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# ***Detection of Borrelia bacteria using Real-time PCR***

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## Abstract

Lymes disease (LD) caused by bacteria in the *Borrelia* genus is the most commonly known vector borne disease on the northern hemisphere. There are at least 10 human pathogenic *Borrelia* species known to date, five of which have been identified in Norwegian ticks. Diagnosis of LD is routinely done by serological tests such as ELISA. However, this method of diagnosis is not well suited to distinguish between a wide range of LD symptoms and the disease causing bacterial species. Serological tests are dependent on an immune response, which may not always occur. Furthermore the immune response is a slow process, typically occurring 6 weeks after infection. A direct method of detection based on DNA typing would potentially allow earlier detection of bacterial infection as well as identify the actual disease causing *Borrelia* species.

Currently there is no direct testing method commercially available, and the *Borrelia* bacteria have proven difficult to work with. In the scientific community, there is controversy as to which is the most optimal sample material for bacterial DNA isolation, as well as which method of detection is the most suitable to apply. In this thesis the development of a direct detection method for the *Borrelia* bacteria by the *hbb* gene for a histone-like protein in human plasma and serum samples with real-time PCR (qPCR) was attempted.

Blood samples from medical patients who had tested positive for LD with ELISA antibody detection was used for isolation of *Borrelia* bacteria. The *hbb*-gene used for primer and probe design is highly conserved between species of *Borrelia* bacteria. A sufficient extraction method and qPCR optimisation was considered essential for *Borrelia* detection.

*Borrelia* was not detected in any of the samples tested. Both the DNA extraction and the qPCR assay applied for the testing needs to be further optimised. If we had succeeded in isolating bacterial DNA from blood samples, we would have proceeded to develop a melting curve qPCR analysis to distinguish between different strains of the disease causing bacteria.

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## Abbreviations

AD	Alzheimer Disease
BLAST	Basic Local Alignment Search Tool
bp	Base pairs
BSK media	Barbour-Stoenner Kelly media
CNF	Central Nervous System
CSF	Cerebrospinal Fluid
C <sub>t</sub>	Threshold cycle
C <sub>q</sub>	Quantification point
DNA	Deoxyribonucleic acid
dsDNA	Double stranded Deoxyribonucleic acid
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme Linked Immunosorbent Assay
EM	Erythema Migranes
FHI	Norwegian institute of public health (Folkehelse instituttet)
IgG	Immunoglobulin G
IgM	Immunoglobulin M
LD	Lyme Disease
MGB	Minor Groove Binding molecule
NCBI	National Centre for Biotechnology Information
Osp	Outer surface protein
PCR	Polymerase Chain Reaction
PTLD	Post-Treatment Lyme Disease
qPCR	Real-time Polymerase Chain Reaction
REK	Regional Ethic Committee (Regional Etisk Komité)
ssDNA	Single stranded Deoxyribonucleic acid
TE buffer	Tris-EDTA buffer
T <sub>m</sub>	Melting temperature
UNG	Uracil-N-Glycosylase
WB	Western Blot

# 1 Introduction

## 1.1 Background

Lyme disease (LD) is the most common vector borne disease in the northern hemisphere. LD is known to spread through ticks in the *Ixodes* family (Tijssse-Klasen, Sprong, & Pandak, 2013). In Norway LD has become endemic as far north as Brønnøysund (63°), and has also been found sporadically above the Arctic Circle (71°) (Kjelland, Stuen, Skarpaas, & Slettan, 2010). LD is caused by at least 10 *Borrelia* species, where *B. burgdorferi*, *B. afzelii* and *B. garinii* are the most common in Norway (Kjelland et al., 2010). LD is as diverse as the bacteria causing it, the symptoms may include a bulls eye rash called erythema migrans (EM), muscle stiffness, pain in joints, face palsy, headache and if left untreated arthritis often occur (Murray & Shapiro, 2010). LD is treated with antibiotic therapy.

Diagnosis of LD is done by indirect serological tests like Enzyme-Linked Immunosorbent Assay (ELISA) and western blot (WB) that detect antibodies produced by an infected person. The antibodies are produced by the immune system after the onset of an infection. This is a slow process and can take up to 6 weeks, before this false negatives can occur (Mygland et al., 2010). Serological tests are less specific than tests based on detecting the *Borrelia* spirochete directly. The ELISA and WB tests can detect an infection, but they cannot differentiate between different bacteria causing LD. Furthermore, it can be difficult to assign symptoms to a specific LD causing bacteria.

A method of detecting the *Borrelia* bacteria directly in human blood samples has proven to be difficult. *Borrelia* bacteria thrive in tissues and are not abundant in the blood (Committee on Lyme Disease and Other Tick-Bourne Diseases, 2011). However *Borrelia* DNA isolation from human blood has been done (Aguero-Rosenfeld, Wang, Schwartz, & Wormser, 2005; Santino, Berlutti, Pantanella, Sessa, & del Piano, 2008). PCR testing of erythema migrans skin biopsies have also been successful, but it's more expensive and invasive than the serological tests. In addition the EM rash is not always present (Tijssse-Klasen et al., 2013). The cerebrospinal fluid (CSF) has also been tested with PCR in some cases if a patient displays neurological symptoms like facial palsy with varying success

(Gooskens, Templeton, Claas, & van Dam, 2006; Kruger & Pulz, 1991). Because of the parasitic lifestyle of the bacteria cultivating it on medium is difficult. They require an expensive nutrient rich growth medium and even then it takes up to 16 weeks to get colonies, even in the right conditions it is difficult to grow (Sapi *et al.*, 2013). To grow the spirochetes from human serum and plasma samples is tedious work, and thus less useful as a diagnostic tool. Also it is likely that the spirochete will express itself different in the human body than in a culture or in the tick.

In this thesis I will try to detect *Borrelia* directly in human blood samples using real-time polymerase chain reaction (qPCR) and distinguish the different *Borrelia* bacteria by making a melting curve analysis. The qPCR setup is adapted from an article by Portnoi *et al.* (2006). His group cultivated *Borrelia* bacteria on growth medium and analysed them with qPCR using a sequence from a histone-like protein called *hbb* that is conserved among the most common disease inducing *Borrelia* spirochetes in Eurasia (Valsangiacomo, Balmelli, & Piffaretti, 1997). The research team was able to make a successful melting curve analysis that I will try to reproduce using human blood samples. Bacterial detection by qPCR from a blood sample would be specific for LD causing bacteria, and could differentiate between the different known *Borrelia* spirochetes.

The blood samples are anonymously donated and was provided by Dr. Med. D. Øgreid from Kolibri medical. The research project was approved by the regional Ethic committee (REK, appendix 5). The samples were IgM and IgG positive by serological testing.



## 1.2 *Borrelia sensu stricto* complex

*Borrelia Sensu stricto* complex is a diverse group of bacteria. According to the National Centre for Biotechnology Information (NCBI) 37 species of *Borrelia* is classified at present time. At least 10 of these are pathogenic to humans; *B. burgdorferi*, *B. afzelii*, *B. garinii*, *B. miyamotoi*, *B. valaisiana*, *B. bavariensis*, *B. bissetii*, *B. spielmanii*, *B. lusitaniae* and *B. kurtenbachii*, respectively (Rudenko, Golovchenko, Grubhoffer, & Oliver, 2011). The different species of *Borrelia* is geographically restricted since they are dependent on ticks in the *Ixodes* family and their vertebrate hosts for pathogenesis (Rudenko *et al.*, 2011). In USA, *B. burgdorferi* is the main cause of LD. In Norway and in the rest of Eurasia the disease picture is more complex, *B. afzelii*, *B. garinii*, *B. burgdorferi*, *B. valaisiana* and the recently discovered *B. miyamotoi* can all cause LD in Norway (Kjelland *et al.*, 2010; Kjelland, Rollum, Korslund, Slettan, & Tveitnes, 2015).

The *Borrelia* bacteria are members of the phylum spirochetes. They have a characteristic spiral structure comprised of a cylinder shaped protoplasmic space covered by an inner and outer membrane. In between the membrane layers there is a periplasmic space containing a peptidoglycan layer and flagellar filaments (Figure 1.1) (Merilainen, Herranen, Schwarzbach, & Gilbert, 2015). Unique for the spirochete is their internal flagella, unlike external flagella the periplasmic flagella gives the spirochete its shape in addition to aid in its movements. It moves in a corkscrew motion, which helps the bacteria to bore into tissues and makes it move efficiently in viscous liquids (Strelkauskas, Strelkauskas, & Moszyk-Strelkauskas, 2009).

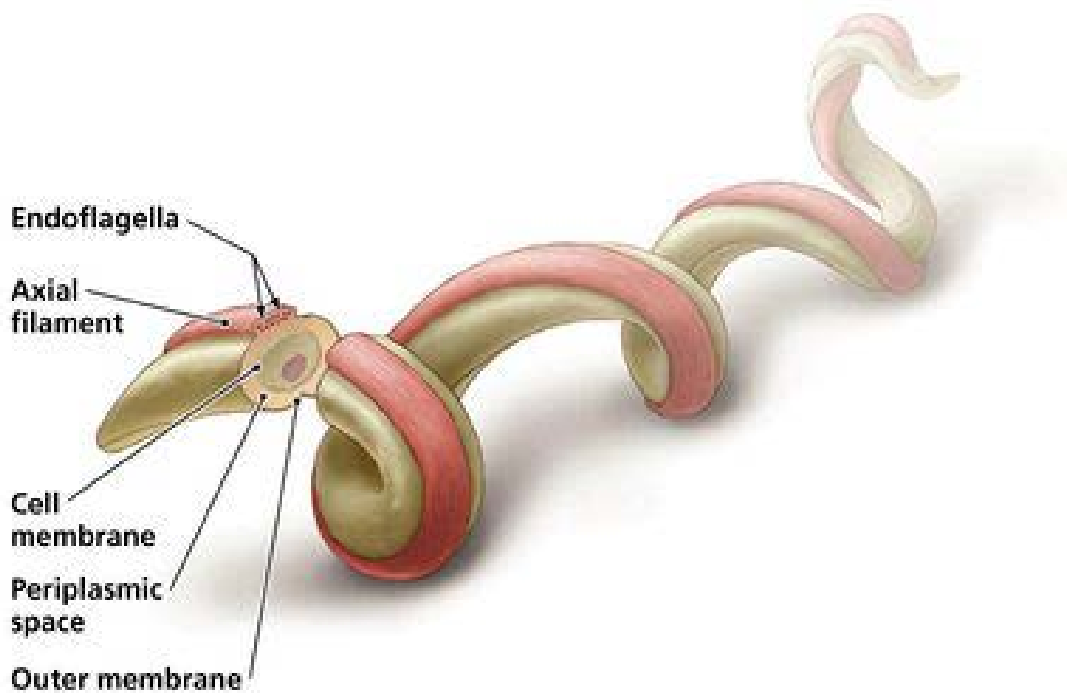


Figure 1.1: *Borrelia spirochete*. Reproduced with the permission of Pearson Education, inc.

