(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
11 May 2018 (11.05.2018)

W!P O | PCT

(10) International Publication Number
WO 2018/083491 Al

(51) International Patent Classification:
C12Q 1/68 (2018.01) *G01N 33/569* (2006.01)

(21) International Application Number:
PCT/GB2017/053323

(22) International Filing Date:
03 November 2017 (03.1 1.2017)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
1618565.4 03 November 2016 (03.11.2016) GB

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(81) Designated States *(unless otherwise indicated, for every kind of national protection available):* AE, AG, AL, AM, AO, AT, AU, AZ, BA, BB, BG, BH, BN, BR, BW, BY, BZ, CA, CH, CL, CN, CO, CR, CU, CZ, DE, DJ, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IR, IS, JO, JP, KE, KG, KH, KN, KP, KR, KW, KZ, LA, LC, LK, LR, LS, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PA, PE, PG, PH, PL, PT, QA, RO, RS, RU, RW, SA, SC, SD, SE, SG, SK, SL, SM, ST, SV, SY, TH, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.

(84) Designated States *(unless otherwise indicated, for every kind of regional protection available):* ARIPO (BW, GH, GM, KE, LR, LS, MW, MZ, NA, RW, SD, SL, ST, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, RU, TJ, TM), European (AL, AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV, MC, MK, MT, NL, NO, PL, PT, RO, RS, SE, SI, SK, SM, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, KM, ML, MR, NE, SN, TD, TG).

(54) Title: PHAGE-BASED DETECTION OF BORRELIOSIS AND MEANS THEREFOR

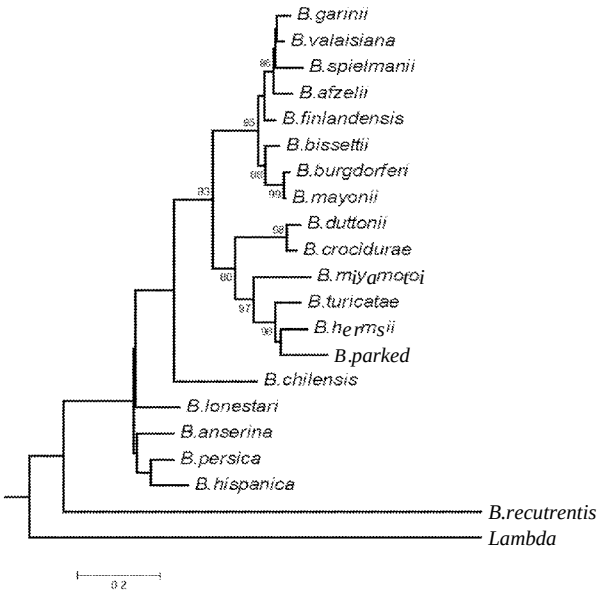


FIG. 1

(57) Abstract: This invention relates to methods of detecting Borrelia burgdorferi sensu lato or for detecting Borrelia associated with Relapsing Fever (RF), kits for carrying out such methods, and methods of treating Borrelia burgdorferi sensu lato or RF infections in a subject. Uses of phage specific for Borrelia are also provided.

WO 2018/083491 A1

[Continued on next page]

Published:
— *with international search report (Art. 21(3))*
— *with sequence listing part of description (Rule 5.2(a))*

PHAGE-BASED DETECTION OF BORRELIOSIS AND MEANS THEREFOR

Field of the Invention

This invention relates to methods of detecting *Borrelia burgdorferi sensu lato or for detecting Borrelia* associated with Relapsing Fever (RF), kits for carrying out such methods, and methods of treating *Borrelia burgdorferi sensu lato* or RF infections in a subject. Uses of phage specific for *Borrelia* are also provided.

Background

Borrelia burgdorferi sensu lato (s.l.) is a group of bacterial species of the *Borrelia* genus of the spirochete phylum, and that are known to be the causative agent in Lyme Disease (LD). Among them, three main species that are commonly found in Lyme patients in Europe are *B. burgdorferi sensu stricto* (s.s.), *B. afzelii,* and *B. garinii.* While *B. burgdorferi* s.s. causes LD both in the USA and in Europe. The other less commonly encountered LD species include *Borrelia spielmanii, Borrelia valaisiana, Borrelia bissettii, Borrelia lusitaniae, Borrelia finlandensis, Borrelia bavariensis, Borrelia japonica, Borrelia sinica, Borrelia spielmanii, Borrelia tanukii, Borrelia turdi, Borrelia Yangtze, Borrelia mayonii, Borrelia carolinensis, Borrelia andersonii, Borrelia lonestari, and Borrelia Americana.*

Bacteria that cause LD are spread to humans through the bite of bacterially infected ticks (and less frequently through other insects). It is the most commonly reported tick-born disease in the United States, and Centres for Disease Control and Prevention (CDC) records approximately 30 000 confirmed cases of LD annually. However, the number of suspected *Borrelia* infection is around 300 000 every year according to CDC. In Europe, the number of LD cases has increased steadily over the last two decades, with an estimation of about 85 000 cases every year. In England and Wales, it is estimated that around 3,000 new cases of LD are diagnosed each year.

Clinical diagnosis of LD is often problematic because the symptoms of LD are easily confused with other diseases. The manifestations of infection can range from a distinctive rash, tiredness, muscle pain, headaches, to severe arthritic, neurologic

and cardiac conditions. Consequently, lab-based determination of *Borrelia* infection plays a vital role in LD diagnosis. Current lab-based diagnostic methods focus on indirectly determining the presence or absence of the bacteria in the body of the patient based on the human immune response to that bacterium. The current method of direct detection of *Borrelia* presence in patient suffers from low sensitivity, and therefore is regarded as of no diagnostic value.

A first problem associated with all known methodologies is as a result of the fact that the bacteria are only easily accessible from blood samples during the early stages of infection. In later stages of infection, the bacteria tend to migrate to the nervous system, where they can remain sequestered and so difficult to locate and identify with conventional diagnostic tools. Consequently, identification of infection in the later stages by attempting to detect the presence of the bacteria often has to involve the analysis of cerebrospinal fluid or the like, rather than the simpler analysis of blood samples.

The current "gold standard" technique for identifying presence of the bacteria involves growing a suspended bacterial culture from a sample from an individual suspected of contracting Lyme disease, and later characterising the bacteria in that culture. Often the characterisation is based on microscopy evaluation of morphological features of the bacteria. However, growing cultures takes up to 7 weeks before a sufficiently large and developed cultured population has been obtained from which to confirm the presence of the bacteria. Even then, such tests may not enable differentiation between Lyme disease and other tick-borne bacterial infections. For example, the bacteria associated with Relapsing Fever (RF) are morphologically similar to the bacteria that cause LD. LD and RF also have overlapping clinical symptoms. Consequently, a patient may be diagnosed as suffering from LD by this method, when in fact they suffer from RF; this being a problem as treatment methodologies may differ for each disease.

A second diagnostic methodology involves antibody based procedures. Antibodies (i.e. diagnostic antibodies) specific for the infected host's own antibodies that are raised as part of the immune response within the host to the infection (i.e. immune response antibodies) are used to detect the presence or absence of the infection. Unfortunately, however, such antibody based methodologies have only a short

window of opportunity to accurately identify the presence of the infection. During the early stage of LD (2-3 weeks post infection), the antibody response in the host as part of their own immune reaction to infection is not normally sufficiently developed to be clearly detected by the diagnostic antibodies. In later stage LD, the production of immune response antibodies is suppressed by the bacteria, and so again the antibody signal may be too weak to be detected with a reasonable degree of accuracy. Consequently, antibody-based diagnostic methods are estimated to falsely identify 54% of patients as un-infected.

The third known diagnostic method involves the PCR-based detection of the bacteria. Due to the often extremely low concentration of the relevant bacteria in a patient's sample, such methods have a poor level of sensitivity. It has been calculated that only one third of patients suffering from LD in the USA showed a positive PCR result when their Cerebrospinal fluid samples were tested. Half of patients in the early stage of LD showed a negative PCR-derived result for the presence of *the* bacteria in blood samples.

As an example of current difficulties with diagnosing and treating LD, the veterinary profession report that tests used by them and based on the use of diagnostic antibodies commonly provides large number of false positive results for *Borrelia burgdorferi sensu lato* infection in horses. As a result, it is generally accepted that a large number of horses are needlessly treated with antibiotics such as oxytetracyline.

Consequently, there is a need for alternative methods of detecting the presence of *Borrelia burgdorferi s.l.,* the causative agent of LD. Because of the similar difficulties faced with diagnosing relapsing fever (RF) which is also caused by Borrelia species and is often wrongly diagnosed as LD and vice versa, there is also a need for alternative methods of detecting the presence of *Borrelia* which are the causative agents of Relapsing Fever (herein referred to as Relapsing Fever Borrelia (RF Borrelia) such as *Borrelia miyamotoi* and *Borrelia hermsii.*

Summary of the Invention

Bacteriophages (or phages) are viruses that can infect and multiply in a bacterium. Commonly they consist of a core of nucleic acid enclosed within a protein coat (i.e. the capsid). Phage that infect *Borrelia burgdorferi s.l.* and RF Borrelia are very poorly understood. The lack of research effort/output concerning *Borrelia* phages is mainly due to the following two hurdles. Firstly, growing *Borrelia* strains *in vitro* needs a complicated medium to mimic their *in vivo* conditions. This coupled with the demand for highly skilled, 'purpose-trained' scientists, who need to possess a good level of specialist knowledge concerning both *Borrelia* and phages, means that the academic output concerning *Borrelia* phages has been inhibited. Secondly, the commonly used phage characterisation methods, such as plaque assays, can't be simply translated into a *Borrelia* phage study because *Borrelia* have not previously been observed to grow on a solid agar surface to form a confluent cell growth on which phages could be observed. The inventors consider that they are the first to consistently grow viable lawns of *Borrelia,* on which a study of *Borrelia* phage can be conducted.

For an agent to be suitable for use in detecting the presence or absence of a bacterial infection, the agent should be capable of specifically identifying the bacteria associated with the infection. Advantageously, that agent should also be able to detect the bacterial infection in the least invasive manner possible. Phage have to-date not been suggested as useful in identifying the presence or absence of *B burgdorferi* s.l. infection. Even if they had, due to the limited understanding of phage that are known to infect *B. burgdorferi s.l,* there has been insufficient information to determine if phage could be used in such a diagnostic method. The art has continued to develop all relevant diagnostics by directing methodologies to the direct identification of the bacteria, or identification of immune response antibodies directly raised against the bacteria.

However, after extensive experimentation, it has surprisingly been found by the inventors that phage can be found that are specific to their *B. burgdorferi* s.l. or Relapsing Fever *Borrelia* host. As a result, successful methodologies have been provided for identifying infection of such bacteria and based on identifying the presence or absence of such phage.

Relapsing Fever *Borrelia* are any *Borrelia* species which are the causative agents of Relapsing Fever, for example, *Borrelia miyamotoi, Borrelia hermsii, Borrelia recurrentis, Borrelia crocidurae, Borrelia duttoni, Borrelia hispanica, Borrelia parkeri* and *Borrelia turicatae* or any combination thereof.

Therefore, in a first aspect of the present invention, there is provided a method of determining the presence or the absence of *B. burgdorferi* s.l. or RF Borrelia in a sample, the method comprising the steps of:-

a) detecting the presence or absence of a phage specific for B. burgdorferi s.l or RF *Borrelia* in the sample; and

b) determining the presence of *B. burgdorferi* s.l. or RF *Borrelia* in the sample on the basis of the detection of the phage, or the absence of *B. burgdorferi* s.l. or RF *Borrelia* in the sample on the basis of the lack of detection of the phage.

The inventors have surprisingly found that *B burgdorferi s.l.* and RF *Borrelia* even those in sequestered states, dispense large numbers of phage. Consequently, for example, even when the bacteria are sequestered and so difficult to detect with little or no bacteria to be found in the blood, a blood sample from an infected subject does contain large numbers of phage or phage fragments originating from the bacteria. Consequently, a particularly advantageous aspect of the present invention is that the methods of the present invention can be practiced on a non-neuronal sample such as blood sample (e.g. whole blood, serum, plasma), urine sample, faecal sample, skin sample, lymph sample, or combinations thereof and still provide useful conclusions on the presence of absence of bacterial infection irrespective of the stage of infection; something that is not possible with known diagnostic methodologies.

Phage specific for *Borrelia burgdorferi* sensu lato *or* RF *Borrelia*

The inventors have found that phylogenetic analysis of for example the terminase genes of the phage revealed a tight correlation between the terminase gene sequences and the identity of Borrelia species (Fig. 1). An independent sub-group of Borrelia species causing LD was well-separated from other Borrelia strains with

statistically significant boot strap values. This demonstrated that the terminase genes were good molecular markers in identifying Lyme Borrelia strains. This is also the case for RF Borrelia as shown in Fig. 1. For example, with the tests provided herein, it is possible to distinguish between Borrelia which cause Lyme disease (B. burgdorferi s.l.) and RF Borrelia.

The same analysis has been carried out for other phage genes. For example, holins and endolysins. Phylogentic analyses based on holins (Fig. 2) endolysins (Fig. 3), integrase (Fig. 4) and portal proteins (Fig. 5) also show the potential of these genes in detecting Borrelia species.

Specificity can therefore be defined as a method which distinguishes between the phage of *B. burgdorferi s.l.* and the phage from other Borrelia species. For example, a nucleotide primer or antibody which binds preferentially to the phage of B. burgdorferi sensu lato; or does not cross-react with a nucleotide sequence or amino acid sequence from another Borrelia species. The method also may distinguish between the phage of RF Borrelia and the phage from other species.

Phage

A bacteriophage, also commonly called a phage, is a virus which infects and replicates within a bacterium. The phages described herein can be pro-phages, temperate/lysogenic phages, phage-like particles (such as plasmids) or lytic phages.

A prophage/temperate/lysogenic phage is a bacteriophage particle made of either double or single strand DNA or RNA. Phage genomes can be inserted and integrated into the circular bacterial DNA chromosome or existing as an extrachromosomal plasmid. This is a latent form of a phage, in which the viral genes are present in the bacterium without causing disruption of the bacterial cell and sometimes may provide competitive advantage to the overall fitness of the bacterial host.

A lytic or virulent phage contains viral DNA/RNA which exists separately from the host bacterial DNA and replicates separately from the host bacterial DNA. Lytic phage are released upon destruction of the infected cell and its membrane.

Sample

The sample may be derived from a number of origins. The sample may therefore comprise or consist of plasma, serum, whole blood, cerebrospinal fluid, urine, faecal matter, skin, brain tissue, glial cells, lymph, sweat or amniotic fluid, or any combination thereof.

A sample may therefore be one that is taken from the subject at any time after infection and consistently provide the correct determination of the presence of the infection. Most surprisingly, the sample may be obtained shortly after infection (i.e. early stage infection), or after the infection has been well developed and that bacteria have become sequestered (i.e. late stage infection). This demonstrates a technical advantage associated with the present invention over known methodologies. Early stage infection would generally be considered to be less than 2 or 3 weeks from infection. Late stage infection would generally be considered to occur when the infected subject begins to suffer from neurological disorders (i.e. brain fog), which can be from 6 months after infection.

The subject from which the sample may be obtained is any animal that suffers from LD or RF. For example, the subject may be human, equine, canine, feline, ovine, caprine, ticks, lice, or any combination thereof. The subject may therefore be human, insect or animal.

Detection of phage

By detection is meant determining if an interaction between the phage and a detection molecule specific for Borrelia burgdorferi sensu lato or Relapsing Fever Borrelia, for example a primer or antibody specific for a phage nucleic acid or protein respectively, is present or absent. These methods may be carried out ex *vivo* or *in vitro.* Detection may also be *in vivo,* for example via a dye probe.

The detection molecule which specifically binds the phage specific for the Lyme or RF Borrelia may be tagged or labelled. For example, detection may include the use of an agent which is capable of detection (a label) using for example spectrophotometry, flow cytometry, or microscopy. Exemplary labels include radioactive isotopes (such as 3H, 14C, 15N, 35S, 90V, 99Tc, ^{111}Ln, ^{1251}, or ^{1311}), fluorophores (such as fluorescein, fluorescein isothiocyanate, rhodamine or the like),

chromophores, ligands, chemiluminescent agents, bioluminescent agents (such as luciferase, green fluorescent protein (GFP) or yellow fluorescent protein), enzymes that can produce a detectable reaction product (such as horseradish peroxidise, luciferase, alkaline phosphatase, beta-galactosidase) and combinations thereof.

Phages can also be labelled with any DNA dye, such as SYBR Green, SYBR Gold, EB. The next generation sequencing technologies, such as short-read sequencing technologies (e.g. 454, lllumina, SOLiD and Ion Torrent) and long-read sequencing technology (e.g. PacBio sequencing) can also be used in identifying phage via whole genome sequencing.

Detection of phage nucleic acid

Detection of the phage may comprise detection of the phage gene or gene fragment.

The phage gene may be defined by a nucleic acid sequence for the region of a phage genome that is specific to a *B burgdorferi s.l.* host in the case of Lyme disease diagnosis (i.e. the nucleic acid sequence is, or is part of, a phage gene that is found in only the target host, e.g. *B. burgdorferi s.l.,* or in a phage specific for such a host, or part thereof). Alternatively, the phage gene may be defined by a nucleic acid sequence for the region of a phage genome that is specific to a RF *Borrelia* host in the case of RF diagnosis (i.e. the nucleic acid sequence is, or is part of, a phage gene that is found in only the target host, e.g. RF *Borrelia* or in a phage specific for such a host, or part thereof).

