

Method for detecting microorganisms

The present invention relates to a method for detecting microorganisms, for example mycoplasma or mouse minute virus (MMV), in a sample by performing PCR with specific primers and optionally additional probes. The present invention also provides primer pairs and a kit for performing the method.

Background of the invention

Cell lines used for the manufacturing of drug substances intended for clinical trial or commercial use must be tested and cleared from contamination by microorganisms, for example mycoplasma or parvoviruses (see European Pharmacopoeia and ICH Guideline Q5 on viral safety). Besides mandatory testing requested by health authorities, it is in the interest of industries to perform regular testing in order to protect facilities from contamination by testing samples prior to their introduction into production facilities, and to detect contamination early on and protect downstream equipment. Several methods can be used to document the status of cell substrates in terms of microorganism contamination, such as mycoplasma or virus contamination, which mainly include sample cultivation in medium suitable for microorganism growth and colony identification and nucleic acid technologies (NAT) such as quantitative PCR (qPCR) and droplet digital PCR ddPCR for the detection of bacterial or viral genetic material derived for example from mycoplasma or parvovirus. Because of the diversity of e.g. mycoplasma strains, health agencies expect assays to be able to detect a sub group of germs, based on their frequency and concerns for human health.

Mouse minute virus (MMV), alternately known as Minute Virus of Mice, is a member of the parvovirus family and is a non-enveloped virus approximately 20 nm in diameter. MMV contains a single-stranded DNA that is roughly 5 kb in size, and 2 genes *ns1* (Non Structural 1) and *vp1* (Viral Protein 1). There are four strains of MMV (i, p, m and c) and seven genomic sequences reported in the NCBI database. MMV is known to have contaminated production processes at multiple companies through extrinsic sources. MMV can infect humans, although it is not considered a significant risk to operators and patient safety. However, the risk to the production process is significant as an MMV contamination will likely result in the disruption of supply to patients, loss of product, costs of cleaning facilities and equipment, and the potential disposal of expensive chromatography resin.

Cell culture-based methods are difficult to implement due to the necessity to use optimal cell growth medium where all mycoplasma strains mentioned in guidelines, as well as parvoviruses, such as MMV, might grow. In addition, due to the slow replication of mycoplasma, the completion of these assays can take up to several weeks during which contamination from external sources might happen and lead to false positive results and long investigations. These assays are therefore outsourced to

contract manufacturing organizations but their long turn-around times are not compatible with short timelines required in development and manufacturing, where products should be released with no delays.

NAT represents an interesting alternative solution to cell culture-based techniques. Because
5 PCR devices are commonly found in all labs, many kits have been developed that guarantee detection of microorganisms, for example mycoplasma contamination or MMV contamination with limit of detection (LOD) matching guidelines' requirements. Existing solutions, however, rely on complex and suboptimal designs. For example, because the assays must be able to detect different mycoplasma strains, some kits require the use of several primer sets specific to each strain. The use of SYBR green
10 in kits, that allows for the detection of amplicons, is also a concern in the sense that non-specific DNA amplification might be detected and lead to false positive signals and trigger long investigation campaigns. This was confirmed recently in Applicant's own lab where the analysis with the MycoTOOL kit (Roche) of a sample contaminated with bacteria, but no mycoplasma, returned positive results.

15 The present invention therefore addresses the need for an easy, fast and cost-effective method for detecting microorganisms, such as mycoplasma or virus contaminations in a sample. The method allows for highly specific and sensitive detection of microorganisms, such as mycoplasma or virus by using novel primers targeting mycoplasma regions not targeted before.

Summary of the invention

20 The objective underlying the present invention is solved by the provision of a method for detecting microorganisms in a sample, comprising: (i) performing PCR on a sample suspected of comprising mycoplasma DNA with a PCR reaction mixture comprising (1) a first primer (P1) annealing to the reverse complement of a nucleic acid sequence of SEQ ID NO: 1 and a second primer (P2) annealing to a nucleic acid sequence of SEQ ID NO: 2; or (2) a third primer (P3) annealing to the
25 reverse complement of a nucleic acid sequence of SEQ ID NO: 109 and a fourth primer (P4) annealing to a nucleic acid sequence of SEQ ID NO: 110; and (ii) detecting a PCR product, wherein the detection of a PCR product indicates that the sample comprises microorganisms, and wherein the absence of a PCR product indicates that the sample does not comprise microorganisms.

According to one aspect, the present invention provides a method for detecting mycoplasma in
30 a sample, comprising: (i) performing PCR on a sample suspected of comprising mycoplasma DNA with a PCR reaction mixture comprising a first primer (P1) annealing to the reverse complement of a nucleic acid sequence of SEQ ID NO: 1 and a second primer (P2) annealing to a nucleic acid sequence of SEQ ID NO: 2; and (ii) detecting a PCR product, wherein the detection of a PCR product indicates that the sample comprises mycoplasma, and the absence of a PCR product indicates that the sample
35 does not comprise mycoplasma.

According to one embodiment, (i) P1 has a length between 18 and 24 nucleotides, preferably between 19 and 21 nucleotides and most preferably 19 nucleotides and/or P2 has a length between 17 and 22 nucleotides preferably between 18 and 20 nucleotides, most preferably 20 nucleotides; and/or (ii) P1 has a T_m of between 60°C and 70°C, preferably between 63°C and 69°C, more preferably between 66°C and 68°C, and/or P2 has a T_m of between 60°C and 67°C, preferably of between 62 and 66°C and most preferably between 64°C and 65°C; and/or (iii) P1 comprises or consists of a nucleic acid sequence of SEQ ID NO: 3 to 49 and/or P2 comprises or consists of a nucleic acid sequence of SEQ ID NO: 50 to 52.

According to another embodiment, the reaction mixture further comprises at least a first nucleic acid probe (Probe 1) annealing or binding to a nucleic acid sequence of SEQ ID NO: 53 or its reverse complement.

According to one embodiment, (i) the reaction mixture further comprises at least a second nucleic acid probe (Probe 2) annealing to SEQ ID NO: 53; and/or (ii) Probe 1 and/or Probe 2 each has a length between 20 and 42 nucleotides, preferably between 21 and 30 nucleotides and most preferably 22 and 24 nucleotides; and/or (iii) Probe 1 and/or Probe 2 each has a T_m of between 55°C and 70°C, preferably between 58°C and 69°C, more preferably between 59°C and 68°C; and/or (iv) Probe 1 comprises or consists of a nucleic acid sequence of SEQ ID NO: 54 to 65 and/or Probe 2 comprises or consists of a nucleic acid sequence of SEQ ID NO: 66; and/or (v) Probe 1 and/or Probe 2 each comprises one or more locked nucleic acids (LNA) and/or a minor groove binding (MGB) moiety; and/or (vi) Probe 1 and/or Probe 2 each comprises a detectable label, wherein preferably Probe 1 and Probe 2 comprise the same detectable label.

According to one embodiment, (i) the PCR is qPCR, dPCR, ddPCR or cdPCR; and/or (ii) the PCR uses annealing temperature of between 55°C and 65°C, preferably between 58°C and 60°C; and/or (iii) the PCR comprises an activation step and/or a degradation step; and/or (iv) annealing and elongation are performed at the same temperature; and/or (v) the PCR comprises between 40 and 50 cycles of denaturation and annealing/elongation, preferably 45 cycles.

According to a preferred embodiment, the reaction mixture further comprises an internal positive control nucleic acid sequence comprising at least 18 consecutive nucleic acids of the nucleic acid sequence of SEQ ID NO: 1 and at least 18 consecutive nucleic acids of the nucleic acid sequence of SEQ ID NO: 2, and between these two nucleic acid sequences an intervening nucleic acid sequence of a length of at least 18 nucleotides, wherein the intervening nucleic acid sequence preferably differs from SEQ ID NO: 53 in that neither Probe 1 nor Probe 2 anneals to the intervening nucleic acid sequence, wherein the reaction mixture preferably further comprises at least a third nucleic acid probe (Probe 3) annealing to the intervening nucleic acid sequence or to its reverse complement.

According to a further embodiment, i) Probe 3 has a length between 20 and 42 nucleotides, preferably between 21 and 30 nucleotides and most preferably 22 and 24 nucleotides; and/or (ii) Probe 3 has a T_m of between 55°C and 70°C, preferably between 58°C and 69°C, more preferably between

59°C and 68°C; and/or (iii) Probe 3 comprises or consists of a nucleic acid sequence of SEQ ID NO: 69 or its reverse complement; and/or (iv) Probe 3 comprises one or more locked nucleic acids (LNA) and/or a minor groove binding (MGB) moiety; and/or (v) Probe 3 comprises a detectable label different from the label of Probe 1 and Probe 2.

5 According to a preferred embodiment, the internal positive control nucleic acid sequence comprises or consists of a nucleic acid sequence at least 90% identical to the nucleic acid sequence of SEQ ID NO: 68 or its reverse complement.