The spirochetes can survive in several different host animals and evade their immune system (Singh & Girschick, 2004). Many different outer surface proteins (Osp) play a major role in the success of LD. They have the ability to up- and down regulate the different surface proteins when needed (Schwan, Piesman, Golde, Dolan, & Rosa, 1995). The spirochete express little Osp A in a mammalian host, but it is heavily present in the tick host (Crowley & Huber, 2003). Some surface proteins like Osp C are regulated by temperature (Kenedy, Lenhart, & Akins, 2012; Stevenson, Schwan, & Rosa, 1995). The ability to regulate Osp expression may play a role in the establishment of LD in different species. Almost 15% of its plasmid DNA and 5% of the chromosomal DNA encodes for outer surface proteins (Templeton, 2004).

The genome for *Borrelia burgdorferi* is very segmented and difficult to manipulate (Kung, Anguita, & Pal, 2013). It consists of a larger chromosomal genome and up to 21 linear and circular plasmids, the GC percentage in the entire genome is lower than 30% (Brisson, Drecktrah, Eggers, & Samuels, 2012). Like a true parasite, *Borrelia* DNA encodes for very little metabolites, and it's dependent on a host to survive (Brisson *et al.* 2012). This trait makes it difficult to culture the bacteria, since they need a growth medium high in nutrients.

The *Borrelia* bacteria thrive in host tissues like the skin, joints and heart (Schwarz *et al.*, 2012). It can also cross the blood-brain barrier and enter the central nervous system (CNS) (Pulzova *et al.*, 2011). There is some speculation if a correlation is possible between bacteria and Alzheimer disease (AD). *Borrelia* spirochetes have been found in brain biopsies from people who suffered from AD (Miklossy, 2011).

Eva Sapi a researcher from Newhaven University argues that *Borrelia* spirochetes has the ability to make biofilm and cysts *in vivo* (Sapi *et al.*, 2012). She was successful in making biofilm *in vitro*. Cystic forms of *Borrelia* spirochetes have also been made and reverted back to a vegetative state *in vitro* by inducing unfavourable conditions. If the *Borrelia* spirochete has the ability of biofilm and cyst formation, a short antibiotic cure might not be sufficient to cure LD.

## 1.2 Lifecycle and prevalence of Ixodes ticks

LD is a zoonotic disease dependent on ticks in the *Ixodes* family for propagation. In Europe *I. ricinus* is the most common vector organism (Schwarz *et al.*, 2012). *I. ricinus* are a species found along the coast of Norway. Their lifecycle consists of four stages, egg, larvae, nymph and adult (Figure 1.2). With the exception of the egg stage they need a blood meal to advance to every new life stage, meaning a tick can be a vector organism for *Borrelia* spirochetes as early as in the larvae stage (Schwarz *et al.*, 2012).

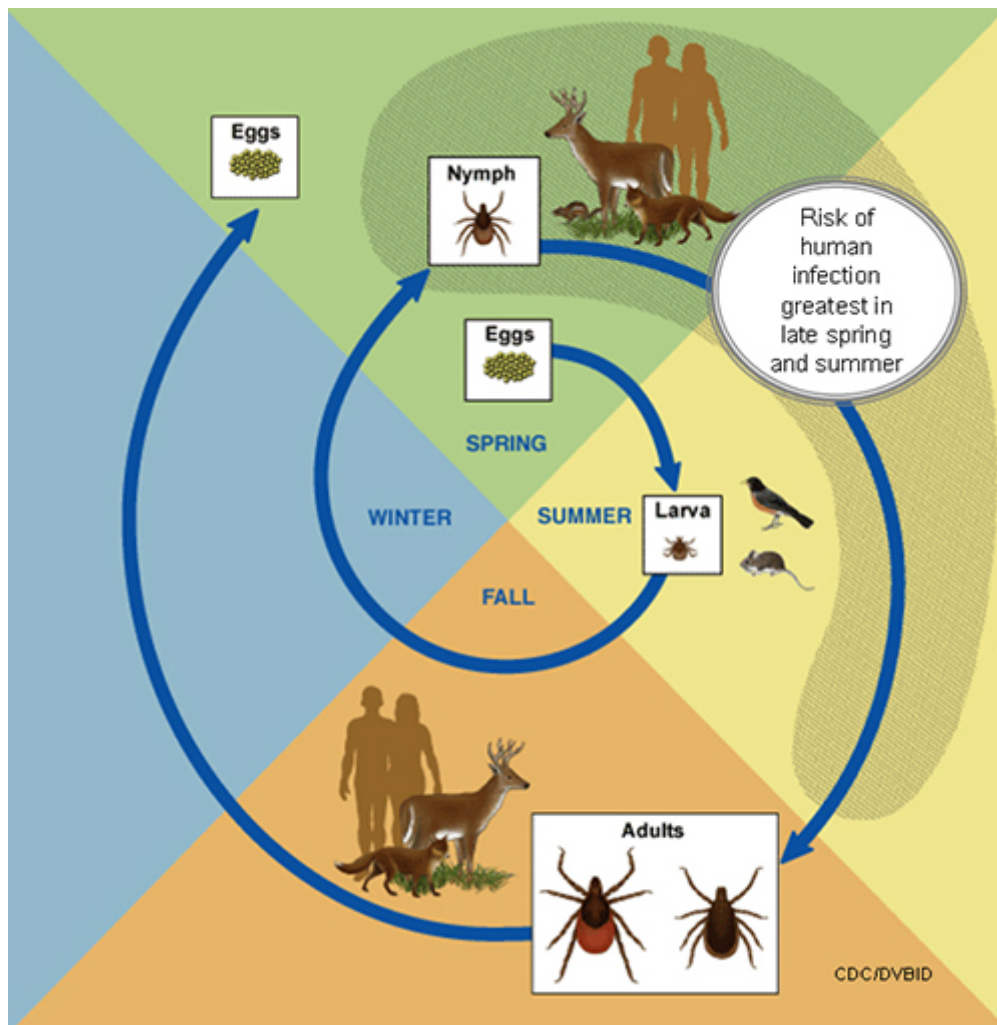


Figure 1.2: Illustration of the tick life cycle and transmission route of *Borrelia spirochetes*. Reproduced with permission from Centres for Disease Control Division of Vector-Borne Infectious Diseases: Lyme Disease Transmission. ([http://www.cdc.gov/ncidod/dvbid/lyme/ld\\_transmission.htm](http://www.cdc.gov/ncidod/dvbid/lyme/ld_transmission.htm).)

The spirochetes are located in the mid-gut in the tick and moves up to the salivary glands during a blood meal to be transferred to a new host organism. The ticks can feed on a wide range of vertebrate hosts which becomes infected as well as reservoirs for LD. qPCR has been used in analysis of *Borrelia*-infected ticks and the results indicates that different spirochetes have a vertebrate host preference. *B. garinii* and *B. valaisiana* is associated with birds, *B. garinii* with small rodents and *B. burgdorferi* with roe deer (Dubska, Literak, Kocianova, Taragelova, & Sychra, 2009; Margos, Vollmer, Ogden, & Fish, 2011).

## 1.4 Lyme disease (LD)

LD also known as borreliosis or lyme borreliosis has existed for a long time, Ötzi the iceman mummy who lived around 3300B.C is the first recorded human with LD (Hall, 2011). LD is a multi-systemic inflammatory disease (Wilhelmsson *et al.*, 2013). The first clinical manifestation of LD is often a bull's eye rash called erythema migrans (EM) which presents itself after 3-30 days, the rash can look like a bulls eye hence the common name (Murray & Shapiro, 2010). However there is a disagreement on how often the rash presents itself in borreliosis patients, it varies from 60-90% according to different sources (Murray & Shapiro, 2010; Norwegian health directorate, 2009). Therefore it is not common to make a diagnosis solely based on the presence of EM. Flu like symptoms often accompanies the rash. LD is associated with several symptoms, which becomes more severe if antibiotic treatment is not started. Face palsies can occur, meningitis like symptoms, radiculoneuritis, memory loss and impairment of motor or sensory functions are some of the many symptoms that may occur. Solely based on the clinical assessment LD is very similar to other autoimmune diseases.

Different *Borrelia* spirochetes are associated with different symptoms. *B. burgdorferi* is associated with arthritis, *B. garinii* with neuroborreliosis and *B. afzelii* with dermatoborreliosis (Kjelland *et al.*, 2010). Current standard diagnostic tests cannot differentiate between the *Borrelia* spirochetes infecting patients making it difficult to be certain. This is one reason why it's important to develop a melting curve analysis for *Borrelia* spirochetes and this is possible with qPCR analysis.

### 1.4.1 Chronic borreliosis or Post Lyme disease

Chronic borreliosis is a controversial diagnosis and is currently not accepted as a disease in the Norwegian health community. After antibiotic treatment there are some patients who claim they are not fully recovered. Many physicians call this condition Post-Treatment Lyme Disease Syndrome (PTLDS). The cause of PTLDS is not well understood, it is believed to be damage on tissues by the immune system, and some doctors argue it's a psychotic condition caused by depression and that it has nothing to do with LD at all (Aucott, Rebman, Crowder, & Kortte, 2013). Symptoms for PTLDS may

include fatigue, widespread pain, sleep disturbance and memory loss (Aucott *et al.*, 2013).

However, if the research on biofilm and cyst formation can be proven *in vivo* a short antibiotic treatment might not cure LD, and a chronic form of the disease could develop (Sapi *et al.*, 2012). A qPCR test on human blood samples would be able to detect whether the infection current. This is difficult with the current diagnostic tests.

### 1.5 Diagnosis of Lyme disease in Norway

LD is diagnosed by serological tests like ELISA and WB. In Norway it's less common to confirm a positive test with WB, but in for instance USA and Germany it is practiced. Unfortunately serological tests are associated with several shortcomings. They don't detect the bacteria, but instead rely on antibodies produced by the immune system against infection. It is no way of knowing based on serological tests which *Borrelia* bacteria a patient is infected with. Both ELISA and WB detect immunoglobulin M (IgM) and immunoglobulin G (IgG) antibodies that the body produce after the onset of the disease. For a positive result both IgM and IgG must be present, and for this to happen can take up to 6 weeks (Mygland *et al.*, 2010). In the incubation period false negative results are a concern. IgM antibodies are produced early in the infection, this antibody is not specific for LD, and can be produced by an autoimmune disease (Racine *et al.*, 2011). IgG is produced later in the course of the illness and can be present in the body many years after a patient recovers from the disease (Nadelman & Wormser, 2007). Some people do not produce antibodies as a response to the bacteria (Murray & Shapiro, 2010). The serological tests are therefore not very specific, and false positives and false negative responses can occur.

