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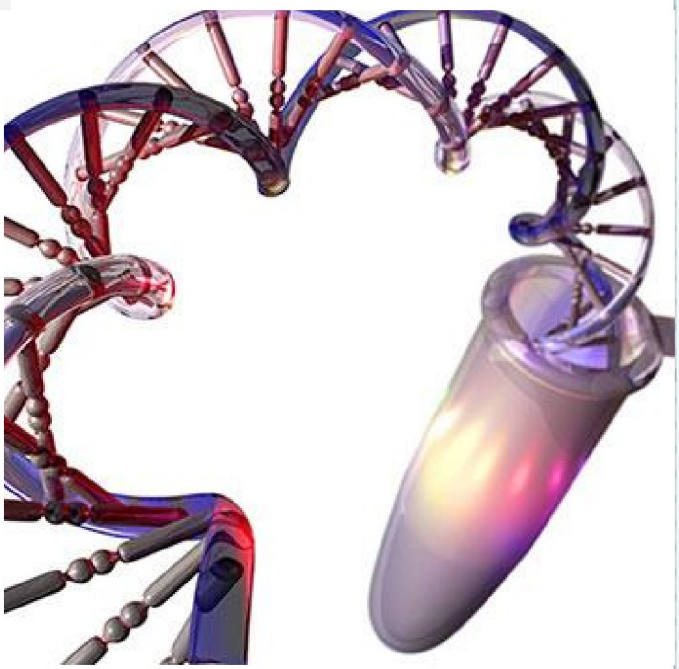
Nasir Sultan

In silico evaluation of PCR-primers for detection of Lyme *Borrelia*.



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This thesis is worth 60 study points

Cover-pictures

Ixodex scapularis: pbase.com
PCR: unlockinglifescode.org

Abstract

Lyme borreliosis (LB) or Lyme disease is the most prevalent vector-borne disease in US and Europe. The etiologic is some species of tick-borne spirochetes *Borrelia burgdorferi* sensu lato (*B. burgdorferi* sl) complex. The most common clinical symptoms of LB is the erythema migrans (EM). The pathogen is transmitted to humans through the tick bite of *Ixodes* species, and spread to cause more severe manifestations such as Acrodermatitis Chronica Atrophicans (ACA), Lyme arthritis, and neuroborreliosis.

Although polymerase Chain reaction (PCR) assay is an important molecular analysis for detection of DNA-pathogen in infected organisms, an apparent discrepancy about its accuracy and reliability is still existing. In order to validate PCR assay for detection of LB, 77 PCR-primer pairs were assembled from previous publications, and investigated for specificity and sensitivity using bioinformatics applications. The primers targeted genes coding for outer surface proteins *ospA* (29), *ospB* (2), and *ospC* (11), flagellin *flaB* (25), outer membrane protein *p66* (5), genetic recombination protein *recA* (2), and plasmid-specific sequence (3).

Basic Local Alignment Search Tool (BLAST) was employed to search homology between sequence of primers and sequences of GenBank database to find out cross-reactivity with non-targeted taxa of Lyme *Borrelia* or relapsing fever *Borrelia*. The primer-set that showed similarity only for the targeted species was considered a specific primer-set, while the primer-set that homologous with untargeted *Borrelia* species was in cross-reaction and was considered unspecific for the target species or PCR analysis. The sensitivity of primer-sets was rational designed and presented by proportion of specific hits for particular species to the total hits number for the same species, which is diagnostic sensitivity (coverage).

The results showed 25 (32%) specific primer-sets, 40 (52%) unspecific primer-sets, and 12 (16%) specific primer-sets but showed limited cross-reaction with untargeted *Borrelia* species. High proportion of *ospA*- primers were specific, most of *flaB*-primers were unspecific, and half of *ospC*-primers was specific. Most primers for remaining genes showed specificity for the species of interest. The vast majority of primer-sets ranged from low to moderate sensitive for the species of interest. This study demonstrated most PCR-primers that have been used previously for detection of Lyme *Borrelia* were suboptimal to be specific for taxon of interest and unsuitable to be specifically used in PCR for detection *B. burgdorferi* sl complex.

Glossary and Abbreviations

(Tick-borne spirochetes) - A spirochete bacteriae that are transmitted or carried by ticks.

Algorithm - A fixed procedure embodied in a computer program.

Alignment - The process or result of matching up the nucleotide or amino acid sequences of two or more biological sequences to achieve maximal levels of identity.

Amplicon - The specific DNA product generated by PCR using one pair of PCR primers.

DNA (deoxyribonucleic acid) - The genetic material of most living organisms, and consists of two complementary chains of nucleotides.

Downloaded amplicon – A sequence of nucleotides was obtained in this master project. This sequence is containing the forward and reverse primers, and is corresponding the amplified segment of certain gene in the source reference of the primers.

E value - The Expectation value or Expect value is the number of alignments with a particular score that are expected to occur by chance.

Entrez - An integrated search and retrieval system that integrates information from various databases at NCBI, including nucleotide.

Enzootic life cycle - A life cycle of microbial pathogen inside living organisms in an endemic area.

Gap - A space introduced into an alignment to compensate for insertions and deletions in one sequence relative to another.

GenBank - Primary nucleotide sequence database produced and maintained at the National Center for Biotechnology Information (NCBI).

Gene - The unit of inheritance. Genes are sections of DNA which code for the production of a particular protein or protein subunit.

Genome - The entire collection of genes in an organism.

Hematophagous arthropod - Blood-feeding insects/ticks.

Homocoel -The body cavity found in many invertebrates where the hemolymph circulates through.

Homologous – two or more nucleotide sequences are similar or having a corresponding.

Homology - A degree of similarity between two or more nucleotide sequences, as in position or structure, and that may indicate a common origin; a correspondence of structure.

Host - A host organism is a living cell in which a microbial pathogen lives and reproduces using cell nourishment and components. In this study mammalians and rodents.

Identity - The extent to which two nucleotide sequences have the same residues at the same positions in an alignment, often expressed as a percentage.

MegaBLAST- A local pairwise nucleotide alignment tool that is optimized for finding long alignments between nearly identical sequences.

Motif: A short conserved region in a nucleotide sequence. Motifs are frequently highly conserved parts of domains.

Nucleotides - Are the building unit of DNA molecule and consisting of a sugar molecule (pentose), a phosphate group and one of four different bases: Adenine (A), Thymine (T), Cytosine (C) and Guanine (G).

PCR: Polymerase Chain Reaction - A technique for amplifying DNA sequences in vitro by separating the DNA into two strands and incubating it with oligonucleotide primers and DNA polymerase. It can amplify a specific sequence of DNA by as many as one billion times

Primer - Short synthetic single-stranded DNA fragment which is required to induce the synthesis of DNA in the PCR reaction.

(Primer-pair) – The sequences of the individual primers (forward and reverse) without their downloaded amplicon.

(Primer-set) – A primer set in this master thesis is presented by the forward and reverse primers and their corresponding downloaded amplicon.

Query coverage (Qc)– The percentage that the query sequence is covered by the sequence of matched database.

Query sequence - The input sequence (or other type of search term) to which all of the entries in a database are to be compared.

Score (S) - A numerical value that describes the overall quality of an alignment or similarity between two or more sequences.

Similarity - The extent to which nucleotide sequences are related. Similarity between two sequences can be expressed as percent sequence identity and/or percent positive substitutions.

Taxon - A taxonomic group of any rank, such as a species, family, or class.

Vector - A vector is an organism that carries / transmits amicrobial pathogen from among hosts. In this study are the ticks.

Zoonotic disease - A disease can be spread from animals to humans.

ACA- Acrodermatitis Chronica Atrophicans.

BLAST - Basic Local Alignment Search Tool.

CSF- Cerebrospinal fluid.

CSF- Chronic fatigue syndrome.

DDBJ - The DNA Data Bank of Japan.

EM - Erythema Migrans.

flaB- gene coding for flagellin B.

LB - Lyme borreliosis.

LD - Lyme disease.

NCBI - The National Center for Biotechnology Information.

Osp - outer surface protein.

ospA, B. C - gene coding for outer surface protein A, B, C, respectively.

p66 - gene coding for outer membrane protein.

recA - gene recombination A.

RFB - Relapsing fever *Borrelia*

RMBL - The European Molecular Biology Laboratory.

SF- synovial fluid.

I. - Ixodes

bp – base pair

B. - Borrelia

B.bu sl - *B. burgdorferi* sl - *B. burgdorferi* sensu lato.

B.bu ss - *B. burgdorferi* ss - *B. burgdorferi* sensu stricto

B.ga - B. garinii

B.af- B. afzelii

B.va - B. valaisiana

B.spl - B. spielmanii

B.bis - B. bissttii

B.amr - B. americana

B.bv - B. bavariensis

B.jpn - B. japonica

B.clf - B. californiensis

B.fin - B. finlandensis

B.chl - B. chilensis

B.crl - B. carolinensis

B.and - B. andersonii

B.okn - B. okinawa

B.lst - B. lusitaniae

B.tnk - B. tanuki

B.trd -B. turdii

B.tur - B. turcica

B.snc - B. sinica

B.yng - B. yangtzensis

B.dtn - B. duttonii

B.prs - B. persica.

B.crd - B. crocidurae.

B.mty - B. miyamotoi.

B.trc - B. turicatae.

B.hsp - B. hispanica.

B.rcnt - B. recurrentis

B.hrm - B. hermsii

B.prk - B. parkeri

B.ans - B. anserina

B.spp. - Undefined *Borrelia* species

Contents

Foreword

This thesis is part of Master Degree project. It is written for Institute for Environmental and Health studies/Institutt for Natur-, Helse- og Miljøvernfag (INHM) at Bø at University College of Southeast Norway, Faculty of Art and Sciences.

This thesis is intended for readers who are interesting in PCR molecular detection of pathogens, particularly *Borrelia burgdorferi* sl genospecies, and for designers of PCR-primers. It also was prepared for bioinformatician who are concerning with homology between gene sequences of different genospecies.

This master project assumes some knowledge about amplified genes of *B. burgdorferi* sl species that used for detection of Lyme disease by PCR. The utility of bioinformatics applications and local sequence alignments is to determine the conserved and variable regions in the gene sequences of different species of *B. burgdorferi* sl and search for specificity of particular sequences.

The aim of this project is to evaluate the specificity and sensitivity of PCR-primers that were used previously for detection of Lyme *Borrelia* genospecies in humans and other organisms.

I would like to thank my supervisor, the molecular microbiologist, Professor Andrew Jenkins. Thanks for your patience, good advices and feedbacks, and to teach me how to write the master thesis. You have been very enthusiastic and motivated me through the process with your strong technical expertise.

I would like to thank my wonderful wife Shahed who has supported me to perform this project. You were patient, helpful, and being with me in all ups and downs of life.

Skien, 1.5.2016

Nasir Sultan

1 Introduction

1.1 *Borrelia*

The bacterial genus *Borrelia* belongs to the eubacterial phylum *Spirochaetes*. This phylum is sharing: (i) Long and thin serpentine cell shape with three modes of movement. (ii) An outside of the periplasmic membrane. (iii) Flagella which are architecturally similar to other bacterial flagella, but located in the periplasmic space and attached near each end of the protoplasmic or cell cylinder [1, 2]. Other features like gene-organization, lifestyle, and pathogenicity are mostly varying. The *Borrelia* species are the only spirochetes transmitted by the hematophagous arthropod vectors between vertebrate hosts, including humans [2, 3].

Borrelia is divided into two phylogenetic groups, *Borrelia* of relapsing fever and Lyme disease *Borrelia*.

Relapsing fever has two variants. Tick-borne relapsing fever (TBRF), which caused by a group of *Borrelia* e.g. *B. hermsii*, *B. turicatae*, *B. recurrentis*, *B. parkeri*, *B. anserina*, *B. hispanica*, *B. crocidurae*, *B. duttonii*, *B. coriaceae*, *and B. miyamotoi* [4-6]. They are transmitted to human through a bite of the fast feeding ticks "soft ticks" the genus *Ornithodoros* which feeds on sleeping rodents and humans [4]. The second relapsing fever is solely caused by *B. recurrentis* that transmitting by human lice *Pediculus humanus* and called louse-borne relapsing fever (LBRF) [7]. Several geographical areas throughout the world including North America, Canada, Central and South America, Central Asia, Africa, and Russia are exposed to TBRF [4, 6, 8].

Lyme Borreliosis (LB) or **Lyme disease** (LD), which is a zoonotic disease, caused by genospecies of *B. burgdorferi* sl [9-11] that are transmitted to humans through bite of an infected "hard ticks" or "slow feeding ticks" of the genus *Ixodes.* The species of *Ixodes* thrive in grassy, low sunlight and high humidity areas, and are the essential vector for disease transmission in North America and Eurasia [12, 13].

The main vector of *B. burgdorferi* sl in Europe is *I. ricinus* and, less frequently, *I. persulcatus* [11]. The blacklegged tick *I. scapularis* and *I. pacificus* in northeastern of USA and Pacific coast, respectively [3]. LB is the most frequently reported tick-borne disease in North America and Europe since 2001, 300,000 estimated infections per year in USA [12].

1.1.1. Lyme disease Symptoms

The manifestations of LD are varying in the untreated infected person according to stages of infection and the genospecies of pathogen. In this regard, LD is classified into three stages early, late, and post infection.

(i) **Early signs** appear approximately 3-30 days after tick bite. The symptoms start with influenza-like illness accompanied with the most definitive sign, Erythema Migrans (EM). EM is also called bull's-eye rash, starts at the site of tick after approximately 7 days of infection, then expands gradually to reach up to 30 cm resulting in a target-formed red rash (see figure 1-1) [14]. However, symptoms of this stage can resolved in 10-20 days antibiotic treatment [11].

(ii) **Late signs** appear one to several months after the tick bite. In this stage the symptoms appear in different areas and organs of the body such as mild stiff neck, severe headache, appearance of additional EM rashes, facial palsy in one or both sides and swelling joints with severe pain especially knees, causing Arthritis [15]. If the disease is not treated, more severe complications appear like heart problems, and neurologic symptoms, and can be fatal.

(iii) **Post Lyme disease syndrome** occurs rarely in patients severe with prolonged and none-specific symptoms even they exposed to antibiotic therapy, such as Acrodermatitis Chronica Atrophicans (ACA) [16], muscles aches, fatigue, and cognitive problems.

Eventually, the disease and its symptoms are probable correlated with the pathogenic species; Arthritic symptoms correlated with *B. burgdorferi* ss [17], neurological symptoms with *B. garinii* [18], and cutaneous manifestations with *B. afzelii* [19].

1.1.2. Lyme *Borrelia* Species

Lyme borreliae belong to the *B. burgdorferi* sl complex which consists of more than 24 confirmed or proposed genospecies including American and European *Borrelia* species [20, 21]. *B. burgdorferi* ss are the species most often reported as a causative of LB in USA. At least six additional genospecies are observed in North America such as *B. americana*, *B. bissettii*, *B. californiensis*, *B. chilensis* and *B. carolinensis*. *B. burgdorferi* ss was the first genospecies reported, possibly it was the first to be investigated [22-24].

In Europe, a greater diversity among genospecies of *B. burgdorferi* sl leads to important differences in the clinical presentations. The main species responsible of LB in Western Europe and Asia are *B. burgdorferi* ss, *B. garinii* and *B. afzelii* [18, 25]. In addition, species such as *B. spielmanii*, *B. bavariensis*, *B. valaisiana*, *B. bissettii*, and *B. lusitaniae* are occasionally reported [24, 26].

New genospecies belonged to *B. burgdorferi* sl were recently identified in different states such as *B. finlandensis* in Finland [27], *B. turdi*, *B. japonica*, *B. sinica*, *B. yangtzensis*, *B. tanukii*, *and B. sinicia* in Asia, but have not been confirmed as pathogenic to humans [28, 29].

1.1.3. Life Cycle

In nature, *B. burgdorferi* sl maintains itself by an enzootic life cycle between the vector ticks and vertebrate hosts. Ticks have three life stages: larva, nymph and adults, with one blood meal per stage [2, 9, 10].

The larva ticks acquire *B. burgdorferi* during their first blood meal on an infected reservoir host (e.g. white-footed mouse, rodents and migratory) [30-32]. *B. burgdorferi* persists in the tick midgut [3, 33]. The spirochetes are transmitted to the mammalian host with tick saliva of the nymph during the second blood meal. The infection often occurs in 48 hours or more after attachment [34]. In this period the spirochetes immigrate from the tick's midgut to the host's hemocoel where the blood triggers bacteria replication. Human infections occur often by nymphs bite. Humans are a dead end host [10]. (Figure 1-1)

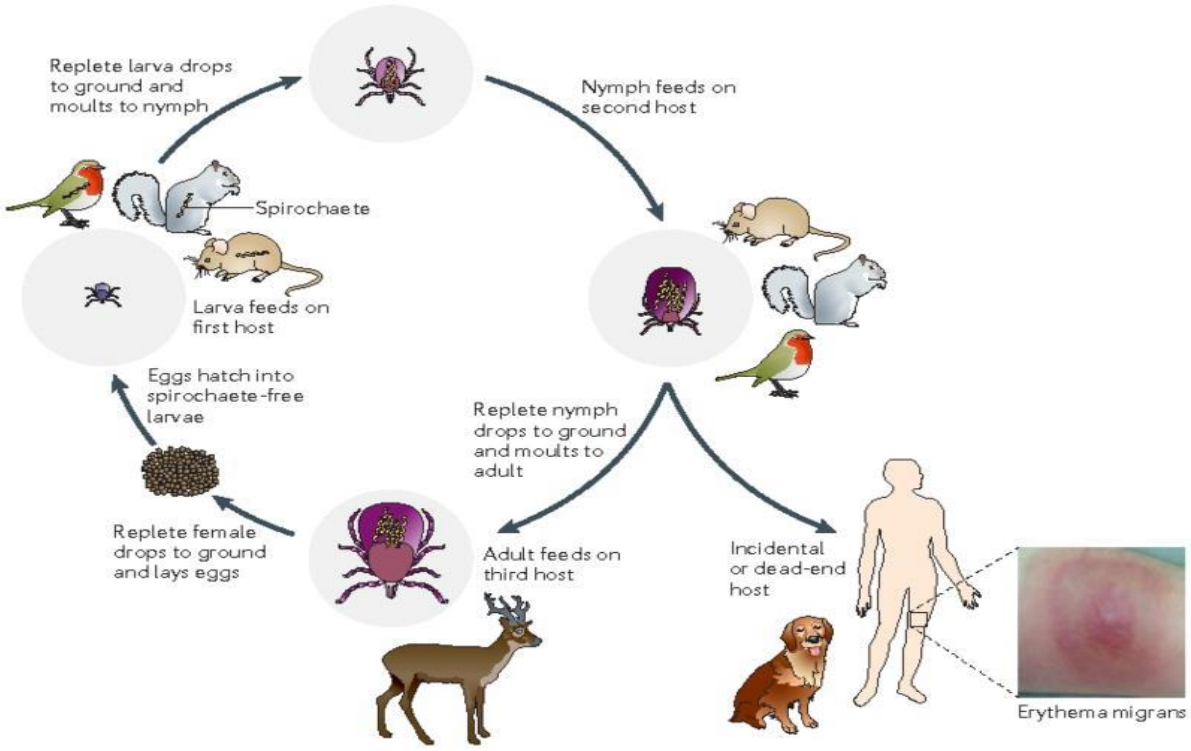


Figure 1-1: Life cycle of B. burgdorferi [10].

1.1.4. Genomic Architecture and Cellular Adaptation

The genome of *B. burgdorferi* (1,521,419-bp for strain B31) [35] is one of the most complicated bacterial genomes. It composed of approximately 1Mb linear chromosome that carries most of housekeeping genes and constant in content and organization cross genus. The second components are assortments of linear and circular plasmids 90-620 kb in size [21, 27, 35]. The plasmids are encoding for majority of outer-surface lipoproteins which are expressed differentially perhaps to facilitate the transition during the enzootic life stages [10, 36].

The genome is low CG content and the plasmid is sealed in telomere region. Currently, there are around 12,500 identified genes distributed in the chromosome and plasmids among numerous strains of *B. burgdorferi* [35, 37].

The metabolic activity is extremely limited in *B. burgdorferi*; it lacks the ability to synthesize amino and fatty acids, nucleotides, and enzyme cofactors [35, 38]. Instead, *B. burgdorferi* utilizes a dynamic mechanism of at least 52 transporter proteins to import the nutritional elements from the host [39]. Further modifications, *B. burgdorferi* derives necessary energy by

fermentation because it lacks the enzymes for citric acid cycle and oxidative phosphorylation [2]. Moreover, instead of iron which is sequestered by the hots as a defense strategy, *B. burgdorferi* use manganese and zinc [38]. Genes for motility and chemotactic function are abundant [40].

The cell envelop of *B. burgdorferi* is distinct in structure and physical attributes from other bacteria membranes. There is a fluid and fragile outer membrane with high density of outer surface lipoprotein, but low density of membrane-spanning channel proteins. The inner membrane abundant integral-membrane proteins most of which are transporter proteins [3, 41], figure (1-2).

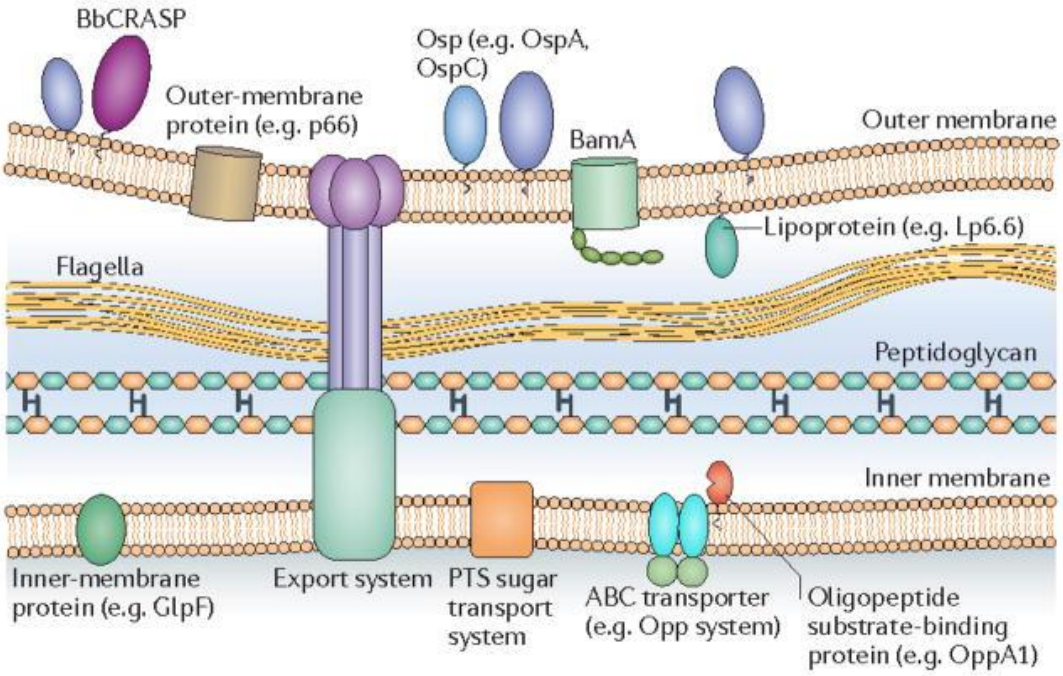


Figure 1-2: Cell envelop of B. burgdorferi [10].

In addition to motility, the flagella grants to spirochetes their length and shape. Spirochetes have 7-11 periplasmic flagella, in contrast to other organisms where flagella is external [2]. Since the flagella are immunogenic and highly conserved, this concealment of flagella helps to shield *B. burgdorferi* of host immune system. Furthermore, spirochetes motility and morphology aid these organisms to move in high viscosity media and may help pathogenic *Borrelia* species to penetrate tissues, invade, and disseminate host tissues [41, 42].

1.2 Polymerase Chain Reaction (PCR)

1.2.1 Definition and Methodology

PCR is an *in vitro* series of replication reaction that amplifies a particular segment of DNA. This DNA segment locates between two regions of defined sequence whose be flanked by two oligonucleotide primers. The main product of PCR is called an amplicon. The amplicon is copied segment of double stranded DNA that its length is defined by the distance between the two oligonucleotide primers and whose termini is recognized by the 5'-end of the primers [43, 44].

The process of PCR is normally composed of three steps: (A) Denaturation (94-98°C), breaks the weak hydrogen bonds between DNA strands and separates them into tow single-stranded DNA (template), (B) Annealing (40-70°C), the primers anneal/bind on either side of the target DNA region (C) Elongation (approximately 72°C), the nucleotides in the solution are added to the annealed primers by DNA polymerase, the addition occurs in (5'→ 3') in the newly synthesized strand; (3'→ 5') in the antiparallel, complementary template strand [45] . (Figure 1-3).

The cycle of denaturation, annealing and elongation is began again using the product of the previous cycle as a template, and so on to accumulate the defined length of DNA in an exponential style in the following rounds of amplification to form a dominant product of the reaction [43]. This cyclic reaction is repeated for 35-45 times, and because it is exponential reaction, more than one billion copies of the targeted DNA segment will be produced in the cycle 30^{th} [46, 47].

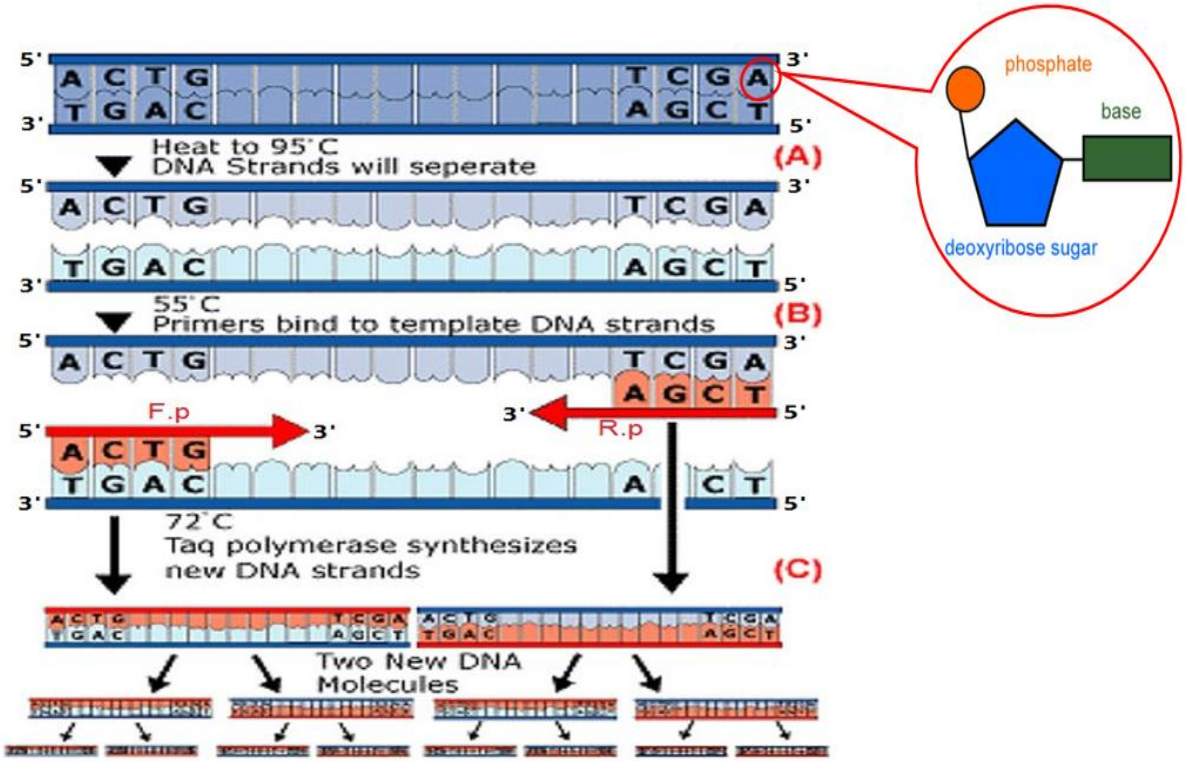


Figure 1-3: Steps of PCR reaction and nucleotide structure. (The primers are 4 nucleotides for simplicity- it would not use these in a real PCR reaction) [48].

1.2.2 PCR-primers

A pair of oligonucleotides (forward and reverse) is necessary for priming PCR reaction by serve as a start point for amplification process. The primers are short (15 to 30-mers), single-stranded DNA oligomers with defined sequence that bind to opposite strands of the target DNA. The sequence of primers is complementary to 3' end of the target sequence/DNA template [45].

