

(19) World Intellectual Property
Organization
International Bureau



WIPO | PCT



(10) International Publication Number
WO 2017/178587 A9

(43) International Publication Date
19 October 2017 (19.10.2017)

(51) International Patent Classification:
C12Q 1/68 (2006.01)

(21) International Application Number:

PCT/EP2017/058928

(22) International Filing Date:

13 April 2017 (13.04.2017)

(25) Filing Language:

English

(26) Publication Language:

English

(30) Priority Data:

16305435.6

14 April 2016 (14.04.2016)

EP

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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, 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).

Published:

— with international search report (Art. 21(3))

— with sequence listing part of description (Rule 5.2(a))

(48) Date of publication of this corrected version:

04 January 2018 (04.01.2018)

(15) Information about Correction:

see Notice of 04 January 2018 (04.01.2018)

(54) Title: METHOD FOR UNIVERSAL DETECTION AND QUANTIFICATION OF MYCOPLASMA (MOLLICUTES) 16S RDNA BY QUANTITATIVE POLYMERASE CHAIN REACTION AMPLIFYING A 1.5 KILOBASE FRAGMENT

(57) Abstract: The present invention relates to a method for detecting mycoplasma 16S rDNA 1.5 kilobase fragment amplified by quantitative PCR. One of the most critical limitation of the qPCR is the DNA fragment length to amplify. The existing qPCR methods does not permit the amplification of long DNA fragments. Here the inventors demonstrate for the first time the amplification of mycoplasma 16S rDNA 1.5 kilobase fragment using qPCR based on real-time polymerase chain reaction. In particular, the present invention relates to a method for universal detection and quantification of mycoplasma 16S rDNA 1.5 kilobase fragment in a sample comprising contacting said sample with degenerate primers amplified by quantitative PCR or qPCR and using a DNA loading probe.



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**METHOD FOR UNIVERSAL DETECTION AND QUANTIFICATION OF
MYCOPLASMA (MOLLICUTES) 16S rDNA BY QUANTITATIVE POLYMERASE
CHAIN REACTION AMPLIFYING A 1.5 KILOBASE FRAGMENT**

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FIELD OF THE INVENTION:

The present invention relates to a method for universally detecting and quantifying mycoplasma (Mollicutes) 16S rDNA by amplifying a 1.5 kilobase fragment by quantitative polymerase chain reaction.

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BACKGROUND OF THE INVENTION:

The ever-growing usage of cell lines to understand every biological process at the molecular and cellular level and the ability of microbes and parasites to invade them put these tools under stringent quality scrutiny to ensure unbiased interpretation of cell-based experiments. One of the major recognized pitfalls in cell culture is the adventitious contamination by mycoplasma. A recent survey of transcriptomic data from the NCBI Sequence Read Archive has revealed that 11% of the samples analysed by hundreds of laboratories were contaminated by mycoplasma RNA, a finding that invalidated many reported findings (Olarerin-George, A. O., and J. B. Hogenesch. 2015. Assessing the prevalence of mycoplasma contamination in cell culture via a survey of NCBI's RNA-seq archive. Nucleic Acids Res 43:2535-2542). Mycoplasma (Mollicutes) are among the smallest prokaryotes and are characterized by a lack of rigid cell wall. Their abundance in varied plant and animal hosts as well as their easily unnoticed presence favour their surreptitious cohabitation with culture cells from which they mostly benefit owing to their frequent axenic growth requirements. Mycoplasma (Mollicutes) have numerous and variable impacts on cell biology making them a dreadful cause in obscuring the data obtained in cell culture. Indeed, they interfere with cellular gene expression (Olarerin-George, A. O., and J. B. Hogenesch. 2015. Assessing the prevalence of mycoplasma contamination in cell culture via a survey of NCBI's RNA-seq archive. Nucleic Acids Res 43:2535-2542.), cellular metabolism, cell activation, apoptosis, RNA and DNA synthesis, signal transduction, cell growth, biochemical and biological assays and increased or decreased virus growth (Drexler, H. G., and C. C. Uphoff. 2002. Mycoplasma contamination of cell cultures: Incidence, sources, effects, detection, elimination, prevention. Cytotechnology 39:75-90). In addition, several

mycoplasma species are pathogenic in their natural host, and as such are regular targets for detection in human and veterinary medicine.

Several techniques have been developed to detect mycoplasma including broth and agar culture, DNA staining after Hoechst labelling, ELISA, enzymatic assay and PCR.

5 Johansson and al. have validated a set of degenerate universal primers to detect 16S rDNA of mycoplasma by nested PCR (Johansson, K. E., M. U. Heldtander, and B. Pettersson. 1998. Characterization of mycoplasmas by PCR and sequence analysis with universal 16S rDNA primers. *Methods Mol Biol* 104:145-165). These available assays suffer from being either cumbersome, difficult to interpret, time consuming (DNA imaging, culture on agar), operator
10 dependent (DNA labelling), limited to the detection of only a limited range of species or/and not quantitative (Young, L., J. Sung, G. Stacey, and J. R. Masters. 2010. Detection of Mycoplasma in cell cultures. *Nat Protoc* 5:929-934).

SUMMARY OF THE INVENTION:

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The present invention relates to a method for detecting mycoplasma 16S rDNA 1.5 kilobase fragment amplified by quantitative polymerase chain reaction (qPCR).

In particular, the invention is defined by the claims.

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DETAILED DESCRIPTION OF THE INVENTION:

Here the inventors demonstrate for the first time the amplification of mycoplasma 16S rDNA 1.5 kilobase fragment using qPCR based on real-time polymerase chain reaction. One of the most critical limitation of the qPCR is the DNA fragment length to amplify. Indeed
25 qPCR is usually performed on 100-250 bp long DNA fragments to improve PCR efficiency. The maximum DNA fragment length used in qPCR was recently reported as being 0.8-0.9 kb long (Li, H., J. Chen, M. Zhou, X. Geng, J. Yu, W. Wang, X. E. Zhang, and H. Wei. 2014. Rapid detection of Mycobacterium tuberculosis and pyrazinamide susceptibility related to pncA mutations in sputum specimens through an integrated gene-to-protein function
30 approach. *J Clin Microbiol* 52:260-267 and Huang, L., X. Hu, M. Zhou, Y. Yang, J. Qiao, D. Wang, J. Yu, Z. Cui, Z. Zhang, X. E. Zhang, and H. Wei. 2014. Rapid detection of New Delhi metallo-beta-lactamase gene and variants coding for carbapenemases with different activities by use of a PCR-based in vitro protein expression method. *J Clin Microbiol* 52:1947-1953). The existing qPCR methods thus does not permit the amplification of long DNA fragments.

The first aspect of the present invention refers to a method for universal detection and quantification of mycoplasma (Mollicutes) 16S rDNA 1.5 kilobase fragment in a sample comprising contacting said sample with degenerate primers of SEQ ID N°3 and SEQ ID N°4 amplified by PCR or quantitative PCR and using a DNA loading probe. Thus, this technique permits the quantitative detection of the presence of mycoplasma strains.

As used herein, the term “sample” must be understood in its broadest sense. For instance, the term “sample” refers to cell culture extract such as supernatant, to human or animal biological sample, water sample or food sample such as milk for example.

As used herein the term “16S rDNA” has its general meaning in the art and refers to the gene encoding for ribosomal 16S RNA. The term “16S rDNA gene” refers to nucleic acid sequence SEQ ID N°1 as defined below.