The *Borrelia* spirochete is seldom cultured in diagnostic labs. To grow the *Borrelia* bacteria in growth medium is slow and tedious work. The spirochete needs a nutrient rich growth medium modified from the Barbour-Stoenner-Kelly (BSK). Eva Sapi from the New Haven University improved cultivation of *Borrelia* spirochetes from human serum samples in 2013, however it took 16 weeks to get a 94% positive result from the control group (Sapi *et al.*, 2013).

qPCR can detect the bacteria itself based on a DNA/RNA segment. And it's possible to differentiate between the different species of *Borrelia* in addition to prove an on going disease. qPCR is sometimes used in diagnosis of LD, but because of low efficiency it is not common (The national academies, 2011). qPCR has potential, but needs improvement to become a new diagnosis method.

### 1.6 *Hbb* histone-like protein

The *hbb*-gene sequence for a histone-like protein was used to design the primer and probe. Because of its close interactions with the genome it is highly homologous between *Borrelia* species (Portnoi *et al.*, 2006; Valsangiacomo *et al.*, 1997). A synthetic 171bp fragment from the 327bp *hbb* gene was utilized as a positive control (table 1.1). The synthetic plasmid DNA was made by Life technologies. Reference gene sequence and accession number is enclosed in appendix 1.

*Table 1.1: Hbb positive control. For complete reference sequence see appendix 1.*

<b><i>Hbb</i> Positive control</b>	GCTATTAAAGTAAGGAAATTAGTTTATGTCTTTTTCAAGAAG
Sequence oligo (5`-...-3`)	ACCAAAGGTTACTAAGTCAGACATTGTTGATCAAATAGCTTT
	GAATATTAATAATAATAATCTGAAATTAGAAAAAAAAATACAT
	AAGACTTGTAATAGATGCTTTTTTTTGAAGAGCTTAAAAGTAA
	TCT

## 2 Aims for the study

The main objective for this thesis was to develop a qPCR assay for the precise, fast and sensitive detection of *Borrelia* bacteria in human serum and plasma samples. First of all the qPCR setup must be optimized then a sufficient DNA extraction method must be achieved.

If this is accomplished the next aim is to develop a melting curve analysis to differentiate between the most common *Borrelia* species in Norway. The spirochetes are not abundant in the blood therefore it is important that the test can detect even the smallest amounts of *Borrelia* bacteria. As a positive control, a conserved sequence in the histone-like protein *hbb* was used (Portnoi *et al.*, 2006). A successful melting curve analysis was made by Portnoi *et al.* (2006), who detected *Borrelia* bacteria with qPCR by the *hbb* gene, and ran a SYBR® Green assay on the qPCR product. The result showed that different *Borrelia* species had different  $T_m$  (Table 2.1).

Table 2.1: **Melting curve results for *Borrelia* species.** Listed is the  $T_m$  for the *Borrelia* strains, results provided from Portnoi *et al.* (2006).

Species	$T_m$ (C°)
<i>B. afzelii</i> (VS461)	64.13 ± 0.41
<i>B. garinii</i> (20047)	51.60 ± 0.07
<i>B. burgdorferi</i> (B31)	67.80 ± 0.09
<i>B. valaisiana</i> (VS116)	58.80 ± 0.14
<i>B. lusitaniae</i> (PotiB2)	63.23 ± 0.47



## 3 Methods

### 3.1 Sample material

Gena collected blood samples at Kolibri medical twice a week, the blood samples were taken in EDTA and gel tubes. At Gena the plasma and serum were transferred to Eppendorf tubes in 200µl and 600µl samples and stored in a -20°C freezer. The samples were never thawed more than once.

### 3.2 Hbb

#### 3.2.1 Plasmid DNA description

*Table 3.1: Short description of plasmid DNA with hbb insertion. Summary of information provided by Life technologies.*

<b>Referance number</b>	1362127
<b>Designation</b>	E. coli K12 (dam+dcn+tonA)
<b>Gene name</b>	<i>Hbb</i> positive ctrl
<b>Gene size</b>	171bp
<b>Vector backbone</b>	pMA-RQ (ampR)
<b>Cloning sites</b>	Sfil/Sfil
<b>Quantity</b>	5µg Plasmid DNA
<b>Stock concentration</b>	0.1µg/µl

The synthetic *hbb* positive control was made with synthetic oligonucleotides and cloned into pMA-RQ (ampR) using Sfil(361) and Sfil(556) as cloning sites (figure 3.1). The plasmid was then purified from the transformed bacteria and DNA concentration determined by UV spectroscopy. The final product was then verified by sequencing.

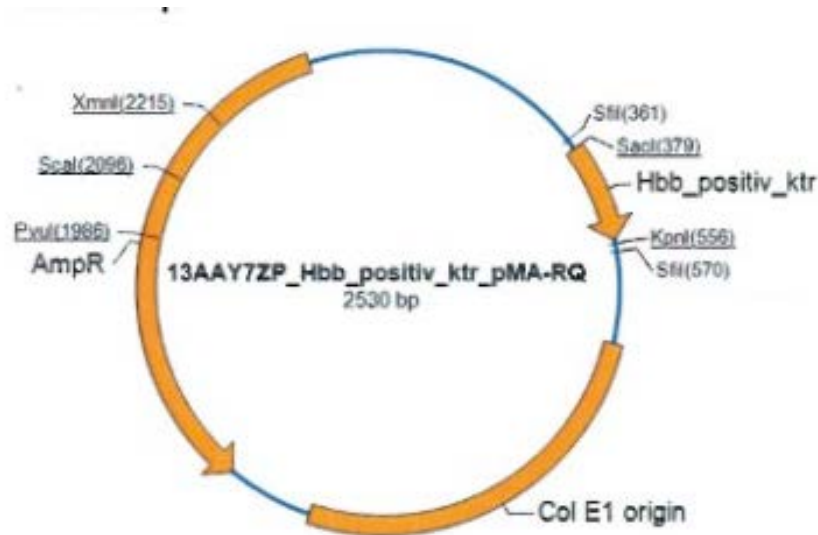


Figure 3.1: **Plasmid map.** *Hbb* positive control inserted in the *SfiI/SfiI* cloning site in a *pMA-RQ* (*ampR*) vector.

### 3.2.2 *Hbb* Sequence analysis

The *hbb* positive control sequence was aligned to *Borrelia* genomes using a Basic Local Alignment Search Tool (BLAST) at the national centre for biotechnology (NCBI) database (<http://www.ncbi.nlm.nih.gov>). The obtained sequences were then aligned in Clustal x a multiple sequence alignment tool.

### 3.3 DNA extraction methods

The principle of DNA extraction is to extract the DNA from the rest of the cell components. First the cell membrane and nucleus membrane have to be lysed, and thereafter the proteins and cell components have to be separated from the DNA and remove potential PCR inhibitors. Once the DNA is isolated downstream applications like qPCR can be done.

As a routine DNA extraction was always done with two parallels.

### 3.3.1 QIAamp® DNA Mini Kit

The protocol for *blood and body fluid spin protocol* in QIAamp® DNA Mini Kit from Qiagen was used to isolate DNA from serum and plasma samples.

Proteinase K and buffer AL were incubated with the sample to ensure cell lysis and protein degradation. The silica-gel membrane in the spin columns absorbs DNA while pH and salt concentration ensures PCR inhibitors to be washed through the column. In the final step Buffer AE elute DNA from the column.

The protocol was modified according to the article written by Girard *et al.* (2011). The start volume was increased from 200µl to 600µl (Girard, Fedorova, & Lane, 2011).

#### *Modified QIAamp® DNA Mini Kit protocol*

*A start volume of 600µl was used, and volumes of reagents were increased as recommended by the protocol for samples larger than 200µl. Both serum and plasma samples were utilized.*

- 1. 60µl proteinase K (20mg/ml, Qiagen) and 34.5µl carrier RNA (1µg/µl, Qiagen) was pipetted into the bottom of a 1.5ml micro centrifuge tube.*
- 2. 660µl Serum/plasma was added to the micro centrifuge tube.*
- 3. 600µl Buffer AL were mixed with the sample to lyse the cells and adjust conditions like salt and pH allowing for optimal DNA binding to the QIAamp membrane spin columns.*
- 4. The incubation time was increased from 10 minutes to 1 hour at 56°C.*
- 5. The samples were briefly centrifuged to remove drops from inside the lid.*
- 6. 600µl 100% EtOH was added to the samples and mixed by vortexing for 15 seconds.*
- 7. The mixture from step 6 was then carefully added to the QIAamp Mini Spin column. The caps were closed and the samples centrifuged 8000rpm for 1 min. Because of a large initial sample size the mixture from step 6 was centrifuged in two steps using the same QIAamp Mini spin column. The collection tube was emptied after each centrifugation.*
- 8. 500µl Buffer AW1 was added to the QIAamp Mini spin columns, the caps were closed and the samples centrifuged at 8000rpm for 1 minute. The QIAamp Mini spin column was then placed in a clean collection tube.*

9. *500µl buffer AW2 was added and centrifuged at 13 000rpm for 3 minutes.*
10. *The samples was then centrifuged for an additional minute at 13 000 rpm to remove all AW2 carryover.*
- *Buffer AW1 and AW2 are washing buffers that remove residual contaminants without affecting DNA binding. Improving the purity of eluted DNA.*
11. *The QIAamp Mini spin column was then placed in a clean collection tube and 50µl buffer AE was added and the sample was incubated for 5 minutes at room temperature before it was centrifuged at 8000rpm for 1 minute. The QIAamp was then placed in a new collection tube and the step repeated once more to elute the rest of the DNA.*

### 3.3.2 Ammonium hydroxide DNA extraction

DNA extraction with ammonium hydroxide is rapid and inexpensive. The protocol used was modified from the article by Santino *et al.* (2008). DDT, proteinase K and carrier RNA were added and incubated for 1 hour at 56°C.

The DNA precipitation method was changed by adding a step with 100% EtOH instead of 70% EtOH, and the centrifugation after overnight incubation was done for 20 instead of 15 minutes.

#### *Modified ammonium hydroxide protocol*

*100µl serum/plasma samples were used.*

- 1. 10µl DDT (1M, Sigma-Aldrich®), 10µl Proteinase K (20mg/ml, Qiagen) and 5µl carrier RNA (1µg/µl, Qiagen) were added to the 100µl samples.*
- 2. 200µl Ammonium hydroxide (0.7M, Sigma-Aldrich®, appendix 4) was then added to the serum/plasma sample and boiled at 100°C for 5 minutes on a heating block (Dry block heating systems, QBD/QBH series, Grant) with the lids closed.*
- 3. Then the samples were centrifuged quickly to remove droplets from the lid.*
- 4. The samples were then boiled for an additional 10 minutes at 100°C with the lids open.*

#### *DNA precipitation*

- 5. 650µl 100% EtOH and 32,5µl sodium acetate (3M, Sigma-Aldrich®) were added to the samples.*
- 6. The samples were then left in a -20°C freezer overnight.*
- 7. The next day the samples were centrifuged for 20 minutes at 13 000rpm (4°C)*
- 8. The supernatant was discarded*
- 9. 100µl 70% EtOH was added*
- 10. The samples were centrifuged for 5 minutes at 13 000rpm*
- 11. The supernatant was discarded and the pellets were left to dry for approximately 15 minutes.*
- 12. The pellets were then re-suspended in 50µl TE-buffer (Appendix 4).*

## 3.4 Developing qPCR protocols

### 3.4.1 Primer and probe design

Primers are short synthetic oligo sequences that bind specifically to a DNA strand and act as a binding site for DNA polymerase to start to synthesize a new DNA strand. Life technologies produce two types of fluorescent dyes TaqMan<sup>®</sup> and SYBR<sup>®</sup> Green. For this thesis TaqMan<sup>®</sup> MGB probes was utilized.

