk factors. [8, 12]

It is possible to try to prevent the early-onset disease in newborns by giving intrapartum antibiotic prophylaxis (IAP) to the mother. IAP should be given at latest 4 hours after labor has been induced, and it should last at least 4 hours. The challenge is to identify the women, who IAP should be given to. There are two prevention strategies for GBS; screening-based and risk-based approach. In screening-based approach, the mother is tested for GBS colonization either during pregnancy weeks 35-37 with cultivation or during the labor with a PCR-test. In the risk-based approach, IAP is targeted to the women, who, on the basis of the risk factors, have higher risk for having GBS colonized newborn. [8, 11, 12] USA, Australia and most of the EU-countries use screening-based GBS prevention approach. The risk-based prevention approach is used in the Netherlands, Great Britain, Sweden, and Finland. [8]

The late onset disease occurs between the 7^{th} and 89^{th} days of life. The source of bacteria in LOD is, in most cases, unknown. The late onset disease causes the same conditions as the early-onset disease even though meningitis and pneumonia are more common in LOD than in EOD. [34] In 2015, 11 LOD cases were detected in Finland and 1265 cases in the USA. [10,12] The amount of the LOD cases in the UK was 339 in 2015 [35]. The late onset disease can't usually be prevented by IAP [14]. The early- and late onset diseases are treated with antibiotics, such as penicillin or ampicillin, through a vein [12].

2.2.2 GBS in adults

Although infections caused by GBS are notably more common in newborns, it can also cause infections in adults. In adults, it can cause, for instance, a urinary tract infection, sepsis, arthritis, pneumonia, and meningitis. For pregnant women, GBS can cause uterus infection and premature labor. [2]

The elderly and people with some chronic disease, such as diabetes, cancer, or HIV, are more exposed to severe infections caused by GBS. GBS is not a sexually transmitted disease, and the sources of diseases caused by GBS are mostly unknown with adults. Symptoms of the disease depend significantly on which part of the body is infected. The treatment depends on the disease that is caused by GBS. GBS itself is treated by using antibiotics, such as penicillin or ampicillin. [9, 12]

2.3 Diagnosis of GBS

GBS can be diagnosed from a clinical sample in several different ways. Cultivation is still the most commonly used and reliable way to identify GBS. It can also be identified by nucleic acid testing techniques, such as PCR, LAMP, or probe hybridization methods. Immunological assays can be used mostly for presumptive identification of GBS. [2, 16]

2.3.1 Clinical specimens

Vaginal and rectal swabs are used for testing GBS colonization of pregnant women during 35 to 37 weeks of pregnancy. Vaginal sample should be taken from the lower third of vagina and rectal swab should pass through the anal sphincter. The sample should not be taken with speculum, but with swab. Swabs are then transported in a nonnutritive transport medium, which keeps the sample viable up to 4 days. However, the results are more sensitive when the sample is tested within 24 hours of collection. [9, 11, 12]

GBS colonization of infants or adults can be tested from urine or from sterile body fluid, such as blood or spinal fluid [9, 12].

2.3.2 Current methods of GBS detection

GBS bacterium is generally detected from cultivated samples. It can be grown either on an agar plate or in liquid growth medium. It is usually grown on a blood agar medium to see the possible hemolytic reaction. Optimum temperature for growth is around 37°C [1.] GBS can be growth from sterile samples on nonselective blood agar. The use of selective broth medium that contains antibiotics, which inhibit the growth of other bacteria, increases the specificity with genital and rectal samples. [30]

Cultivation is usually done by using a streak plate method, which is used to isolate individual colonies [4]. After inoculating the growth medium is incubated at 35°C to 37°C for 18 to 24 hours [1].

Special procedures are followed when screening GBS in pregnant women. According to the recommendation given by a group of experts in Finland, the samples should be inoculated on a selective blood agar plate, which contains colisitin and oxcolinic acid. Selective growth medium prevents the growth of gram-negative rods, staphylococci, and bacilli. Culture is incubated at 37°C for 18 to 24 hours and after that GBS is identified from the culture. If there is not any growth on the plate after 24 hours of incubation, the plate is further incubated for 24 hours before giving a GBS negative result. If there still is not any growth on the plate after 48 hours of incubations, the result is GBS negative. [5]

GBS can be identified from culture plates, for example, by enzyme immunoassay test (ELISA), Lancefield antigen immunoassay, CAMP, catalase, Hippurate, and latex agglunation tests. Lancefield grouping is a reliable way to identify GBS, since GBS is the only *Streptococcus species* having Lancefield group B antigen on the surface of the bacteria. [1, 12] CAMP test can be used for presumptive identification of GBS. Most GBS isolates produce extracellular protein called the CAMP factor. Beta hemolysin of *Staphylococcus aureus* and CAMP factor of GBS lyse erythrocytes synergistically, which can be seen on a blood agar plate as enhanced beta-hemolysis. [1, 16] GBS bacterium has the ability to hydrolyze Hippurate, but so does also some other Streptococci species, therefore, Hippurate test can be used only for presumptive identification [1].

Even though culturing is the current gold standard method for the diagnosis of GBS, it is not 100 % sensitive and false negative results can occur since other possible bacteria of the sample can inhibit the growth of GBS, even in selective medium [14, 25]. In addition,

culture has rather a long turnaround time (24 to 72 hours), therefore, other ways for the diagnosis has been developed [14].

Currently, there is no vaccine available for GBS [23]. Especially with EOD (early-onset disease), fast diagnosis is important and therefore there is a need for a specific and rapid method for GBS diagnosing. Number of various nucleic acid based test have been developed for the detection of GBS. There are multiple commercial PCR assays targeting different genes, such as *dltR, sip, cfb and cylB*, available for detection of GBS. Their sensitivity and specificity are in most cases above 90 %. [14, 17, 24] In most PCR assays, DNA should be extracted before PCR, which takes an hour. Nevertheless, PCR is much faster than culturing since it gives the result within 2 hours of receiving the sample. At its best PCR can give a result within 45 minutes of receiving the sample when one-step lysis is performed to the sample instead of DNA extraction. [17] However, PCR requires heavy and special equipment for performing the reactions. Most of the PCR assays are designed for vaginal samples rather than blood samples. [24]

Another nucleic acid based test developed for detection of GBS is a loop- mediated isothermal amplification assay (LAMP), where DNA target is amplified at 60 – 65 °C without thermal cycles. Assay can target different genes, such as *cfb* and *sip*. Sensitivity and specificity of LAMP assay are above 95 % and it takes 45 to 75 minutes to get a result. [31, 32]

2.3.3 Challenges of GBS diagnosis

In treatment of diseases caused by GBS, a fast and accurate diagnosis is important. In prevention of EOD pregnant women are screened for GBS colonization during 35 to 37 weeks of pregnancy. However, GBS colonization is a dynamic state, which means colonization may come and go during months. Therefore, even with screening during pregnancy weeks 35 to 37, there still is not 100 % reliable knowledge of mother's GBS colonization stage during the labor. The current standard method for diagnosing of GBS, i.e. culturing, takes several days to get the result. As a result, some women and newborn infants may get antibiotics treatment unnecessarily since treatment is started if there is even a slightly suspicion of GBS colonization. [8,11, 12]

2.4 SIBA

SIBA®, i.e. Strand Invasion Based Amplification, is an isothermal nucleic acid amplification technology, which is owned by Orion Diagnostica. SIBA technology is highly sensitive and does not require target-specific probes, and, at its best, it can detect target analyte with the accuracy of one molecule. In SIBA reaction, a single stranded invasion oligonucleotide (IO) recognizes the complementary area of the target sequence and penetrates double stranded target DNA with the help of recombinase enzyme and adenosine triphosphate (Figure 3). The penetration dissociates flanking areas and single stranded target DNA is exposed. Amplification primers, which are specific to target sequence, anneal to single stranded target DNA. After this, DNA polymerase synthesizes complementary strands from free nucleotides. The invasion oligo dissociates and the process starts over. This leads to the exponential amplification of the target DNA. [21, 22]

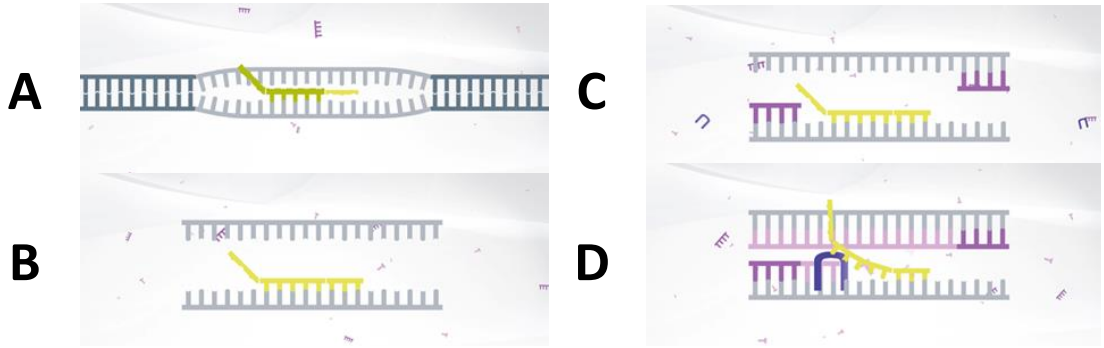

Figure 3. SIBA reaction
A: Invasion oligo (yellow) penetrates dsDNA.
B: The penetration dissociates flanking areas and ssDNA is exposed.
C: Amplification primers bind to ssDNA.
D: DNA polymerase synthesizes complementary strands. IO dissociates and process starts over. [7]

In SIBA reaction, primers are not substrates for the recombinase, therefore, they are unable to attach the target sequence in the absence of the IO. This guarantees that amplification is possible only with the right target DNA. 2`-O- methyl RNA is added to the IO to ensure, that the IO cannot act as a template or substrate for the polymerase. [21, 22]

The SIBA reaction consist of a recombinase, recombinase cofactors, a system producing ATP, polymerase, and nucleic acid components. UvsX can be used as a recombinase

since it ensures efficient turnover of ATP. However, it also produces inorganic phosphate, which inhibits the reaction. Sucrose and sucrose phosphorylase added to the reaction remove inorganic phosphate produced by UvsX. Creatine phosphokinase and phosphocreatine are needed in ATP regeneration system. Polyethylene glycol (PEG) and gp32-protein improve the functioning of recombinase enzyme in SIBA reaction. Concentrations of recombinase, UvsX, and other components used in SIBA depend on the target analyte. SIBA can be performed by using wet mix reagents or by using freeze-dried reagents. [21, 22]

SIBA reaction is isothermal, i.e. the temperature of the reaction stays the same at 40°C during whole amplification reaction. SIBA can, therefore, be performed, for example, in an incubator set at 40°C. Real-time progress of the reaction can be monitored by using fluorescent dyes and qPCR equipment. [22]

3 Materials and methods

3.1 Materials

Primers and qPCR probe used in this thesis were ordered from Eurofins Genomic (Germany). Invasive oligonucleotides were ordered from Integrated DNA Technologies (IDT, USA). Primers and IOs used in oligoscreening are presented in Table 1.

Table 1. Primers and IOs used in oligoscreening.