According to yet another embodiment, the reaction mixture further comprises a discriminatory positive control nucleic acid sequence comprising at least 18 consecutive nucleic acids of the nucleic acid sequence of SEQ ID NO: 1 and at least 18 consecutive nucleic acids of the nucleic acid sequence of SEQ ID NO: 2 and between these two nucleic acid sequences an intervening nucleic acid sequence of a length of at least 18 nucleotides, wherein the intervening nucleic acid sequence preferably does not differ from SEQ ID NO: 53 so that Probe 1 and Probe 2 are capable of annealing to the intervening nucleic acid sequence, wherein the reaction mixture preferably further comprises at least a fourth nucleic acid probe (Probe 4) annealing to the intervening nucleic acid sequence or to its reverse complement at a sequence that is different from the sequence to which Probe 1 and/or Probe 2 anneals.

According to an embodiment, i) Probe 4 has a length between 20 and 42 nucleotides, preferably between 21 and 30 nucleotides and most preferably 22 and 24 nucleotides; and/or (ii) Probe 4 has a T_m of between 55°C and 70°C, preferably between 58°C and 69°C, more preferably between 59°C and 68°C; and/or (iii) Probe 4 comprises or consists of a nucleic acid sequence of SEQ ID NO: 71 or its reverse complement; and/or (iv) Probe 4 comprises one or more locked nucleic acids (LNA) and/or a minor groove binding (MGB) moiety; and/or (v) Probe 4 comprises a detectable label different from the label of Probe 1, Probe 2, and Probe 3.

According to a preferred embodiment, the discriminatory positive control nucleic acid sequence comprises or consists of a nucleic acid sequence at least 90% identical to the nucleic acid sequence of SEQ ID NO: 70 or its reverse complement.

According to yet another embodiment, the sample is a biological sample preferably selected from the group consisting of a cell bank, a cell culture, a culture medium, a cell culture supernatant, a medicinal product, a vaccine preparation, blood, saliva, and sputum.

30 According to yet another aspect, the present invention provides a pair of primers for detecting mycoplasma in a sample, comprising a primer P1 annealing to the reverse complement of a nucleic acid sequence of SEQ ID NO: 1, and a primer P2 annealing to a nucleic acid sequence of SEQ ID NO: 2.

According to a yet a further aspect, the present invention provides a kit for detecting mycoplasma in a sample, wherein the kit comprises the pair of primers of the present invention and a first nucleic acid probe (Probe 1) annealing to a nucleic acid sequence of SEQ ID NO: 53 or its reverse complement, and optionally a second nucleic acid probe (Probe 2) annealing to SEQ ID NO: 53 or its

reverse complement. The kit preferably further comprises one or more of (i) a PCR reaction mixture; (ii) an internal positive control nucleic acid sequence comprising at least 18 consecutive nucleic acids of the nucleic acid sequence of SEQ ID NO: 1 and at least 18 consecutive nucleic acids of the nucleic acid sequence of SEQ ID NO: 2 and between these two nucleic acid sequences an intervening nucleic acid sequence of a length of at least 18 nucleotides, wherein the intervening nucleic acid sequence preferably differs from SEQ ID NO: 53 in that neither Probe 1 nor Probe 2 anneals to the intervening nucleic acid sequence, wherein the kit preferably further comprises at least a third nucleic acid probe (Probe 3) annealing to the intervening nucleic acid sequence or to its reverse complement; (iii) a discriminatory positive control nucleic acid sequence comprising at least 18 consecutive nucleic acids of the nucleic acid sequence of SEQ ID NO: 1 and at least 18 consecutive nucleic acids of the nucleic acid sequence of SEQ ID NO: 2 and between these two nucleic acid sequences an intervening nucleic acid sequence of a length of at least 60 nucleotides, wherein the intervening nucleic acid sequence preferably does not differ from SEQ ID NO: 53 so that Probe 1 and Probe 2 are capable of annealing to the intervening nucleic acid sequence, wherein the kit preferably further comprises at least a fourth nucleic acid probe (Probe 4) annealing to the intervening nucleic acid sequence or to its reverse complement that is different from the sequence to which Probe 1 and/or Probe 2 anneals; (iv) means for extracting and/or purifying DNA from the sample; (v) instructions for performing PCR with the primers.

According to one embodiment, in the method of the invention, in the pair of primers of the invention, or in the kit of the invention, the mycoplasma is one or more *Mycoplasma* species selected from the group consisting of *Mycoplasma arginini*, *Mycoplasma buccale*, *Mycoplasma hominis*, *Mycoplasma orale*, *Mycoplasma salivarium*, *Mycoplasma fermentans*, *Mycoplasma hyorhinis*, *Mycoplasma synoviae*, *Mycoplasma pneumoniae*, *Acholeplasma laidlawii*, *Mycoplasma gallisepticum* and *Spiroplasma citri*.

According to a further aspect, the present invention provides a method for detecting virus in a sample, comprising: (i) performing PCR on a sample suspected of comprising virus DNA with a PCR reaction mixture comprising a third primer (P3) annealing to the reverse complement of a nucleic acid sequence of SEQ ID NO: 109 and a fourth primer (P4) annealing to a nucleic acid sequence of SEQ ID NO: 110; and (ii) detecting a PCR product, wherein the detection of a PCR product indicates that the sample comprises virus, and the absence of a PCR product indicates that the sample does not comprise virus.

According to one embodiment, (i) P3 has a length between 20 and 25 nucleotides, preferably between 21 and 24 nucleotides and most preferably 23 nucleotides, and/or P4 has a length between 15 and 21 nucleotides preferably between 16 and 20 nucleotides, most preferably 23 nucleotides; and/or (ii) P3 has a T_m of between 60°C and 70°C, preferably between 63°C and 69°C, more preferably between 66°C and 68°C, and/or P4 has a T_m of between 60°C and 67°C, preferably of between 62 to 66°C and most preferably between 64°C and 65°C; and/or (iii) P3 comprises or consists of a nucleic

acid sequence of SEQ ID NO: 104, and/or P4 comprises or consists of a nucleic acid sequence of SEQ ID NO: 105.

According to one embodiment, the reaction mixture further comprises at least one nucleic acid probe (Probe 5) annealing to a nucleic acid sequence of SEQ ID NO: 111 or its reverse complement.

5 According to a further embodiment, (i) Probe 5 has a length between 15 and 30 nucleotides, preferably between 18 and 22 nucleotides and most preferably 19 and 21 nucleotides; and/or (ii) Probe 5 has a T_m of between 59°C and 71°C, preferably between 60°C and 70°C, more preferably between 63°C and 67°C; and/or (iii) Probe 5 comprises or consists of a nucleic acid sequence of SEQ ID NO: 106; and/or (iv) Probe 5 comprises one or more locked nucleic acids (LNA) and/or a minor groove binding (MGB) moiety; and/or (v) Probe 5 comprises a detectable label.

10 According to yet another embodiment, (i) the PCR is qPCR, dPCR, ddPCR or cdPCR; and/or (ii) the PCR uses an annealing temperature of between 55°C and 65°C, preferably between 58°C and 60°C; and/or (iii) the PCR comprises an activation step and/or a degradation step; and/or (iv) annealing and elongation are performed at the same temperature; and/or (v) the PCR comprises between 40 and 50 cycles of denaturation and annealing/elongation, preferably 45 cycles.

15 According to a further embodiment, the reaction mixture further comprises an internal positive control sequence comprising at least 18 consecutive nucleic acids of the nucleic acid sequence of SEQ ID NO: 68 or 112 or 115.

20 According to another embodiment, the internal positive control nucleic acid sequence comprises or consists of a nucleic acid sequence at least 90% identical to the nucleic acid sequence of SEQ ID NO: 68 or 112 or 115, or its reverse complement.

25 According to a further embodiment, the reaction mixture further comprises a discriminatory positive control nucleic acid sequence comprising at least 18 consecutive nucleic acids of the nucleic acid sequence of SEQ ID NO: 109 and at least 18 consecutive nucleic acids of the nucleic acid sequence of SEQ ID NO: 110 and between these two nucleic acid sequences an intervening nucleic acid sequence of a length of at least 18 nucleotides.

30 According to an embodiment, the reaction mixture further comprises at least a third nucleic acid probe (Probe 3) and/or a fourth nucleic acid probe (Probe 4) annealing to the intervening nucleic acid sequence or to its reverse complement at a sequence that is different from the sequence to which Probe 5 anneals.

35 According to a preferred embodiment, Probe 3 has a length between 20 and 42 nucleotides, preferably between 21 and 30 nucleotides and most preferably 22 and 24 nucleotides, and/or has a T_m of between 55°C and 70°C, preferably between 58°C and 69°C, more preferably between 59°C and 68°C, and/or comprises or consists of a nucleic acid sequence of SEQ ID NO: 69 or its reverse complement, and/or comprises one or more locked nucleic acids (LNA) and/or a minor groove binding (MGB) moiety. According to a further preferred embodiment, Probe 4 has a length between 20 and 42 nucleotides, preferably between 21 and 30 nucleotides and most preferably between 22 and 24

nucleotides, and/or has a T_m of between 55°C and 70°C, preferably between 58°C and 69°C, more preferably between 59°C and 68°C, and/or comprises or consists of a nucleic acid sequence of SEQ ID NO: 71 or its reverse complement, and/or comprises one or more locked nucleic acids (LNA) and/or a minor groove binding (MGB) moiety, and/or comprises a detectable label different from the label of Probe 3 and Probe 5. According to one embodiment, the discriminatory positive control nucleic acid sequence comprises or consists of a nucleic acid sequence at least 90% identical to the nucleic acid sequence of SEQ ID NO: 113 or its reverse complement.

