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Development of a multiplex real time PCR assay to target bacteria causing meningitis

Johan Lagmo

UPPSALA UNIVERSITET

Molecular Biotechnology Programme

Uppsala University School of Engineering

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Author

Johan Lagmo

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Abstract

Meningitis is a severe disease with fatal outcome if not treated properly. This project evaluate and recommend a variation of multiplex qPCR assays which identify three bacteria causing meningitis, *Neisseria meningitidis*, *Haemophilus influenzae* and *Streptococcus pneumoniae*. The evaluation was based on the analytical sensitivity and specificity of the assays and a comparative analysis on clinical specimens. The qPCR assay was also prepared to be a routine method for the Academic Hospital in Uppsala.

Keywords

Meningitis, Real Time PCR, qPCR, multiplex PCR, *Neisseria meningitidis*, *Haemophilus influenzae*, *Streptococcus pneumoniae*, analytical sensitivity

Supervisors

Björn Herrmann
Uppsala University

Scientific reviewer

Jonas Blomberg
Uppsala University

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Biology Education Centre Biomedical Center Husargatan 3 Uppsala
Box 592 S-75124 Uppsala Tel +46 (0)18 4710000 Fax +46 (0)18 471 4687

Utvecklandet av en multiplex realtids PCR för att detektera bakterier som orsakar hjärnhinneinflammation.

Johan Lagmo

Populärvetenskaplig sammanfattning

Hjärnhinneinflammation är en mycket allvarlig sjukdom som framförallt drabbar äldre och unga barn. Om sjukdomen inte behandlas har den ofta dödlig utgång eller ger stora men för livet. Om sjukdomen upptäcks i ett tidigt stadium och behandlas korrekt är långvariga men och mortalitet betydligt ovanligare.

Inflammationen kan orsakas av bakterier, virus, svampar eller parasiter. Den här studien fokuserar endast på bakterier som kan orsaka sjukdomen. Dessa är *Neisseria meningitidis*, *Haemophilus influenzae* och *Streptococcus pneumoniae*.

För att upptäcka sjukdomen i ett tidigt stadie krävs bra detektionsmetoder. Tidigare har odling använts för att bestämma vad som har orsakat en infektion. Idag används den betydligt snabbare metoden Polymerase Chain Reaction (PCR) som undersöker om vissa DNA sekvenser (arvsmassa) är närvarande i provet. Det prov som undersöks är ett likvorprov. Likvorvätska finns i det centrala nervsystemet. Provet tas från ryggraden på patienten som man misstänker ha hjärnhinneinflammation. PCR metoden ger utslag på bara några timmar och säger även vilken bakterie som orsakar inflammationen.

Om metoden ger utslag kan olika antibiotika sättas in snabbt, anpassat efter vilken bakterie som har orsakat inflammationen. Provet kan också bli negativt vilket skulle visa att inflammationsorsaken med största sannolikhet inte är bakteriell, och då behöver inte antibiotika användas.

Det här projektet gick ut på att minska arbetstiden för PCRmetoden, underlätta för de som arbetar med processen och göra detektionen känsligare. Allt detta uppnås genom att köra en multiplex PCR. Man kör då flera olika PCR reaktioner i ett och samma rör. En multiplex PCR tappar ofta känslighet jämfört med att köra den i singleplex-format. Delarna i en multiplex assay är mer benägna att reagera med varandra och mängden reagens måste därför hållas till ett minimum.

Med teoretiska och praktiska metoder kan en multiplex assay presenteras som uppfyller kraven för projektet och som kringgår problemen.

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Abbreviations

9802	Target gene for *Spn*
ctrA	Target gene for *Nm*
ΔG	Change in Gibbs free energy
DNA	Deoxyribonucleic acid, contains our genetic information
fucK	Target gene for *Hi*
Hi	*Haemophilus influenzae*
hpd	Target gene for *Hi*
lytA	Target gene for *Spn*
Mnc	Meningococcus
Nm	*Neisseria meningitidis*
PCR	Polymerase Chain reaction
qPCR	Real-Time PCR
Spn	*Streptococcus pneumoniae*

Technical terms

Assay	In this report "assay" refers to a pair of primers and a fluorescent probe binding within the amplified target of the primers.
CP or Ct value	The number of cycles required for detection of a fluorescence signal. The signal strength needs to pass a set threshold. Higher value means lower concentration of DNA.
CSF	Cerebrospinal fluid: The liquid in subarachnoid space and ventricular system.
Fluorophore	A chemical compound that can emit light.
Hedberg primers	Primers that target *lytA*, obtained from Hedberg *et al.* 2009.
Multiplex PCR	A PCR that has more than one target in the same reaction solution.
Oligonucleotide	Short single stranded DNA or RNA sequence.
Polymerase	The enzyme that incorporates nucleotides into a DNA-sequence.
Primer	A short oligonucleotide which serves as a starting point for the polymerase in a PCR.
Primer dimer	When two primers can bind to each other in a way, which enables the polymerase to amplify the product.
Probe	An oligonucleotide labeled with a fluorophore, which can be activated when binding to its target.
Real-Time PCR	A PCR that enables detection of the amplified DNA during the progress of the PCR.
SYBR green	A cyanine dye used to stain double stranded DNA. Emits light of wavelength λ=520 nm.
Taq polymerase	A heat stable polymerase usually used in PCR.
Visual OMP	Software to design primers and probes.

Introduction

This study sat up a real time PCR for the detection of *Neisseria meningitides (Nm or Mnc), Haemophilus influenza (Hi)* and *Streptococcus pneumonia (Spn),* the three major causes of bacterial meningitis in the world. Meningitis is inflammation of the membranes of the brain and spinal cord. The disease can be fatal and a fast and proper treatment is needed for the safety of the patient. Post infection, a patient can suffer long term health illnesses such as deafness, epilepsy and hydrocephalus. These detriments can be reduced if treated when the first symptoms appear. If left untreated meningitis is fatal in almost all cases. Mortality is dropped greatly with good treatment, down to 20-30% for newborns and 20-40% in elder adults (Sáez-Llorens *et al.* 2003) (van de Beek *et al.* 2006). For older children and young adults the risk for fatality is very low with proper treatment.

Antibiotics are used against infections of bacterial meningitis with good results. However, different bacteria might need different antibiotics to be eliminated. A fast characterization of the bacteria is therefore important. Since the 1980's several countries have included immunization against *Hi* type B in their routine vaccinations for children. This has greatly reduced fatal infections in young children and will reduce them for older generations in the future.

Meningitis is a global problem and affects both economically well developed countries as well as resource poor countries. However, in economically well developed countries less than 0,01% is effected by the disease, while up to 1% may be affected in some developing countries during epidemics.

This study started with a theoretical investigation to find suitable target genes. These were studied in vitro to analyze the primer and probe interactions and crosstalk. There was a need to keep the crosstalk between the oligonucleotides down to keep high specificity and guarantee certain detection of the target. After the method worked in vitro, I tested the method on cerebrospinal fluid (CSF) samples.

A multiplex PCR can be used for rapid detection if any of the target genes are in the sample. To have a standardized multiplex PCR, targeting these meningitis causing bacteria, would greatly facilitate the laboratory work and even shorten the detection time. If used as the routine detection procedure it will reduce the long term health problems caused by meningitis.

A multiplex PCR is a PCR which is able to run several different assays in the same well. This allows screening for several different targets in the same reaction solution. Each target assay uses its own forward primer, reverse primer and fluorescence probe. If the target DNA is present, the primers will enable amplification of the DNA template. The probes will then bind in to the template and emit light. Different probes will have different wavelengths of their emitted fluorescence. Different probes can have different ways to trigger the reaction to emit light. The LightCycler® 480 Real-Time PCR System enables detection of different wavelengths in a sample. This makes it possible to detect if there is an infection and also from what bacteria.

Target genes

A multiplex PCR needs assays that target genes which uniquely separate the bacteria from each other. Strong primers and good probes need to be available for all genes, the oligonucleotides combined from different assays should not interact with each other. To target *Haemophilus influenzae* the *fucK* gene has been suggested as a reliable target gene, the *fucK* gene separates *Hi* from other *Haemophilus* species (Nørskov-Lauritsen *et al.* 2009). *hpd* has also been suggested as a good target gene and has been used in a multiplex assay (Wang *et al.* 2011). The *ctrA* gene has been established to be a good target for *Neisseria meningitis* (Abdeldaim *et al.* 2010), and to work in multiplex assays (Wang *et al.* 2012). Both assays were considered for our multiplex assay. *lytA* is known to be a specific gene for targeting *Streptococcus pneumoniae* (Wang *et al.* 2012), (Hedberg *et al.* 2009) and *Spn*9802 is another gene used for targeting *Spn* (Abdeldaim *et al.* 2010).

A multiplex real time PCR benefits to have as little unwanted interactions/crosstalk as possible. Interaction in this thesis refers to the possibility for one oligonucleotide sequence to interact/bind to another oligonucleotide sequence. All primers, probes and templates in the PCR have the possibility to interact with each other. Sequence resemblances between two oligonucleotides are proportional to problems caused for the PCR by the interaction. An unwanted interaction between two nucleotides is referred to as crosstalk. 3' end binding possibilities causes more problem than 5' end interactions due to the possibility of polymerase binding, leading to amplification of the misfit sequence.

Materials and methods

Real time PCR (qPCR) – Real time PCR was central for this project. qPCR is a standard PCR with a real time tracking of the DNA amplification occurring in the wells. The method has several different uses depending on the approach. In this project we used it as a tool for detecting human pathogens.

Real time PCR uses probes with fluorophores which enables the use of multiplex assays and faster diagnostics. A multiplex PCR refers to a PCR with several assays in the same tube. In this study I used sequence specific probes for the detection of DNA. If DNA was amplified, the complement probe binds to its target site and emit light. Each probe emits light of different wavelengths. The different wavelengths can be identified by the LC480 in real time. Therefore, detection of multiple organisms in the same reaction was possible. A multiplex PCR reduces the cost and time consumption of the method by reducing manpower and reagents needed.

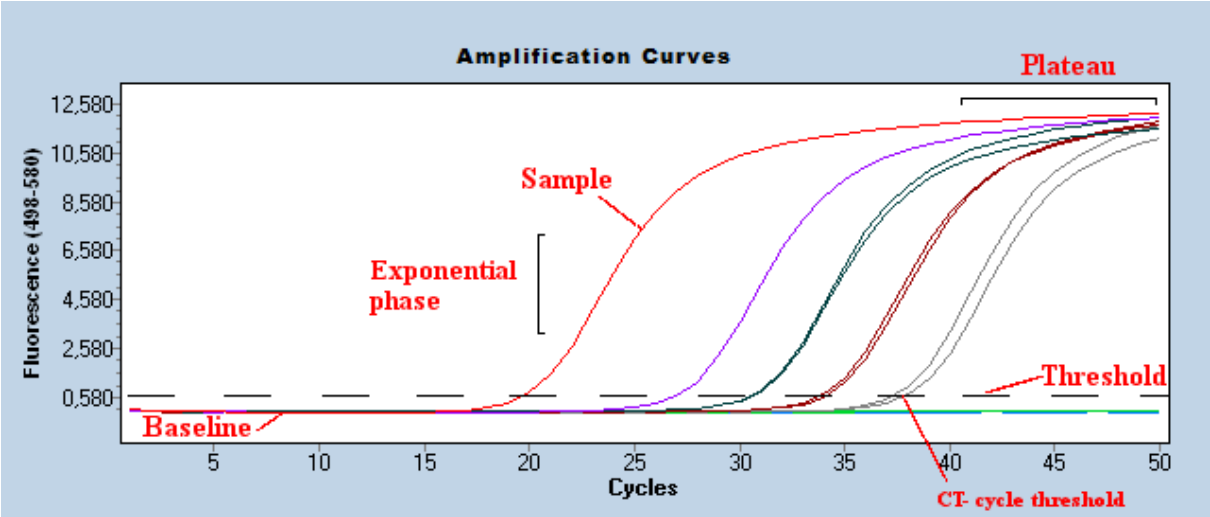


Figure 1: Explanation of the different parts of the amplification curve chart. The X-axis shows the number of cycles, which is a parallel to time. The Y-axis shows the fluorescence signal strength in watt for a specific wavelength. The exponential phase should be steep and reach a plateau in as short time as possible. The plateau should have the same signal strength for all samples due to resources being the limitation and resources was always the same for all reactions. The number of cycles it takes to break the set threshold is called CP or CT value. This value is used to calculate concentrations of target DNA In the sample. We use a combination of second derivative (CP) and signal strength to define if a sample is positive.