B1-F1	B1-F14	B1-R4	B1-IO2	B2-F12	B2-R6	B2-R19	B3-F8	B3-R4	B4-F3	B4-R3	B4-IO1	B5-F12	B5-R8
B1-F2	B1-F15	B1-R5	B1-IO3	B2-F13	B2-R7	B2-R20	B3-F9	B3-R5	B4-F4	B4-R4	B4-IO2	B5-F13	B5-R9
B1-F3	B1-F16	B1-R6	B2-F1	B2-F14	B2-R8	B2-R21	B3-F10	B3-R6	B4-F5	B4-R5	B5-F1	B5-F14	B5-R10
B1-F4	B1-F17	B1-R7	B2-F2	B2-F15	B2-R9	B2-IO1	B3-F11	B3-R7	B4-F6	B4-R6	B5-F2	B5-F15	B5-R11
B1-F5	B1-F18	B1-R8	B2-F3	B2-F16	B2-R10	B2-IO2	B3-F12	B3-R8	B4-F7	B4-R7	B5-F3	B5-F16	B5-R12
B1-F6	B1-F19	B1-R9	B2-F4	B2-F17	B2-R11	B2-IO3	B3-F13	B3-R9	B4-F8	B4-R8	B5-F4	B5-F17	B5-R13
B1-F7	B1-F20	B1-R10	B2-F5	B2-F18	B2-R12	B3-F1	B3-F14	B3-R10	B4-F9	B4-R9	B5-F5	B5-R1	B5-R14
B1-F8	B1-F21	B1-R11	B2-F6	B2-F19	B2-R13	B3-F2	B3-F15	B3-R11	B4-F10	B4-R10	B5-F6	B5-R2	B5-R15
B1-F9	B1-F22	B1-R12	B2-F7	B2-R1	B2-R14	B3-F3	B3-F16	B3-R12	B4-F11	B4-R11	B5-F7	B5-R3	B5-R16
B1-F10	B1-F23	B1-R13	B2-F8	B2-R2	B2-R15	B3-F4	B3-F17	B3-IO1	B4-F12	B4-R12	B5-F8	B5-R4	B5-R17
B1-F11	B1-R1	B1-R14	B2-F9	B2-R3	B2-R16	B3-F5	B3-R1	B3-IO2	B4-F13	B4-R13	B5-F9	B5-R5	B5-R18
B1-F12	B1-R2	B1-R15	B2-F10	B2-R4	B2-R17	B3-F6	B3-R2	B4-F1	B4-R1	B4-R14	B5-F10	B5-R6	B5-IO1
B1-F13	B1-R3	B1-IO1	B2-F11	B2-R5	B2-R18	B3-F7	B3-R3	B4-F2	B4-R2	B4-R15	B5-F11	B5-R7	B5-IO2
F= Forward primer		R= Reverse primer		IO= Invasion oligonucleotide									
B1= Assay 1	B2= Assay 2	B3= Assay 3	B4= Assay 4	B5= Assay5									

The synthetic template used in oligoscreening and double stranded synthetic DNA template used in qPCR quantification were ordered from Invitrogen (Thermo Fischer Scientific, USA). Three clinical samples (serum, sputum, and throat swab) used in this study were ordered from Discovery Life Sciences (USA). Table 2 lists materials used in this thesis.

Table 2. Reagents used in this thesis.

Product	Manufacturer
SIBA regent kit	Orion Diagnostica Oy, Finland
Water- Molecular Biology Reagent	Sigma Aldrich, USA
Magnesium acetate solution	Sigma Aldrich, USA
iTaq Universal Probe Supermix	Bio-rad, USA
Qiagen EZ1 DNA Tissue kit	Qiagen, Germany
QIAmp DNA mini kit	Qiagen, Germany
Ethanol	Altia, Finland
Luria broth	Sigma Aldrich, USA
Blood agar COH	Biomerieux, France
PBS	Orion Diagnostica Oy, Finland
Enzyme 1	Sigma Aldrich, USA
Enzyme 2	Sigma Aldrich, USA
Enzyme 3	Sigma Aldrich, USA
Enzyme 4	Sigma Aldrich, USA
Enzyme 5	Orion Diagnostica Oy, Finland
PEG-400	Sigma Aldrich, USA
DMSO	Sigma Aldrich, USA
Triton X-100	Sigma Aldrich, USA

3.2 Methods

The workflow of this thesis is presented in Figure 4.

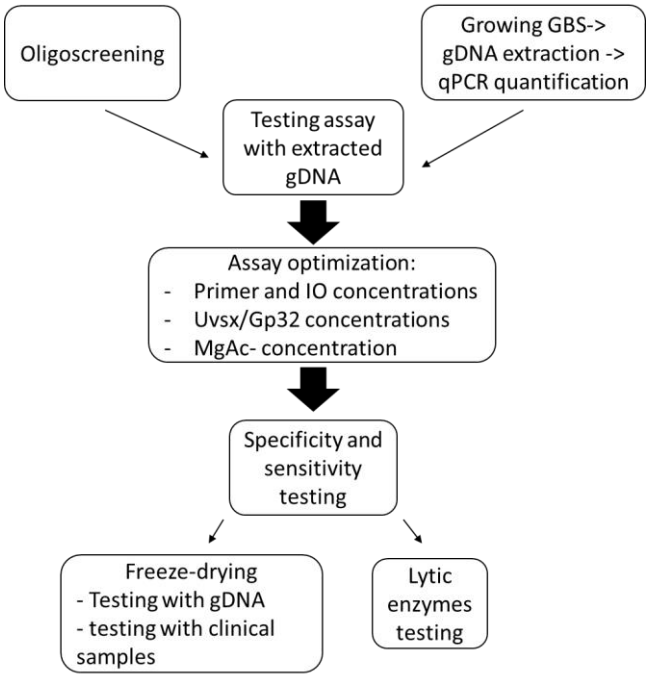

Figure 4. Workflow of this thesis.

3.2.1 Bacterial strains and cultivation

ATCC's strain 12386 was used in this thesis. *Str. Agalactiae* strain was cultured to two COH blood agar plates. The plates were incubated at 37˚C for 24 hours.

One bacterial colony was suspended to 5 ml of Luria broth. Part of the same bacterial colony was subcultured to a new blood agar plate to make a pure culture. Suspension was incubated in a mixer at 37˚C for 16.5 hours. Pure culture was incubated at 37˚C for 23 hours and then transferred into fridge for later use.

Ten different Streptococcus strains from ATCC were used for specificity testing (Table 3).

Table 3. *Streptococcus* strains used in this thesis.

Species	Strain name
S.pyogenes	ATCC 19615
S.dysgalactiae	ATCC 9926
S.dysgalactiae	ATCC 12388
S.dysgalactiae	ATCC 12394
S.agalactiae	ATCC 13813
S.agalactiae	ATTC 12386
S.agalactiae	ATCC 27956
S.intermedius	ATCC 27335
S.mutans	ATCC 31377
S.pneumoniae	ATCC 6305

3.2.2 DNA templates and genome extraction

In this thesis, the genomic DNA of *Streptococcus agalactiae* was extracted from bacterial suspension culture and from clinical samples.

Str. agalactiae from bacterial suspension culture was extracted by using Qiagen EZ1 Advanced XL- robot. Qiagen EZ1 Advanced XL is an automated technique for purification of genomic DNA from any biological sample. Qiagen EZ1 Advanced XL is used for both diagnostic and scientific use. [19] EZ1® DNA Tissue Kit contains all the reagents needed for the bacterial DNA isolation, and it was used in this experiment. Extraction was made according to the instructions in EZ1® Advanced XL user manual. 200 µl of bacterial suspension culture was used for isolation. Elution volume used in first extraction was 200 µl and in second extraction 50 µl to increase the final DNA concentration.

One extraction of *Str. agalactiae* from bacterial suspension culture was made manually with QIAmp® DNA mini kit. Protocol used was DNA Purification from Tissues (Spin protocol). Extraction was made as per instructions in QIAmp® DNA Mini and Blood Mini Handbook [20].

<u>DNA extraction from clinical samples</u>

DNA was extracted from three *Streptococcus agalactiae* positive clinical samples. There were one throat swab, one serum sample and one sputum sample, all from different

people. QIAmp® DNA Mini Kit was used for the extraction, and the extraction was made as per instructions in QIAmp® DNA Mini and Blood Mini Handbook following protocol DNA Purification from Blood or Body Fluids. Throat swab was dipped in 200 µl of PBS and used for extraction. In addition, 150 µl of serum and sputum were used for extraction. Elution volume used with all three samples was 100 µl. One negative extraction control was made.

3.2.3 qPCR quantification

PCR method for detection of GBS was set up to determine the copy number of extracted gDNA. All qPCR quantifications were made using Bio-rad's CFX96™ Real Time System C1000™ Thermal Cycler. The PCR program, probe and primers were designed based on an article [15]. Primers and probes used are presented in Table 4. Used PCR assay targets *cfb* gene, which encodes a diffusible extracellular protein called the CAMP factor [16].

Table 4. Probe and primers used in this study and their sequences.

Oligos	Sequence
strepB-Forward	GGGAACAGATTATGAAAACCG
strepB-Reverse	AAGGCTTCTACACGACTACCAA
strepB-probe	FAM-AGACTTCATTGCGTGCCAACCCTGAGAC-BHQ1

The PCR reaction was performed in a 20 µl volume containing 10 µl iTaq Universal Probes Supermix, 2 µl DNA template, 0,4 µM both primers and 0,2 µM probe. The reaction volume was completed to 20 µl with a molecular grade water. The PCR program consisted of one cycle of 45°C for 10 min and 94°C for 10 min and 45 cycles of 94°C for 30 s, and 60°C for 60 s.

Synthetic double stranded DNA template ordered from a subcontractor was used for quantification. Sequence length and quantity of the synthetic dsDNA was known, and the copy number (cp) was calculated by using Formula 1.

$$\frac{amount\ (ng) * 6.022{*}10^{23}}{length\ (bp) * 1{*}10^{9} * 650}. \quad [1]$$

DNA concentrations used in quantification were 10^6 cp/µl, 10^5 cp/µl, 10^4 cp/µl, 10^3 cp/µl, 10^2 cp/µl, 10 cp/µl and 1 cp/µl. Two replicates were made of each synthetic dsDNA reaction. Extracted gDNA concentrations used were stock GBS gDNA, and dilutions 1:10, 1:100, 1:10^3, 1:10^4, 1:10^5, 1:10^6, 1:10^7 and 1:10^8. Four replicates were made of each gDNA reaction.

3.2.4 SIBA®

Oligo screening

SIBA® reactions were performed using the SIBA® reagent kit. Forward and reverse primer concentrations used were 200 nM and IO concentration used was 200 nM. Uvsx- and Gp32- concentrations used were 0.25 mg/ml. Total volume of reaction was 20 µl.

In this thesis, five different assays were screened. The first step of oligo screening is to screen assay oligos for self-priming and unspecific oligo-oligo interactions in SIBA reaction. Forward and reverse primers were screened individually in the presence of IO using SYBR Green detection. If an unspecific signal was detected, the primer in question was discarded.

Primers without unspecific signal were taken further to be screened together in the presence of IO. Primer combinations that caused unspecific signal, were discarded and combinations without unspecific signal were taken further to be screened with template.

Primer combinations were tested with a template to see if they can amplify the specific target. First the combinations were tested with a synthetic DNA template. Fastest and most specific combinations with synthetic DNA template were then tested with gDNA, which was extracted from *Streptococcus agalactiae*. In screening, amplification time was 90 min at 41 °C with SYBR Green detection.

Melt curve analysis was used to confirm that the amplification product was correct. The melt curve analysis was run straight after SIBA run. Temperature was increased from 41°C gradually to 95°C and kept at 95°C for 15 seconds to denature amplicons. It was known that the correct product has a melting temperature around 70 °C, and if the T_m of amplification product differed from that, it was considered unspecific.

Assay optimization

Assay optimization was done by using SIBA® reagent kit. SIBA reactions were performed in a 20 µl volume containing 2 µl gDNA template and 18 µl mastermix (SIBA® reagent kit). Primer-, IO-, Uvsx/Gp32-, and magnesium concentrations varied depending on what component was optimized. Amplification time with assay optimization tests was 60 minutes at 41°C with SYBR Green detection and melt curve analysis was run straight after SIBA run.

Primer and IO concentrations used in oligo screening were 200 nM in one reaction. The aim was to get the assay faster and more specific without unspecific amplification. Oligo concentrations of the assay were optimized by increasing simultaneously both forward and reverse primer concentrations from 200 nM to 400 nM and by changing forward and reverse primer concentrations between 300 nM and 400 nM. IO concentration was increased gradually from 200 nM to 400 nM. The tested concentration mixes are presented in Table 5. The template concentration used was 1000 cp GBS gDNA per reaction.