According to one embodiment, the sample is a biological sample preferably selected from the group consisting of a cell bank, a culture medium, a cell culture, a cell culture supernatant, a medicinal product, a vaccine preparation, blood, saliva, and sputum.

According to a further aspect, the present invention provides a pair of primers for detecting MMV in a sample comprising: a primer P3 annealing to the reverse complement of a nucleic acid sequence of SEQ ID NO: 109, and a primer P4 annealing to a nucleic acid sequence of SEQ ID NO: 110. Primer P3 preferably comprises or consists of a nucleic acid sequence of SEQ ID NO: 104. Primer P4 preferably comprises or consists of a nucleic acid sequence of SEQ ID NO: 105.

According to a further aspect, the present invention provides a kit for detecting MMV in a sample, wherein the kit comprises the pair of primers of the invention and a nucleic acid probe (Probe 5) annealing to a nucleic acid sequence of SEQ ID NO: 111 or its reverse complement, preferably wherein the kit further comprises one or more of: (i) a PCR reaction mixture; (ii) an internal positive control nucleic acid sequence comprising at least 18 consecutive nucleic acids of the nucleic acid sequence of SEQ ID NO: 109 and at least 18 consecutive nucleic acids of the nucleic acid sequence of SEQ ID NO: 110 and between these two nucleic acid sequences an intervening nucleic acid sequence of a length of at least 18 nucleotides; (iii) a discriminatory positive control nucleic acid sequence comprising at least 18 consecutive nucleic acids of the nucleic acid sequence of SEQ ID NO: 109 and at least 18 consecutive nucleic acids of the nucleic acid sequence of SEQ ID NO: 110 and between these two nucleic acid sequences an intervening nucleic acid sequence of a length of at least 29 nucleotides, wherein the intervening nucleic acid sequence preferably does not differ from SEQ ID NO: 111 so that Probe 5 is capable of annealing to the intervening nucleic acid sequence; (iv) means for extracting and/or purifying DNA from the sample; (v) instructions for performing PCR with the primers.

According to one embodiment, in the method according to the invention, in the pair of primers according to the invention or in the kit according to the invention, the virus is selected from the group consisting of Anelloviridae, Inoviridae, Parvoviridae, preferably Parvovirus, Erythrovirus, Dependovirus, Amdovirus and Bocavirus, more preferably Parvovirus, and most preferably Mouse Minute Virus.

Further aspects and embodiments of the invention will become apparent from the appending claims and the following detailed description.

Description of the drawings

The invention is further illustrated by the following figures and examples without being limited thereto.

Fig. 1 illustrates the results of the search for 100 bp stretches with 80% homology in mycoplasma 16s rRNA sequences across species, where primers and probes could align and bind, respectively. The figure shows the similarity of RNA of all species tested with +1 denoting 100% similarity. Identified sequence stretches fulfilling the criteria are highlighted: in the upper row between about 500 and 600 bp, in the middle row at around 800 bp, and in the bottom row at around 1100 bp.

Fig. 2 shows sequence details of the regions on which forward primers, reverse primers and probes were designed. Sequences of the corresponding species were aligned by subgroup for clarity. A: all mycoplasma species. B: bacteria and mycoplasma species. C: *Mycoplasma hyorhinis*, prokaryotic and eukaryotic species.

Fig. 3 shows the analysis of Fwd7/Rev1* PCR products on mycoplasma DNA samples. A: PAGE 15% analysis of PCRs performed on reference DNA from the indicated organisms. B: melting curves (left panel) and peaks (right panel) of the PCR products.

Fig. 4 shows a schematic positioning of primers and probes on control constructs. A: Positions of primers and probes on the discriminatory positive control (DPC). B: Positions of primers and probes on the internal positive control (IPC).

Fig. 5 shows a comparison of MMV serotype genomes. Sequences from the MMVp, MMVi, MMVc & MMVm listed in Table 24 were aligned and similarities calculated.

Fig. 6 shows the sequence details of the regions on which forward primers, reverse primers and probes were designed. Sequences were aligned by subgroup for clarity. A) all MMV serotypes; B) MMV and parvoviruses of interest; C) MMV and parvoviruses with higher sequence homology in target region.

Fig. 7 schematically shows the positioning of primers and probes on control constructs. A) Positions of primers and probes on the MMV discriminatory positive control (DPC). B) Positions of primers and probes on the internal positive control v2 (IPC MMV). C) Positions of primers and probes on the internal positive control v3 (IPC Myco / MMV).

Fig. 8 shows the results of the amplification of various gDNA with MMV primer set using a first PCR mix containing all the reagents for specific MMV detection. A) MMV and DPC amplification on FAM channel; B) DPC amplification only on HEX channel; C) IPC amplification for all samples on Cy5 channel. X-axis shows the fluorescence intensity and y-axis shows the number of PCR cycles.

Fig. 9 shows the results of the amplification of various gDNA with their own primer set using a second PCR mix containing all the reagents for specific MMV detection. A) Human amplification on

FAM channel; B) E. coli amplification on HEX channel; C) CHO amplification on Cy5 channel. X-axis shows the fluorescence intensity and y-axis shows the number of PCR cycles.

Fig. 10 shows the visualization of amplification products in 15% PAGE using the two different PCR mixes targeting MMV matrixes and other gDNA matrixes, respectively. A) relevant amplifications from MMV using PCR Mix 1; B) relevant amplifications from gDNA using PCR Mix 2.

Fig. 11 is a representation of the calibration curve for two different data units. After data reformatting, a 10 Cq drift is observed meaning about 3Log10 difference in absolute value.

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List of sequences

The sequences referred to herein are disclosed in detail in the accompanying sequence listing. Sequences of the present invention are also listed in the following table 1.

Table 1: Examples of nucleic acid sequences of the present invention, all in 5' → 3' orientation.

SEQ ID NO	Name	Sequence
1	Region3 fw template	GGAATTGACGGGRMYCCGCACAAGYGGTGGAKCATGT KGYTTAATTYGA
2	Region3 rev template	TGGTGCA YGGTTGTCTCGTCAGYTCGTGYCGTGAGRTGTT
3	FwMyco1	CCGCACAAGCGGTGGAGCATGTGG
4	FwMyco2	ACCCGCACAAGCGGTGG
5	FwMyco3	ACCCGCACAAGCGGTG
6	FwMyco4	CCCGCACAAGCGGTG
7	FwMyco5	CCCGCACAAGCGGTGG
8	FwMyco6	CCGCACAAGCGGTGGA
9	FwMyco7	CACAAGCGGTGGAGCATGT
10	FwMyco8	ACAAGCGGTGGAGCATGTG
11	FwMyco9	ACAAGCGGTGGAGCATGTGG
12	FwMyco10	CAAGCGGTGGAGCATGTG
13	FwMyco11	AAGCGGTGGAGCATGTGG
14	FwMyco12	CGGTGGAGCATGTGGTTTAATT
15	FwMyco13	CACAAGTGGTGGAGCATGTG
16	FwMyco14	CACAAGTGGTGGAGCATGTGG
17	FwMyco15	CACAAGTGGTGGAGCATGTGGT
18	FwMyco16	ACAAGTGGTGGAGCATGTG
19	FwMyco17	ACAAGTGGTGGAGCATGTGG
20	FwMyco18	ACAAGTGGTGGAGCATGTGGT
21	FwMyco19	ACAAGTGGTGGAGCATGTGGTT
22	FwMyco20	CAAGTGGTGGAGCATGTGG
23	FwMyco21	CAAGTGGTGGAGCATGTGGT
24	FwMyco22	CAAGTGGTGGAGCATGTGGTT
25	FwMyco23	CAAGTGGTGGAGCATGTGGTTT
26	FwMyco24	GCACAAGCGGTGGATCATG
27	FwMyco25	GCACAAGCGGTGGATCATGT