SEQ ID N°1:

Genbank accession n°NR_036952.1 (*Mycoplasma capricolum subsp. capricolum strain California kid* 16S ribosomal RNA gene, complete sequence, 1524 bp)

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1  aaaatgagag tttgatcctg gctcaggata aacgctggcg gcatgcctaa tacatgcaag
   61  tcgaacgggg gtgcttgcaac ctcagtggcg aacgggtgag taacacgtat ctaacctacc
20  121  ttatagcggg ggataacttt tggaaacgaa agataatacc gcatgtagat cttattatcg
   181  catgagaaaa gatcaaaaga accgtttggt tcactatgag atgggggatgc ggcgtattag
   241  ctagtaggtg agataatagc ccacctaggc gatgatacgt agccgaactg agaggttgat
   301  cggccacatt gggactgaga tacggcccag actcctacgg gaggcagcag tagggaattt
   361  ttcacaatgg acgaaagtct gatgaagcaa tgccgcgtga gtgatgacgg ccttcggggt
25  421  gtaaagctct gttgtaaggg aagaaaaaat agagtaggaa atgactttat cttgacagta
   481  ccttaccaga aagccacggc taactatgtg ccagcagccg cggtaataca taggtggcaa
   541  gcgttatccg gatattattg gcgatatagg tgcgtagggc gttttgcaag tttgaggtta
   601  aagtcgggag ctcaactccg gttcgccttg aagactgttt tactagaatg caagagaggt
   661  aagcgggaatt ccatgtgtag cggtgaaatg cgtagatata tggagaaca cctgtggcga
30  721  aagcggccta ctggcttggt attgacgctg aggcacgaaa gcgtggggag caaataggat
   781  tagataccct agtagtccac gccgtaaacg atgagtacta agtggtggg taactcagcg
   841  ctgcagctaa cgcattaaat actccgcctg agtagtatgc tcgcaagagt gaaactcaaa
   901  ggaattgacg gggaccgcga caagtggtag agcatgtggt ttaattcgaa gcaacacgaa
   961  gaaccttacc agggcttgac atccagtgcg aagctataga gatatagtag aggttaacat
35  1021  tgagacaggt ggtgcatggt tgtcgtagt tcgtgccgtg aggtgttggg ttaagtcctg
   1081  caacgaacgc aaccttgctc gttagttact aacattaagt tgagaactct aacgagactg
   1141  ctagtgtgaa ctgagaggaag gtggggatga cgtcaaatca tcatgcccct tatgtcctgg
   1201  gctacacacg tgctacaatg gctggtacaa agagttgcaa tcctgtgaag gggagctaag
   1261  ctcaaaaaac cagtctcagt tcggattgaa gtctgcaact cgacttcatg aagccggaat
40  1321  cactagtaat cgcgaatcag ctatgtcgcg gtgaatacgt tctcgggtct tgtacacacc
   1381  gcccgctaca ccatgagagt tggtaatacc agaagtaggt agcttaacca tttggagagc
   1441  gcttcccaag gtaggactag cgattggggg gaagtcgtaa caaggtatcc gtacgggaac
   1501  gtgcggatgg atcacctcct ttct

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16S rDNA gene is present in all Prokaryotes, so it is a useful marker of its presence. It is a conserved gene among prokaryotic cells and presents a functional constancy. The DNA

sequence of the 16S rDNA gene has been determined for a large number of species. It is approximately 1500 base pair long, existing as a multigene family or operons, and codes for a ribosomal 16S RNA. 16S RNA is a catalytic RNA which is part of the 30S unit of the prokaryotic ribosome. 16S DNA gene has strongly conserved sequence regions and variable sequence regions. The nucleotide sequences of the rRNA molecules contain well-defined segments of different evolutionary variability, which in the 16S rRNA molecule are referred to as universal (U), semiconserved (S), and variable (V) regions (Gray, M. W., Sankoff, D., and Cedergren, R. J. (1984) On the evolutionary descent of organisms and organelles: a global phylogeny based on a highly conserved structural core in small subunit ribosomal RNA. Nucleic Acids Res. 12, 5837-5852.). The universal regions are numbered U1-U8 from the 5'-terminus.

As used herein, the term "16S rDNA 1.5 kilobase fragment" refers to any nucleic acid sequence as defined below.

SEQ ID N° 2:

Genbank accession n°JN935890.1 (*Acholeplasma laidlawii* strain *Algen* 16S ribosomal RNA gene, partial sequence; 16S-23S ribosomal RNA intergenic spacer, complete sequence; and 23S ribosomal RNA gene, partial sequence, 1578 bp)

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1 ggatgaacgc tggcggcgtg cctaatacat gcaagtcgaa cgaagcatct tcggatgctt
  61 agtggcgaac gggtagtaaa cacgtagata acctaccttt aactcgagga taactccggg
 121 aaactggagc taatactgga taggatgtgt gcatgaaaaa aacacattta aagattttatc
 181 ggtttaagag gggctctgcg cgcattagtt agttgggtgg gtaagagcct accaagacga
 241 tgatgcgtag ccggaactgag aggtctaccg gccacattgg gactgagaac ggcccaaact
 301 cctacgggag gcagcagtag ggaatttttc gcaatggggg aaacctgac cgagcaacgc
 361 cgcgtgaacg acgaagtact tcggtatgta aagttctttt atatgggaag aaaaattaaa
 421 aattgacggg accatatgaa taagccccgg ctaactatgt gccagcagcc gcggtataac
 481 atagggggcg agcgttatcc ggatttactg ggcgtaaaag gtgcgtaggt ggttataaaa
 541 gtttgtggtg taagtgcagt gcttaacgct gtgaggctat gaaaactata taactagagt
 601 gagacagagg caagtggaaat tccatgtgta gcggtaaaat gcgtaaatat atggaggaac
 661 accagtggcg aaggcggctt gctgggtcta tactgacact gatgcacgaa agcgtgggga
 721 gcaaacagga ttagataccc tggtagtcca cgccgtaaac gatgagaact aagtgttggc
 781 cataaggtca gtgctgcagt taacgcatta agttctccgc ctgagtagta cgtacgcaag
 841 tatgaaactc aaaggaattg acgggacccc gcacaagcgg tggatcatgt tgtttaattc
 901 gaagatacac gaaaaacctt accaggtctt gacatactct gcaaaggctt agaaataagt
 961 tcggaggcta acagatgtac aggtgggtgca cggttgtcgt cagctcgtgt cgtgagatgt
1021 tgggttaagt cccgcaacga gcgcaaccct tattgctagt taccatcatt aagttgggga
1081 ctctagcgag actgccagtg ataaattgga ggaagggtgg gatgacgtca aatcatcatg
1141 ccccttatga cctgggctac aaacgtgata caatggctgg aacaaagaga agcrataggg
1201 tgacctggag cgaaactcac aaaaacagtc tcagttcgga ttggagtctg caactcgact
1261 ccatgaagtc ggaatcgcta gtaatcgcaa atcagcatgt tcgggtgaat acgttctcgg
1321 ggtttgtaca caccgcccgt caaacacga aagtgggcaa taccacacgc cggtagccta
1381 acccgaaagg gagggagccg tctaaggtag ggtccatgat tgggggttaag tcgtaacaag
1441 gtatccctac ggaacgtggg ggatggatca cctcctttct aaggagaaaag gctaactaac

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1501 acttagcaca agatgactac tagtaagtag taacattctc taaatttggt catcatattc
1561 agtttttgara gacttaaa
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As used herein, the term “16S rDNA 1.5 kilobase fragment” refers also to any nucleic acid sequence having at least 90% of identity to the nucleic acid sequence of SEQ ID N°2. The term “16S rDNA 1.5 kilobase fragment” refers to any nucleic acid sequence having 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100% of identity to the nucleic acid sequence of SEQ ID N°2.

The method for detecting a mycoplasma 16S rDNA 1.5 kilobase fragment by quantitative PCR according to the invention is made possible by using degenerate primers. By “primer” is meant an oligonucleotide that binds to a specific sequence of nucleic acid and can be elongated by a polymerase under appropriate conditions. By “degenerate primer” is meant a primer sequence that contains several possible bases in one or more positions. The degeneracy of the primer is the total number of sequence combinations it contains. Thus, degenerate primers are actually mixtures of similar, but not identical primers.

More particularly, a primer pair complementary to the universal regions U1 and U8 are used according to the present invention. These degenerate primers used according to the present invention are called U1 primer and U8 primer (Johansson, K. E., M. U. Heldtander, and B. Pettersson. 1998. Characterization of mycoplasmas by PCR and sequence analysis with universal 16S rDNA primers. *Methods Mol Biol* 104:145-165).

The sequence of U1 primer is the following sequence:

SEQ ID N°3: 5'- GTTTGATCCTGGCTCAGGAYDAACG - 3'

The sequence of U8 primer is the following sequence:

SEQ ID N°4: 5' - GAAAGGAGGTRWTCCAYCCSCAC - 3'

Degenerated positions are indicated with the corresponding ambiguity code according to the International Union of Biochemistry as following:

W represents A or T.

S represents C or G.

M represents A or C.

K represents G or T.

R represents A or G.

Y represents C or T.

B represents C, G or T.

D represents A, G or T.

H represents A, C or T.

5 V represents A, C or G.

N represents A, C, G or T.

The method according to the invention shares the advantage of using universal 16S rDNA primers that have been validated by Johansson and al. (Johansson, K. E., M. U. Heldtander, and B. Pettersson. 1998. Characterization of mycoplasmas by PCR and sequence analysis with universal 16S rDNA primers. *Methods Mol Biol* 104:145-165.) and the Applicant (Audrey J., Florence Tardy F., Allatif O., Grosjean I., Blanquier B., Gerlier D. 2017. Assessing mycoplasma contamination of cell cultures by qPCR using a set of universal primer pairs targeting a 1.5 kb fragment of 16S rRNA genes. *PLoS ONE* 12(2): e0172358. doi:10.1371/journal.pone.0172358) to detect any of several tens of mycoplasma strains that have been tested, whilst other PCR methods that amplify highly conserved short 16S cDNA detect only subsets of mycoplasma strains.