The applicant has found that a suitable phage gene is the gene that encodes the holin, endolysin, integrase, capsid, portal or terminase protein, or combinations thereof.

The following describes nucleic acid sequences specific to *B. burgdorferi s.l.* phage.

For example, the phage gene may be the B. burgdorferi s.l. terminase gene, when this is the case the gene may be a nucleic acid according to the sequence of SEQ ID NO.s 1-10 or a gene capable of encoding a protein according to the sequence of SEQ ID NO.s 36-45. Optionally, the terminase gene may be a nucleic acid with a

greater than or equal to 70-100 % sequence homology with SEQ ID NO.s 1-10 or a gene capable of encoding a protein with a greater than 70-100% sequence homology with SEQ ID NO.s 36-45. For example, greater than or equal to 70, 75, 80, 85, 90 or 95, 96, 97, 98, 99 or 99.5% sequence homology with SEQ ID NO.s 1-10 or 46-45. With regards to homologous genes and proteins, preferably these retain the function of the terminase.

SEQ ID NO. 1

5'GTGAACTTATATCAAACAAAACTTTTTACAACACTACAAAAGGAATACAAAAAT
AAATATGGAGTTGATATATCACAATTTGTAAAGCTAACAAATTCTTCAATTAATTT
TGATAAGTTTGAAGAAGAACAGTTAACTTTAAAACAAAAAAATGTGATAAAAAGC
ATTAAAAAGAATAATGAAAAGAAGATTATACTCAGCGGAGGCATAGCTAGTGGC
AAAACGTATCTTGCATGTTATCTTTTTCTAAAAAGTTTAATTGAAATTAAAAAGTT
ATACTCTAGTGATACTAATAATTTCATTATAGGGAATTCACAACGTTCAGTTGAA
GTTAATGTTTTGGGGCAATTTGAAAAGCTATGTAAACTTCTTAAAATTCCTTATAT
TCCAAGACATACAAATAATTCATATATTCTGATTGATTCACTACGTATTAATCTAT
ATGGAGGAGATAAGGCAAGTGATTTTGAAAGATTTAGGGGAAGTAATTCGGCAC
TTATTTTTGTTAATGAGGCTACAACTTTACACAAGCAAACTTTAGAGGAAGTCTT
AAAAAGACTAAGATGCGGGCAAGAAACTATTATTTTTGATACTAATCCTGATCAT
CCAGAACACTATTTTAAAACCGATTATATTGATAATATAGCGACCTTTAAGACATA
TAAGTTTACAACTTATGATAATGTGCTACTTAGTAAAGGATTTGTCGAAACACAA
GAAAAGCTATATAAAGATATACCATCATATAAAGCAAGAGTTTTGTTAGGTGAGT
GGATAGCAAGCACTGATTCAATTTTTACACAAATAAATATTACTGATGATTATGTA
TTTACTAGCCCGATAGCATATTTAGACCCAGCATTTAGTGTTGGCGGGGATAAC
ACTGCATTATGTGTTATGGAGCGAGTTGATGATAAGTATTATGCTTTTGTATTTC
AAGACCAAAGACCAGCTAATGATCCTTATATTATGAATATGGTAAAGACTGTTAT
AGAAAATTTCAATGTGCATACACTGTATTTAGAGGATAGAGATAATACAAAAGGT
GCTGGTGGATTGACCCGTGAATACATCTTGCTAAGAAGTAATATAAGCCAATATT
TTAGAATTGTTCCAGTTAAGCCAAAGTCTAATAAATTTAGCAGAATAACAACGTT
AATTACGCCGTTTACTTACAAAAAACTTTATATTACAAAGTACAGTAGTTCTTCCG
TATTTAATGATATTTATTCGTATAAGGGGGATAATAAAACCCATGATGACGCTCT
TGATGCAATATCTGCAGCATATTTGATGTTGTCTTTAGGATATAGAGAGCGAAGT
GTTCACTTTGGCAATCAAAGATTTTTGTAA3'.

Alternatively or additionally, the phage gene may be the holin (SEQ ID NO.s 17-25), endolysin (SEQ ID NO,s 26-34), integrase (SEQ ID NO. 16), capsid and/or portal (SEQ ID NO. 11-15) gene(s). Alternatively, the phage gene may be a gene capable of encoding a holin protein according to the sequence of SEQ ID NO.s 52-60 or an endolysin protein (SEQ ID NO.s 61-69) or an integrase protein (SEQ ID NO 51) or a capsid and/or portal protein (SEQ ID NO.s 46-50). Optionally, the gene detected may be a nucleic acid with a greater than 70-100 % sequence homology with any of SEQ ID NO.s 11-34) or a gene capable of encoding a protein with a greater than 70-100% sequence homology with SEQ ID NO.s 36-69. For example, greater than or equal to 70, 75, 80, 85, 90 or 95, 96, 97, 98, 99 or 99.5% sequence homology with SEQ ID NO.s 11-34 or 36-69. With regards to homologous genes and proteins, preferably these retain the function of the holin, portal, endolysin, integrase or capsid protein.

Instead of percentage sequence homology, a homologue of the phage gene may alternatively be defined as one with addition, substitution and/or deletion of 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 30, 40 or 50 contiguous or non-contiguous nucleotides, preferably whilst retaining function of the protein encoded by the gene, the addition, substitution and deletion being relative to the unmodified sequence of SEQ ID NO.s 1-34.

Detection may also involve detection of a fragment of any of the holin, endolysin, integrase, portal, capsid or terminase protein, or combinations thereof. For example, detection of a 50-1300 base pair fragment. For example, 50, 100, 150, 200, 250, 300, 350, 400, 450, 500, 550, 600, 650, 700, 750, 800, 850, 900, 950, 1000, 1050, 1100, 1150, 1200, 1250 or 1300 contiguous base pairs or any range of base pairs based on these values. Optionally, the gene fragment to be detected may be a nucleic acid with equal to or greater than 70-100 % sequence homology with any fragment of SEQ ID NO.s 1-34. For example, equal to or greater than 70, 75, 80, 85, 90 or 95, 96, 97, 98, 99 or 99.5% sequence homology with any fragment of SEQ ID NO.s 1-34, preferably whilst retaining function of the protein encoded by the gene.

For example, a fragment of the terminase gene detected may be:

GAGTGGATAGCAAGCACTGATTCAATTTTTACACAAATAAATATTACTGATGATT

ATGTATTTACTAGCCCGATAGCATATTTAGACCCAGCATTTAGTGTTGGCGGGG

ATAACACTGCATTATGTGTTATGGAGCGAGTTGATGAT (SEQ ID NO. 35) or any sequence with equal to or greater than 70-100 % sequence homology with SEQ ID NO. 35). For example, equal to or greater than 70, 75, 80, 85, 90 or 95, 96, 97, 98, 99 or 99.5% sequence homology with SEQ ID NO. 35.

The following describes nucleic acid sequences specific to RF *Borrelia* phage.

The phage gene may be the RF *Borrelia* terminase gene, when this is the case the gene may be a nucleic acid according to the sequence of SEQ ID NO.s 84 or 86 or a gene capable of encoding a protein according to the sequence of SEQ ID NO.s 85 or 87. Optionally, the terminase gene may be a nucleic acid with equal to or greater than 70-100 % sequence homology with SEQ ID NO.s 84 or 86; or a gene capable of encoding a protein with equal to or greater than 70-100% sequence homology with SEQ ID NO.s 85 or 87. For example, equal to or greater than 70, 75, 80, 85, 90 or 95, 96, 97, 98, 99 or 99.5% sequence homology with SEQ ID NO.s 84 or 86, preferably whilst retaining function of the terminase protein encoded by the gene.

Alternatively or additionally, the phage gene may be the holin (SEQ ID NO.s 97-100), endolysin (SEQ ID NO. 101), integrase (SEQ ID NO.103), capsid and/or portal (SEQ ID NO.s 102) gene(s). Alternatively, the phage gene may be a gene capable of encoding a holin protein according to the sequence of SEQ ID NO.s 104-107 or an endolysin protein (SEQ ID NO. 108) or an integrase protein (SEQ ID NO. 110) or a capsid and/or portal protein (SEQ ID NO. 109). Optionally, the gene detected may be a nucleic acid with a greater than 70-100 % sequence homology with any of SEQ ID NO.s 97-103) or a gene capable of encoding a protein with a greater than 70-100% sequence homology with SEQ ID NO.s 104-1 10. For example, greater than or equal to 70, 75, 80, 85, 90 or 95, 96, 97, 98, 99 or 99.5% sequence homology with SEQ ID NO.s 97-103 or 104-1 10. With regards to homologous genes and proteins, preferably these retain the function of the holin, portal, endolysin, integrase or capsid protein.

Instead of percentage sequence homology, a homologue of the phage gene may alternatively be defined as one with addition, substitution and/or deletion of 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 30, 40 or 50 contiguous or non-contiguous nucleotides, preferably whilst retaining function of the

protein encoded by the gene, the addition, substitution and deletion being relative to the unmodified sequence of SEQ ID NO.s 84, 86 or 97-103.

Detection may also involve detection of a fragment of any of the holin, endolysin, integrase, portal, capsid or terminase protein, or combinations thereof. For example, detection of a 50-1300 base pair fragment. For example, 50, 100, 150, 200, 250, 300, 350, 400, 450, 500, 550, 600, 650, 700, 750, 800, 850, 900, 950, 1000, 1050, 1100, 1150, 1200, 1250 or 1300 contiguous base pairs or any range of base pairs based on these values. Optionally, the gene fragment to be detected may be a nucleic acid with equal to or greater than 70-100 % sequence homology with any fragment of SEQ ID NO.s 84, 86 or 97-103. For example, equal to or greater than 70, 75, 80, 85, 90 or 95, 96, 97, 98, 99 or 99.5% sequence homology with any fragment of SEQ ID NO.s 84, 86 or 97-103, preferably whilst retaining function of the protein encoded by the gene.

The following description applies to the detection of the entire phage gene or a gene fragment for a phage specific for *B. burgdorferi s.l.* or RF *Borrelia.*

Isolation of the nucleic acid from a sample

In order to assist molecular biological tools to practice the step of detecting, step a) may be preceded by the step of isolating nucleic acid from the sample, and step a is practiced on the isolated nucleic acid. Any method available to the skilled person that is capable of isolating nucleic acid from the sample would be appropriate for use in the present application. Not wishing to be restricted further, but in the interest of clarity, isolation of the nucleic acid from the sample may be performed using phenol chloroform extraction and isopropanol precipitation, or using membrane based nucleic acid isolation kits (such as QIAGEN® kits; QIAAmp DNA stool mini kit®).

The inventors have devised an efficient method for isolation of nucleic acid from a sample containing *Borrelia.* Therefore, also provided is a method of extracting phage DNA from *Borrelia,* the method comprising: a) incubating the Borrelia in ammonium hydroxide; and b) adding phenol-chloroform to the *Borrelia* and ammonium hydroxide mixture.

The method may further comprise centrifugation to remove bacterial debris. The resulting supernatant following removal of the bacterial debris may be mixed with sodium acetate. Isopropanol may then be added to the supernatant and sodim acetate mixture. A further step of centrifugation may then be carried out to precipitate the phage DNA

The same volume of phenol-chloroform may be used as the volume of ammonium hydroxide.

Part a) may be carried out at a temperature of 50-150°C. For example, 70-130°C, 80-120°C or 90-110°C.

The ammonium hydroxide concentration may be 0.5-1 M. For example, 0.6M, 0.7M, 0.8M, 0.9M.

Further exemplary details are provided in the examples section under the heading "PCR and sequencing".

Detection of the nucleic acid

Any method known to the skilled person and capable of detecting the presence or absence of a gene (optionally in an isolated nucleic acid) may be suitable for use in step a) of the presently claimed invention. As the phage gene may be present in relatively low copy number, in order to make detection easier, in one embodiment of the present invention the method of detecting involves subjecting the isolated nucleic acid to amplification of the phage gene, e.g. by real time polymerase chain reaction (qPCR).

Alternatively or additionally detection of the phage gene may be confirmed by nucleic acid sequence analysis, by detection of labelled nucleotides inserted in the amplified product, or by detection of hybridisation of probe that is specific to the phage gene (hybridisation being detected, for example, by use of labelled probes).

Apart from Taqman-based qPCR platform, other methods of nucleic acid detection that can be used include SYBR green-based real time PCR assay, digital PCR which involves splitting the same qPCR mix into a large number of individual wells. The

endpoint PCR products are determined using Poisson statistical analysis according to the presence (scored as '1') and absence (scored as '0') of fluorescent signal in each well. Other possible methods include Loop mediated isothermal amplification (LAMP), an isothermal nucleic acid amplification technique that does not require a thermal cycler. LAMP could be employed to target terminase, holin, endolysin, integrase, capsid, and portal proteins; and DNA hybridisation based methods such as Fluorescence in situ hybridisation (FISH), which works by performing a DNA/DNA hybridisation using fluorescently labelled short DNA strands (the phage genes) as probes to hybridise to its complementary parts on genomic DNA.

Detection may include the use of an agent which is capable of detection (a label) using for example spectrophotometry, flow cytometry, or microscopy. Exemplary labels include radioactive isotopes (such as 3H, 14C, 15N, 35S, 90V, 99Tc, 111Ln, 1251, or 1311), fluorophores (such as fluorescein, fluorescein isothiocyanate, rhodamine or the like), chromophores, ligands, chemiluminescent agents, bioluminescent agents (such as luciferase, green fluorescent protein (GFP) or yellow fluorescent protein), enzymes that can produce a detectable reaction product (such as horseradish peroxidise, luciferase, alkaline phosphatase, beta-galactosidase) and combinations thereof.

The following relates to primers specific for *B. Burgdorferi s.l.* phage.

The step of amplification may involve the use of a forward primer selected from the group consisting of nucleic acids comprising or consisting of SEQ ID NO 70:
5'GTGAACTTATATCAAAC3'

Alternatively, or additionally, the step of amplification involves the use of a reverse primer selected from the group consisting of nucleic acids comprising or consisting of SEQ ID NO. 71:
5'ATAATCTTCTTTTCATT3'

Alternatively or additionally, the step of amplification may involve the use of any one or more of the primers of SEQ ID NO.s 73-77 and/or 79-83.

Where identification of the phage gene is achieved by the use of hybridisation probes, suitable probes could be any capable of specifically binding to the phage gene, or portion thereof. For example, appropriate probes may be any one or more of the aforementioned primers comprising or consisting of SEQ ID NO.s 70-77 and/or 79-83, or nucleic acids comprising or consisting of SEQ ID NO.s 1-34 or fragment thereof, any homologue thereof, or specifically binding portions thereof, or any combination thereof.

The following relates to primers specific for RF Borrelia phage.

The step of amplification may involve the use of any of primers comprising or consisting of SEQ ID NO.s 88-96, any homologue thereof, or specifically binding portions thereof, or any combination thereof.

Where identification of the phage gene is achieved by the use of hybridisation probes, suitable probes could be any capable of specifically binding to the phage gene, or portion thereof. For example, appropriate probes may be any one or more of the aforementioned primers comprising or consisting of SEQ ID NO.s 88-96, or nucleic acids comprising or consisting of SEQ ID NO.s 84, 86 and/or 97-103 or fragment thereof, any homologue or thereof, or specifically binding portions thereof, or any combination thereof.

Detection may also comprise detection of a phage RNA or RNA fragment. For example, an RNA fragment of 50, 100, 150, 200, 250, 300, 350, 400, 450, 500, 550, 600, 650, 700, 750, 800, 850, 900, 950, 1000, 1050, 1100, 1150, 1200, 1250 or 1300 base pairs or any range of base pairs based on these values.

For example, presence of the phage gene may also be detected by detection of the corresponding mRNA. This can be achieved by performing a reverse transcriptase real time PCR by performing a real time PCR against cDNA synthesised from total RNA. For example, by hybridisation of primers to mRNA. The methodology used in PCR methods, for example RT-PCR, will be well known to those skilled in the art.

Apart from PCR, there are probe-based methods such as Northern blotting, in situ hybridisation. There are also RNA sequencing method that can be used in detecting phage RNA via sequencing the total RNA.

Some methods may require the isolation of RNA from a sample. Such isolation techniques are known in the art and may utilise commercially available RNA isolation kits from manufacturers such as Qiagen or Maxwell viral total nucleic acid purification kit from Promega or the isolation methods described above with regards to gene sequence detection.

Detection of phage proteins

Detection may also comprise detection of a phage protein or protein fragment.

Other ways of detecting the Borrelia phage include detection of the phage specific proteins.

In the case of LD, the phage protein is one specific to a *B burgdorferi s.l.* host (i.e. the amino acid sequence is found in only the target host, e.g. *B. burgdorferi s.l.,* or in a phage specific for such a host, or part thereof).

Suitable proteins for LD detection include holin (SEQ ID NO.s 52-60), endolysin (SEQ ID NO. 61-69), integrase (SEQ ID NO. 51), portal (SEQ ID NO. 46-50), capsid and/or terminase (SEQ ID NO.s 36-45).