Since DNA polymerase adds the nucleotides only in a preexisting 3'-OH end, the 3' end of the primers has to be in the direction of the extension. This means, the direction of extension from each primer will be toward the other primer (converging directions); the extended and template strands are antiparallel (figure 1-3) [49, 50].

The complementarity between primers and their binding regions on the template of DNA is essential for annealing [50]. (Figure 1-4)

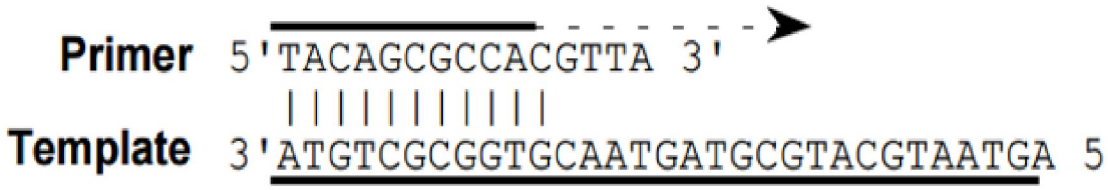


Figure 1-4: An example for primer-template complementarity. Pairs of bases without lines between indicate the new synthesized DNA.

Primer-template complementarity has an important role in primer specificity [49]. However, the primer and template do not have to be 100% homologous to give a product, depending on location, number, and type of mismatch. Single internal or 5' end mismatches affect PCR slightly. By contrast mismatch at or near 3' end of the primer has more effect and can decrease amplification. No or inefficient amplification likely with three or more mismatches between the primer and template sequences. All types of nucleotide mismatch (except G:T) are destabilizing [51, 52] (figure 1-5).

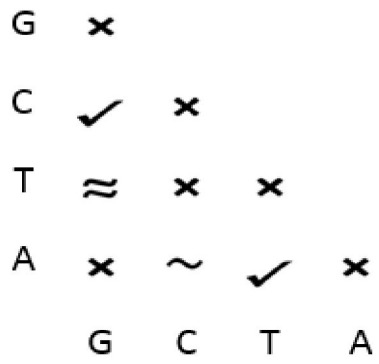


Figure 1-5: Binding relations between the nucleotides. (✓): natural, (×): destabilizing. (≈) unnatural but stabilizing, (~) unnatural but slightly destabilizing

1.2.3 Real time-PCR

Also known quantitative PCR (qPCR). As the name suggests, this method of PCR, allows to monitor the progress of amplification in each cycle, as well as, to quantify the amplified DNA [47].

The technique was developed from conventional PCR, based principally on tracking the emission of fluorescent reporters, which is directly proportional with the increasing amount of the amplified DNA. These fluorescent reporter molecules are either fluorochromes that bind to the double-stranded DNA (e.g. SYBR® Green) or fluorescently labeled sequence-specific probes (e.g. TaqMan® Probes). The fluorochromes method is considered less specific than fluorescent probe, which is, however, somewhat more expensive and complex [46, 53].

The qPCR can detect the product during the early cycles of the reaction. This provides a high-throughput automation and distinctive advantage compared with the traditional PCR technique that uses the gel electrophoresis to visualize the product at the end-point of the reaction. This feature makes qPCR is high sensitive and can detect less than five copies of DNA. Finally, qPCR minimizes chance of cross contamination since it performed in close vessels and does not need post-PCR manipulations [53-55].

Noteworthy, the abbreviation RT-PCR is commonly usedto indicate to the reverse transcription PCR more than real time PCR, this is a kind of terminological confusion.

1.2.4 Nested PCR

Nested PCR is composed of two sequential PCR reactions. The product of the first PCR reaction is utilized as template and re-amplified by a second PCR [56]. Two primer pairs are needed (outer and inner), of which the second pair is specific to the internal amplified sequence of the first PCR reaction.

Concepts for specificity of nested PCR are debatable. Some consider nested PCR is specific since the specificity of the first amplification will be supplemented by the second PCR reaction [57, 58]. whereas, other consider it highly unspecific [52]. Nested PCR is valuable if there were small amounts of target DNA/RNA [56, 59], but is labor intensive and rather prone to false-positive results because of carry-over contamination [52].

1.2.5 PCR for Detection of *B. burgdorferi* sl and Lyme disease

The first PCR-detection of *B. burgdorferi* sl was reported in 1989 [60]. Thereafter, different protocols of PCR were developed for detection of causative agent of Lyme disease. In 1990, detection of *B. burgdorferi* was performed by amplification of a gene coding for the outer surface proteins OspA and OspB [61], In the same year, other study was able to amplify 145-bp DNA of the *ospA* gene [62]. Thereafter, the gene coding for flagellin was sequenced, this gene was described as excellent target for PCR [63].

The PCR has proved to be sensitive and fast molecular method for detection, species typing, and quantification of Lyme *Borrelia* species. It has been applied for detection of *B. burgdorferi* sl species in infected ticks [64-66] and different clinical samples of mammalians and humans, with varying degrees of success [67-69]. PCR protocols amplified different borrelial genes, chromosomal such as flagellin gene [70, 71], 23S rRNA [72], *p66* gene [73], *recA* gene [74], and plasmids-encoded *osp* genes [75, 76]. The PCRs for detection of *B. burgdorferi* sl showed remarkable variation in results over the last 25 years [77-80].

Contamination with nucleic acids or presence of inhibitory substances, are the most known problems in PCR and cause false positive results. DNA instability, is another obstacle that causes false-negative results [81]. Sensitivity of PCR may be reduced because of DNA degradation during storage, transport and processing of samples. PCR that used fresh frozen skin specimens has higher yields than that used paraffin-embedded or formalin-fixed skin biopsies [82].

The primers and gene target are the most important factors in development of new PCR protocols. The primers that produce amplicon of 100-300-bp can give high amplification efficiency and minimize the effects of DNA fragmentation during specimens processing [50, 83].

1.2.6 The clinical samples and PCR analysis for detection of *B. burgdorferi* sl

Although the number of spirochetes in infected ticks is about 4,500, number of spirochetes in clinical samples of Lyme disease patients is extremely low compared with the other bacterial or viral diseases. The number of spirochetes in urine or plasma samples of infected person is often less than 50 per ml, whilst CSF samples might contain even fewer [84, 85].

PCR sensitivity was usually ranging 36-88% and 54-100%, for EM and ACA, respectively, in skin samples [83]. Detection of *B. burgdorferi* sl in skin samples of ACA-patients by PCR assay was more sensitive when targeting *ospA* gene than 5S-23S rRNA gene intergenic spacer, and PCR targeting *p66* was more sensitive than 23S rRNA gene or *recA* [86-88]. The sensitivity of skin-PCR for EM-patients is inversely correlated to duration of infection [83].

PCR assay can detect DNA of *B. burgdorferi* sl in blood samples of EM [84], carditis, and neuroborreliosis patients [18, 89]. However, blood-PCR was not sensitive, perhaps due to the low level of spirochetes in the blood, and/or to PCR-inhibitors in host blood [84, 90]. The latter can also affect the detection of *B. burgdorferi* sl in engorged ticks that fed on human blood [91]. Blood-PCR was negative for patients with post Lyme disease syndrome, although the same samples were positive in immunoglobulin (IgG) test [92].

B. burgdorferi sl has been detected in cerebrospinal fluid (CSF) specimens by PCR analysis in patients with neurological symptoms. Disease duration, CSF white cell counts, clinical manifestations, and antibiotic therapy, are the main parameters that correlated with the PCR findings [83, 93]. Other studies showed higher PCR sensitivity in the early neuroborreliosis stage and in LB-patients with CSF pleocytosis [93, 94]. The reported sensitivities of CSF-PCR show very poor concordance [83]. Most studies described CSF-PCR as insensitive, and attributed this for the low number of spirochete cells in CSF samples, or because of DNA degradation during storage [95-98]. However, a meta-analysis study described CSF-real time-PCR of neuroborreliosis patients as specific and sensitive [78].

PCR analysis showed high sensitivity (up to >90%) in synovial fluid (SF) samples and tissue from patients with Lyme arthritis [99] especially those who were newly treated with antibiotics, or untreated [83, 100]. The gene target has an effect on SF-PCR analysis; *ospA* primers showed more PCR positivity than primers of gene coding for 16S rRNA [100].

The first detection of *B. burgdorferi* sl in urine samples from Lyme arthritis patients was in 1991 by Goodman et al. [101]. Thereafter, PCR assay detected *B. burgdorferi* ss in urine of EM patients [102] and neuroborreliosis [103]. Since that time, many studies were carried out to estimate specificity and sensitivity of urine-PCR [81, 104, 105]. The comparison was very complicated due to the difference in study design, DNA extraction, PCR methods and patient selection. A meta-analysis showed 99% specificity and 68% sensitivity of urine-PCR analysis [105]. However, the urine samples are still not recommended for PCR analysis for detection of *B. burgdorferi* sl because of the extremely variable sensitivity [83].

In general, the diagnostic value of PCR analysis for detection of LB in blood and CSF specimens is low. By contrast, sensitivity is high of PCR analysis for skin biopsy samples from patients with EM lesion and synovial fluid samples from patients with Lyme arthritis.

1.2.7 The gene target and PCR analysis for detection of *B. burgdorferi* sl

PCR tests target a variety of genes (e.g. 16S rRNA gene, *flaB*, *p66*, *recA*, *ospA*, and 5S rRNA-23S rRNA gene spacer) for detection DNA of *B. burgdorferi* sl in skin biopsy samples in patients with EM lesion or ACA [83]. Genes coding for flagellin and outer surface proteins were the most frequently targeted [70, 78].

For synovial fluid-PCR, *ospA* was preferred because it showed higher sensitivity than the 16S rRNA [100]. It has been pointed out that genetic stability is an important consideration in selecting a target for PCR [81].

In this master project, five groups of genes were investigated for the specificity and sensitivity of their primers.

1.2.7.1 Genes of outer surface proteins *osp*:

Since the outer surface membrane is the interface between pathogen and its host, proteins within the outer membrane have an important role in virulence, dissemination, immune evasion, and tissue tropism. Due to these potential roles of outer surface proteins, several studies have recently placed much attention to identify the uncharacterized borrelial outer surface proteins [35, 106].

The *osp* genes, particularly *ospA*, have been successfully utilized as target for detection of *B. burgdorferi* sl by PCR assay [18, 62, 75, 86, 103, 107-109]. The European species of *Borrelia* (especially *B. garinii*) have heterogeneous sequences of *ospA*, this was utilized for differentiation and specification of *Borrelia* using PCR assay [94, 110].

1.2.7.2 Flagellin B gene (*flaB*):

The periplasmic flagellar filaments in spirochetes are comprised of two classes of protein: outer-layer proteins (FlaA) and core proteins (flaB). *B. burgdorferi* contains much more FlaB than FlaA. *B. burgdorferi* is the only spirochete that has only one type of *flaB* [42, 63, 111, 112].

Wallich et al. sequenced the gene coding for flagellin [63], thereafter the gene was selected as PCR target in several studies [71, 113-115]. PCR targeting the chromosomal *flaB* gene showed specificity, sensitivity, and rapidity for detection *B. burgdorferi* sl species [113, 116, 117]. Furthermore, the flagellin gene is valuable PCR target in taxonomy and phylogenetic relationship among *Borrelia* species [112, 118, 119].

1.2.7.3 Gene of outer membrane protein (OMP/*p66*)

Most of the known OMPs of *B. burgdorferi* sl are coded by stable chromosomal loci [35]. OMPs provide a range of functions, such as antibiotic resistance by drug efflux pumps, nutrition acquisition by porins, cellular adhesion and protein transport. Furthermore, OMPs have been implicated as an important virulence factor for Lyme disease [120].

The *p66* gene, also known as *oms66*, encodes a 66 kD outer membrane protein and is located on the chromosome. It has been identified in several species of *Borrelia*, with approximately 60 - 94% identity across *Borrelia* spp [120, 121]. In one study, p66 nested primer sets have shown more sensitivity in PCR protocols compared to other genes such as ospA [103]. PCR targeting p66 and recA genes was reliable and fast for detection of Borrelia in skin samples, as well as differentiation of three species of Borrelia commonly associated with Lyme disease [88, 122].

1.2.7.4 Gene of recombination protein A (recA)

Genetic recombination, is certainly involved in antigenic variation. This feature is attributed to recA protein, which associated with genetic recombination to DNA replication and bacterial reparations especially in mammalian hosts. The *recA* gene has a central role in genetic recombination and DNA repair [123, 124].

Sequence of *recA* gene for *B. burgdorferi* Sh-2-82, was firstly identified by Dew-Jager et al. using PCR-based approaches. The gene encodes a protein of 365 amino acid, which is highly homologous to other bacterial RecA [123, 125].

By real time-PCR, *recA* gene was reliable and fast for detection of *B. burgdorferi* sl species in skin samples, as well as differentiation of three species of *Borrelia* commonly associated with Lyme disease (*B. burgdorferi* ss, *B. garinii*, and *B. afzelii*) [88, 126].

1.2.7.5 Genes of species-specific sequence

Three primer pairs investigated here were derived from species-specific sequences on plasmids. The primer sequences targeted 3-kb, 25-kb, and 16-kb plasmids of *B. garinii*, *B. afzelii*, and *B. burgdorferi* ss, respectively. The primers showed high specificity in identification of *Borrelia* genospecies in biological samples [127].

1.3 Bioinformatics

1.3.1 Definition and overview

Bioinformatics is defined as collection, storage, classification, and analysis the biochemical and biological information using computers especially as applied in molecular genomics and genetics [128].

In general, bioinformatics has three aims: (a) Organize data in a style that allows access to the information and to record the new entries. (b) Development of new tools and resources that assist in data analysis such as PSI-BLAST [129] for protein sequences comparison. (c) Employing of tools to analyze the data and interpret the outcomes in biological understanding [128, 130].

1.3.2 Bioinformatics Database

Databases are large, organized repositories of data associated with software allowing to update, retrieval, analysis, and output of the information. Different models of database were designed according to how the data are stored and retrieved such as flat file database model, hierarchical database model, relational database model, object oriented database model, and other database systems [131-133]. The database are stored either in public repositories such as GenBank, or as private database like those used by research groups of biotech companies.

According to the level of complexity, biological database is divided into three groups: primary, secondary, and composite database [131, 134]. Since this master project concerns with the nucleotide sequence of PCR-primers, which is primary database, explanation below will focus only on this type of biological data.

A Primary database, also known archival database [134], contains information about sequence and structure alone, which is directly submitted into database by researchers. The primary nucleotide or proteins sequence is an example for this type of database including the sequences of DNA, RNA, or amino acids as a raw material as they were extracted by the experimental studies [134]. For nucleic acid sequence database GenBank [135], EMBL (European Molecular Laboratory) [136], and DDBJ (DNA Databank of Japan) [137] are the most popular repositories.

1.3.3 Sequence alignment

Sequence alignment or sequence comparison lies at the heart of bioinformatics. It is a powerful tool to compare DNA/RNA or protein sequences in order to identify conserved and homologous regions. Conserved sequences may be assumed to have specific function. Sequence alignment used also to estimate the functional, structural, and evolutionary relationship between sequences by common patterns [131, 138].

Alignment finds similarity between query sequences and a target database sequences or between searched query sequences. The algorithms work by dynamic programming approaches which divide the problem into smaller independent sub problems [139]. It rates the alignment quantitatively by assigning positive scores or higher value for matches and negative scores or lower value for mismatches. The highest score in this score matrix indicates the most homologous alignment [139, 140].

1.3.3.1 Global sequence alignment

Global alignment detects matches between sequences in their entirety and tries to optimize alignment along the whole length of both sequences. Global alignment involves the Needleman-Wunsch algorithm [141] to find best alignment between two sequences. The global alignment is suitable to find similarity from beginning till end between two closely related sequences of the same length [139] (Figure 1-6).

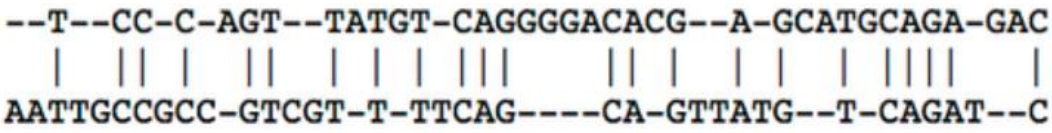


Figure 1-6: An example for global sequence alignment.

1.3.3.2 *Local sequence alignment*

The local alignment is based on the Smith-Waterman algorithm [142]. It targets the similarity between specific regions within long sequences that would be more divergent if compared in their entirety (figure 1-7); similarity may be clearly apparent between two genes in different species over short conserved regions, while the remaining regions are dissimilar. It is powerful tool for finding DNA sequences that share a common motif but are otherwise different [139, 143, 144].

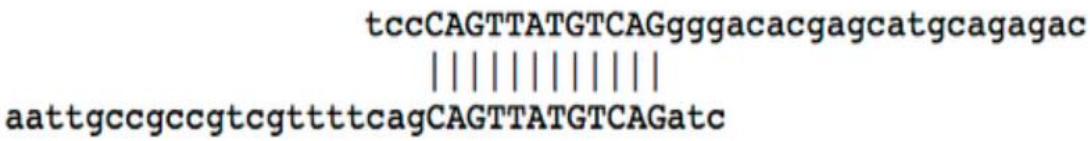


Figure 1-7: An example for local sequence alignment.

1.3.4 The GenBank database

The GenBank database is one of NCBI's products. It is a comprehensive annotated collection of publicly available nucleotide sequences and their corresponding protein sequences. Since its inception in 1982, GenBank has grown exponentially, in August 2013, over 167 million sequences for more than 380000 organisms [135].

Enormous amount of data is produced by batch submissions from large-scale sequencing projects, sequencing centers, and from individual laboratories to NCBI which synchronizes the data with EMBL and DDBJ according to the International Nucleotide Sequence Database Collaboration (INSDC) [145].

1.3.4.1 *Identification and citation of GenBank records*

A unique sequence identifier called "accession number" is assigned to each sequence and its annotations recorded in the GenBank. This number is shared across all database collaborators (NCBI, EMBL and DDBJ) [145]. In NCBI, the accession number is located on the ACCESSION line of each recorded GenBank sequence, remains unchanged although changes may be made with the sequence or its annotations. In this case, the version number will be

notated [135, 145]. For instance, AF095941 is an accession number for sequence of *B. valaisiana*, while AF095941.1 is the accession version, where the integer 1 at the end indicates the initial version of the sequence, which will be updated over time if the record is changed [135].

The GenBank accessions are the most reliable and practical citing method for sequence records in GenBank [135], especially since NCBI has decided to phase out GI number in September 2016 [146]. The accession number was utilized in this master project to cite and reference nucleotide sequences of GenBank database.

1.3.4.2 Retrieving GenBank data

The sequences identifiers and annotation in GenBank are accessible and can be retrieved through the NCBI Entrez Nucleotide [147]. The sequence of nucleotides and protein in GenBank database can also be retrieved as results using the Basic Local Alignment Search Tool (BLAST) [129] which will be explained below.

For more detailed information about conducting NCBI database include GenBank is found in the NCBI Help Manual http://www.ncbi.nlm.nih.gov/books/NBK3831/

All analyzed nucleotide sequences in this master project were retrieved from Entrez nucleotide or from BLAST alignment results.

1.3.5 BLAST software for sequence similarity search

The Basic Local Alignment Search Tool BLAST comes under the category of homology search tools. BLAST is a family of heuristic programs that are provided by NCBI to investigate similarity between two or more sequences and to search the homology between queries of DNA, RNA or protein sequences in nucleotide or amino acid database in NCBI. It also calculates the statistical significance of homology/similarity [129, 144].

Several versions of BLAST are available on BLAST homepage and can be used via interface (http://blast.ncbi.nlm.nih.gov/Blast.cgi) or downloaded as stand-alone tool. The nucleotide BLAST (BLASTn) searches for similarity between a nucleotide query sequence and the

nucleotide database, which contains approximately 35 610 311 nucleotide sequences [135, 148]. BLASTn was used for method in this master project.

BLAST scans for statistically significant local alignments, then displays a table of results and a set of gapped alignments with links to full sequence records [149, 150]. BLAST has several features that make it one of the most sensitive, fast, and flexible sequence similarity search programs [143, 150].

1.3.5.1 BLAST algorithm parameters and statistical significance

BLAST offers a range of possibilities for altering algorithm parameters, but it was found that the default parameters for short sequences worked best.

Table 1-1 shows an example for automatically adjusted values of algorithmic and scoring parameters that were used in similarity search for primer sequences.

Parameter	Value
Word size	7
Expect value	1000
Hitlist size	1000
Match/Mismatch scores	1,-3
Gapcosts	5,2

Table 1-1: An example for values of automatically adjustment for algorithmic and scoring parameters.

Different algorithm and scoring parameters influence of BLAST search. The "word size" is the number of nucleotides that form the seed (word) in the sequence, it regulates sensitivity and speed of the search [150]. The cutoff of "Expectation value" (E-value) represents the number of matches are expected to be found merely by chance; the false-positive rate [143]. E-value is inversely correlated with score value and the reliability of the hit/alignment2. The hitlist size is the maximum number of hits that will be displayed in BLAST result report.

The match/mismatch scores is a scoring system of reward for match nucleotide and penalty for mismatch nucleotide to calculate the statistical significance of similarity. The gap cost is a value of exist and extend a gap in an alignment. Increasing value of gap cost will decrease the number of gaps in the alignment (figure 1-8).

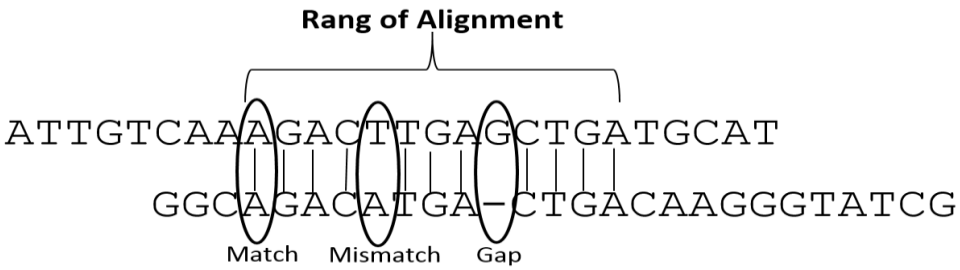


Figure 1-8: Relations between nucleotides of two sequences in an alignment pairwise.

BLAST search can be restricted into subsets by exclude or include the taxon of interest in the database. [148, 149]. For more information

All definitions and explanations about algorithmic parameters and statistical significance is present in the link http://www.ncbi.nlm.nih.gov/BLAST/tutorial/Altschul-1.html. However, analysis of the short queries that less 50-mers (PCR-primers) relied on automatically adjustment for algorithm parameters. BLASTn is optimized according to three selectable sub-programs (megablast, discontiguous megablast, and blastn). The "megablast" able to compare a query to closely related sequences and works best if the target percent identity is 95% or more and is very fast [150].

1.3.5.2 BLAST output format

Standard BLAST output consists of three parts: graphic summary, descriptions, and alignments. Each part shows different aspects of the same results. For more information about BLAST procedure and results see links:
ftp://ftp.ncbi.nlm.nih.gov/pub/factsheets/HowTo_BLASTGuide.pdf ,
ftp://ftp.ncbi.nlm.nih.gov/pub/factsheets/HowTo_NewBLAST.pdf

- **Graphic summary**

The graph is simply an overview in lines form for the databases that aligned to the query sequence (figure 1- 9). The five colored regions indicate score values for each hit/alignment. Thus, blue lines beneath represent alignments that in score 40-50. By mousing over these lines, definition of alignment including accession number and parameters (Score & E-value) will be appear in the text box at the top of graph.

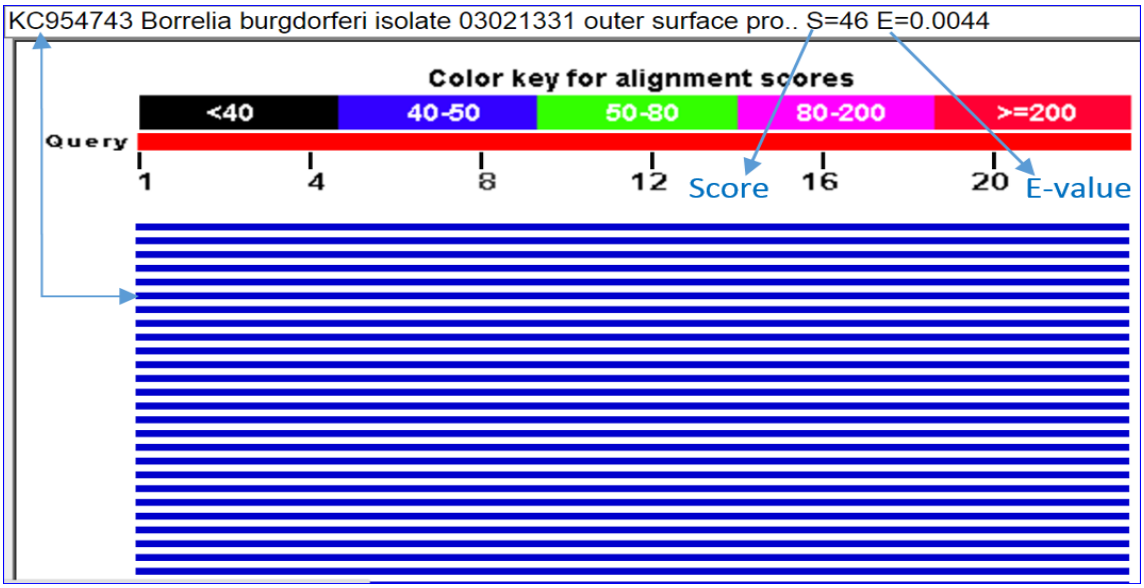


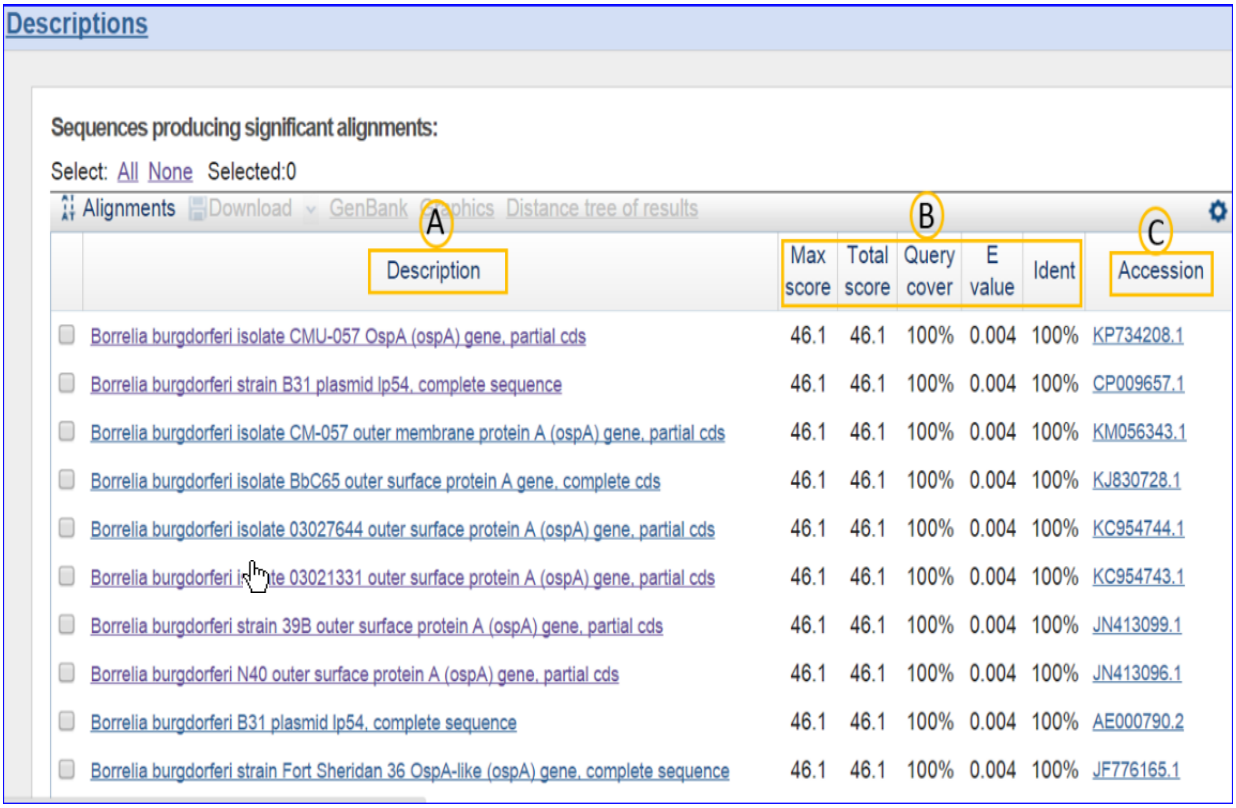
Figure 1-9: The graph summary of BLAST results report.