The use of this degenerate primer pair permits the quantitative detection of mycoplasma 16S rDNA 1.5 kilobase fragment by quantitative Polymerase Chain Reaction.

Thus, in one embodiment, the method according to the invention may be used for detecting the presence of mycoplasma (Mollicutes) strains in a sample.

Theoretically, the pair of degenerate primers could have been used for the detection of Phytoplasma strains. However, because they detect as well DNA from chloroplasts there are practically useless with plant derived samples.

More particularly, due to the high conservation of the 16S RNA gene between species, U1/U8 degenerate primers are predicted to be complementary to a wide range of Prokaryote species. In a further embodiment, the method according to the invention may be used for detecting the presence of Prokaryote strains in a sample.

In a further embodiment, the sample tested according to the invention for detecting the presence of mycoplasma strains or Prokaryote strains is, for example, a cell culture supernatant sample, food sample, livestock sample, or human sample.

- 5 In a further embodiment, the invention refers to a method for detecting any homologue gene of mycoplasma 16S rDNA in a sample comprising contacting said sample with degenerate primers of SEQ ID N°3 and SEQ ID N°4 amplified by qPCR.

- The Prokaryote strains (Tenericutes, incl mollicutes) predicted to be detected with no mismatch with U1/U8 degenerated primers with Silva Test Prime Algorithm (Quast C,
10 Pruesse E, Yilmaz P, Gerken J, Schweer T, Yarza P, Peplies J, Glöckner FO (2013), The SILVA ribosomal RNA gene database project: improved data processing an web-based tools. Nucl. Acids Res. 41 (D1): D590-D596) and used in the present invention are, for example:

- Ureaplasma urealyticum serovar 8 str. ATCC 27618
Ureaplasma urealyticum serovar 4 str. ATCC 27816
15 Ureaplasma urealyticum serovar 7 str. ATCC 27819
Ureaplasma urealyticum serovar 9 str. ATCC 33175
Ureaplasma parvum serovar 6 str. ATCC 27818
Ureaplasma urealyticum serovar 5 str. ATCC 27817
Ureaplasma urealyticum serovar 11 str. ATCC 33695
20 Ureaplasma urealyticum serovar 12 str. ATCC 33696
Ureaplasma parvum serovar 14 str. ATCC 33697
Ureaplasma urealyticum serovar 13 str. ATCC 33698
Ureaplasma urealyticum serovar 2 str. ATCC 27814
Ureaplasma parvum serovar 3
25 Ureaplasma parvum serovar 3 str. ATCC 27815
Ureaplasma urealyticum serovar 10 str. ATCC 33699
Mycoplasma hyopneumoniae J
Mycoplasma hyopneumoniae 7448
Mycoplasma mobile 163K
30 Mycoplasma hyopneumoniae 232
Mycoplasma hominis
Mycoplasma sp. HRC689

- Mycoplasma collis
Mycoplasma cynos
Mycoplasma molare
Mycoplasma spumans
5 Mycoplasma opalescens
Mycoplasma flocculare ATCC 27716
Mycoplasma anatis 1340
Mycoplasma iowae 695
Mycoplasma canis PG 14
10 Mycoplasma canis UF31
Mycoplasma pulmonis
Mycoplasma fermentans PG18
Mycoplasma californicum HAZ160_1
Mycoplasma canadense
15 Mycoplasma arginini
Mycoplasma vulturii
Mycoplasma sp. VJC358
Mycoplasma orale
Mycoplasma arthritis 158L3-1
20 Mycoplasma bovis HB0801
Mycoplasma bovis PG45
Mycoplasma hyopneumoniae 168
Mycoplasma fermentans M64
Mycoplasma bovis Hubei-1
25 Mycoplasma hyorhinis MCLD
Mycoplasma hyopneumoniae 168-L
Mycoplasma genitalium M2321
Mycoplasma genitalium M6282
Mycoplasma genitalium M6320
30 Mycoplasma genitalium M2288
Mycoplasma hyopneumoniae 7422
Mycoplasma pneumoniae M129-B7
Mycoplasma bovis CQ-W70
Mycoplasma gallisepticum S6

- Mycoplasma bovoculi M165/69
Mycoplasma californicum
Mycoplasma flocculare ATCC 27399
Mycoplasma hominis ATCC 27545
- 5 Mycoplasma agalactiae
uncultured Mycoplasma sp.
Mycoplasma hominis ATCC 23114
Mycoplasma neophronis
Mycoplasma cynos C142
- 10 Mycoplasma hyosynoviae
Mycoplasma bovis
Mycoplasma iowae
Mycoplasma flocculare
Mycoplasma hyopneumoniae
- 15 Spiroplasma sp.
Spiroplasma sp. Anisosticta MK
Spiroplasma alleghenense
Spiroplasma chinense
Spiroplasma chrysopicola
- 20 Spiroplasma corruscae
Spiroplasma culicicola
Spiroplasma diminutum
Spiroplasma helicoides
Spiroplasma insolitum
- 25 Spiroplasma lampyridicola
Spiroplasma leptinotarsae
Spiroplasma litorale
Spiroplasma montanense
Spiroplasma sabaudiense
- 30 Spiroplasma turonicum
Spiroplasma velocicrescens
Spiroplasma sp. 277F
Spiroplasma sp. LB-12
Spiroplasma sp. TAAS-1

- Spiroplasma sp. CB-1
- Spiroplasma sp. Ar-1357
- Spiroplasma sp. W115
- Spiroplasma sp. TIUS-1
- 5 Spiroplasma sp. BIUS-1
- Spiroplasma sp. BARC 1901
- Spiroplasma melliferum
- Spiroplasma phoeniceum
- Spiroplasma mirum ATCC 29335
- 10 Spiroplasma kunkelii CR2-3x
- Spiroplasma sp. BARC 1357
- Spiroplasma sp. BARC 2649
- Spiroplasma tabanidicola
- Spiroplasma citri
- 15 Mesoplasma florum L1
- Mycoplasma monodon
- Mycoplasma capricolum subsp. capripneumoniae
- Mycoplasma mycoides subsp. mycoides SC str. PG1
- Mycoplasma capricolum subsp. capricolum ATCC 27343
- 20 Mycoplasma mycoides subsp. mycoides SC str. Gladysdale
- Mycoplasma leachii PG50
- Mycoplasma putrefaciens KS1
- Mycoplasma putrefaciens Mput9231
- Mycoplasma yeatsii GM274B
- 25 Mycoplasma mycoides subsp. capri
- Mycoplasma mycoides subsp. mycoides
- Mycoplasma capricolum subsp. capricolum
- Entomoplasma freundtii
- Acholeplasma laidlawii PG-8A
- 30 Acholeplasma oculi

In one embodiment, the method according to the invention is suitable for the detection of any Prokaryote contamination, especially mycoplasma contamination, in cell cultures.

Another application of the method of the present invention may be its use in diagnosis and follow up of contamination in human, livestock, food, or water for example.

In a further embodiment, the method according to the invention comprises the following steps:

- (a) Adding to the sample to be tested a known amount of a DNA loading probe;
- (b) Extracting and purifying DNA;
- (c) Quantifying DNA and determining DNA extraction yield and DNA purity (i.e. absence of contaminants that are inhibitors of the PCR reaction) by DNA loading probe-specific qPCR;
- (d) Performing 16S rDNA-specific Quantitative PCR;
- (e) Using the melting curve of the amplified DNA fragment as a first screening step indicative of 16S rDNA amplicon;
- (f) Determining 16S DNA copy number by plotting cycling quantitation values on a standard curve.

As described in steps “a” and “b”, DNA extracting method from sample can be optimized with internal DNA loading probe. During the DNA extraction step, a known amount of DNA loading probe (DLP) is added to the sample to be tested so as to check the efficiency of DNA recovery measured by DLP-specific qPCR. The DNA loading probe is so quantified by DLP-specific qPCR. Thus, DNA extraction yield is determined by DNA loading probe-specific qPCR (step “c”).

In a particular embodiment, the extraction and purification of DNA is carried out using a commercial kit.

As described in step “e”, it is possible to detect the presence of the 16S rDNA amplicon by analysing the melting curve of the final amplicon as a first filter.

As described above in step “f”, in order to quantify the initial amount of 16S rDNA in the sample, the method according to the invention comprises a step of determination of 16S DNA copy number by plotting C_q (cycling quantitation) values on a standard curve obtained by qPCR of an internally deleted 16S rDNA source.