28	FwMyco26	CACAAGCGGTGGATCATGT
29	FwMyco27	CACAAGCGGTGGATCATGTT
30	FwMyco28	CACAAGCGGTGGATCATGTTG
31	FwMyco29	ACAAGCGGTGGATCATGTTG
32	FwMyco30	ACAAGCGGTGGATCATGTTGT
33	FwMyco31	ACAAGCGGTGGATCATGTTGTT
34	FwMyco32	CAAGCGGTGGATCATGTTG
35	FwMyco33	CAAGCGGTGGATCATGTTGT
36	FwMyco34	GCACAAGTGGTGGAGCATGT
37	FwMyco35	GCACAAGTGGTGGAGCATGTT
38	FwMyco36	GCACAAGTGGTGGAGCATGTTG
39	FwMyco37	CACAAGTGGTGGAGCATGT
40	FwMyco38	CACAAGTGGTGGAGCATGTT
41	FwMyco39	CACAAGTGGTGGAGCATGTTG
42	FwMyco40	CACAAGTGGTGGAGCATGTTGC
43	FwMyco41	ACAAGTGGTGGAGCATGTT
44	FwMyco42	ACAAGTGGTGGAGCATGTTG
45	FwMyco43	ACAAGTGGTGGAGCATGTTGC
46	FwMyco44	ACAAGTGGTGGAGCATGTTGCT
47	FwMyco45	CAAGTGGTGGAGCATGTTG
48	FwMyco46	CAAGTGGTGGAGCATGTTGC
49	FwMyco47	CAAGTGGTGGAGCATGTTGCT
50	RevMyco1*	CTGACGACAACCATGCACCA
51	RevMyco2*	ACGACAACCATGCACCA
52	RevMyco3*	TGACGACAACCATGCACCA
53	Region3 pb template	AACCTTACCHRSDYTTGACATMYHBBGCRAWRBYDTRG ARAYAHRDYNRGAGGYAWCVBDDDKACAG
54	SMyco1 ZEN	GCAAAGCTATAGAGATATAGTGGAGGTTAACA
55	SMyco2 ZEN	GCAAAGCTATAGAGATATAGTGGAGGTTAAC
56	SMyco3 ZEN	CAAAGCTATAGAGATATAGTGGAGGTTAACA
57	SMyco4 ZEN	CAAAGCTATAGAGATATAGTGGAGGTTAACAGATGAC AG
58	SMyco4bis ZEN	CAAAGCTATAGAGATATAGTGGAGGTTAACAGAGTGAC AG
59	SMyco5 LNA	TATAGTGGAGGTTAACAGAAATG
60	SMyco5bis LNA	TATAGTGGAGGTTAACAGAGTG
61	SMyco5	TATAGTGGAGGTTAACAGAAATG
62	SMyco5bis	TATAGTGGAGGTTAACAGAGTG
63	SMyco6	TATAGAGATATAGTGGAGGTTAACAGAGTG
64	SMyco7	TATAGAGATATAGTGGAGGTTAACAGA
65	SMyco8	TATAGAGATATAGTGGAGGTTAACAG
66	SAcho1	TAAGTTCGGAGGCTAACAGATGT
67	SAcho1 LNA	TAAGTTCGGAGGCTAACAGATGT
68	Myco IPC; MMV IPC v3	CGTCATCGGATCTCGAGGACCTGGCTTTAGAGCCTTGGA GCACACCAAATACTCCTGTTGCGGGCACTGCAGAAACC CAGAACACTGGGGAAGCTGGTTCCAAAGCCTGCCAAGA TGGTCAACTGAGCCCAACTGGTTTCACAAGCGGTGGA GCATGTGACAGAGATCGAGGAGGATTTGAGAGGCGCCC GTCAAAAAGAAGTCCGTCACACGCAGTGATCCCGGCTG CTGCGTCACATTCTGATGATGTTCTTCCCGCTAAGACTT CAGCGAGCCGCTTGGTGCATGGTTGTCGTCAGTTTGAAC TTGGACTAAGGTACGATGGCGCCTCCAGCTAAAAGAGC TAAAAGAGGTAAGGGTTTAAGGGATGGTTGGTTGGTGG

		GGTATTAATGTTTAATTACCTGTTTTACAGGCCTGAAAT CACTTGGTTTTAGGTTGGGTGCCTCCTGGCTACAAGTAC CTGGGACCACGT
69	StraA	CGCAGTGATCCCCGGCTGCTG
70	Myco DPC	CGTCATCGGATCTCGAGGACCTGGCTTTAGAGCCTTGGA GCACACCAAATACTCCTGTTGCGGGCACTGCAGAAACC CAGAACACTGGGGAAGCTGGTTCCAAAGCCTGCCAAGA TGGTCAACTGAGCCCAACTGGTTTCACAAGCGGTGGA GCATGTGGTTTAATTTGAAGATACGCGTAGAACCTTACC CACTCTTGACATCTTCTGCAAAGCTATAGAGATATAGTG GAGGTTAACAGAATGATTTAAGTTCGGAGGCTAACAGA TGTATCCGACTCGCAGAACCGGAACGATTGATGGTGC ATGGTTGTCGTCAGTTTGAAGTTGACTAAGGTACGATG GCGCCTCCAGCTAAAAGAGCTAAAAGAGGTAAGGGTTT AAGGGATGGTTGGTTGGTGGGGTATTAATGTTTAATTAC CTGTTTTACAGGCCTGAAATCACTTGGTTTTAGGTTGGG TGCCTCCTGGCTACAAGTACCTGGGACCACGT
71	SAtPSY	CCGACTCGCAGAACCGGAACGA
104	FwMMV	CAGAGATCGAGGAGGATTTGAGA
105	RevMMV	GCGGCTCGCTGAAGTCT
106	Probe MMV	CGTGCTTCGGTGCAGAACCG
107	FtraA12	GCGCCCGTCAAAAAGAAGT
108	RtraA11	GCGGGAAGAACATCATCAGAA
109	FwMMV template	AAGCTGGTTCCAAAGCCTGCCAAGATGGTCAACTGAGC CCAAGTTGGTCAGAGATCGAGGATTTGAGA
110	RevMMV template	AGACTTCAGCGAGCCGCTGAACTTGGACTAAG
111	MMV probe region	AGCGTGCTTCGGTGCAGAACCGTTGAAGA
112	MMV IPC v2	CAGAGATCGAGGAGGATTTGAGAGGCGCCCGTCAAAAA GAAGTCGTTTTTTTCCAAATTCCTCGTCTGAATATGCTT CGCCTGGCTCGCGCAGTGATCCCGGCTGCTGTTCTGATG ATGTTCTTCCCGCTTGAAGAAAGACTTCAGCGAGCCGC
113	MMV DPC	CAGAGATCGAGGAGGATTTGAGAGCGTGCTTCGGTGCG GAACCGTTGCCGACTCGCAGAACCGGAACGAAGAAAGA CTTCAGCGAGCCGC
114	MMV DPC full length	AAAGGTGCCAACTCCTATAAATTTACTAGGTTCCGGCAGC CTCACCATTACGACACCGAAAAGTACGCCTCTCAGCC AGAACTATGCACTAACTCCACTTGCATCGGATCTCGAGG ACCTGGCTTTAGAGCCTTGGAGCACACCAAATACTCCTG TTGCGGGCACTGCAGAAACCCAGAACACTGGGGAAGCT GGTTCCAAAGCCTGCCAAGATGGTCAACTGAGCCCAAC TTGGTCAGAGATCGAGGAGGATTTGAGAGCGTGCTTCG GTGCGGAACCGTTGCCGACTCGCAGAACCGGAACGAAG AAAGACTTCAGCGAGCCGCTGAACTTGGACTAAGGTAC GATGGCGCCTCCAGCTAAAAGAGCTAAAAGAGGTAAGG GTTTAAGGGATGGTTGGTTGGTGGGGTATTAATGTTTAA TTACCTGTTTTACAGGCCTGAAATCACTTGGTTTTAGGT TGGGTGCCTCCTGGCTACAAGTACCTGGGACCAGGG
115	IPCv2 full length	AAAGGTGCCAACTCCTATAAATTTACTAGGTTCCGGCAGC CTCACCATTACGACACCGAAAAGTACGCCTCTCAGCC AGAACTATGCACTAACTCCACTTGCATCGGATCTCGAGG ACCTGGCTTTAGAGCCTTGGAGCACACCAAATACTCCTG TTGCGGGCACTGCAGAAACCCAGAACACTGGGGAAGCT GGTTCCAAAGCCTGCCAAGATGGTCAACTGAGCCCAAC TTGGTCAGAGATCGAGGAGGATTTGAGAGGCGCCCGTC

		AAAAAGAAGTCGTTTTTTTCCAAATTCACCTCGTCTGAAT ATGCTTCGCCTGGCTCGCGCAGTGATCCCGGCTGCTGTT CTGATGATGTTCTTCCCGCTTGAAGAAAGACTTCAGCGA GCCGCTGAACCTGGACTAAGGTACGATGGCGCCTCCAG CTAAAAGAGCTAAAAGAGGTAAGGGTTTAAGGGATGGT TGGTTGGTGGGGTATTAATGTTTAATTACCTGTTTT
116	MMV IPC v3	CACAAGCGGTGGAGCATGTGACAGAGATCGAGGAGGAT TTGAGAGGCGCCCGTCAAAAAGAAGTCCGTCACACGCA GTGATCCCGGCTGCTGCGTCACATTCTGATGATGTTCTT CCCGCTAAGACTTCAGCGAGCCGCTTGGTGCATGGTTGT CGTCAG

SEQ ID NOs: 72 to 103 shown in the figures are listed in the sequence listing, as are SEQ ID NOs: 117 to 192.