Figure 1 shows a run with detected target DNA in different dilutions. A sample is considered positive when its signal strength breaks a set threshold. The number of cycles it takes for a sample to reach the threshold is called the CT or CP value. A strong positive reaction is indicated as the red curve to the left. The other four colored curves were dilutions with ten-fold dilution between each step. On the y-axis is the fluorescence strength, and the x-axis is the number of cycles in the PCR. The CT (or CP) value difference between the dilutions should here be ~3.3 since the dilution steps were ten-fold in concentration. The samples reach maximum signal strength when all the fluorophore labeled probes in the well had been consumed. This caused a plateau in signal strength. This also means that all samples should have the same signal strength independent of starting concentration of the template DNA. The curve shown and used during the analysis steps in this study was usually the second derivative of the raw data.

The form of the curve is important. An exponential rise of the curve was expected and a plateau should be reached a short time after the rise was started as shown in Figure 1.

My project was to design a multiplex qPCR to fit Roche's LightCycler® 480. All PCR runs were performed in 96 well plates. The solution volumes in the PCR wells were always 25 µL.

Master Mix – All master mixes were prepared in a room dedicated for Master Mix preparations to prevent DNA contaminations. In probe solutions we used Roche LC480 ProbeMaster 2x, diluted to 1x. Standard protocols were made, we used 0.75 µL of a forward + reverse primer mix and 0.25 µL of a diluted probe mix. This volumes were the standard volumes at the facility and all mixes used the same volumes. The concentrations of the mixes were changed, never the volume. The primers and probe concentrations were optimized to fit the multiplex PCR protocol. All primer and probe concentrations were kept as low as possible to reduce unwanted crosstalk in the multiplex PCR. The methods detection safety and specificity was prioritized over low concentrations.

For the PCRs with SYBR green we used Roche's LightCycler® 480 SYBR Green I QPCR MM instead of the ProbeMaster. Also a passive reference dye was used. The protocol followed was Stratagene's Introduction to Quantitative PCR -Methods and Application Guide (page 39-40) when

using SYBR green. The exception to the protocol was that 50 nM was considered to be too low on beforehand and was skipped. The concentrations used were 100, 300, 600 and 900 nM.

DNA Template – For standard protocols 5 µL DNA template and 20 µL master mix were used, if not noted otherwise. The dilution steps of the templates were always made just before pipetting to get the dilution steps as even and accurate as possible. The dilutions were made on a biosafety bench or on a bench under a cupboard which was thoroughly cleaned after each use.

DNA extraction – Three different extraction methods were used in this project. MagNA Pure Compact from Roche, DNA extraction M48 and easyMag.

MagNA Pure Compact is an instrument for DNA extraction from Roche. We followed the instrument instructions in "MagNA Pure Compact System – Versatile Nucleic Acid Purification" and used prefilled reagents supplied by Roche. Everything is automated and everything is delivered packed in sealed plastics which reduces the risk for contamination.

DNA extraction was also done with a genovision M48 robot. Samples were first centrifuged at more than 500xg for 10 minutes. 180 µL ATL buffer was added followed by 20 µL Proteinase K. This was incubated at 60^{o}C for 15 minutes. After that, 200 µL AL buffer was added and the sample was immediately vortexed. The sample was then incubated at 70^{o}C for 10 minutes. 200 µL ethenol (95%) was added. After a quick centrifugation the samples were collected in a QIAamp colon with centrifugation in several steps. We also used easyMag which has a standardized program which we followed, it was called "Extraktionsrobot, NucliSens easyMAG Biomerieux".

Centrifugation – All 96 well plates were centrifuged in a Sigma 4K15 centrifuge at RCF 1499 xg for 2 minutes, to gather the PCR solution in the bottom of the wells.

Roche LightCycler® 480 – Two different run protocol templates were used for the LC480 during the development of this method, LC480 ProbesMaster Meningit BH and LC480 SybrGreen Mix Meningit BH. These were run in 3 different formats, SYBR Green I/HRM DYE, 3 Color Hydrolysis Probe and 4 Color Hydrolysis Probe.

The 3 and 4 color formats capture the probe fluorophores and can distinguish the different wavelengths from each other. 3 color and 4 color differ in colors they detect and how many (3 and 4, respectively). The second was a SYBR green protocol. The SYBR green cyanine dye was not sequence specific and binds to all double stranded DNA. It can bind to single stranded DNA with a much lower affinity. Since it was not sequence specific it could be used to measure all DNA amplifications in the PCR.

During analysis of PCR we mainly used the 2nd derivative from the LC480 curves and Ct values as raw data. Often a threshold was added to exclude false positives.

Gel electrophoresis – Two different gel electrophoresis methods were used during this project. I used Invitrogen E-gel® or a self-made 1% agarose TAE gel run in a 1% TAE buffer. The gels were set up and run in a special post PCR lab room to keep other labs from getting contaminated with PCR products. For all important experiments during the project, Invitrogen E-gel was used and the 1% self-molded gels were only used when a minor confirmation was needed.

The Invitrogen E-gel® has two different programs you can use. One program was using 12 wells or less and the other 13-24 wells. The 12 wells program was 30 minutes long while the 13-24 wells program was 15 minutes. A better resolution is achieved with the smaller 12 well gels since it can be

run for a longer time. For self-casted gels the voltage was set to 90V over the gel, and the gel run duration differed depending on the resolution needed.

Results

Primer optimization Hedberg lytA

Two primers, lytA_F and lytA_R previously described by (Hedberg *et al.* 2009) were ordered. I needed to optimize the ratio and concentration of the primers. I measured and compared the DNA concentrations generated by different ratios and concentrations of primers and then followed the SYBR green protocol in Stratagene's Methods and application guide: Introduction to quantitative PCR, as described in Materials and methods.

The melting curves aggregated in two groups. The group with lower signal strength included reactions where one of the primer concentrations was 100 nM. The group with higher signal strength included all samples where both primer concentrations were higher than 100 nM. The curves with higher concentrations of primer were somewhat comparable to each other. 900 nM was not necessary to generate enough DNA.

A new run was set up using the four different variations of 300 nM and 600 nM. The four primer mixes concentrations were as follows R300/F300; R300/F600; R600/F300; R600/F600. The PCR run showed that there were small variations using the four new primer mixes. We therefore decided that we would use a primer mix with 300 nM forward primer and 300 nM reverse primer in the future. This is to keep the concentrations of all primers as low as possible.

Primer optimization Wang ctrA

Optimization of the ratio and concentration of the Wang *ctrA* primers had been done at the department before but needed an update. The currently used concentration of the dual primer mix was 300 nM reverse primer and 900 nM forward primer in the final solution. I followed the SYBR green protocol in Stratagene's Methods and application guide: Introduction to quantitative PCR, as described in Materials and methods.

The curves were divided into two major groups. The group with lower CP value has a reverse primer of 300 nM or higher. There were no major differences between reverse primer 300 nM and higher concentrations of the reverse primer. There were small differences between forward primer 600 nM and 900 nM when mixed with reverse primer 300 nM. For further dilutions I recommended using a R300/F600 concentration mix to keep the concentrations as low as possible to save resources and optimize the assay for use in multiplex PCR.

lytA cyan500 evaluation, Hedberg primer standard curve.

The probes were confirmed by PCR. The assay with *lytA* cyan500 probe was run with *Spn* template and with a negative water control. In the same run we investigate primer dimer formation in Wang *Spn* and Wang *Mnc*. The result is presented in Figure 2.

Figure 2: A gel electrophores was run on the product from four different assays, Wang assays for *Spn, Mnc* and *Hi* and Lagmo lytA. The upper part of the gel is not used in this discussion but we confirmed primer dimer formation in Wang Mnc. The red box shows the primer dimer formation in Wang HI and Lagmo lytA. Highest DNA template concentration was to the left and dropping going right. The upper band show the wanted product while the lower represent primer dimer. The strength of the upper band decresaes with decreasing DNA concentration while the lower band increased.

In the qPCR the signal was high and the CP values were spread with approximately 3.3 cycles between them. A spread of CP values around 3.3 was expected from tenfold dilution steps. The 10^{-6} dilution was close to negative since it varies a lot between the two reaction curves of that dilution. The negative control was negative in the qPCR. Since light was emitted of the expected wavelength, I concluded that the probe worked well.

A standard curve will help to determine the DNA concentration in a sample. It will also help to ensure that the method is sensitive enough to capture even low concentrations of DNA. A standard curve was made for the Lagmo assay in two steps. First a dilution series was created by using template DNA diluted 1000 times, called S*pn* 10E3. Evenly spread dilution intervals, 10 000 (10E4), 100 000, (10E5) and 1 000 000 (10E6) times, was then diluted. We added appropriate concentrations of primers and probes, decided in the projects primer and probe optimization parts. Figure 3.

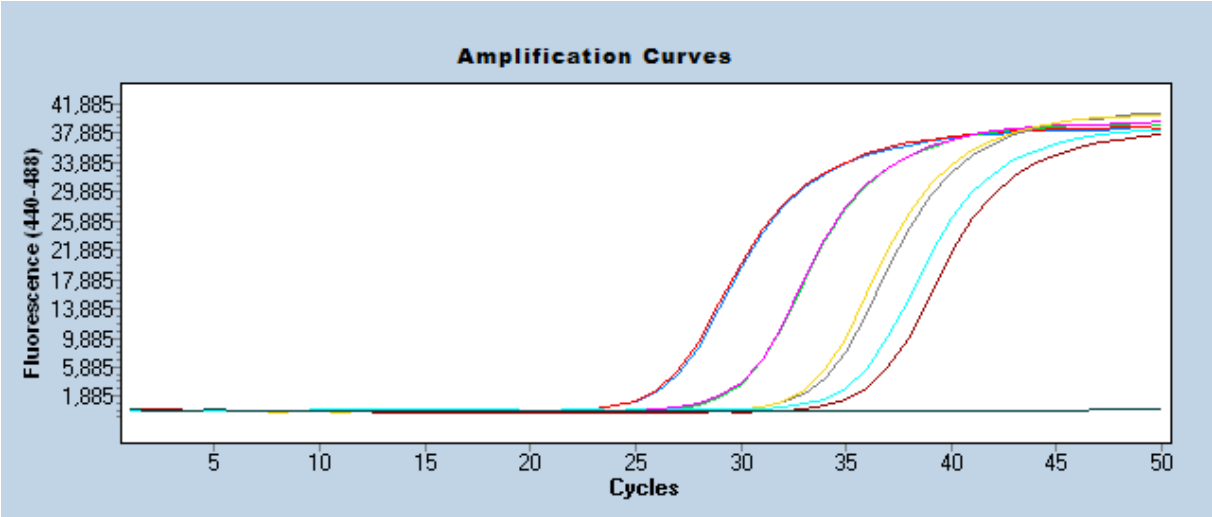


Figure 3: PCR run with the Lagmo assay and DNA Spn template diluted 1:100; 1:1 000; 1:10 000; 1:100 000; 1:1000 000, all run as duplicates.