In a particular embodiment, the standard curve is obtained by qPCR of an internally deleted 16S rDNA source.

5 In some embodiment, the method according to the invention further comprises using a traceable internal PCR positive control. In a particular embodiment, the positive control is a known and traceable 16S rDNA source.

10 In a particular embodiment, the method according to the invention further comprises a step of detecting inhibitors of the PCR contaminating the sample.

In one embodiment, the method according to the invention further comprises a step of visualizing the 1.5 kilobase size of the amplicon by electrophoresis on agarose gel.

15 In still another embodiment, the method according to the invention comprises a further step consisting of identifying the mycoplasma (Mollicutes) strain by sequencing of the DNA amplicon.

In a particular embodiment, the method according to the invention comprises a further step consisting of identifying the Prokaryote strain by sequencing of the DNA amplicon.

20 In a further embodiment, the sequence of the DNA amplicon is blasted against a gene sequences database. The gene sequences database may be Genbank. This further step is useful to trace the source of the contamination.

25 In a further embodiment, the sample tested according to the invention is for example a cell culture supernatant sample, food sample, livestock sample or human sample. In particular, the sample is Biosafety Level 2 to Biosafety Level 4 pathogenic sample.

30 As used herein, the term "biosafety level" has its general meaning in the art and refers to a set of biocontainment precautions required to isolate dangerous biological agents in an enclosed laboratory facility. The levels of containment range from the lowest biosafety level 1 (BSL-1) to the highest at level 4 (BSL-4). These levels have been defined in reference documents in the USA (Richmond JY, McKinney RW, Biosafety in Microbiological and Biomedical Laboratories (4th ed.)(1999), ISBN 0-7881-8513-6) and in the European Union (Council Directive 90/679/EEC of 26 November 1990 on the protection of workers from risks related to exposure to biological agents at work, OJ No. L 374, p. 1)

In a further embodiment, the method according to the invention comprises the steps as described in Figure 1.

5 The qPCR technique is detailed below.

 The template nucleic acid need not be purified. Nucleic acids may be extracted from a sample by routine techniques such as those described in Diagnostic Molecular Microbiology: Principles and Applications (Persing et al. (eds), 1993, American Society for Microbiology,
10 Washington D.C.).

 U.S. Pat. Nos. 4,683,202, 4,683,195, 4,800,159, and 4,965,188 disclose conventional PCR techniques. PCR typically employs two oligonucleotide primers that bind to a selected target nucleic acid sequence. Primers useful in the present invention include oligonucleotides
15 capable of acting as a point of initiation of nucleic acid synthesis within the target nucleic acid sequence. A primer can be purified from a restriction digest by conventional methods, or it can be produced synthetically. If the template nucleic acid is double-stranded (e.g. DNA), it is necessary to separate the two strands before it can be used as a template in PCR. Strand separation can be accomplished by any suitable denaturing method including physical,
20 chemical or enzymatic means. One method of separating the nucleic acid strands involves heating the nucleic acid until it is predominately denatured (e.g., greater than 50%, 60%, 70%, 80%, 90% or 95% denatured). The heating conditions necessary for denaturing template nucleic acid will depend, e.g., on the buffer salt concentration and the length and nucleotide composition of the nucleic acids being denatured, but typically range from about 90° C. to
25 about 105° C. for a time depending on features of the reaction such as temperature and the nucleic acid length. Denaturation is typically performed for about 30 sec to 4 min (e.g., 1 min to 2 min 30 sec, or 1.5 min). If the double-stranded template nucleic acid is denatured by heat, the reaction mixture is allowed to cool to a temperature that promotes annealing of each primer to its target sequence on the target nucleic acid sequence. The temperature for
30 annealing is usually from about 35° C. to about 65° C. (e.g., about 40° C. to about 60° C.; about 45° C. to about 50° C.). Annealing times can be from about 10 sec to about 1 min (e.g., about 20 sec to about 50 sec; about 30 sec to about 40 sec). The reaction mixture is then adjusted to a temperature at which the activity of the polymerase is promoted or optimized, i.e., a temperature sufficient for extension to occur from the annealed primer to generate

products complementary to the template nucleic acid. The temperature should be sufficient to synthesize an extension product from each primer that is annealed to a nucleic acid template, but should not be so high as to denature an extension product from its complementary template (e.g., the temperature for extension generally ranges from about 40° C. to about 80° C. (e.g., about 50° C. to about 70° C.; about 60° C.). Extension times can be from about 10 sec to about 5 min (e.g., about 30 sec to about 4 min; about 1 min to about 3 min; about 1 min 30 sec to about 2 min).

qPCR involves use of a thermostable polymerase. The term “thermostable polymerase” refers to a polymerase enzyme that is heat stable, i.e., the enzyme catalyzes the formation of primer extension products complementary to a template and does not irreversibly denature when subjected to the elevated temperatures for the time necessary to effect denaturation of double-stranded template nucleic acids. Generally, the synthesis is initiated at the 3' end of each primer and proceeds in the 5' to 3' direction along the template strand. Thermostable polymerases have been isolated from *Thermus fiavus*, *T. ruber*, *T. thermophilus*, *T. aquaticus*, *T. lacteus*, *T. rubens*, *Bacillus stearothermophilus*, and *Methanothermus fervidus*. Nonetheless, polymerases that are not thermostable also can be employed in PCR assays provided the enzyme is replenished. Typically, the polymerase is a Taq polymerase (i.e. *Thermus aquaticus* polymerase).

The primers are combined with PCR reagents under reaction conditions that induce primer extension. Typically, chain extension reactions generally include 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 15 mM MgCl₂, 0.001% (w/v) gelatin, 0.5-1.0 µg denatured template DNA, 50 pmoles of each oligonucleotide primer, 2.5 U of Taq polymerase, and 10% DMSO). The reactions usually contain 150 to 320 µM each of dATP, dCTP, dTTP, dGTP, or one or more analogs thereof.

The newly synthesized strands form a double-stranded molecule that can be used in the succeeding steps of the reaction. The steps of strand separation, annealing, and elongation can be repeated as often as needed to produce the desired quantity of amplification products corresponding to the target nucleic acid sequence molecule. The limiting factors in the reaction are the amounts of primers, thermostable enzyme, and nucleoside triphosphates present in the reaction. The cycling steps (i.e., denaturation, annealing, and extension) are preferably repeated at least once. For use in detection, the number of cycling steps will

depend, e.g., on the nature of the sample. If the sample is a complex mixture of nucleic acids, more cycling steps will be required to amplify the target sequence sufficient for detection. Generally, the cycling steps are repeated at least about 20 times, but may be repeated as many as 40, 60, or even 100 times.

5

Quantitative PCR is typically carried out in a thermal cycler with the capacity to illuminate each sample with a beam of light of a specified wavelength and detect the fluorescence emitted by the excited fluorophore. The thermal cycler is also able to rapidly heat and chill samples, thereby taking advantage of the physicochemical properties of the nucleic acids and thermal polymerase.

10

In order to detect and measure the amount of amplicon (i.e. amplified target nucleic acid sequence) in the sample, a measurable signal has to be generated, which is proportional to the amount of amplified product. All current detection systems use fluorescent technologies. Some of them are non-specific techniques, and consequently only allow the detection of one target at a time. Alternatively, specific detection chemistries can distinguish between non-specific amplification and target amplification. These specific techniques can be used to multiplex the assay, i.e. detecting several different targets in the same assay.

15

SYBR® Green I: SYBR® Green I is the most commonly used dye for non-specific detection. It is a double-stranded DNA intercalating dye, that fluoresces once bound to the DNA. A pair of specific primers is required to amplify the target with this chemistry. The amount of dye incorporated is proportional to the amount of generated target. The dye emits at 520 nm and fluorescence emitted can be detected and related to the amount of target. The advantage of this technique is that the SYBR®-Green-I will bind to any amplified dsDNA. However, consequently, primer dimers or unspecific products will be also detected, thus introducing a possible bias in the quantification. Thus it is essential to check for the specificity of the system by running a meltcurve at the end of the PCR run. The principle is that every product has a different dissociation temperature, depending of the size and base contents, so it is still possible to check the number of products amplified. A valid SYBR® assay - primer pair - should produce a unique, well defined peak on the meltcurve. For these reasons, SYBR® Green I is rarely used for qualitative PCR. However, SYBR® Green I is often used as the first step to optimize a specific detection system assay, to check the specificity of the primers and validate the design.