Detailed description of the invention

5 Before the present invention is described in detail below, it is to be understood that this invention is not limited to the particular methodology, protocols and reagents described herein as these may vary. It is also to be understood that the terminology used herein is for the purpose of describing particular embodiments only, and it is not intended to limit the scope of the present invention which will be limited only by the appended claims. Unless defined otherwise, all technical and scientific
10 terms used herein have the same meanings as commonly understood by one of ordinary skill in the art.

Preferably, the terms used herein are defined as described in "A multilingual glossary of biotechnological terms: (IUPAC Recommendations)", Leuenberger, H.G.W, Nagel, B. and Klbl, H. eds. (1995), Helvetica Chimica Acta, CH-4010 Basel, Switzerland).

Throughout this specification and the claims which follow, unless the context requires
15 otherwise, the word "comprise", and variations such as "comprises" and "comprising", will be understood to imply the inclusion of a stated integer or step or group of integers or steps but not the exclusion of any other integer or step or group of integers or steps. In the following passages, different aspects of the invention are defined in more detail. Each aspect so defined may be combined with any other aspect or aspects unless clearly indicated to the contrary. Any feature indicated as being
20 optional, preferred or advantageous may be combined with any other feature or features indicated as being optional, preferred or advantageous.

In the following, the elements of the present invention will be described. These elements are listed with specific embodiments; however, it should be understood that they may be combined in any manner and in any number to create additional embodiments. The variously described examples and
25 preferred embodiments should not be construed to limit the present invention to only the explicitly described embodiments. This description should be understood to support and encompass embodiments which combine the explicitly described embodiments with any number of the disclosed and/or preferred elements. Furthermore, any permutations and combinations of all described elements

in this application should be considered disclosed by the description of the present application unless the context indicates otherwise.

Several documents are cited throughout the text of this specification. Each of the documents cited herein (including all scientific publications, manufacturer's specifications, instructions etc.), whether supra or infra, is hereby incorporated by reference in its entirety.

Definitions

In the following, some definitions of terms frequently used in this specification are provided. These terms will, in each instance of its use, in the remainder of the specification have the respectively defined meaning and preferred meanings.

As used in this specification and the appended claims, the singular forms "a", "an", and "the" include plural referents, unless the content clearly dictates otherwise.

The term "nucleic acid" or "polynucleotide" as used in this specification comprises polymeric or oligomeric macromolecules, or large biological molecules, essential for all known forms of life. Nucleic acids, which include DNA (deoxyribonucleic acid) and RNA (ribonucleic acid), are made from monomers known as nucleotides. Most naturally occurring DNA molecules consist of two complementary biopolymer strands coiled around each other to form a double helix. The DNA strand is also known as polynucleotides consisting of nucleotides. Each nucleotide is composed of a nitrogen-containing nucleobase as well as a monosaccharide sugar called deoxyribose or ribose and a phosphate group. Naturally occurring nucleobases comprise guanine (G), adenine (A), thymine (T), uracil (U) or cytosine (C). The nucleotides are joined to one another in a chain by covalent bonds between the sugar of one nucleotide and the phosphate of the next, resulting in an alternating sugar-phosphate backbone. If the sugar is deoxyribose, the polymer is DNA. If the sugar is ribose, the polymer is RNA. Typically, a polynucleotide is formed through phosphodiester bonds between the individual nucleotide monomers. In the context of the present invention the term "nucleic acid" includes but is not limited to ribonucleic acid (RNA), deoxyribonucleic acid (DNA), and mixtures thereof such as e.g. RNA-DNA hybrids (within one strand), as well as cDNA, genomic DNA, recombinant DNA, crRNA and mRNA. The nucleic acid can also be an artificial nucleic acid. Artificial nucleic acids include polyamide or peptide nucleic acid (PNA), morpholino and locked nucleic acid (LNA), as well as glycol nucleic acid (GNA) and threose nucleic acid (TNA). Each of these is distinguished from naturally-occurring DNA or RNA by changes to the backbone of the molecule. The nucleic acids can e.g. be synthesized chemically, e.g. in accordance with the phosphotriester method (see, for example, Uhlmann, E. & Peyman, A. (1990) Chemical Reviews, 90, 543-584).

The term "nucleic acid" and "nucleic acid molecule" are used synonymously herein and are understood as well-accepted in the art, *i.e.* as single or double-stranded oligo- or polymers of deoxyribonucleotide or ribonucleotide bases or both. The term "nucleic acids" as used herein includes not only deoxyribonucleic acids (DNA) and ribonucleic acids (RNA), but also all other linear

polymers in which the bases adenine (A), cytosine (C), guanine (G) and thymine (T) or uracil (U) are arranged in a corresponding sequence (nucleic acid sequence). The invention also comprises the corresponding RNA sequences (in which thymine is replaced by uracil), complementary sequences and sequences with modified nucleic acid backbone or 3' or 5'-terminus. Nucleic acids in the form of DNA are however preferred.

The nucleotide base symbols used throughout the present disclosure are in line with the symbols according to the WIPO ST.26 standard, as outlined in the following table 2.

Table 2: standard abbreviations for nucleotides

Symbol	Definition
a	adenine
c	cytosine
g	guanine
t	thymine in DNA/uracil in RNA (t/u)
m	a or c
r	a or g
w	a or t/u
s	c or g
y	c or t/u
k	g or t/u
v	a or c or g; not t/u
h	a or c or t/u; not g
d	a or g or t/u; not c
b	c or g or t/u; not a
n	a or c or g or t/u

The term "primer" has the meaning as it would be understood by the person of ordinary skill in the field of genetics. It denotes a short single-stranded nucleic acid molecule usually having a length of between 18 and 24 nucleotides and being capable of binding to a target nucleic acid (also referred to as template) by annealing (hybridization with the template through Watson-Crick base pairing).

The "percentage of sequence identity" is determined by comparing two optimally aligned sequences over a comparison window, wherein the portion of the sequence in the comparison window can comprise additions or deletions (*i.e.* gaps) as compared to the reference sequence (which does not comprise additions or deletions) for optimal alignment of the two sequences. The percentage is calculated by determining the number of positions at which the identical nucleic acid base or amino acid residue occurs in both sequences to yield the number of matched positions, dividing the number of matched positions by the total number of positions in the window of comparison and multiplying the result by 100 to yield the percentage of sequence identity.

The term "identical" is used herein in the context of two or more nucleic acids to refer to two or more sequences or subsequences that are the same, *i.e.* that comprise the same sequence of nucleotides or amino acids. Sequences are "identical" to each other if they have a specified percentage of nucleotides or amino acid residues that are the same. According to the present invention, at least 90% identical includes at least at least at least 91%, at least 92%, at least 93%, at least 94%, at least

95%, at least 96%, at least 97%, at least 98%, or at least 99% identity over the specified sequence, when compared and aligned for maximum correspondence over a comparison window, or designated region as measured using one of the following sequence comparison algorithms or by manual alignment and visual inspection. These definitions also refer to the complement of a test sequence.

5 Accordingly, the term "at least XY% sequence identity" is used throughout the specification with regard to polynucleotide sequence comparisons. In the context of the present invention, a nucleic acid sequence having at least 90% sequence identity to a given SEQ ID NO or a nucleic acid sequence reverse complementary thereto thus preferably means that said nucleic acid has a sequence having at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least

10 97%, at least 98%, at least 99% or 100% sequence identity to the given SEQ ID NO or a nucleic acid sequence reverse complementary to said SEQ ID NO.

The term "sequence comparison" is used herein to refer to the process wherein one sequence acts as a reference sequence, to which test sequences are compared. When using a sequence comparison algorithm, test and reference sequences are entered into a computer, if necessary,

15 subsequence coordinates are designated, and sequence algorithm program parameters are designated. Default program parameters are commonly used, or alternative parameters can be designated. The sequence comparison algorithm then calculates the percent sequence identities for the test sequences relative to the reference sequence, based on the program parameters. In case where two sequences are compared and the reference sequence is not specified in comparison to which the sequence identity

20 percentage is to be calculated, the sequence identity is to be calculated with reference to the longer of the two sequences to be compared, if not specifically indicated otherwise. If the reference sequence is indicated, the sequence identity is determined on the basis of the full length of the reference sequence indicated by one of the SEQ ID NOs of the present invention, if not specifically indicated otherwise.

Methods of alignment of sequences for comparison are well known in the art. Optimal

25 alignment of sequences for comparison can be conducted, for example, by the local homology algorithm of Smith and Waterman (Adv. Appl. Math. 2:482, 1970), by the homology alignment algorithm of Needleman and Wunsch 1970, by the search for similarity method of Pearson and Lipman 1988, by computerized implementations of these algorithms (e.g., GAP, BESTFIT, FASTA, and TFASTA in the Wisconsin Genetics Software Package, Genetics Computer Group, 575 Science

30 Dr., Madison, Wis.), or by manual alignment and visual inspection (see, e.g., Ausubel et al., Current Protocols in Molecular Biology (1995 supplement)). Algorithms suitable for determining percent sequence identity and sequence similarity are the BLAST and BLAST 2.0 algorithms, which are described in Altschul et al. (Nuc. Acids Res. 25:3389-402, 1977), and Altschul et al. (J. Mol. Biol. 215:403-10, 1990), respectively. Software for performing BLAST analyses is publicly available

35 through the National Center for Biotechnology Information (<http://www.ncbi.nlm.nih.gov/>). This algorithm involves first identifying high scoring sequence pairs (HSPs) by identifying short words of length W in the query sequence, which either match or satisfy some positive-valued threshold score T

when aligned with a word of the same length in a database sequence. T is referred to as the neighborhood word score threshold (Altschul et al., supra). These initial neighborhood word hits act as seeds for initiating searches to find longer HSPs containing them. The word hits are extended in both directions along each sequence for as far as the cumulative alignment score can be increased.