TaqMan<sup>®</sup> probes binds to a specific sequence in the template strand. The probe is bound to a fluorescent reporter dye and a quencher. The quencher will inhibit the reporter dye from emitting a signal when they are close to each other (figure 3.2). Once the DNA polymerase starts to elongate the template strand the TaqMan<sup>®</sup> probe will hydrolyse and the reporter dye will generate a signal. There are two kinds of TaqMan<sup>®</sup> probes MGB and non-MGB.

MGB TaqMan<sup>®</sup> probes has a minor groove binding (MGB) molecule at the 3' end, this molecule increase the probes  $T_m$  by strengthening binding to the template strand. This means that these kinds of probes can be shorter than non-MGB probes and still have a higher  $T_m$  than longer probes without MGB.

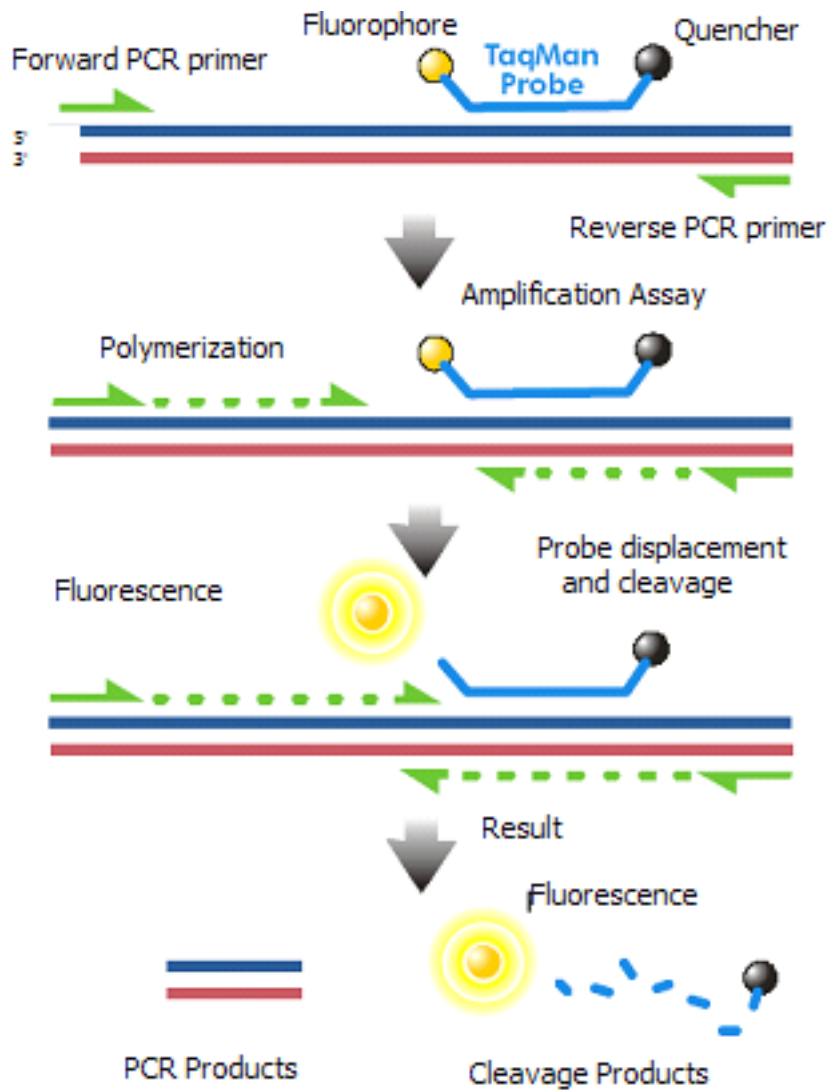


Figure 3.2: **Taqman probe.** The probe and primers binds to the template strand. A quencher molecule inhibits the fluorophore/reporter dye until the new DNA strand hydrolyse the probe and separates the reporter dye from the quencher allowing it to fluoresce (<http://en.wikipedia.org/wiki/TaqMan>).

All primers and probes for this project were designed in Primer Express® version 3.0.1, a software developed by Life technologies. The primers and probes were designed according to Real-time PCR handbook (2014) provided by Life Technologies. The sequences for the primers and probes used are listed in table 3.2. 4bp was added at the start of the *hbb* positive control. The bp added is not a part of the *hbb* DNA sequence, but is still highly conserved in *Borrelia* genome (Appendix 3).

Life primers and probes were designed by Bjørn Rosén a technician from Life technologies. Portnoi primers and probes were based on the same sequences used by Portnoi *et al.* (2006). See appendix 2 for amplicon binding and length.

*Table 3.2: qPCR primers and probes used for qPCR. All probes were MGB TaqMan® probes with FAM™ reporter dye and TAMRA™ quencher.*

Assay		Sequence oligo (5`-...-3`)	T <sub>m</sub> (°C)
Life	Probe	6-FAM-GCT ATT TGA TCA ACA ATG-MGB	69.0
	F. primer	TGA CTT AGT AAC CTT TGG TCT TCT	52.4
	R. primer	GTC TTA TGT ATT TTT TTT CTA ATT TCA G	52.3
Portnoi	Probe	6-FAM- CAA TGT CTG ACT TAG TAA CCT TTG GTC TTC TTG A-MGB	77
	F. primer	AAA GAC ATA AAC TAA TTT CCT TAC	47.1
	R. primer	TAA GCT CTT CAA AAA AAG CAT CTA	49.0
Modified Portnoi	Probe	6-FAM-ACC TTT GGT CTT CTT G-MGB	62.0
	F. primer	AAA GAC ATA AAC TAA TTT CCT TAC TTT AA	49.7
	R. primer	TAA GCT CTT CAA AAA AAG CAT CTA	49.0

### 3.4.2 Real-time PCR

TaqMan® Fast Advanced master mix and 96-well clear reaction plates for qPCR were provided by Life Technologies. The *hbb* positive control was diluted with TE-buffer (Appendix 4).

qPCR was carried out in QuantStudio™ 6 Flex provided by Life technologies, and is based on the same principle as conventional PCR where a target gene sequence is amplified. In qPCR, DNA concentration is measured after each cycle unlike conventional PCR where a



post PCR-analysis such as gel-electrophoresis must be done. This enables us to monitor the PCR progress in real time. The amplification normally occurs in three steps denaturation, annealing and extension/elongation, or two steps where the annealing and extension step is combined (table 3.3). According to the real-time PCR handbook (2014) from Life technologies the reaction is usually run for maximum 40 cycles.

*Table 3.3: Description of the steps in qPCR. The steps for qPCR and PCR are the same. The reaction time for each step is amplicon size dependent, generally 1000bp/min for TaqMan DNA polymerase ([http://en.wikipedia.org/wiki/Taq\\_polymerase](http://en.wikipedia.org/wiki/Taq_polymerase)). GC content also increase reaction time.*

<b>Denaturation</b>	Separation of the two DNA strands at 95°C to disrupt the hydrogen bonds between base pairs.
<b>Annealing</b>	The reaction temperature is lowered to 50-65°C and the primers and probe binds to their template strands. The temperature used in this step depends on the melting temperature ( $T_m$ ) of the primers. If the temperature is too high the primers cannot bind to the template, but if it's too low unspecific binding can occur.
<b>Extension/elongation</b>	DNA polymerase adds complementary base pairs to the primers and two new DNA strands are made. The temperature is increased for the DNA polymerase to work optimally. For Taq DNA polymerase the optimal temperature is 72°C.
<b>Combined annealing and extension</b>	Since the Taq DNA polymerase still function under optimal temperatures although slower a combined annealing and extension step can be done to save time.

In the reaction, a forward and reverse primer and a probe bind to the target sequence. The qPCR machine emits a light at a certain wavelength that will excite a reporter dye on the probe when it's hydrolysed. The qPCR instrument then measures the change in fluorescence intensity. When the signal exceeds the threshold value, the point of detection is called a threshold cycle ( $C_t$ ) or quantification point ( $C_q$ ). The threshold value is set to distinguish relevant amplification signal from the background. The threshold is automatically set at 10 times the standard deviation of the fluorescence value of the

baseline. A standard curve can be made based on the  $C_t$  value and the known amount of DNA concentration from a control sample. Unknown samples can be calculated using the standard curve and the  $C_t$  value.

### qPCR Setup for Life primers and probes

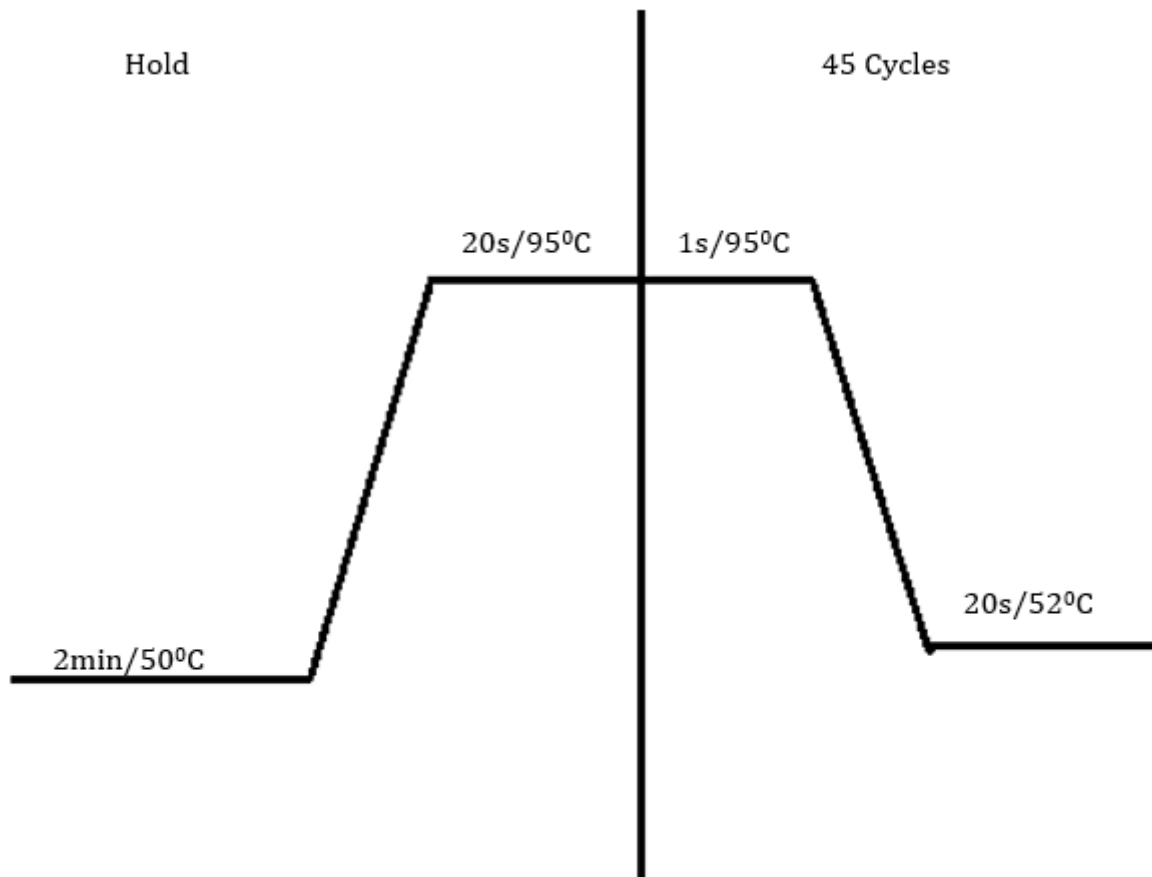
15 $\mu$ l of PCR mix and 5 $\mu$ l of template DNA was added to each well.