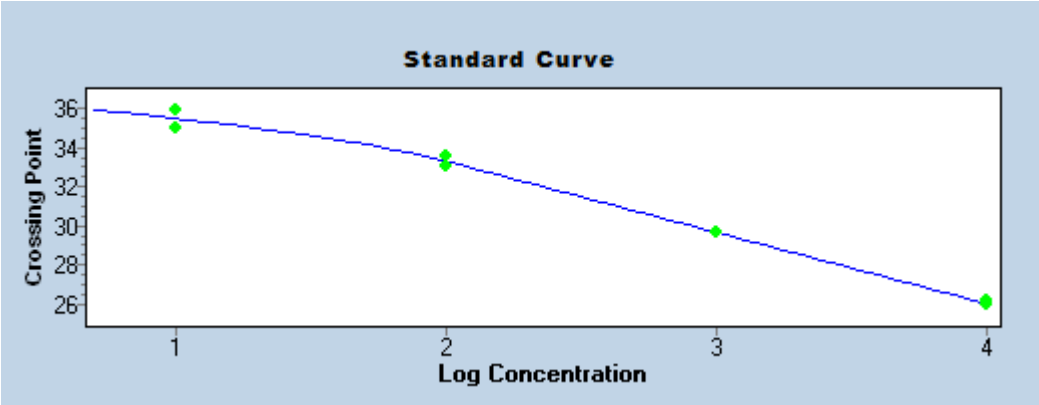
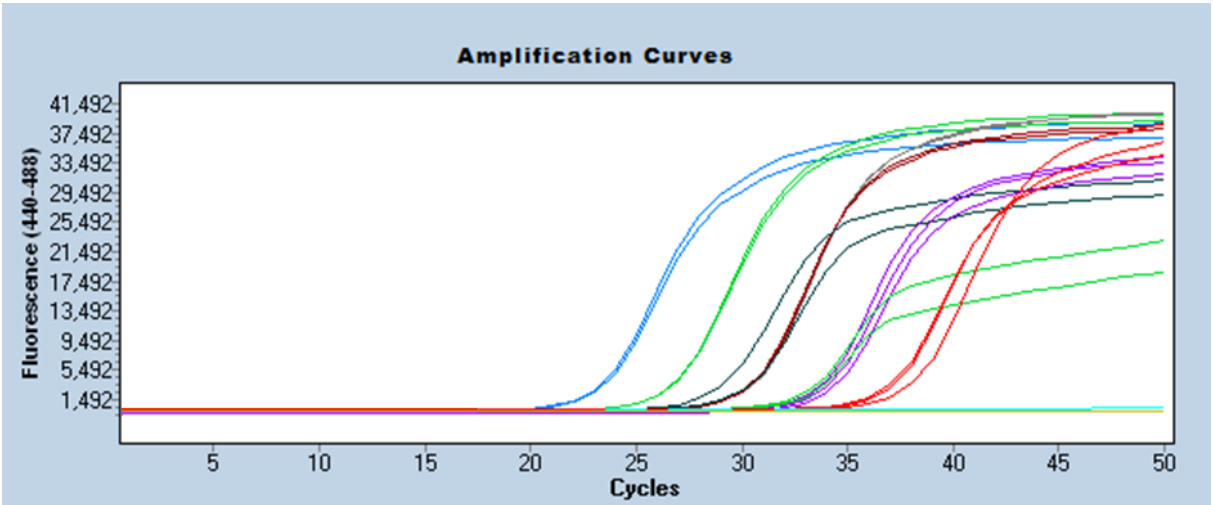


Figure 4: The standard curve serving as an outline. You can see that it was not straight due to lack of data points and the most diluted concentration was variable. A straight line was expected.

The first experiment was made as an outline for the next standard curve seen in Figure 4. I confirmed that the CP values were as designed in the primer optimization experiment earlier. I went on with the larger standard curve generation.

In the larger standard curve I used duplicate measure points of a ten-fold DNA template dilution series in dilution 1:100 to $1:10^6$ of the stock target DNA solution. In this experiment I also investigated how the multiplex oligo nucleotides will affect the amplification of DNA in the PCR run. I used oligo nucleotides from the assays currently used in the multiplex real time PCR, Wang *ctrA*, Hedberg *lytA* and Abdeldaim *fucK* (Figure 5 and Table 1). All sequences can be found in "References" table 22. In Figure 5 the reduction in signal strength is seen, e.g. when comparing Lagmo assay+temp SpnE-5 (purple) with Multiplex assay+temp *Spn* E-5 (green), but the CP value does not differ much.



COLOR (FROM LEFT TO RIGHT)	SAMPLE	CP
BLUE	Lagmo assay + temp Spn E^{-2}	~23
GREEN	Lagmo assay + temp Spn E^{-3}	~27
GREY	Lagmo assay + temp Spn E^{-4}	~30
WINE RED	Lagmo assay + temp Spn unknown	~30
DARK GREEN	Multiplex assay + temp Spn E^{-4}	~29
PURPLE	Lagmo assay + temp Spn E^{-5}	~33
GREEN	Multiplex assay + temp Spn E^{-5}	~32
RED	Lagmo assay + temp Spn E^{-6}	~37
TEAL	Neg Lagmo assay	-
YELLOW	Neg Multiplex assay	-

Figure 5: Some signal strength reduction was observed.

The standard curve was generated from the run. The standard curve can be used to predict the target DNA concentration in a sample using the CP value. The new standard curve can be seen in Figure 6 and notice that the line was straighter than in Figure 4.

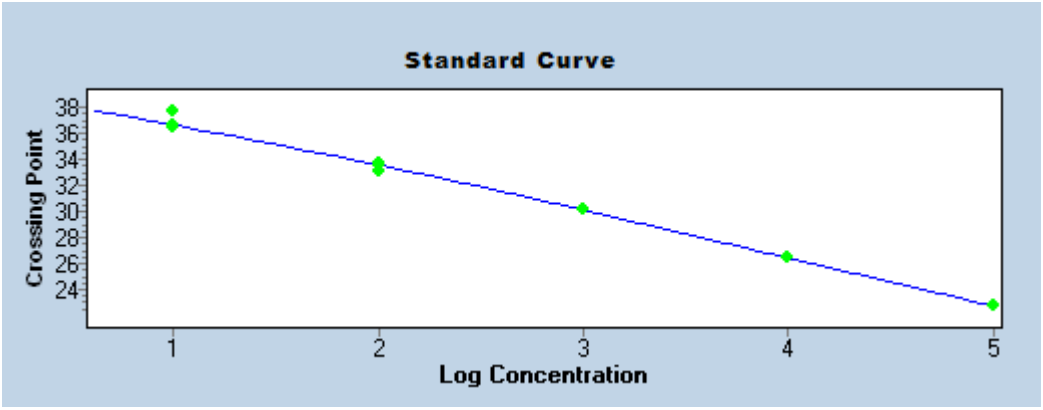


Figure 6: The second standard curve was straighter and can be used as intended. Note that the last point in the dilution was more stable here.

Multiplex efficiency

To obtain the best possible detection of target bacteria it was important to have the same sensitivity and specificity and the same efficiency regarding amplification in a multiplex run as in a singleplex run. This was ensured by running the multiplex assay parallel to all three singleplex assays. This was done various times during the project. Different setups of multiplex assays were used.

Lagmo lytA efficiency in multiplex

I ran a multiplex qPCR containing *Spn* Lagmo l*ytA*, the *HI fucK* and *Nm* Wang *ctrA* with target DNA *Spn*. For parallel comparison I ran a positive control of the *Spn* Lagmo assay including the Hedberg *lytA* primers and the *lytA* Cyan500 probe. Some kind of negative inhibition took place in the multiplex assay. All singleplex reactions showed high signal strength and expected CP values through all concentrations. For the multiplex assay the CP values remained almost the same but the signal strength was reduced significantly when the concentration of target DNA was lowered. The reaction curves from the most diluted concentrations were not as expected.

For the next reaction I used two duplex runs with the *HI* Abdeldaim *fucK* assay and the *Spn* Lagmo *lytA* assay in one and the *Nm* Wang *ctrA* assay and the *Spn* Lagmo *lytA* assay in the other. The template DNA used was still *Spn*. The parallel reference reaction was set by a positive run of *Spn* Lagmo *LytA*. The result showed that the Wang *ctrA* assay does not affect the *Spn* Lagmo l*ytA* assay in any way. The signal strength remained the same for the Wang *ctrA* duplex assay reaction as the Lagmo singleplex reference reaction, through all concentrations.

The result showed that the Abdeldaim *fucK* assay was the cause for the reduction in signal strength for the Lagmo assay seen in the multiplex assay. The curves had the same character as seen in the multiplex experiment, with very low signal strengths in reactions with the most diluted DNA template. The next experiment was aimed for further diagnosis of the *fucK* problem. A reference curve was set by a positive *Spn* Lagmo *lytA* assay reaction. Three other reactions ran parallel to it, each including one of the parts from the Abdeldaim's *fucK* assay (*fucK* F primer, *fucK* R primer and the *fucK* probe). The parts of the assay were added separately to a pure *Spn* Lagmo *lytA* assay positive reaction. This showed that the inhibition of *Spn* Lagmo *lytA* comes from the *fucK* F primer. The other two reactions worked well with expected signal strengths. The one including the *fucK* F primer showed the same reduction in signal strength as seen earlier. *fucK* was during this time of the project the most likely gene to be used as a target for *Hi*. The rationale for this choice is described in the section "Choice of target gene for *HI*" under Discussion.

A multiplex reaction was again set up with *Spn* as template. This reaction included the assays for *Spn* Lagmo *lytA*, *HI* Wang *hpd* and *Nm* Wang *ctrA*. The run showed that there was no inhibition from *Nm* Wang *hpd* on *Spn* Lagmo *lytA* either. The following PCR experiment was set up as a duplex assay with Wang *ctrA* and Lagmo *lytA* since we made a decision to split the multiplex into two duplex methods. One method targeted *HI*, *fuck* and *hpd,* with two different assays and one targeted *Mnc ctrA* and *Spn lytA*. The template DNA concentration during these studies was lower than those used before. At low concentrations we showed that there was inhibition of the Lagmo assay from Wang *ctrA*. The inhibition mainly lowered the signal strength but also affected the ability to detect DNA at low concentrations. After revising we changed the assay again. The full Wang assay was retested and confirmed to work as said in their paper (Wang *et al.* 2012) but contrasted to our own initial results.

Wang ctrA efficiency in multiplex

Wang *ctrA* has been tested for crosstalk with Wang *hpd* (Wang *et al.* 2012). It showed no inhibition of the PCR product and it was concluded that a multiplex format did not impair the detection capacity. No crosstalk analysis had previously been made between the *Spn* Lagmo *lytA* assay and the Wang *ctrA* assay. Therefore, a test was needed to investigate eventual inhibition of Wang *ctrA*'s PCR product from the *lytA* primers/probe. We ran a duplex PCR containing the Wang *ctrA* assay, and the Lagmo assay with the target *Mnc's* DNA. Parallel to this we ran a positive control of *ctrA* with Wang *ctrA* primers and the Wang *ctrA* probe with the target *Mnc's* DNA. The signal strength and CP values were the same in the positive run as in the duplex run. This indicated that no inhibition took place from the Lagmo *lytA* assay on the PCR product from Wang *ctrA*'s assay.

I ran a multiplex reaction with the Wang *ctrA* assay, the Lagmo *lytA* assay and the Wang *hpd* assay. The aim for the PCR was to analyze the analytical sensitivity between multiplex and singleplex assays. The target DNA concentrations were 1x10-fold dilution steps lower than in previous experiments. There was some kind of inhibition in the last few dilution steps but it was not significant. It affected signal strength, CP value and the appearance of the curve, but there was no loss of positive detection.

A new run of the experiment was set up with the same assays but with a new prep of DNA dilution series. This clearly indicated inhibition of Wang *ctrA* when in multiplex compared to singleplex. The signal strength was greatly affected and so was the CP value in the last dilution steps. The CP value was not higher but change in appearance of the curve change the CP values. The reaction curves were flattened out, meaning that signal strength and CP values were lowered. When the curves are too flattened out they lose the characteristics of a qPCR curve and it is hard to tell if the sample is positive or not. The lowering of CP values was a result from the flattening of the curves which can be removed by increasing the CP value threshold. The analytical sensitivity remains the same, though the last dilution step was arguable negative for the multiplex assay. The signal strength reduction you can see in Figure 7 was a result from primer dimer formation caused by the Lagmo assay discussed in "Choice of target gene for *Spn*" under Discussion.