Optionally, the amino acid sequence detected may have equal to or greater than 70-100 % sequence homology with SEQ ID NO.s 36-69 or may be encoded by a gene having equal to or greater than 70-100 % sequence homology with SEQ ID NO.s 1-34. For example, equal to or greater than 70, 75, 80, 85, 90 or 95, 96, 97, 98, 99 or 99.5% sequence homology with SEQ ID NO.s 36-69 or 1-34. With regards to homologous genes and proteins, preferably these retain the function of the holin, portal, endolysin, integrase or capsid protein.

In the case of RF, the phage protein is one specific to a RF *Borrelia* host (i.e. the amino acid sequence is found in only the target host, e.g. RF *Borrelia* or in a phage specific for such a host, or part thereof).

Suitable proteins for RF detection include terminase (SEQ ID NO.s 85 or 87), holin (SEQ ID NO.s 104-107), endolysin (SEQ ID NO. 108), portal protein (SEQ ID NO. 109) and/or integrase (SEQ ID NO. 110).

Optionally, the amino acid sequence detected may have equal to or greater than 70-100 % sequence homology with SEQ ID NO.s 85, 87 or 104-110 or may be encoded by a gene having equal to or greater than 70-100% sequence homology with SEQ ID NO.s 84, 86 or 97-103. For example, equal to or greater than 70, 75, 80, 85, 90 or 95, 96, 97, 98, 99 or 99.5% sequence homology with SEQ ID NO.s 84-87 or 97-110. With regards to homologous genes and proteins, preferably these retain the function of the holin, portal, endolysin, integrase or capsid protein.

Instead of percentage sequence homology, a homologue may be defined as a protein with addition, substitution and/or deletion of 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 30, 40 or 50 contiguous or non-contiguous amino acids, preferably whilst retaining function of the protein, the addition, substitution and deletion being relative to the unmodified sequence of SEQ ID NO.s 85, 87 or 104-100.

Detection may also involve detection of a fragment of any of the holin, endolysin, integrase, portal, capsid or terminase proteins, or combinations thereof. For example, detection of an epitope on any of these proteins by an antibody specific for Borrelia burgdorferi sensu lato. The epitope may be a linear epitope. For example, a fragment may comprise a stretch of amino acid residues of at least 5 to 10 contiguous amino acids, 10 to 15 contiguous amino acids, 15 to 20 contiguous amino acids, or 20 to 30 or more contiguous amino acids. Or the epitope may be a non-contiguous epitope specific to the phage protein. For example, a non-contiguous epitope comprising 5, 10, 15 or 20 amino acids.

Optionally, the protein fragment to be detected may be an amino acid with equal to or greater than 70-100 % sequence homology with any fragment of SEQ ID NO.s 36-

69, 85, 87 or 104-1 10). For example, equal to or greater than 70, 75, 80, 85, 90 or 95, 96, 97, 98, 99 or 99.5% sequence homology with any fragment of SEQ ID NO.s 36-69, 85, 87 or 104-1 10 whilst retaining the function of the protein.

Protein specific detection can be carried out by any method known in the art. For example, Immunohistochemistry (IHC) can be used to detect protein expression. Western blotting and ELISA are also methods useful for detecting protein expression and secretion. Antibodies or other proteins or molecules capable of selective binding to the phage proteins can be used for detection. Protein can also be detected by MALDI-TOF mass spectrometry. Protein sequencing, for example by mass spectrometry or Edman degradation, can also be used for detection. Linear fragments of 10, 20, 30, 40, 50, 60, 70, 80, 90, 100, 110, 120, 130, 140, 150, 160, 170, 180, 190, 200, 300, 400, 500 amino acids or any range of these numbers may be sequenced.

Phage protein can be detected using a primary antibody with binding specificity for the phage protein. The primary antibody can be labelled with a detectable moiety or can be conjugated to a hapten (such as biotin or the like) wherein the hapten is detectable by a detectably labelled cognate hapten binding molecule, for example streptavidin horseradish peroxidase. Alternatively, a secondary antibody can be used which specifically binds the first primary antibody and instead this secondary antibody may be detectable as described above for the primary antibody.

The binding specificity of phage antibodies (antibodies with binding specificity to a phage protein) can be established using, for example, Western blotting.

The term antibody refers to an immunoglobulin molecule or combinations thereof that specifically binds to or is immunologically reactive with a particular antigen and includes polyclonal, monoclonal, genetically engineered and otherwise modified forms of antibodies, not limited to chimeric antibodies, humanised antibodies, heteroconjugate antibodies (for example bispecific antibodies, diabodies, triabodies, and tetrabodies), single chain Fv antibodies (scFv), or polypeptides that contain at least a portion of immunoglobulin that is sufficient to confer specific antigen binding to the polypeptide. Antibody fragments include proteolytic antibody fragments such as F(ab')2 fragments, Fab' fragments, Fab'-SH fragments, Fab fragments, FV, rlgG,

recombinant antibody fragments such as sFv fragments, dsFv fragments, bispecific sFv fragments, bispecific dsFv fragments, complementarity determining region (CDR) fragments, camelid antibodies and antibodies produced by cartilaginous and bony fishes and isolated binding domains thereof. A Fab fragment is a monvalent fragment consisting of the VL, VH, CL and CH1 domains; a F(ab')2 fragment is a bivalent fragment comprising two Fab fragments linked by a disulphide bridge at the hinge region, an Fd fragment consists of the VH and CH1 domains; an FV fragment consists of the VL and VH domains of a single arm of an antibody; and a dAb fragment consists of a VH domain. A single chain antibody (scFv) is an antibody in which a VL and VH region are paired to form a monovalent molecule via a synthetic linker that enables them to be made as a single protein chain. Diabodies are bivalent, bispecific antibodies in which the VH and VL domains are expressed on a single polypeptide chain, but using a linker that is too short to allow for pairing between the two domains on the same chain, thereby forcing the domains to pair with complementary domains of another chain and creating two antigen binding sites. A chimeric antibody is an antibody that contains one or more regions from one antibody and one or more regions from one or more other antibodies. An antibody may have one or more binding sites. If there is more than one binding site, the binding sites may be identical to one another or may be different. For instance, a naturally occurring immunoglobulin has two identical binding sites, a single chain antibody or Fab fragment has one binding site, while a bispecific or bifunctional antibody has two different binding sites.

Alternatively, other proteins which are capable of selective binding may also be used for detection.

For example, the phage protein may be detected using aptamers (for example a single stranded nucleic acid molecule (such as, DNA or RNA) that assumes a specific, sequence dependent shape and binds to the phage protein with high affinity and specificity), mirror image aptamers (SPIEGELMER™), engineered nonimmunuoglobulin binding proteins, for example nonimmunoglobulin binding proteins based on scaffolds including fibronectin (ADNECTINS™), CTLA-1 (EVIBODIES™), lipocalins (ANTICALINS™), protein A domain (AFFIBODIES™) or the like.

Homology

With regards to nucleic acid sequences, homology may be defined as to a nucleotide sequence which encodes a protein with a similar function. With regards to protein sequences, homology may be defined as to a protein with a similar function. For example, a protein with the same function from another *Borrelia burgdorferi sensu lato or* RF *Borrelia* species.

Percentage homology can be defined as the percentage of identical residues and the percentage of residues conserved with similar physiochemical properties. The degree of homology between sequences may be determined using any suitable method known in the art (See e.g., Smith and Waterman, Adv. Appl. Math., 2:482 [1981]; Needleman and Wunsch, J. Mol. Biol, 48:443 [1970]; Pearson and Lipman, Proc. Natl. Acad. Sci. USA 85:2444 [1988]; programs such as GAP, BESTFIT, FASTA, and TFASTA in the Wsconsin Genetics Software Package (Genetics Computer Group, Madison, Wl); and Devereux et al., Nucl. Acid Res., 12:387-395 [1984]).

The above percentages of homology may equally apply to percentage sequence identity only. For example, the percentage of nucleic acid residues which are identical between the terminase nucleic acid sequence and another terminase sequence, which may for example, be from a different *Borrelia* species.

Instead of percentage sequence homology, a homologue of the phage gene may be defined as one with addition, substitution or deletion of 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 30, 40 or 50 contiguous or non-contiguous nucleotides, preferably whilst retaining function of the protein encoded by the gene, the addition, substitution and deletion being relative to the unmodified sequence.

Sequences

In a further aspect of the present invention there is provided the phage genes, primers, nucleic acids and hybridising probes; and amino acid sequences as defined above.

For reference, a table of the various gene sequences isolated from various *B. burgdorferi s.l.* in the sequence listing is provided below along with the corresponding proteins.

SEQ ID NO.	Description	Corresponding protein SEQ ID NO.
1-10	Terminase gene sequences	36-45
11-15	Portal gene sequences	46-50
16	Integrase gene sequence	51
17-25	Holin gene sequences	52-60
26-34	Endolysin gene sequences	61-69
35	Terminase 147bp fragment	

The SEQ ID NO.s of the primers used for gene sequence detection are also provided:

SEQ ID NO.	Description
70 and 71	Terminase Forward and Reverse Primers (these are primers for end-point, conventional, gel-based PCR)
72 and 73	Holin Forward and Reverse Primers
74 and 75	Endolysin Forward and Reverse Primers
76 and 77	Forward and Reverse Primers to amplify SEQ ID NO. 78
78	Gene sequence of combined Holin and Endolysin
79	Forward primer for Terminase 147bp fragment
80	Reverse primer for Terminase 147bp fragment
81	Probe for Terminase 147bp fragment
82	forward primer for SEQ ID NO. 1 (terminase); 721 bp fragment
83	reverse primer for SEQ ID NO. 1 (terminase); 721 bp fragment

Provided below is a table of the various gene sequences isolated from various *Borrelia* species in the sequence listing which are causative agents of RF:

SEQ ID NO.	Description	Corresponding protein SEQ ID NO.
84	Borrelia miyamotoi terminase	85
86	Borrelia hermsii terminase	87
97	Borrelia parkeri holin	104
98	Borrelia turicatae holin	105
99	Borrelia hermsii holin	106
100	Borrelia coriaceae holin	107
101	Borrelia hermsii endolysin	108
102	Borrelia turicatae portal	109
103	Borrelia turicatae integrase	110

The SEQ ID NO.s of the primers used for gene sequence detection are also provided:

SEQ ID NO.	Description
88	B. miyamotoi terminase probe (120bp amplicon)
89 and 90	B. miyamotoi terminase forward and reverse primers (120bp amplicon)
91	B. miyamotoi terminase probe (87bp amplicon)
92 and 93	B. miyamotoi terminase forward and reverse primers (87bp amplicon)
94	B. hermsii terminase probe
95 and 96	B. hermsii forward and reverse primers (124 bp amplicon)

Homologues and fragments of these nucleic acid and protein sequences are also provided in accordance with the description above for the detection method.

Also provided are use of the above sequences, homologues and fragments as diagnostic markers for LD or RF.

Species detection

The *Borrelia burgdorferi sensu lato* may be any that are the causative agent for LD.

For example any of *Borrelia afzelii, Borrelia spielmanii, Borrelia valaisiana, Borrelia garinii, Borrelia finlandensis, Borrelia bugdorferi sensu strictu, Borrelia bissettii, Borrelia bavariensis, Borrelia japonica, Borrelia lusitaniae, Borrelia sinica, Borrelia spielmanii, Borrelia tanukii, Borrelia turdi, Borrelia valaisiana, Borrelia Yangtze, Borrelia mayonii, Borrelia carolinensis, Borrelia andersonii, Borrelia lonestari, and Borrelia Americana* or any combination thereof. For example, it may be *Borrelia afzelii, Borrelia bugdorferi sensu strict, Borrelia garinii,* or any combination thereof. For example, it may be *Borrelia bugdorferi sensu strictu,* or any combination of *Borrelia bugdorferi sensu strictu* with any of the above.

The RF Borrelia may be any that are the causative agent for RF. For example, any of *Borrelia miyamotoi, Borrelia hermsii, Borrelia recurrentis, Borrelia crocidurae, Borrelia duttoni, Borrelia hispanica, Borrelia parkeri* and *Borrelia turicatae* or any combination thereof.

The inventors were surprised that when analysing *Borrelia burgdorferi sensu lato* species specific phage genes further, these genes not only showed specificity to *Borrelia burgdorferi sensu lato,* but also specifically to species within that group. The RF Borrelia specific phage genes were also found to be specific to particular species.

None of the known methods can distinguish between different species of *Borrelia.* This can be an important advantage as knowing what species of *Borrelia* is present can educate the treatment regimen and thereby optimise the success of treatment (as some species of *Borrelia* respond better to specific antibiotics than do others).

Consequently, the method may determine the presence or the absence of a species of *Borrelia burgdorferi sensu lato or* RF *Borrelia* in the sample, the method comprising the steps of:-

a) detecting the presence or absence of the *Borrelia burgdorferi sensu lato* or RF *Borrelia* species specific phage gene in the sample;

b) determining the presence of the species of the *Borrelia burgdorferi sensu lato* or RF *Borrelia* in the sample on the basis of the detection

of the *species* specific phage gene, or the absence of the species of *Borrelia burgdorferi sensu lato or* RF *Borrelia* in the sample on the basis of the lack of detection of the species specific phage gene.

In order to assist molecular biological tools to practice the step of detecting, step a) may be preceded by the step of isolating nucleic acid from the sample, and step a) is practiced on the isolated nucleic acid. Any method available to the skilled person that is capable of isolating nucleic acid from the sample would be appropriate for use in the present application. Not wishing to be restricted further, but in the interest of clarity, isolation of the nucleic acid from the sample may be performed using phenol chloroform extraction and isopropanol precipitation, or using membrane based nucleic acid isolation kits (such as QIAGEN® kits; QIAAmp DNA stool mini kit®).

For example, when the bacteria is *Borrelia bugdorferi sensu strictu* the *Borrelia bugdorferi sensu strictu* specific phage gene may be any of those discussed above. For example, the gene may be a terminase gene (i.e. the phage gene), which can be that according to SEQ ID NO 1. Optionally the gene is a nucleic acid with equal to or greater than 70-99.5% sequence homology to any one of SEQ ID No. 1. The forward primer used to amplify the *Borrelia bugdorferi sensu strictu* specific terminase gene is a nucleic acid comprising or consisting of SEQ ID NO 70. The reverse primers may be a nucleic acid comprising or consisting of SEQ ID NO 71. Where identification of this gene is achieved by the use of hybridisation probes, suitable probes would be any of the aforementioned primers, nucleic acids comprising or consisting of SEQ ID NO 1-10 or 35, any homologue thereof, or specifically binding portions thereof, or any combination thereof.

For example, when the bacteria is *Borrelia miyamotoi,* the specific phage gene may be any of those discussed above. For example, the gene may be a terminase gene (i.e. the phage gene), which can be that according to SEQ ID NO 84. Optionally the gene is a nucleic acid with equal to or greater than 95, 96, 97, 98, 99 or 99.5% sequence homology to SEQ ID No. 84. The forward primer used to amplify the *Borrelia miyamotoi* specific terminase gene is a nucleic acid comprising or consisting of SEQ ID NO 89 or 92. The reverse primers may be a nucleic acid comprising or consisting of SEQ ID NO 90 or 93. Where identification of this gene is achieved by the use of hybridisation probes, suitable probes would be nucleic acids comprising or

consisting of SEQ ID NO 84, or any primer comprising or consisting of SEQ ID NO.s 88-93; any homologue thereof, or specifically binding portions thereof, or any combination thereof.

Kit

In a further aspect of the present invention there is a kit for determining the presence or the absence of *Borrelia burgdorferi sensu lato and or* RF *Borrelia* in a sample, the kit may comprise one or more of the aforementioned primers for specifically hybridising with nucleic acid sequence of a Borrelia specific phage gene. The selection of the primer, or preferably primer pairs (i.e. forward and reversed primers) would be guided by the target *Borrelia burgdorferi sensu lato* or RF *Borrelia* species. The kit may further comprise hybridisation probes which may be the aforementioned primers, or any of the aforementioned probes. Again, the choice of probes would be selected on the basis of the target species to be identified by the kit. The choice of hybridisation probes for a kit for determining the presence of *Borrelia burgdorferi sensu lato,* or indeed a kit for identifying a specific species of such bacteria can be extrapolated from the details of appropriate hybridisation probes discussed above. As an alternative to nucleic acid probes, antibodies which bind to phage specific proteins may be included in a kit. Such antibodies are discussed above.

Monitoring the progression of infection

The method may be applied to monitoring the progression of an infection from *Borrelia burgdorferi sensu lato or* RF *Borrelia;* or a species of such bacteria based on the teachings of the earlier aspects of the present invention.

The terms used in this aspect of the present invention are the same as, and so refer back to, the terms used in earlier aspects of the present invention.

In a further aspect of the present invention there is provided a method of monitoring the progression of infection from Borrelia burgdorferi sensu lato or RF *Borrelia.* The method comprising steps of:-

a) determining the amount of phage in a first sample obtained from the subject;

b) detecting the amount of phage in a second sample obtained from the subject at a second time point;

c) comparing the amount of phage in the first sample identified in step a) with that identified for the second sample in step b).

The method may include the step of isolating a first population of nucleic acid from a first sample obtained from the subject at a first time period, prior to step a). The method may include the step of isolating a second population of nucleic acid from a second sample obtained from the subject at a second time point, prior to step b).