The qPCR setup solution used is listed in table 3.4. The final primer and probe concentration was 0.9 and 0.25 $\mu$ M, respectively. For the standard curve DNA concentration between 0.1 and 0.0001ng was utilized in a 1:10 dilution series.

*Table 3.4: qPCR set-up solution for Life primers and probe.*

Reagent	Volume ( $\mu$ l)	Final concentration
TaqMan® Fast Advanced Master Mix	10	
F. primer	1.8	0.9 $\mu$ M
R. primer	1.8	0.9 $\mu$ M
Probe	0.5	0.25 $\mu$ M
Nuclease free water (Sigma-Aldrich®)	0.9	
Template DNA	5	0.1-0.0001ng
Final volume	20	

The qPCR program utilized (figure 3.3) was recommended by Rosen and optimized by Elin Skårland Frøyland.



*Figure 3.3: qPCR program for Life primers and probe. Initial annealing at 50°C for 2 minutes and initial denaturation at 95°C for 20 seconds. Followed by 45 cycles with denaturation at 95°C for 1 second and a combined annealing and extension step at 52°C for 20 seconds.*

Since the qPCR master mix contained Uracil-N-Glycosylase (UNG) an initial solitary annealing step was added to break down unspecific DNA. The initial denaturation step helps denaturation of a larger initial genome size.

### qPCR setup for Portnoi and modified Portnoi primers and probes

The qPCR setup was inspired by the article, "A single-run, real-time PCR for detection and identification of *Borrelia burgdorferi sensu lato* species, based on the *hbb* gene sequence" (Portnoi *et al.*, 2006).

The Portnoi probe was shortened because of its high  $T_m$ . The Portnoi forward primer was elongated with 5bp. The qPCR setup solutions were optimized from the qPCR setup solution used in the article by Portnoi *et al.* (2006).

18 $\mu$ l of PCR mix and 2 $\mu$ l of template DNA was added to each well. A DNA concentration of 200-1ng was used for the standard curve. The setup solution was optimized several times, increasing the primer and probe concentration from 0.5 and 0.2 $\mu$ M to 0.9 and 0.5 $\mu$ M, respectively (table 3.5).

Table 3.5: Modified Portnoi qPCR set-up solution.

Reagent	Volume ( $\mu$ l)	Final concentration
TaqMan® Fast Advanced Master Mix	10	
F. primer	1.8	0.9 $\mu$ M
R. primer	1.8	0.9 $\mu$ M
Probe	1	0.5 $\mu$ M
Nuclease free water (Sigma-Aldrich®)	3.4	
Template DNA	2	200, 100, 10, 1ng
Final volume	20	

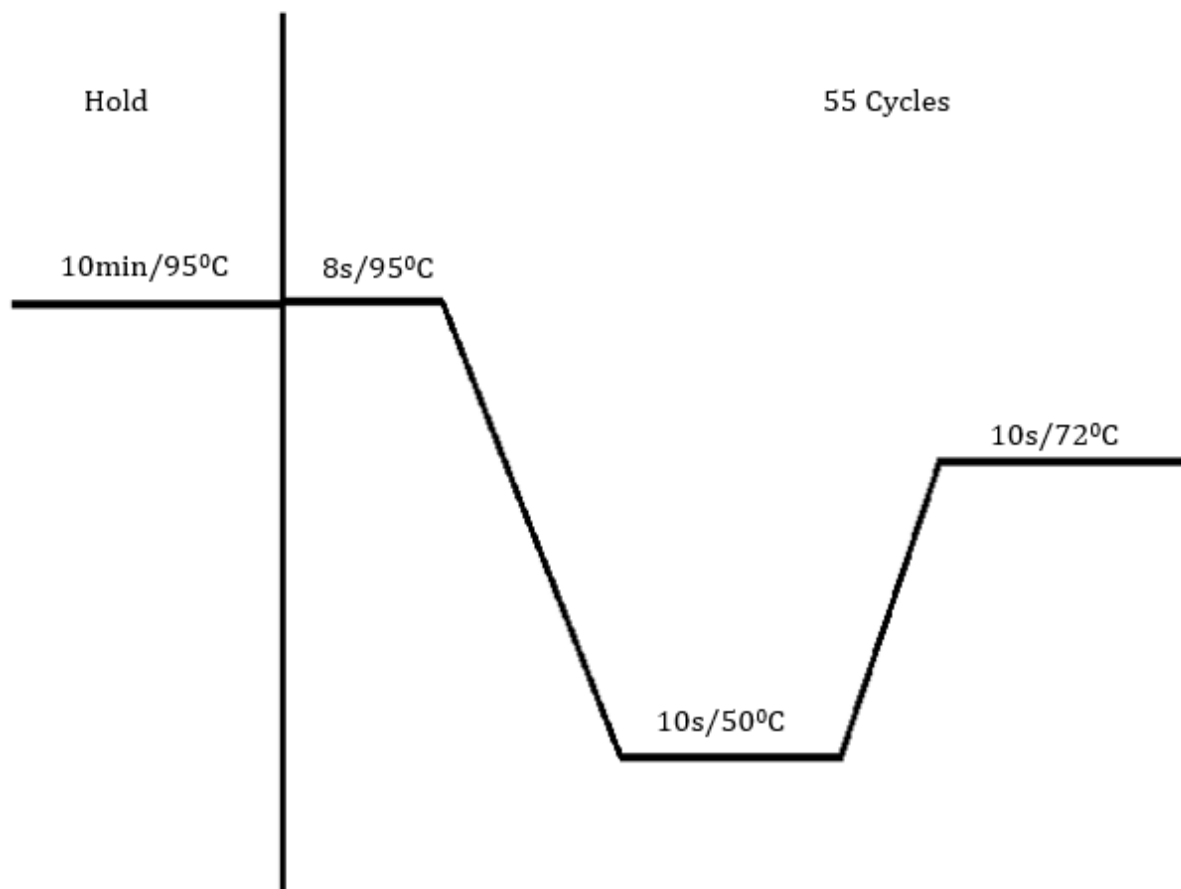
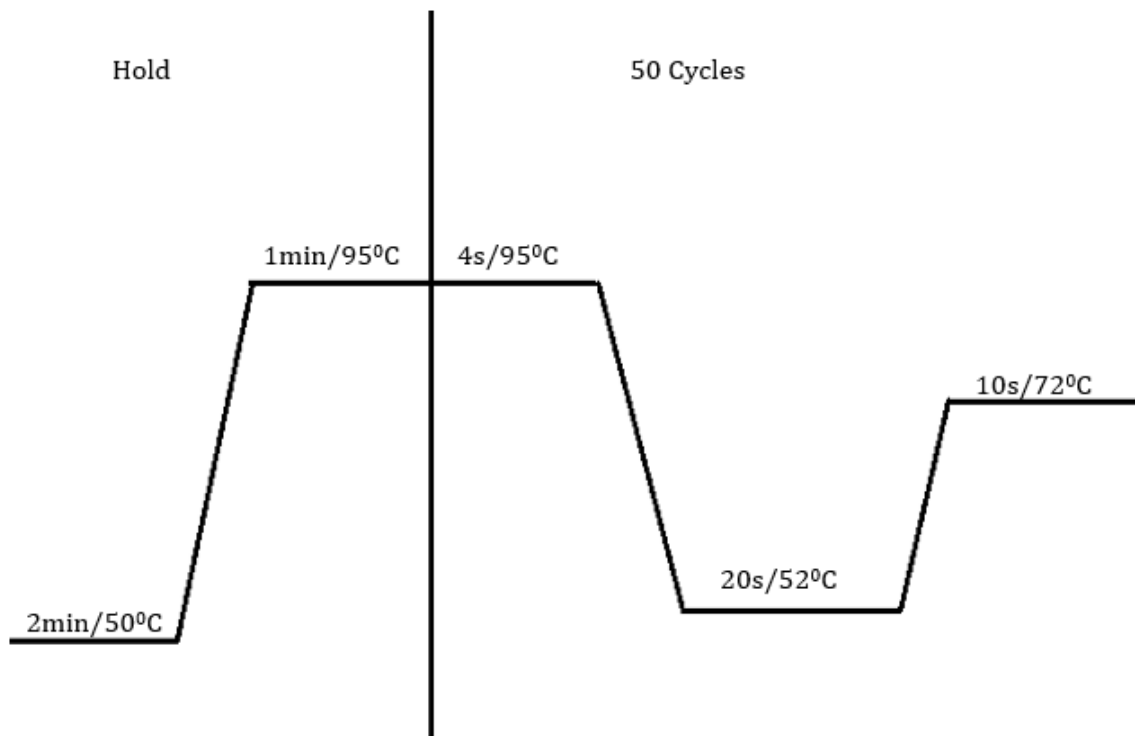


Figure 3.4: qPCR program ran in the article by Portnoi *et al.* (2006). Initial denaturation at 95°C for 10 minutes followed by 55 cycles with denaturation at 95°C for 8 seconds, Annealing at 50°C for 10 seconds and extension at 72°C for 10 seconds.

The qPCR program from Portnoi *et al.* was the basis of the optimizing steps (Figure 3.4).

Several modification steps were tried. An initial annealing step at 50°C was among others added to activate UNG in the TaqMan® Fast Advanced master mix. The time intervals in each qPCR step were changed one at the time and the annealing temperature was tried altered to optimize primer-binding efficiency (Figure 3.5).