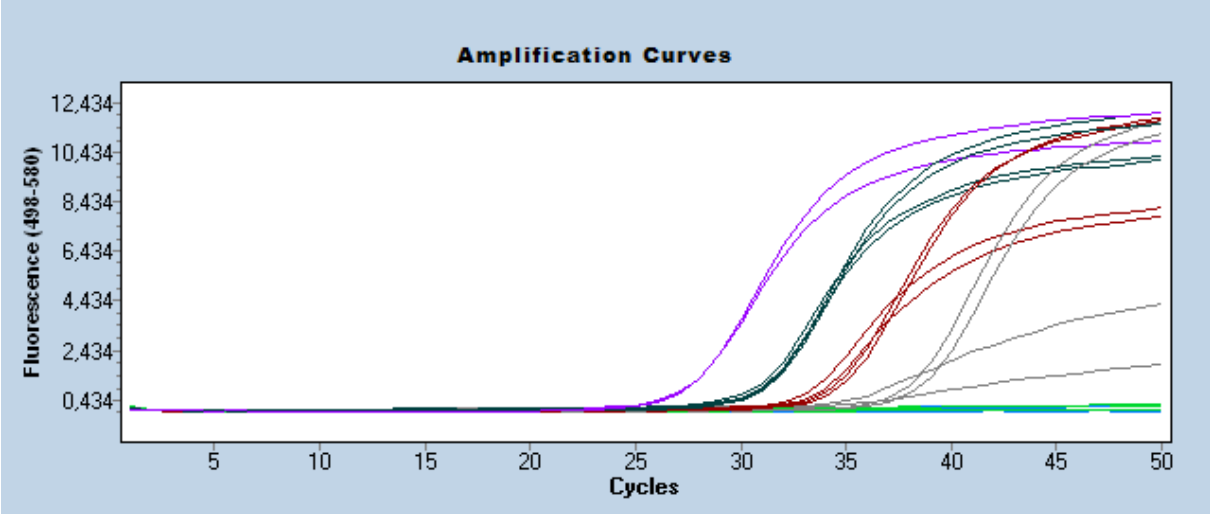


Figure 7: The different colors of the curves indicate different concentration of target DNA. Purple was run as a solo reaction while the others were run in duplicate reactions. The curves with lower signal strengths are always the multiplex assay. We could see a clear reduction in signal strength with decreasing DNA concentration for the multiplex assays. This was a sign for primer dimer formation.

fucK efficiency in multiplex

The Abdeldaim *fucK* assay showed no complications when run in multiplex qPCR with Lagmo *lytA* and Wang *ctrA* compared to singleplex. However, the assay does not reach a plateau for an unknown reason. See Figure 8 for an example. The reason could be faulty quenching of the probe at the temperatures used in the qPCR.

Wang ctrA and Lagmo lytA singleplex/duplex cerebrospinal fluid test

The specificity of the method needed confirmation by CSF tests. Already known positive samples of *Spn* and *Mnc* were collected from the Department of Clinical Microbiology, Uppsala University Hospital. No *HI* samples were found due to the rarity of the infection. 18 samples were collected, ten *pneumococcus* samples and eight *meningococcus* samples. The samples consisted of CSF from patients which were stored in a -20^{o}C freezer. All samples were previously positive for either *Nm* or *Spn.* The samples DNA were extracted using Easymag NucliSENS from Biomerieux. All *Mnc* and *Spn* samples were tested both with singleplex and duplex assays for their respective target. As a positive control we used the target DNA used in the departments routine work. The results of the CSF test can be seen in Table 1.

Table 1: The data set from the CSF test of the duplex assay (the Wang ctrA assay and the Lagmo lytA assay) versus singleplex of the Wang ctrA and the Lagmo lytA assays, respectively.

Sample ID	**Bacterial species**	**PCR results** (singleplex/duplex)		**Signal strength reduction in duplex PCR****
		ctrA	*lytA*	
BART-0800124	*S.pneumoniae*	-/-	-/- *	No
BART-0800205	*S.pneumoniae*	-/-	+/+	No
(BART-0800273)	*S.pneumoniae*		Missing data	
BART-0900324	*S.pneumoniae*	-/-	+/+	No
BART-0900513	*S.pneumoniae*	-/-	-/- *	No
BART-0900557	*S.pneumoniae*	-/-	+/+	No
BART-0900750	*S.pneumoniae*	-/-	+/+	No
BPCR-1100313	*S.pneumoniae*	-/-	+/+	Yes
BPCR-1100494	*S.pneumoniae*	-/-	+/+	No
BPCR-1215070	*S.pneumoniae*	-/-	-/- *	No
BPCR-1300294	*S.pneumoniae*	-/-	+/+ **	Yes
BART-0800504	*N.meningitidis*	-/- *	-/+ *	No
BART-0900512	*N.meningitidis*	+/+	-/-	No
BPCR-1000026	*N.meningitidis*	+/+	-/-	No
BPCR-1000527	*N.meningitidis*	+/+	-/-	No
BPCR-1100196	*N.meningitidis*	+/+	-/-	Yes
BPCR-1100594	*N.meningitidis*	+/+	-/-	No
BPCR-1100772	*N.meningitidis*	+/+	-/-	No
BPCR-1200674	*N.meningitidis*	+/+	-/-	Yes
Pos control *Spn*	*S.pneumoniae*	-/-	+/+	No
Pos control *Mnc*	*N.meningitidis*	+/+	-/-	Yes
Neg control	No template	-/-	-/-	No

*** The result varies from that expected.**

**** Signal strength reduction refers to a signal strength loss of more than 30% in the multiplex PCR compared to the parallel singleplex signal strength.**

One of the *Mnc* samples was negative. (BART-0800504). The sample was negative both for the singleplex and the duplex assays. However, BART-0800504 was strongly positive for *Spn,* a result that called for further investigation. One of the positive samples had greater signal strength reduction

in the duplex assay. Three of the *Spn* samples were negative both in the duplex and the singleplex qPCR run.

The cells had been stored at -20°C since 2008. This might have damaged the cells, but it should not affect the DNA concentration. Ensuring the viability of the cells was needed, and therefore, another PCR was run using the same setup. The negative samples, and samples where the signal strength was not the same for the duplex and the singleplex, were run again. Three of the negative samples remained negative. The PCR did change the result for BPCR-1215070 where the signal strength for the duplex assay was below that of the singleplex, but both were positive at the repeated testing.

Full Wang multiplex assay cerebrospinal fluid test

The same samples as in "*Mnc* and *lytA* singleplex/duplex cerebrospinal fluid test" were used in this test. The same procedure was used with the exception that as target assay we used the full multiplex Wang assay. The results are shown in Table 2.

Table 2: The data set from the CSF test of the full Wang multiplex assay.

Sample ID	**Bacterial species**	**PCR results**		
		ctrA	*lytA*	*hpd*
BART-0800124	*S.pneumoniae*	-	-*	-
BART-0800205	*S.pneumoniae*	-	+	-
(BART-0800273)	*S.pneumoniae*	Missing data		
BART-0900324	*S.pneumoniae*	-	+	-
BART-0900513	*S.pneumoniae*	-	(+)**	-
BART-0900557	*S.pneumoniae*	-	+	-
BART-0900750	*S.pneumoniae*	-	+	-
BPCR-1100313	*S.pneumoniae*	-	+	-
BPCR-1100494	*S.pneumoniae*	-	+	-
BPCR-1215070	*S.pneumoniae*	-	+	-
BPCR-1300294	*S.pneumoniae*	-	+	-
BART-0800504	*N.meningitidis*	-*	+*	-
BART-0900512	*N.meningitidis*	+	-	-
BPCR-1000026	*N.meningitidis*	+	-	-
BPCR-1000527	*N.meningitidis*	+	-	-
BPCR-1100196	*N.meningitidis*	+	-	-
BPCR-1100594	*N.meningitidis*	+	-	-
BPCR-1100772	*N.meningitidis*	+	-	-
BPCR-1200674	*N.meningitidis*	+	-	-
Pos control *Spn*	*S.pneumoniae*	-	+	-
Pos control *Mnc*	*N.meningitidis*	+	-	-
Neg control	No template	-	-	-

*** The result varies from the expected.**
**** The sample was weakly positive.**

All *Nm* samples were positive with the exception of BART-0800504, which was strongly positive for *Spn*. The sample was strongly positive for *Spn* instead of *Mnc* in both the multiplex Wang assay and the duplex Lagmo assay. This made me think that the sample was archived incorrectly. There was no time to investigate the sample further and therefore it was ignored in this study.

All except one (BART-0800124) of the *Spn* samples were positive, thus the multiplex Wang assay appeared to be slightly more sensitive than the Lagmo *lytA* assay. We also detected some weak false positives which were discarded since the threshold was increased. The reason for the false positive

was found in the negative control, which was weak positive. The curve looked the same for all false positives, a slow rising linear curve reaching a signal strength around 0.45 fluorescence units.

Analytical sensitivity

The analytical sensitivity indicates the lowest copy number of target DNA that can be detected by the assay. It was usually measured in DNA copies/ PCR reaction. Our method needs to be as sensitive as possible since preparations from CSF samples may have very low concentrations of target DNA.

The experiment was done the same way for all three target bacteria. A new DNA preparation was made. The concentration was measured using NanoDrop® ND-1000 spectrophotometer. The concentration was then calculated into DNA copy number/µL using: http://www.thermoscientificbio.com/webtools/copynumber/ and the calculated extracted DNA concentrations are presented in Table 3.

Table 3: Measured and calculated target DNA concentrations of two different DNA preparations. These values were used for analytical sensitivity.

#	Bacterial species	Genome size [Mbp]	Measured DNA conc. [ng/µL]	Genome copies/ng	Calculated conc. [copies/µL]*
1	*H. influenzae*	1.8	124	506263	~$6.3*10^7$
1	*N. meningitidis*	2.3	80	402809	~$3.2*10^7$
1	*S. pneumoniae*	2.1	-	441172	-
2	*H. influenzae*	1.8	121	506263	~$6.1*10^7$
2	*N. meningitidis*	2.3	58	402809	~$2.4*10^7$
2	*S. pneumoniae*	2.1	64	441172	~$2.8*10^7$

*"Calculated concentration" was calculated by multiplying Measured DNA concentration with genome copies/ng.

The genome sizes used were 1.83 Mbp for *Hi*; 2.1Mbp for *Spn* and 2.3Mbp for *Mnc*. The first DNA preparation failed for *Spn* because I missed a pretreatment with lysozyme and proteinase K incubation at room temperature for at least ten minutes. This was done for the later set, and that data set was a success and was subsequently used for the analytical sensitivity tests.

With the copy number known the dilution series were made. The dilution series ranged from copy numbers around 100 000 to below one copy of DNA per reaction. A PCR's detecting capacity can theoretically not be stronger than that. The detection probability in the last dilution steps will be more dependent on chance than anything else. Negative values were expected in the last step to confirm that the concentration estimation was accurate.

Primers and probes in a reaction mixture may interact and inhibit the DNA amplification. Therefore, the analytical sensitivity of the multiplex and singleplex methods needs to be compared. We ran the multiplex assays parallel to the singleplex assays of their respective targets. Two different dilution series were used, one for *Mnc* and one for *HI*. Determination of the *Spn* solution's DNA concentration was not possible, because it was too low for the first prep. This was remade in a later experiment.

The analytical sensitivity for the full Wang assay was the same while run in multiplex and while run as singleplex for all targets (Wang *et al.* 2012). Therefore, no comparison between the singleplex assay and multiplex assays were run when testing the sensitivity for Wang.