- **The description**

A tabular part of BLAST output (figure 1-10) shows the results in easily-parsable Hit Table that contains annotation and statistical significance for each matched sequence of databases (hit). The hits table is sorted by default according to the highest score value and lowest E-value; the first hit is the most significant homology. Order of the hits can be changed by clicking on the column headers.

The column header (B) contains similarity statistics. The “Max score” and “Total score” indicate calculation of similarity score. It is directly proportional with reliability of hits and inversely with the “E-value”. The “query cover” indicates what percent the matched database sequence covers the query sequence. Finally, “Identity” is the percentage of alignment

similarity. Query cover and Identity start with the highest percentage and decreasing downward.



Descriptions

Sequences producing significant alignments:

Select: All None Selected:0

Alignments Download GenBank Graphics Distance tree of results

	A Description	B Max score	Total score	Query cover	E value	Ident	C Accession
	Borrelia burgdorferi isolate CMU-057 OspA (ospA) gene, partial cds	46.1	46.1	100%	0.004	100%	KP734208.1
	Borrelia burgdorferi strain B31 plasmid lp54, complete sequence	46.1	46.1	100%	0.004	100%	CP009657.1
	Borrelia burgdorferi isolate CM-057 outer membrane protein A (ospA) gene, partial cds	46.1	46.1	100%	0.004	100%	KM056343.1
	Borrelia burgdorferi isolate BbC65 outer surface protein A gene, complete cds	46.1	46.1	100%	0.004	100%	KJ830728.1
	Borrelia burgdorferi isolate 03027644 outer surface protein A (ospA) gene, partial cds	46.1	46.1	100%	0.004	100%	KC954744.1
	Borrelia burgdorferi isolate 03021331 outer surface protein A (ospA) gene, partial cds	46.1	46.1	100%	0.004	100%	KC954743.1
	Borrelia burgdorferi strain 39B outer surface protein A (ospA) gene, partial cds	46.1	46.1	100%	0.004	100%	JN413099.1
	Borrelia burgdorferi N40 outer surface protein A (ospA) gene, partial cds	46.1	46.1	100%	0.004	100%	JN413096.1
	Borrelia burgdorferi B31 plasmid lp54, complete sequence	46.1	46.1	100%	0.004	100%	AE000790.2
	Borrelia burgdorferi strain Fort Sheridan 36 OspA-like (ospA) gene, complete sequence	46.1	46.1	100%	0.004	100%	JF776165.1

Figure 1-10: Description part of BLAST output. Lines under (A) provide title for sequence, (B) statistical significance for similarity between the query sequence and database sequence, (C) the accession version for the entire sequence of gene.

- **The alignments**

The alignments (figure 1-11) is the detailed part in BLAST report that shows the results of similarity search in pairwise sequence alignments. The alignment shows the places of match, mismatch, and gap between the query sequence and database sequence. The alignment shows also the positions/coordinates of database sequence within the sequence of entire gene. The coordinates were used to determine the start and end positions for all analyazed ampliocn. Alignments are sorted correponding with the hits in the privious part of results.

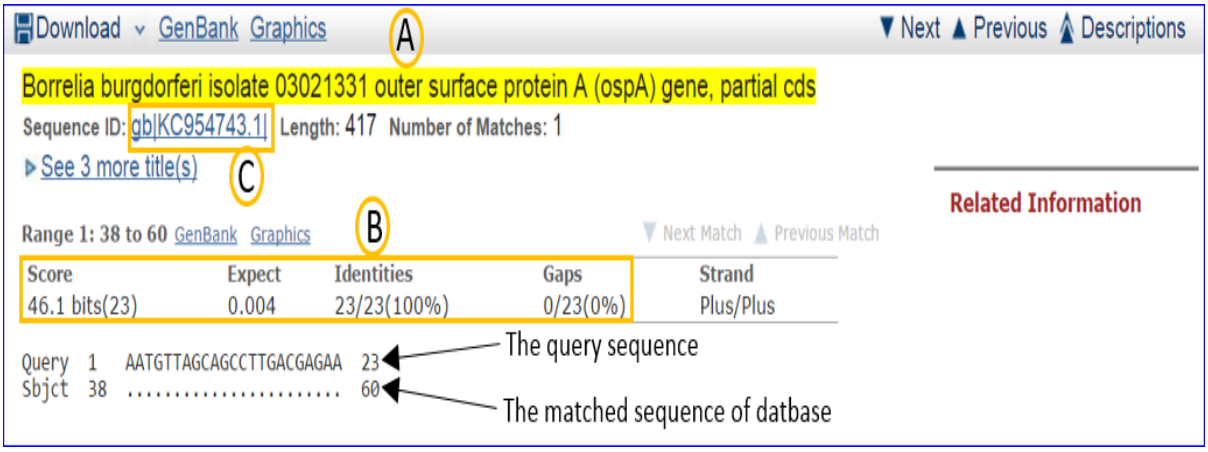


Figure 1-11: An example of alignment for a primer search. (A) sequence title, (B) statistical significance, and (C) accession version.

2 Material

The material in this master project is the sequences of 77 primer pairs (oligonucleotides) with their downloaded sequence of amplicons/samples. The primer pairs were assembled from approximately 60 references/sources. All these sources used the primer pairs to amplify a particular segment of an interested gene for Lyme *Borrelia* genospecies for detection, differentiation/classification, or genotyping of *B. burgdorferi* sl complex by PCR assay.

Studies that used primer pairs in conventional PCR, real time-PCR, and quantitative (qPCR) were preferred. Thus, sizable of the primer pairs that used here were used in one of the preferred PCRs. The primer pairs that were used in nested PCR were avoided as far as possible. However, five internal primer pairs of nested PCR were used here.

Table 1, shows sequences of the primer pairs and their target species and gene, and references. It also contains all information about the downloaded amplicons by Entrez as they were reported in GenBank database. Arrangement of the primer-pairs in this table was according to their references.

Primer-set [Ref]	Species target/ Gene Target	Primers Fw. primer/ length (bp)/ name Re. primer/ length (bp) / name	Amplicon accession nr./ Coordinates/length (bp)
1[18]	*B.bu* sl/ *ospA*	AATAGGTCTAATAATAGCCTTAATAGC/ 27/ SL+ CTAGTGTTTTGCCATCTTCTTTGAAAA/ 27/ SL-	CP009657/ 9477–9784 / 308 CP001653/ 7186-7493 / 308
2 [18]	*B.bu* ss/ *ospA*	AACAAAGACGGCAAGTACGATCTAATT/ 27/ GI+ TTACAGTAATTGTTAAAGTTGAAGTGCC/ 28/ GI-	CP009657/ 9595-10135 / 541 KM676017/ 57-597/ 541
3 [18]	*B.ga*/ *ospA*	TGATAAAAACAACGGTTCTGGAAC/ 24/ GII+ GTAACTTTCAATGTTGTTTTGCCG/ 24/ GII-	HM623301/ 1-345/ 345 GU826976/ 113-457/ 345
4 [18]	*B.af*/ *ospA*	TAAAGACAAAACATCAACAGATGAAATG/ 28/ GIII+ TTCCAATGTTACTTTATCATTAGCTACTT/ 29/ GIII-	Z29087/ 348-537/ 190 CP001247/ 16073-16262/190
5 [100]	*B.bu* ss/ *ospA*	CTGCAGCTTGGAATTCAGGCACTTC/ 25/ OspA4 GTTTTGTAATTTCAACTGCTGACC/ 24/ OspA2	JF776165 / 638-793/ 156 JN413099/ 631-786/ 156
6 [100, 151]	*B.bu* ss/ *ospA*	TTATGAAAAAATATTTATTGGGAAT/25/ OspA149 CTTTAAGCTCAAGCTTGTCTACTGT/25/ OspA319	AE000790/ 9455-9649/ 195 X16467/ 119-313/ 195
7 [69]	*B.bu* ss N40/ *ospA*	AATGTTAGCAGCCTTGACGAGAA/23/ OspA-N40.seq-17F GATCGTACTTGCCGTCTTTGTTT/23/ OspA-N40.seq-119R	JN413096/ 45-147/ 103 CP009657/ 9514-9616/ 103
8 [152]	*B.bu* ss/ *ospA*	GGGAATAGGTCTAATATTAGCC/22/ bba15F TTTCAACTGCTGACCCCTC/ 19/ bba15R	JF776165/ 18-784/ 767 JN413099/ 11-777/ 767

9 [152]	*B.bu* ss/ *ospA*	TGAAGGCGTAAAAGCTGACAAA/ 22/ bba15FF TTCTGTTGATGACTTGTCTTTGGAA/ 25/ bba15RR	GU826949/ 171-312/ 142 JN413098/ 138-279/ 142
10 [152]	*B.bu* ss/ *ospC*	GGGAAAGATGGGAATACATCTGC/ 23/ bbb19F CTGCCACAACAGGGCTTGTAAGC/ 23/ bbb19R	JQ308224/ 37-583/ 547 GU142954/ 31-577/ 547
11 [152]	*B.bu* ss/ *ospC*	CAGGGAAAGATGGGAATACATCTGC/25/ bbb19FF CGCTTCAACCTCTTTCACAGCAAG/24/ bbb19RR	DQ437455/ 26-153/ 128 EF537413/ 11-138/128
12 [152]	*B.bu* ss/ *flaB*	GCAGCTAATGTTGCAAATCTTTTC/ 24/ bb014f TGAGCTCCTTCCTGTTGA/ 18/ bb0147r	KF422803/ 452-543/ 92 KC003470/ 210-301/ 92 *B.am* EU081295/ 301-392/ 92
13[153]	*B.bu* ss/ *ospA*	AAGTACGATCTAATTGCAACAGT/23/OSP-A1 GTTTTGTAATTTCAACTGCTGACC/24/OSP-A12	CP009657/ 9607-10249/ 643 JN413098/ 61-703/ 643
14 [61]	*B.bu* ss/ *flaB*	GATGATGCTGCTGGCATGGGAGTTTCTGG/ 29/ fla1 CTGTCTGCATCTGAATATGTGCCGTTACCT/ 30/ fla3	X15660/ 121-320/ 200 KF422801/ 2-201/ 200
15 [154]	*B.bu* X14407/ *ospA*	TATTTATTGGGAATAGGTC/ 19/ ¤X14407-F GACTCAGCACCTTTTTG/ 17/ ¤X14407-R	X14407/ 160-1049/ 890 L19701/ 160-1049/ 890
16 [155, 156]	*B.bu* ss/ *ospA*	AAGTACGATCTAATTGCAACAG/ 22/ OspA01 TTCCTTCTTTAACCACCAATGT/ 22/ OspA02	X85442/ 151-550/ 400 KM676016/ 56-455/ 400
17 [31]	*B.bu* sl/ *ospA*	ATGAAAAAATATTTATTGGGAATAGGT/ 27/ ¤OspA1F GCTCAAGCTTGTCTACTGTTGC/ 22/ ¤OspA1F	Z29086/ 1-187/ 187 *B.ga* GU906888/ 1-187/ 187
18 [31]	*B.bu* B31/ *flaB*	ATATTTATGCAGCTAATGTTGCAA/ 24/ F TATTAGCATCAACTGTAGTTGT/ 22/ 7C	KF422803/ 444-623/ 180 *B.va* KF990324/ 326-505/ 180
19 [157]	*B.va* VS116/ *ospA*	TGCTGAAAATGCTACAAAAGCAGT/ 24/ Bval 1F CAAGACAAAACTTGTATTTACAAAAC/ 26/ Bval 1R	AF095940/ 453-721/ 269 AF095941/ 453-721 / 269
20 [158]	*B.bu* ss/ *ospA*	CTGCAGCTTGGAATTCAGGC/ 20/ BAE-1 ATTTGGTGCCATTTGAGTCG/ 20/ BAE-2	GQ443122/ 608-733/ 126 JN413099/ 631-756/ 126
21 [159]	*B.bu* ss/ *ospA*	GCGTTTCAGTAGATTTGCCTGGTG/ 24/ OspA/BBA15F ACGCCTTCAAGTACTCCAGATCCA/ 24/ OspA/BBA15R	KM676013/ 4-154/ 151 JN413099/ 79-229/ 151 *B.clf* DQ393324/ 39-189/ 151
22 [159]	*B.bu* B31/ *ospC*	CGGATTCTAATGCGGTTTTACTTG/ 24/ OspC/BBB19F CAATAGCTTTAGCAGCAATTTCATCT/ 26/ OspC/BBB19F	JQ951145/ 71-154/ 84 EU377749/ 56-139/ 84
23 [78]	*B.bu* sl/ *ospA*	ATATTTATTGGGAATAGGTCTAATAT/ 26/ BORs CTTTGTCTTTTTCTTT**R**CTTACAAG/ 25/ BORas	X68542/ 9-145/ 137 AF230516/ 9-145/ 137 *B.af* CP000396/ 11899-12035/ 137
24 [94, 160]	*B.bu* sl/ *ospC*	CGTTTCAGTAGATTTACCTGG/ 21/ ipF-OspA ACTAATGTTTTGCCATCTTCT/ 21/ ipR-OspA	Z29086/ 87-329/ 243 *B.ga* DQ479286/ 87-329/ 243
25 [161]	*B.spl*/ *ospA*	CAGTAGATGTACCTGGGGAACTT/ 23/ ¤OspA151-F GCTTTTACGCCTTCCAGTACA/ 21/ ¤OspA151-R	*B.spl* EU545183/ 13-163/ 151 *B*.spp. AF102057/ 92-242/ 151

26 [162, 163]	*B.va/ ospA*	GGAGAATATATTATGAAA/ 18/ OspA1 CTCCTTATTTTAAAGCG/ 17/ OspA2	*B.va* AB016979/ 76-916/ 841 *B.va* CP001433/ 9243-10083/ 841 X14407/ 139-976/ 838 CP001199/ 9401-10238/ 838
27 [164]	*B.bu* ss/ *ospA*	CTTGGAATTCAGGCACTTCAACTT/ 24/ OspA F ATTGTTGTACTGTAATTGTGT/ 21/ OspA R	GQ443108/ 614-712/ 99 GU815347/ 614-712/ 99
28 [165]	*B.bu* ss/ *ospA*	CAAGTACGATCTAATTGC/ 18/ ¤OspA138-F TGACCTAGATCGTCAGAAAT/ 20/ ¤OspA138-R	EU564839/ 49-186/ 138 HQ434099/ 54-191/ 138
29 [165]	*B.bis/ ospA*	TAAGTACAGTCTAATGGC/ 18/ ¤Biss138-F GTGCTGAGATCCTCAGAAAC/ 20/ ¤Biss138-R	*B.bis* CP002761/ 9653-9790/ 138 *B.bis* DQ393323/ 103-240/ 138 U65801/ 109-246/ 138
30 [166, 167]	*B.bu* ss/ *ospA*	TTCTGACGATCTAGGTCAAA/ 20/ primer 3 GCAGTTAAAGTTCCTTCAAG/ 20/ primer 4	AY597034/ 61-309/ 249 GQ443114/ 240-488/ 249
31 [18, 168]	*B.bu* ss/ *ospA*	AGCCTTAATAGCATG**C**TAAGCAAAATG/ 27/ OspA iLC CTAGTGTTTTGCCATCTTCTTTGAAAA/ 27/ SL-	JN413096/ 23-315/ 293 KJ830728/ 36-328/ 293 Z29087/ 36-324/ 289
32 [169]	*B.bu* ss *B.and B.bis /ospA*	G**Y**AAAGTAAAATTAACA**R**T/ 19/ MOspAF TGTTTT**R**CCATCTTCTTT/ 18/ MOspAR	U96258/32-105/ 74 GQ247743/221-294/ 74 *B.and* AY654919/ 17-90/ 74 CP001651 (N40)/9757-10625/ 869 *B.bis* AF186846/ 298-1166/ 869
33 [170, 171]	*B.bu* ss/ *ospB*	GGTGCTGAGTCAATTGGTTCT/ 21/ OspB-1 TTCTAGGCTGGTTCCAGCTGT/ 21/ OspB-2	AY498727/ 58-837/ 780 L23140/ 820-1599/ 780
34 [151]	*B.bu* ss/ *ospB*	AAACGCTAAACAAGACCTTCCTG/ 23/ OspB-1110 AGCTTTGAGAGTTTCCTCTGTTATTGA/ 27/ OspB-1411	AY498726/ 129-456/ 328 L23137/ 891-1218/ 328
35 [172]	*B.bu* ss/ *ospC*	AAGTGC(**AG**)ATATTAATGACTTTA/22/OspC3 TTTTTTGGACTTTCTGCCACA/21/ OspC4	AF467875/ 18-623/ 606 JN969070/ 18-632/ 615 AE000792/ 16920-17528/ 609
36 [173]	*B.bu* ss/ *ospC*	TGTTAAAGGGCCTAATCTTACAGAAATAA/29/OSPC-108F TACCAATAGCTTTGGTAGCAAGTTCAT/27/OSPC-235R	EU482045/30-157/128 L42894/54-181/128
37 [174]	*B.va/ ospC*	CACAAATTAATGAAAAAGAATACA/ 24/ OspC-N CCAGTTACTTTTTTAAAACAAATTA/ 25/ OspC-C	L42874/ 213-905/ 693 GU569091/ 16885-17569/ 685 AE000792/ 16894-17581/ 688 *B.va* CP001432/ 17292- 17975/ 684
38 [175]	*B.bu* ss/ *ospC*	TAATGAAAAAGAATACATTAAGTG/ 24/ OspC1 TTAAGGTTTTTTTGGACTTTCTGC/ 24/ OspC2	*B.af* CP002934/ 16929-17569/ 641 *B.ga* CP000014/ 17529-18154/ 626 *B.ga* JN828669/1-626/ 626
39 [118]	*B.ga/ flaB*	GCAGTTCAATCAGGTAACGG/ 20/ C AGGTTTTCAATAGCATACTC/ 20/ D	AY342019/ 1-584/ 584 *B.ga* X75203/ 356-939/ 584
40 [71]	*B.bu* ss/ *flaB*	GCTCAAATAAGAGGTTTGTC/ 20/ ¤FlaB299-F ATTCCAAGCTCTTCAGCTG/ 19/ ¤FlaB299-R	X16833/ 343-641/299 KF422801/41-339/299

41 [71, 76]	*B.bu* ss/ *flaB*	TTGCTGATCAAGCTCAATATAACCA/ 25/ FlaB134-F TTGAGACCCTGAAAGTGATGC/ 21/ FlaB134-R	JQ711236/ 55-188/ 134 KC607896/ 72-205/ 134 *B.ga* GU826819/ 62-195/ 134 *B.am* HM802232/ 101-234/ 134
42 [76]	*B.bu* ss/ *ospC*	ATACCGAAAATAATCACAATGGA/ 23/ ¤OspC231-F CTGAATTAGCAAGCATCTCTTTAG/ 24/ ¤OspC231-R	U91798/ 222-542/ 231 GU142954/ 227-457/ 231
43 [176]	*B.bu* ss *B.ga* *B.bv* *B.af*/ *ospC*	TTGTTAGCAGGAGCTTATGCAATATC/ 26/ ¤OspC314-F GGGCTTGTAAGCTCTTTAACTG/ 22/ ¤OspC314-R	FJ932735/ 196-515/ 320 X84765/ 208527/ 320 *B.ga* AY150196/ 249-574/ 326 *B.ga* AJ841695/ 280-605/ 326
44 [177, 178]	*B.af* *B.bu* sl/ *ospC*	F.p(A): AAAGCTATTGGTAAAGTAAT/ 20/ SC3 F.p(B): AAAGCTATTGGTAAAAAAAT/ 20/ SC3 R.p: GTTTTTAAAATAGCTTTTTTTG/ 22/ OspC92	KM676046/ 149-414/ 266 KP644292/ 115-377/ 263 *B.ga* KP644251/ 115-377/ 263 *B.ga* F331425/ 163-401/ 239 *B.af* AY363719/ 172-434/ 263 *B.af* KP644288/ 115-380/ 266
45 [176, 179]	*B.bu* ss/ *ospA*	AAAGAATACATTAAGTGCGATATT/ 24/ ¤OspC600-F GGGCTTGTAAGCTCTTTAACTG/ 22/ ¤OspC600-R	JQ253803/ 1-600/ 600 *B.ga* AJ841695/ 6-605/ 600 *B.af* D49501/ 6-605/ 600
46 [115]	*B.bu* sl/ *flaB*	GTGCATTTGGTTATATTGAG/ 20/ ¤FlaB89-F CAGACAGAGGTTCTATACA/ 19/ ¤FlaB89-R	*B.ga* KF894057/ 80-168 /89 AM159177/ 23-111/ 89
47 [70]	*B.bu sl*/ *flaB*	TCTTTTCTCTGGTGAGGGAGCT/ 22/ ¤flaB70- F TCCTTCCTGTTGAACACCCTCT/ 22 / ¤flaB70- R	JQ711236/ 272-341/ 70 X15660/ 588-657/ 70 LC018212/ 295-364/ 70
48 [180, 181]	*B.bu* GeHo/ *flaB*	AGCAAATTTAGGTGCTTTCCAA/ 22/ FlaF1A GCAATCATTGCCATTGCAGA/ 20/ FlaR1	X15660/ 792-965/ 174 *B.ga* L42885/ 904-1077/ 174 *B.af* X75202/ 792-965/ 174
49 [182]	*B.bu* ss/ *flaB*	CGGCACATATTCAGATGCAGACAG/ 24/ FLA 297 CCTGTTGAACACCCTCTTGAACC/ 23/ FLA 652	KC494770/ 18-373/ 356 KF990319/ 60-415/ 356
50 [183]	*B.bu* ss/ *flaB*	TGAAATAGAGCAACTTACAGACGAAATT/ 28/ ¤Fla98-F CATTTTGAGAAGCAGATTTGTTTGA/ 25/ ¤Fla98-R	KF422803/ 217-314/ 98 *B.ga* KF422758/ 217-314/ 98
51 [116, 184]	*B.bu* ss/ *flaB*	AGAGCAACTTACAGACGAAATTAAT/ 25/ FLA1F CAAGTCTATTTTGGAAAGCACCTAA/ 25/ FLA2R	KF918617/ 122-603/ 482 *B.ga* L42885/ 342-823/ 482 *B.af* AY342020/ 63-544/ 482 *B.lst* DQ016623/ 122-603/ 482
52 [65]	*B.bu* sl/ *flaB*	GGGAAGCAGATTTGTTTGACA/ 21/ B.398f ATAGAGCAACTTACAGACGAAATTAATAGA/30/B.484r	X63413/ 340-427/ 88 *B.ga* JQ711239/ 24-111/ 88 *B.af* KF894064/ 112-199/ 88
53 [113]	*B.bu* sl/ *flaB*	AACACACCAGCATCACTTTCAGGGT/ 25/ Bb-1 GAATTAACTCCGCCTTGAGAAGGTG/ 25/ Bb-2	FJ871030/ 163-395/ 233 KM875674/ 2-234/ 233
54 [113, 185]	*B.bu sl*/ *flaB*	AACACACCAGCATCACTTTCAGG/ 23/ Bbs1-1 GAGAATTAACTCCGCCTTGAGAAGG/ 25/ Bbsl-3c	X16833/ 658-892/ 235 *B.ga* D82846/ 475-709/ 235

55 [186]	*B.bu* sl/ *flaB*	GCATTAACGCTGCTAATC/ 18/ 1F TTGCAGGCTGCATTCCAA/ 18/ 2F	X75200/ 156-575/ 420 X15660/ 50-469/ 420
56 [82]	*B.bu* sl/ *flaB*	ATTAACGCTGCTAATCTTAGT/ 21/ primer Fl GTACTATTCTTTATAGATTC/ 20/ primer F3	X16833/ 235-1025/ 791 AB022133/ 52-842/ 791
57 [187]	*B.bu* ss/ *flaB*	TTCAGGGTCTCAAGCGTCTTGGACT/ 25/ FL6 GCATTTTCAATTTTAGCAAGTGATG/ 25/ FL7	LC018214/ 196-471/ 276 AF264884/ 190-465/ 276
58 [188]	*B.bu* sl/ *flaB*	AACAGCTGAAGAGCTTGGAATG/ 22/ fla I TTATCTAAGCAATGACAAAACATAT/ 25/ fla II	X15660/ 438-1011/ 574 FJ874924/ 448-1021/ 574 *B.ga* JX570875/ 438-1011/ 574 *B.va* AB022139/ 403-976/ 574
59 [63]	*B.bu* ss/ *flaB*	TCAATTGCATACTCAGTACT/ 20/ prB31/41-5 CTGCTGGCATGGGAGTTTCT/ 20/ prB31/41-4	CP001205/ 147500 -148229/ 730 X15660/ 128-857/ 730
60 [66]	*B.bu* ss/ *flaB*	CAAACCAAGATGAAGCTATTGCTGTA/ 26/ ¤FlaB120-F CTTCCTGTTGAACACCCTCTTGAA/ 24/ ¤FlaB120-R	KM875675/ 63-182/ 120 KC246025/ 257-376/ 120
61 [189]	*B.bu* ss/ *flaB*	TTCTCTGGTGAGGGAGCTCAAAC/ 23/ ¤FlaB75-F CTGTTGAGCTCCTTCCTGTTG/ 21/ ¤FlaB75-R	KM875674/ 119-193/ 75 DQ867082/ 293-367/ 75
62 [15, 190]	*B.bu* ss/ *flaB*	AGCTGAAGAGCTTGGAATGC/ 20/ FlaB103-F TTGGTTTGCTCCAACATGAA/ 20/ FlaB103-R	KF836508/ 204 -306/ 103 *B.bis* FJ231346/ 168-270/ 103 *B.crl* EU076499/ 171-273/ 103
63 [32]	*B.bu* ss/ *flaB*	CTTTTCTCTGGTGAGGGAGCTC/ 22/ primer 10 GCTCCTTCCTGTTGAACACCC/ 21/ primer 9	KM875670/ 116-186/ 71 EU220786/ 319-389/ 71
64 [73, 122]	*B.bu* B31/ *p66*	GATCAAATATTTCAGCTT/ 18/ a' CGAAGATACTAAATCTGT/ 18/ a	CP002228/ 626557-626927/ 371 AE000783/ 627874-628244/ 371
65 [122]	*B.bu* ss/ *p66*	TGCAGAAACACCTTTTGAAT/ 20/ f AATCAGTTCCCATTTGCA/ 18/ f'	X87725/ 952-1187/ 236 *B.am* HM802238/ 1-236/ 236
66 [73, 122]	*B.bu* ss/ *p66*	CCAACTTTATCAAATTCTGC/ 20/ c AGGATCTATTCCAAAATC/ 18/ c'	AY654938/ 105-230/ 126 *B.ga* KF844227/ 853-978/ 126 *B.bis* KM269454/ 145-270/ 126 *B.af* KF844231/ 853-978/ 126
67 [122, 191]	*B.bu* ss/ *p66*	GATAAAAACGAAGATAATCG/ 20/ b ACTAGGATCTGTGGATATTC/ 20/ b'	*B.ga* KF844225/ 697-1053/ 357 *B.af* KF844220/ 697-1053/ 357
68 [15, 126]	*B.bu* ss/ *recA*	GTGGATCTATTGTATTAGATGAGGCTCTCG/ 30/ nTM17.F GCCAAAGTTCTGCAACATTAACACCTAAAG/ 30/ nTM17.R	U23457/ 194-415/ 222
69 [74]	*B.bu* ss/ *recA*	GCAAGAGTTCAAATAGAAAA/ 20/ RecF3 AAAGCTTTTGCATAAACAG/ 19/ RecR3	U23457/ 103-389/ 287 *B.af* CP009058/ 126938-127224/ 287
70 [127]	*B.bu* ss/ 16-kb plasmid species-specific plasmid sequences.	TAAAGTTTTGCATAAGC/ 17/ MC16+ TACTAAAGGTGTTTCTCC/ 18/ MC16-	CP001564/ 2424-2818/ 395 U43414/ 1837-2230/ 394 AE000793/ 1808-2201/ 394

71 [127]	*B.ga*/ 33-kb plasmid species-specific plasmid sequences.	CTAACCGCACTAACAGCAGCAAT/23/ MC33+ AGTTTTCATTAGCAGCAA/18/ MC33-	U83998/ 10-245/ 236 CP001302/ 14220-14459/ 240
72 [127]	*B.af*/ 25-kb plasmid species-specific plasmid sequences.	AGAAGGAGATAAAAGAAC/ 18 /MC25+ AAAAAGGTATAGCACAGT/18/ MC25-	CP002944/ 2634-2753/ 120 U84145/ 196-315/ 120
73 [192]	*B.bu* ss/ *ospA*	GGAGTACTTGAAGGCG/ 16/ ¤OspA345-F GCTTAAAGTAACAGTTCC/ 18/ ¤OspA345-R	JF776165/ 220-564/ 345 GQ443122/ 220-564/ 345
74 [193]	*B.bu* sl/ *ospA*	ATGGATCTGGAGTACTTGAA/ 20/ OspA-N2 CTTAAAGTAACAGTTCCTTCT/ 21/ OspAC2	JN413099/ 205-556/ 352 GQ433632/ 182-533/ 352
75 [103, 194]	*B.bu* sl/ *ospA*	GCAAAATGTTAGCAGCCTTGAT/ 22/ Osp6 CGTTGTATTCAAGTCTGGTTCC/ 22/ Osp8	X60300/ 198-586/ 389 *B.ga* AB007102/ 40-428/ 389 *B.bv* JX889254/ 4-392/ 389
76 [195, 196]	*B.bu* sl/ *flaB*	ACATATTCAGATGCAGACAGAGGTTCTA/ 29/ FLA1 GAAGGTGCTGTAGCAGGTGCTGGCTGT/ 27/ FLA2	KJ676826/22-410/ 389 *B.am* HM802232/31-419/ 389 *B.crl* KF793050/ 29-417/ 389
77 [197]	*B.bu* ss/ *p66*	CAAAAAAGAAACACCCTCAGATCC/ 24/ ¤P66684-F CCTGTTTTTAAATAAATTTTTGTAGCATC/ 29/ ¤P66684-R	KM676037/ 26-709/ 684 CP001205/ 623268-623951/ 684

Table 2-1. Primer-sets including individual primers and corresponding amplicons. ¤ indicates the name was given in this master project. The undefended accession numbers indicate sequences of B. burgdorferi. Coordinates for amplicon are representing the 5' end of forward and reverse primers.