*Figure 3.5: Modified qPCR program. Initial annealing at 50°C for 2 minutes and initial denaturation at 95°C for 1 second. This was followed with 50 cycles of denaturation at 95°C for 4 seconds, annealing at 52°C for 20 seconds and elongation at 72°C for 10 seconds.*

### 3.5 Melting curve analysis

A melting curve analysis differentiates between the *Borrelia* bacteria species. The Samples who tests positive for the *hbb* DNA sequence in the initial qPCR run, will be run again in a new qPCR assay with SYBR® Green. SYBR® Green is a non-specific fluorescent dye who bind to double stranded DNA (dsDNA). The dye binds to the minor groove of dsDNA. Instead of replicating the target sequence any further the temperature where DNA goes from double stranded to single stranded DNA (ssDNA) will be determined. This will be done by a gradual increase in temperature until there is no fluorescence detected by the qPCR sensor.  $T_m$  can be calculated based on the resulting graph.

## 4 Results

### 4.1 qPCR results

The *hbb* DNA sequence was not detected in any of the patient samples. The modified Portnoi and Life qPCR setups generated standard curves. Portnoi primers and probe did not generate a standard curve with the qPCR setup used by Portnoi *et al.* (2006).

Based on the standard curve primer binding efficiency was calculated (formula 4.1). The binding efficiency should be 100% ±10%. 100% efficiency corresponds to a slope of -3.32 (Real-Time PCR handbook, 2014). None of the standard curves produced was within this range.

$$E = ((10^{-1/slope}) - 1) * 100$$

*Formula 4.1: Primer-binding efficiency. The slope corresponds to the slope of the standard curve.*

#### 4.1.1 Life qPCR setup

The amplification plot for Life primers and probe had a threshold line of 0.206 for this qPCR setup (Table 3.4, figure 3.3). The patient sample ran with the same assay did not cross the threshold line (figure4.1).



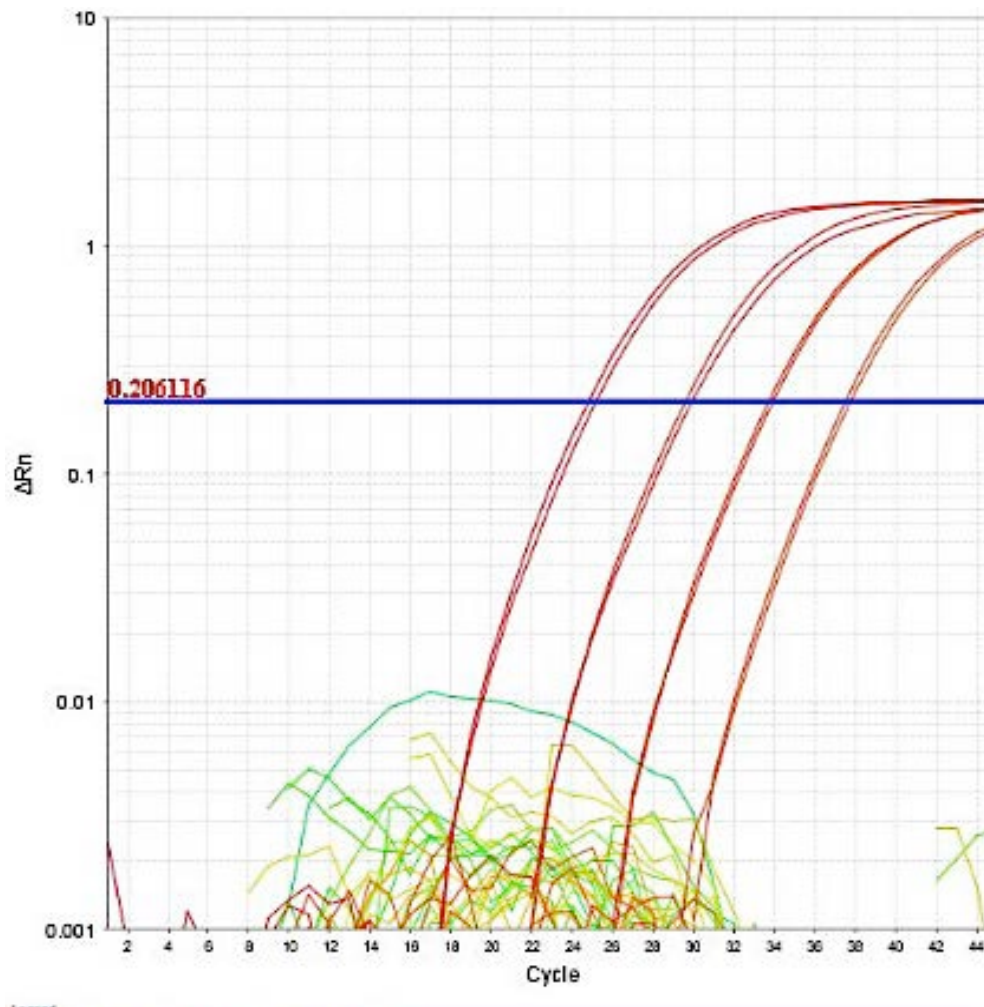


Figure 4.1: Amplification plot for Life qPCR setup.  $\Delta Rn$  plotted against cycle number. The strong background noise is largely due to patient samples. Threshold line is 0.206.

Based on the standard curve primer binding efficiency was calculated with formula 4.1 to be 73.2%. The  $R^2$  value, Y-intercept and the slope of the curve are 0.997, 25.22 and -4.19, respectively (figure 4.2).

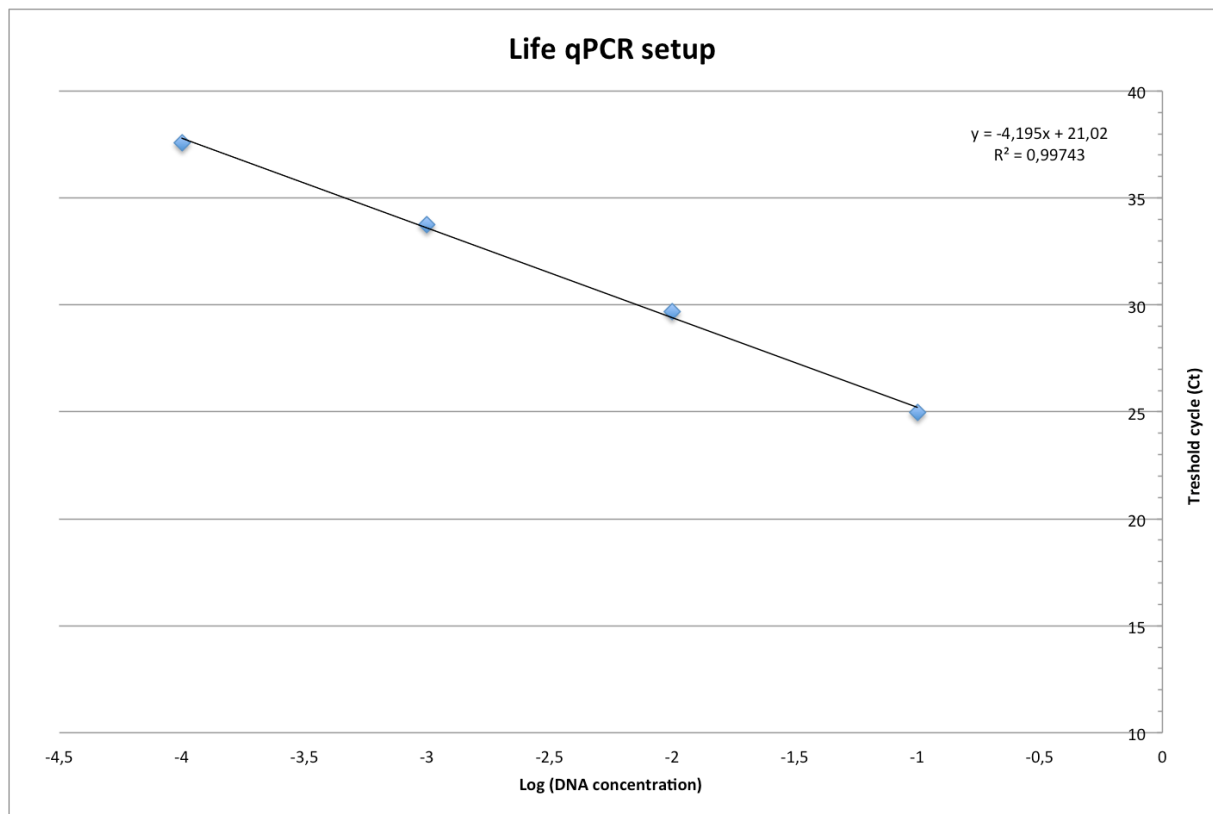


Figure 4.2: *Standard curve for Life qPCR assay.  $C_q$  plotted against Log (DNA concentration). The slope of the curve is -4.19.  $R^2$  and Y-intercept is 0.997 and 25.22 respectively.*

#### 4.1.2 Modified Portnoi qPCR setup

The minimum start concentration of *hbb* positive control that produced a signal was 1ng. The amplification plot had a threshold line of 0.0889 with the qPCR program ran (table 3.5, figure 3.5).