Table 5. Oligo concentrations used in optimizing tests.

F	R	IO
200 nM	200 nM	200 nM
200 nM	200 nM	300 nM
200 nM	200 nM	400 nM
400 nM	400 nM	200 nM
400 nM	400 nM	300 nM
400 nM	400 nM	400 nM
300 nM	300 nM	300 nM
350 nM	300 nM	300 nM
400 nM	300 nM	300 nM
300 nM	350 nM	300 nM
350 nM	350 nM	300 nM
400 nM	350 nM	300 nM
300 nM	400 nM	300 nM
350 nM	400 nM	300 nM
400 nM	400 nM	300 nM

Best assays from previous test were then optimized by titrating Uvsx and Gp32 concentrations. In this experiment, Uvsx and Gp32 concentrations of 0.25 mg/ml, 0.3 mg/ml and 0.35 mg/ml were tested.

Increasing magnesium concentration usually fastens SIBA reaction, but if the amount of magnesium is too high, false positives can occur. Magnesium concentrations of 8 mM, 10 mM, 12.5 mM, 15 mM, 17.5 mM, 20 mM and 50 mM were tested. The primer concentrations used were F350/R300 nM with IO concentration of 300 nM. *Str.agalactiae* gDNA was used as template with 100 cp per reaction.

Sensitivity and specificity

SIBA reactions were performed in a 20 µl volume containing 2 µl gDNA template and 18 µl mastermix (SIBA® reagent kit). Optimized primer-, IO-, Uvsx/Gp32-, and magnesium concentrations were used. The amplification time was 60 minutes at 41°C with SYBR Green detection and melt curve analysis was run straight after SIBA run.

After oligo and enzyme optimizations, the sensitivity of F17/R7 and F11/R17 assays were determined. Assays were tested with template concentrations of 10^5 cp, 10^4 cp, 10^3, 100 cp, 10 cp and 1 cp per reaction. Four replicates were tested at each template concentration. Extracted and quantified *Str. agalactiae* gDNA was used as template.

The specificity of the F17/R7 assay was determined by testing the assay with ten different *Streptococcus* strains, which are shown in Table 2. Templates used in this experiment were extracted gDNA from each *Streptococcus* strain with concentration 5 ng/ml in one reaction. There were also one gDNA template, from which the copy number was known to be 10 000 cp/reaction. Four replicates of each template were tested.

Enzymatic cell lysis

SIBA reactions were performed in a 20 µl volume containing 2 µl gDNA template, 2 µl lytic enzyme and 16 µl mastermix (SIBA® reagent kit). Optimized primer-, IO-, Uvsx/Gp32-, and magnesium concentrations were used. Amplification time was 60 minutes at 41°C with SYBR Green detection and melt curve analysis was run straight after SIBA run.

The tolerance of SIBA reaction towards lytic enzymes was tested with five different enzymes. Enzymes 1, 2, 3, 4 and 5 were tested. The experiment was done by adding 2 µl of lytic enzyme straight to the SIBA reaction. Different concentrations of the enzymes were used to see if there is a limit where enzyme does not inhibit SIBA reaction. Enzyme

concentrations used were enzyme stock and stock dilutions 1:2, 1:4, 1:8, 1:16, 1:32 and 1:64. The stock concentration of the enzymes was 10 mg/ml except for Enzyme 5, which had the stock concentration of 4.5 mg/ml. Also, the control reaction without an enzyme was made. Three replicates of each enzyme concentrations were tested and 100 cp gDNA per reaction was used as template.

Enzymes that did not inhibit SIBA reaction, were tested with GBS cells. One colony of *Str. agalactiae* from pure culture was suspended in 100 µl of molecular grade water and used as a template. Lysis efficiency was tested by comparing enzymes effect on cells with and without incubation. Cells with enzyme were incubated at 37°C for 10 minutes and then added to SIBA reaction. Since SIBA itself is basically a one hour incubation at 41°C, it was tested if pre-incubation at 37°C was necessary by adding cells and enzyme straight to SIBA reaction without incubation. Control reactions without enzymes were also made with and without incubation. Two replicates were made of each reaction.

GBS cells used as a template were diluted to see at which cell concentration the lysis enzyme is required. Enzymes were added straight to mastermix and cells were heated at 95°C for 5 minutes and then added to SIBA reaction. Reactions were also made without heating the cells. Cell concentrations used in this experiment were 1:10, $1:10^2$, $1:10^3$, $1:10^4$, $1:10^5$ and $1:10^6$. Control reactions without enzymes were also made with and without heating in every cell concentration. Two replicates were made of each reaction.

Freeze drying

A freeze-dried trial batch was made of the assay which had been developed in this thesis. Mastermix was prepared and pipetted to a 96-well reaction plate 70 µl/well. Concentrations of IO, primers, Uvsx and Gp32 are found in Table 6. After pipetting mastermix to wells, it was made sure that there weren't any bubbles. Plate was moved to pre-cooled Julabo-dryer and drying protocol was started.

Table 6. Concentrations used in freeze-drying.

Reagent	Conc.
Forward primer	350 nM
Reverse primer	300 nM
IO	300 nM
Uvsx	300 nM
Gp32	300nM

After drying was done, freeze-dried reagents were tested with two different buffers and extracted GBS gDNA. Freeze-dried SIBA reaction was performed in a 41 µl volume containing 40 µl buffer and 1 µl DNA template. 100 000 cp and 10 000 cp of gDNA per reaction were used as template. Three replicates were made of each copy number with both buffers. NTCs had 1 µl molecular grade water instead of template. Two replicates of NTC were made with both buffers. Amplification time was 60 minutes at 41 °C and melt curve analysis was done straight after SIBA run.

Clinical samples

Three GBS positive clinical samples were tested with freeze-dried reagents. All three samples had different matrixes; there were one serum sample, one sputum sample and one throat swab sample. Throat swab was dipped to 200 µl of PBS and then tested with freeze-dried (FD) reagents. All samples were first heated at 95˚C for 5 min. Four different sample preparation styles were tested with the samples. Sample preparation styles are presented in Figure 5.

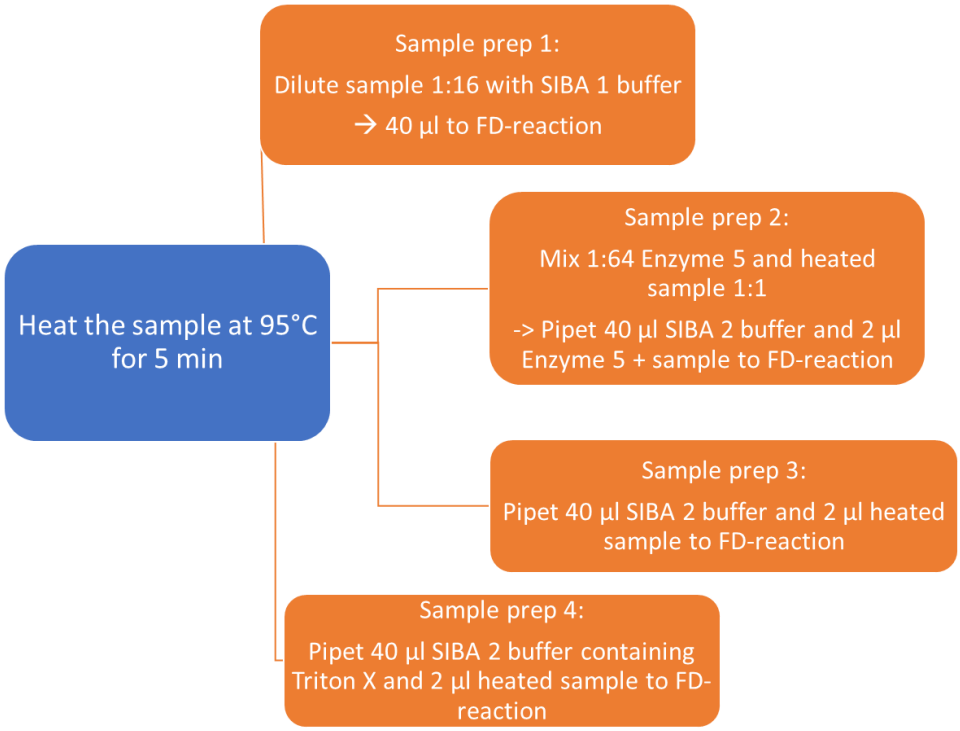

Figure 5. Sample preparation styles tested with clinical samples.

In each condition, there was also one positive control, where the growth GBS cells were used as a template. Also, NTC reaction was made in each condition. All reactions were made with two replicates.

Extracted gDNAs from clinical samples were tested with both SIBA freeze-dried reagents (without heating) and qPCR. In SIBA reaction and qPCR extracted GBS gDNA from bacterial suspension culture was used as a positive control. In addition, qPCR had also synthetic dsDNA as another positive control.

4 Results

4.1 qPCR quantification

GBS gDNA was extracted twice using EZ1 Advanced XL- robot. Copy numbers of extracted GBS gDNA were $2{,}3*10^3$ cp/µl and $5{,}0*10^3$ cp/µl. Elution volume used in first extraction was 200 µl and in second extraction 50 µl to increase the final DNA concentration. Copy number of extracted gDNA was quite low and therefore more GBS gDNA

was extracted manually by using QIAmp® DNA mini kit instead of EZ1 Advanced XL robot.

The sensitivity of the qPCR quantification was 2 cp/reaction since all replicates of all dilutions from $2*10^6$ cp/reaction to 2 cp/reaction amplified (Figure 6). Extracted GBS gDNA amplified from stock to $1:10^5$ dilution. One replicate of $1:10^6$ gDNA dilution amplified. NTCs did not amplify (Figure 6).

The copy number of GBS gDNA from extraction was $4,9 * 10^5$ cp/µl. Concentration was calculated based on samples 1- $1:10^5$, because all their replicates amplified.