Treatment

Detection of the presence of *Borrelia burgdorferi sensu lato* in a sample from a subject confirms a diagnosis that the subject has LD. Treatment for *Borrelia burgdorferi sensu lato* infection is the treatment of Lyme disease.

Detection of the presence of an RF *Borrelia* in a sample from a subject confirms a diagnosis that the subject has RF.

Any of the methods described may include the step of administering an antibiotic or more than one antibiotic. Such a method may be capable of determining the ability for the antibiotic to treat *Borrelia burgdorferi sensu lato or* RF infection, or species specific *Borrelia burgdorferi sensu lato* or RF infection.

The following relates to treatment of LD:

The recommended treatment for infection is a combined therapy comprising one, two, or sometimes three, antibiotics used at the same time. This may be in the form of sequential treatments or synchronously combined long-term antibiotic treatment. Antibiotics such as tetracyclines can be used for treatment alone or in combination with hydroxychloroquine. This may involve simultaneous administration of the antibiotics.

The main antibiotics recommended for use are: 1) pencillins; 2) cephalosporins; 3) macrolides; 4) fluroquinolones and/or 5) cyclines.

Any of the above antibiotics may be combined or used sequentially.

As an example, cephalosporins can be used alone or in combination with minocycline. This treatment may involve alternating between the two antibiotics. Doxycycline and/or minocycline can be combined with azithromycin and/or hydroxychloroquine. Other combinations based on the above classes of antibiotics or other antibiotics are possible.

The terms used in this aspect of the present invention are the same, and so refer back to the same terms used in earlier aspects of the present invention.

The following relates to treatment of RF:

For relapsing fever (RF), treatment uses the same classes of antibiotic as for LD. Treatment for infection may be a combined therapy comprising one, two, or sometimes three, antibiotics used at the same time. This may be in the form of sequential treatments or synchronously combined long-term antibiotic treatment. Any of the above antibiotics may be combined or used sequentially.

For example, oral treatment may include a daily single dose of any of an antibiotic from any of the above classes 1-5. For example, oral treatment may consist of a daily single dose of tetracycline 500 mg, doxycycline 200 mg, or, when tetracyclines are contraindicated, erythromycin 500 mg. Treatment duration may be up to 7-10 days or more owing to reported relapses of 20% or greater after single-sequence. In adults, intravenous therapy with doxycycline, erythromycin, tetracycline, or procaine penicillin G may be used when oral therapy is not tolerated.

Procaine penicillin G may be administered at a single dose of 600,000 IU in adult patients with LBRF or 600,000 IU daily in patients with RF.

For example, in a further aspect of the present invention there is provided a method of treating an infection resulting from *Borrelia burgdorferi sensu lato or* RF *Borrelia,* or species of *Borrelia burgdorferi sensu lato or* RF *Borrelia,* the method comprising steps of:-

a) identifying *Borrelia burgdorferi sensu lato* or *RF Borrelia* infection, or the species of *Borrelia burgdorferi sensu lato or* RF *Borrelia* infection, using the method according to the first aspect of the present invention;

b) selecting at least one antibiotic that is suitable for treating *Borrelia burgdorferi sensu lato* or RF *Borrelia* infection, or the determined species of *Borrelia burgdorferi sensu lato or* RF *Borrelia;*

c) administering the selected antibiotic(s) to the subject identified in step a as being infected by *Borrelia burgdorferi sensu lato or* RF *Borrelia.*

In a further aspect of the present invention there is provided a method of treating an infection of *Borrelia burgdorferi sensu lato or* RF *Borrelia,* or species of *Borrelia burgdorferi sensu lato or* RF *Borrelia,* the method comprising steps of:-

a) selecting at least one antibiotic that is suitable for treating *Borrelia burgdorferi sensu lato or* RF *Borrelia* infection, or the determined species of *Borrelia burgdorferi sensu lato or* RF *Borrelia;*

b) administering the selected antibiotic(s) to a subject that had been identified as having *Borrelia burgdorferi sensu lato* or RF *Borrelia* infection, or the species of *Borrelia burgdorferi sensu lato or* RF *Borrelia* infection, by the method according to the first aspect of the present invention.

Alternatively, the method may be applied for research purposes, for example, research into the physiological effects of *Borrelia burgdorferi sensu lato or* RF *Borrelia.* The method may also be applied in clinical situations when it is important to determine if a subject has been infected with the *Borrelia burgdorferi sensu lato* or RF *Borrelia.* Consequently, in one embodiment in the present invention, the method is a method of diagnosing infection of *Borrelia burgdorferi sensu lato* or RF *Borrelia* or species of *Borrelia burgdorferi sensu lato or* RF *Borrelia* in the subject, the method comprising the steps:-

a) detecting the presence or absence of phage or species specific phage in the sample;

b) determining that the subject is infected with *Borrelia burgdorferi sensu lato or* RF *Borrelia,* or a species thereof by detecting the phage or species specific phage in the sample, or determining that the subject is not infected with *Borrelia burgdorferi sensu lato or* RF *Borrelia* or a species thereof, by the lack of detection of the phage, or a species thereof, in the sample.

The terms used in this aspect of the invention are the same as, and so refer back to, the same terms used in earlier aspects of the present invention. For example, samples used in each of the methods of the present invention may be obtained shortly after infection (i.e. early stage infection), or after the infection has been well developed and that bacteria have become sequestered (i.e. late stage infection).

The step of detection for all methods of the invention may be a quantification step, i.e. the amount of phage gene, RNA or protein is calculated. The methods for all methods of the invention may involve the step of taking the sample from a subject. Wherein all the methods of the invention may involve the selection of subjects for treatment for LD when a sample from the subject is determined to be positive for the presence of *Borrelia burgdorferi sensu lato;* or may involve the selection of subjects for treatment for RF when a sample from the subject is determined to be positive for the presence or RF *Borrelia.*

The present invention will now be described, by way of example, with reference to the accompanying figures, in which:-

Fig. 1 shows phylogenetic analysis based on the terminase protein showing that the Lyme Borrelia species are closely related to each other but naturally separated from other Borrelia strains. Scale bar represents 0.2 amino acid changes per site.

Fig. 2 shows phylogenetic analysis based on the holin protein showing that the Lyme Borrelia species are closely related to each other but naturally separated from other Borrelia strains. Scale bar represents 0.2 amino acid changes per site.

Fig. 3 shows phylogenetic analysis based on the endolysin protein showing that the Lyme Borrelia species are closely related to each other but naturally separated from

other Borrelia strains. The top 10 (from *B. Afzelii* to *B. Valaisiana)* are Lyme Borrelia strains, The next 8 (from *B. hermsii* to *B. duttonii)* are Relapsing fever Borrelia strains. Scale bar represents 0.2 amino acid changes per site.

Figure 4 shows phylogenetic analysis based on the portal protein showing that the Lyme Borrelia species are closely related to each other but naturally separated from other Borrelia strains. Scale bar represents 0.2 amino acid changes per site.

Figure 5 shows phylogenetic analysis shows phylogenetic analysis based on the integrase protein showing that the Lyme Borrelia species are closely related to each other but naturally separated from other Borrelia strains. Scale bar represents 0.2 amino acid changes per site.

Figure 6 shows the detection limit of the phage-based method of the present invention, based on analysis of bacterial spiked human blood.

Figure 7 shows the detection limit of the phage-based method of the present invention in comparison to the standard bacterial 16S-based method.

Figure 8 shows the specificity of the phage-based method according to the present invention for four species of *Borrelia burgdorferi sensu lato.*

Figure 9 shows a series of five tenfold dilutions (10^6 down to 10^2 copies) of plasmid DNA carrying the terminase gene fragment. 10 technical repeats were performed for each dilution. As shown, positive amplifications were observed from four diluted plasmid DNA samples down to 10^2 copies.

Figure 10 shows a linear relationship between the concentration of plasmid DNA template and Ct values across with a strong correlation coefficient (R2=0.99). In addition, the amplification efficiency of BbFAM was 100%.

Figure 11 shows the performance of the phage-based assay against 222 serum samples of different background (Lyme positive, Lyme negative, Lyme borderline, healthy volunteers).

The present invention will now be described with reference to the following examples, which are by way of illustration alone. The following examples are not intended to completely define or otherwise limit the scope of the invention.

Examples

Lyme disease

Borrelia burgdorferi sensu lato isolates

Lab cultures of Borrelia burgdorferi sensu lato (s.l.) strains were provided by professor Sven Bergstrom (Umeå University, Sweden). Two Borrelia miyamotoi strains were provided by the CDC (Centers for Disease Control and Prevention, USA). The lab strains were maintained in BSKII medium. Routine characterisation was carried out using phase contrast microscope.

PCR and sequencing

DNA was extracted from serum samples using a novel method of combining ammonium hydroxide and phenol chloroform. 600 µl of samples (was incubated in the presence of 1.2 ml 0.7 M ammonium hydroxide at 100 °C for 5 min, followed by 10 min at 100 °C with the tube open. After the tube was cooled to room temperature, the samples were extracted with the same volume of phenol-chloroform (1:1). After incubation time of 5 min at RT, the solution was centrifuged for 10 min at 18 000 g. The clear supernatant was transferred into a new 2 ml tube and mixed with 0.1 volume of 3 M sodium acetate. This suspension was then mixed with 0.7 volumes of room-temperature isopropanol. DNA was precipitated down by centrifuging at 21 000 g for 10 min at 4°C. After decanting the supernatant, 1.5 ml of room-temperature 70% ethanol was added followed by centrifuging at 21 000 g for 10 min at 4°C. The resulting DNA pellet was briefly air dried for 5 min, and dissolved in 50-100 µl of a suitable buffer (such as elution buffer, EB, which is 10 mM Tris-Cl, pH 8.5).

PCR primers were designed manually against conserved regions in all known *Borrelia* phage terminase gene sequences. The primers amplify a 194 bp product from 8 lab all Lyme Borrelia burgdorferi s.l. strains. PCRs were carried out in a

LabCycler (SensoQuest GmbH, Gottingen, Germany) in a total volume of 50 µl, containing 0.25 mM dNTPs, 3 mM MgCl2, 2 µM primers, 50 ng of template DNA, 0.5 unit of Taq polymerase (Bioline), and 5 µl 10* Taq buffer (Bioline). Amplification conditions were: 94°C for 2 min, 30 cycles of 94°C for 45 sec, 48°C for 45 sec, 72°C for 1 min, with a final extension of 10 min at 72°C. PCR products were gel-purified using a Qiagen gel extraction kit, and subjected to TOPO TA cloning (Invitrogen). Sequencing was carried out by GATC Biotech. Sequencing results were edited using Chromas 2.33, searched using a nucleotide BLAST (NCBI).

***Borrelia burgdorferi sensu lato* species identification**

A previous reported Multi locus sequence typing (MLST) scheme was used to distinguish different genotypes of *Borrelia.*

Phylogenetic analysis

Phylogenetic analysis were constructed using the program Molecular Evolutionary Genetics Analysis (MEGA) package version 4.1 (Beta) (Tamura et al., 2007; Kumar et al., 2008). Alignment Explorer/CLUSTAL in MEGA 4.1 (Beta) was used to align the DNA sequences. NJ and MP analysis were conducted on a nucleotide data set; for NJ a maximum composite likelihood model was used and for MP a close-neighbour-interchange with a search level of 3 was used. Supports for clades were estimated using a bootstrap analysis implemented in MEGA using 1, 000 replicates. The trees were rooted with phage Lambda (NC_001416) as an outgroup.

Nucleic Acid Hybridisation

"Hybridization" refers to the association of two nucleic acid sequences to one another by hydrogen bonding. Typically, one sequence will be fixed to a solid support and the other will be free in solution. Then, the two sequences will be placed in contact with one another under conditions that favour hydrogen bonding.

Factors that affect this bonding include: the type and volume of solvent; reaction temperature; time of hybridization; agitation; agents to block the non-specific attachment of the liquid phase sequence to the solid support (Denhardt's reagent or BLOTTO); concentration of the sequences; use of compounds to increase the rate of

association of sequences (dextran sulphate or polyethylene glycol); and the stringency of the washing conditions following hybridization.

"Stringency" refers to conditions in a hybridization reaction that favour association of very similar sequences over sequences that differ. For example, the combination of temperature and salt concentration should be chosen that is approximately 120 to 200°C below the calculated Tm of the hybrid under study. The temperature and salt conditions can often be determined empirically in preliminary experiments in which samples of genomic DNA immobilized on filters are hybridized to the sequence of interest and then washed under conditions of different stringencies. See Sambrook et al. at page 9.50.

Variables to consider when performing, for example, a Southern blot are (1) the complexity of the DNA being blotted and (2) the homology between the probe and the sequences being detected. The total amount of the fragment(s) to be studied can vary a magnitude of 10, from 0.1 to 1mg for a plasmid or phage digest to 10-9 to 10-8 g for a single copy gene in a highly complex eukaryotic genome. For lower complexity polynucleotides, substantially shorter blotting, hybridization, and exposure times, a smaller amount of starting polynucleotides, and lower specific activity of probescan be used. For example, a single-copy yeast gene can be detected with an exposure time of only 1 hour starting with 1 mg of yeast DNA, blotting for two hours, and hybridizing for 4-8 hours with a probe of 108 cpm/mg.

For a single-copy mammalian gene a conservative approach would start with 10 mg of DNA, blot overnight, and hybridize overnight in the presence of 10% dextran sulphate using a probe of greater than 108 cpm/mg, resulting in an exposure time of 24 hours.

Several factors can affect the melting temperature (Tm) of a DNA-DNA hybrid between the probe and the fragment of interest, and consequently, the appropriate conditions for hybridization and washing. In many cases the probe is not 100% homologous to the fragment. Other commonly encountered variables include the length and total G+C content of the hybridizing sequences and the ionic strength and formamide content of the hybridization buffer. The effects of all of these factors can be approximated by a single equation: where Ci is the salt concentration

(monovalent ions) and n is the length of the hybrid in base pairs (slightly modified from Meinkoth & Wahl (1984) Anal. Biochem. 138: 267-284).

In designing a hybridization experiment, some factors affecting nucleic acid hybridization can be conveniently altered. The temperature of the hybridization and washes and the salt concentration during the washes are the simplest to adjust. As the temperature of the hybridization increases (i.e. stringency), it becomes less likely for hybridization to occur between strands that are no homologous, and as a result, background decreases. If the radiolabeled probe is not completely homologous with the immobilized fragment (as is frequently the case in gene family and interspecies hybridization experiments), the hybridization temperature must be reduced, and background will increase. The temperature of the washes affects the intensity of the hybridizing band and the degree of background in a similar manner. The stringency of the washes is also increased with decreasing salt concentrations.

In general, convenient hybridization temperatures in the presence of 50% formamide are 42°C for a probe with is 95% to 100% homologous to the target fragment, 37°C for 90% to 95% homology, and 32°C for 85% to 90% homology. If the homology between the probe and the target fragment are not known, the simplest approach is to start with both hybridization and wash conditions which are nonstringent. If non-specific bands or high background are observed after autoradiography, the filter can be washed at high stringency and re-exposed. If the time required for exposure makes this approach impractical, several hybridization and/or washing stringencies should be tested in parallel. Nucleic acid Probe Assays.

Example 1: Efficacy of Phage-based test, as shown in spiked blood

A known number of cells of *Borrelia burgdorferi s.s.* B31 strain (1000, 100, 10, and 1 cell/cell) were added into 1 ml of commercial available healthy human whole blood (3 replicas). Total DNA was extracted from 100 µl of the spiked blood, respectively using the Qiagen blood and tissue kit. The extracted DNA was then used as a template for PCR amplification of the terminase gene (primers used were as shown in SEQ ID No. 70 and 71 - forward and reverse).

As clearly demonstrated in the gel picture shown in Figure 6, the PCR products were seen in the DNA from blood samples spiked with 100 cells or more of the B31 strain. This showed that 100 Borrelia cells will be needed to generate a positive terminase PCR test. This also demonstrated that the terminase PCR detection limit is 100 Borrelia/ml of blood, this concentration reflects the normal Borrelia concentration in Lyme patients. No terminase PCR product can be seen from the total DNA extracted from whole blood without Borrelia spiking (see lanes marked blood only). A false signal for the presence of detection was not found.

Comparison with Known Methodologies

The present invention, as described above, using the terminase PCR targeting *B. burgdorferi s.l.* was practiced on samples with known concentrations of Borrelia burgdorferi strain B31. The sensitivity of the present test was compared with bacteria- based PCR, our terminase PCR targeting *B. burgdorferi s.l.* phage-based PCR . The test of the present invention showed a markedly higher sensitivity compared to the known test, and could detect bacteria at a concentration ~ 1 bacterium per ml (see Table 1).

Number of *Borrelia* bacteria/ml	**Current bacterial PCR based on bacterial 5S-23S**	**Leicester phage PCR**
<100	Positive	Positive
<10	Weak signal	Positive
<1	Negative	Positive

Table 1:

Conclusion: The phage-based PCR was positive against Lyme Borrelia suspension with a concentration of less than 1 bacterium per ml, while the bacterial PCR was negative.