The species of the downloaded amplicons were selected corresponding to which species was amplified in the references. For instance, if a primer pair was used to amplify *B. burgdorferi* ss in a reference, an amplicon for this species was downloaded and analyzed in this study. Similarly, several amplicons of different species were downloaded in case that reference intended to detect different species of Lyme *Borrelia* using one primer pair.

2.1 References of primer pairs.

In the source studies, DNA sample was extracted from different organisms, e.g. humans, rodents, birds, dogs, ticks, laboratory cultures [18, 31, 69, 82, 86, 116, 188], and others. Furthermore, wide spectrum of specimens was used, depended variously on researcher's concerns, purpose of the amplification, geographical dissemination of pathogens, and which *B. burgdorferi* sl genospecies intended to be amplified or detected.

Specimens of human and mammalian infections were often cerebrospinal fluid (CSF), synovial fluid (SF), synovial tissue (ST), Blood, skin biopsy, urine [15, 100, 113, 189]. Specimens of rodents (e.g. white-footed mouse), birds and dogs, were blood, tissues of kidney, spleen and liver, and skin biopsies [158]. Finally, specimens of ticks were either midgut tissues or the whole tick [175].

During the search for primer pairs among several hundred sources, it became apparent some primer pairs were designed for nested PCR, but they were posteriorly exploited as standard primers for quantitative or real time-PCR. For instance, at 2002, Ornstein et al. used a primer pair in nested PCR for identification of *B. burgdorferi* [94]. Thereafter, the same primer pair was used by Brandt et al. at 2014 for genotyping of four *B. burgdorferi* sl species using conventional PCR [160]. However, the publication dates of primer pairs are ranged from 1989 as earliest until 2014 as latest reference.

2.2 Genes Target

Selection of primer pairs for this master project was mainly depending on the gene that intended to be amplified by PCR in the sources. Most of those sources amplified genes that encoded the outer surface proteins (*osps*) and flagellin B (*flaB*), which were In this master project, the gene that were mainly preferred are the outer surface preferred to investigate in this master project. In addition, primers for outer membrane protein (*OMP/p66*), and *recA* were analyzed. Pus, 16-kb, 30-kb, and 25-kb plasmid species-specific plasmid sequences for *B. burgdorferi*, *B. garinii* and *B. afzelii*, respectively.

In the analysis, the presented forward primer, reverse primer, and amplicon sequences were used; these sequences are referred to as a 'primer-set'

2.3 Primer Pairs (Oligonucleotides)

Seventy seven primer pairs (samples) were analyzed.

2.3.1 Names of the primers

The vast majority of the primers have a name in their references. This name as a distinctive identity for each primer, it wasn't be changed when the primer was used by other studies. The same name was also used to refer the primers here too, such as [18, 63, 158]. In case that the reference did not name the primers such as [115, 176], they were presumptively named in this master project to distinguish them. The name given composed of gene target, length of amplicon, and primer. For instance, primer pair amplified *ospA* gene with amplicon length 345-bp, it was named *ospA*345-F for forward primer, and ospA345-R for reverse primer [192]. In some studies, both forward and reverse primers have exactly the same name, in this case, F or R letter were added to the original name to indicate forward or reverse primer, respectively [152, 163].

2.3.2 Length of primers

The length of primers ranged from 16 as shortest to 30-mers for longest primer, depending on terms and conditions that primers were designed with, e.g. melting temperature (T_m) which is directly proportional with length of primer, template complexity, and length of the target on complementary strand, plus other concerns such as gene target, and the length of DNA segment that supposed to be amplified [45, 51, 52]. In several primer pairs, lengths of forward and reverse primers were substantially different [71, 157].

2.3.3 Nucleotide codes

In some primers' references, common nucleotide code was used within some primer sequences; R for (A/G) and Y for (C/T) [78, 169]. More investigation for nucleotide substitutions was required to determine which one is suitable for similarity search. BLAST was also used for this purpose by searching all probabilities for the sequence. For instance, primer-set 32 was G**Y**AAAGTAAAATTAACA**R**T and TGTTTT**R**CCATCTTCTTT for the forward and reverse primer, respectively [169]. According to nucleotides code, four probabilities for the forward primer and two for the reverse primer have to be searched by BLAST to find the most correct sequence.

2.4 Amplicon Sequence.

The sequence of amplified amplicons in the reference for each primer pair were downloaded from Entrez Nucleotide in this master study using BLAST results and NCBI database in order to analyze forward and reverse primers together within amplicon sequence; the amplicon sequence with its primers as one unit (figure.2-1). This was the second stage/part of each sample in this master project. Noteworthy, in some references, a specific probe was designed for amplification, as well as. In this case, sequence of this probe was searched to find whether it actually exists within the downloaded amplicon sequence or not.

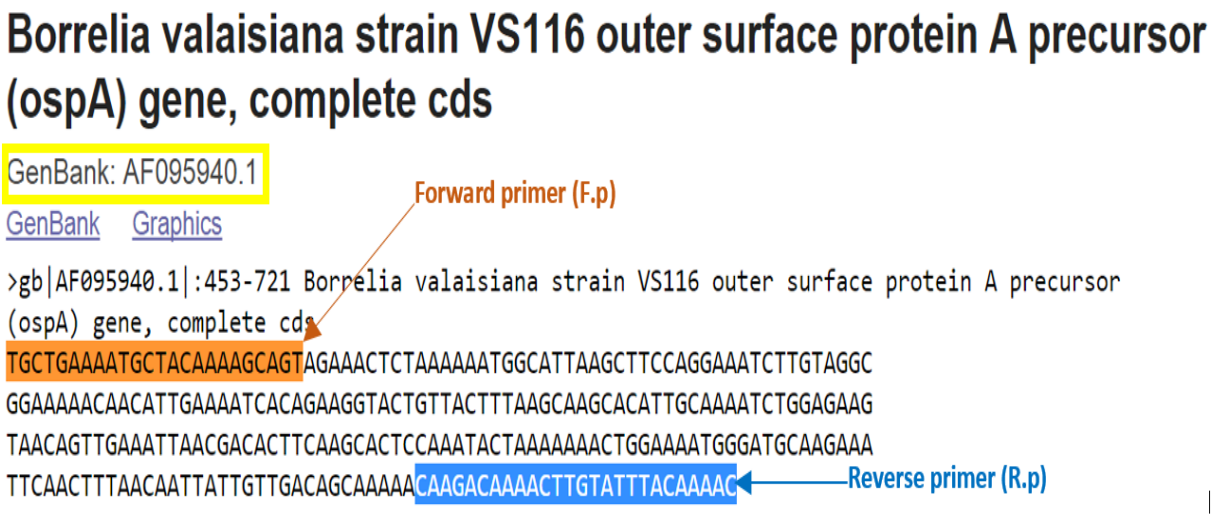


Figure 2-1: An example for the downloaded amplicon for primer-set 19, shows the forward primer (F.p) and the complementary sequence for the reverse primer (R.p) on the sides of the downloaded amplicon.

2.4.1 Names of amplicons

In this master study, each downloaded sequence of amplicon was identified by the GenBank accession number, coordinates/positions within the entire gene sequence, and length. For instance, an amplicon of *B. burgdorferi* with GenBank accession number AY597034 and 249-bp length and coordinates 61-309, this sequence was downloaded using primer-set 30.

2.4.2 Length of amplicons

Most of the downloaded amplicons were at the same length of amplified amplicon in the references such as [122, 154, 195], except some cases that showed difference in lengths between the reference and the downloaded amplicons [74, 163]. Moreover, in some primer-sets, the downloaded amplicons showed different lengths for the same primer-set within strains and between genospecies of *B. burgdorferi* sl [162, 175, 176, 198]. There was a special case for amplicons in the primers general nucleotide codes, such as in primer-set 32, two lengths of amplicons were downloaded, 834 and 74-bp, both were analyzed [169]. However, 890-bp [154], the longest amplicon that was downloaded, and 71-bp was the shortest [32].

3 Methods

In order to investigate specificity and sensitivity of 77 primer-sets, **B**asic **L**ocal **A**lignment **S**earch **T**ool (BLAST®) software http://blast.ncbi.nlm.nih.gov/Blast.cgi was the main method in this master project. The nucleotide BLAST (BLASTn) that searches similarity of nucleotide query sequence in **nucleotide** database sequences was particularly employed. In addition to BLAST, the Entrez GenBank database was used to obtain and download the amplicon sequence corresponding each primer pair that targeted certain genospecies of *B. burgdorferi* sl. The downloaded sequence of amplicons were analyzed with their forward and reverse primers.

3.1 Analysis of the primers individually

Each single primer of each primer pair was separately searched for homology against nucleotide sequences of GenBank database. The search was simply performed by entering the sequence of primer in "BLAST text box", regulated the algorithmic parameters, and ran the program.

The format "bare sequence" was used to enter primer sequences for searching by BLAST. Furthermore, the "Automatically adjustment" for the "Algorithmic parameters" was selected to enhance efficiency of BLAST for significance similarity. For instance, in parameter "Word size", the shortest available size of word that can be manually selected was 16 letters, whilst it supposed to be much lesser (for example 7) for a primer length with 20-mers. Using of "Automatically adjustment", BLAST enhance the manual selected values into parameters that can give stronger or more significant results.

3.2 Analysis of primers within the downloaded amplicon.

In this step, the forward and reverse primers were once more searched for similarity but together within their amplicon sequence that was actually amplified in the reference; the downloaded amplicon sequence in this master study including the primer pair is corresponding the amplified amplicon in the reference. This was the second stage of assessment specificity of each primer pair.

After searched the primers separately and find the results for both, a common accession number of usually 100% similarity alignment for both primers was identified. Thereafter, the positions of 5' end of each alignment for forward and reverse primers in the results of BLST represented the coordinates of the amplicon. These coordinates were entered in the subsequence tool in ENTREZ nucleotide to obtain the amplicon sequence and download it.

The figures below illustrate the method whereby amplicon sequences were obtained and downloaded from Entrez nucleotide library in NCBI database.

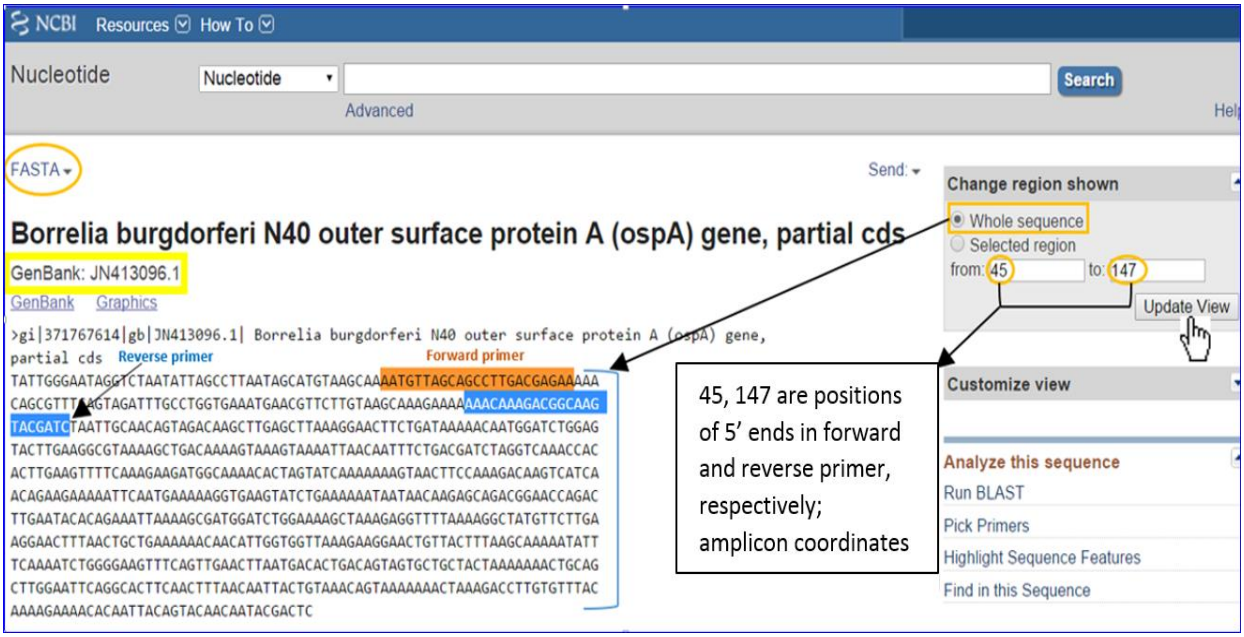


Figure 3-1: An example shows the entire sequence of ospA gene of B. burgdorferi N40 with accession number JN413096. It also shows the coordinates of the interested amplicon sequence to be downloaded and analyzed.

The sequence of the interested amplicon will be solely displayed by clicking on “Update View”. See figure 2.

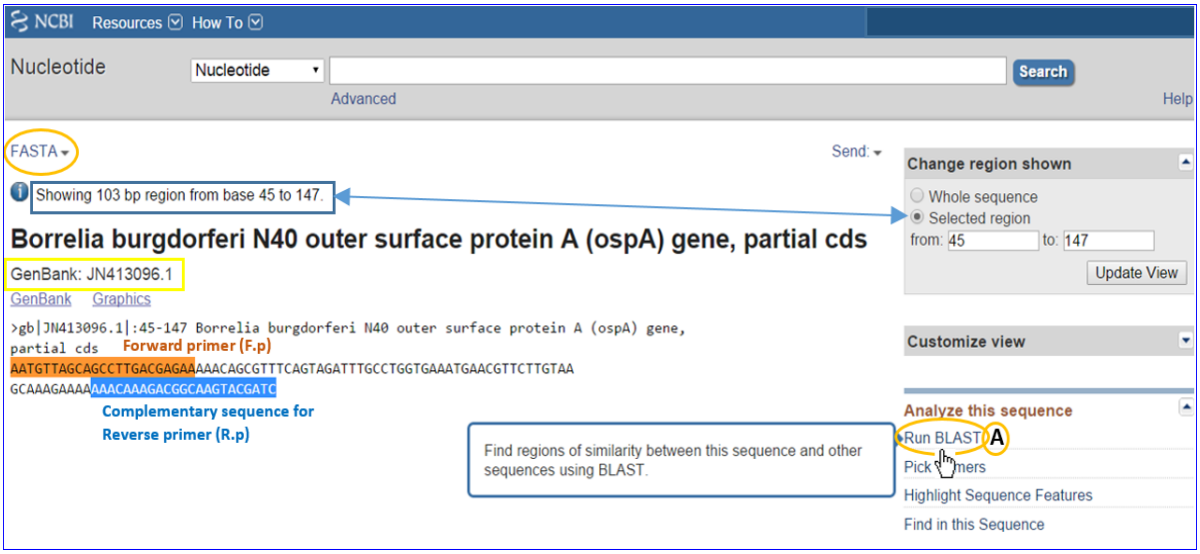


Figure 3-2. The sequence of amplicon as was downloaded. It shows the forward and reverse primers within amplicon sequence, and in some cases a specific probe.

Noteworthy, reverse primer within in amplicon sequence was represented by the complementary nucleotides for this primer, but not the primer itself. Furthermore, in some primer sets such as 59, the forward primer altered its position with reverse primer; the primers were reciprocal in positions.

3.3 Reading of the Results

Sequences of the individual forward and reverse primers were firstly tested separately and result was recorded. Thereafter, in order to investigate the specificity of the primers in more accurate manner, both primers were tested together as part of the amplicon sequence. Thus, results for each primer-set consist of three parts corresponding to forward primer, reverse primer and amplicon.

Two main parameters were considered in all searches: **Identity** and **Coverage**.

The "**Identity**" (Id), denotes the percentage of similarity between the query sequence and the matched sequence of database. Thus, alignment of two sequences in 100% identity indicate identical match or 100% similarity. Whereas, alignment with mismatch nucleotides will reduce the identity percent depending on length of the query sequence and number of mismatches.

The second parameter is the "**Query cover**" (Qc) which indicates what percentage of the query sequence is covered by the matched sequence of database. A query cover of 100% indicates that the entire query sequence is contained within the matched database sequence. Query cover lower than 100%, indicates that part of the query sequence is missing from the matched sequence of database; the result is a match alignment that is shorter than the query sequence. In most cases, reduced Qc involves the ends of the sequence, because the sequenced region is shorter or only partially overlaps the query sequence - although gaps in the central part may also reduce the Qc score. Besides, some alignments termination occurred due to mismatch near the end of sequence.

3.3.1 Primers Search Results

In primer searches, only hits/alignments showing 100% identity and 100% query cover (figure3-3) were considered homologous/identical match and counted in the tables of results. Hits showing mismatch could be identified by reading the score of identity (figure 3-4). Incompleteness in the coverage of a query sequence could be identified by examine the score of query cover or the numbering of nucleotide in the alignment (figure 3-5).

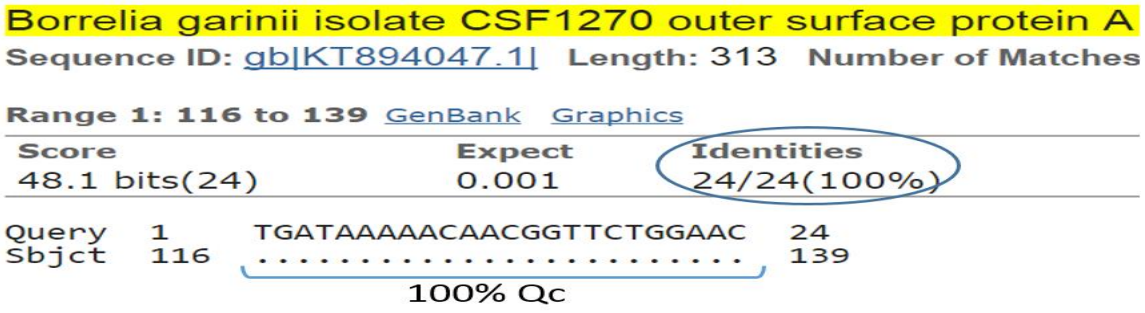


Figure: 3-3. An alignment of primer results in 100% identity (Id) and 100% Query cover (Qc). This alignment is 100% homologous/identical.

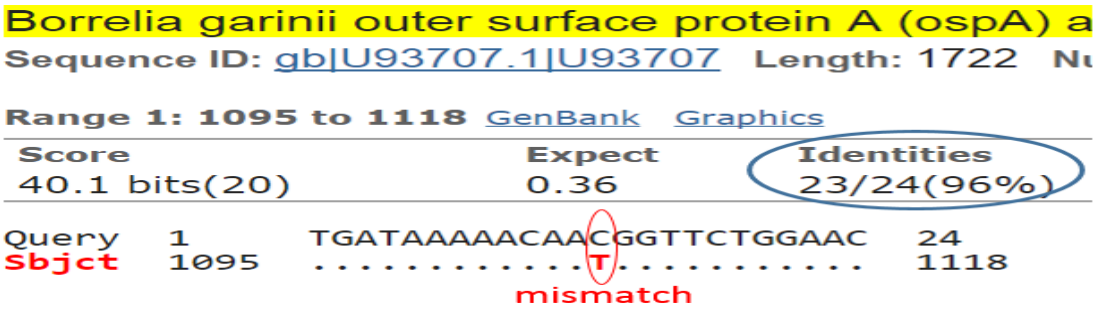


Figure 3-4: An alignment of primer results in 96% identity (Id) with one base mismatch. Such alignment considered suboptimal homologous and counted as mismatch in results of primer.

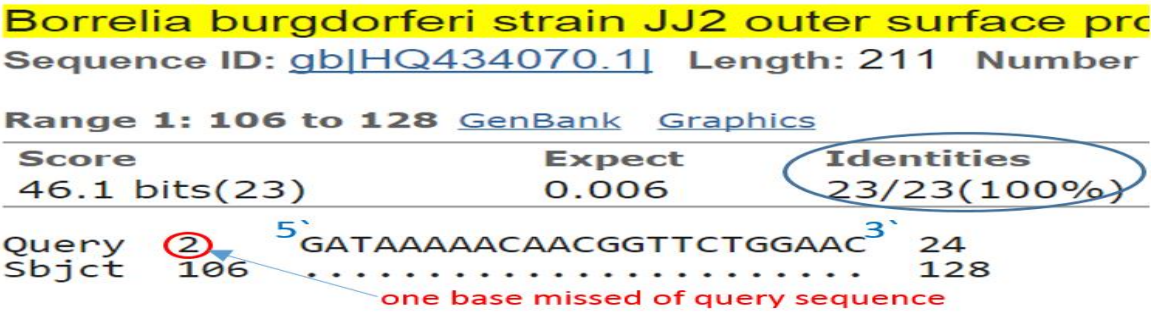


Figure 3-5: An alignment of primer results in 95% Query cover (Qc) with one missed base at 5`. Such alignment was considered as heterologous/mismatch.

Actually, several examples of alignments that showed more than one mismatch or missed nucleotides were displayed in BLAST results. All these alignments considered mismatch for the queried primer sequence.

3.3.2 Amplicon Search Results.

In the amplicon search, forward and reverse primers were tested within sequence amplicon. The focus was on similarity of the primers, but sequence between them was disregarded. The hits should be in 100% Qc and maximum two mismatch nucleotides in either primer, to be considered as significance similarity. By these criteria, several cases of similarity for the primers within the amplicon, depending on how many mismatch nucleotides was detected in primers.

I. **Identical match (100% similarity)**: is shown by an alignment of amplicon that contains primers in 100% query cover and no mismatch nucleotide, (figure 3-6).

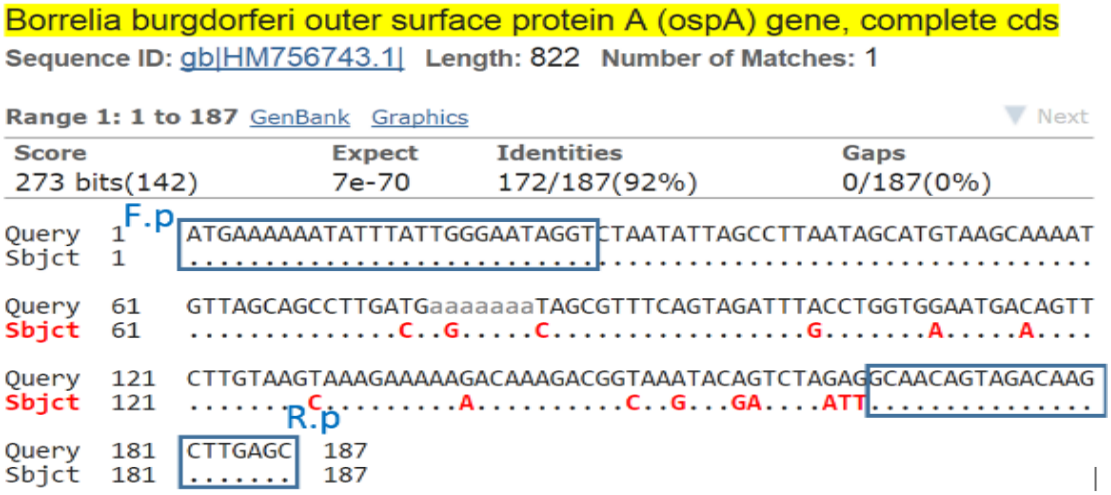


Figure 3-6. An alignment of amplicon search results shows identical match by 100% similarity for the forward primer (F.p) and reverse primer (R.p). .

II. **Lowest similarity**: Alignment in 100% Qc and showing maximum two nucleotides mismatch in either primer, (figure 3-7).

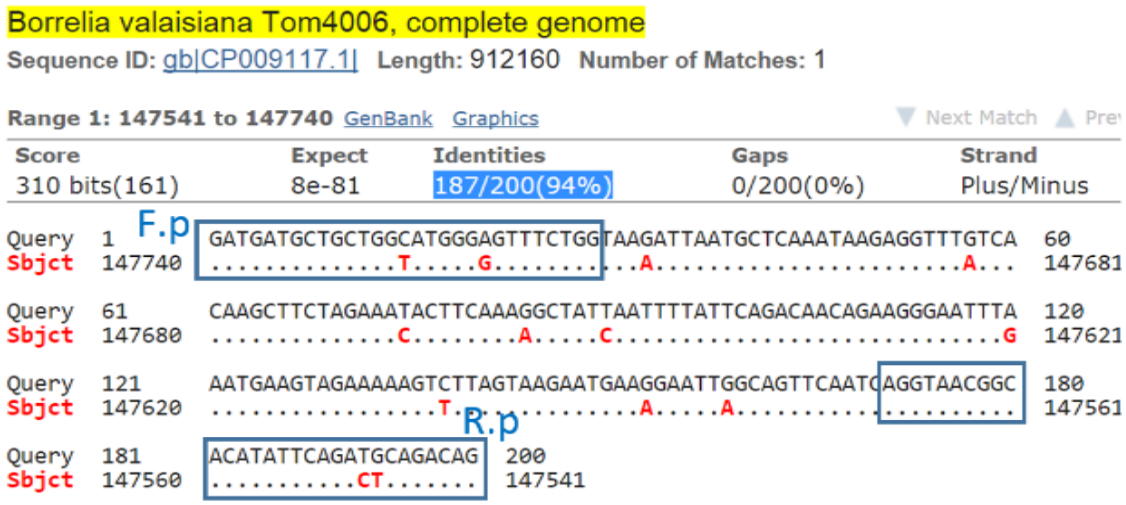


Figure 3-7: An alignment of amplicon search results in 100% Qc and shows two nucleotides mismatch in forward primer (F.p) and reverse primer (R.p). Such alignment was considered lowest significant similarity for the primers.

III. **Mismatch Alignments:** This results shown in alignment with at least three mismatch nucleotides in one of the primers, (figure 3-8).