5 Cumulative scores are calculated using, for nucleotide sequences, the parameters M (reward score for a pair of matching residues; always >0) and N (penalty score for mismatching residues; always <0). For amino acid sequences, a scoring matrix is used to calculate the cumulative score. Extension of the word hits in each direction are halted when: the cumulative alignment score falls off by the quantity X from its maximum achieved value; the cumulative score goes to zero or below, due to the

10 accumulation of one or more negative-scoring residue alignments; or the end of either sequence is reached. The BLAST algorithm parameters W, T, and X determine the sensitivity and speed of the alignment. The BLASTN program (for nucleotide sequences) uses as defaults a wordlength (W) of 11, an expectation (E) of 10, M=5, N=-4 and a comparison of both strands. For amino acid sequences, the BLASTP program uses as defaults a wordlength of 3, and expectation (E) of 10, and the BLOSUM62

15 scoring matrix (see Henikoff and Henikoff, 1989) alignments (B) of 50, expectation (E) of 10, M=5, N=-4, and a comparison of both strands. The BLAST algorithm also performs a statistical analysis of the similarity between two sequences (see, e.g., Karlin and Altschul, Proc. Natl. Acad. Sci. USA 90:5873-87, 1993). One measure of similarity provided by the BLAST algorithm is the smallest sum probability (P(N)), which provides an indication of the probability by which a match between two

20 nucleotide or amino acid sequences would occur by chance. For example, a nucleic acid is considered similar to a reference sequence if the smallest sum probability in a comparison of the test nucleic acid to the reference nucleic acid is less than about 0.2, typically less than about 0.01, and more typically less than about 0.001.

The term "anneals to" as used herein refers to pairing of complementary sequences of single-stranded nucleic acids to form a double-stranded polynucleotide preferably under stringent conditions. The term "subject" as used herein, refers to an animal, preferably a mammal, most preferably a human.

25

The term "melting temperature" or "T_m" as used herein refers to the temperature at which half of the DNA strands are in the random coil or single-stranded (ssDNA) state. T_m depends on the length of the DNA molecule and its specific nucleotide sequence. In the present invention, the T_m of a given

30 nucleotide sequence is calculated using the software PrimerExpress[®] 3.0 (Life Technologies Corporation) using standard settings, the software Geneious Prime 2023.2 (Dotmatics) using the settings Monovalent ions 50 mM, Divalent ions 3 mM, Primer 200 nM, dNTP 0.8 mM, or the Primer design tools for PCR & qPCR (Integrated DNA Technologies) using the parameter set for qPCR:

35 Monovalent ions 50 mM, Divalent ions 3 mM, Primer 200 nM, dNTP 0.8 mM, further using the SantaLucia 1998 thermodynamics & salt correction (SantaLucia, Proc Natl Acad Sci USA, 1998, Vol.

95(4): 1460-1465), or the SantaLucia 1998 thermodynamics and the Owczarzy 2004 salt correction (Owczarzy et al., Biochemistry 2004, Vol. 43(12): 3537-3554).

Description of embodiments

5 The present invention is in the field of microorganism detection, and provides a method for detecting microorganisms such as mycoplasma or mouse minute virus, specific primers and probes, as well as kits for detecting microorganisms such as mycoplasma or mouse minute virus.

 In the experiments leading to the present invention, it was surprisingly found that although three regions of sufficient homology between mycoplasma species for primer and probe design were identified (Fig. 1), only one region turned out to be suitable for primer and probe design, which further required a widening to accommodate the forward primer, a probe, and the reverse primer. It was further surprisingly found that only one genomic region turned out to be suitable for primer and probe design for detecting mouse minute virus (MMV). The present invention describes the identification of these specific regions for primer and probe design, which primers and probes can be used for mycoplasma detection or MMV detection, optionally together with other probes for positive control and for internal control. The primers and probes of the invention guarantee a high degree of specificity (no detection of bacterial DNA, no detection of CHO DNA, no detection of Human DNA) and sensitivity as required by established guidelines, e.g. for detecting as little as 10 CFU/mL of mycoplasma in a sample.

20 Thus, the present invention provides a method for detecting microorganisms in a sample, the method comprising the steps of (i) performing PCR on a sample suspected of comprising microorganism DNA with a PCR reaction mixture comprising: (1) a first primer (P1) annealing to the reverse complement of a nucleic acid sequence of SEQ ID NO: 1 and a second primer (P2) annealing to a nucleic acid sequence of SEQ ID NO: 2, or (2) a third primer (P3) annealing to the reverse complement of a nucleic acid sequence of SEQ ID NO: 109 and a fourth primer (P4) annealing to a nucleic acid sequence of SEQ ID NO: 110; and (ii) detecting a PCR product. Since the method aims at detecting microorganisms in the sample, the detection of a PCR product that was produced in the PCR reaction using the two primers of (1) and/or (2) indicates that the sample comprises microorganisms. Likewise, the absence of such a PCR product indicates that the sample does not comprise microorganisms such as mycoplasma and MMV.

30 The sample used in the context of the present invention is preferably a biological sample. According to a preferred embodiment, the sample is selected from the group consisting of but not limited to a sample from a cell bank, a culture medium (such as but not limited to a cell culture medium), a cell culture, a cell culture supernatant, a medicinal product, a vaccine preparation, and a body fluid such as blood, saliva or sputum. The sample can be used in the method of the present invention in essentially untreated form. For example, the sample can be derived or obtained from an article, object, item or a subject. The sample may be directly added to the reaction mixture without

requiring any preceding purification steps. Methods for obtaining such a sample are well known to the skilled person. Alternatively, the sample can be pretreated before being added to the reaction mixture. Such a pretreatment may include methods for purifying or isolating DNA from the sample, which can then be used in the method of the invention.

5 The method of the present invention allows for detecting microorganisms, such as bacteria or virus in the sample.

 According to one aspect, the present invention provides a method for detecting mycoplasma in a sample, comprising: (i) performing PCR on a sample suspected of comprising mycoplasma DNA with a PCR reaction mixture comprising a first primer (P1) annealing to the reverse complement of a
10 nucleic acid sequence of SEQ ID NO: 1 and a second primer (P2) annealing to a nucleic acid sequence of SEQ ID NO: 2; and (ii) detecting a PCR product, wherein the detection of a PCR product indicates that the sample comprises mycoplasma, and the absence of a PCR product indicates that the sample does not comprise mycoplasma.

 This method allows for detecting bacteria in the sample, in particular mycoplasma selected
15 from the group consisting of Mycoplasma species *Mycoplasma arginini*, *Mycoplasma buccale*, *Mycoplasma hominis*, *Mycoplasma orale*, *Mycoplasma salivarium*, *Mycoplasma fermentans*, *Mycoplasma hyorhinitis*, *Mycoplasma synoviae*, *Mycoplasma pneumoniae*, *Acholeplasma laidlawii*, *Mycoplasma gallisepticum* and *Spiroplasma citri*.

 According to a further aspect, the present invention provides a method for detecting virus in a
20 sample, comprising: (i) performing PCR on a sample suspected of comprising virus DNA with a PCR reaction mixture comprising a third primer (P3) annealing to the reverse complement of a nucleic acid sequence of SEQ ID NO: 109 and a fourth primer (P4) annealing to a nucleic acid sequence of SEQ ID NO: 110; and (ii) detecting a PCR product, wherein the detection of a PCR product indicates that the sample comprises virus, and the absence of a PCR product indicates that the sample does not
25 comprise virus.

 This method allows for detecting virus in the sample selected from the group consisting of single-stranded DNA virus, such as Anelloviridae, Inoviridae, Parvoviridae. The family of Parvoviridae can be further subdivided into Parvovirinae comprising Parvovirus, Erythrovirus, Dependovirus, Amdovirus and Bocavirus. Preferably, the method of the present invention allows for
30 the detection of Parvovirus, in particular of Mouse Minute Virus (MMV).

 The reaction mixture used for the PCR is not particularly limited to a specific type and may be selected by the person of ordinary skill in the art or by following the instructions of a manufacturer of a component of a PCR reaction mixture. A PCR reaction mixture typically contains in addition to the primers and the DNA template a DNA polymerase, deoxynucleoside triphosphates (dNTPs), a buffer
35 solution and bivalent cations such as Mg^{2+} or Mn^{2+} .

 Preferred characteristics of the primers P1 and P2 to be used in the context of the invention are disclosed in the following.