Example 2: Comparative Study of methods of present invention with conventional 16sPCR method

Four individual Borrelia burgdorferi B31 cultures were diluted to 10 Borrelia/ml, 100 µl of these diluted cultures were then subjected to DNA extraction using the Qiagen

blood and tissue kit. The resulting extracted DNA was then used as a template for PCR amplification of the terminase gene (primers used were as shown in SEQ ID No. 70 and 71 - forward and reverse) and as a template for PCR amplification of the 16S (a bacterial gene for ribosomal RNA, commonly as a molecular diagnostic tool for detecting presence of absence of bacteria). As can be seen in the gel picture shown in figure 7, two strong positive results were seen with terminase PCR, while only one weak positive with 16S PCR was identified. The leftmost two lanes are PCR negatives (ie did not include starting material derived from cultures), the rightmost lane is PCR positive (ie included a high concentration of bacterial cells).

Conclusion: This example demonstrated that the efficiency of terminase PCR is higher than 16S PCR.

Example 3: **Demonstrate of Test according to present invention on different *Borrelia Burgdorferi s.l. species***

Terminase PCR was carried out against a set of different Borrelia genotypes (different isolates).

The gel picture of Figure 8 shows the results of this PCR: B31=Borrelia burgdorferi B31 ; the numbers are Borrelia strains as shown in the table below:

	Isolate names	***Scientific names***
3	VS185 P9	*Borrelia burgdorferi s.s.*
4	NE218	*Borrelia valaisiana*
5	ACA1	*Borrelia afzelii*
6	UK filtered	*Borrelia burgdorferi s.s.*
7	190 P9	*Borrelia garinii*
8	China23	*Borrelia burgdorferi s.s.*

Table 2.

This demonstrated that this terminase PCR technique of the present invention can amplify the four key strains of *Borrelia burgdorferi s.l.* group, which are *Burgdorferi, grinii, afzelii,* and *valaisiana.* This terminase PCR technique was also applied to other bacteria, such as *Clostridium difficile, Burkholderia thailandensis, E. coli, Salmonella,*

legionellae, and *haemophilia* strains. None of these bacteria generated any PCR product with terminase primer.

Further analysis was carried out to determine the efficiency of the method to distinguish between Lyme Borrelia and relapsing fever Borrelia strains.

Primers and TaqMan probes (Table 3) were designed based on the *B. burgdorferi* terminase gene sequence (GenBank accession NC_000948.1) using PrimerQuest® Tool (IDT). To ensure the specificity of the primer/probe combinations (referred to as 'BbFAM'), BLAST analysis using sequences submitted to GenBank was performed. All hits with e-value < 0.01 were Lyme *Borrelia* species dominated by *B. burgdorferi* with one hit of each of the following *Borrelia* strains: *B. mayonii, B. garinii, B. afzelii, B. bisettii,* and *B. valasiana.* In addition, *'In silico'* (http://insilico.ehu.es/PCR/) was performed against all the available bacterial species, PCR product of the correct sizes was only observed from plasmid fractions of *Borrelia burgdorferi.* This demonstrated that the primer/probe combinations can detect the Lyme *Borrelia* strains. The TaqMan probe was labelled 5' with 6-carboxyfluorescein (FAM) fluorescent dye and a double-quencher with a ZEN™ Quencher and Iowa Black FQ to the 3' (5'FAM/ZEN/3'IBFQ). These double-quenched probes generate less background and increased signal compared to probes containing a single quencher. Both primers, the probe and PrimeTime Gene Expression Master Mix were supplied by IDT.

	Sequence (5' to 3')	**Expected amplicon size (bp)**	**GeneBank accession no.**
Probe (SEQ ID NO. 81)	TGCTGGGTCTAAATATGCTATCGGGC	147	NC_000948.1
Primer F (SEQ ID NO. 79)	GAGTGGATAGCAAGCACTGAT		
PrimerR (SEQ ID NO. 80)	ATCATCAACTCGCTCCATAACA		

Table 3 Sequences of primers and probes for terminase real-time PCR (BbFAM)

As seen in table 4, the primer/probe were tested against DNA extracted from different genotypes of *Borrelia* strains. All Lyme *Borrelia* strains (1-6) tested generated a positive PCR with a threshold cycle (Ct) value <30. No Ct value can be detected from Relapsing fever *Borrelia* strains. Apart from *'in silico'* PCR, 'wet experiment' was also carried out to confirm that no PCR products were observed when BbFAM PCR was performed against a range of different bacterial DNA including, *E. coli, Pseudomonas, Clostridium, Haemophilus, Burkholderia,* and *Salmonella.*

	Isolate Names	Scientific Names	BbFAM PCR results
1	VS185 P9	*Borrelia burgdorferi*	Positive
2	NE218	*Borrelia valasiana*	Positive
3	ACA1	*Borrelia afzelii*	Positive
4	UK filtered	*Borrelia burgdorferi*	Positive
5	190 P9	*Borrelia garinii*	Positive
6	China23	*Borrelia burgdorferi*	Positive
7	1120	*Borrelia duttonii*	Negative
8	Her HS1	*Borrelia hemsii*	Negative
9	CA128	*Borrelia bisettii*	Negative
10	HT31	*Borrelia miyamotoi*	Negative
11	FR64b	*Borrelia miyamotoi*	Negative

Table 4 BbFAM PCR against different Lyme Borrelia and Relapsing fever Borrelia strains.

To test the robustness, efficiency and the limit of detection (LOD) of the BbFAM PCR, a series of five tenfold dilutions of a plasmid carrying terminase gene fragment were amplified with BbFAM. To construct a standard for real time PCR, a plasmid carrying the terminase gene fragment was made. This plasmid served as the positive control and helped the calculations of the copy numbers in each PCR. The terminase plasmid was constructed as described below:

PCR primers were designed using Primer Blast against terminase gene sequence (SEQ ID No 1). The primers were FTer721:AGACTAAGATGCGGGCAAGA (SEQ ID NO. 82) and RTer721:TTGCATCAAGAGCGTCATCA (SEQ ID NO. 83). A 721 bp

PCR product was generated. PCRs were carried out in a LabCycler (SensoQuest GmbH, Gottingen, Germany) in a total volume of 50 μl, containing 0.25 mM dNTPs, 3 mM MgCl2, 3 μM primers, 50 ng of template DNA, 0.5 unit of Taq polymerase (Bioline), and 5 μl 10* Taq buffer (Bioline). Amplification conditions were: 94°C for 2 min, 30 cycles of 94°C for 30 sec, 50°C for 30 sec, 72°C for 1 min, with a final extension of 10 min at 72°C. PCR products were gel-purified using a Qiagen gel extraction kit, and subjected to cloning using NEB® PCR Cloning Kit according to the standard protocol. The positive clones were confirmed by PCR and sequencing. The resulting plasmid carrying terminase gene fragments were purified using Qiagen plasmid kit and used as positive controls. The concentration of DNA was measured with a Qubit Fluorometer (Invitrogen). The copy number of the plasmid DNA with the terminase gene fragment was calculated according the following formula (available online):

$$\text{Number of copies} = \frac{4022 \times 10^{23}\,(\text{copies/mol}) \times \text{DNA amount}\,(\text{ng})}{\text{DNA length}\,(\text{bp}) \times 10^{9}\,(\text{ng/g}) \times 650\,(\text{g/mol/bp})}$$

To work out the limit of detection (LOD) of the Taqman real time PCR, firstly 10-fold serial dilutions of the plasmid DNA carrying terminase fragment were tested to evaluate the performance of Taqman PCR. Each dilution (10^6-10^2) was tested by BbFAM PCR with five replicates (Table 5).

Next, the plasmid DNA was diluted from 1000 copies numbers to 100, 80, 60, 40, 20, 10, 5, and 1 copies/PCR. Ten replicates were used for each dilution. Probit analysis in SPSS was performed to calculate the LOD of 32 copies of plasmids with 95% probability.

The results were summarised in the following two tables:

Copy number/PCR	Average Ct
10^6	20.63
10^5	24.32
10^4	28.03
10^3	31.12
10^2	34.27

Table 5 Taqman real time PCR performance on plasmid DNA carrying terminase gene fragment

Copy number/PCR	Number of replicates	Number of PCR positive replicates (% of positive)
100	10	10 (100%)
80	10	10 (100%)
60	10	10 (100%)
40	10	10 (100%)
20	10	6 (60%)
10	10	5 (50%)
5	10	2 (20%)
1	10	0

Table 6 Determination of LOD of Taqman real time PCR (10 replicates for each dilution)

The LOD determined by Probit analysis with 95% probability was 30 copies of plasmid DNA.

The copy numbers of plasmid DNA template per PCR ranged from 10^6 to 10^2. As shown in Fig. 10, positive amplifications were observed from all the plasmid DNA samples. As shown in figure 11, a linear relationship between the concentration of DNA template and Ct values across with a strong correlation coefficient (R^2=0.99). In addition, the amplification efficiency of BbFAM was 100%. This demonstrated the high sensitivity and efficiency of the PCR.

To rule out the possibility of human DNA interference, human DNA and healthy whole blood were purchased from Sigma. Total DNA was extracted from the healthy whole blood. Both DNAs were examined using BbFAM PCR. No Ct values were observed from any PCRs, while positive PCR targeting human housekeeping genes RNase P produced positives. This confirmed that the human DNA had no effect on BbFAM PCR.

Conclusion: This experiment demonstrated that this terminase PCR is specific for Borrelia burgdorferi sensu lato and that using real time sequencing, the test has a very low limit of detection (LOD).

PCR primers for holin, endolysin and for amplifying the region which contains both these genes (SEQ ID NO. 72-77; the region containing both genes = SEQ ID NO. 78) have also been demonstrated to be able to amplify the correct regions of Lyme Borrelia.

Example 4: Comparative Study of methods of present invention with conventional 16sPCR method practiced in serum sample

Five Lyme-positive serum samples (S1-S5) (as tested and confirmed by antibody and clinical presentation tests) were subjected to DNA extraction using Qiagen Blood and tissue kit. The DNAs were then analysed using both terminase (top panel) and 16S (bottom panel) PCR primers, respectively (in the case of terminase, primers used were as in SEQ ID No. 70 and 71). As seen in the gel pictures shown in figure 9, four PCR positive results were observed for terminase (S1, S2, S4 and S5), while only two positive can be seen for 16S (S2 and S3). This was a pilot study prior to real time PCR experiments performed with large scale clinical trials (see below).

Conclusion: This example demonstrated that terminase PCR has a much higher sensitivity than techniques based on 16s. + = control, a high concentration of bacterial cells.

Example 5: Clinical results

The sensitivity of the BbFAM PCR was also investigated against 222 serum samples, which are all derived from clinically-confirmed Lyme patients (among those patients, 91 of patients have ELISA and or Western Blot data).

207 out of the 222 patients showed BbFAM PCR positive, representing a sensitivity of 93.2%.

91 out of 222 patients had been examined by either ELISA and/or WB.

Out of the 91 patients, 16 of them showed ELISA and/or WB positive, representing a sensitivity of 17.6%. Out of these 91 patients, 85 showed positive to BbFAM PCR, representing a sensitivity of 93.4%, which agrees well with the sensitivity calculated based on 222 patients. If you look into the correlation between BbFAM PCR results to the ELISA/WB data, you will find that the 16 ELISA and/or WB positive patients all showed BbFAM PCR positive. In addition, 6 patients from the 91 patient cohort who displayed BbFAM PCR negative also showed ELISA/WB negative. The vast majority of clinically confirmed patients in the 91 cohort only showed positive to BbFAM PCR, but negative to ELISA/WB, an indication of high sensitivity and reliability of BbFAM

PCR. To compare the sensitivity of BbFAM with the current commercial Lyme PCR detection kit, GeneProof Borrelia burgdorferi PCR Kit was applied to 65 serum samples that were randomly selected from the 222 cohort. Only 7 out of 65 serum samples showed positive to GeneProof kit (a sensitivity of 10.8%).

Summary

This is the first study to use a molecular marker to investigate the distribution and diversity of Borrelia phages.

A phylogentic tree constructed by the inventors on phage terminase gene shows that it is a good phylogenetic marker because the Borrelia phage sequences form a discrete yet genetically diverse group which is clearly separated from other spirochetes. Lyme disease infection (ie Borrelia burgdorferi sensu lato infection) forms a discrete well supported clade and these correlate well with bacteria.

Summary for Lyme Disease

1) Overall ability to identify LD much higher sensitivity compared to bacterial 16S method. In addition, the clinical results also demonstrate that the phage terminase-based real time PCR is significantly more sensitive than bacterial 16S-based PCR (GeneProof PCR kit).
2) Relates to multiple species of *Borrelia burgdorferi sensu lato* (Table 4: Phage terminase-based real time PCR against different Lyme *Borrelia* and Relapsing fever *Borrelia* strains)
3) Early detection possible as the phage based test can detect a low concentration of bacteria. The real time PCR has a low detection limit of 30 copies of terminase gene.

Relapsing Fever

Example 6: Differentiation of Relapsing Fever *(Borellia Hermsii)* from Lyme Disease

Primers and TaqMan probes (Table 8) were designed based on the *B. hermsii* terminase gene sequence using PrimerQuest® Tool (IDT). To ensure the specificity of the primer/probe combinations (referred to as 'BhFAM'), BLAST analysis using sequences submitted to GenBank was performed. Big E-value drops were observed from 0.000004 to 0.004 between B. hermsii hit and the next closest blast hit of *B. turicatae* and *B. parkeri.*

'In silico PCR' (http://insilico.ehu.es/PCR/) was performed against all the available bacterial species, PCR product of the correct sizes was only observed from *Borrelia hermsii.* The TaqMan probe was labelled 5' with 6-carboxyfluorescein (FAM) fluorescent dye and a double-quencher with a ZEN™ Quencher and Iowa Black FQ to the 3' (5'FAM/ZEN/3'IBFQ). These double-quenched probes generate less background and increased signal compared to probes containing a single quencher. Both primers, the probe and PrimeTime Gene Expression Master Mix were supplied by IDT.

	Sequence (5' to 3')	**Expected amplicon size (bp)**	**GeneBank accession no.**
Probe	AGGCACCAATAGCATATTTAGATCCTGCA	124	CP014792.1
Primer F	GGAGAATGGGTTGCGTCATA		
Primer R	GCGCAGTATTATCACCTCCAATA		

Table 8 Sequences of primers and probe targeting terminase gene in *B. hermsii* (BhFAM)

As seen in table 9, the primer/probe were tested against DNA extracted from different genotypes of *Borrelia* strains. No positive can be seen from all Lyme *Borrelia* strains (1-6) and other relapsing fever *Borrelia* strains. Only *B. hermsii* generated the correct PCR product. Apart from *'in silico'* PCR, 'wet experiment' was also carried out to confirm that no PCR products were observed when BhFAM PCR was performed against a range of different bacterial DNA including, *E. coli, Pseudomonas, Clostridium, Haemophilus, Burkholderia,* and *Salmonella.*

	Isolate Names	Scientific Names	BbFAM PCR results
1	VS185 P9	*Borrelia burgdorferi*	Negative
2	NE218	*Borrelia valasiana*	Negative
3	ACA1	*Borrelia afzelii*	Negative
4	UK filtered	*Borrelia burgdorferi*	Negative
5	190 P9	*Borrelia garinii*	Negative
6	China23	*Borrelia burgdorferi*	Negative
7	1120	*Borrelia duttonii*	Negative
8	Her HS1	*Borrelia hermsii*	Positive
9	CA128	*Borrelia bisettii*	Negative
10	HT31	*Borrelia miyamotoi*	Negative
11	FR64b	*Borrelia miyamotoi*	Negative

Table 9 BhFAM PCR against different Lyme Borrelia and Relapsing fever Borrelia strains

Example 7: Differentiation of Relapsing Fever (Borrelia miyamotoi) from Lyme Disease

Two sets of primers and TaqMan probes were designed based on two *B. miyamotoi* terminase genes using PrimerQuest® Tool (IDT) with manual inspection (referred to as 'BmFAM' and 'Bm-2FAM', respectively as shown in the Table 10). Both sets target different versions of terminase genes located on different plasmids, therefore they are complementary to each other in detecting B. miyamotoi. To ensure the specificity of the primer/probe combinations, BLAST analysis using sequences submitted to GenBank was performed. Big E-value drops were observed for both sets of Taqman probes. For example, BmFAB and Bm-1FAM showed E-value drops from 0.0002 to 0.79 and 0.006 to 1.6, respectively, between miyamotoi hits and the next closest blast hit. *'In silico PCR'* (http://insilico.ehu.es/PCR/) was performed against all the available bacterial species, PCR product of the correct sizes was only observed from *Borrelia miyamotoi.* This demonstrated specificity of the primer/probe combinations in detecting B.miyamotoi strains. The TaqMan probe was labelled 5' with 6-carboxyfluorescein (FAM) fluorescent dye and a double-quencher with a ZEN™ Quencher and Iowa Black FQ to the 3' (5'FAM/ZEN/3'IBFQ). These double-quenched probes generate less background and increased signal compared to probes containing a single quencher. Primers, probes and PrimeTime Gene Expression Master Mix were supplied by IDT.