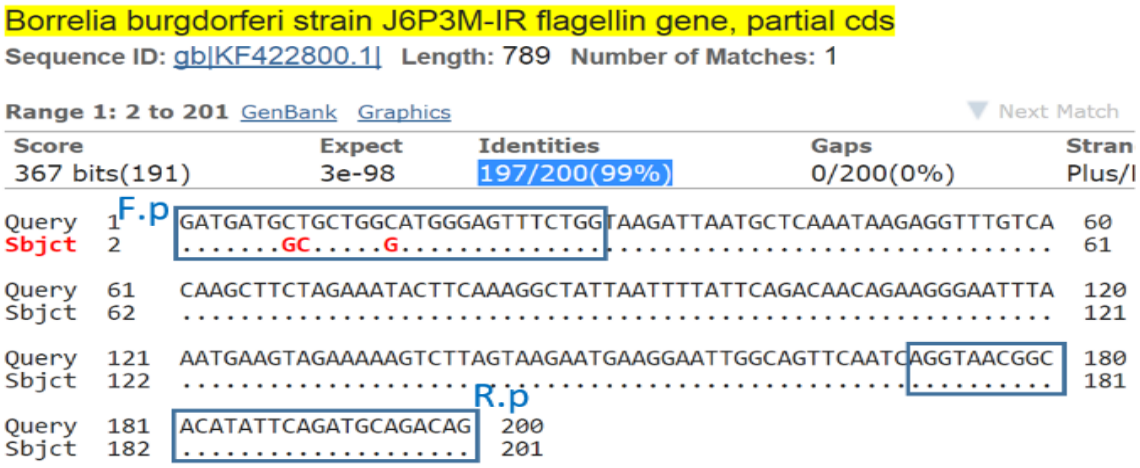


Figure 3-8: An alignment of amplicon results in 100% Qc but three mismatches in forward primer (F.p). The amplicon is counted as mismatch: F.p; match: R.p.

Deficiency in the coverage of the query sequence gives mismatch alignment due to lacking nucleotides from the primers, (figure 3-9).

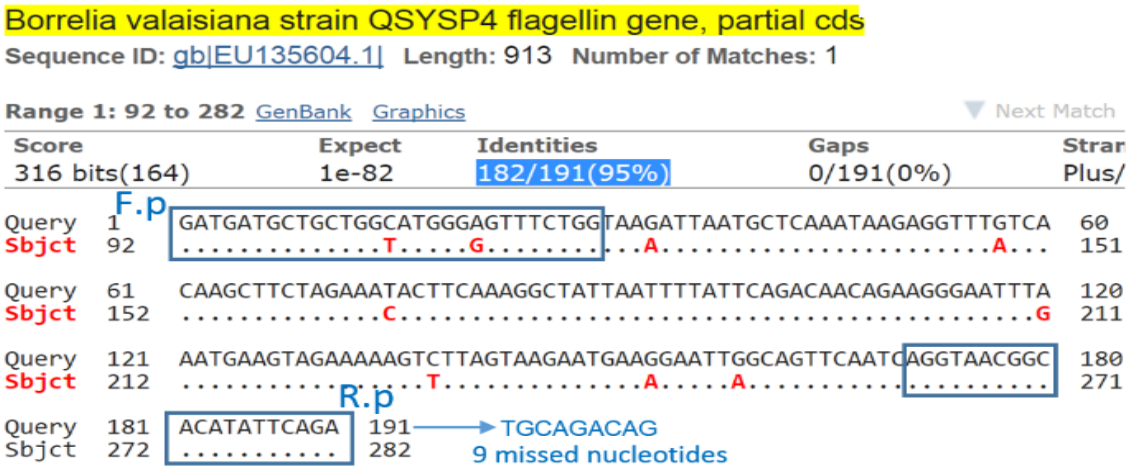


Figure 3-9: An alignment in 95% Qc truncates nine nucleotides from reverse primer (R.p). Such alignment was considered as mismatch for reverse primer.

A sequence of amplicon that truncates one or two nucleotides in either end was considered as a partial similarity

4 Results

Seventy-seven primer-sets were searched for specificity and sensitivity using BLASTn software. Each primer pair and its downloaded amplicon sequence was named “primer-set”.

Although the individual primers were analyzed separately, the evaluation of specificity and sensitivity for primer-sets relied only on the results of amplicon analysis, taking into account the results of search the individual primers. The results will be presented below in one main table, table 4-1.

Results of amplicons search revealed that 25 primer-sets (32%) are specific for the target species, 40 primer-sets (52%) are non-specific by cross-reaction with untargeted species, and 12 primer-sets (16%) show limited cross-reaction and may be considered specific primers or partial specific. PCR-primers targeting *ospA* gene are more specific than primers targeting *flaB* gene, which most are non-specific for the target species.

Details about each primer-set is presented in an independent tables containing the number of hits/alignments for analysis of primers and amplicon sequences by BLASTn searches. Thus, the results in this master thesis will be also presented in 77 tables, for 77 analyzed primer-sets/samples, see annex 1.

Table 4-1 shows summary of BLAST search results for amplicons of the primer-sets. Elements in the table were sorted according to the target species of *B. burgdorferi* sl complex that was amplified in the reference of the primers.

Target species	**Target gene/ Amplicon size (bp)**	**Cross-reaction**	**Sensitivity for target spp.**	**Comment**	**Primer-set**	**Ref.**
B.bu ss	*ospA*/541	X	44%	Not for all strains	2	[18]
B.bu ss	*ospA/*156	*B.*spp	70%	limited cross-reaction	5	[100]
B.bu ss	*ospA/*195	5 LB spp.	10%	All low sensitivity	6	[100, 151]

B.bu N40	*ospA/*103	*B.and*	70%	Limited cross-reaction	7	[69]
B.bu ss	*ospA/*767	X	12%		8	[152]
B.bu ss	*ospA/*142	4 LB spp.	68%	Low hits number	9	[152]
B.bu ss	*ospA/*643	X	33%	For all target strains	13	[153]
B.bu ss	*ospA/*890	*B.trd*	6%	Limited cross-reaction	15	[154]
B.bu ss	*ospA/*400	X	44%	High percent of hits for *B.ga*	16	[155, 156]
B.bu ss	*ospA/*126	X	58%	Specific in all tests	20	[158]
B.bu ss	*ospA/*151	3 LB spp.	50%		21	[159]
B.bu ss	*ospA/*99	*B.fin*	66%	One hit cross-reaction	27	[164]
B.bu ss	*ospA/*138	*B.*spp	38%	One hit cross-reaction	28	[165]
B.bu ss	*ospA/*294	X	48%	Specific in all tests	30	[166, 199]
B.bu ss *(nested)*	*ospA/*345	*B.amr*/B.spp	53%	One hit cross-reaction	73	[192]
B.bu ss *(nested)*	*ospA/*352	3 LB spp	49%	Not for all strains	74	[193]
B.bu ss	*ospB/*780	*B.*spp	100%	One hit cross-reaction	33	[170, 171]
B.bu ss	*ospB/*328	X	16%		34	[151]
B.bu ss	*ospC/*547	X	6%		10	[152]
B.bu ss	*ospC/*128	X	33%	Specific in all tests	11	[152]
B.bu B31	*ospC/*84	X	45%	Specific in all tests	22	[159]
B.bu ss	*ospC/*128	X	14%	Specific in all tests	36	[173]
B.bu ss	*ospC/*231	X	22%	Prevalent in all tests	42	[76]

B.bu ss	*ospC/*600	5 LB spp.	9%	All low sensitivity	45	[176]
B.bu ss	*flaB/*92	6 LB spp.	92%	Sensitive cross-reaction	12	[152]
B.bu ss	*flaB/*299	7 LB, 2 RFB.	16%	Uneven sensitivity	40	[71]
B.bu (B31,N40)	*flaB/*134	7 LB spp.	86%	More sensitive for *B.bu* sl	41	[71, 76]
B.bu ss	*flaB/*356	*B.*spp	90%	Limited cross-reaction	49	[182]
B.bu ss	*flaB/*791	3 LB spp.	13%	Low sensitivity for all	56	[82]
B.bu ss	*flaB/*730	4 LB spp.	8%	Low sensitivity for all	59	[63]
B.bu ss	*flaB/*120	5 LB spp.	85%	Moderate sensitivity	60	[66]
B.bu ss	*flaB/*75	5 LB spp.	67%	*B.bu* is dominant	61	[189]
B.bu ss	*flaB/*103	7 LB spp.	78%	Sensitive for *B.af* & *B.lst*	62	[15, 190]
B.bu ss	*flaB/*71	*B.crl*, *B.*spp	90%	Low hits cross-reaction	63	[32]
B.bu GeHo	*flaB/*70	4 LB spp.	90%		47	[70]
B.bu ss	*p66/*371	X	27%	Low hits number	64	[122]
B.bu ss	*p66/*236	6 LB spp.	58%	Preference for all spp.	65	[122]
B.bu ss *(nested)*	*p66/*684	X	68%	Low hit number	77	[197]
B.bu ss	*recA/*222	X	17%	Specific in all tests	68	[15, 126]
B.bu ss	16-kb /395	X	53%	Specific in all tests	70	[127]
B.ga	*ospA/*345	*B.bu* ss, *B.bv*	31%	High sensitive of *B.bv*	3	[18]
B.ga	33-kb/236,240	X	66%	Very low hits number not for target strains	71	[127]
B.af	*ospA/*190	*B.bu* ss	52%	3 of 7 target strains	4	[18]

B.af	25-kb/120	X	100%	Low hits number, 1 of 3 target strains	72	[127]
B.va	*ospA/*269	*B.bu* ss	87%	Limited cross-reaction	19	[157]
B.va	*ospC/*684	*B.bu* ss, *B.af*	16%	Low sensitivity for all	37	[174]
B.spl	*ospA/*151	*B.*spp	99%	limited cross-reaction low hits number	25	[161]
B.bis	*ospA/*138	*B.bu* ss	62%	Low hits number	29	[165]
B.bu ss, *B.va*	*ospA/* 838, 841	*B.bis, B.trd*	9%, 10%	Limited cross-reaction Low hits number	26	[162, 163]
B.bu sl	*ospA/*200		9%-100%	Low hits number for 5 spp. of *B.bu* sl	14	[61]
B.bu sl	*flaB/*276	X	43%	Preference for *B.af*	57	[187]
B.bu sl	*flaB/*574	3 RFB spp.	5%-40%	Low sensitive for all	58	[188]
B.bu sl (nested)	*flaB/*389	X	13%-100%	Preference for *B.ga*	76	[195, 196]
B.bu ss, *B.ga, B.af*	*ospA/*308	3 LB spp.	23%, 32%, 86%		1	[18]
B.bu ss, *B.ga, B.af*	*ospA/*187	3 LB spp.	40%, 22%, 13%		17	[31]
B.bu ss, *B.ga, B.af*	*ospA/* 289, 293	X	20%, 0%, 1%	No *B.ga*	31	[18, 168]
B.bu ss, *B.ga, B.af*	*ospC/* 320, 326	3 LB spp.	26%, 19%, 8%	Low hits number	43	[160, 176]
B.bu ss, *B.ga, B.af*	*ospC/* 266	5 LB spp.	36%, 12%, 28%	Low hits number	44	[177, 178]
B.bu ss, *B.ga, B.af*	*flaB/* 174	3 LB spp.	12%, 12%, 12%	Low hits number	48	[180]

B.bu ss, *B.ga, B.af*	*flaB/* 98	6 LB spp.	91%, 82%, 91%	Sensitive for all	50	[183]
B.bu ss, *B.ga, B.af*	*flaB/* 233	5 LB spp.	65%, 6%, 0%	Preference for *B.bu* ss	53	[113]
B.bu ss, *B.ga, B.af*	*recA/* 287	2 LB spp.	15%, 8%, 62%	Limited cross-reaction Low hits number	69	[74]
B.bu ss, *B.ga, B.af* (nested)	*ospA/* 389	3 LB spp.	31%, 46%, 0%	Preference for *B.ga* and *B.bv*	75	[103, 194]
B.ga, B.bu ss, *B.af*	*flaB/*180	7 LB, 4 RFB	85%, 70%, 0%		18	[31]
B.ga, B.bu ss, *B.af*	*flaB/ B* 584	*B.va, B.bis*	30%, 27%, 52%		39	[118]
B.ga, B.bu ss, *B.af*	*flaB/*89	7 LB spp.	82%, 82%, 94%	Preferred for *B. bu*. sl	46	[115]
B.ga, B.bu ss, *B.af*	*flaB/*88	*B.va*	79%, 8%, 93%	Limited cross-reaction Preference for *B.ga*, *B.af*	52	[65]
B.af, B.ga, B.bv, B.bu ss	*ospA/*234	X	0%, 42%, 91%, 35%	Preference for *B.bv*	24	[94, 160]
B.bu ss, *B.ga, B.af, B.jpn*	*ospC/*641, 626	*B.va, B.spl*	4%, 6%, 5%, 0%	Wrong accession numbers	38	[175]
B.bu ss, *B.bis, B.and*	*ospA/869, 74*	X	27%, 1%, 0%	Low hits number	32	[169]
B.bu ss, *B.ga, B.af, B.va, B.lst.*	*flaB/*482	4 LB spp.	28%, 57%, 66%, 42%, 33%	Low sensitive for all	51	[116, 184]
B.bu ss, *B.ga, B.af, B.va, B.jpn, B.spl,*	*ospA/*137	3 LB spp.	25%, 36%, 14%, 36%, 0%, 100%	Limited cross-reaction	23	[78]
B.bu ss, *B.ga, B.af, B.jpn, B.and, B.va, B.lst, B.bis*	*flab/*235	4 LB spp.	50%, 62%, 0%, 0%, 80%, 50%, 0%, 50%	*B.bu* ss is dominant	54	[185]

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13 European of *B.bu* sl isolates	*ospA/ 606, 615, 609*	X	22%-100%	Only 6 isolates	35	[172]
European strains	*p66/*357	X	35%-43%	Only 3 species	67	[122, 191]
American & European strains	*flaB/*420	3 RFB spp.	6%-100%	Only 4 spp. of *B.bu* sl	55	[186]
All universal types	*p66/*126	X	75%-100%	Sensitive for all but low hits number	66	[73, 122]

Table 4-1. Results of amplicon search for 77 primer-sets.

5 Discussion

Based on amplicon comparisons, the specificity of 77 primer-sets to target species of *B.burgdorferi* sl, were classified into three groups: (i) **specific, 25** (**32%**) primer-sets that showed similarity only for the target species; (ii) **nonspecific, 40 (52%)** primer-sets that showed clearly cross-reaction with at least one untargeted species; (iii) **partially specific, 12 (16%)** primer-sets that showed specificity for the target species but cross-reacted to a limited degree with one or two untargeted species. Ten primer-sets were high specific for the target species in all searches (the individual primers and amplicon).

Some primer-sets were specific in all searches (individual primers and amplicons) such as 11, 20, 22, 36, 68, 70, and 77, while some others showed specificity only in the amplicon comparisons. This may reflect the strength of primers specificity since the primer-set was specific in the individual primers and amplicon experiments. Species of relapsing fever *Borrelia* were detected in only 4 of the nonspecific primer-sets. The partially specific primer-sets intended to be specific rather non-specific by very low hits number (mostly only one) for one or two untargeted *Borrelia* species.

B. burgdorferi ss, *B. garinii* and *B. afzelii* were the dominant species that detected in BLAST results, they seldom showed high sensitivity. The moderate species of *B. burgdorferi* sl: *B. americana*, *B. valaisiana*, *B. bavariensis*, *B. bissettii*, *B. spielmanii*, *B. lusitaniae*, and *B.* spp. They showed either low-moderate sensitivity or undetected in amplicon searches such as primer-sets 7, 8, 14, and 16. Finally the scarce or modern species of *B. burgdorferi* sl e.g. *B. tanuki*, *B. turdi*, *B. finlandensis*, *B. yangtzensis*, *B. Okinawa*, *B. californiensis*, *B. carolinensis*, and *B. chilensis*, they were detected in very low hits number.

Most primer-sets that target *ospA* of *B. burgdorferi* ss were specific, while, those that target *ospA* of *B. burgdorferi* sl or the three main species together (*B. burgdorferi* ss, *B. garinii*, and *B. afzelii*) were not specific or able to detect all the target species. For instance, primer-set 24 was designed to amplify the three main species. In BLAST, the primer-set was homologous to *B. burgdorferi* ss and *B. garinii* but failed to match *B. afzelii*. In general, *ospA-primer*-sets showed low sensitivity. Both primer-sets 33 and 34 for *ospB* were specific, but only one (33) was sensitive. Of eleven *ospC* primer-sets, five were specific, but all low sensitive.

Primer-sets targeting *flaB* were mostly for detection of either *B. burgdorferi* ss or the three main species together. Of 25 primer-sets for *flaB* were analyzed, only 4 were specific. However, most of them showed moderate to high sensitivity compared with primer-sets targeting *ospA*.

Five primer-sets targeted *p66* of *B. burgdorferi* ss, European genospecies of *B. burgdorferi* sl, or all universal types of Lyme *Borreliae*. Four were specific and moderately sensitive. One of two primer-sets for *recA* was specific and moderately sensitive. Finally, primer-sets targeting 16-kb, 33-kb, and 25-kb plasmids of *B. burgdorferi* ss, *B. garinii*, and *B. afzelii*, respectively, showed high specificity for the target species, but low sensitivity, although this conclusion is based on a very number of BLAST hits.

Not all strains used in the published studies of primer-sets could be detected in BLAST results. There are several possible reasons for this, of which, the strain has not been sequenced. Another reason is concerned with the technicalities of sequencing PCR products. In a PCR product, the ends are either primers, or complements of primers. Thus, if the tested primer was used for sequencing, then the primer sequence should have been trimmed of the published sequence. This case was observed in several primer-sets although they were specific. For instance, primer-set 3, was used to amplify 7 strains of *B. garinii* [18], whilst, in BLAST, only 3 strains of *B. garinii* were detected. The same case was observed in further primer-sets: 1, 2, 4, 19, 23, 26, 31, 32, 37, 44, 52, 53, 54, 56, 57, 70, 72, 74, 74, and 76. Other primer-sets such as 13, 25, 33, 48, 51, and 73, showed sensitivity for all strains of *Borrelia* amplified in their references.

BLAST results for some primer-sets contradicted published in vitro findings. For example, the main species that was amplified in vitro by the primer-set 24 was *B. afzelii*, then *B. garinii* but not *B. burgdorferi* ss [94]. According to BLAST results, the primer-set was mainly sensitive for *B. burgdorferi* ss then *B. garinii* but not *B. afzelii*. In another case, primer-set 61 was reportedly derived from *B. burgdorferi* ss (GenBank AF244889) [189], this is a sequence of mRNA for “like protein Kinase” from Glycine max. Furthermore, ten accession numbers (AY662999 to AY663008) of *flaB B. burgdorferi* sl were deposited into GenBank database using the primer-set 76 [196], all these accession numbers were not detected in forward primer searches. This is probably due to primer sequence being trimmed off.

The identity (Id) of alignment in amplicon searches does not always reflect the homology of the primers to the amplicon. For instance, amplicon of primer-set 14 showed mismatch to the primers alignments with 99% and 98% overall Id alignments; primer-sets 57 and 60 showed mismatch to the primers in 96% Id overall alignments with the amplicon. On the other hand, amplicon searches for other primer-sets, such as 15, 24, 31, 44, and 45, showed similarity for the primers even at over all Id as low as 78%. In general, however, mismatch in primers started at over all Id 93%. The explanation for primers that keep homologous at low overall Id us that they are derived from conserved regions. Conversely, primers that showed mismatches at high overall Id were derived from variable regions.

There was an association between primer homology/heterology and which genospecies of *B. burgdorferi* sl the alignment was for. The primers in 90% Id alignment of the prime-set 12 were heterologous for *B. japonica*, but homologous for *B. valaisiana* in the same identity. Another example, amplicon of primer-set 74 was homology for alignment of *B. garinii* in 89% Id, but mismatched *B, yangtzensis* in the same identity. This may explain the homologous species is more related to the species of the search sequence than the heterologous. However, it was observed that which genospecies of *B. burgdorferi* sl is searched, can affect the amplicon identity but mostly cannot change the homology of primers.

BLAST prioritizes the identity of alignment over query cover, and prefers to the truncate terminal nucleotides if they contain mismatch rather to present them in the alignment. An alignment for *B. afzelii* showed 98% query coverage for the amplicon of primer-set 1 due to absence of 4 terminal bases of the reverse primer. These four bases contained only one mismatch, so the alignment is actually homologous for the reverse primer of amplicon but BLAST truncated these four bases rather to display them with one mismatch. The same case was observed in primer-sets 31.

On the other hand, BLAST was correctly truncated some based that contained genuine mismatch, such as alignment of *B. garinii* in only 94% query coverage with the amplicon of primer-set 58 because it contained more than three mismatches. Further, incomplete coverage of query sequence may occurs because of insufficiency in the target gene sequence. Alignment of *B.burgdorferi* (KM069288) covered only 98% of queried amplicon in primer-set 13, lacking 7 bases; this was because of incompleteness of the gene sequence, not mismatch. However, further investigations were necessary to find out whether the truncated bases are homologous for the primers or not.

The expectation value (E-value) is an important parameter to evaluate the reliability of alignment homology. In general, hits of amplicon experiments showed E-value lower than 0, while experiments of the individual primer showed some hits with E-value higher than 0, included hits of cross-reactions with Relapsing fever *Borrelia* and other unrelated bacteria and organisms. Eventually, hits with E-value lower than 0 were more reliable and were recorded in the results.

In BLAST results, almost all hits that showed similarity for the query sequence belonged to the gene of interest (*ospA*, *ospB, flaB*, etc.). In rare cases, some hits belonged to undefined or unrelated sequences, such as some hits of primer-sets 15 and 33. The most hits of cross-reaction with relapsing fever *Borrelia* or unrelated organisms belonged to undefined or unrelated genes.

Not all primer-sets that showed specificity in amplicon experiment, were also specific in searches with the individual primers. Most of primers found to be specific in the amplicon searches. Most of the primers found to be specific in amplicon searches showed cross-reaction with untargeted *Borrelia* species when searched individually as independent primers. Moreover, many hits of uncultured *B. burgdorferi* showed homology for individual primers in primer-sets such as 12, 41, 60, and 63. These hits were not recorded in results for individual primer, and they disappeared in the amplicon search. That another reason for the results here relied on the amplicon search since they were more reliable to evaluate the specificity of the primer-sets.

Since NCBI is an accessible website and addition of new sequences in GenBank is possible, it is important to know that GenBank database is under a continuous updating. This was evidently observed by variance in the hits number of searches in two different time periods (October 2015 and January 2016). *B. finlandensis* and *B. yangtzensis* were not detected in the search of 2015, perhaps because they were not yet registered or entered into GenBank database. Thus, it is important to remember that number of hits in this master study was dependent on the date of project performance. Furthermore, not all entered *Borrelia* sequences in GenBank database are trustworthy or dependable. The comparison between the two searches revealed the increase in total hits is much more than the increase in the specific hits. This may explain the low sensitivity of most primer-sets. Finally, BLAST reveals that some accession numbers did not correctly correspond to *Borrelia* strains that were sequenced in the references such as strains in primer-set 37 and 38.

Although all primer-sets were specific according to the source references, BLAST results showed that 52% of them were nonspecific. This discordance in the results may be due to primer-design methods less developed than these now available. New genospecies of *B. burgdorferi* sl have been defined and classified recently and primer-sets for specific certain species for ten years ago may cross-react with the newly defined *Borrelia* species.

Interestingly, most primers designed after 2000 (except *flaB* primers) e.g. 8-11, 7, 15, 19, 22, 24, 25, 27 and others, all were specific in BLAST searches. This underscores the importance of increasing availability in bioinformatics and sequence information, which may lead to better primer design in future.

Although BLAST is useful tool, there are some limitations. It was designed to search similarity between sequences of protein and DNA/RNA, but not to estimate the specificity of primers. BLAST can detect and report mismatches within a sequence, but it cannot arrange the results according to whether primer works or not because it is indistinguishable for BLAST which mismatch is destabilizing and which is not. The alignments in BLAST need to be examined for interpretation according to PCR-primers criteria. Furthermore, because of identity criteria of BLAST search, BLAST may ignore an alignment if the region between primers contains many mismatch nucleotides (high variable) although the primers may bind stably show significance similarity. It would be of enormous help if BLAST has a matrix that weights substitutions according to their distance from the 3’ end and their destabilizing effects.

6 Conclusion

PCR assay is an important analysis for detection of *B. burgdorferi* sl genospecies in infected organisms. In order to guarantee the accuracy of this assay and avoid false results, PCR-primers must be specific for the target taxon. All references of PCR-primers reported specificity that all their primers showed specific amplification of their primers.

According to BLAST results in this master project, specificity of PCR-primers varies substantially from the published result. 40 of the presently available PCR primer-sets failed to do the job that they were designed for. They were not specific enough for specific amplification of their target *B. burgdorferi* sl species because they showed cross-reaction with untargeted taxa.

At the same time, 25 primer-sets were specific for the target species and can be used for PCR-amplification of their target species of *B. burgdorferi* sl. These primer-sets showed no cross-reaction with untargeted species of *B. burgdorferi* sl. Most of the specific primers amplified genes coding for service proteins such outer surface protein A, while primers of flagellin B were mostly non-specific. The remaining genes showed variable specificity.

B. burgdorferi ss, was the most dominant species that detected in the results of BLAST. This indicate to the high proportion of sequence entries for this species compared with the others. B. garinii nd B. afzelii, were the second dominant species in the results. For these species, cross-reaction with uncommon species in few hits may not reduce specificity or sensitivity of the primers.

According to BLAST results, the primers targeting genes of outer surface proteins (*ospA*, *ospB*, and *ospC*) were more specific comparing with the other genes. Some of they showed high specificity and they can be used specifically to amplify a certain species of *Borrelia*.

This study tried underscore the importance of bioinformatics tools to gain more knowledge about gene sequences and their alignments to find out conserve and variable regions. We sought to use standard tools to evaluate specificity of PCR-primers. It seems likely that these tools were suitable for this task since they compared sequence of primers with a massive database of nucleotide sequences, and all published sequences of Lyme *Borrelia* species for particular target genes were covered by this search.

7 References

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8 Annexes

8.1 Detailed tables for results of analysis 77 primer-sets including the individual primers and corresponding amplicons.

Table 8-1 shows BLAST results for primer-set 1 using 308-bp *ospA* amplicon of *B. burgdorferi* sl gene (KC954744) and sequences of the individual primers. In amplicon results, 23% of *B. burgdorferi*, 32% of *B. garinii*, 86% of *B. afzelii*, 13% of *B. valaisiana*, 8% of *B. spielmanii* and 72% of other LB specie show ≤ 2 mismatches to either primer and may be expected to amplify efficiently. In individual primers, very few hits have 100% similarity for the primers.

Sequence	**primer-set 1**	**Number of BLAST hits**						
	Mismatches	*B.bu*	*B.ga*	*B.af*	*B.va*	*B.spl*	*B.*spp	Sum
Amplicon	≤ 2 in either primer	62	61	13	4	1	8	149
	>2 in either primer	197	127	2	25	11	3	523
Forward	0	2	1	9	0	0	2	14
	Total	69	92	24			9	194
Reverse	0	150	2	7	7	1	0	167
	Total	210	207	97	13	9		536

Table 8-1: Calculation of the hits in BLAST results for primer-set 1.

The primers amplified *B. burgdorferi* ss, *B. garinii* and *B. afzelii* [18]. According to BLAST three other species show similarity for the primers; the primers are unspecific for the target species, and low sensitive except for *B. afzelii* (86%).

Table 8-2 shows BLAST Results for primer-set 2 using 541-bp *ospA* amplicon of *B. burgdorferi* ss (KM676017) and sequences of the individual primers. Amplicon results detect only *B. burgdorferi* ss in 44% of hits show ≤ 2 mismatches to either primer and may be expected to amplify efficiently. In the individual primers, *B. burgdorferi* ss is predominantly in 100% similarity to the primers sequences.

Sequence	primer-set 2	Number of BLAST hits					
	Mismatches	*B.bu*	*B.ga*	*B.bis*	*B.amr*	*B.*spp	Sum
Amplicon	≤ 2 in either primer	105	0	0	0	0	105
	>2 in either primer	132					132
Forward	0	132	1	0	0	1	135
	Total	151	1			2	153
Reverse	0	99	0	1	1	0	101
	Total	120		4	1		125

Table 8-2: Calculation of the hits in BLAST results for primer-set 2.

The primers were used to amplify *B. burgdorfer*i ss [18]. BLAST shows this primer-set is specific but moderate sensitive.