Analytical sensitivity *Mnc*

Experiment 1and 2 – The analytical sensitivity for *Mnc* Wang *ctrA* was compared while run in multiplex and while run as singleplex. The multiplex assay contained assays: Wang *hpd,* Wang *ctrA* and Lagmo *lytA*. The results are shown in Table 4.

Table 4: CP value comparison for the Wang *ctrA* assay while in singleplex and multiplex.

Concentration of target DNA *Mnc* [copies/reaction]	**Active target assay**	**Average CP value**	
		Singleplex	*Multiplex**
~15000	Wang *ctrA*	27.4	27.5
~1500	Wang *ctrA*	30.7	30.6
~150	Wang *ctrA*	34.2	33.0
~15	Wang *ctrA*	37.7	34.8**
~1,5	Wang *ctrA*	-	-
Negative control	Wang *ctrA*	-	-
Positive control	Wang *ctrA*	26.3	25.9

***The multiplex assay contained the Wang *ctrA,* Wang *hpd* and Lagmo *lytA* assays.**
**** The signal strength of this reaction was significantly affected compared to singleplex.**

The CP values of the multiplex and singleplex run followed each other closely. However, the signal strength curve was not shared. The last positive dilution was 15 copies/ 25µL well. Both of the two wells of 1.5 copies/well were negative for singleplex as well as for the multiplex. The detection threshold of the method was between 0.3-3 copies/uL or 1.5-15 copies of DNA/reaction. The signal strength of the multiplex methods seems to be affected by one of the others present. The detection capacity of the method was not worth risking.

Experiment 3 – The analytical sensitivity of Wang *ctrA* was compared while run in singleplex and duplex with Lagmo *lytA*. The Wang *ctrA* assay showed no signs of inhibition from the Lagmo *lytA* assay. Both the curve and CP values followed each other closely. The data of experiment 3 is shown in Table 5.

Table 5: The Wang *ctrA* assay's sensitivity was compared while run as singleplex and while run as duplex with Lagmo *lytA*.

Concentration of target DNA *Mnc* [copies/reaction]	**Active target assay**	**Average CP value**	
		Singleplex	*Duplex*
~150	Wang *ctrA*	33.3	33.1
~15	Wang *ctrA*	37.4	37.9
~1.5	Wang *ctrA*	-	39.5*
Negative control	Wang *ctrA*	-	-
Positive control	Wang *ctrA*	26.3	26.0

***One of the duplicate reactions was negative.**

Experiment 4 – Determination of analytical sensitivity with higher resolution was obtained by running a singleplex run for the Wang *ctrA* assay. The data of experiment 4 is shown in Table 6.

Table 6: The Wang *ctrA* assay was run as singleplex with higher resolution to get more specific values of the sensitivity.

Concentration of target DNA *Mnc*[copies/reaction]	**Active target assay**	**Average CP value** *Singleplex*
~12000	Wang *ctrA*	27.5
~1200	Wang *ctrA*	30.9
~120	Wang *ctrA*	34.4
~43	Wang *ctrA*	36.6
~12	Wang *ctrA*	38.3
~4.3	Wang *ctrA*	37.7*
~1.2	Wang *ctrA*	39.1*
Negative control	Wang *ctrA*	-

*Values were weakly positive and only one of the duplicate values was positive. The reaction curves were also flattened.

Experiment 5 – Determination of the analytical sensitivity.The *ctrA* targeting assay was run in multiplex with the other assays from Wang, the results are shown in Table 7.

Table 7: CP values of the full Wang multiplex assay targeting the *Mnc* gene *ctrA*.

Concentration of target DNA *Mnc*[copies/reaction]	**Active target assay**	**Average CP value** *Multiplex**
~12000	Wang *ctrA*	27.1
~1200	Wang *ctrA*	30.6
~120	Wang *ctrA*	34.4
~12	Wang *ctrA*	36.9
~1,2	Wang *ctrA*	-
Negative control	Wang *ctrA*	-

*The multiplex assay contained assays Wang *ctrA*, Wang *hpd* and Wang *lytA*.

This assay showed positive results when the DNA concentration was 12 copies in 25 µL, with full signal strength. All curves had a nice exponential rise to maximum signal strength, even those with ~12 copies/ well. The analytical sensitivity was shown to be 12 copies per reaction for several experiments.

Analytical sensitivity *Hi*

Experiment 1 – The analytical sensitivity was compared for *Hi* Wang *hpd* and *Hi* Abdeldaim *fucK* while run in multiplex and while run as singleplex. The multiplex assays contained additional assays: Wang *ctrA* and Lagmo *lytA*. The analytical sensitivity of *Hi* experiment 1 is presented in Table 8.

Table 8: CP value comparison in singleplex versus multiplex between Abdeldaim target gene assay *fucK* and Wang target gene assay *ctrA*.

Concentration of target DNA *Hi*[copies/reaction]	**Active target assay**	**Average CP value**	
		Singleplex	*Multiplex**
~32 000	Wang *hpd*	30.3	30.3
~3 200	Wang *hpd*	34.6	34.2
~320	Wang *hpd*	38.5	38.0
~32	Wang *hpd*	41.0**	41.0**
~3	Wang *hpd*	-	-
Negative control	Wang *hpd*	-	-
~32 000	Abdel. *fucK*	26.5**	27.5**
~3 200	Abdel. *fucK*	31.2**	31.3**
~320	Abdel. *fucK*	33.8**	33.7**
~32	Abdel. *fucK*	39.1**	34.1**

~3	Abdel. *fucK*	-	-
Negative control	Abdel. *fucK*	-	-

*In addition to the assay in "Assay" the multiplex assay contained Wang *ctrA* and Lagmo *lytA* assays.
** The value presented was connected to a reaction curve which was too gradual. The curves should have had a quicker rise in signal strength to be fully acceptable.

The CP values of the methods were comparable between multiplex and singleplex. However, the signal strength was not (See figure 10 and 11). The assays do seem to be inhibited while being in a multiplex assay. The CP values of the singleplex dilution step shows positive values down to 30 copies /25 µL-well for both assays. When analyzing the curves I could only accept positive values down to 300 copies per 25 µL-well for the multiplex assays. The mostly diluted reactions signal strengths, were too low.

Experiment 2 – The analytical sensitivity was compared for *Hi* Wang *hpd* while run in multiplex and while run as singleplex. The multiplex assay contained assays: Wang *hpd* Wang *ctrA* and Lagmo *lytA*. The results are presented in Table 9.

Table 9: CP value comparison for the Wang *hpd* assay in singleplex and multiplex.

Concentration of target DNA *Hi*[copies/reaction]	**Active target assay**	**Average CP value** *Singleplex*	*Multiplex**
~30 000	Wang *hpd*	27.3	27.5
~3 000	Wang *hpd*	30.9	31.0
~300	Wang *hpd*	34.3	32.7**
~30	Wang *hpd*	37.8	-
~3	Wang *hpd*	40.7***	-
Negative control	Wang *hpd*	-	-
Positive control	Wang *hpd*	32.2	32.0

* The multiplex assay consisted of the Wang *ctrA,* Wang *hpd* and Lagmo *lytA* assays.
** The signal strength of this reaction was significantly affected compared to singleplex.
***This value was really high and one of the duplicates was negative, but the curve had high signal strength and cannot be considered as negative.

Experiment 3 – To further determine the analytical sensitivity of the multiplex assay, the experiment was repeated. An analytical sensitivity of 30/25 µL well was also gained for Wang *hpd* while run as multiplex in the full Wang assay. The results are presented in Table 10.

Table 10: Full Wang multiplex assay with target DNA HI.

Concentration of target DNA *Hi*[copies/reaction]	**Active target assay**	**Average CP value** *Multiplex**
~30 000	Wang *hpd*	27.6
~3 000	Wang *hpd*	31.1
~300	Wang *hpd*	34.8
~30	Wang *hpd*	36.2**
~3	Wang *hpd*	-
Negative control	Wang *hpd*	-

*The multiplex contained Wang *hpd* Wang *ctrA* and Wang *lytA*.
**Reaction showed low signal strength.

Experiment 4 – The Wang *hpd* assay was compared to the Abdeldaim *fucK* assay while run in duplex and singleplex. The duplex assay consisted of the *hpd* assay and the *fucK* assay. Wang *hpd* showed positive results down to 30 copies/well in singleplex, and down to 300 copies/well (300/25 copies/µL) in duplex. The results are shown in Table 11 and Figure 8.

Table 11: Comparison of CP value between the Wang hpd assay and the Abdeldaim fucK assay run in duplex with each other.

Concentration of target DNA *Hi*[copies/reaction]	**Active target assay**	**Average CP value**	
		Singleplex	*Duplex**
~30 000	Wang *hpd*	29.1	28.8
~3 000	Wang *hpd*	33.4	31.9
~300	Wang *hpd*	36.0	34.7**
~30	Wang *hpd*	40.5**	-
~3	Wang *hpd*	-	-
Negative control	Wang *hpd*	-	-
~30 000	Abdel. *fucK*	26.9	27.1
~3 000	Abdel. *fucK*	29.8	29.8
~300	Abdel. *fucK*	33.1	33.3
~30	Abdel. *fucK*	36.6	36.4**
~3	Abdel. *fucK*	-	-
Negative control	Abdel. *fucK*	-	-

*The duplex was a mix of the two assays.
**Signal strengths were low but positive.

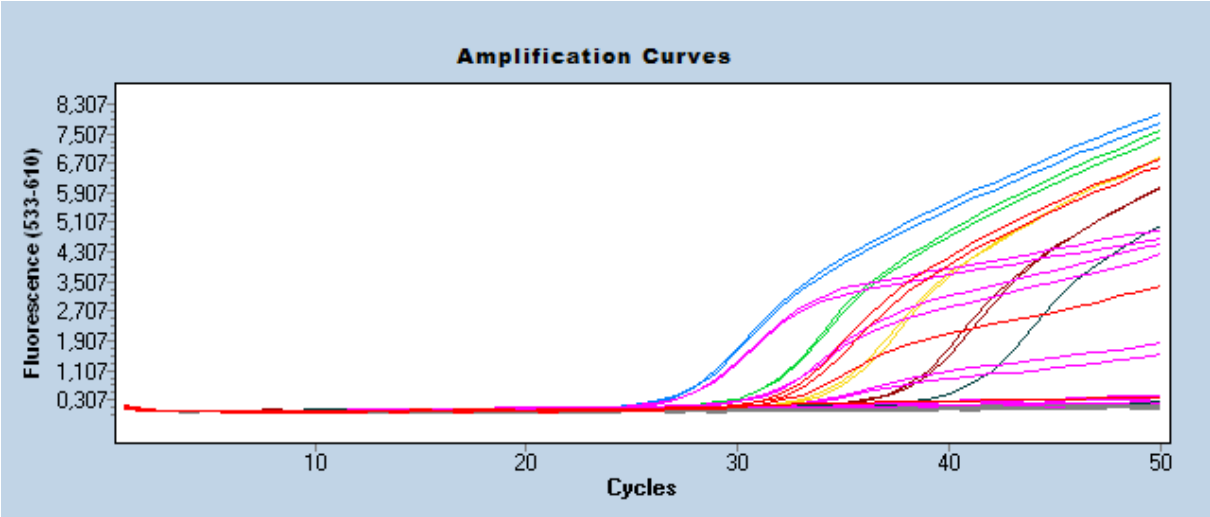


Figure 8: Curves not colored in pink were fucK singleplex assays, the curves in pink were the duplex assays with the same template DNA concentration as the differently colored ones. The duplex assays signal strengths were lower and the highest dilution were negative in the duplex assay. We noticed a reduction in signal strength for the multiplex assays.

The *fucK* assay show positive results down to 30 copies /25 µL per well for both the duplex PCR and the singleplex PCR. This confirms the previously reported sensitivity of 5 to 50 copies per PCR reaction (Abdeldaim *et al*. 2013).