		Sequence (5' to 3')	**Expected amplicon size (bp)**	**GeneBank accession no.**
BmFAM	Probe	AGTGCACTTTGTGTGCTTGAAATGGT	120	CP004220.1
	Primer F	AGCCTACCTAGATCCTGCTTAT		
	Primer R	GGGTCACTTGCTGGTAGTTT		
Bm-2FAM	Probe	ACGCTTCAGAGGCTCTAATTCTG	87	CP017131.1
	Primer F	GTGGAGATAAGGCAAGTGA		
	Primer R	CTTTATGAAGAGTAGTTGCTTC		

Table 10 Two sets of primer/probes (BmFAM and Bm-2FAM) targeting terminase genes in Borrelia miyamotoi strains

As seen in table 11, the primer/probe were tested against DNA extracted from different genotypes of *Borrelia* strains. No Ct value can be detected from all Lyme *Borrelia* strains (1-6) and relapsing fever *Borrelia* strains. PCR positive can only be observed in B. miyamotoi DNA. In addition, no PCR products were observed when both sets of primer/probe were performed against a range of different bacterial DNA including, *E. coli, Pseudomonas, Clostridium, Haemophilus, Burkholderia,* and *Salmonella.*

	Isolate Names	Scientific Names	BbFAM PCR results
1	VS185 P9	*Borrelia burgdorferi*	Negative
2	NE218	*Borrelia valasiana*	Negative
3	ACA1	*Borrelia afzelii*	Negative
4	UK filtered	*Borrelia burgdorferi*	Negative
5	190 P9	*Borrelia garinii*	Negative
6	China23	*Borrelia burgdorferi*	Negative

7	1120	*Borrelia duttonii*	Negative
8	Her HSI	*Borrelia hermsii*	Negative
9	CA128	*Borrelia bisettii*	Negative
10	HT31	*Borrelia miyamotoi*	Positive
11	FR64b	*Borrelia miyamotoi*	Positive

Table 11 BmFAM and Bm-2FAM PCR against different Lyme Borrelia and Relapsing fever Borrelia strains.

As a pilot study, 43 tubs of blood/serum samples derived from healthy volunteers and Lyme patients were randomly chosen and subjected to test agasint BmFAM and BbFAM, respectively. Results were as follows: 7 (16.3%) tubes showed positive to BmFAM, while 26 (60.5%) showed positive to BbFAM. This demonstrated a potential low level of *B. miyamotoi* carriage in the population. Large scale clinical validation is on-going.

Summary for Relapsing Fever

1) The phage-based test can specifically amplify RF Borrelia strains, and didn't amplify LD Borrelia strains. This makes it possible to design a phage terminase-based duplex PCR to diagnose LD and RF at the same time.
2) The phage-based RF tests have the ability to distinguish different RF Borrelia strains, such as *B. miyamotoi* and *B. hermsii.* This would enable clinicians to better manage and prescribe antibiotics once the RF species was known. With a fully validated phage-based RF test, clinicians would be better informed about which Borrelia species are causing the symptoms.
3) As a result of these successful initial validation results, large scale clinical validation is currently underway.

Claims

1. A method of determining the presence or the absence of *Borrelia burgdorferi sensu lato or* Relapsing Fever *Borrelia* in a sample, the method comprising the steps of:-

   a) detecting the presence or absence of a phage specific for *Borrelia burgdorferi sensu lato or* Relapsing Fever *Borrelia* in the sample; and

   b) determining the presence of *Borrelia burgdorferi sensu lato or* Relapsing Fever *Borrelia* in the sample on the basis of the detection of the phage, or the absence of *Borrelia burgdorferi sensu lato or* Relapsing Fever *Borrelia* in the sample on the basis of the lack of detection of the phage.

2. The method of claim 1, wherein detection of the phage comprises detection of a phage gene or gene fragment.

3. The method of claim 1 or 2, wherein step a) is preceded by the step of isolating nucleic acid from the sample, and step a) is practiced on the isolated nucleic acid.

4. The method of claim 2 or 3, wherein the phage gene or gene fragment encodes the terminase, holin, endolysin, integrase, portal or capsid protein, or combinations thereof.

5. The method of claim 4, wherein the phage gene or gene fragment encodes terminase protein.

6. The method of any of claims 2 to 5, wherein the phage gene comprises a nucleic acid according to the sequence of:
   a)SEQ ID NO.s 1-34; or a nucleic acid with greater than or equal to 70-99.5% sequence homology with SEQ ID NO.s 1-34; or a fragment thereof; or wherein the phage gene is capable of encoding a protein according to any one of SEQ ID NO.s 36-69 or a fragment thereof; or

b) SEQ ID NO.s 84, 86 or 97-103; or a nucleic acid with greater than or equal to 70- 99.5% sequence homology with SEQ ID NO.s. 84, 86 or 97-103; or a fragment thereof; or wherein the phage gene is capable of encoding a protein according to any one of SEQ ID NOs 85, 87 or 104-1 10 or a fragment thereof.

7. The method of claim 6, wherein the phage gene fragment detected comprises SEQ ID NO. 35; or a nucleic acid with greater than or equal to 70-99.5% sequence homology to SEQ ID NO. 35.

8. The method of claim 1, wherein detection of the phage comprises detection of a phage protein or fragment thereof.

9. The method of claim 8, wherein the phage protein comprises the amino acid sequence of:

a) SEQ ID NO.s 36-69 or is encoded by a gene according to SEQ ID NO.s 1-34; or an amino acid sequence with 70-99.5% sequence homology with any of SEQ ID NO.s. 36-69 or is encoded by a gene with 70-99.5% sequence homology with any of SEQ ID NO.s. 1-34; or is encoded by a gene fragment according to SEQ ID NO. 35; or

b) SEQ ID NO.s 85, 87 or 104-1 10 or is encoded by a gene according to SEQ ID NO. 84, 86 or 97-103; or an amino acid sequence with 70-99.5% sequence homology with any of SEQ ID NO.s 85, 87 or 104-1 10; or is encoded by a gene with 70-99.5% sequence homology with SEQ ID NO.s. 84, 86 or 97-103.

10. The method of claim 1, wherein detection of the phage comprises detection of a phage RNA or RNA fragment.

11. The method of any of the preceding claims, wherein the sample is plasma, whole blood, cerebrospinal fluid, urine, faecal matter, skin, brain tissue, glial cells, lymph, sweat, amniotic fluid or any combination thereof.

12. The method of any of the preceding claims, wherein the sample is one that has been obtained in the early or late stage of *Borrelia burgdorferi sensu lato* or Relapsing Fever *Borrelia* infection.

13. The method of any of the preceding claims, wherein the *Borrelia burgdorferi sensu lato* may be any of *Borrelia afzelii, Borrelia spielmanii, Borrelia valaisiana, Borrelia garinii, Borrelia finlandensis, Borrelia bugdorferi sensu strictu, Borrelia bissettii, Borrelia bavariensis, Borrelia japonica, Borrelia lusitaniae, Borrelia sinica, Borrelia spielmanii, Borrelia tanukii, Borrelia turdi, Borrelia valaisiana, Borrelia Yangtze,Borrelia mayonii, Borrelia carolinensis, and Borrelia andersonii, Borrelia lonestari, and Borrelia Americana* or any combination thereof.

14. The method of any of the preceding claims wherein the Relapsing Fever Borrelia may be any of *Borrelia miyamotoi, Borrelia hermsii, Borrelia recurrentis, Borrelia crocidurae, Borrelia duttoni, Borrelia hispanica, Borrelia parkeri* and *Borrelia turicatae* or any combination thereof.

15. The method of any of the preceding claims, wherein the method may determine the presence or the absence of a species of *Borrelia burgdorferi sensu lato* or Relapsing Fever *Borrelia* in the sample, the method comprising the steps of:-

a) detecting the presence or absence of the *Borrelia burgdorferi sensu lato* or Relapsing Fever *Borrelia* species specific phage in the sample;

b) determining the presence of the species of the *Borrelia burgdorferi sensu lato* or Relapsing Fever *Borrelia* in the sample on the basis of the detection of the *species* specific phage, or the absence of the species of *Borrelia burgdorferi sensu lato or* Relapsing Fever *Borrelia* in the sample on the basis of the lack of detection of the species specific phage.

16. A method of monitoring the progression of infection from *Borrelia burgdorferi sensu lato* or Relapsing Fever *Borrelia,* the method comprising the steps of:

a) determining the amount of phage in a first sample obtained from the subject;

b) detecting the amount of phage in a second sample obtained from the subject at a second time point;

c) comparing the amount of phage in the first sample identified in step a) with that identified for the second sample in step b).

17. The method of 16, wherein determining the amount of phage comprises the method of any of claims 1 to 15.

18. The method of any of claims 1 to 17, wherein the method additionally comprises treatment of a patient for Lyme disease, the patient's sample having tested positive for *Borrelia burgdorferi sensu lato.*

19. The method of any of claims 1 to 17, wherein the method additionally comprises treatment of a patient for Relapsing Fever, the patient's sample having tested positive for Relapsing Fever *Borrelia.*

20. The method of claim 18 or 19, wherein treatment comprises administering at least one antibiotic.

21. A kit for detecting phage specific for *Borrelia burgdorferi sensu lato and/or* Relapsing Fever *Borrelia* in a sample, the kit comprising one or more detection molecules specific for *Borrelia burgdorferi sensu lato and/or* Relapsing Fever *Borrelia.*

22. The kit of claim 21, wherein the one or more detection molecule(s) specifically bind: a) a phage gene or gene fragment encoding the terminase, holin, endolysin, integrase, portal or capsid protein, or combinations thereof; or b) a phage terminase, holin, endolysin, integrase, portal or capsid protein, or combinations thereof.

23. The kit of claim 22, wherein the detection molecule specifically binds: a) SEQ ID NO.s 1-35, 84, 86 or 97-103; or b) SEQ ID NO.s 36-69, 85, 87 or 104-1 10.

24. The kit of claim 22 or 23, wherein the kit comprises one or more primers of: a) SEQ ID NO.s 70-77 and/or 79-83; and/or b) SEQ ID NO.s 88-96.

25. The kit of claims 22 or 23, wherein the detection molecule is an antibody specific for a phage terminase, holin, endolysin, integrase, portal or capsid protein, preferably an antibody specific for a protein according to SEQ ID NO.s 36-69, 85, 87 or 104-1 10.

26. Use of a phage specific for *Borrelia burgdorferi sensu lato* as a diagnostic marker for Lyme Disease or as a diagnostic marker for *Borrelia burgdorferi sensu lato* infection.

27. Use according to claim 26, wherein said phage specific for *Borrelia burgdorferi sensu lato* comprise a nucleic acid according to any of SEQ ID NO.s 1-34 or a fragment thereof or SEQ ID NO: 35 or a protein according to any of SEQ ID NO.s 36-69 or a fragment thereof.

28. Use of a phage specific for Relapsing Fever *Borrelia* as a diagnostic marker for Relapsing Fever.

29. Use according to claim 28, wherein said phage specific for Relapsing Fever *Borrelia* comprises a nucleic acid according to any of SEQ ID NO.s 84, 86 or 97-103 or a fragment thereof; or a protein according to any of SEQ ID NO.s 85, 87or 104-1 10,or a fragment thereof.

30. A *Borrelia* phage nucleic acid comprising: a) SEQ ID NO.s 1-34 or a fragment thereof; or a phage nucleic acid sequence with equal to or greater than 70-99.5% homology with SEQ ID NO.s 1-34; or b) SEQ ID NO.s 84, 86 or 97-103 or a phage nucleic acid sequence with equal to or greater than 70-99.5% homology with SEQ ID NO.s 84, 86 or 97-103.

31. A *Borrelia* phage nucleic acid fragment comprising SEQ ID NO. 35 or a phage nucleic acid sequence with equal to or greater than 70-99.5% homology with SEQ ID NO. 35.

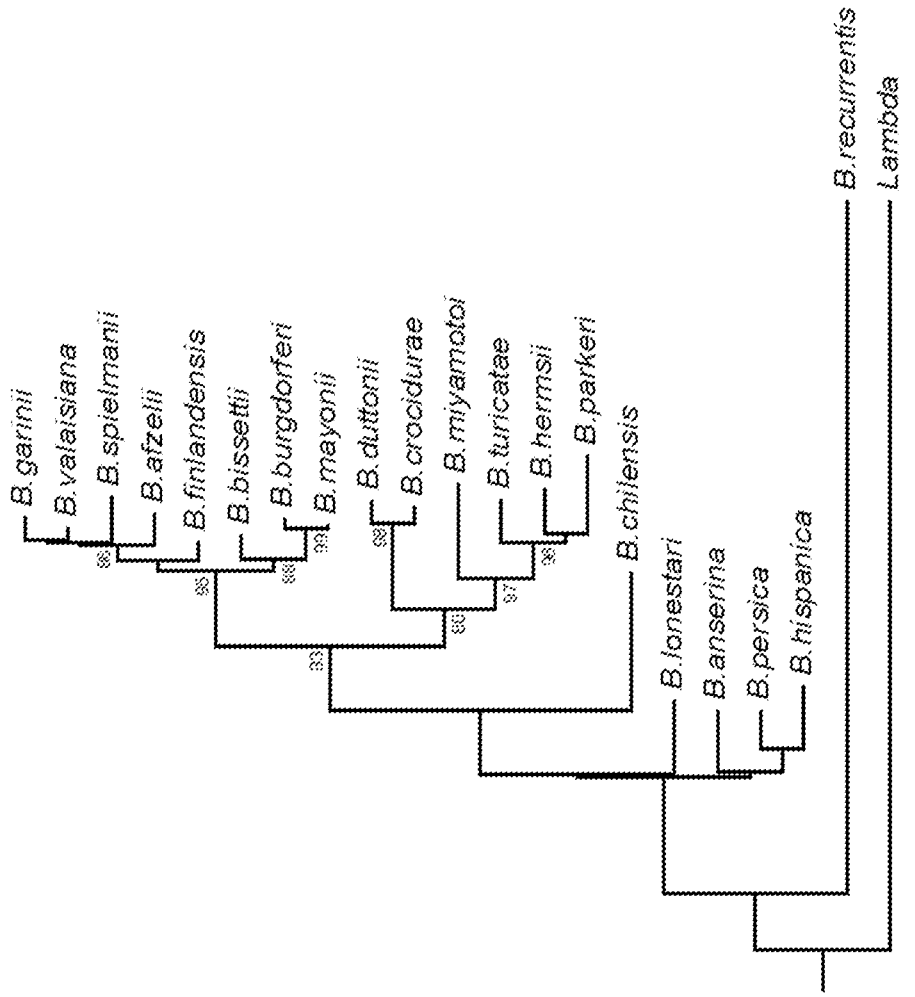
32. A DNA primer specific for the phage nucleic acid of claims 30-31 .

33. The DNA primer of claim 32, comprising any one or more of: a) SEQ ID NO.s 70-77, and /or 79-83; or b) 88-96.

34. A *Borrelia* phage protein comprising: a) SEQ ID NO.s 36-69 or a fragment thereof; or a phage protein with equal to or greater than 70-99.5% homology with SEQ ID NO.s 36-69; or b) SEQ ID NO.s 85, 87 or 104-1 10 or a fragment thereof; or a phage protein with equal to or greater than 70-99.5% homology with SEQ ID NO.s 85, 87 or 104-1 10.