Table 8-3 shows BLAST results for primer-set 3 using 345-bp *ospA* amplicon of *B. garinii* (HM62330) and sequences of the individual primers. In amplicon results, 31% of *B. garinii*, 4% of *B. burgdorferi* ss, and 90% of *B. bavariensis* show ≤ 2 mismatches to either primer. In the individual primers, *B. garinii* is prevalent.

Sequence	Primer-set 3	Number of BLAST hits				
	Mismatches	*B.ga*	*B.bu*	*B.bv*	*B.jpn*	Sum
Amplicon	≤ 2 in either primer	69	10	20	0	78
	>2 in either primer	150	225	2		389
Forward	0	102	31	21	4	158
	Total	124	91	22	9	246
Reverse	0	54	10	17	0	81
	Total	67	14	22		103

Table 8-3: Calculation of the hits in BLAST results for primer-set 3.

The primers were designed to amplify *B. garinii* but not *B. burgdorferi* or *B. afzelii* [18]. BLAST shows cross-reaction with two species. The primes are unspecific for *B. garinii*, and low sensitive for this species. They are preferred for *B. bavariensis*.

Table 8-4 shows BLAST results using 190-bp *ospA* amplicon of *B. afzelii* (CP001247), and sequences of the individual primers. In amplicon results, 52% of *B. afzelii* and *B. burgdorferi* ss show ≤ 2 mismatches to either primer, and may expected to be amplify efficiently.

Sequence	**Primer-set 4**	**Number of BLAST hits**		
	Mismatches	*B.af*	*B.bu*	Sum
Amplicon	≤ 2 in either primer	47	9	56
	>2 in either primer	43	8	51
Forward	0	67	9	76
	Total	89	10	99
Reverse	0	47	9	81
	Total	90	17	103

Table 8-4: Calculation of the hits in BLAST results for primer-set 4.

The primers in their reference were for specific amplification of *B. afzelii* [18]. BLAST results show cross-reaction with *B. burgdorferi* ss. The primers are unspecific for *B. afzelii*, and moderate sensitive.

Table 8-5 shows the results of BLAST searches using 156-bp *ospA* amplicon of *B. burgdorferi* ss (JF776165) and sequences of the individual primers. In amplicon results, 70% of *B. burgdorferi* ss and 66% of other LB species show ≤ 2 mismatches to either primer.

Sequence	**Primer-set 5**	**Number of BLAST hits**		
	Mismatches	*B.bu*	*B.*spp	Sum
Amplicon	≤ 2 in either primer	77	2	79
	>2 in either primer	30	1	31
Forward	0	102	2	104
	Total	106	2	108
Reverse	0	77	1	78
	Total	94	2	96

Table 8-5: Calculation of the hits in BLAST results for primer-set 5.

The primers amplified *B. burgdorferi* ss [100]. According to BLAST, they are partially specific for this species because of limited cross-reaction with non-target species. The primers are moderate sensitive.

Table 8-6 shows BLAST results using 195-bp of *ospA* amplicon of *B. burgdorferi* ss (AE000790) and sequences of the individual primers. In amplicon results, 10% of *B. burgdorferi* ss, 3% of *B. garinii*, 27% *B. afzelii*, 11% of *B. valaisiana*, 14% of *B. bissettii*, 8% of *B. spielmanii*, and 40% of undefined LB species show ≤ 2 mismatches to either primer.

Sequence	**Primer-set 6**	**Number of BLAST hits**										
	Mismatches	*B.bu*	*B.ga*	*B.af*	*B.va*	*B.bis*	*B.spl*	*B.bv*	*B.fin*	*B.clf*	*B.*spp	Sum
Amplicon	≤ 2 in either primer	25	5	5	3	1	1	0	0	0	4	44
	>2 in either primer	205	148	13	24	6	11				6	413
Forward	0	24	6	6	3	1	1	0	0	0	4	45
	Total	65	48	14	10	2	1				7	147
Reverse	0	167	72	0	0	7	0	21	2	1	6	276
	Total	244	144			7		22	2	1	14	434

Table 8-6: Calculation of the hits in BLAST results for primer-set 6.

The primers were used in two studies to amplify *B. burgdorferi* ss [100, 151]. BLAST results show further species in similarity for the primers. The primers are unspecific for the target species and very low sensitive.

Table 8-7 shows BLAST result using 103-bp of *ospA* amplicon of *B. burgdorferi* ss (JN413096) and sequences of the individual primers. The amplicon search reveals that 70% of *B. burgdorferi* ss and 100% of *B. andersonii* show ≤ 2 mismatches to either primer and can be amplified efficiently.

Sequence	**Primer-set 7**	**Number of BLAST hits**				
	Mismatches	*B.bu*	*B.ga*	*B.bis*	*B.and*	Sum
Amplicon	≤ 2 in either primer	87	0	0	2	89
	>2 in either primer	37			0	37
Forward	0	93	0	3	2	98
	Total	136		6	2	144
Reverse	0	164	1	0	0	165
	Total	263	1			264

Table 8-7: Calculation of the hits in BLAST results for primer-set 7.

The primers were used to amplify *B. burgdorferi* ss [69]. BLAST results show limited cross-reaction with non-target species *B. andersonii*. The primers are moderate sensitive.

Table 8-8 shows BLAST results of search 767-bp ospA amplicon of *B. burgdorferi* ss (JF776165) and the individual primers. Amplicon results reveal that 12% of *B. burgdorferi* ss show ≤ 2 mismatches to either primer and may be expected to amplify efficiently. Results of the individual primers show further species in 100% similarity for the forward primer.

Sequence	**Primer-set 8**	**Number of BLAST hits**					
	Mismatches	*B.bu*	*B.ga*	*B.af*	*B.va*	*B.chl*	Sum
Amplicon	≤ 2 in either primer	33	0	0	0	0	33
	>2 in either primer	221					221
Forward	0	60	52	14	7	1	111
	Total	72	92	23	13	1	211
Reverse	0	94	0	0	0	0	165
	Total	95					264

Table 8-8: Calculation of the hits in BLAST results for primer-set 8.

The primers were designed to amplify *B. burgdorferi* ss [152]. The results of BLAST reveal that the primer-set is specific for this species, but low sensitive.

Table 8-9 shows BLAST results of search 142-bp *ospA* amplicon of *B. burgdorferi* ss (GU826949) and sequences of the individual primers. In amplicon results, 68% of *B. burgdorferi* ss, 3% of *B. garinii*, 100% of *B. americana* and *B. finlandesis*, and 25% of other LB species show ≤ 2 mismatches to either primer. The individual primer show few hits for most species above have 100% similarity, but *B. burgdorferi* ss is prevalent.

Sequence	**Primer-set 9**	**Number of BLAST hits**							
	Mismatches	*B.bu*	*B.ga*	*B.bis*	*B.crl*	*B.am*	*B.fin*	*B.*spp	Sum
Amplicon	≤ 2 in either primer	135	3	0	0	1	2	2	140
	>2 in either primer	63	111			0	0	6	221
Forward	0	166	1	7	3	1	0	0	178
	Total	196	8	7	3	1			215
Reverse	0	131	2	0	0	1	2	3	138
	Total	178	206			1	2	14	401

Table 8-9: Calculation of the hits in BLAST results for primer-set 9.

The primers amplified *B. burgdorferi* ss [152]. BLAST shows this species is prevalent, but also cross-reaction with other *Borrelia* species. The primers are moderate specific.

Table 8-10 shows BLAST results of 547-bp ospC amplicon of *B. burgdorferi* ss (JQ308224) and the individual primers. *B. burgdorferi* ss with 6% show ≤ 2 mismatches to either primer. In forward primer, only *B. burgdorferi* ss shows 100% similarity for.

Sequence	**Primer-set 10**	**Number of Blast hit**				
	Mismatches	*B.bu*	*B.ga*	*B.af*	*B.jpn*	Sum
Amplicon	≤ 2 in either primer	24	0	0	0	24
	>2 in either primer	383				383
Forward	0	82	0	0	0	82
	Total	221				221
Reverse	0	50	7	2	3	62
	Total	171	43	42	4	260

Table 8-10: Calculation of the hits in BLAST results for primer-set 10.

The primer-set was designed to amplify *B. burgdorferi* ss [152]. The results of BLAST show specificity of primers for this pieces, but low sensitivity.

Table 8-11 shows BLAST results using 128-bp *ospC* amplicon of *B. burgdorferi* ss (DQ437455) and the individual primers. In amplicon results only this species in 33% shows ≤ 2 mismatches to either primer. The same species shows 100% similarity in the individual primers search.

Sequence	**Primer-set 11**	**Number of BLAST hits**		
	Mismatches	*B.bu*	*B.fin*	Sum
Amplicon	≤ 2 in either primer	80	0	80
	>2 in either primer	340		340
Forward	0	83	0	83
	Total	235		235
Reverse	0	106	1	107
	Total	127	1	128

Table 8-11: Calculation of the hits in BLAST results for primer-set 11.

The primer-set was designed specifically to amplify *B. burgdorferi* ss [152]. BLAST reveals that primers are specific for this species, but low sensitive.

Table 8-12 shows BLAST results using 92-bp *flaB* amplicon of *B. burgdorferi* ss (KF422803) and *B. americana* (EU081295), plus sequences the individual primers. In amplicon results, 92% of *B. burgdorferi*, 33% of *B. garinii*, 11% of *B. americana*, 70% of *B. valaisiana*, 100% of *B. bissettii*, *B. andersonii* and *B. carolinensis* show ≤ 2 mismatches to either primer. The individual primers show different species in 100% similarity

Sequence	**Primer-set 12**	**Number of BLAST hits**									
	Mismatches	*B.bu*	*B.ga*	*B.bis*	*B.am*	*B.and*	*B.va*	*B.crl*	*B.jpn*	*B.prs*	Sum
Amplicon	≤ 2 in either primer	199	1	15	1	9	17	7	0	0	249
	>2 in either primer	15	2	0	8	0	7	0			32
Forward	0	155	0	7	2	0	0	7	0	11	182
	Total	271		14	9			7		15	316
Reverse	0	207	1	5	8	8	17	0	4	0	250
	Total	209	1	15	8	8	19		5		265

Table 8-12: Calculation of the hits in BLAST results for primer-set 12.

B. burgdorferi ss was amplified using this primer pair [152]. The primers are not specific but sensitive for this species.

Table 8-13 shows BLAST results using 643-bp *ospA* amplicon of *B. burgdorferi* ss (CP009657) and the individual primers. Only *B. burgdorferi* shows ≤ 2 mismatches to either primer in amplicon search with 33%, and can be amplified efficiently. Results of the individual primers, the reverse primer has 100% similarity only for *B. burgdorferi* ss.

Sequence	**Primer-set 13**	**Number of BLAST hits**		
	Mismatches	*B.bu*	*B.ga*	Sum
Amplicon	≤ 2 in either primer	80	0	80
	>2 in either primer	340		340
Forward	0	139	9	148
	Total	146	9	155
Reverse	0	80	0	80
	Total	96	0	96

Table 8-13: Calculation of the hits in BLAST results for primer-set 13.

The primer-set was used to amplify 646-bp of *ospA* gene for four strains of *B. burgdorferi* ss [153]. BLAST results show that primers are specific for *B. burgdorferi* ss but low sensitive.

Table 8-14 shows BLAST results using 200-bp *flaB* amplicon of *B. burgdorferi* (X15660) and sequences of the individual primers. In amplicon results, 35% of *B. burgdorferi*, 27% of *B. bissettii*, 9% *B. afzelii*, 14% of *B. carolinensis*, and 100% of *B. andersonii* show ≤ 2 mismatches to either primer. Results of individual primers show few hits have 100% similarity for the primer sequences.

Sequence	**Primer-set 14**	**Number of BLAST hits**					
	Mismatches	*B.bu*	*B.bis*	*B.af*	*B.crl*	*B.and*	Sum
Amplicon	≤ 2 in either primer	27	3	1	1	3	35
	>2 in either primer	49	8	10	6	0	73
Forward	0	20	0	1	0	3	24
	Total	35	0	21	0	3	59
Reverse	0	46	11	0	7	0	64
	Total	107	11	0	7	0	125

Table 8-14: Calculation of the hits in BLAST results for primer-set 14.

The primers used for *B. burgdorferi* sl [61]. BLAST is concordance with that, but low sensitive.

Table 8-15 shows BLAST results for 890-bp ospA of *B. burgdorferi* ss (X14407) and the individual primer sequences. Only this species in amplicon search in 6% show ≤ 2 mismatches to either primer. The individual primers show further 100% similarity species.

Sequence	**Primer-set 15**	**Number of BLAST hits**							
	Mismatches	*B.bu*	*B.ga*	*B.af*	*B.va*	*B.jpn*	*B.bis*	*B.trd*	Sum
Amplicon	≤ 2 in either primer	17						1	18
	>2 in either primer	261						0	261
Forward	0	60	47	14	9	4	4	1	139
	Total	67	47	14	9	6	4	1	148
Reverse	0	44						1	45
	Total	45						1	46

Table 8-15: Calculation of the hits in BLAST results for primer-set 15.

The primers amplified 794-bp of *B. burgdorferi* ss [154]. BLAST results showed specificity for this species but limited cross-reaction with *B. turdi* by one hit. The sensitivity is low.

Table 8-16 shows BLAST results of 400-bp *ospA* of *B. burgdorferi* ss (X85442) and the individual primers. Only this species is detected in the amplicon test with 44% hits that show ≤ 2 mismatches to either primer.

Sequence	**Primer-set 16**	**Number of BLAST hits**				
	Mismatches	*B.bu*	*B.ga*	*B.bis*	*B.*spp	Sum
Amplicon	≤ 2 in either primer	107				107
	>2 in either primer	131				131
Forward	0	121	9	0	0	130
	Total	125	9	0	0	134
Reverse	0	109	0	4	3	116
	Total	111	0	6	3	120

Table 8-16: Calculation of the hits in BLAST results for primer-set 16.

The primers used for amplification of *B. burgdorferi* ss [155, 156]. BLAST shows the primers are specific for this species and moderate sensitive.

Table 8-17 shows BLAST results for 187-bp *ospA* of *B. burgdorferi* sl including *B. burgdorferi* ss (Z29086) and *B. garinii* (GU906888), plus the individual primers. For amplicon, 40% of *B. burgdorferi* ss, 22% of *B. garinii*, 13% of *B. afzelii*, 53% of *B. valaisiana* and *B. bissettii*, and 55% of undefined species show ≤ 2 mismatches to either primer. The individual primers show further species in 100% similarity.

Sequence	**Primer-set 17**	**Number of BLAST hits**								
	Mismatches	*B.bu*	*B.ga*	*B.af*	*B.va*	*B.bis*	*B.spl*	*B.bv*	*B.*spp	Sum
Amplicon	≤ 2 in either primer	59	43	13	10	2	0	0	5	132
	>2 in either primer	86	144	87	8	2			4	331
Forward	0	59	47	14	10	2	1	0	0	133
	Total	65	48	14	10	4	1	0	0	142
Reverse	0	167	67	0	0	7	0	21	6	268
	Total	229	143	0	0	7	0	22	11	412

Table 8-17: Calculation of the hits in BLAST results for primer-set 17.

The primers were derived from B. burgdorferi B31 to amplify the three main species *of B. burgdorferi* sl [31]. BLAST shows the primers are not specific and low sensitive.

Table 8-18 shows BLAST results of 180-bp *flaB* for *B. burgdorferi* sl including *B. burgdorferi* ss (KF422803) and *B. valaisiana* (KF990324), and sequences of the individual primers. In amplicon, 85% of *B. garinii*, *B. spielmanii*, and *B. bissettii*, 70% of *B. burgdorferi* and *B. valaisiana*, 47% of *B. lusitania*, and *B. spielmanii*, 100% for *B. carolinensis*, and 70% of undefined species, and 90% of four RFB species, all show ≤ 2 mismatches to either primer.

Sequence	**Primer-set 18**	**Number of BLAST hits**															
	Mismatches	*B.bu*	*B.ga*	*B.lst*	*B.va*	*B.and*	*B.spl*	*B.bis*	*B.crl*	*B.spp*	*B.dtn*	*B.prs*	*B.crd*	*B.mty*	*B.trc*	*B.hsp*	*B.rcnt*	Sum
Amplicon	≤ 2 in either primer	192	220	33	29	8	8	13	7	44	13	0	12	0	0	1	3	584
	>2 in either primer	86	37	36	11	1	9	2	0	19	1	0	0	0	0	3	0	205
Forward	0	231	3	54	43	19	10	8	0	0	17	12	12	11	6	5	3	435
	Total	261	226	57	44	21	32	14	0	0	20	23	12	20	6	5	3	754
Reverse	0	153	6	0	34	5	10	0	0	0	0	0	0	0	0	0	0	208
	Total	214	155	0	34	7	10	0	0	0	0	0	0	0	0	0	0	420

Table 8-18: Calculation of the hits in BLAST results for primer-set 18.

The primer pair was derived from *B. burgdorferi* ss B31 to amplify 156-bp of *flaB* for *B. burgdorferi* sl [31]. BLAST results show cross-reaction with species of RFB; the primers are not specific for *B. burgdorferi* sl, but sensitive for *B. burgdorferi* ss and *B. garinii*.

Table 8-19 shows BLAST results of 269-bp *ospA* of *B. valaisiana* (AF095940) and the individual primers. In amplicon, 87% of *B. valaisiana*, and 1% of *B. burgdorferi* ss show ≤ 2 mismatches to either primer.

Sequence	**Primer-set 19**	**Number of BLAST hits**			
	Mismatches	*B.va*	*B.bu*	*B.*spp	Sum
Amplicon	≤ 2 in either primer	14	1	0	15
	>2 in either primer	2	53		55
Forward	0	16	1	1	18
	Total	16	47	1	64
Reverse	0	12	1	0	13
	Total	13	1	0	14

Table 8-19: Calculation of the hits in BLAST results for primer-set 19.

The primer pair was designed to amplify three strains of *B. valaisiana* [157]. In BLAST results, the primers are specific and sensitivity for this species, but one hit cross-reaction with *B. burgdorferi* ss.

Table 8-20 shows BLAST results of 126-bp *ospA* of *B. burgdorferi* ss (GQ443122) and sequences of the individual primers. The amplicon results reveal that 58% *B. burgdorferi* ss shows ≤ 2 mismatches to either primer and may be expected to amplify efficiently.

Sequence	**Primer-set 20**	**Number of BLAST hits**
	Mismatches	*B.bu*
Amplicon	≤ 2 in either primer	77
	>2 in either primer	54
Forward	0	86
	Total	90
Reverse	0	164
	Total	263

Table 8-20: Calculation of the hits in BLAST results for primer-set 20.

The primer amplified 123-bp of *B. burgdorferi* ss [158]. BLAST shows only this species in similarity for the primers in all tests; the primer-set is high specific and moderate sensitive.

Table 8-21 shows BLAST results of 151-bp *ospA* of *B. burgdorferi* ss (KM676013) and individual primers. In amplicon, 50% of *B. burgdorferi* ss, 75% of *B. bissettii*, 100% of *B. andersonii* and 22% of undefined LB species show ≤ 2 mismatches to either primer.

Sequence	**Primer-set 21**	**Number of BLAST hits**								
	Mismatches	*B.bu*	*B.ga*	*B.bis*	*B.va*	*B.trd*	*B.and*	*B.am*	*B.*spp	Sum
Amplicon	≤ 2 in either primer	110	0	6	0	0	1	0	2	119
	>2 in either primer	108		2			0		7	117
Forward	0	105	23	6	4	1	0	0	4	143
	Total	155	113	7	4	1	0	0	8	288
Reverse	0	128	1	6	8	32	1	1	8	186
	Total	151	10	15	8	63	2	1	8	259

Table 8-21: Calculation of the hits in BLAST results for primer-set 21.

The primers amplified *B. burgdorferi* ss [159]. BLAST shows limited cross-reaction with non-target species; the primers are not quite specific and moderate sensitive.

Table 8-22 shows BLAST results of 84-bp *ospC* of *B. burgdorferi* ss (JQ951145) and the individual primers. The results show only *B. burgdorferi* ss in all searches. 45% of hits in amplicon test show ≤ 2 mismatches to either primer.

Sequence	**Primer-set 22**	**Number of BLAST hits**
	Mismatches	*B.bu*
Amplicon	≤ 2 in either primer	56
	>2 in either primer	66
Forward	0	115
	Total	151
Reverse	0	59
	Total	129

Table 8-22: Calculation of the hits in BLAST results for primer-set 22.

The primers used to detect *B. burgdorferi* ss [159].According to BLAST, the primers are high specific for this species, but moderate sensitive.

Table 8-23 shows BLAST results using 137-bp *ospA* of *B. burgdorferi sl*: *B. burgdorferi* ss (X68542), and *B. afzelii* (CP000396), and the individual primers. For amplicon, 25% of *B. burgdorferi*, 36% of *B. garinii*, *B. valaisiana*, 14% of *B. afzelii*, 42% of *B. bissettii*, and 100% of *B. chilensis*, *B. turdi*, and *B. bavariensis*, all show ≤ 2 mismatches to either primer and may be expected to amplify efficiently. Results of primers show the same species 100% similarity.

Sequence	**Primer-set 23**	**Number of BLAST hits**									
	Mismatches	*B.bu*	*B.ga*	*B.af*	*B.va*	*B.bis*	*B.chl*	*B.trd*	*B.spl*	*B.bv*	Sum
Amplicon	≤ 2 in either primer	61	62	15	10	7	1	1	1	0	158
	>2 in either primer	182	106	89	19	4	0	0	0		410
Forward	0	69	47	14	13	2	1	1	1	0	148
	Total	83	64	14	13	4	1	1	1		181
Reverse	0	68	144	101	13	7	1	1	0	22	357
	Total	236	168	102	29	7	1	1		22	566

Table 8-23: Calculation of the hits in BLAST results for primer-set 23.

The primer set was mainly used to amplify several species of *B. burgdorferi* sl [78]. BLAST shows limited cross-reaction with untargeted species of *B. burgdorferi* sl.

Table 8-24 shows BLAST results using 234-bp *ospA* of *B. burgdorferi* ss (Z29086) and *B. garinii* (DQ479286), plus individual primers. In amplicon, 35% of *B. burgdorferi* ss, 42% of *B. garinii*, 91% of *B. bavariensis* show ≤ 2 mismatches to either primer and may be expected to amplify efficiently. For individual primers, two more species have 100% similarity.

Sequence	**Primer-set 24**	**Number of BLAST hits**					
	Mismatches	*B.bu*	*B.ga*	*B.bv*	*B.bis*	*B.af*	Sum
Amplicon	≤ 2 in either primer	110	103	22	0	0	235
	>2 in either primer	108	141	2			251
Forward	0	24	72	21	0	0	118
	Total	132	108	21			262
Reverse	0	31	176	22	1	84	314
	Total	194	206	22	7	88	517

Table 8-24: Calculation of the hits in BLAST results for primer-set 24.

The references of primers showed *B. afzelii* is prevalence, *B. garinii* and *B. bavariensis* are moderate, but no *B. burgdorferi ss* was detected [94, 160]. In BLAST, *B. afzelii* was not detected, and the primers was mostly sensitive for *B. bavariensis*.

Table 8-25 shows BLAST results of 151-bp *ospA B. spielmanii* (EU545183) and sequences of individual primers. This species is prevalent in all tests. In amplicon, 99% of *B. spielmanii* and 14% of undefined LB species show ≤ 2 mismatches to either primer.

Sequence	**Primer-set 25**	**Number of BLAST hits**		
	Mismatches	*B.spl*	*B.*spp	Sum
Amplicon	≤ 2 in either primer	10	1	11
	>2 in either primer	1	6	7
Forward	0	9	1	10
	Total	11	1	12
Reverse	0	12	1	13
	Total	12	2	14

Table 8-25: Calculation of the hits in BLAST results for primer-set 25.

The primers amplified specifically *B. spielmanii* [161]. In BLAST, the primers are specific and sensitive for this species, but one hit cross-reaction with non-target species.

Table 8-26 shows BLAST results for 841-bp and 838-bp *ospA* amplicon of *B. valaisiana* (AB016979) and *B. burgdorferi* ss (X14407), respectively, and sequences of the individual primers. For amplicon, 9% of *B. burgdorferi* ss, 10% of *B. valaisiana*, and 14% of *B. bissettii* and *B. turdi* show ≤ 2 mismatches to either primer.

Sequence	**Primer-set 26**	**Number of BLAST hits**						
	Mismatches	*B.bu*	*B.ga*	*B.af*	*B.va*	*B.bis*	*B.trd*	Sum
Amplicon	≤ 2 in either primer	23	0	0	3	1	1	28
	>2 in either primer	237			29	7	0	273
Forward	0	24	6	5	3	0	1	39
	Total	24	6	6	3	0	1	40
Reverse	0	25	12	0	3	3	1	44
	Total	26	22	0	3	3	1	55

Table 8-26: Calculation of the hits in BLAST results for primer-set 26.

The primers were designed to amplify *B. burgdorferi* ss [162, 163], and *B. valaisiana* [165]. BLAST shows cross-reaction with other two species. The primers are not specific and low sensitive.

Table 8-27 shows BLAST results for 99-bp *ospA* of *B. burgdorferi* ss (GQ443108) and individual primers. For amplicon, 66% of *B. burgdorferi* ss and 50% of *B. finlandensis* show ≤ 2 mismatches to either primer and may be expected to amplify efficiently.

Sequence	**Primer-set 27**	**Number of BLAST hits**			
	Mismatches	*B.bu*	*B.crl*	*B.fin*	Sum
Amplicon	≤ 2 in either primer	101	0	1	102
	>2 in either primer	50		1	51
Forward	0	104	0	0	104
	Total	106	0	0	106
Reverse	0	98	4	1	103
	Total	116	4	2	122

Table 8-27: Calculation of the hits in BLAST results for primer-set 27.

The primers were designed to amplify *B. burgdorferi* ss [164]. BLAST results show one hit cross-reaction with non-target species; the primers are partially specific and moderate sensitive for *B. burgdorferi* ss.

Table 8-28 shows BLAST results for 138-bp *ospA* of *B. Burgdorferi* ss (EU564839) and the individual primers. The results show this species is dominant in all tests. In amplicon, 38% of *B. burgdorferi* ss and 5% of undefined *Borrelia* species show ≤ 2 mismatches to either. The results of primers show mostly for *B. burgdorferi* ss in 100% similarity.

Sequence	**Primer-set 28**	**Number of BLAST hits**			
	Mismatches	*B.bu*	*B.ga*	*B.*spp	Sum
Amplicon	≤ 2 in either primer	123	0	1	124
	>2 in either primer	179		17	196
Forward	0	139	1	1	141
	Total	142	9	1	152
Reverse	0	148	0	0	148
	Total	192	0	0	192

Table 8-28: Calculation of the hits in BLAST results for primer-set 28.

The primers amplified *B. burgdorferi* ss [165]. BLAST results show one hit cross-reaction with non-target species; the primers are partially specific and moderate sensitive.

Table 8-29 shows BLAST results for 138-bp *ospA* gene of *B. bissettii* (DQ393323) and *B. burgdorferi* ss (U65801), and the individual primers. In amplicon, 62% of *B. bissettii* and 4% of *B. burgdorferi* ss show ≤ 2 mismatches to either primer and can be amplified efficiently.

Sequence	**Primer-set 29**	**Number of BLAST hits**		
	Mismatches	*B.bu*	*B.bis*	Sum
Amplicon	≤ 2 in either primer	8	5	13
	>2 in either primer	181	3	184
Forward	0	8	5	13
	Total	11	7	18
Reverse	0	2	5	7
	Total	15	6	21

Table 8-29: Calculation of the hits in BLAST results for primer-set 29.

The primer pair was designed to amplify specifically *B. bissettii* [165]. The primer pair is not specific for this species but moderate sensitive.

Table 8-30 shows BLAST results for 249-bp *ospA* of *B. burgdorferi* ss (AY597034) and sequences of the primers. Only this species shows ≤ 2 mismatches to either primer in amplicon search with 48% of total hits. The results of primers show only *B. burgdorferi* ss in 100% similarity for forward primer.

Sequence	**Primer-set 30**	**Number of BLAST hits**		
	Mismatches	*B.bu*	*B.ga*	Sum
Amplicon	≤ 2 in either primer	117	0	117
	>2 in either primer	122		122
Forward	0	131	0	131
	Total	145	0	145
Reverse	0	43	7	50
	Total	118	56	174

Table 8-30: Calculation of the hits in BLAST results for primer-set 30.

The primer pair was used for specific amplification of *B. burgdorferi* ss in two studies [166, 167]. BLAST results show the primers are specific for this species, and moderate sensitive.