Primer P1 preferably has a length of between 18 and 24 nucleotides, such as 18, 19, 20, 21, 22, 23 and 24 nucleotides, preferably between 19 and 23, 19 and 22, 19 and 21, or 19 and 20 nucleotides, and most preferably a length of 19 nucleotides.

Primer P1 preferably has a melting temperature (T_m) of between 60°C and 70°C, such as
5 60°C, 61°C, 62°C, 63°C, 64°C, 65°C, 66°C, 67°C, 68°C, 69°C and 70°C, preferably between 63°C and 69°C, more preferably between 66°C to 68°C, and most preferably of 67°C.

Primer P1 preferably comprises or consists of a nucleic acid sequence of SEQ ID NOs: 3 to 49. More preferably, primer P1 comprises or consists of a nucleic acid sequence according to SEQ ID NO: 9. Most preferably, P1 consists of the nucleic acid sequence according to SEQ ID NO: 9.

10 Primer P2 preferably has a length of between 17 and 22 nucleotides, such as 17, 18, 19, 20, 21 and 22 nucleotides, preferably between 18 and 20 nucleotides, or 19 and 20 nucleotides, and most preferably a length of 20 nucleotides.

Primer P2 preferably has a T_m of between of between 60°C to 67°C, such as 60°C, 61°C, 62°C, 63°C, 64°C, 65°C, 66°C and 67°C, preferably of between 62°C to 66°C, and most preferably
15 between 64°C to 65°C.

Primer P2 preferably comprises or consists of a nucleic acid sequence of SEQ ID NOs: 50 to 52. More preferably, primer P1 comprises or consists of a nucleic acid sequence according to SEQ ID NO: 50. Most preferably, P1 consists of the nucleic acid sequence according to SEQ ID NO: 50.

Preferred characteristics of the primers P3 and P4 to be used in the context of the invention are
20 disclosed in the following.

Primer P3 preferably has a length of between 20 and 25 nucleotides, such as 20, 21, 22, 23, 24 and 25 nucleotides, preferably between 21 and 24, 22 and 23, and most preferably a length of 23 nucleotides.

Primer P3 preferably has a melting temperature (T_m) of between 60°C and 70°C, such as
25 60°C, 61°C, 62°C, 63°C, 64°C, 65°C, 66°C, 67°C, 68°C, 69°C and 70°C, preferably between 63°C and 69°C, more preferably between 66°C to 68°C, and most preferably of 67°C.

Primer P3 preferably comprises or consists of a nucleic acid sequence of SEQ ID NOs: 104. Most preferably, P3 consists of the nucleic acid sequence according to SEQ ID NO: 104.

Primer P4 preferably has a length of between 15 and 21 nucleotides, such as 15, 16, 17, 18,
30 19, 20, and 21 nucleotides, preferably between 16 and 20 nucleotides, or 17 and 19 nucleotides, and most preferably a length of 17 nucleotides.

Primer P4 preferably has a T_m of between of between 60°C to 67°C, such as 60°C, 61°C, 62°C, 63°C, 64°C, 65°C, 66°C and 67°C, preferably of between 62°C to 66°C, and most preferably
between 64°C to 65°C.

35 Primer P4 preferably comprises or consists of a nucleic acid sequence of SEQ ID NOs: 105. Most preferably, P4 consists of the nucleic acid sequence according to SEQ ID NO: 105.

The T_m of primers and in particular of primers P1 and P2 or of primers P3 and P4 is preferably calculated using a software and the settings as described in the section 'Definitions' above.

The method of the present invention comprises performing PCR on a sample suspected of comprising mycoplasma DNA and/or virus DNA such as MMV DNA with a PCR reaction mixture comprising. The type of PCR is preferably real-time quantitative PCR (qPCR) or digital PCR (dPCR). The dPCR can be for example droplet digital PCR (ddPCR) or chip digital PCR (cdPCR). A particularly preferred PCR method according to the present invention is qPCR.

The PCR is preferably performed by using an annealing temperature of between 55°C to 65°C for P1 and P2, such as 55°C, 56°C, 57°C, 58°C, 59°C, 60°C, 61°C, 62°C, 63°C, 64°C and 65°C, preferably between 58°C and 60°C. It is preferred that the annealing temperature of P1 and P2 or P3 and P4 is about the same, such as within the range of about 2°C to 3°C difference between both primers, more preferably within the range of $\pm 1^\circ\text{C}$. For example, if P1 has an annealing temperature of about 59°C, it is preferred that P2 has an annealing temperature of $59^\circ\text{C} \pm 1^\circ\text{C}$. Although it is preferred that the annealing temperature of P1 and P2 or P3 and P4 is about the same, and more preferably identical, this is not mandatory.

The elongation temperature can be readily selected by the person of ordinary skill in the art and may depend on the specific type of polymerase used in the reaction and the length of the expected PCR product to be generated in the reaction. Typical elongation temperatures are between 60°C and 80°C, typically at around 72°C, but may depend on the type of enzyme used.

According to a preferred embodiment of the present invention, the annealing step and the elongation step are performed at the same temperature, and may thus be performed in the same step. For example, the annealing and elongation may take place at a temperature of between about 57°C and 65°C, such as at about 58°C, 59°C, 60°C, 61°C, 62°C, 63°C, 64°C or 65°C. According to a particularly preferred embodiment, the annealing and elongation may take place at a temperature of about 60°C. Such combined annealing and elongation steps are also simply referred to as amplification step and may speed up the time required for running the PCR. The duration of the amplification step depends on the size of the target to be amplified and the DNA polymerase used. For example, the amplification may require between about 20 and 60 seconds, between about 30 and 50 seconds or around 40 seconds. According to a preferred embodiment of the present invention, the duration of the amplification, *i.e.* the annealing and the elongation, takes about 30 seconds.

A typical number of cycles comprising denaturation, annealing and elongation is between about 20 and 50 cycles, such as 20, 25, 30, 35, 40, 45 and 50 cycles, preferably between about 30 and 45 cycles. According to a particularly preferred embodiment, the PCR is run for about 45 cycles.

The PCR may comprise in addition to the standard procedure of multiple cycles of denaturation, annealing and elongation, one or more additional steps preferably preceding said cycles for degrading contaminant DNA, such as contaminant DNA from remnant PCR products. Such a degradation is preferably performed by adding an enzyme such as uracil-DNA glycosylase (UNGase,

e.g. from ArcticZymes Technologies, Norway) to the reaction mixture and applying a degradation step at about 40°C for a time period of between 60 and 180 seconds, preferably for 120 seconds. The temperature and duration of such an additional step may depend on the enzyme and its concentration used in the reaction mixture for degrading contaminant DNA, and may be readily selected by the person of ordinary skill in the art or by following the instructions of the manufacturer of the enzyme(s) used for this purpose.

The PCR may, in addition to or as alternative to the degrading step, comprise a step of activating the reaction before the actual cycles are started. Such a starting or initiation step may comprise applying a temperature of between about 88°C and 98°C, preferably of about 90°C, for about 45 seconds to 10 minutes, preferably for about 60 seconds, to the reaction mixture comprising the sample and primers. The specific parameters of such an activation step may be readily selected by the person of ordinary skill in the art or by following the instructions of the manufacturer of the polymerase or of any of the other reaction mixture components used in the PCR.

A particularly preferred PCR protocol for the method of the present invention is disclosed in the following:

Step	Temperature	Time	Cycle(s)
Enzym. degradation	40°C	120s	1
Activation	90°C	60s	1
Denaturation	90°C	15s	45
Amplification	60°C	30s	

The detection of the PCR product may be performed by any respective method known in the field. For example, the PCR product generated may be detected during the PCR (also referred to as real time techniques) or after the PCR (also referred to as end point techniques). Real time techniques are performed inside the PCR reaction vessel and during PCR thermocycling using for example DNA binding fluorescent dyes (e.g. non-specific fluorescent dyes that intercalate with any double-stranded DNA), DNA hybridization (e.g. sequence-specific DNA probes consisting of oligonucleotides that are labelled e.g. with a fluorescent dye), or dNTP nucleotides with fluorescent dyes. End point techniques include but are not limited to DNA gel electrophoresis and the use of DNA intercalating dyes. A particularly preferred detection method is the use of sequence-specific DNA probes comprising a label directly in the reaction mixture and thus during the PCR.

In accordance with such a particularly preferred embodiment of the present invention, the reaction mixture for detecting mycoplasma may further comprise at least a first nucleic acid probe (herein referred to as Probe 1) in the reaction mixture. Probe 1 anneals to a nucleic acid sequence at least 90% identical to SEQ ID NO: 53, or to the reverse complement of a nucleic acid sequence at least 90% identical to SEQ ID NO: 53. Accordingly, the reaction mixture for detecting MMV may further comprise at least a one nucleic acid probe (herein referred to as Probe 5) in the reaction

mixture. Probe 5 anneals to a nucleic acid sequence at least 90% identical to SEQ ID NO: 111, or to the reverse complement of a nucleic acid sequence at least 90% identical to SEQ ID NO: 111.