35. An antibody or other binding molecule specific for the phage protein or protein fragment of claim 34.

36. A method of extracting phage DNA from *Borrelia,* the method comprising: a) incubating the *Borrelia* in ammonium hydroxide; and b) adding phenol-chloroform to the *Borrelia* and ammonium hydroxide mixture.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B.crocidurae
B.miyamotoi
B.turicatae
B.hermsii
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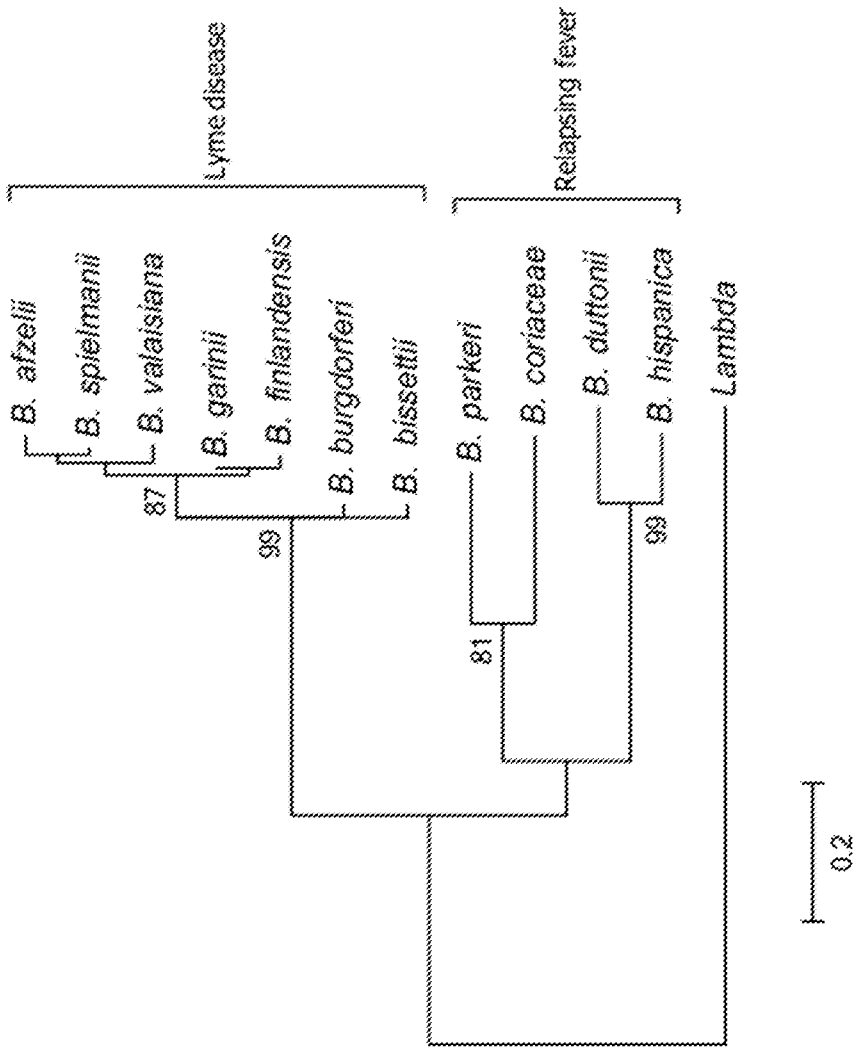
FIG. 1


Lyme disease
Relapsing fever
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B. finlandensis
B. burgdorferi
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B. hispanica
Lambda
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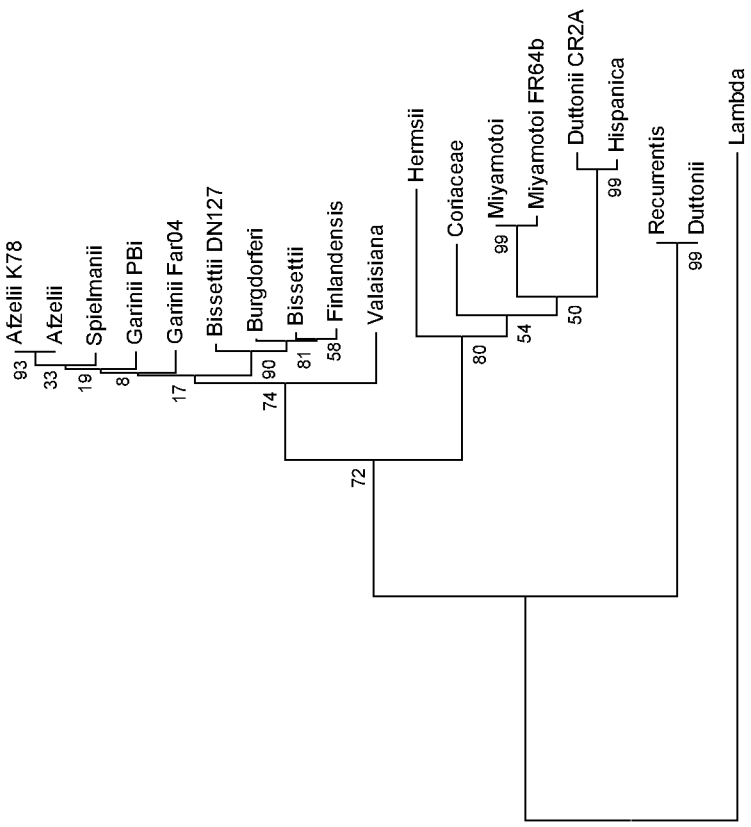
FIG. 2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Afzelii
Spielmanii
Garinii PBi
Garinii Far04
Bissettii DN127
Burgdorferi
Bissettii
Finlandensis
Valaisiana
Hermsii
Coriaceae
Miyamotoi
Miyamotoi FR64b
Duttonii CR2A
Hispanica
Recurrentis
Duttonii
Lambda
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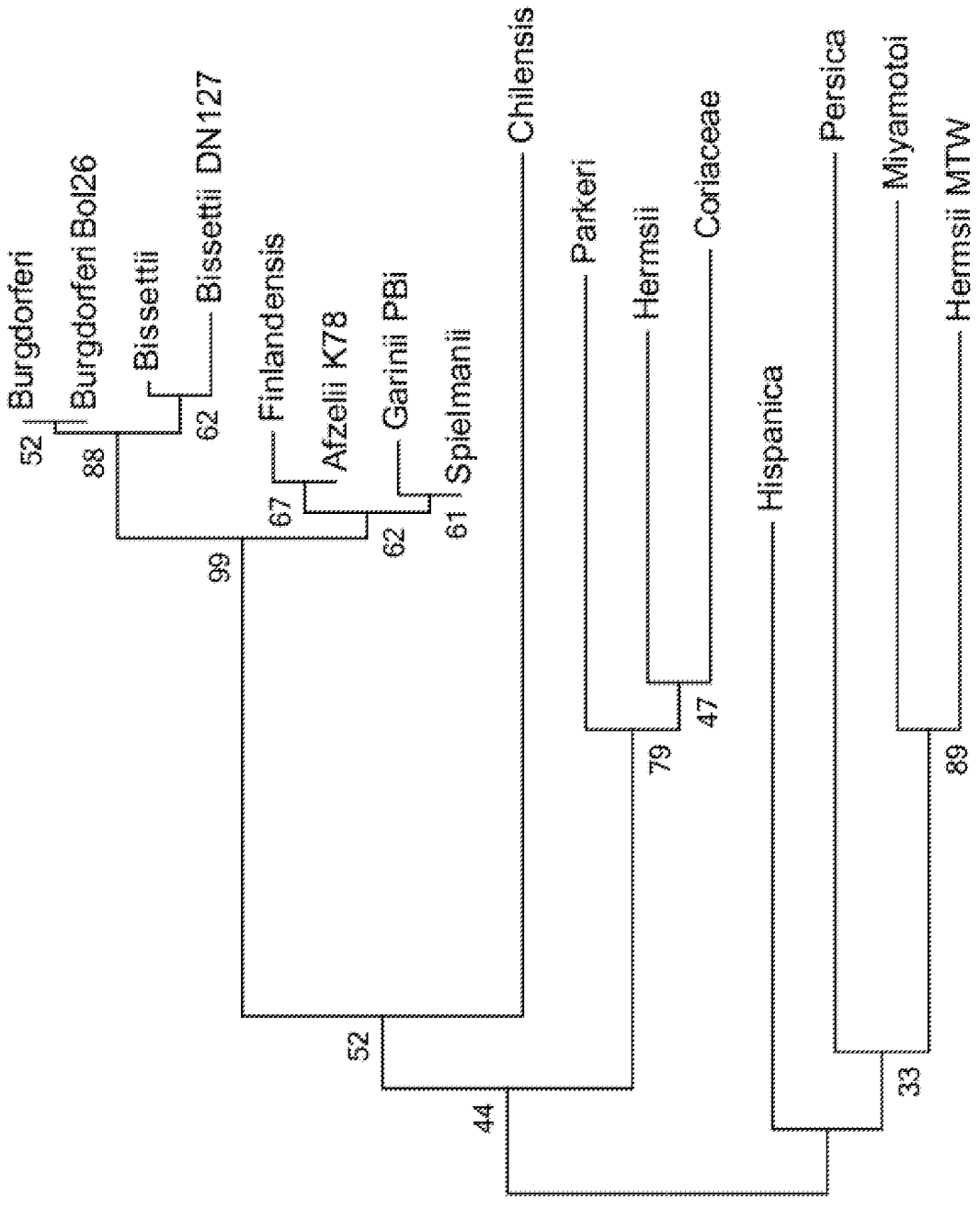
0.2

FIG. 3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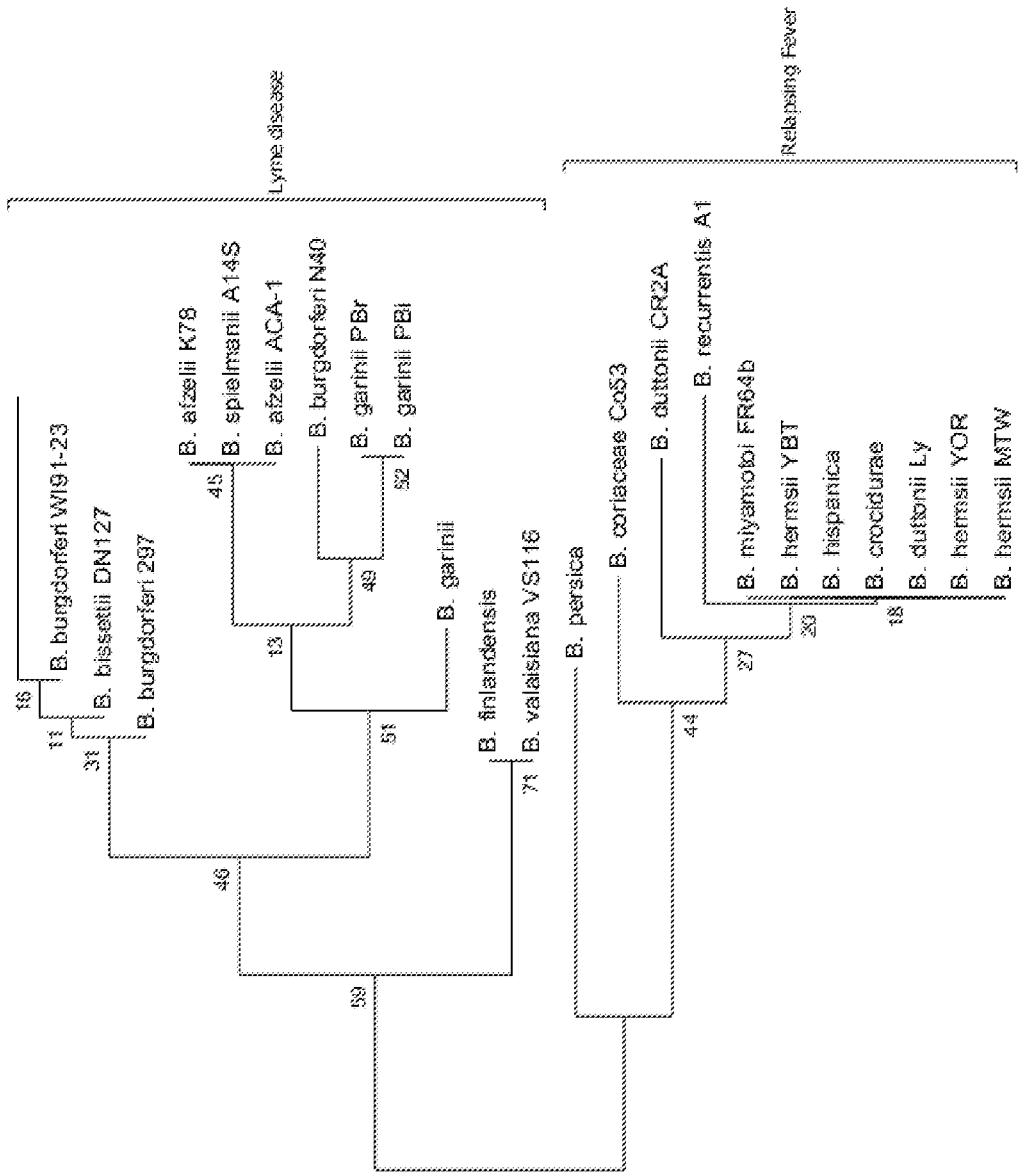
Burgdorferi Bol26
Bissettii
Bissettii DN127
Finlandensis
Afzelii K78
Garinii PBi
Spielmanii
Chilensis
Parkeri
Hermsii
Coriaceae
Hispanica
Persica
Miyamotoi
Hermsii MTW
52
88
62
67
62
61
99
52
44
79
47
89
33
0.1

FIG. 4


Lyme disease
Relapsing Fever
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B. bissettii DN127
B. burgdorferi 297
B. afzelii K78
B. spielmanii A14S
B. afzelii ACA-1
B. burgdorferi N40
B. garinii PBr
B. garinii PBi
B. garinii
B. finlandensis
B. valaisiana VS116
B. persica
B. coriaceae Co53
B. duttonii CR2A
B. recurrentis A1
B. miyamotoi FR64b
B. hermsii YBT
B. hispanica
B. crocidurae
B. duttonii Ly
B. hermsii YOR
B. hermsii MTW
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46
59
44
27
29
18
0.035