Experiment 5 – A sensitivity analysis with higher resolution was set up as a duplex run for the Wang *hpd* assay and the Abdeldaim *fucK* assay. The results are shown in Table 12.

Table 12: Both assays targeted the same DNA and were active during the PCR. This limits both assays and the result was that the curves were flattened.

Concentration of target DNA *Hi*[copies/reaction]	**Active target assay**	**Average CP value** *Duplex*
~30 000	Wang *hpd*	28.3
~3 000	Wang *hpd*	32.2
~300	Wang *hpd*	34.5*
~90	Wang *hpd*	35.1*
~30	Wang *hpd*	-
~9	Wang *hpd*	-
~3	Wang *hpd*	-
Negative control	Wang *hpd*	-
~30 000	Abdel. *fucK*	27.2
~3 000	Abdel. *fucK*	30.8
~300	Abdel. *fucK*	33.9
~90	Abdel. *fucK*	34.3*
~30	Abdel. *fucK*	35.3*
~9	Abdel. *fucK*	-
~3	Abdel. *fucK*	-
Negative control	Abdel. *fucK*	-

*The curve was flattened and the CP value was incorrectly low.

Experiment 6 – The full Wang assay was run again to confirm earlier analytical sensitivity. The results are shown in Table 13.

Table 13: Full Wang *hpd* multiplex assay for HI.

Concentration of target DNA *Hi*[copies/reaction]	**Active target assay**	**Average CP value** *Multiplex**
~30 000	Wang *hpd*	27.8
~3 000	Wang *hpd*	31.2
~300	Wang *hpd*	33.0**
~30	Wang *hpd*	35.9**
~3	Wang *hpd*	-
Negative control	Wang *hpd*	-

*Multiplex consisted of Wang *hpd*, Wang *lytA* and Wang *ctrA*.
** The signal strengths of these were greatly reduced.

The assay showed positive results for 30 copies/ 25µL reaction. This was the same as last time but the two most diluted target DNA reactions signal strengths were below five units. The analytical sensitivity for *Hi* was hard to determine with good specificity. The curves were always flat and lacked the expected exponential rise. The signal strengths were often low for both assays but I concluded that the analytical sensitivity for the Abdeldaim *fucK* assay was ~30 copies per reaction and for Wang *hpd* ~30-90 copies per reaction.

Analytical sensitivity *Spn*

Since *Spn* is a Gram-positive bacterium it needs to be lysed differently from *Hi* and *Mnc* during DNA preparation.

Experiment 1 – The analytical sensitivity was compared for *Spn* Lagmo *lytA* assay while run as singleplex and while run as multiplex with Wang *ctrA* and Abdeldaim *fucK* assays. The analytical sensitivity was determined by three identical experiments. The results are presented in Table 14.

Table 14: *Spn* Lagmo *lytA* was compared while run as singleplex and multiplex with *fucK* and *ctrA* assays.

Concentration of target DNA *Spn* [copies/reaction]	**Active target assay**	**Average CP value** *Singleplex*	*Multiplex*
~14 000	Lagmo *lytA*	26.2	26.2
~1 400	Lagmo *lytA*	29.7	29.7
~140	Lagmo *lytA*	33.0	32.2
~14	Lagmo *lytA*	36.3	33.8**
~1.4	Lagmo *lytA*	39.0*	-
Negative control	Lagmo *lytA*	-	-

*Only one of the two duplicates was positive.
**Reaction curve was flattend out and CP value was pushed lower.

Experiment 2 – The analytical sensitivity was compared for the *Spn* Lagmo *lytA* assay while run as singleplex and while run as multiplex with the Wang *ctrA* and Wang *hpd* assays. The results are shown in Table 15.

Table 15: *Spn* Lagmo *lytA* was compared while run as singleplex and multiplex with *hpd* and *ctrA*.

Concentration of target DNA *Spn* [copies/reaction]	**Active target assay**	**Average CP value** *Singleplex*	*Multiplex*
~14 000	Lagmo *lytA*	26.0	26.8
~1 400	Lagmo *lytA*	29.5	29.9
~140	Lagmo *lytA*	32.8	33.7
~14	Lagmo *lytA*	36.0	37.5*
~1,4	Lagmo *lytA*	-	-
Negative control	Lagmo *lytA*	-	-

*Signal strength was reduced significantly (50%).

Experiment 3– The analytical sensitivity was compared for *Spn* Lagmo *lytA* while run as singleplex and run as duplex with Wang *ctrA*. The results are shown in Table 16.

Table 16: The *Spn* Lagmo *lytA* assay was run as singleplex and duplex with the Wang *ctrA* assay.

Concentration of target DNA *Spn* [copies/reaction]	**Active target assay**	**Average CP value** *Singleplex*	*Duplex*
~14 000	Lagmo *lytA*	24.7	24.7
~1 400	Lagmo *lytA*	27.9	27.8
~140	Lagmo *lytA*	31.5	30.4
~14	Lagmo *lytA*	34.5	31.6*
~1,4	Lagmo *lytA*	36.4	-
Negative control	Lagmo *lytA*	-	-

*Signal strength was reduced but still clearly positive!

The CP value of the Lagmo assay was clearly affected of being in a multiplex PCR with Wang *ctrA*, but only when there was less than 140 DNA copies per reaction. When it was run as singleplex the Lagmo assay showed positive results down to a few copies per well (1.5 copies/ 25 µL). The same well for the multiplex assay was negative. However, the multiplex assays analytical sensitivity was 15 copies per well (15 copies/ 25 µL.) All positive reaction curves had high signal strengths and the curves rose quickly.

At high template DNA concentrations the singleplex and multiplex assays were comparable both in CP values and signal strength. The signal strength of the multiplex assay showed a clear correlation with the reduction in template concentration. When the template DNA concentration was lowered, the signal of the multiplex assay was lowered as well, while the singleplex signal strength remained at the same level as for higher concentrations. This was a sign of primer dimer formation.

Experiment 4 – Determination of the analytical sensitivity with higher resolution was obtained by a singleplex run for the Lagmo *lytA* assay. The results are presented in Table 17.

Table 17: Lagmo *lytA* was run as a singleplex assay to get higher resolution of the analytical sensitivity. The reactions with the most diluted target DNA had flattened reaction curves.

Concentration of target DNA *Spn*[copies/reaction]	**Active target assay**	**Average CP value**
~14 000	Lagmo *lytA*	25.0
~1 400	Lagmo *lytA*	28.2
~140	Lagmo *lytA*	31.6
~42	Lagmo *lytA*	32.7
~14	Lagmo *lytA*	34.6
~4,2	Lagmo *lytA*	34.6
~1,4	Lagmo *lytA*	35.6*

*One of the duplicates was negative.

Experiment 5 – Another analytical sensitivity experiment was set up to investigate primer dimer formation in *Spn* Lagmo *lytA* and compare it to the full Wang multiplex assay. The results are presented in Table 18 and confirmation of the primer dimer formation can be seen in Figure 9.

Table 18: Lagmo *lytA* was run as a singleplex assay and compared to the Wang *lytA* assay while run in the full Wang multiplex assay. The Lagmo assay detected lower target DNA concentrations.

Concentration of target DNA *Spn*[copies/reaction]	**Active target assay**	**Average CP value**
~14 000	Lagmo *lytA*	24.5
~1 400	Lagmo *lytA*	27.8
~140	Lagmo *lytA*	30.8
~14	Lagmo *lytA*	33.5
~1.4	Lagmo *lytA*	35.8
Negative control	Lagmo *lytA*	-
~14 000	Wang *lytA*	25.6
~1 400	Wang *lytA*	29.0
~140	Wang *lytA*	32.6
~14	Wang *lytA*	36.5
~1.4	Wang *lytA*	-
Negative control	Wang *lytA*	-

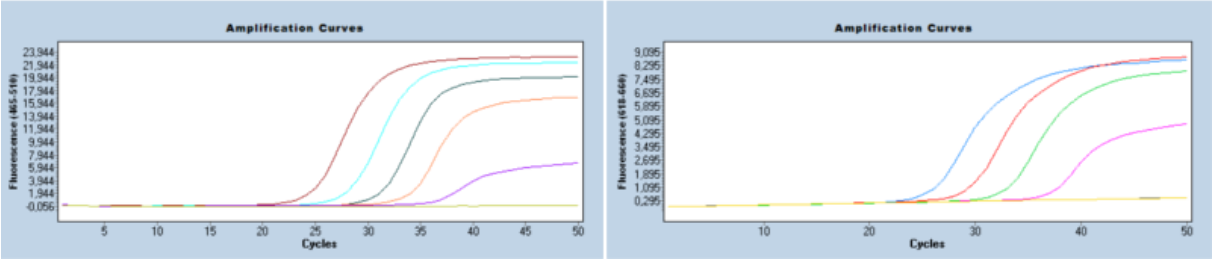


Figure 9: The picture show Lagmo assay to the left and Wang lytA in multiplex to the right. Notice the decreasing signal strength in both cases.

The primer dimer formation was confirmed on E-Gel®. Figure 2.

Experiment 6 – We ran Wang *lytA* in the full Wang multiplex assay. The results are shown in Table 19.

Table 19: The full Wang assay consisted of target assays for *ctrA, lytA* and *hpd*.

Concentration of target DNA *Spn*[copies/reaction]	**Active target assay**	**Average CP value**
~14 000	Wang *lytA*	26.7
~1 400	Wang *lytA*	30.2
~140	Wang *lytA*	34.0
~14	Wang *lytA*	35.8*
~1,4	Wang *lytA*	-
Negative control	Wang *lytA*	-

*Sample had reduced signal strength but was clearly positive.

The qPCR showed clear and quick rising curves with good signal strength when run in multiplex. The assay can identify concentrations with more than 14 DNA molecules per reaction (15/25 copies/µL).

The analytical sensitivity for the Lagmo assay was really high. The sensitivity was so high that we tested it several times but the results were unambiguous. During the tests the Lagmo assay was run parallel to the Wang *Spn* assay. During the experiments the Lagmo assay detected the target DNA concentrations which were ten-fold diluted compared to the most diluted target that Wang *lytA* could detect.

All the assays used in the project were covered by the sensitivity analysis. The values in Table 20 are rough numbers. Further, more thorough studies need to be done to verify and specify the values.

Table 20: Summary of analytical sensitivities for the assays that were used in this project.

Active target assay	**Target bacteria**	**Additional assays**	**Analytical sensitivity [DNA molecules/reaction]**
Wang *ctrA*	*N. meningitidis*	none	~15
Wang *ctrA*	*N. meningitidis*	Lagmo *lytA*	~15
Wang *ctrA*	*N. meningitidis*	Wang *hpd,* Wang *lytA*	~12
Wang *ctrA*	*N. meningitidis*	Wang *hpd*, Lagmo *lytA*	~150
Wang *hpd*	*H. influenzae*	none	~3
Wang *hpd*	*H. influenzae*	Wang *ctrA*, Lagmo *lytA*	~300

Wang *hpd*	*H. influenzae*	Wang *ctrA*, Wang *lytA*	~30
Wang *hpd*	*H. influenzae*	Abdeldaim fucK	~90-300
Lagmo *lytA*	*S. pneumoniae*	none	~2
Lagmo *lytA*	*S. pneumoniae*	Wang *ctrA*	~14
Lagmo *lytA*	*S. pneumoniae*	Wang *ctrA*, Wang *hpd*	~14
Wang *lytA*	*S. pneumoniae*	Wang *ctrA*, Abdeld. *fucK*	~14
Abdeld. *fucK*	*H. influenzae*	none	~30
Abdeld. *fucK*	*H. influenzae*	Wang *hpd*	~30
Abdeld. *fucK*	*H. influenzae*	Wang *ctrA*, Lagmo *lytA*	~300

Discussion

Target genes to use

There were several possible genes that could be used as targets. A target gene should be preserved within the species of bacteria, but for species specific detection it has to be unique for that bacterial species, or at least have species specific sequences. This is somewhat rare, since preserved genes are useful genes, and will therefore remain in the new species when they evolve and they will share the trait with their genus. However, some genes are unique for the species and these are the genes we need to target to get specificity. The perfect target gene would be a unique gene which is closely connected to the pathogenicity of the bacteria. Then there would be a chance that we could target close relatives which share the same pathogenicity traits as well. It is also important that the target gene lack repeats of 3 nucleotides or longer and preferably has 50% GC content.