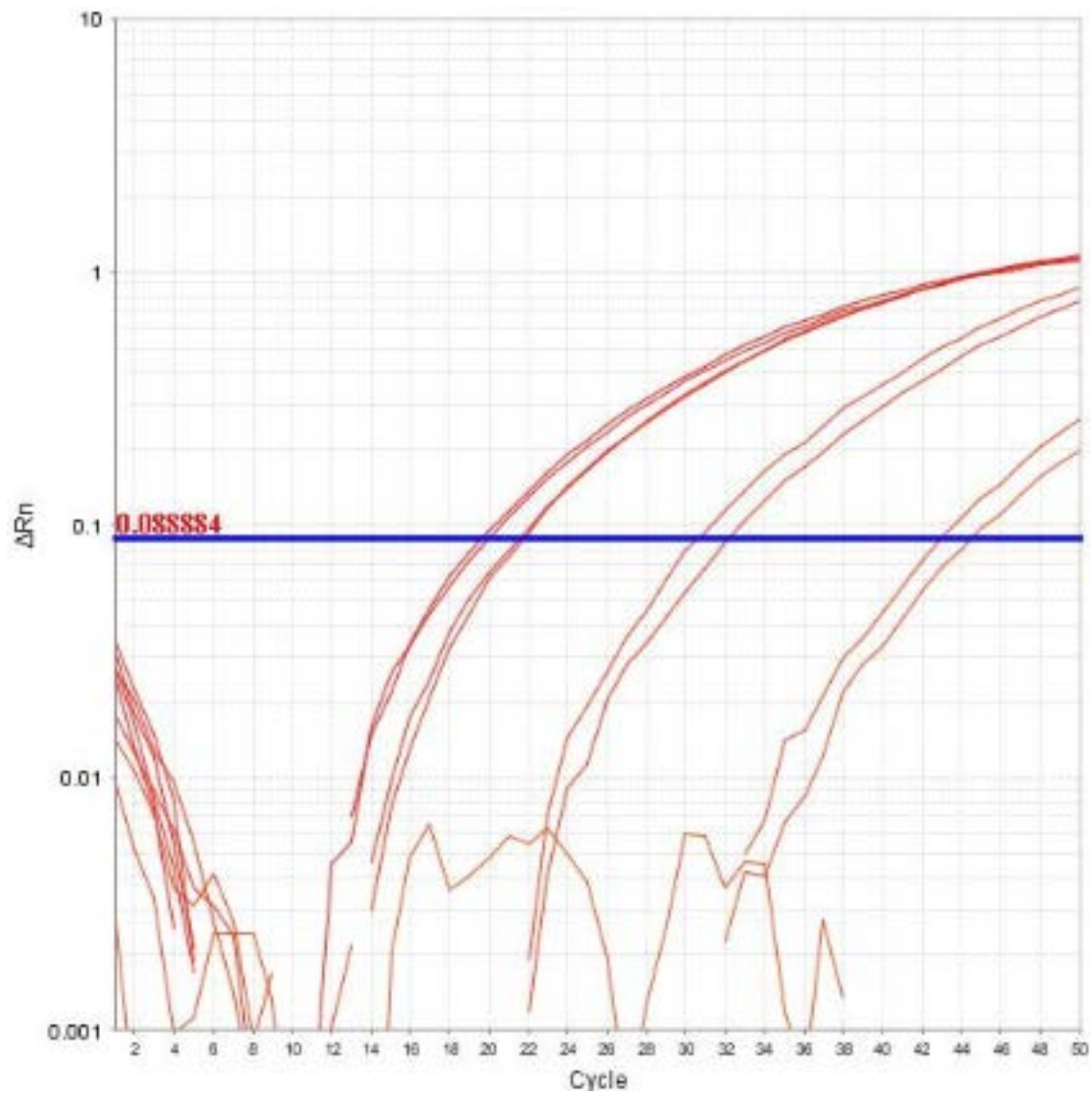


Figure 4.3: Amplification plot for the modified Portnoi qPCR setup.

The generated standard curve had a slope of -10,52 equivalent of a primer binding efficiency of 24,5% (formula 4.1). The Y- intercept and  $R^2$  value are 43,112 and 0.992, respectively (figure 4.4).

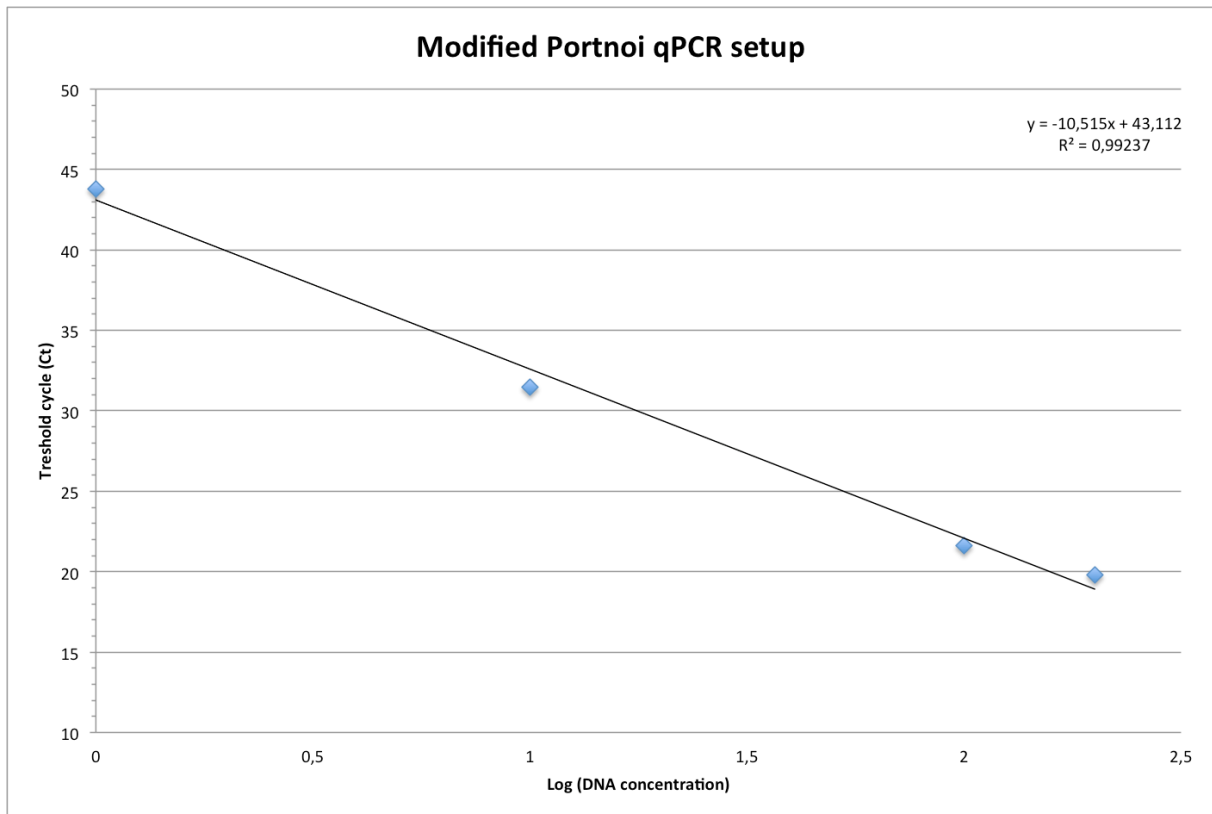


Figure 4.4: *Standard curve for the modified Portnoi qPCR setup. The slope of the curve is -10,52.  $R^2$  and Y-intercept is 0.992 and 43.112, respectively.*

## 5 Discussion

The main goal for this thesis was to develop a qPCR assay to detect *Borrelia* by a histone-like protein (*hbb* gene) in human serum and plasma samples and make a melting curve analysis to differentiate between the different *Borrelia* species. A method for direct detection of *Borrelia* bacteria in blood is not commercially available at present, but would be a positive contribution for LD diagnosis and for further *Borrelia* research.

Development of a sensitive qPCR assay for detection of *Borrelia* spirochetes in blood samples is more specific than the ELISA test. qPCR can quantify the concentration of bacteria in a sample. It would be able to detect an on-going infection and it would be species-specific giving medical staff the possibility of optimizing treatment and researchers the ability to map the different strains. We chose to design primers and probes by using the histone-like protein *hbb* gene, because the *hbb* DNA sequence within the different *Borrelia* species is conserved (Portnoi *et al.*, 2006; Valsangiacomo *et al.*, 1997).

### 5.1 DNA isolation

Serum and plasma were used for DNA isolation of *Borrelia* bacteria. *Borrelia* spirochete DNA has previously been extracted from serum and plasma samples, but there are no commercially available methods of direct detection of *Borrelia* in blood (Aguero-Rosenfeld *et al.*, 2005; Santino *et al.*, 2008; Sapi *et al.*, 2013). The reason for this can be that the amount of circulating spirochetes is low, which makes it difficult to develop a 100% reliable test for direct detection (Eshoo *et al.*, 2012). As mentioned in the introduction the spirochete structure is optimized for boring into tissue and the symptoms associated with the *Borrelia* bacteria are all associated with different kinds of tissue (Kjelland *et al.*, 2010; Strelkauskas *et al.*, 2009). The spirochetes might be using the blood as a transport highway to different tissues. A larger initial sample volume that is filtered to concentrate *Borrelia* spirochetes can be worth trying in the future (Aguero-Rosenfeld *et al.*, 2005).

The blood samples tested in this thesis had previously tested positive for LD with ELISA assay. Kolibri medical could not provide additional information about the course of the

illness or if antibiotic treatment had been started. This made it difficult to discuss the quality of the blood samples, possibly a vital part of the results of this project.

A good DNA isolation method was considered as the first objective in this thesis. It was important that the extraction method could potentially open *Borrelia* bacteria in cystic form (Sapi *et al.*, 2012). If the patient who provided the blood sample had started antibiotic treatment there might not be any *Borrelia* bacteria to detect, or only small amounts of bacteria. The extraction method should therefore be able to concentrate the spirochetes in the serum/plasma samples since there probably is a little amount of spirochetes circulating in the blood.

When a BLAST was done on the *hbb* gene it became clear that there was several mutations at the binding site for Life primers and probe (appendix 3). When this was discovered the focus was shifted to primer and probe design and qPCR optimisation. If the qPCR assay was not binding to the target sequence it is no way of knowing if the extraction method was good.

## 5.2 qPCR analysis

The qPCR setup designed for Life primers and probe produced a standard curve with primer binding efficiency of around 72.3%, indicating the need for further optimization. *Borrelia* bacteria were not detected by *hbb* DNA sequence in any of the plasma or serum samples. Initially it was believed the DNA extraction methods also needed improvement. However the Microbe BLAST of the *hbb* sequence at ncbi.gov revealed that the binding sites for the primers and probe had several mutations between the different species (appendix 3). The best match was *B. burgdorferi* B31 with only one mutation site. However *B. burgdorferi* bacteria are less frequent in Norway than *B. afzelii* and *B. garinii*. In a study by Kjelland *et al.* (2010) 1789 ticks in different life stages were collected all over southern Norway. Only 23.4% were infected with *B. burgdorferi*, indicating a similar distribution among infected humans. Mismatches between primers and probe and the template DNA strand leads to poor binding efficiency. Because of the BLAST result a new qPCR assay similar to Portnoi *et al.* (2006) was utilized instead. The qPCR assay had a primer binding efficiency of 83.77% in the article by Portnoi *et al.* (2006). We hoped this assay could be optimized and transferred to human samples.

The qPCR setup with Portnoi primers and probe generated no standard curve with the *hbb* control. The probe used was an MGB hydrolysis probe giving it a  $T_m$  of 77°C. The forward and reverse primer had a  $T_m$  of 47.1 and 49.0, respectively. The probe  $T_m$  was much higher than the recommended. The probe was therefore shortened from 34 to 16bp and the temperature decreased to 62°C. The primers had  $T_m$  difference of 1.9°C which is higher than recommended according to the Real-time PCR handbook (2014). Therefore 5bp were added to the forward primer increasing  $T_m$  with 1°C. The 5bp were added in an area where there were no mutations between the *Borrelia* species according to the *hbb* microbe BLAST. The primer  $T_m$  was still considerably low increasing the risk of unspecific binding. Portnoi *et al.* (2006) also had low  $T_m$  for his primers and probe, but they were able to detect *Borrelia* with this assay.

The modified Portnoi primers and probe qPCR setup produced a standard curve, but the primer binding efficiency was not within the 90-110% range. Several optimisation steps were attempted. The concentration of *hbb* positive control DNA used for the standard curve dilution was increased from a start concentration of 1ng to 200ng. In the article by Portnoi *et al.* (2006) DNA concentration corresponding to  $10^1 - 10^6$  cells was used for the standard curve.

The final concentration of primer and probe were increased from 0.5 and 0.2 $\mu$ M to 0.9 and 0.5 $\mu$ M, respectively. Primer concentration was not increased from 0.9 $\mu$ M because of the greater risk of unspecific binding. Probe concentration was increased to 0.5 $\mu$ M due to the difference in hybridisation probe and hydrolysis probe reaction chemistry. To increase the hydrolysis probe concentration further could increase the background fluorescence.