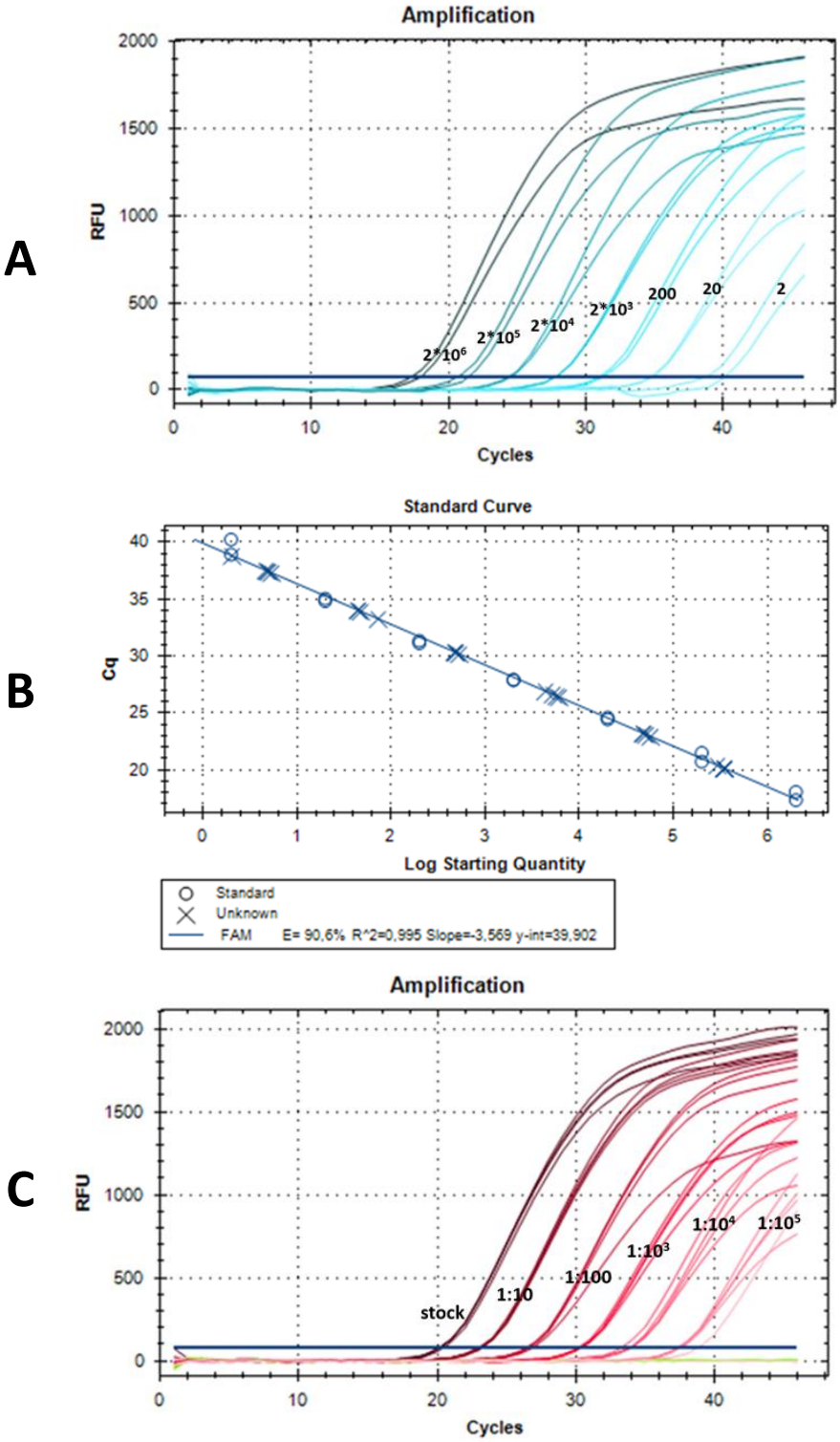

Figure 6. A: Amplification plots of synthetic dsDNA used as a standard. All replicates from $2*10^6$ cp/reaction to 2 cp/reaction amplified.
B: Standard curve used in quantification.
C: Amplification plots of extracted GBS gDNA. All replicates from stock to $1:10^5$ dilution amplified.

4.2 Oligo screening

In total, five different assays for detection of GBS were screened during this thesis and the results are presented in Table 7. Assays 1 and 2 were screened by testing primers individually and together in the presence of IO and the combinations, which passed, were tested with synthetic template. After that, the most promising combinations were tested with gDNA. The fastest combination of Assay 1 detected GBS around 20 minutes and the fastest combination of Assay 2 detected GBS around 25 minutes.

Table 7. Number of screening passed oligo combinations.

Assay	Screening step	Passed screening	Taken further
1	F+IOs	21/23	Not taken further
	R+IOs	9/15	
	F+R+IO1	112/189	
	F+R+IO2	100/189	
	F+R+IO3	13/189	
	F+R+IO1+synthetic template	4/85	
	F+R+IO2+synthetic template	15/84	
	F+R+IO3+synthetic template	5/13	
2	F+IOs	18/19	Not taken further
	R+IOs	21/21	
	F+R+IO1	73/378	
	F+R+IO2	64/378	
	F+R+IO3	232/378	
	F+R+IO1+synthetic template	19/69	
	F+R+IO2+synthetic template	1/43	
	F+R+IO3+synthetic template	1/45	
3	F+IOs	17/17	Not taken further
	R+IOs	12/12	
	F+R+IO1+synthetic template	0/48	
	F+R+IO2+synthetic template	0/48	
4	F+R+IO1	69/96	Not taken further
	F+R+IO2	95/96	
	F+R+IO1+synthetic template	6/96	
	F+R+IO2+synthetic template	0/96	
5	F+R+IO1	160/192	7 primer combinations taken further
	F+R+IO2	100/192	
	F+R+IO1+synthetic template	7/160	
	F+R+IO2+synthetic template	0/100	

To speed up the screening, Assay 3 was screened by testing primers individually in the presence of IO and then primer combinations straight with the template. None of the combinations tested with template passed, therefore, Assay 3 was discarded.

Six primer combinations with IO1 and template passed screening in Assay 4. The fastest combination detected GBS at 22 minutes and other combinations around 25 minutes. In Assay 5, seven primer combinations with IO1 and template passed the screening. All seven primer combinations detected GBS under 20 minutes, at fastest at 14.5 minutes. Assay 5 seemed to be the most promising assay for the detection of GBS based on the detection times and the fact, that there was not any unspecific amplification with these primer combinations. Therefore, the 7 primer combinations that passed screening in Assay 5 were taken further for assay optimization.

4.3 Assay optimization

Seven primer combinations were tested, but only the results of primer combination B5-F17/R7 are presented and results of other combinations are not shown.

Primer and oligo concentrations were optimized by increasing simultaneously both forward and reverse primer concentrations and by gradually increasing IO concentration from 200 nM. Tested concentration mixes and detection times of the B5-F17/R7 assay are presented in Table 8.

Table 8. Used primer and oligo concentrations and detection times of B5-F17/R7 assay.

F	R	IO	Average detection time (min)
200 nM	200 nM	200 nM	17.33
200 nM	200 nM	300 nM	16.13
200 nM	200 nM	400 nM	16.98
400 nM	400 nM	200 nM	12.01
400 nM	400 nM	300 nM	12.08
400 nM	400 nM	400 nM	11.47

It seems that higher primer concentration fastens the detection time, but there was also some unspecific amplification after 40 minutes. With IO concentration 300 nM, there was some unspecific amplification, but not as much as with IO concentrations 200 nM and 400 nM. On the basis of these results, the IO concentration was set to 300 nM and the primer concentrations to 400 nM.

Uvsx and Gp32 concentrations were optimized by testing three different concentrations 0.25 mg/ml, 0.30 mg/ml, and 0.35 mg/ml (Table 9). Primer concentrations used were 400 nM and IO concetration was 300 nM.

Table 9. Used Uvsx- and Gp32 concentrations and detection times of B5- F17/R7 assay.

Uvsx	Gp32	Average detection time (min)
0.25 mg/ml	0.25 mg/ml	13.85
0.3 mg/ml	0.3 mg/ml	10.9
0.35 mg/ml	0.35 mg/ml	12

It seems that the assay is fastest with Uvsx/Gp32 concentration 0.3 mg/ml. There was not any unspecific amplification with any of the enzyme concentrations. On the basis of these results Uvsx/Gp32 concentrations were set to 0.3 mg/ml.

Primer concentrations were further optimized by changing the forward and reverse primer concentrations between 300 nM and 400 nM while the IO concentration was 300 nM and Uvsx/Gp32 concentrations were 0.3 mg/ml. The tested concentration mixes and detection times are presented in Table 10.

Table 10. Used primer and IO concentrations and detection times of B5-F17/R7 assay.

F	R	IO	Average detection time (min)
300 nM	300 nM	300 nM	11.98
350 nM	300 nM	300 nM	11.53
400 nM	300 nM	300 nM	11.75
300 nM	350 nM	300 nM	11.58
350 nM	350 nM	300 nM	11
400 nM	350 nM	300 nM	10.9
300 nM	400 nM	300 nM	11.65
350 nM	400 nM	300 nM	11.06
400 nM	400 nM	300 nM	10.85

On the basis of these results primer concentrations F350/R300 nM and F300/R350 nM are the best, since there was not any unspecific amplification with them. With other concentration mixes, there was some unspecific amplification around 30 minutes. Primer concentration was set to F350/R300, since the detection time is slightly faster with it than with F300/R350.

Magnesium concentration was optimized after IO concentration was set to 300 nM and primer concentrations were set to F350 nM and R300 nM. Magnesium concentrations 8 mM, 10 mM, 12.5 mM, 15 mM, 17.5 mM and 20 mM were tested with the template concentration 100 cp per reaction. The results are shown in Table 11.

Table 11. MgAc concentrations used and corresponding detection times.

MgAc concentration	Average detection time (min)
8 mM	17.7
10 mM	16.18
12.5 mM	15.08
15 mM	12.13
17.5 mM	11.06
20 mM	10.61
50 mM	-

The detection time seems to be faster as the magnesium concentration increases, however, 50 mM is too high since there was nothing amplifying in that concentration. Detection times were at their fastest with MgAc concentrations 17.5 mM and 20 mM, but there was also substantial unspecific amplification occuring around 27 minutes. With MgAc concentrations 8 mM and 10 mM there was not any unspecific amplification. With MgAc concentrations 12.5 mM and 15 mM, there was some unspecific amplification after 40 minutes. The optimal magnesium concentration of this assay is probably somewhere between 10 to 15 mM, and it should be further optimized.

4.4 Sensitivity and specificity

Sensitivity test was done for two GBS assays after optimization. B5-F17/R7 assay detected all replicates from 100 000 cp to 100 cp per reaction (Figure 7). In addition, three of four replicates amplified with 10 cp/reaction. One replicate of 1cp/reaction was detected. None of the NTCs amplified.

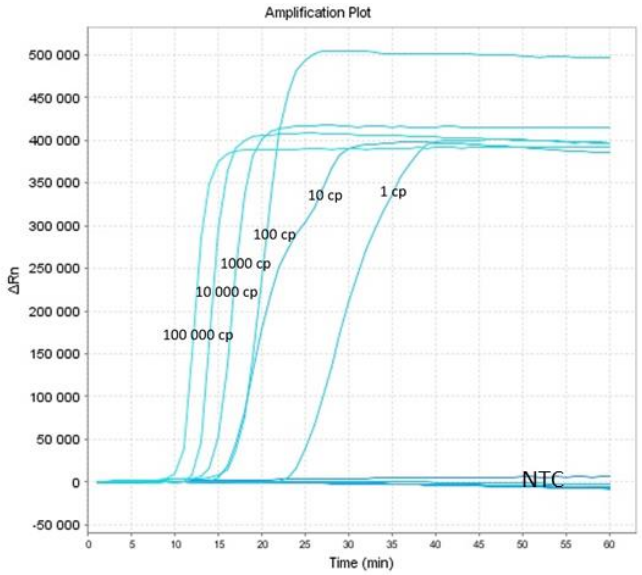

Figure 7. Amplification plots and NTCs of the B5-F17/R7 assay in sensitivity test. 100 000 cp, 10 000 cp, 1000 cp, 100 cp, 10 cp and 1 cp per reaction amplified. There wasn't any unspecific amplification.

B5-F11/R17 assay was more sensitive than B5-F17/R7 assay. It detected all replicates from 100 000 cp to 10 cp per reaction (Figure 8). In addition, 1 cp/reaction was detected in two replicates of four. There was quite a much unspecific amplification and therefore specificity test was done only for the B5-F17/R7 assay, which also had faster detection times than B5-F11/R17 assay.

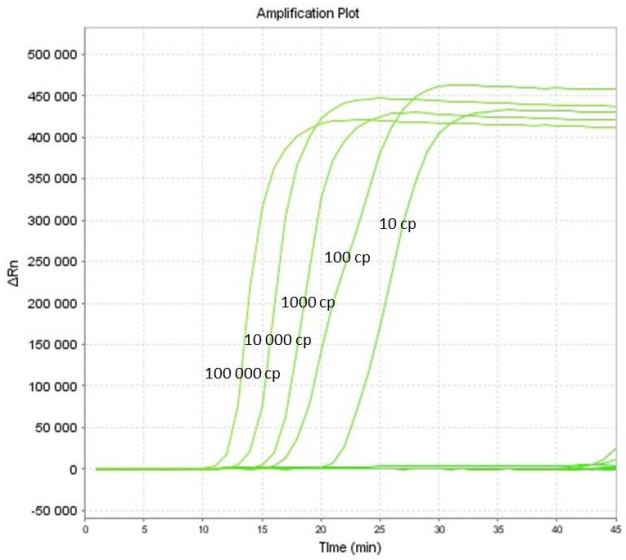

Figure 8. Amplification plots of the B5-F11/R17 assay in sensitivity test. 100 000 cp, 10 000 cp, 1000 cp, 100 cp,and 10 cp per reaction amplified. In addition, there was some unspecific amplification after 45 minutes.