To find species specific genes for the three target organisms *Neisseria meningitidis, Haemophilus influenzae* and *Streptococcus pneumoniae* we used published articles (Abdeldaim *et al.* 2008) (Abdeldaim *et al.* 2009) (Wang *et al.* 2012). These present a variety of different genes. Wang *et al.* published a multiplex method targeting *lytA* (*Streptococcus pneumoniae*), *hpd* (*Haemophilus influenzae*) and *ctrA* (*Neisseria meningitidis*). These three genes have been used in several studies and were specific for the respective species. Previously studied gene *fucK* in *Haemophilus influenzae* was also evaluated as a target (Abdeldaim *et al.* 2013). Also *Spn*9802 was considered as a candidate to target *Spn* (Abdeldaim *et al.* 2009).

Primer dimer formation

A common problem in PCR is primer dimer formation. Primer dimers mainly consume the solutions resources and inhibit proper primer-target binding. This can be devastating to a PCR and a multiplex PCR is more vulnerable for these kinds of interferences. All assays from Wang show primer dimer formation after 35 cycles. It is often difficult to reliably analyze reaction values after 35 cycles since primer dimer formation is common. The Lagmo assay targeting *lytA* also show primer dimer formation after 35 cycles and reduction in signal strength. Even though these primer dimer formations were formed, all methods were able to detect 30 molecules per reaction and are to be considered sensitive enough.

Theoretical analysis

Visual OMP was the program used for a theoretical analysis. The theoretical analysis was focused on the interactions with the components of the multiplex PCR. All strands from the target DNA, primers and probes from the different methods were added to the program. The settings in the program were

set to emulate the multiplex PCR. All interactions between different strands were emulated and information about binding energies, melting temperature and primer dimer risks were presented.

Design of the lytA probe by theoretical analysis of the multiplex qPCR

The Hedberg assay used in this project was missing a fluorophoric probe. To be able to use the Hedberg assay I needed to design the probe. There were things that needed to be taken into consideration when designing a probe for a multiplex real time PCR. The probe needed to have good specificity and binding strength to the target. It was important that there were no G at the 5' end since that would quench the adjacent fluorophore. The maximum run of the same nucleotide was four since a repetition in nucleotides can cause the nucleotide to fold upon itself. Interactions with other oligos in the multiplex was avoided to the extent it was possible.

The program Visual OMP was used for the analysis. The target region was defined by the *lytA* primers called Hedberg F and Hedberg R. Several possible probes were created using Visual OMP and Blast-Primer (which were modified to suit a probe). Visual OMP helped out by constructing possible interaction possibilities between the oligonucleotides. It showed the binding strengths and interaction sites. Visual OMP can also produce suggestion primers and probes. All suggested probes are presented in Table 21.

Table 21: All the probes that were tested theoretically in the program OMP. Probe U3 was the one later chosen for the assay *Spn* Lagmo *lytA*

Name of the probe	Nucleotide sequence
ProbeU1	AACTCTTACGCAATCTAGCAGATGA
ProbeU2	CAATCTAGCAGATGAAGCAGGT
ProbeU3	ACGCAATCTAGCAGATGAAGCAGGT
ProbeU4	CGCAATCTAGCAGATGAAGCAGGT
ProbeD1	GCGTTTTCGGCAAACCTGCT
ProbeD2	CCTGTATCAAGCGTTTTCGGCA
ProbeD3	CCCTGTATCAAGCGTTTTCGGCA
ProbeD4	TCCCTGTATCAAGCGTTTTCGGCA
Probe1 visualOMP	TTGCGTAAGAGTTCGATATAAAGGC
Probe2 visualOMP	GATTGCGTAAGAGTTCGATATAAA
Probe3 visualOMP	TTGCGTAAGAGTTCGATATAAA

These probes along with all existing probes, primers and target genes where loaded into the program. The changeable settings in the program like temperature, salt concentration, nucleotide concentrations etc. were altered to fit our multiplex PCR. The settings are listed in Table 22.

Table 22: Shows the settings used in Visual OMP during interaction simulation.

Setting	Value inserted
Assay temperature	60^{o}C
[Monovalent +]	1 M
[Mg ++]	3.5 mM
Glycerol	0%
DMSO	0%
Formamide	0%
TMAC	0 M
Betaine	0 M
PH	7

The program was run several times slightly altering the settings and oligonucleotides used. The changed settings favor different probes.

When choosing a probe there were factors that I needed to take into consideration. I looked at the overall binding strength (ΔG) of the probe to its target, at the interactions melting temperatures (T_m), efficient T_m, efficient ΔG and if the probe/primer could cause a primer dimer reaction. All these were factors that were taken into consideration. However, the efficient T_m and efficient ΔG were the most valuable since those values considered the other nucleotides in the mix when calculated. That meant I needed to do a separate run for each of the probes so that the probe to probe interactions would not affect the analysis.

Binding strength – A good binding strength was needed for the probe to bind properly and to be activated. In visual OMP the binding strength was measured with ΔG. The efficient ΔG was the binding strength to the target when all nucleotides were present. If several similar sequences were present, it means that the relative strength of all bindings will go down and hence there will be many competing interactions. The binding with the lowest ΔG will be the most likely reaction to take place. No reaction should be stronger than any of the expected bindings. The expected reactions in this case means all primer and probe bindings with their respective target. Those had to be stronger than any primer dimer binding or other unwanted crosstalk.

Melting temperature – The T_m and efficient T_m were important since all of the reactions in the multiplex needed to run at the same temperature. The qPCR run all reactions in one well. If some of the assays were not fit to run at the temperature used, they will not be able to detect their target in the reaction. Therefore, all primers should have the same efficient T_m and all the probes melting temperatures should be approximately 10^{o}C higher. Most of the primers used in this study have the same melting temperature. The new *lytA* probe and other probes melting temperatures needed to fit together to work in a multiplex qPCR.

If an unwanted binding melts at a temperature way below the elongation state temperature of the PCR the binding was no real threat. Strong bindings usually have a higher melting temperature, so not all bindings can be discarded, those which could not be discarded this way, were taken into consideration when choosing an appropriate probe.

Unwanted extensions – During the analysis I also investigated the whole multiplex together to make sure the primers and probes would not form primer dimers. If a nucleotide binds strongly in the 3' end it will attract polymerases. The polymerases can amplify these types of nucleotides causing a so called primer dimer formation. Primer dimers will amplify fast in the solution and will work both as template and primer for the counterpart. Primer dimers will inhibit any other reactions can take place by consuming the PCR reagents and inhibit the primer binding competitively.

ProbeU3 – With all these properties taken into consideration I decided that probeU3 was the best *lytA* probe candidate. It had a melting temperature very close to the other assays probes and a good binding strength. ProbeU3 did not have any threatening binding possibilities with other oligonucleotides in the multiplex mix. The probe needed a fluorophore along with the target sequence and I chose cyan500. The fluorophore was not in use at the facility and was known to give good signal strength. The full probes name was changed to *lytA* Cyan500 and will be called so henceforth.

Several of the candidates were possible to use. After discussing with Björn Herrmann and Christina Öhrmalm it was decided that probeU3 was the overall best choice.

Choice of target gene for Spn

Four different assays were considered in this study. Two different assays targeting *lytA*, one targeting *Spn9802* and one targeting *ply*. The *ply* gene is commonly used but has been shown to be unspecific and was therefore discarded early during the development of this PCR.

The two different *lytA* assays generate strong signals while the amplification efficiency from the *Spn9802* assay has shown to be suboptimal due to its probe design. Gel electrophoresis was used to analyze the amplification of the DNA. There we could see that *Spn9802* and Wang *lytA* gave really strong DNA bands. All samples had the correct length of their respective products. Wang *lytA* and *Spn9802* were used for further investigation. The *Spn9802* assay continued to give negative real time PCR results, hinting that the probe was faulty or old. Therefore, *lytA* was chosen as the target for *Spn.*

Lagmo primers and Wang primers were used to target *lytA*. Both gave good PCR products and had strong signal strengths. The two assays target the same gene but the specificity cannot be tested within the boundaries of this project. Such study would require a great number of representative strains of *Spn* and closely related species such as *Streptococcus mitis.* The sensitivity of the assays can be compared by analytical sensitivity. See section "Analytical sensitivity *Spn*".

The determination of the analytical sensitivity in my study indicates that the singleplex Lagmo assay had higher sensitivity than the Wang *lytA* assay. The assays were run separately and in parallel, both indicating the same thing. The analytical sensitivity of other assays in this project are very similar to previously published data (Abdeldaim *et al.* 2013). The same method was used when determining the DNA concentrations for all three different target DNA. Therefore, my claimed analytical sensitivity should not be far from the truth. If the template DNA concentrations were incorrect the relative sensitivity of the methods were compared in the parallel studies.

To design the assay with highest sensitivity for detection of *Spn* I would use Lagmo *lytA*. Both the assays targeting *lytA* showed signs of primer dimer formation but with such a high sensitivity that this would not cause any problems when run as singleplex. However, the main goal of the project was to create a multiplex assay to save resources. The small advantage in sensitivity for the Lagmo assay diminishes in the advantages of running a multiplex PCR. I recommended the Clinical Microbiology Department at The Academic Hospital in Uppsala to use Wang's multiplex assay since it shows lower primer dimer formation and is a well-tested method developed over a long period of time. The other target genes were more compatible with the Wang *lytA* assay, which makes it a better choice in order to detect bacteria *Spn.*

Choice of target gene for Mnc

CtrA has been used by several groups (Wang *et al.* 2012) (Corless *et al.* 2001) with success, likewise it is in use at this department. During the project this assay was run several times both as multiplex and singleplex and it was always reliable. We choose the Wang *ctrA* assay as target for *Mnc*.

Choice of target gene for HI

The considered targets for *Haemophilus influenzae* were the *hpd* and *fucK* genes. Both genes have proven to be specific targets for *HI* (Abdeldaim *et al.* 2013) (Wang *et al.* 2011) (Nørskov-Lauritsen *et al.* 2009). See figure 10 and 11 in "Supplementary". The figures show that both primer targets give similar but low signals. The amplification curve also show some kind of abnormal amplification. The

singleplex assays works very similar which was shown in the tables and graphs. Figure 10 and 11 show the typical curves generated by the two targeting assays for *H. influenzae.* The reaction curves do not rise to maximum signal strength as fast as expected. The signal strength was relatively low compared to the assays used for other target organisms. The curves flatten out more easily when the target DNA concentration was lowered.

Detection by gel electrophoresis showed that the *fucK* assay generated higher concentration of product DNA. At low target DNA concentration both methods showed signs of primer dimer formation.

The analytical sensitivity showed that Wang *hpd* was more suitable as target assay. Wang *hpd* was more sensitive while in singleplex and was designed for a multiplex assay which has been well analyzed.

To acquire safety for a method in a multiplex environment a lot of tests needs to be done. I feel that this safety exists for the *fucK* assay while run in singleplex but not for any multiplex assay including *fucK* I have run during this project. The Wang *hpd* assay was designed with the other two Wang assays in mind and show reproducible results. The Wang assay was recommended to be used because of the safety that the method brings.

Quality assurance panel

The panel was designed to be used for quality assurance of nucleic acid detection assays for bacterial meningitis used in Swedish hospital laboratories. The panel consisted of cell suspensions of different concentrations and included one strong positive sample, one less strongly positive sample and sample at the detection limit of each of the target organisms. To achieve this I analyzed our analytical sensitivity runs and compare the CP values achieved by different copy numbers. I tried to achieve CP values of 25, 28, 31, and 34 in my first dilution series from the bacteria samples. I calculated the copy number needed to achieve a CP value of 25 for the different bacterium, taking all dilution steps into consideration. The bacterium was diluted in water 1000 times over three steps of ten. This should achieve a CP value difference of 3-4 between the dilutions.