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High Resolution Melting dyes (HRM dyes): High Resolution Meltcurve analysis is a newly emerging technology, which characterizes nucleic acid samples based on their dissociation behaviour. It combines the principle of intercalating dyes, meltcurve analyses and the application of specific statistical analyses. HRM uses the fundamental property of the separation of the two strands of DNA with heat (melting), and the monitoring of this melting with a fluorescent dye. On the contrary of SYBR Green, HRM dyes do not inhibit PCR at high concentration. The dye can consequently saturate the amplified target dsDNA and fluoresces. Melting temperature of a dsDNA target depends on GC content, length, and sequence. Due to the high sensitivity of HRM dyes, even a single base change will induce differences in the melting curve, and consequently in fluorescence (Erali M. et al., 2008). This emerging method is less expensive and as precise as probe-based methods. Only a few thermocyclers on the market currently allow the use of this technology, among them the Roche LightCycler®480, the Quiagen HRM Rotor-Gene™, and the ABI Prism®7500. The main HRM dyes available are EvaGreen, LCGreen®, SYTO® 9 and BEBO.

TaqMan® probes = Double-Dye probes: TaqMan® probes, also called Double-Dye Oligonucleotides, Double-Dye Probes, or Dual- Labelled probes, are the most widely used type of probes and are often the method of choice for scientists who have just started using Real-Time PCR. They were developed by Roche (Basel, Switzerland) and ABI (Foster City, USA) from an assay that originally used a radio- labelled probe (Holland et al. 1991), which consisted of a single-stranded probe sequence that was complementary to one of the strands of the amplicon. A fluorophore is attached to the 5' end of the probe and a quencher to the 3' end. The fluorophore is excited by the machine and passes its energy, via FRET (Fluorescence Resonance Energy Transfer) to the quencher. Traditionally the FRET pair has been FAM as the fluorophore and TAMRA as the quencher. In a well-designed probe, FAM does not fluoresce as it passes its energy onto TAMRA. As TAMRA fluorescence is detected at a different wavelength to FAM, the background level of FAM is low. The probe binds to the amplicon during each annealing step of the PCR. When the Taq polymerase extends from the primer which is bound to the amplicon, it displaces the 5' end of the probe, which is then degraded by the 5'-3' exonuclease activity of the Taq polymerase. Cleavage continues until the remaining probe melts off the amplicon. This process releases the fluorophore and quencher into solution, spatially separating them (compared to when they were held together

by the probe). This leads to an irreversible increase in fluorescence from the FAM and a decrease in the TAMRA.

LNA® Double-Dye probes: LNA® (Locked Nucleic Acid) was developed by Exiqon® (Vedbaek, Denmark). LNA® changes the conformation of the helix and increases the stability of the duplex. The integration of LNA® bases into Double-Dye Oligonucleotide probes, opens up great opportunities to improve techniques requiring high affinity probes as specific as possible, like SNP detection, expression profiling and in situ hybridization. LNA® is a bicyclic RNA analogue, in which the ribose moiety in the sugar-phosphate backbone is structurally constrained by a methylene bridge between the 2'-oxygen and the 4'-carbon atoms. The integration of LNA® bases into probes changes the conformation of the double helix from the B to A type (Ivanova A. et al., 2007). LNA® conformation allows a much better stacking and therefore a higher stability. By increasing the stability of the duplex, the integration of LNA® monomers into the oligonucleotide sequence allows an increase of the melting Temperature (T_m) of the duplex. It is therefore possible to reduce the size of the probe, which increases the specificity of the probe and helps designing it (Karkare S. et al., 2006).

Molecular Beacon probes: Molecular Beacons are probes that contain a stem-loop structure, with a fluorophore and a quencher at their 5' and 3' ends, respectively. The stem is usually 6 bases long, should mainly consist of C's and G's, and holds the probe in the hairpin configuration (Li Y. et al., 2008). The 'stem' sequence keeps the fluorophore and the quencher in close vicinity, but only in the absence of a sequence complementary to the 'loop' sequence. As long as the fluorophore and the quencher are in close proximity, the quencher absorbs any photons emitted by the fluorophore. This phenomenon is called collisional (or proximal) quenching. In the presence of a complementary sequence, the Beacon unfolds and hybridizes to the target, the fluorophore is then displaced from the quencher, so that it can no longer absorb the photons emitted by the fluorophore, and the probe starts to fluoresce. The amount of signal is proportional to the amount of target sequence, and is measured in real time to allow quantification of the amount of target sequence (Takacs T. et al., 2008). The increase in fluorescence that occurs is reversible, (unlike TaqMan® probes), as there is no cleavage of the probe, that can close back into the hairpin structure at low temperature. The stem structure adds specificity to this type of probe, because the hybrid formed between the probe and target has to be stronger than the intramolecular stem association. Good design of

Molecular Beacons can give good results, however the signal can be poor, as no physical separation of fluorophore from quencher occurs. Wavelength-Shifting Molecular Beacons are brighter than standard Molecular Beacons due to an enhanced fluorescence intensity of the emitter fluorophore. These probes contain a harvester fluorophore that absorbs strongly in the wavelength range of the monochromatic light source, an emitter fluorophore of the desired emission color, and a non-fluorescent (dark) quencher. In the absence of complementary nucleic acid targets, the probes are non-fluorescent, whereas in the presence of targets, they fluoresce, not in the emission range of the harvester fluorophore, that absorbs the light, but rather in the emission range of the emitter fluorophore. This shift in emission spectrum is due to the transfer of the absorbed energy from the harvester fluorophore to the emitter fluorophore by FRET, which only takes place in probes that are bound to the targets. Wavelength-Shifting Molecular Beacons are substantially brighter than conventional Molecular Beacons that cannot efficiently absorb energy from the available monochromatic light source (Tyagi S. et al., 2000).

Scorpions® primers: Scorpions® primers are suitable for both quantitative Real-Time PCR and genotyping/end-point analysis of specific DNA targets. They are PCR primers with a “stem-loop” tail consisting of a specific probe sequence, a fluorophore and a quencher. The “stem-loop” tail is separated from the PCR primer sequence by a “PCR blocker”, a chemical modification that prevents the Taq polymerase from copying the stem loop sequence of the Scorpions® primer. Such read-through would lead to non-specific opening of the loop, causing a non-specific fluorescent signal. The hairpin loop is linked to the 5' end of a primer via a PCR blocker. After extension of the primer during PCR amplification, the specific probe sequence is able to bind to its complement within the same strand of DNA. This hybridization event opens the hairpin loop so that fluorescence is no longer quenched and an increase in signal is observed. Unimolecular probing is kinetically favorable and highly efficient. Covalent attachment of the probe to the target amplicon ensures that each probe has a target in the near vicinity. Enzymatic cleavage is not required, thereby reducing the time needed for signaling compared to TaqMan® probes, which must bind and be cleaved before an increase in fluorescence is observed. There are three types of Scorpions® primers. Standard Scorpions®, which consist of a bi-labelled probe with a fluorescent dye at the 5' end and an internal non-fluorescent quencher. FRET Scorpions®, for use on a LightCycler® system. As the capillary system will only excite at 470 nm (FAM absorption wavelength) it is necessary to incorporate a FAM within the stem. A ROX is placed at the 5' end of the Scorpions®

primer, FAM is excited and passes its energy onto the ROX. Duplex Scorpions® have also been developed to give much better signal intensity than the normal Scorpions® format. In Standard Scorpions® the quencher and fluorophore remain within the same strand of DNA and some quenching can occur even in the open form. In the Duplex Scorpions® the quencher is on a different oligonucleotide and physical separation between the quencher and fluorophore is greatly increased, reducing the quenching when the probe is bound to the target.

Hybridization probes (also called FRET probes): Roche has developed hybridization probes (Caplin et al. 1999) for use with their LightCycler®. Two probes are designed to bind adjacent to one another on the amplicon. One has a 3' label of FAM, whilst the other has a 5' LC dye, LC red 640 or 705. When the probes are not bound to the target sequence, the fluorescent signal from the reporter dye is not detected. However, when the probes hybridize to the target sequence during the PCR annealing step, the close proximity of the two fluorophores allows energy transfer from the donor to the acceptor dye, resulting in a fluorescent signal that is detected.

TaqMan® MGB® probes: TaqMan® MGB® probes have been developed by Epoch Biosciences (Bothell, USA) and Applied Biosystems (Foster City, USA). They bind to the minor groove of the DNA helix with strong specificity and affinity. When the TaqMan® MGB® probe is complemented with DNA, it forms a very stable duplex with DNA. The probe carries the MGB® moiety at the 3' end. The MGB strongly increases the probe T_m, allowing shorter, hence more specific designs. The probe performs particularly well with A / T rich regions, and is very successful for SNP detection (Walburger et al., 2001). It can also be a good alternative when trying to design a probe which should be located in the splice junction (for which conventional probes are hard to design). Smaller probes can be designed with T_m as 65-67 °C, which gives a better discrimination (the probe is more specific for single mismatch). A good alternative to MGB probes are LNA® probes where the increase in T_m induced by the addition of LNA® bases is specific, contrary to the MGB moiety (cf. p. 15). During the primer extension step, the hybridized probe is cleaved by the 5' exonuclease activity of Taq polymerase and an increase in fluorescence is seen. Fluorescence of the cleaved probe during PCR is monitored in Real-Time by the thermocycler.