Specificity was tested with ten different *Streptococcus* strains. Four of the strains were *Str.agalactiae* and the assay detected all replicates of them all (Figure 9.). Also, extracted GBS gDNA was detected. None of the other *Streptococcus* strains or unspecific amplification was detected.

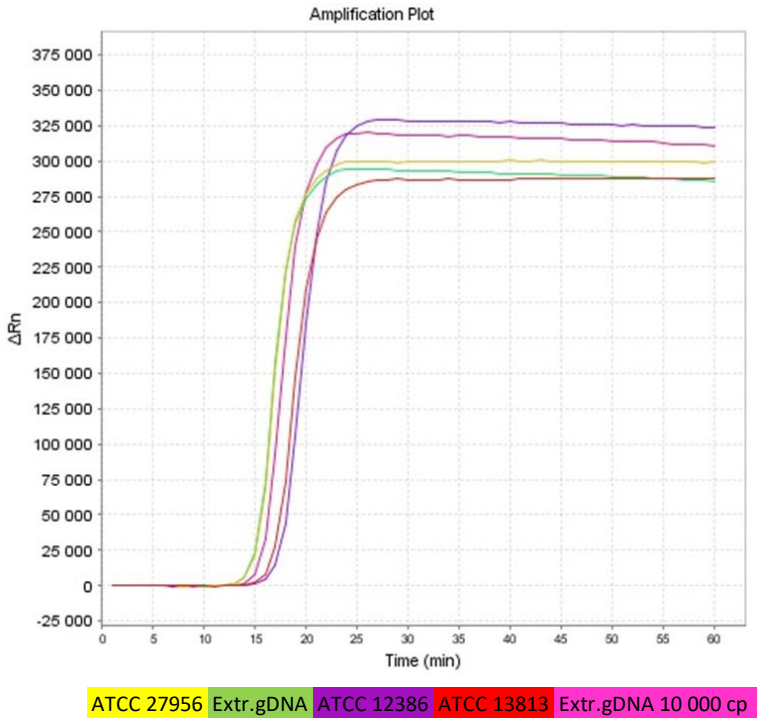

Figure 9. Amplification plots in specificity test. All *Streptococcus agalactiae* strains and extracted GBS gDNA amplified.

4.5 Enzymatic cell lysis

Impact of different enzymes and their concentrations on SIBA reaction is presented in Table 12. Used template was extracted GBS gDNA in concentration 100 cp/reaction. Enzyme 3 inhibited SIBA with all dilutions. Enzymes 2 and 5 inhibited SIBA with higher concentrations, but when they were diluted the inhibition faded. With enzymes 2 and 3 the detection time fastend the more the enzyme was diluted. Enzyme 2 worked fine in SIBA with dilutions 1:16 – 1:64 and enzyme 3 with dilution 1:64. With enzymes 1 and 4 the dilution of enzyme had no significant effect on detection time. Average detection time without any enzyme was 15.46 min.

Table 12. Impact of enzyme concentrations on detection time. Stock concentration of enzymes 1, 2, 3 and 4 was 10 mg/ml and stock concentration of enzyme 5 was 4.5 mg/ml. GBS gDNA 100 cp/reaction was used as a template.

	Average detection times (min)				
Enzyme dilution	Enzyme 1	Enzyme 2	Enzyme 3	Enzyme 4	Enzyme 5
without enzyme	15.46				
stock	15.73	40.37	-	17.27	-
1:2	16	21.1	-	17.03	-
1:4	17.67	21.47	-	19.4	-
1:8	17.63	17.43	-	17.4	31.33
1:16	18.23	17.67	-	19.3	18.1
1:32	16.43	16.87	-	17.27	17.37
1:64	17.36	17.4	-	17.33	18.03

The effect of lytic enzymes to GBS cells was tested with and without incubation (Figure 10). With all tested enzymes, the detection time was faster when the cells and enzymes were not incubated for 10 min at 37˚C. SIBA itself is basically an hour incubation at 41˚C and on the basis of these results a pre-incubation with enzyme before the SIBA run slows down the detection time.

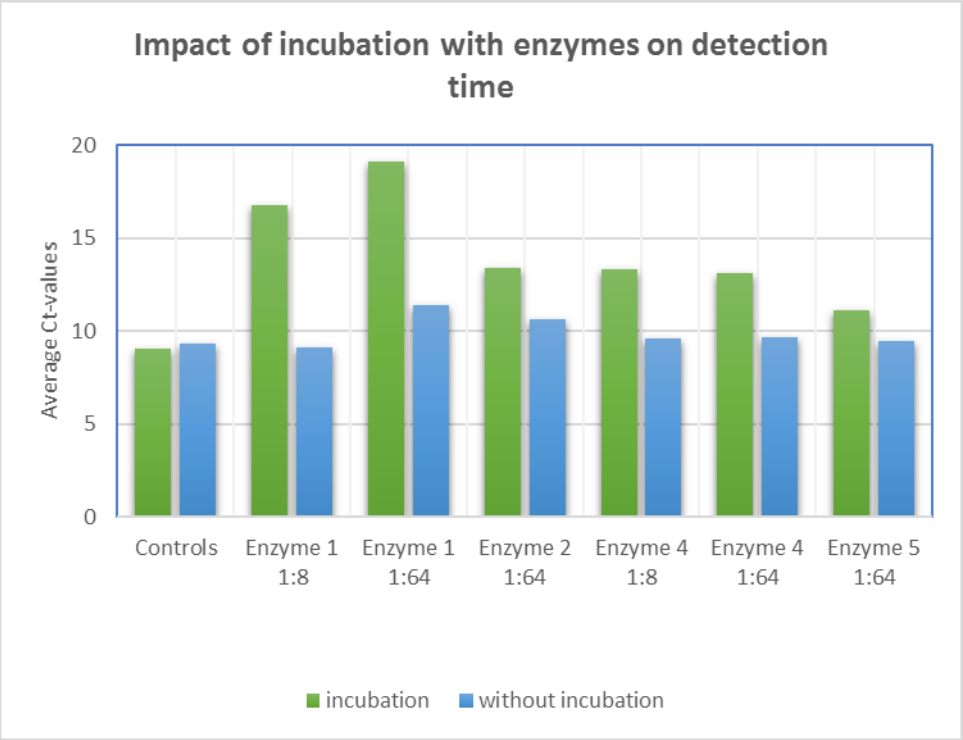

Figure 10. Impact of incubation on detection time. Cells were incubated with different enzymes at 37 °C for 10 minutes before SIBA run.

The effect of heating the cells before adding them to the SIBA reaction was tested by heating the cells for 5 minutes at 95°C. With enzyme 4 heating the sample seems to improve the sensitivity of the assay and speed up detection times (Figure 11). With enzyme 4 dilution 1:8 and heating the assay detected cells until dilution $1:10^4$, but without heating it only detected cells until dilution $1:10^2$. With enzyme 4 dilution 1:64 and heating, the assay detected all replicates of cells until dilution 1:100 and some replicates of cell dilution $1:10^3$ and $1:10^4$. Without heating, it detected all replicates of cells until dilution 1:100 and some replicates of cell dilution $1:10^3$. Without enzyme 4, the assay detected the same cell dilutions as with enzyme 4.

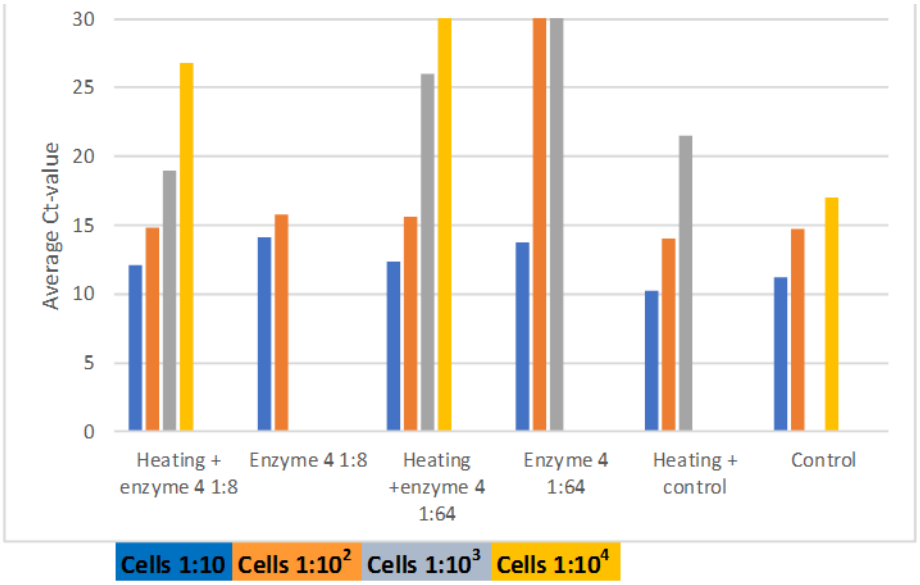

Figure 11. Impact of heating on detection limit with enzyme 4.

With enzyme 1, the detection times were faster when the cells were first heated (Figure 12). Heating did not seem to have any effect on the sensitivity of the assay with enzyme 1, since the assay detected only cell dilutions 1:10 and 1:100 with and without heating. There was not significant difference between enzyme 1 concentrations 1:8 and 1:64. With 1:8 dilution of enzyme 1, the detection times were slightly faster than with 1:64 dilution. Without enzyme 1, the assay detected same cell dilution as with enzyme 1.

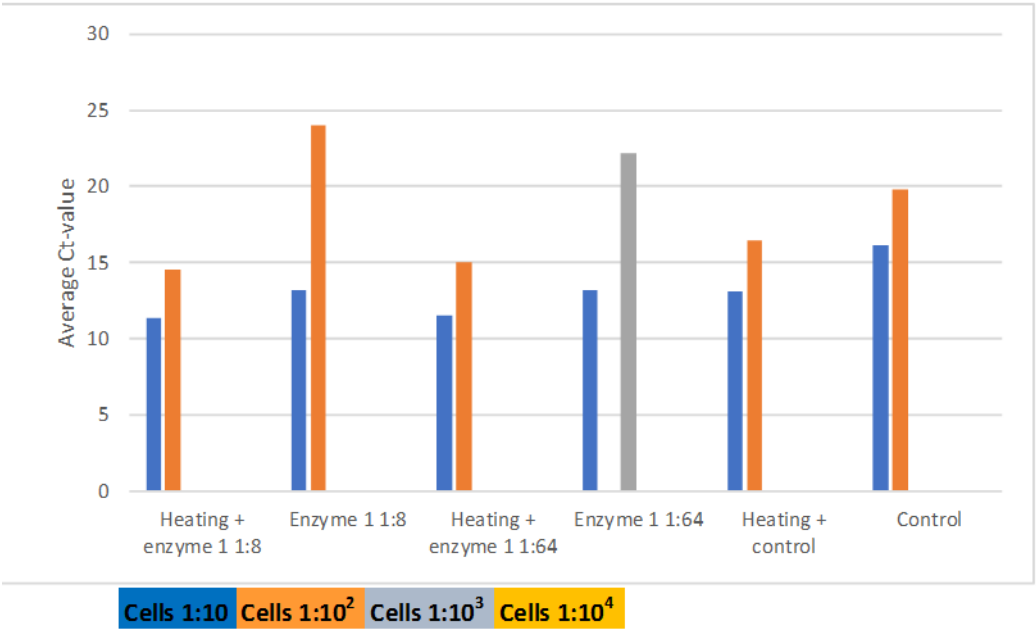

Figure 12. Impact of heating on detection limit with enzyme 1.