DNA preparation protocols prefer 200 µL of sample. The bacterial samples were diluted in fetal bovine serum to simulate CSF to 200 µL. Three different DNA preparation methods were used to extract the DNA from the cells. We used NucliSens easyMAG bioMerieux, Genovision M48 and MagNa pure from Roche. For *Spn* I achieve CP values: 26.2; 24.8 and 25.9 for the three different extraction methods when the copy number in the 200 µL solution was roughly 564 000. For *HI* I achieved CP values: 22.2; 21.7 and 23.1 for the three different extraction methods when the copy number in the 200 µL solution was roughly 6 000 000. Notice that my pre calculations were off. A copy number of 1/10 of the one used seems accurate. For *Mnc* I achieve CP values: 23.3; 23.1 and 26.9 for the three different extraction methods when the copy number in the 200 µL solution was roughly 5 340 000. Notice that the calculations were off here as well, and 1/5 of the concentration should be accurate to hit CP value 25. Later on a further mistake in the calculations was found. When we made the DNA preparation we did not use the full volume of the sample sent, which should set the DNA concentration to approximately one third of that calculated.

The decision to change into one duplex method and two singleplex, a sidestep

HI has been tested using two different target genes, *hpd* and *fucK*. Both were specific and gave amplification curves. The curves were similar and rose slowly and did not reach the maximum signal strength. The curves continued to rise slowly till the end of the PCR. Real Time PCR amplifications

should have quickly rising reaction curves with a signal strength plateau equal for all template dilutions. See figure 10 and 11 in "Supplementary".

The oligonucleotides targeting the *fuck* and *hpd* had shown to inhibit the amplification of Wang *ctrA* and Lagmo *lytA*. See "Analytical sensitivity *Spn*".

It was possible to develop a new target assay for *HI*. A new target would take several weeks to develop. I would have needed a unique gene, developed good primers and an efficient probe. I would have needed to be able to guarantee the specificity and certainty of *HI* detection, which would have required a lot of testing. The decision was made with time and resources in mind, that I could not develop a new target assay for *H. influenzae*. I decided to split the PCR into one duplex method and two singleplex methods. The decision was made in two steps. First we decided to make it into two duplex methods since the Wang *ctrA* assay had shown no inhibition on or from, the Lagmo *lytA* assay. This was however proven not to be the case during further studies with the CSF samples. The two assays did however prove to be very specific and accurate when run as singleplex.

To compensate for the two flawed targeting systems for *H. influenzae* we ran them together as a duplex. The duplex would target two different genes in *H. influenzae*. That would make the *H. influenzae* detection more specific and secure. The idea to split the assay was later revised when the Lagmo assay was discarded due to lack of benefits over Wangs *lytA* target which could be run as a multiplex assay. The two different assays will be active at the same time and inhibit each other. The split method was therefore not a good alternative, and the full multiplex Wang assay is preferable since it is designed to be run as a multiplex assay and it has good sensitivity and specificity.

Conclusion

The main goal for the project was to present the best overall multiplex qPCR assay for targeting the three meningitis causing bacteria *H. influenzae, N.meningitis* and *S. pneumoniae.* The assay would preferably be run on *Roche LightCycler® 480.* I tried some new assays, some self-developed assays and some previously used assays. We compared sensitivity, reliability, primer dimer formation and specificity. As conclusion, the most reliable and safe method to use for the Clinical Microbiology Department at the Academic Hospital in Uppsala, was the method published by Wang *et al.* 2012. Some methods we used were more sensitive than some parts of this multiplex method, but overall, multiplex Wang, consisting of assays for target genes *hpd, lytA and ctrA,* was considered to be the best multiplex assay, and that is what I recommended them to use.

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Supplementary

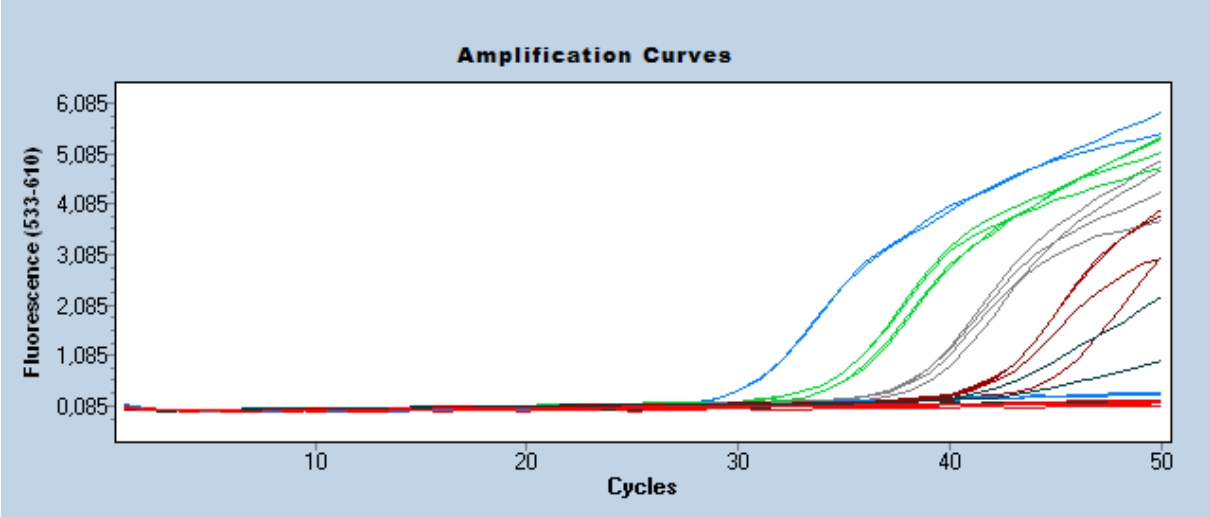

Figure 10

Figure 10 description: Meningit HI fucK versus Wang multiplexTest 130410 Selected Filter: Red 610 (533-610)

Table 23

Name	CP
Wang HI temp HI 10E3	30.25
Wang HI temp HI 10E3	neg? *
Wang HI temp HI 10E4	34.65
Wang HI temp HI 10E4	34.83
Wang HI temp HI 10E5	38.96
Wang HI temp HI 10E5	38.06
Wang HI temp HI 10E6	41.77
Wang HI temp HI 10E6	Neg
TWang HI temp HI 10E7	Neg
Wang HI temp HI 10E7	Neg
Wang HI neg	Neg
Wang HI neg	Neg
WangHI_multi temp HI 10E3	30.31
WangHI_multi temp HI 10E3	Neg
WangHI_multi temp HI 10E4	34.22
WangHI_multi temp HI 10E4	34.33
WangHI_multi temp HI 10E5	37.97
WangHI_multi temp HI 10E5	38.01
WangHI_multi temp HI 10E6	41.82
WangHI_multi temp HI 10E6	41.66
WangHI_multi temp HI 10E7	42.78
WangHI_multi temp HI 10E7	41.87
WangHI_multi neg	Neg
WangHI_multi neg	Neg

*The value is negative for both the *hpd* assay as well as for the *fucK* assay

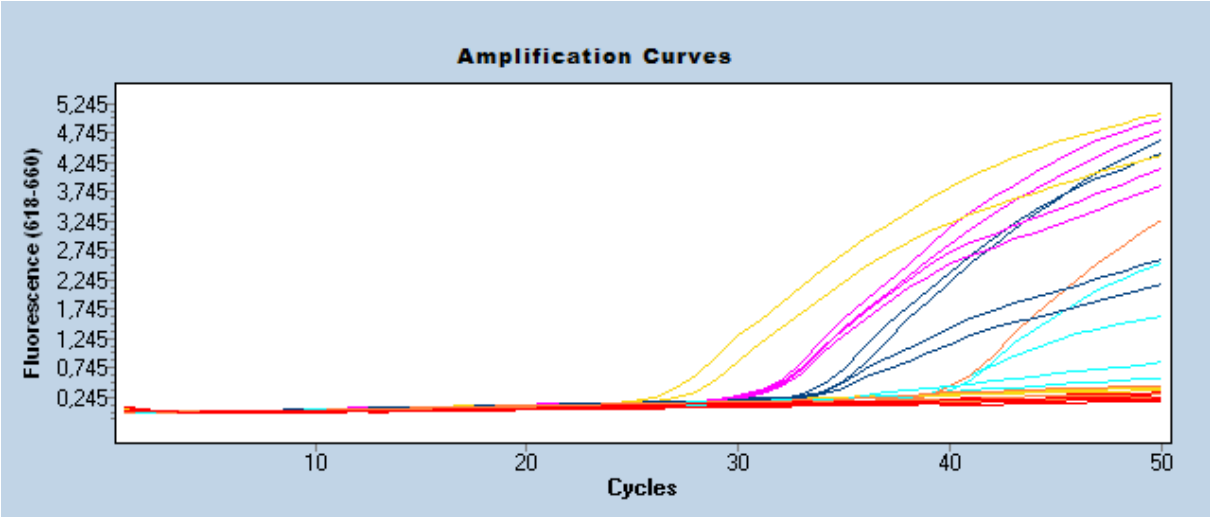

Figure 11

Figure 11 description: Meningit HI fucK versus Wang multiplex Test 130410 Selected Filter: Cy 5 / Cy 5.5 (618-660)

Table 24

Name	Cp
fucK temp HI 10E3	26.55
fucK temp HI 10E3	Neg see above
fucK temp HI 10E4	31.33
fucK temp HI 10E4	31.10
fucK temp HI 10E5	33.31
fucK temp HI 10E5	34.22
fucK temp HI 10E6	39.59
fucK temp HI 10E6	38.30
fucK temp HI 10E7	Neg
fucK temp HI 10E7	39.78
fucK neg	Neg
fucK neg	Neg
fucK_Multi temp HI 10E3	27.57
fucK_Multi temp HI 10E3	Neg
fucK_Multi temp HI 10E4	31.23
fucK_Multi temp HI 10E4	31.47
fucK_Multi temp HI 10E5	33.69
fucK_Multi temp HI 10E5	33.76
fucK_Multi temp HI 10E6	Neg
fucK_Multi temp HI 10E6	34.08
fucK_Multi temp HI 10E7	Neg
fucK_Multi temp HI 10E7	Neg
fucK_Multi Neg	Neg
fucK_Multi Neg	Neg

Table 25: Sequences of primers and probes used.

Name	Sequence
Abdeldaim fucK F primer	TAAGATTTCCCAGGTGCCAG
Abdeldaim fucK R primer	TTCCCTCCTATGCGTTATGC
Abdeldaim fucK probe	ACTTAACACAGGCTGATTCAGCCCTG
Abdeldaim ctrA F primer	GCTGCGGTAGGTGGTTCAA
Abdeldaim ctrA R primer	TTGTCGCGGATTTGCAACTA
Abdeldaim ctrA probe	CATTGCCACGTGTCAGCTGCACAT
Wang hpd F729	AGATTGGAAAGAAACACAAGAAAAAGA
Wang hpd R819	CACCATCGGCATATTTAACCACT
Wang hpd Pbr762i	AAACATCCAATCGTAATTATAGTTTACCCAAT
Wang lytA F373	ACGCAATCTAGCAGATGAAGCA
Wang lytA R424	TCGTGCGTTTTAATTCCAGCT
Wang lytA Pb400	TGCCGAAAACGCTTGATACAGGGAG
Wang ctrA F753	TGTGTTCCGCTATACGCCATT
Wang ctrA R846	GCCATATTCACACGATATACC
Wang ctrA Pb 820i	AACCTTGAGCAATCCATTTATCCTGACGTTCT
Hedberg (Lagmo assay) F primer	CAGCGGTTGAACTGATTGA
Hedberg (Lagmo assay) R primer	TGGTTGGTTATTCGTGCAA