MGB Eclipse® probes: MGB Eclipse® probes also known as QuantiProbes, have originally been developed by Epoch Biosciences (Bothell, USA). MGB Eclipse® probes carry a minor groove binder moiety that allows the use of short probes for very high specificity. These are short linear probes that have a minor groove binder and a quencher on the 5' end and a fluorophore on the 3' end. This is the opposite orientation to TaqMan® MGB® probes and it is thought that the minor groove binder prevents the exonuclease activity of the Taq polymerase from cleaving the probe. The quencher is a Non Fluorescent Quencher also known as Eclipse Dark Quencher. Quenching occurs when the random coiling of the probe in the free form brings the quencher and the fluorophore close to another. The probe is straightened out when bound to its target and quenching is decreased, leading to an increase in fluorescent signal. The technologies that have been discussed above are the most widely used today, but numerous other technologies have occurred in publications, or are available on the market, such as: Resonsense probes, Light-up probes, HyBeacon® probes, LUX primers, Yin-yang probes, or Amplifluor®. You can contact us for more information on any of them.

The majority of the thermocyclers on the market now offer similar characteristics. Typically, thermocyclers involve a format of glass capillaries, plastics tubes, 96-well plates or 384-wells plates. The thermocycler also involve a software analysis.

Typically qPCR involves use of:

- Taq polymerase: A HotStart Taq polymerase is inactive at low temperatures (room temperature). Heating at 95 °C for several - usually 5 to 10 - minutes activates the enzyme, and the amplification can begin once the primers are annealed. The enzyme is not active until the entire DNA is denatured. Two major HotStart modifications exist, the antibody-blocked Taq and the chemically- blocked Taq. The antibody-blocked Taq is inactive because it is bound to a thermolabile inhibitor that is denatured during the initial step of PCR. The chemically-blocked Taq provides one clear advantage over the antibody-blocked Taq, as it is completely inactive at 60 °C, (the hybridization temperature of primers), thus preventing the formation of non- specific amplification and reducing primer dimer formation.
- dNTPs / dUTPs: Some kits contain a blend of dNTPs and dUTPs, other ones contain only dNTPs. Using only dNTPs increases the sensitivity, the reason being that the Taq incorporates more easily dNTPs than dUTPs. However, using a mix

containing dUTPs brings security to the assay, in case of contamination from a previous PCR product. Thanks to the UNG activity in association with incorporated dUTPs, this contamination can be eliminated.

- 5 - Uracil-N-Glycosylase: The Uracil-N-Glycosylase is an enzyme that hydrolyses all single-stranded and double-stranded DNA containing dUTPs. Consequently, if all PCR amplifications are performed in the presence of a dNTPs/dUTPs blend, by carrying a UNG step before every run it is possible to get rid of any previous PCR product.
- 10 - ROX reference dye: Some thermocyclers require MasterMix containing ROX dye for normalization. This is the case for the ABI and Eppendorf machines, and optional on the Stratagene machines. If you work with such machines, it is easier to work with the ROX dye already incorporated in the MasterMix rather than adding it manually. It guarantees a higher level of reproducibility and homogeneity of your assays.
- 15 - Fluorescein: For iCycler iQ®, My iQ® and iQ5 machines (BioRad thermocyclers), the normalization method for SYBR® Green assay uses Fluorescein to create a “virtual background”. As in the case for the ROX, it is better and easier to use a MasterMix that contains pre-diluted Fluorescein, guaranteeing higher reproducibility and homogeneity of your assays.
- 20 - MgCl₂: MgCl₂ is necessary for the Taq activity. MgCl concentration in MasterMixes is optimized according to the amount of Taq and also the buffer composition. However, it may be necessary sometimes to add MgCl₂ and most MasterMixes include an additional tube of MgCl₂.
- 25 - Inert colored dye: Some buffers also include an inert colored dye, to enable visualization of the buffer when loading in the wells. This colored dye has no effect on the sensitivity of the assay and is a convenient working tool. Note that such mixes, in combination with white plastic plates, provide better levels of fluorescence and a really easy way of working.

30 Well-designed primers and probes are a prerequisite for successful qPCR. By using well-designed primers and probes, PCR efficiencies close to 100 % can be obtained. Typically primers are designed using a designing software (for example Beacon Designer™). Most thermocycler softwares now offer tools to help in designing primers with the best

characteristics. Some of the best softwares are Beacon Designer, Primer Express, and DNA Star. Some other tools are freely available on the web, for example:

- <http://medgen.ugent.be/rtpimerdb/> (human primer and probe database)
- <http://www.ncbi.nlm.nih.gov/tools/primer-blast/>
- 5 – <http://frontend.bioinfo.rpi.edu/applications/mfold/> (for testing secondary structures)
- <http://www.ebi.ac.uk/~lenov/meltinghome.html> (Tm calculators)
- http://frodo.wi.mit.edu/cgi-bin/primer/primer3_www.cgi
- <http://bibiserv.techfak.uni-bielefeld.de/genefisher2>
- 10 – <http://www.premierbiosoft.com/qpcr/index>

Typically, qPCR involves the preparation of a standard curve for each amplified target nucleic acid sequence. Preparing a standard curve can indeed provide a good idea of the performance of the qPCR and thus serves as a quality control. The standard curve should cover the complete range of expected expression. Using standard material the standard curve should include at least 5 points of dilution, each of them in duplicate (at least). The 10-fold or 2-fold dilution range should cover the largest range of expression levels. Plotting these points on a standard curve, will determine the linearity, the efficiency, the sensitivity and the reproducibility of the assay. According to the present invention the standard curve is prepared from a “built on purpose” plasmid DNA containing the genomic DNA target. As used herein, "genomic DNA sample" or "gDNA" refers to a genomic DNA sample prepared from a DNA preparation. Methods for DNA purification are well known in the art. The genomic DNA may be prepared from a cell that is of the same organism than the cell that is used for preparing the nucleic acid sample of the invention (i.e. a human cell). Furthermore the cell from which the genomic sample is prepared must present the same ploidy than the cell used for preparing the nucleic acid sample of the invention; i.e. the cells present the same chromosomal abnormalities (e.g. in case of cancer cells).

Another aspect of the present invention relates to a kit for detecting mycoplasma 16S rDNA 1.5 kilobase fragment in a sample, comprising degenerate primers of SEQ ID N°3 and SEQ ID N°4, a thermal cycler, and reagents for performing the quantitative polymerase chain reaction.

The invention will be further illustrated by the following figure and example. However, these example and figure should not be interpreted in any way as limiting the scope of the present invention.

5 **FIGURE:**

Figure 1: Flow diagram of the method for detecting mycoplasma (Mollicutes) 16S rDNA 1.5 kilobase fragment amplified by quantitative PCR or qPCR.

10 **EXAMPLE:**

In a particular embodiment, the method for detecting mycoplasma 16S rDNA 1.5 kilobase fragment amplified by quantitative polymerase chain reaction is described with a flow diagram (figure 1).

The first step of the method consists of DNA extraction from an unknown sample (e.g. cell free supernatant) with addition of DNA loading probe (DLP). During the second step, 16S rDNA quantitative PCR using universal U1/U8 primers (SED ID N°3 and SED ID N°4 respectively) is carried out. The efficiency of DNA recovery is checked: the DNA loading probe is quantified by DLP-specific qPCR in order to determine DNA extraction yield.

If it is concluded a good yield of DLP recovery, the step 3 can be carried out. If it is concluded the yield of DLP recovery is less than 3%, method has to be started again from the first step.

The third step consists in analysing the melting curve of the final amplicon as a first filter for presence of the 16S rDNA amplicons in the sample. If the melting curve is identical to water control, mycoplasma is not detected. If the melting curve is a typical 16S rDNA melting curve, there is mycoplasma in the sample and the method can be continued with the fourth step. If the melting curve is atypical, the method has to be continued directly with the fifth step.

When the melting curve is a typical 16S rDNA melting curve, the copy number of 16S rDNA is determined by plotting C_q (cycling quantitation) values on a standard curve obtained by qPCR of an internally deleted 16S rDNA source (step 4).