Portnoi *et al.* (2006) used reagents and qPCR machine from Roche diagnostics, in principle there should be no difference between qPCR machines from two different companies. A single fluorescein-labelled hybridisation probe and a forward primer with an internal Red640 label at the 3' end was used for *Borrelia* detection by the *hbb* gene. The single labelled hybridisation probe is not hydrolysed by the DNA polymerase and can be reused accounting for the low probe concentration used compared to the cycles

ran. It has been reported that hybridisation probes are more sensitive than hydrolysis probes and produce less background noise than hydrolysis probes (Wilhelm, Pingoud, & Hahn, 2001). Also glass capillary reaction tubes used by Portnoi *et al.* (2006) are reported to be more sensitive than plastic plates for samples with small DNA concentrations (Elenitoba-Johnson, David, Crews, & Wittwer, 2008).

Changes were made on the qPCR program from Portnoi *et al.* (2006). The initial denaturation time was decreased from 10 to 1 minute respectively. The *hbb* positive control template is 171bp long and therefore this was considered as sufficient denaturation time. At the start of this experiment qPCR steps were run for 40 cycles, but this was gradually increased to 50, which is very high. The annealing temperature was increased from 50°C to 52°C because primers bonded better with the template DNA strand.

The low primer binding efficiency for the standard curve (figure 4.4) is a clear indication of the need for further optimisation work for this assay. Human sources of errors cannot be excluded. From the amplification plot for the modified Portnoi qPCR assay (figure 4.3) the parallels are not gathered as close as I would like. It would be better if 3 parallels were used for each dilution instead of 2.



## 6 Conclusion and future perspectives

For further research on qPCR detection of *Borrelia* in human blood samples it would be beneficial to have a better understanding on *Borrelia* pathogenesis in the human body. Is there a constant flow of spirochetes circulating in the blood or are they only present during certain stages of the disease? To figure this out an idea can be to monitor a control group who has tested positive for LD. Blood samples could be taken regularly and cultured in the modified growth media developed by Eva Sapi *et al.* (2013). After sufficient incubation time (approximately 16 weeks) qPCR followed by sequencing can be done to confirm the presence of *Borrelia* bacteria. Another idea worth exploring could be to concentrate the spirochetes present in a blood sample by filtrating a larger volume of blood before DNA isolation, followed by a nested PCR. Increasing the target DNA sequence with PCR followed by qPCR for detection and quantification. But before this the qPCR assay have to work properly.

The *hbb* gene proved to be difficult to work with and the modified Portnoi qPCR assay needs further optimisation to detect *Borrelia* in human samples. The *hbb* gene had little GC percentage and thus the primers had to be very long to obtain a sufficient  $T_m$ , making them difficult to work with. Increasing sensitivity with hybridisation probes and glass capillary tubes could be worth exploring in the future.

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## Appendix

### 1 *Hbb* positive control reference gene

#### *Hbb* fragment used as positive control

The initial 4bp that was added to the *hbb* fragment is marked in yellow.

(5`-...-3`)

**GCTA**TTAAAGTAAGGAAATTAGTTTATGTCTTTTTCAAGAAGACCAAAGGTTACTAAGTCAGACATTG  
TTGATCAAATAGCTTTGAATATTAATAATAATCTGAAATTAGAAAAAATACATAAGACTTGTA  
ATAGATGCTTTTTTTGAAGAGCTTAAAAGTAATCT

#### Reference gene

#### *Borrelia burgdorferi* strain B31 histone-like protein HBbu (*hbb*) gene, complete cds

Accession number: U48648.1 ([www.ncbi.gov](http://www.ncbi.gov))

(5`-...-3`)

TTAAAGTAAGGAAATTAGTTTATGTCTTTTTCAAGAAGACCAAAGGTTACTAAGTCAGACATTGTTGA  
TCAAATAGCTTTGAATATTAATAATAATCTGAAATTAGAAAAAATACATAAGACTTGTAATAG  
ATGCTTTTTTTGAAGAGCTTAAAAGTAATCTTTGTTTCGAATAATGTTATTGAGTTTAGATCTTTGGTA  
CATTTGAAGTTAGAAAAGAAAGGGACGCTTAAATGCTCGAAATCCTCAAACAGGGGAGTATGTTA  
AGGTCCTAGATCATCACGTTGCGTATTTTCGTCCAGGCAAAGATTTGAAAGAGAGAGTGTGGGGTAT  
CAAAGGTAA

## 2 Primer and probe binding sites

*Hbb* positive control with bound primers and probe. Forward primer, Reverse primer and probe

### Life primers and probe

Amplicon length: 94bp

Oligo sequence (5'-...-3')

GCTATTAAAGTAAGGAAATTAGTTTATGTCTTTTTCAAGAAGACCAAAGGTTACTAAGTCAGACATTG  
TTGATCAAATAGCTTTGAATATTAATAATAATAATCTGAAATTAGAAAAAATACATAAGACTTGTA  
ATAGATGCTTTTTTTGAAGAGCTTAAAAGTAATCT

### Portnoi primers and probe

Amplicon length: 152bp

Oligo sequence (5'-...-3')

GCTATTAAAGTAAGGAAATTAGTTTATGTCTTTTCAAGAAGACCAAAGGTTACTAAGTCAGACATTG  
TTGATCAAATAGCTTTGAATATTAATAATAATAATCTGAAATTAGAAAAAATACATAAGACTTGTA  
ATAGATGCTTTTTTTGAAGAGCTTAAAAGTAATCT

### Modified Portnoi primer and probe

Amplicon length: 157bp

Oligo sequence (5'-...-3')

GCTATTAAAGTAAGGAAATTAGTTTATGTCTTTTCAAGAAGACCAAAGGTTACTAAGTCAGACATTG  
TTGATCAAATAGCTTTGAATATTAATAATAATAATCTGAAATTAGAAAAAATACATAAGACTTGTA  
ATAGATGCTTTTTTTGAAGAGCTTAAAAGTAATCT



### 3 Microbe BLAST result

Table A1: *Microbe BLAST results*. Data obtained from NCBI.gov. See figure A1 for Clustal x alignment.

Species	Accession number
<b>B. afzelii (HLJ01)</b>	NC_018887.1
<b>B. valaisiana (VS116)</b>	NZ_ABCY02000001.1
<b>B. burgdorferi (B31)</b>	NC_001318.1
<b>B. garinii (NMJW1)</b>	NC_018747.1
<b>B. garinii (BgVir)</b>	NC_017717.1
<b>B. garinii (PBi)</b>	NC_006156.1

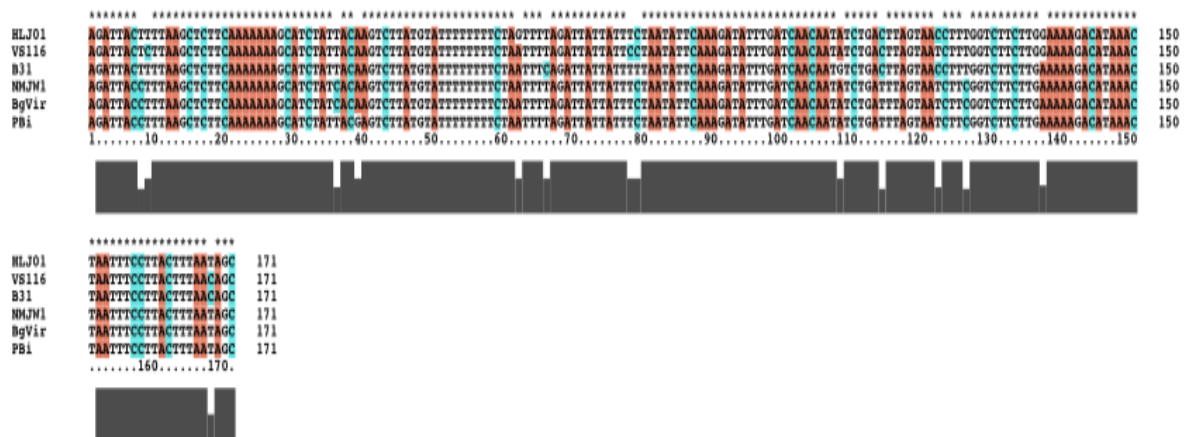


Figure A1: *Clustal x alignment of Microbe BLAST*. See table A1 for species accession number. Sequence oligo 5`-...-`3.

### 4 Buffers and solutions

#### Tris-EDTA buffer

Prepared by Elin skårland Frøyland

EDTA 10µl (10.5M, pH 8.0, Gbiochem) was mixed with Tris-HCl 2µl (1M, pH8.0, Life technologies). And then volume was adjusted to 1000µl with distilled water. The final solution had a pH of 8.0 optimal for DNA isolation. TE-buffer was stored at 4°C.

### Ammonium hydroxide

A stock solution of ammonium hydroxide (5M, Sigma-Aldrich®) was diluted with sterile water to a 0.7M solution.

## Forskningsprosjekt

### Påvisning av *Borrelia* bakterier ved bruk av real-time PCR metode

#### Prosjektbeskrivelse:

Lyme Borreliose har siden 1990 tallet vært registrert i Norwegian Surveillance System for Communicable Diseases (MSIS) som en smittsom sykdom. I Norge innrapporteres tilfeller av nevroborreliose, omtrent 300 tilfeller årlig, men som kun er en av mange Borreliose sykdommer. Borreliose skyldes betennelse med spiroketen *Borrelia burgdorferi sensu lato* som kan overføres gjennom flåttbitt. Utgangspunktet for denne studien er en påstand om at eksisterende tester er for dårlige og gir for mange falske negative svar. I dette prosjektet vil man «utvikle en metode for real-time PCR påvisning av *Borrelia*». Deltakerne vil være pasienter som går til lege for testing av mulig borreliose – ca 40 personer. Rekruttering skjer i utgangspunktet ved plakat på det aktuelle legekantoret. Legen spør muntlig om de vil delta (avgi blodprøve) og deretter skriftlig. Deltakelse innebærer at det tas en blodprøve som sendes til GENA for «metodeutvikling» i aidentifisert form. Prøven destrueres umiddelbart etter analyse. Data anonymiseres etter prosjektslutt.

(Redigert av REK)

Ref. nr.: 2014/596

Prosjektstart: 02.06.2014

Prosjektslutt: 01.06.2015

Behandlingsstatus: Godkjent

Forskningsstatus: Pågående

Prosjektleder: Ragne Farnen

Forskningsansvarlig(e): GENA- institutt for DNA analyse

Initiativtaker: Bidragsforskning

Finansieringskilder: Forskningen foregår hos GENA, finansiert med interne prosjektmidler.

Forskningsdata: Humant biologisk materiale

Utvalg: Allmennbefolkning

Forskningsmetode: Både statistiske og fortolkende analysemetoder

Antall forskningsdeltakere (Norge): 40

#### Behandlet i REK

Dato REK

14.08.2014REK vest

08.05.2014REK vest