With enzyme 5 heating the cells seems to improve the sensitivity of the assay (Figure 13). Without heating, the cells assay detected all replicates until cell dilution 1:100, but with heating, it detected also all replicates of 1:1000 cell dilution and one replicate of 1:10 000 cell dilution. Also with enzyme 2, the sensitivity is improved when cells are heated. The assay detected all replicates until cell dilution 1:1000 and one replicate of 1:10 000 cell dilution when cells were heated first. Without heating, the cells assay detected only all replicates until cell dilutions 1:100.

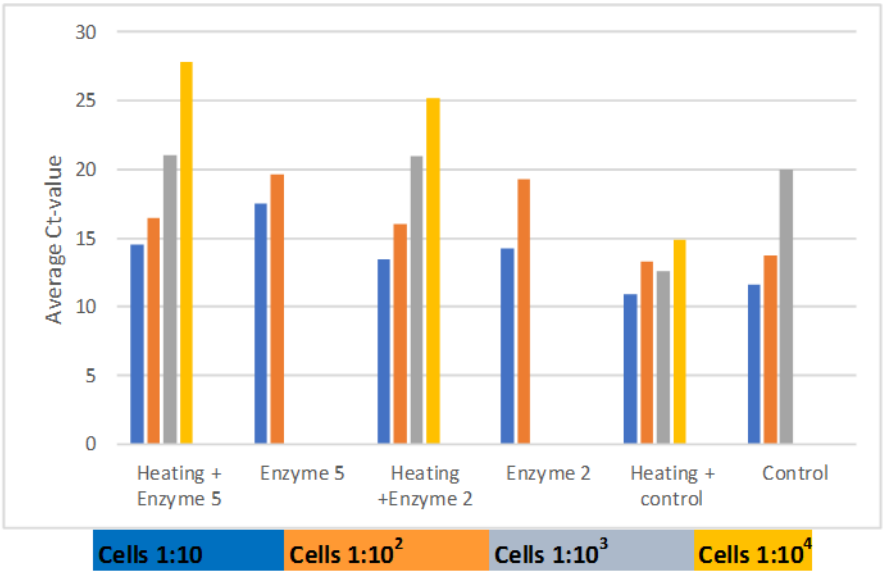

Figure 13. Impact of heating on detection limit with enzymes 2 and 5. Used concentration with both enzymes is 1:64.

When cells were heated, and there was not any lytic enzyme in the SIBA reaction, the assay detected all replicates until cell dilution 1:10^3 and one replicate of 1:10^4 cell dilution. Without heating, the cells and without any lytic enzyme the assay detected all replicates until cell dilution 1:100 and one replicate of 1:1000 cell dilution.

On the basis of these results, enzyme 5 with dilution 1:64, enzyme 2 with dilution 1:64 and enzyme 4 with dilution 1:8 seem to be the best. With each enzyme, it was seen that the assay detected cells also without the enzyme, thus, the enzymes did not really improve the sensitivity of the assay. Lytic enzymes should be tested more to find out at which cell concentration the lysis enzyme is required.

4.6 Freeze drying

Freeze drying was successful. All replicates of 100 000 cp and 10 000 cp amplified with both SIBA 1 and SIBA 2 buffers and there wasn't any unspecific amplification (Figure 14).

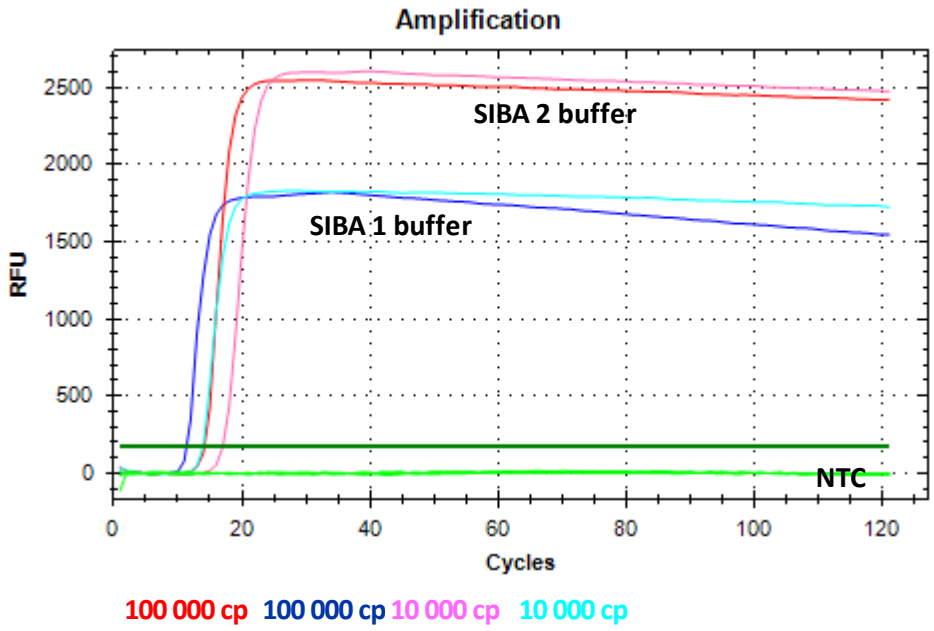

Figure 14. Amplification plots of freeze-dried trial batch testing. One cycle was 30 s.

Detection times were faster with SIBA 1 buffer than with SIBA 2 buffer, but signal levels were higher with SIBA 2 buffer. Average detection times (min) are presented in Table 13.

Table 13. Average detection times (min) of GBS gDNA with freeze-dried reagents.

	SIBA 1 buffer	SIBA 2 buffer
100 000 cp	5,69 min	7,29 min
10 000 cp	7,05 min	8,3 min

4.7 Clinical samples

qPCR

Extracted gDNA from serum and throat swab samples did not amplify at all in qPCR, which could mean they were low positive samples and qPCR was not sensitive enough to detect them, or the sample preparation method was not proper. Extracted gDNA from sputum sample amplified in all replicates around 24 cycles (Figure 15). All replicates of both positive controls amplified around 20 cycles. Negative control from extraction didn't amplify. None of NTC reactions amplified.

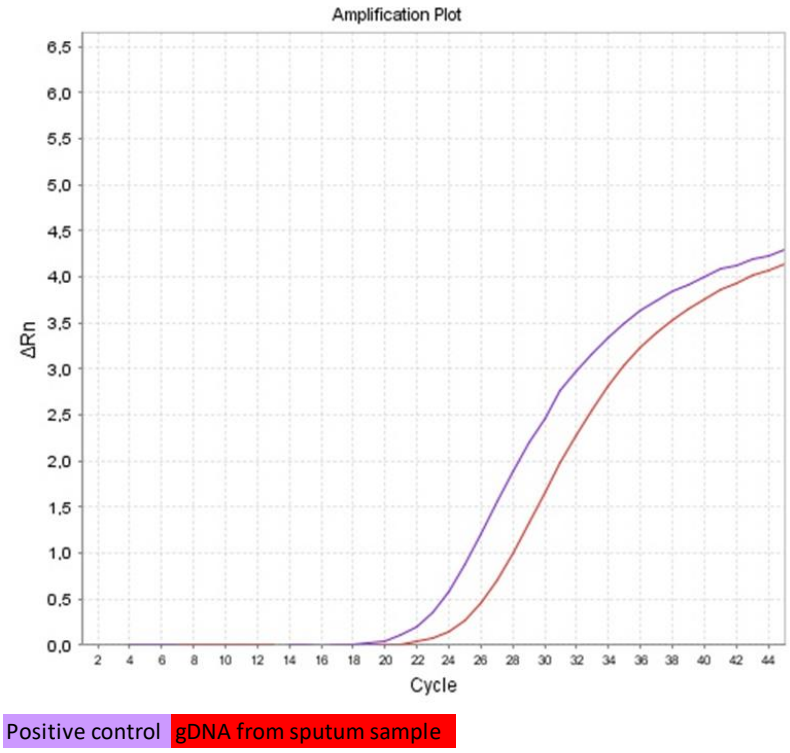

Figure 15. qPCR amplification plots of extracted gDNA from sputum sample and positive control.

Freeze-dried SIBA reagents

Likewise, extracted gDNA from throat swab sample did not amplify at all in FD-SIBA reaction. 1/2 replicate of gDNA from serum sample amplified at 22.7 min with Sample Prep 2. All replicates of gDNA from sputum sample amplified in all sample prep conditions (Figure 16). There was not significant difference between Sample Prep 1 and Sample Prep 3 when comparing detection times and signal levels. With Sample Prep 4 detection times were slowest and signal levels weakest. Sample Preparation Style 2 gave the highest signal levels. Sample preparation styles are presented in Figure 5. All control GBS gDNAs amplified between 7 and 10 min (Figure 16). PCR took over an hour to detect GBS, but SIBA took only 10 mins.

None of the throat swab samples amplified in FD-SIBA reaction or in qPCR. It may be that swab sample was not handled correctly, therefore, it did not amplify in both SIBA and qPCR. Only one replicate of the serum sample amplified with sample prep 2 at 13.8 min. After heating the serum sample, it became clumpy and therefore pipetting the sample to wells was challenging. It is possible that there was not enough serum sample in the wells, which would explain that only one of the replicates amplified. Sputum sample

amplified in all other sample prep conditions except in Sample Prep 1. Signal levels were highest and detection times fastest with Sample Preparation Style 2. With Sample Prep 4 the detection times and signal levels were the weakest.

Both NTC reactions with Sample Prep 1 amplified between 23 and 29 min. Also with sample prep 3 one NTC reaction amplified at 25.9 min. Other NTC reactions didn't amplify. The melt analysis of amplified NTCs suggests that there is some unspecific amplification. NTCs have not appeared in any of the previous experiments with this assay, so it is possible that the unspecific amplification in NTCs is caused by the buffers that were used in sample preparation. Optimization of the freeze-drying for this assay has not been done yet, so specificity could be improved by optimization.

All replicates of GBS cell controls amplified in all sample preparation conditions between 7 and 9 min (Figure 16). There was not significant difference between sample preparation styles, when looking at the detection times and signal levels of cell controls.