The fifth step consists of the visualization of the length of the amplicons by electrophoresis on agarose gel. If only primer/dimer DNA band is detected (i.e 1.5 kb DNA band is not detected), there is no mycoplasma detected in the sample. If the 1.5 kb DNA band is detected, there is mycoplasma in the sample and the method can be continued with the sixth step. If the 0.9 kb DNA band of the positive 16S rDNA control is detected, method has to be

started again from the first step because it reflects accidental contamination when preparing the sample.

5 The sixth step, comprising DNA sequencing and blasting against Genbank, is carried out when there is mycoplasma in the sample, i.e. a 1.5 kb DNA band is detected during the fifth step. This finally leads to the last step of the method which is the identification of the mycoplasma (Mollicutes) strain.

REFERENCES:

10 Throughout this application, various references describe the state of the art to which this invention pertains. The disclosures of these references are hereby incorporated by reference into the present disclosure.

CLAIMS:

1. A method for universal detection and quantification of mycoplasma 16S rDNA 1.5 kilobase fragment in a sample comprising contacting said sample with degenerate primers of SEQ ID
5 N°3 and SEQ ID N°4 amplified by quantitative PCR or qPCR and using a DNA loading probe.
2. The method according to claim 1, wherein it further comprises the following steps:
 - (a) Adding to the sample to be tested a known amount of a DNA loading probe;
 - (b) Extracting and purifying DNA;
 - 10 (c) Quantifying DNA and determining DNA extraction yield and DNA purity (i.e. absence of contaminants that are inhibitors of the PCR reaction) by DNA loading probe-specific qPCR;
 - (d) Performing 16s rDNA-specific Quantitative PCR;
 - (e) Using the melting curve of the amplified DNA fragment as a first screening step
15 indicative of 16S rDNA amplicon;
 - (f) Determining 16S DNA copy number by plotting cycling quantitation values on a standard curve.
3. The method according to claim 2, wherein extracting and purifying DNA is carried out using a commercial kit.
- 20 4. The method according to one of the preceding claims, wherein the standard curve is obtained by qPCR of an internally deleted 16S rDNA source.
5. The method according to one of the preceding claims, wherein it further comprises using a traceable internal PCR positive control.
6. The method according to claim 5, wherein the positive control is a known and traceable
25 16S rDNA source.
7. The method according to one of the preceding claims, wherein it further comprises a step of detecting inhibitors of the PCR contaminating the sample.
8. The method according to one of the preceding claims, wherein it further comprises a step of visualizing the 1.5 kilobase size of the amplicon by electrophoresis on agarose gel.

9. The method according to one of the preceding claims, wherein it further comprises a step of identifying the Prokaryote species by sequencing of the DNA amplicon.
10. The method according to claim 9, wherein the DNA amplicon sequence is blasted against a gene sequences database.
- 5 11. The method according to one of the preceding claims for detecting the presence of mycoplasma (Mollicutes) strains in a sample.
12. The method according to one of the preceding claims for detecting the presence of Prokaryote strains in a sample.
- 10 13. The method according to anyone of claims 11 or 12, wherein the sample is a cell culture supernatant sample, food sample, livestock sample or human sample.
14. the method according to claim 13, wherein the sample is Biosafety Level 2, Biosafety Level 3 or Biosafety Level 4 pathogenic sample.
- 15 15. A kit for detecting mycoplasma 16S rDNA 1.5 kilobase fragment in a sample, comprising degenerate primers of SEQ ID N°3 and SEQ ID N°4, a thermal cycler, and reagents for performing the qPCR.

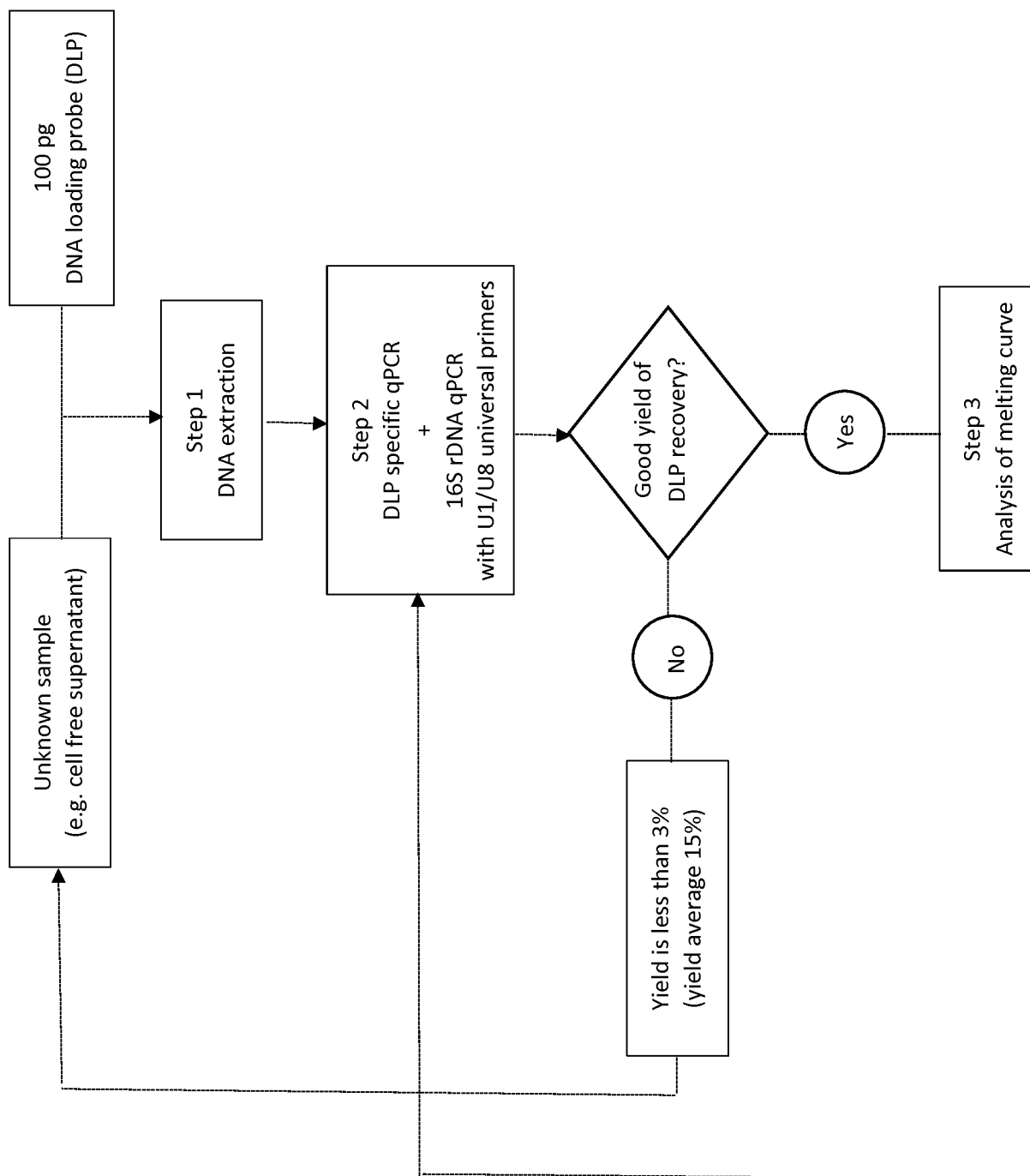


Figure 1 (1/3)