FIG. 5

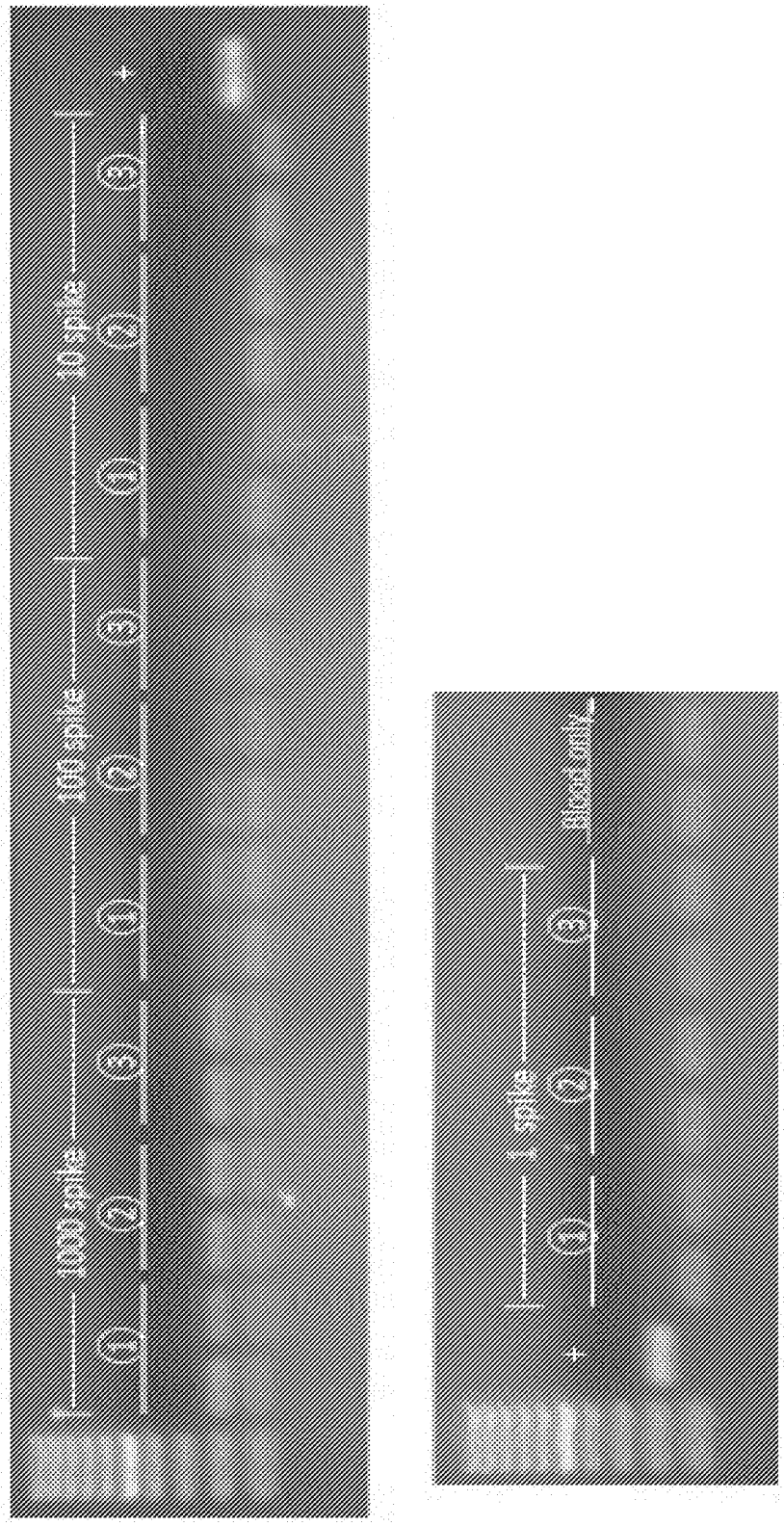


FIG. 6

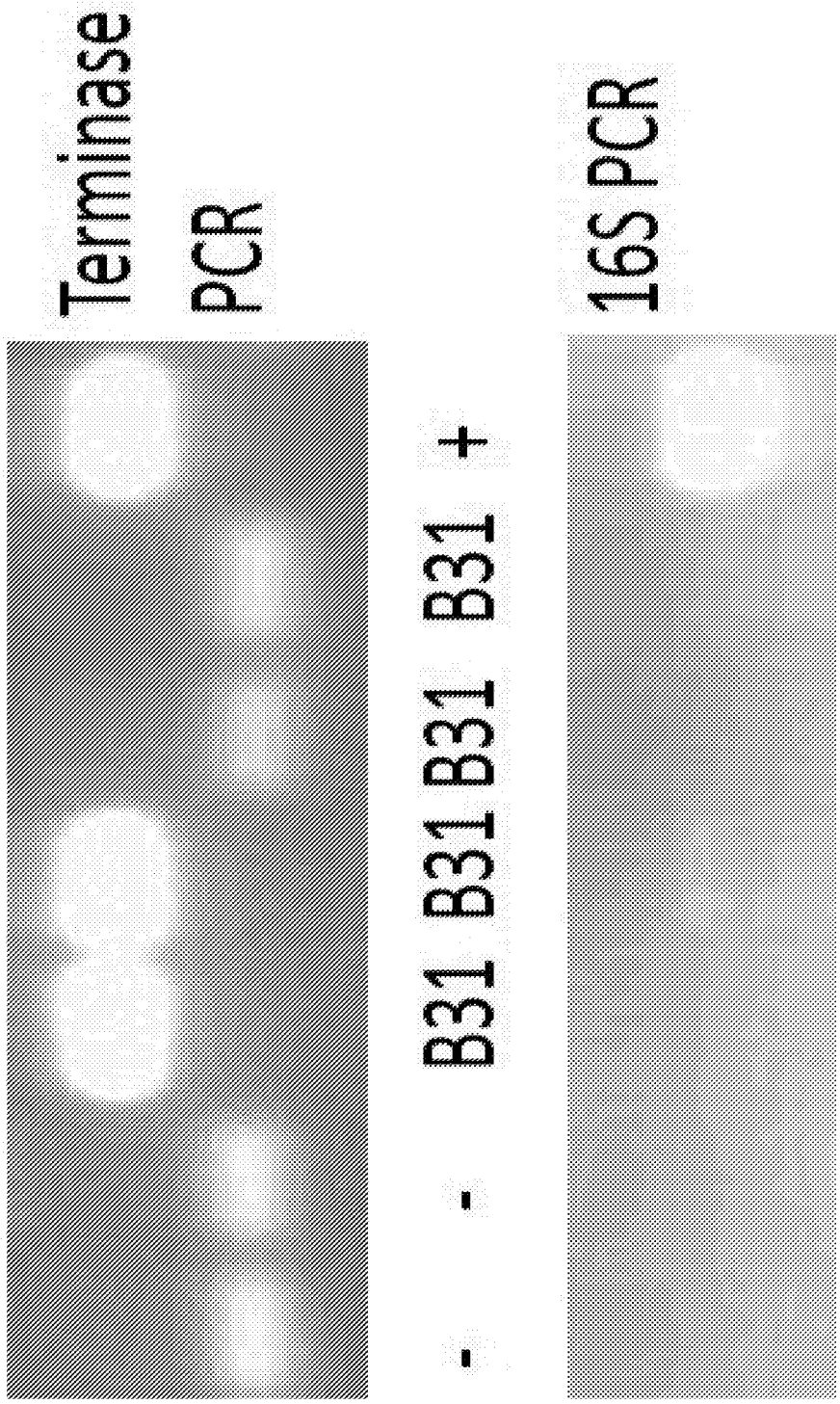


FIG. 7

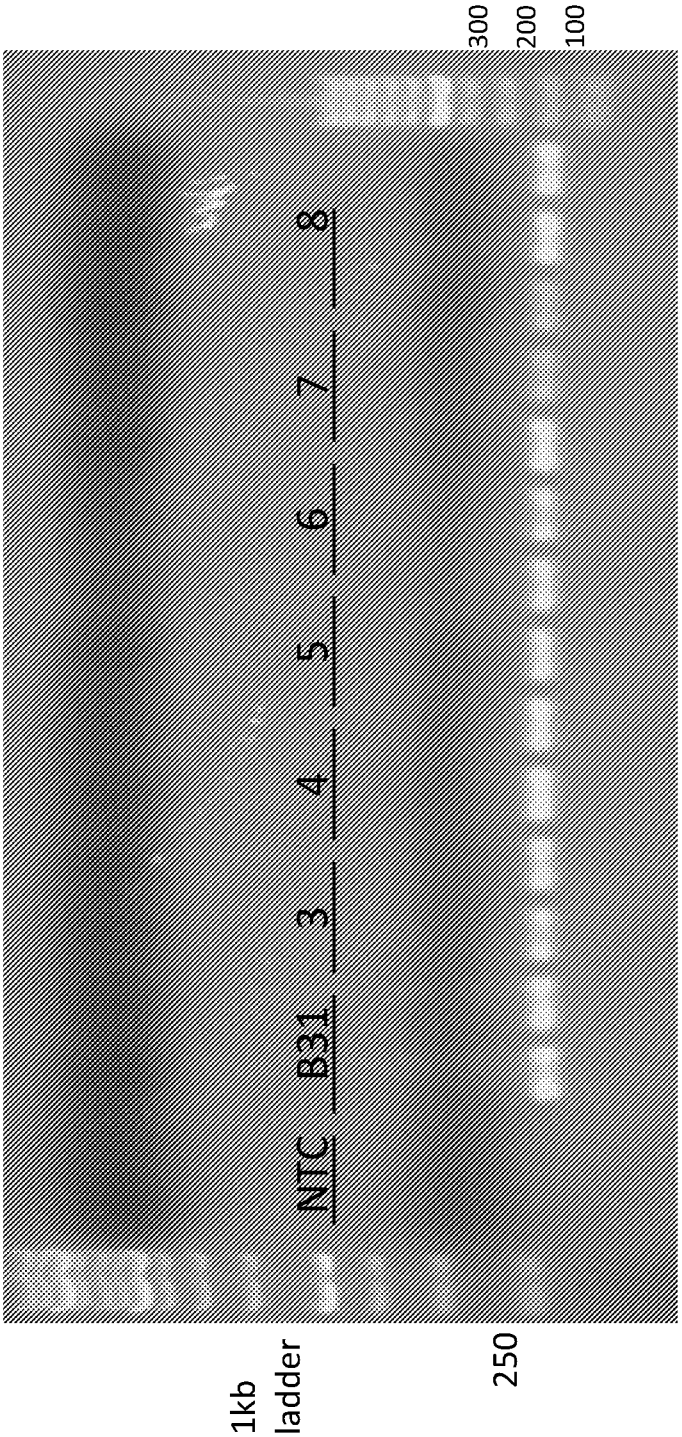


FIG. 8

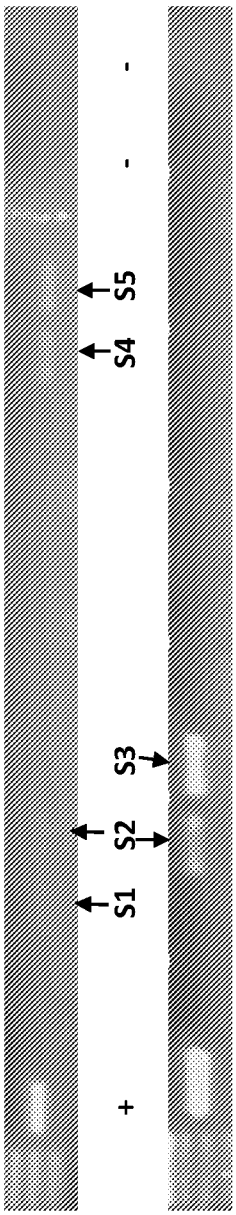
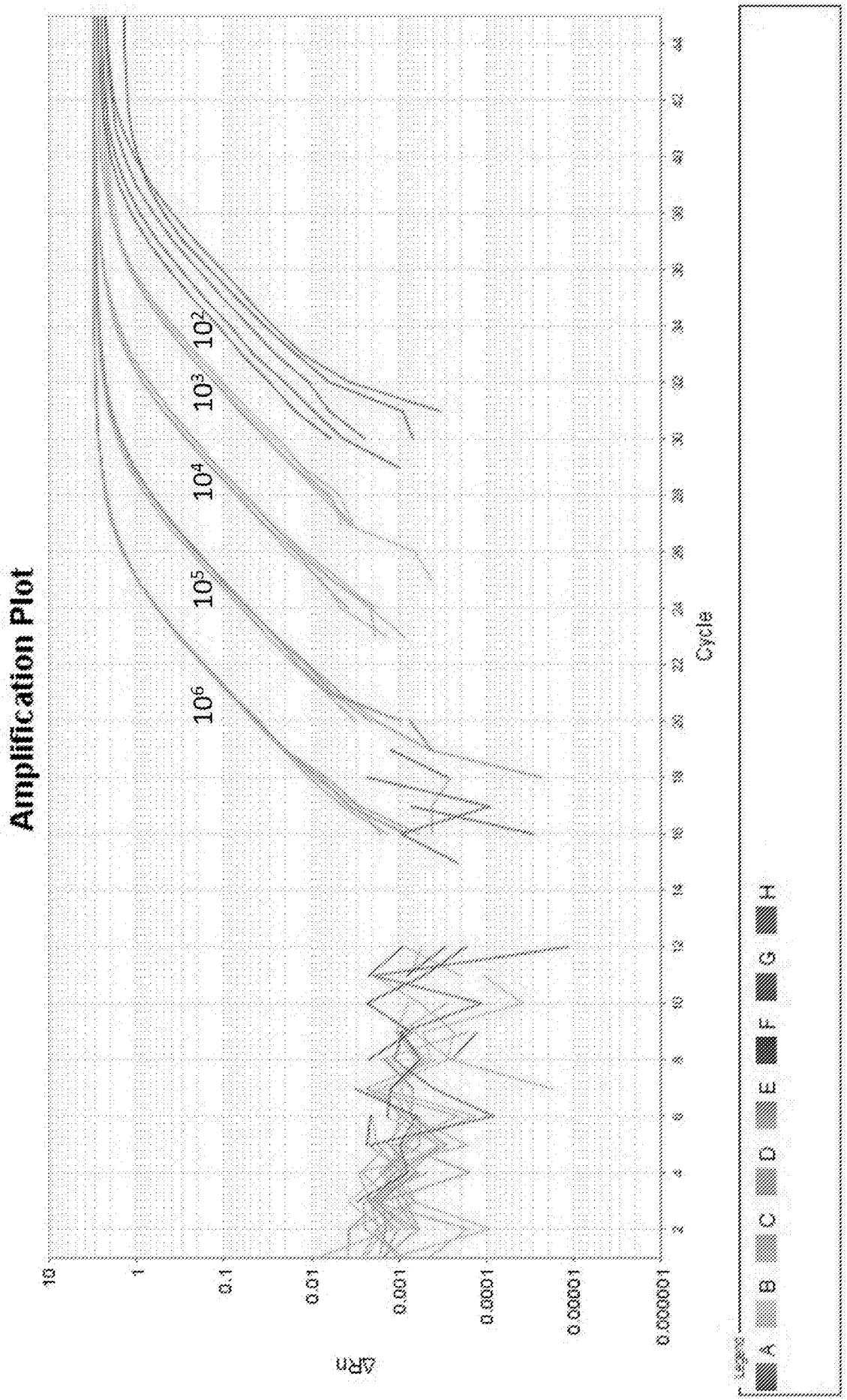


FIG. 9


Amplification Plot
ΔRn
Cycle
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1
0.1
0.01
0.001
0.0001
0.00001
0.000001
2
4
6
8
10
12
14
16
18
20
22
24
26
28
30
32
34
36
38
40
42
44
10^6
10^5
10^4
10^3
10^2
Legend
A
B
C
D
E
F
G
H

FIG. 10

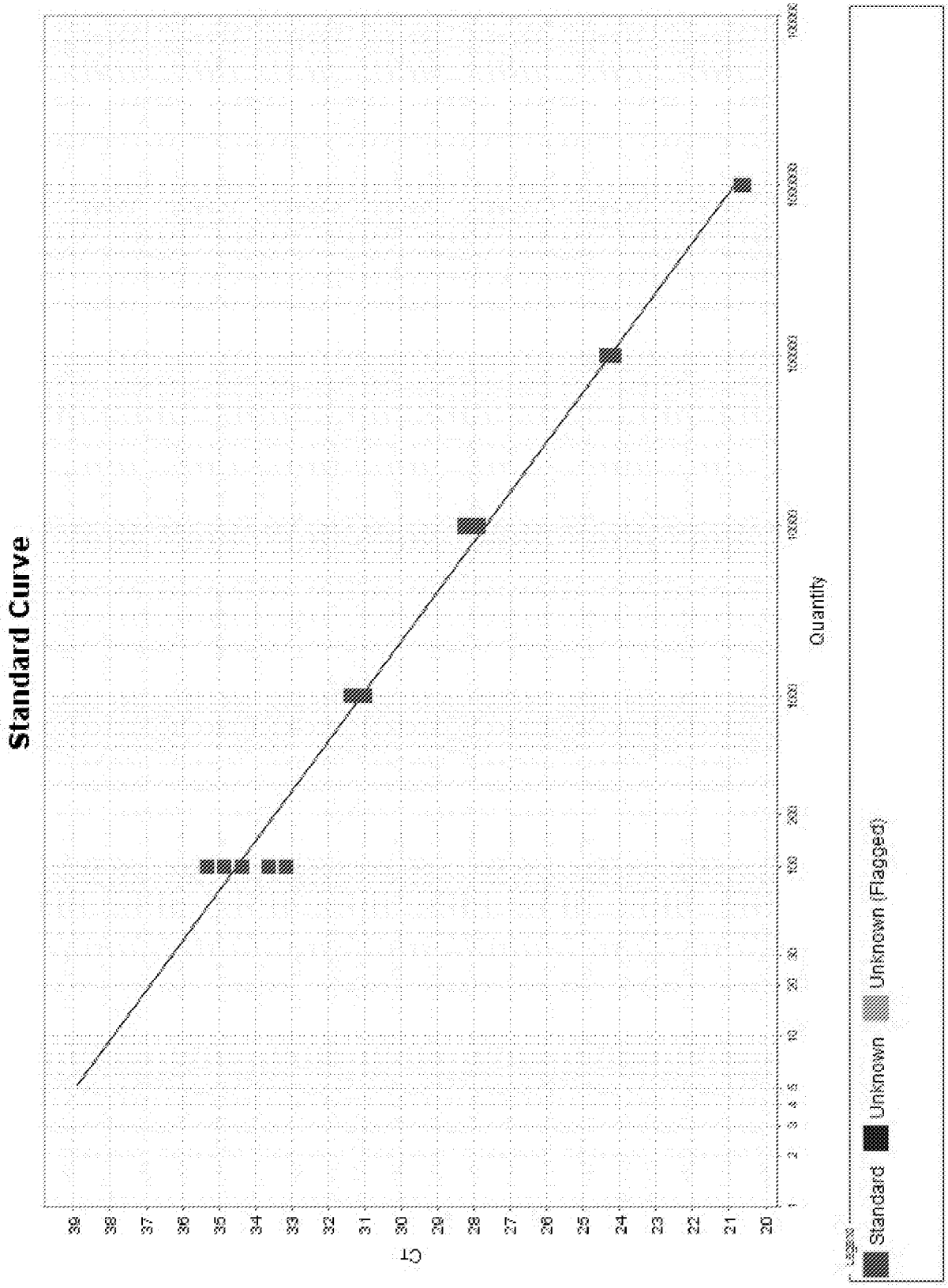
Standard Curve
CT
Quantity
Standard
Unknown
Unknown (Flagged)

FIG. 11

INTERNATIONAL SEARCH REPORT

International application No
PCT/GB2017/053323

A. CLASSIFICATION OF SUBJECT MATTER
INV. C12Q1/68 G01N33/569
ADD.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
C12Q G01N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
EPO-Internal , WPI Data, CHEM ABS Data, EMBASE

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	K. BABB ET AL: "Borrelia burgdorferi EbfC, a Novel, Chromosomally Encoded Protein, Binds Specific DNA Sequences Adjacent to erp Loci on the Spirochete's Resident cp32 Prophages", JOURNAL OF BACTERIOLOGY, vol. 188, no. 12, 15 June 2006 (2006-06-15), pages 4331-4339, XP055434657, US ISSN: 0021-9193, D0I: 10.1128/JB.00005-06 abstract	1-36

-/- ·

[X] Further documents are listed in the continuation of Box C.

[X] See patent family annex.

* Special categories of cited documents :

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"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
15 December 2017	22/12/2017

Name and mailing address of the ISA/	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016	Cel ler, Jakub

1

Form PCT/ISA/210 (second sheet) (April 2005)

INTERNATIONAL SEARCH REPORT

International application No
PCT/GB2017/053323

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 98/58943 A1 (HUMAN GENOME SCIENCES INC [US]; MEDIMMUNE INC [US]; FRASER CLAIRE [US]) 30 December 1998 (1998-12-30)	1-35
Y	abstract; claims 8,10,11,19,20, 21; sequences 4,5 page 1, line 6 - line 13 page 35, line 31 - line 36 page 35, line 27 - line 30	2-7, 10-12, 14, 16-25, 28-33, 35,36

Y	W0 03/033724 A2 (MOLECULAR STAGING INC [US]) 24 April 2003 (2003-04-24)	36
A	abstract; claims 1,52, 135	1-35

X	US 2012/184710 A1 (LUNDBERG URBAN [AT] ET AL) 19 July 2012 (2012-07-19)	1-6, 8-10, 12-28, 30,32, 34,35
Y	abstract; table 2; sequences 354, 482	7,11,12, 16-25, 28,29, 31,33,36

X	EP 1 394 254 A1 (HARTUNG THOMAS DR RER NAT DR M [DE]) 3 March 2004 (2004-03-03)	1-6, 8-13, 15-23, 26,27, 30,32, 34,35
Y	abstract; claims 1,12; sequences 3,4 paragraph [0026]	2-7, 10-12, 14, 16-25, 28-33, 35,36

1

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
PCT/GB2017/053323

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W0 03033724 A2	24-04-2003	CA 2463933 A1	24-04-2003
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		US 2012184710 A1	19-07-2012
		US 2013116406 A1	09-05-2013
		WO 2008031133 A2	20-03-2008
EP 1394254 A1	03-03-2004	NONE	