Table 8-31 shows BLAST results for two ospA of *B. burgdorferi* ss (Z29087) and (JN413096) in lengths 289-bp and 293-bp, respectively, plus the individual primers. For amplicons, 20% of *B. burgdorferi* ss, 45% of *B. garinii*, 60% of *B. afzelii*, and 100% of *B. japonica* show ≤ 2 mismatches to either primer and can be amplified efficiently.

Sequence	**Primer-set 31**	**Number of BLAST hits**						
	Mismatches	*B.bu*	*B.ga*	*B.af*	*B.va*	*B.jpn*	*B.*spp	Sum
Amplicon	≤ 2 in either primer	64	96	66	0	3	0	229
	>2 in either primer	234	114	44		0		392
Forward	0	86	79	1	7	4	5	182
	Total	122	93	26	11	9	10	271
Reverse	0	150	2	7	7	0	4	170
	Total	210	207	97	13		8	535

Table 8-31: Calculation of the hits in BLAST results for primer-set 31.

The primers amplified 296-bp of three main species of *B. burgdorferi* sl [18, 168]. BLAST shows limited cross-reaction with non-target species.

Table 8-32 shows BLAST results for 869-bp *ospA* of *B. burgdorferi* ss (CP001651) and sequence of the individual primers. In amplicon results, 27% of *B. burgdorferi* ss, 1% of *B. bissettii* show ≤ 2 mismatches to either primer. The individual primers show different LB species in 100% similarity for primers sequences.

Sequence	**Primer-set 32**	**Number of BLAST hits**											
	Mismatches	*B.bu*	*B.ga*	*B.bis*	*B.and*	*B.fin*	*B.clf*	*B.spl*	*B.af*	*B.bv*	*B.crl*	*B.*spp	Sum
Amplicon	≤ 2 in either primer	64	0	1	0	0	0	0	0	0	0	0	65
	>2 in either primer	234		110									344
Forward	0	52	1	3	3	2	1	0	0	0	0	1	63
	Total	224	114	7	3	2	1					14	365
Reverse	0	193	205	7	16	2	0	10	95	24	4	93	171
	Total	193	206	7	16	2		10	95	24	4	93	544

Table 8-32: Calculation of the hits in BLAST results for primer-set 32.

The primer set amplified *B. burgdorferi ss* N40, *B. andersonii* CO-501, and *B. bissetti* 21308 [169]. In BLAST, the primers show similarity for *B. burgdorferi* ss and B. *bissettii* but not B. andersonii. The primers are not specific for all these species and very low sensitive.

Table 8-33 shows BLAST results of 780-bp *ospB* of *B. burgdorferi* ss (AY498727) and the individual primers. In amplicon, 100% of *B. burgdorferi* ss, and one LB species show ≤ 2 mismatches to either primer. The primers test show same species have 100% similarity.

Sequence	**Primer-set 33**	**Number of BLAST hits**		
	Mismatches	*B.bu*	*B.*spp	Sum
Amplicon	≤ 2 in either primer	41	1	42
	>2 in either primer	0	0	0
Forward	0	41	1	42
	Total	41	1	42
Reverse	0	41	1	42
	Total	41	1	42

Table 8-33: Calculation of the hits in BLAST results for primer-set 33.

The primers amplified *B. burgdorferi* ss in two studies [170, 171]. In Blast, the primers are specific and sensitive for this species, but one hit cross-reaction with undefined species.

Table 8-34 shows BLAST results of 328-bp *ospB* of *B. burgdorferi* ss (AY498726) and sequences of the individual primers. Only *B. burgdorferi* ss shows ≤ 2 mismatches to either primer in 16% in amplicon search result and can amplified with optimum efficiency. Two species in the primers search show 100% similarity to primer sequences.

Sequence	**Primer-set 34**	**Number of BLAST hits**			
	Mismatches	*B.bu*	*B.bis*	*B.*spp	Sum
Amplicon	≤ 2 in either primer	41	0	0	41
	>2 in either primer	247			247
Forward	0	44	2	1	47
	Total	46	3	1	50
Reverse	0	42	2	1	47
	Total	46	3	1	50

Table 8-34: Calculation of the hits in BLAST results for primer-set 34.

The primer pair was used to amplify specifically *B. burgdorferi* N40 [151]. The results of BLAST show specificity for this species but low sensitivity.

Table 8-35 shows BLAST results for 606-bp (AF467875), 615-bp (JN969070), and 609-bp (AE000792) of *ospC* of *B. burgdorferi* ss, plus the individual primers. In amplicon results, 22% of *B. burgdorferi* ss and *B. afzelii*, 76% of *B. valaisiana*, 48% of *B. garinii*, 100% *of B. yangtzensis* and *B. bavariensis*, and 40% of undefined LB species show ≤ 2 mismatches to either primer and can be amplified. The results of individual primers search show few sequences have 100% similarity to the primers.

Sequence	Primer-set 35	Number of BLAST hits										
	Mismatches	*B.bu*	*B.va*	*B.ga*	*B.yng*	*B.spl*	*B.af*	*B.bv*	*B.fin*	*B.hrm*	*B.*spp	Sum
Amplicon	≤ 2 in either primer	86	19	33	3	0	13	5	0	0	4	134
	>2 in either primer	301	6	35	0		49	0			6	446
Forward (A)	0	43	9	4	3	1	0	0	0	0	3	63
	Total	104	10	43	3	2					9	171
Forward (B)	0	59	1	43	0	1	51	5	0	13	6	179
	Total	111	11	49		2	51	5		33	10	272
Reverse	0	74	2	34	0	0	10	3	2	0	3	128
	Total	135	5	44			27	4	2		5	222

Table 8-35: Calculation of the hits in BLAST results for primer-set 35.

The primer-set 35 was designed to amplify 606 to 618-bp length amplicons of 13 European *B. burgdorferi* sl isolate [172]. According to BLAST, several LB species show similarity for the primers, all in low sensitivity, the primer pair is not specific for certain species.

Table 8-36 shows the results of BLAST searches using 128-bp fragment of *ospC* gene of *B. burgdorferi* (EU482045) and sequences of the individual primers. The results of amplicon reveal that 14% of *B. burgdorferi* show ≤ 2 mismatches to either primer and may be expected to amplify efficiently. The results individual primers show two other species in 100% similarity for the forward primer.

Sequence	Primer-set 36	Number of BLAST hits			
	Mismatches	*B.bu*	*B.am*	*B.fin*	Sum
Amplicon	≤ 2 in either primer	64	0	0	64
	>2 in either primer	376			376
Forward	0	301	3	1	305
	Total	304	6	2	50
Reverse	0	17	0	0	17
	Total	122			122

Table 8-36: Calculation of the hits in BLAST results for primer-set 36.

The primers amplified 730-bp of *B. burgdorferi* ss [173]. Different length of amplicon was downloaded by Entrez. According to BLAST, the primers were specific for *B. burgdorferi* ss, but low sensitive.

Table 8-37 shows BLAST results of search 684-bp *ospC* of *B. valaisiana* (CP001432) and the individual primers. The results of amplicon show that 13% of *B. burgdorferi* ss, 6% of *B. afzelii*, and 16% of *B. valaisiana* in ≤ 2 mismatches to either primer and may be expected to amplify efficiently. The results of individual primers show few hits have 100% similarity for the primers sequences.

Sequence	**Primer-set 37**	**Number of BLAST hits**					
	Mismatches	*B.bu*	*B.af*	*B.ga*	*B.va*	*B.bis*	Sum
Amplicon	≤ 2 in either primer	27	5	0	3	0	35
	>2 in either primer	178	67		15		260
Forward	0	29	9	8	2	1	49
	Total	39	10	11	2	1	63
Reverse	0	16	5	1	1	0	23
	Total	19	5	4	3		31

Table 8-37: Calculation of the hits in BLAST results for primer-set 37.

The primers amplified 630-636-bp amplicon of eight strains of *B. valaisiana* [174]. The results by BLAST show several other species in similarity for the primers; the primer-set is not specific for *B. valaisiana* and low sensitive for all the detected species.

Table 8-38 shows BLAST results of two *ospC* amplicons for *B. afzelii* (CP002934) and B. *garinii* (JN828669) in 641-bp and 626-bp, respectively. In amplicon results, 4% of *B. burgdorferi* ss, 6% of *B. garinii*, 5% of *B. afzelii*, 9% *B. valaisiana*, and 50% of other LB species show ≤ 2 mismatches to either primer and may be expected to amplify efficiently.

Sequence	**Primer-set 38**	**Number of BLAST hits**					
	Mismatches	*B.bu*	*B.ga*	*B.af*	*B.va*	*B.spl*	Sum
Amplicon	≤ 2 in either primer	22	9	7	2	1	64
	>2 in either primer	456	125	113	20	1	715
Forward	0	31	15	12	2	1	61
	Total	85	49	31	10	2	177
Reverse	0	54	34	26	4	0	118
	Total	125	41	29	7		202

Table 8-38: Calculation of the hits in BLAST results for primer-set 38.

The primer pair was derived from *B. afzelii* PKo to amplify *B. burgdorferi ss*, *B. garinii*, *B. afzelii*, and *B. japonica* [175]. According to BLAST, all these species except *B. japonica* show similarity for the primers sequences, but cross-reaction with other LB species; the primers not specific for the target species and low sensitive.

Table 8-39 shows BLAST results of 584-bp *flaB* of *B. garinii* (X75203) and *B. burgdorferi* ss (AY342019). In amplicon results, 30% of *B. garinii* and *B. valaisiana*, 27% of *B. burgdorferi* ss, 52% of *B. afzelii*, and 13% of *B. bissettii* show ≤ 2 mismatches to either primer.

Sequence	**Primer-set 39**	**Number of BLAST hits**						
	Mismatches	*B.ga*	*B.bu*	*B.af*	*B.va*	*B.bis*	*B.lst*	Sum
Amplicon	≤ 2 in either primer	97	34	65	11	2	0	209
	>2 in either primer	217	179	59	25	13		493
Forward	0	167	80	65	30	11	7	360
	Total	218	80	69	30	11	7	415
Reverse	0	78	13	4	2	0	0	97
	Total	85	24	28	16			153

Table 8-39: Calculation of the hits in BLAST results for primer-set 39.

The primers were derived from *B. garinii* HT22 to amplify this species plus *B. burgdorferi* ss and *B. afzelii* [118]. In BLAST, *B. garinii* HT22 was not detected in any search. However, further species of LB show similarity for the primers. The primers are not specific for target species and low sensitive.

Table 8-40 shows BLAST results of search 299-bp *flaB* of *B. burgdorferi* ss (X16833), and sequences of the individual primers. For amplicon, 16% of *B. burgdorferi* ss, 45% of *B. afzelii* and *B. andersonii*, 25% of *B. valaisiana*, *B. bissettii, and B. garinii*, and 11% of undefined LB species, and finally two species of RFB, all show ≤ 2 mismatches to either primer.

Sequence	primer-set 40	Number of BLAST hits													
	Mismatche	*B.bu*	*B.af*	*B.va*	*B.bis*	*B.spl*	*B.ga*	*B.and*	*B.spp*	*B.hrm*	*B.crd*	*B.dtn*	*B.prk*	*B.ans*	Sum
Amplicon	≤ 2 in either primer	31	58	10	4	1	72	3	9	16	0	0	1	0	205
	>2 in either primer	155	69	33	10	7	244	4	72	1			5		600
Forward	0	23	0	0	1	0	1	3	0	0	0	0	0	0	28
	Total	74			3		73	3							153
Reverse	0	172	81	37	14	8	4	0	71	17	8	6	6	4	428
	Total	174	81	37	14	8	4		85	17	9	14	6	4	453

Table 8-40: Calculations of the hits in BLAST results for primer-set 40.

The primer pair was designed to amplify *B. burgdorferi* N40 [71]. BLAST results show further several species of *B. burgdorferi* sl and RFB in similarity for the primers; the primers neither specific for *B. burgdorferi* ss nor for LB species.

Table 8-41 shows BLAST results for analysis 134-bp *flaB* of *B. burgdorferi* ss (JQ711236) and *B. garinii* (GU826819), plus the individual primers. In amplicon, 62% of *B. garinii*, 86% of *B. burgdorferi* ss and *B. lusitaniae*, 96% of *B. afzelii*, and 100% for *B. americana*, *B. spielmanii*, and *B. tanuki* show ≤ 2 mismatches to either primer. In the individual primers most of these species have 100% similarity for the primers sequences.

Sequence	Primer-set 41	Number of BLAST hits											
	Mismatches	*B.ga*	*B.bu*	*B.va*	*B.lst*	*B.bis*	*B.amr*	*B.spl*	*B.crl*	*B.af*	*B.tnk*	*B.trd*	B.spp	Sum
Amplicon	≤ 2 in either primer	200	196	0	40	0	9	8	0	123	4	0	29	609
	>2 in either primer	119	27		6		0	0		4	0		42	198
Forward	0	287	181	35	25	14	9	8	7	6	4	3	55	634
	Total	298	210	38	25	14	9	8	7	88	4	3	56	760
Reverse	0	3	136	0	4	0	9	0	0	6	0	0	29	187
	Total	35	234		49		9			114			64	505

Table 8-41: Calculation of the hits in BLAST results for primer-set 41.

The primers were used to amplify *B. burgdorferi* ss B31 and N40 [71, 76]. In BLAST, the primer-set is preferred for the three main species of *B. burgdorferi* sl (*B. burgdorferi* ss, *B. garinii*, *B. afzelii*). The primers are not specific for *B. burgdorferi* ss, but they are sensitive.

Table 8-42 shows BLAST results for 231-bp *ospC* amplicon of *B. burgdorferi* ss (U91798) and sequence of primers. The results of amplicon reveal 22% of *B. burgdorferi* show ≤ 2 mismatches to either primer and may be expected to amplify efficiently. In the individual primers, *B. burgdorferi* ss is dominant.

Sequence	**Primer-set 42**	**Number of BLAST hits**			
	Mismatches	*B.bu*	*B.am*	*B.fin*	Sum
Amplicon	≤ 2 in either primer	47	0	0	47
	>2 in either primer	158			158
Forward	0	58	0	0	58
	Total	63			63
Reverse	0	135	2	1	138
	Total	198	2	1	201

Table 8-42: Calculation of the hits in BLAST results for primer-set 42.

The primer pair was used to amplify 397-bp fragment of *B. burgdor2feri* B31 [76]. A different length of amplicon was downloaded by Entrez. However, the primers are specific for the target species but low sensitive.

Table 8-43 shows BLAST results of 320-bp of *B. burgdorferi* ss (FJ932735) and 326-bp of *B. garinii* (AY150196) of *ospC* gene, plus sequences of the individual primers. The results for amplicon reveal that 26% of *B. burgdorferi* ss, 19% of *B. garinii*, 8% of *B. afzelii*, and 50% of *B. bissettii and B. americana* show ≤ 2 mismatches to either primer.

Sequence	**Primer-set 43**	**Number of BLAST hits**							
	Mismatches	*B.bu*	*B.ga*	*B.af*	*B.va*	*B.jpn*	*B.bis*	*B.amr*	Sum
Amplicon	≤ 2 in either primer	57	16	4	0	4	2	2	85
	>2 in either primer	156	65	43	15	1	1	1	282
Forward	0	4	4	4	5	5	1	0	23
	Total	300	114	93	15	7	8		537
Reverse	0	84	9	4	0	0	12	4	113
	Total	220	52	47			14	4	337

Table 8-43: Calculation of the hits in BLAST results for primer-set 43.

The primer pair was used to amplify 314-bp of *ospC* of *B. burgdorferi*, *B. garinii* and *B. afzelii* [176]. In BLAST, other three LB species are in similarity for the primers; the primers are not specific for the target species and low sensitive.

Table 8-44 shows BLAST results of search 266-bp *ospC* of *B. burgdorferi* ss (KM676046), *B. garinii* (JQ951097), and *B. afzelii* (KP644288), plus sequences of the individual primers. In amplicons, 36% of *B. burgdorferi* ss, 12% of *B. garinii*, 28% of *B. afzelii*, 8% of *B. bissettii* and 100% of *B. americana*, *B. bavariensis*, and *B. japonica*, *all* show ≤ 2 mismatches to either primer. In individual primers, few hits have 100% similarity for the primer sequences.

Sequence	**Primer–set 44**	**Number of BLAST hits**										
	Mismatches	*B.bu*	*B.ga*	*B.af*	*B.bis*	*B.va*	*B.amr*	*B.tnk*	*B.bv*	*B.ipn*	*B.mty*	*Sum*
Amplicon	≤ 2 in either primer	29	9	14	2	0	6	3	6	6	0	75
	>2 in either primer	46	62	36	21		0	4	0	0		169
Forward (A)	0	2	16	11	0	0	0	0	0	0	0	29
	Total	16	26	4								46
Forward (B)	0	321	41	61	19	10	7	7	6	6	2	480
	Total	330	72	86	19	12	7	7	6	6	2	547
Reverse	0	51	52	40	2	8	6	3	6	5	0	173
	Total	186	18	16	3	5	1	1	0	0		230

Table 8-44: Calculation of the hits in BLAST results for primer-set 44.

The forward primer (A) and (B) were used by in two different studies, but the reverse primer was common in both. Three main species of *B. burgdorferi* sl was amplified [177, 178]. BLAST shows that primers are not specific for these species and low sensitive.

Table 8-45 shows BLAST results of 600-bp *ospC* for *B. burgdorferi ss* (JQ253803) and *B. garinii* (AJ841695), plus the individual primers. In amplicon results, 9% of *B. burgdorferi* ss, 17% of *B. garinii and B. afzelii*, 33% of *B. bissettii*, and 71% for *B. americana* show ≤ 2 mismatches to either primer. The results individual primers show most of these species have 100% similarity for the primers, but in low hits number.

Sequence	Primer-set 45	Number of BLAST hits									
	Mismatches	*B.bu*	*B.ga*	*B.af*	*B.bis*	*B.amr*	*B.trd*	*B.spl*	*B.hrm*	*B.*spp	Sum
Amplicon	≤ 2 in either primer	42	22	13	6	5	0	0	0	1	89
	>2 in either primer	402	105	60	12	2				9	590
Forward	0	41	42	29	13	5	2	1	2	5	140
	Total	90	50	34	16	5	2	2	28	9	236
Forward	0	98	9	14	12	4	0	1	0	1	139
	Total	220	51	49	14	4		2		7	347

Table 8-45: Calculation of the hits in BLAST results for primer-set 45.

The primer pair was used in in two studies [176, 179] to amplify *B. burgdorferi* ss. BLAST results show other LB species in similarity for the primers; the primer set was not specific for *B. burgdorferi* ss, and low sensitive.

Table 8-46 shows the results of BLAST tests using 89-bp amplicon of *flaB* for *B. garinii* (KF894057) and *B. burgdorferi* (AM159177), plus sequence of the individual primers. In amplicon, 82% of *B. garinii*, *B. burgdorferi* ss *and B. valaiasiana*, 94% of *B. afzelii*, *B. lusitaniae* and *B. bissettii*, 100% of *B. spielmanii and B. carolinensis*, all show ≤ 2 mismatches to either primer. In primer results, same species have 100% similarity for the primer sequences.

Sequence	Primer-set 46	Number of BLAST hits									
	Mismatches	*B.ga*	*B.bu*	*B.af*	*B.va*	*B.lst*	*B.bis*	*B.spl*	*B.crl*	*B.and*	*B.*spp	Sum
Amplicon	≤ 2 in either primer	265	167	111	28	25	13	10	7	3	44	673
	>2 in either primer	51	39	3	6	11	1	0	0	3	15	129
Forward	0	307	190	123	33	39	14	10	7	6	59	788
	Total	310	200	124	33	39	14	10	7	6	59	802
Forward	0	250	142	110	15	10	13	8	8	6	21	583
	Total	255	152	113	18	11	13	8	8	6	36	620

Table 8-46: Calculation of the hits in BLAST results for primer-set 46.

The primers amplified several species of *B. burgdorferi* sl especially *B. garinii* SZ then *B. burgdorferi B31* and *B. afzelii BO23* [115]. In BLAST; the primers are not specific for the target species, but high sensitive. The primers are preferred for *B. burgdorferi* sl.

Table 8-47 shows BLAST results using 70-bp *flaB* of *B. burgdorferi* ss (JQ711236) and the individual primers. In amplicon, 90% of *B. burgdorferi* ss, 88% of *B. americana*, 16% of *B. carolinensis*, 73% of *B. andersonii* and undefined species show ≤ 2 mismatches to either primer. The individual primers show only *B. burgdorferi* ss.in similarity for reverse.

Sequence	**Primer-set 47**	**Number of BLAST hits**							
	Mismatches	*B.bu*	*B.bis*	*B.amr*	*B.crl*	*B.ga*	*B.and*	*B.*spp	Sum
Amplicon	≤ 2 in either primer	223	0	8	1	0	6	28	266
	>2 in either primer	23		1	5		2	11	42
Forward	0	155	14	9	6	2	14	35	235
	Total	242	15	9	7	5	14	39	331
Reverse	0	188	0	0	0	0	0	0	188
	Total	218							218

Table 8-47: Calculation of the hits in BLAST results for primer-set 47.

The primer pair and its specific probe was designed to amplify *B. burgdorferi* GeHo [70]. According to BLAST, the primers show similarity for further LB species; the primers are not specific for *B. burgdorferi* ss, but they are sensitive.

Table 8-48 shows BLAST results using 174-bp *flaB* of *B. burgdorferi* ss (X15660), *B. garinii* (L42885), and *B. afzelii* (X75202), plus the individual primers. In amplicon, 12% of *B. burgdorferi* ss, *B. garinii*, and *B. afzelii*, 43% of *B. valaisiana*, and 71% of undefined LB species show ≤ 2 mismatches to either primer and may be expected to amplify efficiently

Sequence	**Primer-set 48**	**Number of BLAST hits**									
	Mismatches	*B.ga*	*B.bu*	*B.af*	*B.va*	*B.spl*	*B.lst*	*B.okn*	*B.tnk*	B.spp	Sum
Amplicon	≤ 2 in either primer	16	18	9	7	0	0	0	2	8	60
	>2 in either primer	135	103	58	9				2	6	313
Forward	0	161	79	62	17	9	6	2	0	59	395
	Total	183	126	62	19	9	13	2		59	473
Reverse	0	17	25	9	4	0	0	0	2	8	65
	Total	19	26	9	6				2	12	74

Table 8-48: Calculation of the hits in BLAST results for primer-set 48.

The primers amplified *B. burgdorferi* sl [180, 181]. BLAST shows primer are not specific.

Table 8-49 shows BLAST results of 356-bp *flaB* of *B. burgdorferi* ss (KC494770) and the individual primers. In amplicon results, 27% of *B. burgdorferi* ss, and 2% of undefined species show ≤ 2 mismatches to either primer and may be expected to amplify efficiently.

Sequence	**Primer-set 49**	**Number of BLAST hits**				
	Mismatches	*B.bu*	*B.bis*	*B.crl*	*B.*spp	Sum
Amplicon	≤ 2 in either primer	71	0	0	2	73
	>2 in either primer	191			84	275
Forward	0	68	12	7	2	89
	Total	98	12	7	26	143
Reverse	0	175	0	0	1	176
	Total	229			33	262

Table 8-49: Calculation for results of BLAST hits for primer-set 49.

The primers were designed for specific amplification of *B. burgdorferi* ss [182]. BLAST shows limited cross-reaction with undefined LB species; the primers are not specific for *B. burgdorferi* ss and low sensitive.

Table 50 shows BLAST results using 98-bp *flaB* of *B. burgdorferi* ss (KF422803) and *B. garinii* (KF422758), plus the individual primers. In amplicon, 82% of *B. garinii*, 91% of *B. burgdorferi* ss and *B. afzelii*, 55% of *B. valaisiana*, 67% of *B. lusitaniae*, 100% of *B. carolinensis and B. americana*, and 85% of undefined LB species, all show ≤ 2 mismatches to either primer. In primers, all these species have 100% similarity for the primer sequences.

Sequence	**Primer-set 50**	**Number of BLAST hits**								
	Mismatches	*B.ga*	*B.bu*	*B.af*	*B.va*	*B.bis*	*B.lst*	*B.crl*	*B.amr*	B.spp	Sum
Amplicon	≤ 2 in either primer	260	190	105	19	14	27	7	9	51	682
	>2 in either primer	55	17	8	15	0	13	0	0	9	117
Forward	0	216	163	66	26	12	12	7	0	46	548
	Total	262	190	81	33	14	16	7	0	58	661
Reverse	0	1	175	0	1	14	26	7	9	22	255
	Total	285	155	0	31	14	27	7	9	33	561

Table 8-50: Calculations for results of BLAST hits for primer-set 50.

The primers amplified species of *B. burgdorferi* sl [183]. In BLAST, further species are in similarity for the primers.

Table 8-51 shows BLAST results of 482-bp *flaB* of *B. burgdorferi* ss (KF918617), *B. garinii* (L42885), *B. afzelii* (AY342020), and *B. lusitaniae* (DQ016623), plus the individual primers. In, 28 % of *B. burgdorferi* ss, 57% of *B. garinii*, 66% of *B. afzelii*, 42% of *B. valaisiana* and *B. lusitaniae*, 33% of *B. bissettii*, and 16% of undefined *Borrelia* species, all show ≤ 2 mismatches to either primer and may be expected to amplify efficiently.

Sequence	**Primer-set 51**	**Number of BLAST hits**								
	Mismatches	*B.bu*	*B.ga*	*B.af*	*B.va*	*B.bis*	*B.lst*	*B.crl*	*B.spl*	*B.*spp	Sum
Amplicon	≤ 2 in either primer	82	182	85	15	5	21	1	7	12	414
	>2 in either primer	201	132	43	21	10	27	6	5	59	504
Forward	0	169	240	111	32	12	13	7	0	49	637
	Total	185	284	119	33	14	16	7	0	59	721
Reverse	0	114	153	91	14	0	5	1	7	12	397
	Total	126	198	98	19	0	12	1	7	71	532

Table 8-51: Calculation for results of BLAST hits for primer-set 51.

The primers amplified five European genospecies of *B. burgdorferi* sl [116, 184]. In BLAST, all they, and others species are in similarity for the primers; the primer set is not specific for the target species, and moderate sensitive

Table 8-52 shows BLAST results for 88-bp *flaB* of *B. burgdorferi* ss (X63413), *B. garinii* (JQ711239) and *B. afzelii* (KF894064), plus the primers individually. The amplicons reveal 79% of *B. garinii*, 8% of *B. burgdorferi* ss, 93% of *B. afzelii* and 2% of *B. valaisiana* show ≤ 2 mismatches to either primer and may be expected to amplify efficiently

Sequence	**Primer-set 52**	**Number of BLAST hits**								
	Mismatches	*B.ga*	*B.bu*	*B.af*	*B.va*	*B.bis*	*B.lst*	*B.tnk*	*B.snc*	Sum
Amplicon	≤ 2 in either primer	247	15	118	1	0	0	0	0	381
	>2 in either primer	63	164	8	33					268
Forward	0	216	9	83	1	0	0	0	0	309
	Total	291	194	88	31	0	0	0	0	604
Reverse	0	263	158	72	35	11	12	4	2	557
	Total	284	187	8 1	38	13	18	4	2	627

Table 8-52: Calculation for results of BLAST hits for primer-set 52.

Several species of *B. burgdorferi* sl were amplified by this primer pair [65]. In BLAST, they are specific for these species but low sensitive except *B. garinii*.

Table 8-53 shows BLAST results of search 233-bp *flaB* of *B. burgdorferi* sl (FJ871030) and the individual primers. In amplicon, 65% of *B. burgdorferi* ss, 6% of *B. garinii*, 80% of *B. bissettii*, 20% of *B. valaisiana*, 100% for *B. carolinensis*, and 41% of undefined LB species, all show ≤ 2 mismatches to either primer and may be expected to amplify efficiently.

Sequence	**Primer-set 53**	**Number of BLAST hits**										
	Mismatches	*B.bu*	*B.ga*	*B.bis*	*B.crl*	*B.va*	*B.clf*	*B.amr*	*B.tnk*	*B.and*	*B.chl*	*B.*spp	Sum
Amplicon	≤ 2 in either primer	173	5	12	7	8	1	0	0	0	0	32	238
	>2 in either primer	100	63	3	0	31	2					45	244
Forward	0	146	0	11	7	4	1	0	0	0	0	6	175
	Total	237	0	14	7	39	1					99	397
Reverse	0	146	3	46	17	17	3	6	4	3	1	17	263
	Total	191	95	180	24	33	3	8	4	8	1	70	617

Table 8-53: Calculation for results of BLAST hits for primer-set 53.