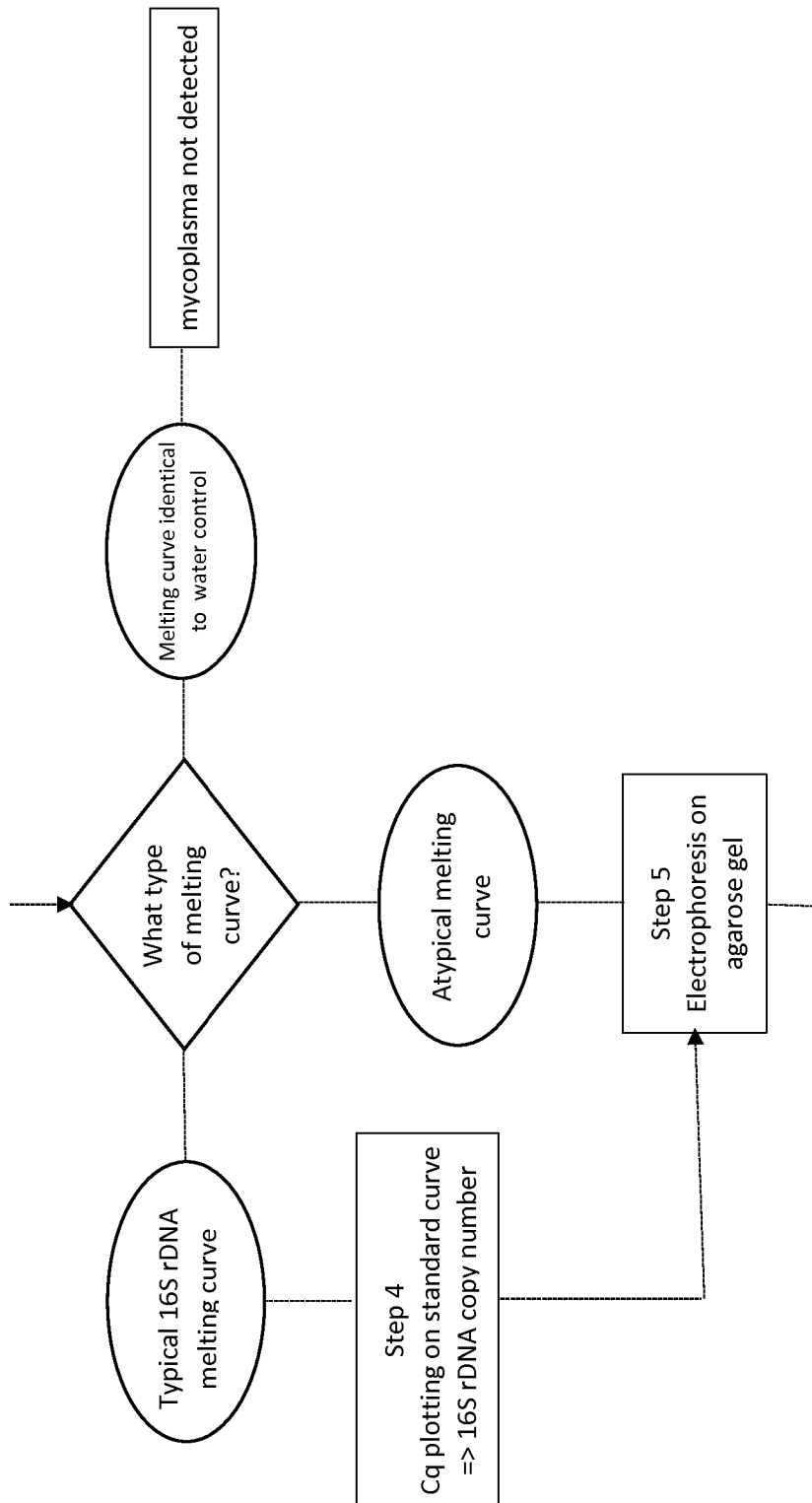


Figure 1 (2/3)

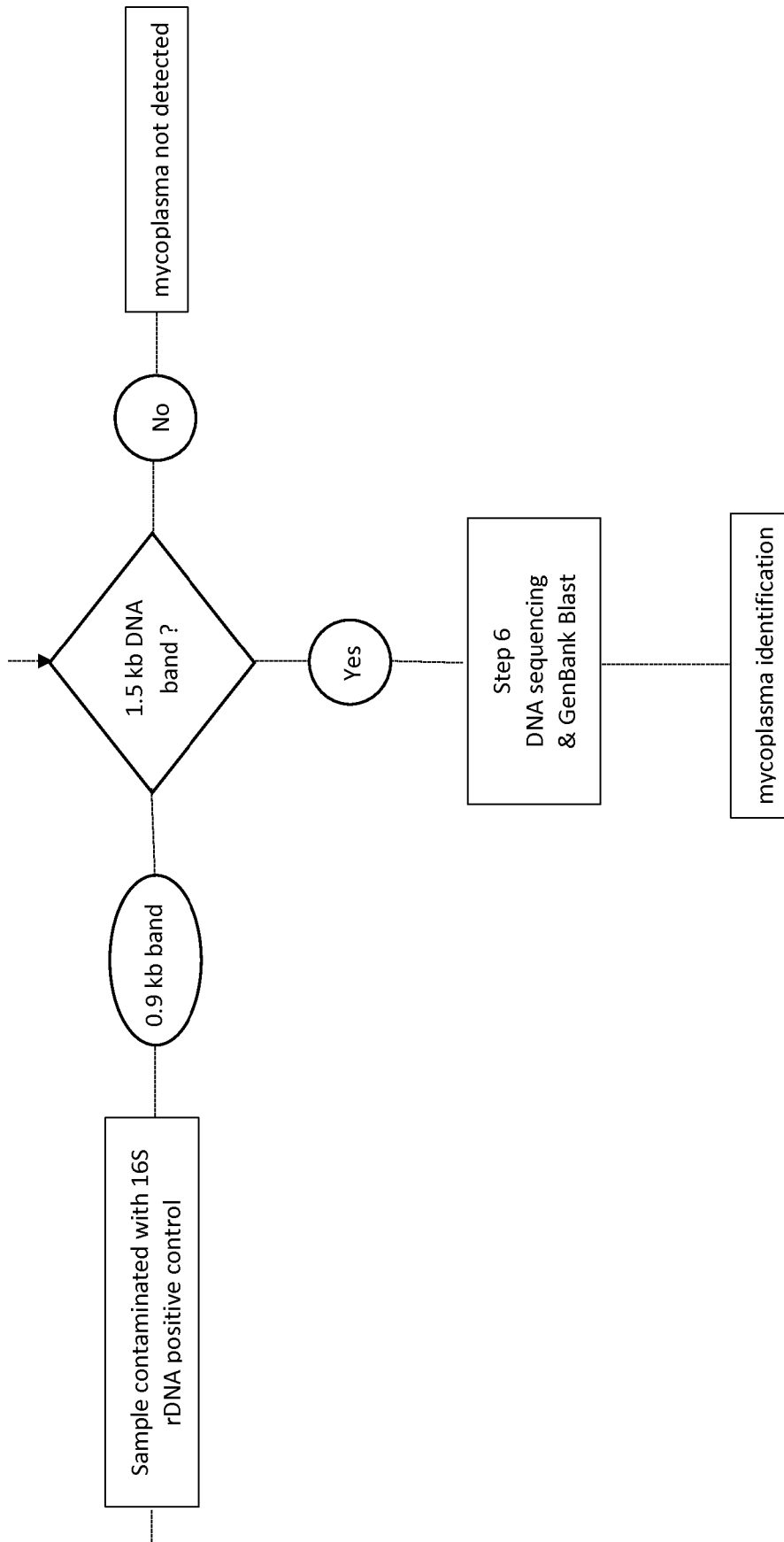


Figure 1 (3/3)

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2017/058928

A. CLASSIFICATION OF SUBJECT MATTER
INV. C12Q1/68
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

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

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	US 2011/104692 A1 (RUDI KNUT [NO] ET AL) 5 May 2011 (2011-05-05) [0006], [0008], [0010], Fig. 1-7. p. 20 tab. 11 and p. 10 tab. 10 -----	1-15
A	UPHOFF CORD C ET AL: "Comparative PCR analysis for detection of mycoplasma infections in continuous cell lines", IN VITRO CELLULAR & DEVELOPMENTAL BIOLOGY. ANIMAL, SPRINGER US, NEW YORK, vol. 38, no. 2, 1 February 2002 (2002-02-01), pages 79-85, XP002541349, ISSN: 1071-2690 abstract, tab. 1 and Fig. 1 ----- -/-	1-15



Further documents are listed in the continuation of Box C.



See patent family annex.

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Date of the actual completion of the international search

21 June 2017

Date of mailing of the international search report

29/06/2017

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INTERNATIONAL SEARCH REPORT

International application No

PCT/EP2017/058928

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

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X	JOHANSSON ET AL: "Characterisation of mycoplasmas by PCR and sequence analysis with universal primer 16S rDNA primers", METHODS IN MOLECULAR BIOLOGY, vol. 104, 1 January 1998 (1998-01-01), pages 145-165, XP009190925,	3-15
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Y	WO 01/71033 A2 (FORINNOVA AS [NO]; AARSKOG NINA K [NO]; VEDELER CHRISTIAN A [NO]) 27 September 2001 (2001-09-27) p. 3 l. 21-35 and p. 3 l. 13 and l. 29-31 -----	1
Y	ARCENAS R C ET AL: "A real-time polymerase chain reaction assay for detection of Pneumocystis from bronchoalveolar lavage fluid", DIAGNOSTIC MICROBIOLOGY AND INFECTIOUS DISEASE, ELSEVIER, AMSTERDAM, NL, vol. 54, no. 3, 1 March 2006 (2006-03-01), pages 169-175, XP028005607, ISSN: 0732-8893, DOI: 10.1016/J.DIAGMICROBIO.2005.08.006 [retrieved on 2006-03-01] abstract, p. 170 col. 1 and 2, p. 171, col. 2 -----	2

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No

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