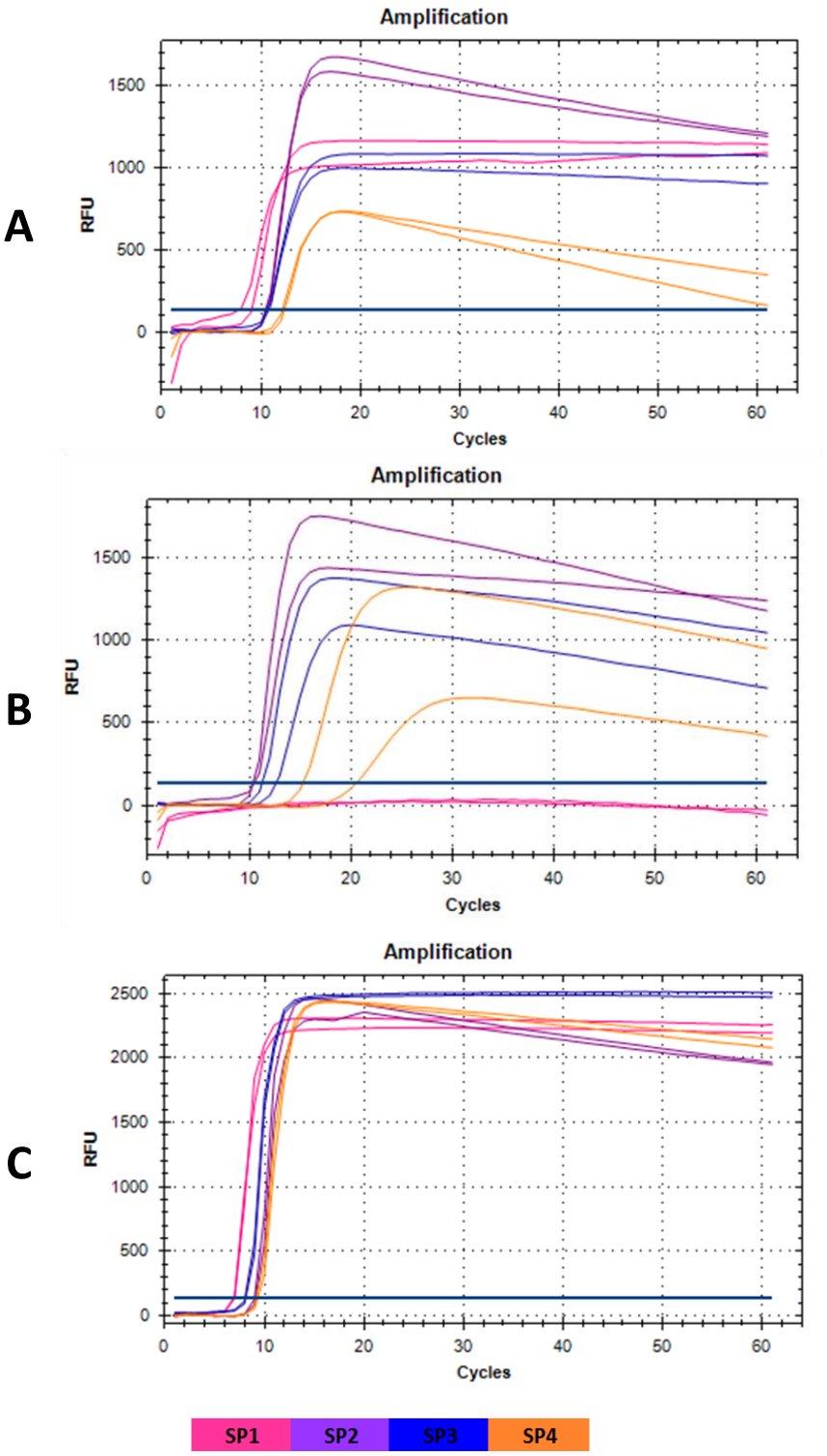

Figure 16. A: Amplification of extracted gDNA from sputum sample in different sample prep (SP) conditions.
B: Amplification of the sputum sample in different sample prep conditions.
C: Amplification of GBS cell controls in different sample prep conditions.
Two replicates were made of each reaction.

5 Discussion

The aim of this thesis was to develop a rapid and specific method for detection of *Streptococcus agalactiae* by using isothermal SIBA®-technology. SIBA is a nucleic acid method that is fast and can be performed on relatively small and low cost devices. Unlike PCR, SIBA does not require special equipment, which allows molecular diagnostic to be performed at the point of care.

Another aim was to study the effect of different lytic enzymes to SIBA® reactions and evaluate the lysis efficiency. The aim was to perform lysis and SIBA assay at the same time without separate lysis step.

As a result of the experimental part of this thesis, a rapid and specific assay for detection of GBS was successfully developed. The best developed SIBA assay detected 100 copies of GBS in 10.9 minutes in a wet mix. For PCR, it took over an hour to detect the same amount of GBS, thus, SIBA is significantly faster than PCR.

SIBA assay is specific, and it does not detect any other species than *Str.agalactiae* from *Streptococcus* genus. The sensitivity of the assay was 10 copies, and it should be further optimized so that also lower copy numbers could be detected.

When the effect of lytic enzymes on SIBA reactions was studied, it was noticed that enzymes 2, 4 and 5 work the best with SIBA®. On the basis of the experiments, it was discovered that heating the sample at 95°C was enough for cell lysis, at least in higher concentrations. Lytic enzyme testing should be continued to find at which cell concentration the lysis enzyme is required. Also, the need for lytic enzymes with clinical samples should be further studied. Growth cells may function better than clinical samples that have more cells and other components, which may inhibit SIBA.

The original aims of this thesis did not include freeze-drying, but because a good assay was successfully developed, freeze-drying was also done. Freeze-drying of the assay was successful, and with freeze-dried reagents, the assay detected GBS in 5.7 minutes at its best. Freeze-dried reagents were also tested with three GBS positive clinical samples and four different sample preparation methods. The assay detected part of the clinical samples, and there was some unspecific amplification. Sample preparation should

be developed further, since not all positive samples were detected. Also sample preparation buffers may have caused unspecific amplification.

In conclusion, the developed SIBA assay for the detection of GBS displays good analytical sensitivity and specificity. It is significantly faster than culturing or PCR. The assay could be suitable for screening GBS at the point of care or, for example, in the maternity or pediatric wards.

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Appendix 1. qPCR raw data

Table 1. Raw data from first qPCR quantification

	Well	Content	Cq	Cq Mean	Cq Std. Dev	Starting Quantity (SQ)	Log SQ	SQ Mean	SQ Std. Dev
GBS stock	A01	Unkn-1	28,91	28,73	0,14	1,43E+03	3,155	1,60E+03	1,39E+02
	B01	Unkn-1	28,78	28,73	0,14	1,55E+03	3,19	1,60E+03	1,39E+02
	C01	Unkn-1	28,61	28,73	0,14	1,72E+03	3,236	1,60E+03	1,39E+02
	D01	Unkn-1	28,63	28,73	0,14	1,70E+03	3,231	1,60E+03	1,39E+02
GBS 1:10	E01	Unkn-2	32,02	32,03	0,197	2,03E+02	2,307	2,03E+02	2,47E+01
	F01	Unkn-2	32	32,03	0,197	2,05E+02	2,311	2,03E+02	2,47E+01
	G01	Unkn-2	31,8	32,03	0,197	2,32E+02	2,365	2,03E+02	2,47E+01
	H01	Unkn-2	32,28	32,03	0,197	1,72E+02	2,235	2,03E+02	2,47E+01
GBS 1:100	A02	Unkn-3	35,45	35,38	0,494	2,35E+01	1,371	2,55E+01	8,21E+00
	B02	Unkn-3	35,39	35,38	0,494	2,43E+01	1,386	2,55E+01	8,21E+00
	C02	Unkn-3	35,93	35,38	0,494	1,73E+01	1,239	2,55E+01	8,21E+00
	D02	Unkn-3	34,73	35,38	0,494	3,69E+01	1,567	2,55E+01	8,21E+00
GBS $1:10^3$	E02	Unkn-4	38,35	38,35	0	3,80E+00	0,58	3,80E+00	0,00E+00
	F02	Unkn-4	N/A	0	0	N/A	N/A	0,00E+00	0,00E+00
	G02	Unkn-4	N/A	0	0	N/A	N/A	0,00E+00	0,00E+00
	H02	Unkn-4	N/A	0	0	N/A	N/A	0,00E+00	0,00E+00
GBS $1:10^4$	A03	Unkn-5	42,82	40,81	2,833	2,29E-01	-0,641	1,53E+00	1,84E+00
	B03	Unkn-5	38,81	40,81	2,833	2,84E+00	0,453	1,53E+00	1,84E+00
	C03	Unkn-5	N/A	0	0	N/A	N/A	0,00E+00	0,00E+00
	D03	Unkn-5	N/A	0	0	N/A	N/A	0,00E+00	0,00E+00
GBS $1:10^5$	E03	Unkn-6	N/A	0	0	N/A	N/A	0,00E+00	0,00E+00
	F03	Unkn-6	N/A	0	0	N/A	N/A	0,00E+00	0,00E+00
	G03	Unkn-6	N/A	0	0	N/A	N/A	0,00E+00	0,00E+00
	H03	Unkn-6	N/A	0	0	N/A	N/A	0,00E+00	0,00E+00
GBS $1:10^6$	A04	Unkn-7	N/A	0	0	N/A	N/A	0,00E+00	0,00E+00
	B04	Unkn-7	N/A	0	0	N/A	N/A	0,00E+00	0,00E+00
	C04	Unkn-7	N/A	0	0	N/A	N/A	0,00E+00	0,00E+00
	D04	Unkn-7	N/A	0	0	N/A	N/A	0,00E+00	0,00E+00
GBS $1:10^7$	E04	Unkn-8	N/A	0	0	N/A	N/A	0,00E+00	0,00E+00
	F04	Unkn-8	N/A	0	0	N/A	N/A	0,00E+00	0,00E+00
	G04	Unkn-8	N/A	0	0	N/A	N/A	0,00E+00	0,00E+00
	H04	Unkn-8	N/A	0	0	N/A	N/A	0,00E+00	0,00E+00
GBS $1:10^8$	A05	Unkn-9	N/A	0	0	N/A	N/A	0,00E+00	0,00E+00
	B05	Unkn-9	N/A	0	0	N/A	N/A	0,00E+00	0,00E+00
	C05	Unkn-9	N/A	0	0	N/A	N/A	0,00E+00	0,00E+00
	D05	Unkn-9	N/A	0	0	N/A	N/A	0,00E+00	0,00E+00
NTC	E12	NTC	N/A	0	0	N/A	N/A	0,00E+00	0,00E+00
	F12	NTC	N/A	0	0	N/A	N/A	0,00E+00	0,00E+00
	G12	NTC	N/A	0	0	N/A	N/A	0,00E+00	0,00E+00
	H12	NTC	N/A	0	0	N/A	N/A	0,00E+00	0,00E+00
Standards	A06	Std	17,52	17,52	0	2,00E+06	6,301	2,00E+06	0,00E+00
	A07	Std	21,12	21,12	0	2,00E+05	5,301	2,00E+05	0,00E+00
	A08	Std	24,63	24,63	0	2,00E+04	4,301	2,00E+04	0,00E+00
	A09	Std	28,26	28,26	0	2,00E+03	3,301	2,00E+03	0,00E+00
	A10	Std	32,34	32,34	0	2,00E+02	2,301	2,00E+02	0,00E+00
	A11	Std	36,17	36,17	0	2,00E+01	1,301	2,00E+01	0,00E+00
	A12	Std	N/A	0	0	2,00E+00	0,301	0,00E+00	0,00E+00
	B06	Std	17,2	17,2	0	2,00E+06	6,301	2,00E+06	0,00E+00
	B07	Std	21,5	21,5	0	2,00E+05	5,301	2,00E+05	0,00E+00
	B08	Std	24,39	24,39	0	2,00E+04	4,301	2,00E+04	0,00E+00
	B09	Std	28,14	28,14	0	2,00E+03	3,301	2,00E+03	0,00E+00
	B10	Std	31,72	31,72	0	2,00E+02	2,301	2,00E+02	0,00E+00
	B11	Std	35,51	35,51	0	2,00E+01	1,301	2,00E+01	0,00E+00
	B12	Std	N/A	0	0	2,00E+00	0,301	0,00E+00	0,00E+00

Table 2. Raw data from second qPCR quantification.