The primers amplified 230-bp of the three main species of *B. burgdorferi* sl [113]. BLAST shows no sequences of *B. afzelii* in the results. The primers are not specific for these species, and low sensitive for all except *B. burgdorferi* ss.

Table 8-54 shows BLAST results of search 235-bp *flaB* amplicon of *B. burgdorferi* sl (X16833) and *B. garinii* (D82846), and sequences of the individual primers. For the amplicons, 50% of *B. burgdorferi* ss, *B. valaisiana*, and *B. bissettii*, 62% of *B. garinii*, 80% for *B. carolinensis* and *B. andersonii*, and 45% of undefined LB species show ≤ 2 mismatches to either primer and may be expected to amplify efficiently. For the individual primers, most species have 100% similarity for primer sequences.

Sequence	Primer-set 54	Number of BLAST hits										
	Mismatches	*B.bu*	*B.ga*	*B.af*	*B.va*	*B.bis*	*B.crl*	*B.and*	*B.yng*	*B.tnk*	*B.amr*	*B.*spp	Sum
Amplicon	≤ 2 in either primer	138	50	0	20	8	6	6	4	4	8	38	282
	>2 in either primer	135	30		19	7	1	2	5	0	1	46	246
Forward	0	162	16	80	37	11	7	6	5	4	0	31	359
	Total	221	259	96	39	13	7	6	5	4	0	67	717
Reverse	0	157	3	0	17	0	5	5	7	4	6	41	245
	Total	237	216	0	34	0	12	8	8	4	8	77	604

Table 8-54: Calculation for results of BLAST hits for primer-set 54.

The primers amplified *B. burgdorferi ss, B. garinii, B. afzelii, B. japonica, B. andersonii, B. valaisiana, B. lusitaniae*, and *B. bissettii* [113, 185]. Not all these species show similarity for the primers in BLAST, this primer-set was not specific for all target species, and show mostly moderate sensitivity.

Table 8-55 shows BLAST results using 420-bp *flaB* of *B. burgdorferi* sl (X75200) and sequences of the individual primers. In results of amplicon, 6% of *B. burgdorferi* ss, 22% of *B. afzelii*, 15% of *B garinii*, 100% of *B. turcica*, and 73% of *B. hermsii*, *B. anserine* (RFB), and 10% of undefined *Borrelia* sequences show ≤ 2 mismatches to either primer and may be expected to amplify efficiently. The results of the individual primers show only *B. burgdorferi* ss in 100% similarity for the forward primer.

Sequence	**Primer-set 55**	**Number of BLAST hits**										
	Mismatches	*B.bu*	*B.af*	*B.bis*	*B.spl*	*B.crl*	*B.ga*	*B.*spp	*B.hrm*	*B.ans*	*B.tur*	Sum
Amplicon	≤ 2 in either primer	12	10	1	0	0	32	7	12	3	2	79
	>2 in either primer	174	45	6			170	60	5	1	0	461
Forward	0	18	0	0	0	0	0	0	0	0	0	18
	Total	18	0	0	0	0	0	0	0	0	0	18
Reverse	0	167	78	13	10	7	3	3	0	4	0	285
	Total	168	82	13	10	7	3	3	0	5	0	291

Table 8-55: Calculation for results of BLAST hits for primer-set 55.

The primers were selected from a conserved sequence of *flaB* in American and European Lyme *Borrelia* [186]. BLAST shows a cross-reaction with three species of RFB; the primer-set is not specific for LB species and very low sensitive for *B. burgdorferi* sl.

Table 8-56 shows BLAST results for 791-bp *flaB* of *B. burgdorferi* ss (X16833) and sequences of the individual primers. In amplicon, 12% of *B. burgdorferi* ss, *B. garinii* and *B. valaisiana*, and 6% *B. bissettii* show ≤ 2 mismatches to either primer and may be expected to amplify efficiently.

Sequence	**Primer-set 56**	**Number of BLAST hits**							
	Mismatches	*B.bu*	*B.va*	*B.bis*	*B.ga*	*B.clf*	*B.chl*	*B.okn*	Sum
Amplicon	≤ 2 in either primer	26	3	1	30	0	0	0	60
	>2 in either primer	174	31	14	257				476
Forward	0	18	0	0	0	0	0	0	18
	Total	10	0	0	0	0	0	0	10
Reverse	0	39	15	3	3	3	2	2	67
	Total	77	17	4	10	3	2	2	115

Table 8-56: Calculation for results of BLAST hits for primer-set 56.

The primer pair was used to amplify *B. burgdorferi* B31 [82]. BLAST search shows the primer-set is not specific for *B. burgdorferi* ss and low sensitive.

Table 8-57 shows BLAST results of 276-bp *flaB* of *B. burgdorferi* ss (LC018214) and sequences of the individual primer set. In amplicon, 43% of *B. burgdorferi* ss and *B. valaisiana*, 66% *B. garinii*, and 82% of *B. afzelii* show ≤ 2 mismatches to either primer and may be expected to amplify efficiently.

Sequence	**Primer-set 57**	**Number of BLAST hits**				
	Mismatches	*B.bu*	*B.ga*	*B.af*	*B.va*	Sum
Amplicon	≤ 2 in either primer	127	170	98	18	413
	>2 in either primer	175	87	21	22	305
Forward	0	186	0	0	0	186
	Total	218				218
Reverse	0	82	0	0	0	82
	Total	153				153

Table 8-57: Calculation for results of BLAST hits for primer-set 57.

The primer pair was used to amplify *B. burgdorferi* sl species and test the cross-reactivity with *B. hermsii* and *B. coriaceae* (RFB) [187]. The results of BLAST shows specificity of the primers for *B. burgdorferi* sl species, and moderate sensitivity.

Table 8-58 shows BLAST results for 574-bp *flaB* of *B. burgdorferi* sl: *B. burgdorferi ss* (X15660), *B. garinii* (JX570875) and *B. valaisiana* (AB022139), plus sequences of the primers. In amplicon, a cross-reaction between LB and RFB by hits that show ≤ 2 mismatches to either primer: 6% of *B. burgdorferi*, 5% of *B. afzelii*, 13% of *B. valaisiana*, 2% for *B. garinii*; 40% of two species of RFB (*B. crocidurae* and *B. recurrentis*) and 60% of *B. anserina*. In primer results show further species in 100% similarity for primer sequences.

Sequences	**Primer-set 58**	**Number of BLAST hits**												
	Mismatches	*B.bu*	*B.af*	*B.va*	*B.lst*	*B.ga*	*B.hrm*	*B.crd*	*B.prk*	*B.trc*	*B.ans*	*B.rcnt*	*B.*spp	Sum
Amplicon	≤2 in either primer	20	7	5	0	8	0	4	0	0	3	1	0	49
	>2 in either primer	282	120	31		264		6			2	2		707
Forward	0	166	81	37	27	8	17	8	6	5	4	3	70	435
	Total	183	87	39	31	281	17	10	6	5	5	3	90	760
Reverse	0	19	7	5	0	7	0	0	0	0	0	0	2	41
	Total	23	7	36	0	8	0	0	0	0	0	0	2	77

Table 8-58: Calculation for results of BLAST hits for primer-set 58.

The primers amplified three strains of *B. burgdorferi* sl [188]. In BLAST, the primers are not specific for LB species, and low sensitive for the target species.

Table 8-59 shows BLAST results of 730-bp *flaB* of *B. burgdorferi* ss (CP001205), and the individual primers. The amplicon results reveal that 8% of *B. burgdorferi* ss, 20% of *B. bissettii*, 33% of *B. andersonii* and *B. californiensis*, and 21% of *B. afzelii* show ≤ 2 mismatches to either primer and may be expected to amplify efficiently.

Sequence	**Primer-set 59**	**Number of BLAST hits**					
	Mismatches	*B.bu*	*B.bis*	*B.and*	*B.clf*	*B.af*	Sum
Amplicon	≤ 2 in either primer	24	3	3	1	20	51
	>2 in either primer	249	12	6	2	72	341
Forward	0	45	4	3	1	0	53
	Total	68	4	5	1	0	78
Reverse	0	20	0	3	0	1	24
	Total	21	0	3	0	3	27

Table 8-59: Calculation for results of BLAST hits for primer-set 59.

The primers amplified two strains of *B. burgdorferi* ss [63]. BLAST shows that primers are not specific for this species and low sensitive.

Table 8-60 shows BLAST results using 120-bp *flaB* of *B. burgdorferi* ss (KM875675) and sequences of the individual primers. The amplicon results reveal that 85% of *B. burgdorferi* ss and *B. americana*, 13% of *B. bissettii*, 77% of *B. andersonii*, 57% of *B. carolinensis*, 46% of undefined *Borrelia* species show ≤ 2 mismatches to either primer and may be expected to amplify efficiently.

Sequence	**Primer-set 60**	**Number of BLAST hits**						
	Mismatches	*B.bu*	*B.bis*	*B.amr*	*B.and*	*B.crl*	*B.*spp	Sum
Amplicon	≤ 2 in either primer	193	2	8	7	4	28	242
	>2 in either primer	32	13	1	2	3	32	305
Forward	0	233	35	14	9	8	7	306
	Total	287	46	14	9	8	7	371
Reverse	0	197	0	0	0	0	0	197
	Total	232	0	0	0	0	0	232

Table 8-60: Calculation for results of BLAST hits for primer-set 60.

The primer pair was derived from *B. burgdorferi* (X15660) to amplify this strain [66]. BLAST shows the primer-set is not specific for *B. burgdorferi* ss, but was relatively sensitive.

Table 8-61 shows BLAST results for 75-bp *flaB* of *B. burgdorferi* ss (KM875674), and the individual primers. In amplicon, 67% of *B. burgdorferi* ss, 59% of *B. bissettii*, 88% of *B. americana*, 33% of *B. carolinensis*, and 89% of undefined *Borrelia* species in ≤ 2 mismatches to either primer and may be expected to amplify efficiently

Sequence	**Primer-set 61**	**Number of BLAST hits**						
	Mismatches	*B.bu*	*B.bis*	*B.amr*	*B.and*	*B.crl*	*B.*spp	Sum
Amplicon	≤ 2 in either primer	144	7	8	1	2	26	188
	>2 in either primer	68	7	1	8	4	3	91
Forward	0	169	14	9	0	6	34	232
	Total	264	15	9		7	67	362
Reverse	0	189	0	8	8	0	31	236
	Total	236		8	8		44	296

Table 8-61: Calculation for results of BLAST hits for primer-set 61.

The primer are derived from *B. burgdorferi* ss (AF244889) to amplify a 100-bp amplicon of this species [189]. In BLAST, the accession number is not correct for this species. However, the primer-set is not specific for *B. burgdorferi* ss, but moderate sensitive.

Table 8-62 shows BLAST results for 103-bp *flaB* of *B. burgdorferi* ss (KF836508), *B. bissettii* (FJ231346) and *B. carolinensis* (EU076499), and sequence of primers. In amplicon, 78% of B. *burgdorferi* ss, 96% of *B. afzelii*, 25% of *B. valaisiana* and *B. garinii*, 100% *B. bissettii* and *B. americana*, and 64% of undefined species, all show ≤ 2 mismatches to either primer and may be expected to amplify efficiently.

Sequence	**Primer-set 62**	**Number of BLAST hits**									
	Mismatches	*B.bu*	*B.af*	*B.va*	*B.lst*	*B.bis*	*B.chl*	*B.ga*	*B.amr*	*B.and*	*B.*spp	Sum
Amplicon	≤ 2 in either primer	171	122	8	0	14	0	2	9	4	32	362
	>2 in either primer	48	5	23		0		6	0	3	18	99
Forward	0	172	81	37	35	14	9	8	0	0	74	430
	Total	173	86	38	38	14	9	72			83	513
Reverse	0	219	0	0	0	15	7	0	9	7	35	292
	Total	238				15	7		9	8	73	350

Table 8-62: Calculation for results of BLAST hits for primer-set 62.

The primer amplified *B. burgdorferi* ss [15, 190]. In BLAST, the primers show similarity for further LB species; the primers are not specific for *B. burgdorferi* ss but moderate sensitive. The primers are preferred also for *B. afzelii* and *B. bissettii*.

Table 8-63 shows BLAST results for 71-bp *flaB* of *B. burgdorferi* ss (KM875670) and sequences of the individual primers. In amplicon, 90% of *B. burgdorferi* ss, 16% of *B. carolinensis*, and 86% of undefined *Borrelia* species show ≤ 2 mismatches to either primer and can be amplified efficiently. In individual primers show only *B. burgdorferi* ss in 100% similarity for the reverse primer.

Sequence	Primer-set 63	Number of BLAST hits						
	Mismatches	*B.bu*	*B.bis*	*B.crl*	*B.clf*	*B.ga*	*B*.spp	Sum
Amplicon	≤ 2 in either primer	193	0	1	0	0	25	219
	>2 in either primer	20		5			4	29
Forward	0	183	14	5	27	2	34	265
	Total	252	14	7	31	8	76	388
Reverse	0	193	0	0	0	0	0	193
	Total	232						232

Table 8-63: Calculation for results of BLAST hits for primer-set 63.

The primer pair and its specific probe amplified *B. burgdorferi* stain 297 [32]. According to BLAST, primers show similarity for further species; the primers are not specific for the target species but sensitive.

Table 8-64 shows the results of BLAST tests for 371-bp amplicon *p66* of *B. burgdorferi* ss (CP002228), and sequences of the individual primers. Amplicon results reveal 27% of *B. burgdorferi* ss show ≤ 2 mismatches to either primer and may be expected to amplify efficiently. The results of individual primers show only *B. burgdorferi* ss in 100% similarity for forward primer, while further LB species in 100% similarity for the reverse primer.

Sequence	Primer-set 64	Number of BLAST hits					
	Mismatches	*B.bu*	*B.amr*	*B.crl*	*B.bis*	*B*.spp	Sum
Amplicon	≤ 2 in either primer	9	0	0	0	0	9
	>2 in either primer	24					24
Forward	0	12	0	0	0	0	12
	Total	12					12
Reverse	0	7	6	2	2	1	18
	Total	8	6	2	2	2	232

Table 8-64: Calculation for results of BLAST hits for primer-set 64.

The primer pair performed a species specific PCR that detected *B. burgdorferi* ss [73, 122]. BLAST results show specificity of primers for this species, but low sensitivity.

Table 8-65 shows BLAST results using 236-bp *p66* of *B. burgdorferi* ss (X87725) and *B. americana* (HM802238) and sequences of the individual primers. The amplicon results reveal that 86% of *B. garinii*, 71% of *B. afzelii*, 58% of *B. burgdorferi* ss, and 100% of *B. bissettii*, *B. americana*, and undefined *Borrelia* species, all show ≤ 2 mismatches to either primer.

Sequence	**Primer-set 65**	**Number of BLAST hits**							
	Mismatches	*B.ga*	*B.af*	*B.bu*	*B.va*	*B.amr*	*B.bis*	*B.*spp	Sum
Amplicon	≤ 2 in either primer	20	10	19	2	6	5	10	72
	>2 in either primer	3	4	10	0	0	0	0	17
Forward	0	21	14	12	2	0	0	7	56
	Total	21	14	13	2			9	59
Reverse	0	0	0	23	0	5	1	8	37
	Total			34		6	5	10	55

Table 8-65: Calculation for results of BLAST hits for primer-set 65.

The primer pair was specifically designed to amplify *B. burgdorferi* ss [122]. In BLAST the primers show cross-reaction with non-target species; the primers are not specific for *B. burgdorferi* ss. and low sensitive. They are preferred for a group of *B. burgdorferi* sl species.

Table 8-66 shows BLAST results for 126-bp *p66* of *B. burgdorferi* ss (AY654938), *B. garinii* (KF844227), *B. bissettii* (KM269454), and *B. afzelii* (KF844231), plus the individual primers. In amplicon, 75% of *B. burgdorferi* ss, 100 % of *B. garinii*, *B. afzelii*, *B. bissettii*, *B. americana*, *B. valaisiana*, *B. carolinensis*, and *B. andersonii* show ≤ 2 mismatches to either primer and may be expected to amplify efficiently.

Sequence	**Primer-set 66**	**Number of BLAST hits**									
	Mismatches	*B.bu*	*B.ga*	*B.af*	*B.bis*	*B.amr*	*B.va*	*B.crl*	*B.and*	*B.*spp	Sum
Amplicon	≤ 2 in either primer	21	23	14	2	6	2	2	1	2	73
	>2 in either primer	7	0	0	0	0	0	0	0	7	14
Forward	0	34	21	14	5	5	2	1	0	10	92
	Total	34	21	16	5	6	2	2		10	96
Reverse	0	26	12	14	2	4	0	2	1	3	64
	Total	26	12	14	2	4		2	1	3	64

Table 8-66: Calculations for results of BLAST hits for primer-set 66.

The primer pair was used to amplify all "universal types" of Lyme spirochetes [73, 122]. In BLAST, the primers are specific for the target species, and relatively high sensitive.

Table 8-67 shows BLAST results for 357-bp of *p66* of *B.* garinii (KF844225) and *B. afzelii* (KF844220), plus the individual primers. In amplicon, 43% of *B. garinii*, 35% of *B. afzelii*, and 3% *B. burgdorferi* ss show in ≤ 2 mismatches to either primer and may be expected to amplify efficiently.

Sequence	**Primer-set 67**	**Number of BLAST hits**			
	Mismatches	*B.ga*	*B.af*	*B.bu*	Sum
Amplicon	≤ 2 in either primer	10	5	1	16
	>2 in either primer	13	9	28	50
Forward	0	20	5	1	26
	Total	23	5	1	29
Reverse	0	7	5	1	13
	Total	3	9	28	40

Table 8-67: Calculations for results of BLAST hits for primer-set 67.

The primer pair was designed to amplify European Lyme spirochetes but not the American [122, 191]. In BLAST, the primers were specific for target species and moderate sensitive.

Table 8-68 shows BLAST results for 222-bp *recA* of *B. burgdorferi* ss (U23457) and the individual primers. In BLAST, only *B. burgdorferi* ss was detected in similarity for the primer in all searches; 17% of this species show ≤ 2 mismatches to either primer in amplicon search.

Sequence	**Primer-set 68**	**Number of BLAST hits**
	Mismatches	*B.bu*
Amplicon	≤ 2 in either primer	7
	>2 in either primer	34
Forward	0	6
	Total	50
Reverse	0	6
	Total	43

Table 8-68: Calculation for results of BLAST hits for primer-set 68.

The primers were designed to amplify *B. burgdorferi* N40 [15, 126]. In BLAST, only *B. burgdorferi* ss in similarity for the primers in all tests; the primer-set is specific for *B. burgdorferi* ss, but low sensitive.

Table 8-69 shows BLAST results for 287-bp *recA* of *B. burgdorferi* ss (U23457) and *B. afzelii* (CP009058), plus the individual primers. In amplicon results, 15% of *B. burgdorferi* ss, 62% of *B. afzelii*, 8% of *B. garinii*, 25% of *B. bissettii*, and 33% of *B. valaisiana* show ≤ 2 mismatches to either primer and may be expected to amplify efficiently.

Sequence	**Primer-set 69**	**Number of BLAST hits**									
	Mismatches	*B.bu*	*B.af*	*B.ga*	*B.bis*	*B.va*	*B.yng*	*B.trc*	*B.prk*	*B.*spp	Sum
Amplicon	≤ 2 in either primer	7	5	3	1	1	0	0	0	0	17
	> 2 in either primer	37	3	32	3	2					77
Forward	0	7	5	4	1	1	0	0	0	0	18
	Total	7	5	4	1	1					18
Reverse	0	7	5	24	1	1	4	2	2	2	48
	Total	7	5	25	1	1	4	3	2	2	50

Table 8-69: Calculation for results of BLAST hits for primer-set 69.

The primers amplified *B. burgdorferi ss* N40, *B. afzelii* VS146, and *B. garinii* PBi [74]. In BLST, the primer-set is not specific and low sensitive for the target species.

Table 8-70 shows BLAST results using 395-bp of 16-kb plasmid species-specific plasmid for *B. burgdorferi ss* (CP001564) and sequences of the individual primers. The results show that only *B. burgdorferi* was detected in similarity for the primers in all searches; 53% of this species show ≤ 2 mismatches to either primer in amplicon search.

Sequence	Primer-set 70	Number of BLAST hits
	Mismatches	*B.bu*
Amplicon	≤ 2 in either primer	8
	>2 in either primer	7
Forward	0	14
	Total	14
Reverse	0	7
	Total	7

Table: 8-70 Calculation for results of BLAST hits for primer-set 70.

The primer pair was designed specifically to amplify species-specific of *B. burgdorferi ss* [127]. BLAST showed high specificity and moderate sensitivity of the primers for this species.

Table 8-71 shows BLAST results *B. garinii* 236-bp for (U83998), and 240-bp for (CP001302) of 33-kb plasmid species-specific. The results show only *B. garinii* in similarity for the primers in all searches; 66% of this species show ≤ 2 mismatches to either primer in amplicon search.

Sequence	Primer-set 71	Number of BLAST hits
	Mismatches	*B.ga*
Amplicon	≤ 2 in either primer	2
	>2 in either primer	3
Forward	0	2
	Total	6
Reverse	0	1
	Total	1

Table 8-71: Calculation for results of BLST hits for primer-set 71

The primer-set amplified 236-bp of *B. garinii* 20047, N34, and G25B strains [127]. In BLAST, no one of those strains was detected in any search. However, the primers are specific and low sensitive in few hits numbers.

Table 8-72 shows BLAST results for 120-bp of 25-kb plasmid species-specific of *B. afzelii* for (CP002944), and sequences of the individual primers. The results show only *B. afzelii* in BLAST hits in similarity for the primer in all searches; 100% of this species show ≤ 2 mismatches to either primer in amplicon search.

Sequence	**Primer-set72**	**Number of BLAST hits**
	Mismatches	*B.af*
Amplicon	≤ 2 in either primer	4
	>2 in either primer	0
Forward	0	5
	Total	5
Reverse	0	5
	Total	5

Table 8-72: Calculation for results of BLST hits for primer-set 72.

The primers amplified specifically 125-bp DNA fragment for three strains of *B. afzelii* (VS461, UO1, and Iper3) [127]. In BLAST one strain (UO1) is in similarity for the primers. However, the primer set was high specific but extremely low hits number for *B. afzelii*.

Table 8-73 shows BLAST results for 345-bp *ospA* of *B. burgdorferi* ss (JF776165) and the individual primers. In amplicon results, 53% of *B. burgdorferi* ss, 100% of *B. spielmanii* and *B. americana*, and 33% *of* undefined *Borrelia* show ≤ 2 mismatches to either primer and may be expected to amplify efficiently.

Sequence	**Primer-set 73**	**Number of BLAST hits**				
	Mismatches	*B.bu*	*B.spl*	*B.amr*	*B.*spp	Sum
Amplicon	≤ 2 in either primer	115	7	1	4	127
	>2 in either primer	99	0	0	8	107
Forward	0	134	0	1	0	135
	Total	135		1		136
Reverse	0	103	6	1	2	113
	Total	111	6	1	7	125

Table 8-73: Calculation for results of BLST hits for primer-set 73.

The primer-set was designed for amplification of several strains of *B. burgdorferi* ss [192]. BLAST shows cross-reaction with *Borrelia* species; the primer-set is not specific for *B. burgdorferi* ss and moderate sensitive.

Table 8-74 shows BLAST results for 352-bp of *ospA* of *B. burgdorferi* ss (JN413099) and the individual primers. In amplicon results, 49% of *B. burgdorferi* ss, 10% of *B. garinii*, 37% of *B. bissettii*, and 33% of undefined species show ≤ 2 mismatches to either primer and may be expected to amplify efficiently.

Sequence	**Primer-se 74**	**Number of BLAST hits**						
	Mismatches	*B.bu*	*B.ga*	*B.spl*	*B.lst*	*B.bis*	*B.*spp	Sum
Amplicon	≤ 2 in either primer	106	22	0	0	3	4	135
	>2 in either primer	110	189			5	8	312
Forward	0	124	9	0	0	0	1	134
	Total	173	10				2	185
Reverse	0	106	6	6	7	4	6	135
	Total	123	50	6	7	7	7	200

Table 8-74: Calculation for results of BLST hits for primer-set 74.

The primers amplified specifically five strains of *B. burgdorferi* ss [193]. BLAST shows cross-reaction with further species of Lyme *Borrelia*; the primer-set is not specific for *B. burgdorferi* ss and moderate specific.

Table 8-75 shows BLAST results for 389-bp *ospA* of *B. burgdorferi* ss (X60300) and (AB007102), plus the individual primers. In amplicon, 46% of *B. garinii*, 31% of *B. burgdorferi* ss, 95% of *B. bavariensis*, 16% of *B. valaisiana*, and 54% of undefined species show ≤ 2 mismatches to either primer and may be expected to amplify efficiently.

Sequence	Primer-set 75	Number of BLAST hits							
	Mismatches	*B.ga*	*B.bu*	*B.af*	*B.bv*	*B.va*	*B.jpn*	*B.*spp	Sum
Amplicon	≤ 2 in either primer	111	69	0	21	5	0	6	212
	>2 in either primer	145	150		1	25		5	326
Forward	0	92	34	27	21	12	0	7	193
	Total	99	135	49	21	16		11	331
Reverse	0	120	126	1	21	7	4	9	288
	Total	126	126	1	21	8	4	9	295

Table 8-75: Calculation for results of BLST hits for primer-set 75.

The primer pair was used to amplify 391-bp of the three main species of *B. burgdorferi* sl [103, 199]. BLAST shows primers in similarity for these species except *B. afzelii*; the primer-set is not specific for all the target species and show low sensitivity.

Table 8-76 shows BLAST results of 389-bp *flaB* of *B. burgdorferi* ss (KJ676826), *B. americana* (HM802232), and *B. carolinensis* (KF793050), plus the individual primers. In amplicon results, 13% of *B. burgdorferi* ss, 85% of *B. bissettii* and *B. americana*, 68% of *B. garinii*, 100% of *B. carolinensis* and *B. tanuki*, and 34% of undefined species, all show ≤ 2 mismatches to either primer and may be expected to amplify efficiently.

Sequence	Primer-set 76	Number of BLAST hits									
	Mismatches	*B.bu*	*B.bis*	*B.amr*	*B.crl*	*B.ga*	*B.tnk*	*B.hrm*	*B.prk*	*B.dtn*	*B.*spp	Sum
Amplicon	≤2 in either primer	33	12	8	7	220	4	0	0	0	22	306
	>2 in either primer	204	3	1	0	99	0				41	348
Forward	0	78	12	9	6	5	0	15	6	5	10	146
	Total	118	13	9	8	242		17	6	5	32	449
Reverse	0	164	6	8	7	8	4	0	0	0	32	229
	Total	263	15	9	7	319	4				63	680

Table 8-76: Calculation for results of BLST hits for primer-set 76.

The primer set was designed as nested to amplify *B. burgdorferi* sl species in United States and parts of Europe and Asia [195]. Same primers were used to amplify *B. burgdorferi* sl (*B. andersonii*, *B. bissettii*, and *B. burgdorferi* ss) [196]. According to BLAST, not all target species show similarity for the primers, and extra species were in similarity for the primers. The primer-set is not specific for the target species and low sensitive.

Table 8-77 shows BLAST results for 684-bp *p66* of *B. burgdorferi* ss (KM676037) and the individual primers. The results of amplicon reveal 68% of *B. burgdorferi* ss show ≤ 2 mismatches to either primer and may be expected to amplify efficiently. Results of the individual primers show only *B. burgdorferi* in 100% similarity for forward primer, while further species for reverse primer.

Sequence	**Primer-set 77**	**Number of BLAST hits**				
	Mismatches	*B.bu*	*B.af*	*B.bis*	*B.chl*	Sum
Amplicon	≤ 2 in either primer	11	0	0	0	11
	>2 in either primer	5				5
Forward	0	14	0	0	0	14
	Total	14				14
Reverse	0	11	28	2	1	42
	Total	11	36	2	1	50

Table 8-77: Calculation for results of BLAST hits for primer-set 77.

The primer pair was derived from *B. burgdorferi* (X87725) to amplify a segment of this species by nested PCR [197]. According to BLAST, the primer-set is specific for *B. burgdorferi* and shows moderate sensitivity, but low hits number.