	Well	Content	Cq	Cq Mean	Cq Std. Dev	Starting Quantity (SQ)	Log SQ	SQ Mean	SQ Std. Dev
GBS stock	A01	Unkn-1	27,97	27,64	0,222	3,15E+03	3,499	3,94E+03	5,31E+02
	B01	Unkn-1	27,5	27,64	0,222	4,29E+03	3,633	3,94E+03	5,31E+02
	C01	Unkn-1	27,55	27,64	0,222	4,15E+03	3,618	3,94E+03	5,31E+02
	D01	Unkn-1	27,54	27,64	0,222	4,18E+03	3,622	3,94E+03	5,31E+02
GBS 1:10	E01	Unkn-2	31,1	31,07	0,268	4,08E+02	2,611	4,22E+02	7,74E+01
	F01	Unkn-2	31,14	31,07	0,268	3,98E+02	2,599	4,22E+02	7,74E+01
	G01	Unkn-2	30,69	31,07	0,268	5,32E+02	2,726	4,22E+02	7,74E+01
	H01	Unkn-2	31,33	31,07	0,268	3,50E+02	2,544	4,22E+02	7,74E+01
GBS 1:100	A02	Unkn-3	35,62	34,5	0,795	2,13E+01	1,329	4,84E+01	2,08E+01
	B02	Unkn-3	33,78	34,5	0,795	7,09E+01	1,851	4,84E+01	2,08E+01
	C02	Unkn-3	34,45	34,5	0,795	4,57E+01	1,66	4,84E+01	2,08E+01
	D02	Unkn-3	34,15	34,5	0,795	5,56E+01	1,745	4,84E+01	2,08E+01
GBS $1:10^3$	E02	Unkn-4	38,33	37,49	0,733	3,64E+00	0,561	6,82E+00	3,13E+00
	F02	Unkn-4	37,85	37,49	0,733	4,98E+00	0,697	6,82E+00	3,13E+00
	G02	Unkn-4	36,69	37,49	0,733	1,06E+01	1,025	6,82E+00	3,13E+00
	H02	Unkn-4	37,11	37,49	0,733	8,05E+00	0,906	6,82E+00	3,13E+00
GBS $1:10^4$	A03	Unkn-5	3,63	3,63	0	2,53E+10	10,403	2,53E+10	0,00E+00
	B03	Unkn-5	N/A	0	0	N/A	N/A	0,00E+00	0,00E+00
	C03	Unkn-5	N/A	0	0	N/A	N/A	0,00E+00	0,00E+00
	D03	Unkn-5	N/A	0	0	N/A	N/A	0,00E+00	0,00E+00
GBS $1:10^5$	E03	Unkn-6	N/A	0	0	N/A	N/A	0,00E+00	0,00E+00
	F03	Unkn-6	N/A	0	0	N/A	N/A	0,00E+00	0,00E+00
	G03	Unkn-6	N/A	0	0	N/A	N/A	0,00E+00	0,00E+00
	H03	Unkn-6	N/A	0	0	N/A	N/A	0,00E+00	0,00E+00
GBS $1:10^6$	A04	Unkn-7	6,31	6,31	0	4,40E+09	9,644	4,40E+09	0,00E+00
	B04	Unkn-7	N/A	0	0	N/A	N/A	0,00E+00	0,00E+00
	C04	Unkn-7	N/A	0	0	N/A	N/A	0,00E+00	0,00E+00
	D04	Unkn-7	N/A	0	0	N/A	N/A	0,00E+00	0,00E+00
GBS $1:10^7$	E04	Unkn-8	N/A	0	0	N/A	N/A	0,00E+00	0,00E+00
	F04	Unkn-8	N/A	0	0	N/A	N/A	0,00E+00	0,00E+00
	G04	Unkn-8	N/A	0	0	N/A	N/A	0,00E+00	0,00E+00
	H04	Unkn-8	N/A	0	0	N/A	N/A	0,00E+00	0,00E+00
GBS $1:10^8$	A05	Unkn-9	4,25	4,25	0	1,68E+10	10,226	1,68E+10	0,00E+00
	B05	Unkn-9	N/A	0	0	N/A	N/A	0,00E+00	0,00E+00
	C05	Unkn-9	N/A	0	0	N/A	N/A	0,00E+00	0,00E+00
	D05	Unkn-9	N/A	0	0	N/A	N/A	0,00E+00	0,00E+00
NTC	E12	NTC	N/A	0	0	N/A	N/A	0,00E+00	0,00E+00
	F12	NTC	N/A	0	0	N/A	N/A	0,00E+00	0,00E+00
	G12	NTC	N/A	0	0	N/A	N/A	0,00E+00	0,00E+00
	H12	NTC	N/A	0	0	N/A	N/A	0,00E+00	0,00E+00
Standards	A06	Std	18,6	18,6	0	2,00E+06	6,301	2,00E+06	0,00E+00
	A07	Std	21,38	21,38	0	2,00E+05	5,301	2,00E+05	0,00E+00
	A08	Std	25,32	25,32	0	2,00E+04	4,301	2,00E+04	0,00E+00
	A09	Std	29,09	29,09	0	2,00E+03	3,301	2,00E+03	0,00E+00
	A10	Std	31,42	31,42	0	2,00E+02	2,301	2,00E+02	0,00E+00
	A11	Std	35,29	35,29	0	2,00E+01	1,301	2,00E+01	0,00E+00
	A12	Std	N/A	0	0	2,00E+00	0,301	0,00E+00	0,00E+00
	B06	Std	17,79	17,79	0	2,00E+06	6,301	2,00E+06	0,00E+00
	B07	Std	21,51	21,51	0	2,00E+05	5,301	2,00E+05	0,00E+00
	B08	Std	25,01	25,01	0	2,00E+04	4,301	2,00E+04	0,00E+00
	B09	Std	28,48	28,48	0	2,00E+03	3,301	2,00E+03	0,00E+00
	B10	Std	32,6	32,6	0	2,00E+02	2,301	2,00E+02	0,00E+00
	B11	Std	36,39	36,39	0	2,00E+01	1,301	2,00E+01	0,00E+00
	B12	Std	39,19	39,19	0	2,00E+00	0,301	2,00E+00	0,00E+00

Table 3. Raw data from third qPCR quantification.

	Well	Content	Cq	Cq Mean	Cq Std. Dev	Starting Quantity (SQ)	Log SQ	SQ Mean	SQ Std. Dev
GBS stock	A01	Unkn-1	2,04E+01	2,02E+01	0,134	2,93E+05	5,47E+00	3,35E+05	2,76E+04
	B01	Unkn-1	2,01E+01	2,02E+01	0,134	3,51E+05	5,55E+00	3,35E+05	2,76E+04
	C01	Unkn-1	2,01E+01	2,02E+01	0,134	3,50E+05	5,54E+00	3,35E+05	2,76E+04
	D01	Unkn-1	2,01E+01	2,02E+01	0,134	3,45E+05	5,54E+00	3,35E+05	2,76E+04
GBS 1:10	E01	Unkn-2	2,29E+01	2,31E+01	0,128	5,74E+04	4,76E+00	5,14E+04	4,34E+03
	F01	Unkn-2	2,31E+01	2,31E+01	0,128	5,17E+04	4,71E+00	5,14E+04	4,34E+03
	G01	Unkn-2	2,32E+01	2,31E+01	0,128	4,88E+04	4,69E+00	5,14E+04	4,34E+03
	H01	Unkn-2	2,32E+01	2,31E+01	0,128	4,77E+04	4,68E+00	5,14E+04	4,34E+03
GBS 1:100	A02	Unkn-3	2,69E+01	2,66E+01	0,229	4,38E+03	3,64E+00	5,39E+03	7,64E+02
	B02	Unkn-3	2,64E+01	2,66E+01	0,229	6,14E+03	3,79E+00	5,39E+03	7,64E+02
	C02	Unkn-3	2,65E+01	2,66E+01	0,229	5,77E+03	3,76E+00	5,39E+03	7,64E+02
	D02	Unkn-3	2,66E+01	2,66E+01	0,229	5,25E+03	3,72E+00	5,39E+03	7,64E+02
GBS $1:10^3$	E02	Unkn-4	3,03E+01	3,03E+01	0,077	4,88E+02	2,69E+00	4,98E+02	2,54E+01
	F02	Unkn-4	3,02E+01	3,03E+01	0,077	5,35E+02	2,73E+00	4,98E+02	2,54E+01
	G02	Unkn-4	3,03E+01	3,03E+01	0,077	4,90E+02	2,69E+00	4,98E+02	2,54E+01
	H02	Unkn-4	3,03E+01	3,03E+01	0,077	4,77E+02	2,68E+00	4,98E+02	2,54E+01
GBS $1:10^4$	A03	Unkn-5	3,40E+01	3,38E+01	0,365	4,49E+01	1,65E+00	5,27E+01	1,37E+01
	B03	Unkn-5	3,40E+01	3,38E+01	0,365	4,48E+01	1,65E+00	5,27E+01	1,37E+01
	C03	Unkn-5	3,33E+01	3,38E+01	0,365	7,32E+01	1,86E+00	5,27E+01	1,37E+01
	D03	Unkn-5	3,39E+01	3,38E+01	0,365	4,81E+01	1,68E+00	5,27E+01	1,37E+01
GBS $1:10^5$	E03	Unkn-6	3,74E+01	3,74E+01	0,093	5,05E+00	7,03E-01	5,00E+00	3,04E-01
	F03	Unkn-6	3,75E+01	3,74E+01	0,093	4,69E+00	6,71E-01	5,00E+00	3,04E-01
	G03	Unkn-6	3,75E+01	3,74E+01	0,093	4,87E+00	6,87E-01	5,00E+00	3,04E-01
	H03	Unkn-6	3,73E+01	3,74E+01	0,093	5,40E+00	7,33E-01	5,00E+00	3,04E-01
GBS $1:10^6$	A04	Unkn-7	N/A	0,00E+00	0	N/A	N/A	0,00E+00	0,00E+00
	B04	Unkn-7	3,88E+01	3,88E+01	0	2,05E+00	3,12E-01	2,05E+00	0,00E+00
	C04	Unkn-7	N/A	0,00E+00	0	N/A	N/A	0,00E+00	0,00E+00
	D04	Unkn-7	N/A	0,00E+00	0	N/A	N/A	0,00E+00	0,00E+00
GBS $1:10^7$	E04	Unkn-8	N/A	0,00E+00	0	N/A	N/A	0,00E+00	0,00E+00
	F04	Unkn-8	N/A	0,00E+00	0	N/A	N/A	0,00E+00	0,00E+00
	G04	Unkn-8	N/A	0,00E+00	0	N/A	N/A	0,00E+00	0,00E+00
	H04	Unkn-8	N/A	0,00E+00	0	N/A	N/A	0,00E+00	0,00E+00
GBS $1:10^8$	A05	Unkn-9	N/A	0,00E+00	0	N/A	N/A	0,00E+00	0,00E+00
	B05	Unkn-9	N/A	0,00E+00	0	N/A	N/A	0,00E+00	0,00E+00
	C05	Unkn-9	N/A	0,00E+00	0	N/A	N/A	0,00E+00	0,00E+00
	D05	Unkn-9	N/A	0,00E+00	0	N/A	N/A	0,00E+00	0,00E+00
NTC	E12	NTC	N/A	0	0	N/A	N/A	0,00E+00	0,00E+00
	F12	NTC	N/A	0	0	N/A	N/A	0,00E+00	0,00E+00
	G12	NTC	N/A	0	0	N/A	N/A	0,00E+00	0,00E+00
	H12	NTC	N/A	0	0	N/A	N/A	0,00E+00	0,00E+00
Standards	A06	Std-1	18,05	1,77E+01	0,506	2,00E+06	6,30E+00	2,00E+06	0,00E+00
	B06	Std-1	17,34	1,77E+01	0,506	2,00E+06	6,30E+00	2,00E+06	0,00E+00
	A07	Std-2	21,48	2,11E+01	0,547	2,00E+05	5,30E+00	2,00E+05	0,00E+00
	B07	Std-2	20,71	2,11E+01	0,547	2,00E+05	5,30E+00	2,00E+05	0,00E+00
	A08	Std-3	24,58	2,45E+01	0,115	2,00E+04	4,30E+00	2,00E+04	0,00E+00
	B08	Std-3	24,42	2,45E+01	0,115	2,00E+04	4,30E+00	2,00E+04	0,00E+00
	A09	Std-4	27,91	2,79E+01	0,032	2,00E+03	3,30E+00	2,00E+03	0,00E+00
	B09	Std-4	27,86	2,79E+01	0,032	2,00E+03	3,30E+00	2,00E+03	0,00E+00
	A10	Std-5	31,32	3,12E+01	0,144	2,00E+02	2,30E+00	2,00E+02	0,00E+00
	B10	Std-5	31,11	3,12E+01	0,144	2,00E+02	2,30E+00	2,00E+02	0,00E+00
	A11	Std-6	35,02	3,49E+01	0,148	2,00E+01	1,30E+00	2,00E+01	0,00E+00
	B11	Std-6	34,81	3,49E+01	0,148	2,00E+01	1,30E+00	2,00E+01	0,00E+00
	A12	Std-7	40,21	3,96E+01	0,932	2,00E+00	3,01E-01	2,00E+00	0,00E+00
	B12	Std-7	38,89	3,96E+01	0,932	2,00E+00	3,01E-01	2,00E+00	0,00E+00