According to a further particularly preferred embodiment of the present invention referring to the detection of mycoplasma, the reaction mixture comprises a second nucleic acid probe (Probe 2), which anneals to a nucleic acid sequence at least 90% identical to SEQ ID NO: 53, or to the reverse complement of a nucleic acid sequence at least 90% identical to SEQ ID NO: 53. Probe 2 anneals or binds to a different section on the nucleic acid sequence at least 90% identical to SEQ ID NO: 53 or to the reverse complement thereof than Probe 1. Preferably, Probe 2 anneals to a section on the nucleic acid sequence at least 90% identical to SEQ ID NO: 53 or to the reverse complement thereof separated by at least two nucleotides from the section on the nucleic acid sequence at least 90% identical to SEQ ID NO: 53 or to the reverse complement thereof to which Probe 1 anneals or binds.

Probe 1 and/or Probe 2 or Probe 5 may be present in a pre-mix of the reaction mixture or may be added before the PCR is started.

Preferred characteristics of Probe 1, Probe 2 and Probe 5 are disclosed in the following.

Probe 1 and/or Probe 2 preferably have a length between about 20 and 42 nucleotides, such as about 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41 and 42 nucleotides, more preferably of between about 21 and 30 nucleotides, and most preferably of between about 22 and 24 nucleotides. The most preferred length of Probe 1 and/or Probe 2 is 23 nucleotides. Probe 5 preferably has a length between about 15 and 30 nucleotides, such as about 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, and 30 nucleotides, more preferably of between about 18 and 22 nucleotides, and most preferably of between about 19 and 21 nucleotides. The most preferred length of Probe 5 is 20 nucleotides.

Probe 1 and/or Probe 2 preferably have a T_m of between about 55°C and 70°C, such as about 55°C, 56°C, 57°C, 58°C, 59°C, 60°C, 61°C, 62°C, 63°C, 64°C, 65°C, 66°C, 67°C, 68°C, 69°C and 70°C, more preferably of about between 58°C and 69°C, and most preferably of about between 59°C and 68°C, such as between about 59°C and 67°C, between about 59°C and 66°C, between about 59°C and 65°C, between about 59°C and 64°C, between about 59°C and 63°C, between about 59°C and 62°C, between about 59°C and 61°C.

Probe 5 preferably has a T_m of between about 59°C and 71°C, such as about 59°C, 60°C, 61°C, 62°C, 63°C, 64°C, 65°C, 66°C, 67°C, 68°C, 69°C, 70°C, and 71°C, more preferably of about between 60°C and 70°C, and most preferably of about between 62°C and 68°C, such as between about 63°C and 67°C, between about 64°C and 66°C.

Probe 1 preferably comprises or consists of a nucleic acid sequence of SEQ ID NOs: 54 to 65. More preferably, Probe 1 comprises or consists of a nucleic acid sequence according to SEQ ID NO: 62, most preferably Probe 1 consists of a nucleic acid sequence according to SEQ ID NO: 62. Probe 1 may comprise one or more locked nucleic acids (LNA). In addition to or as an alternative to LNAs, Probe 1 may comprise one or more of a minor groove binding (MGB) moiety. According to one

particularly preferred embodiment, Probe 1 consists of a nucleic acid sequence according to SEQ ID NO: 62 and comprises LNAs, preferably five LNAs as shown in SEQ ID NO: 60 (underlined nucleotides represent LNA modifications).

Probe 2 preferably comprises or consists of a nucleic acid sequence of SEQ ID NO: 66. More preferably, Probe 2 consists of a nucleic acid sequence according to SEQ ID NO: 66. Probe 2 may comprise one or more LNAs. In addition or as an alternative to LNAs, Probe 2 may comprise one or more of a minor groove binding (MGB) moiety. According to one particularly preferred embodiment, Probe 2 consists of a nucleic acid sequence according to SEQ ID NO: 66 and comprises LNAs, preferably three LNAs as shown in SEQ ID NO: 67 (underlined nucleotides represent LNA modifications).

Probe 5 preferably comprises or consists of a nucleic acid sequence of SEQ ID NO: 106. More preferably, Probe 5 consists of a nucleic acid sequence according to SEQ ID NO: 106. Probe 5 may comprise one or more locked nucleic acids (LNA). In addition to or as an alternative to LNAs, Probe 5 may comprise one or more of a minor groove binding (MGB) moiety.

If the length of Probe 1 and/or Probe 2 exceeds about 30 nucleotides, internal quenchers can be used instead of the LNA and/or MEG modifications. Internal quenchers can be for example ZEN and/or TAO modifications. Such internal quenchers are particularly useful in long probes comprising a fluorescent dye at one end and a quencher at the other end, in order to increase assay efficiency by decreasing the number of cycles required to meet the signal threshold compared to single-quenched probes, and by providing greater overall dye quenching, thus resulting in decreased background fluorescence and improved signal to noise ratio.

Probe 1 and/or Probe 2 and/or Probe 5 comprise one or more detectable labels. According to one embodiment, Probe 1 and Probe 2 comprise the same detectable label(s). The detectable label can be any label that allows identification of the probes. A preferred detectable label is a fluorescent dye. Preferred fluorescent dyes include but are not limited to FAM, HEX, NED, TET, VIC, Cy3, Cy5, Texas Red or Tide Fluor™ dyes, more preferably FAM. The fluorescent dye is preferably attached to the 5' end of the nucleic acid probe. In such a case, the probe may further comprise a quencher dye attached to its 3' end. Preferred quencher dyes include but are not limited to DABCYL, TAMRA, BXQ-1, BXQ-2, IABkFQ, IABkRQ or Tide Quencher™ dyes, more preferably IABkFQ. IABkFQ and IABkRQ are Iowa Black® FQ and RQ, respectively, obtainable from Integrated DNA Technologies, Inc., USA. IABkFQ is preferably used in combination with FAM, HEX, NED, TET and VIC, and IABkRQ is preferably used in combination with Cy5 or Texas Red. Probe 1 and/or Probe 2 and/or Probe 5 are preferably hydrolysis probes comprising a fluorescent dye at their 5' end and a quencher dye at their 3' end. A particularly preferred combination for Probe 1, Probe 2 and Probe 5 is FAM / IABkFQ. While the hydrolysis probe is intact, the fluorescent dye and quencher dye remain in close proximity to each other, FRET occurs, and fluorescent dye is quenched. When during PCR cycling, the DNA polymerase binds to and extends the primer upstream of the probe, any probe bound to the

correct target sequence is hydrolyzed. The fluorescent dye fragment is released, resulting in a fluorescence signal proportional to the amount of amplicon produced, thus allowing detection of the PCR product during the reaction.

According to a particularly preferred embodiment, Probe 1 consists of SEQ ID NO: 60 and
5 comprises the fluorochrome/quencher pair FAM / IABkFQ. According to a further particularly preferred embodiment, Probe 2 consists of SEQ ID NO: 67 and comprises the fluorochrome/quencher pair FAM / IABkFQ. According to a further particularly preferred embodiment, Probe 5 consists of SEQ ID NO: 106 and comprises the fluorochrome/quencher pair FAM / IABkFQ.

According to a particularly preferred embodiment of the present invention regarding the
10 detection of mycoplasma, the reaction mixture comprises primer P1, primer P2, Probe 1 and Probe 2 as described herein.

According to a particularly preferred embodiment of the present invention regarding the detection of MMV, the reaction mixture comprises primer P3, primer P4, and Probe 5 as described herein.

15 According to one embodiment, in the method of the present invention, the reaction mixture additionally comprises a positive control nucleic acid sequence, which is also referred to as internal positive control (IPC). The IPC may be present in a pre-mix of the reaction mixture or it may be added to the reaction mixture before the PCR is started.

In the embodiment for detecting mycoplasma, the positive control nucleic acid sequence or
20 myco-IPC comprises at least 18 consecutive nucleic acids of the nucleic acid sequence of SEQ ID NO: 1, at least 18 consecutive nucleic acids of the nucleic acid sequence of SEQ ID NO: 2, and an intervening nucleic acid sequence of a length of at least 18 nucleotides between these two nucleic acid sequences. This intervening sequence of at least 18 nucleotides preferably differs from SEQ ID NO: 53 in such a way that neither Probe 1 (if present) nor Probe 2 (if present) anneals to the intervening nucleic acid
25 sequence. According to a particularly preferred embodiment, a third probe (Probe 3) anneals or binds to said intervening sequence or to its reverse complement. The intervening nucleic acid sequence preferably has a length of about 20 to 150 nucleotides, about 30 to 140 nucleotides, or about 40 to 120 nucleotides, such as 50, 60, 70, 80, 90, 100, 110 or about 120 nucleotides. According to a particularly preferred embodiment, the intervening nucleic acid sequence has a length of about 120 nucleotides.

30 In the embodiment for detecting mycoplasma, the internal positive control nucleic acid sequence or myco-IPC preferably comprises or consists of a nucleic acid sequence at least 90% identical to the nucleic acid sequence of SEQ ID NO: 68, or to the reverse complement of a nucleic acid sequence at least 90% identical to the nucleic acid sequence of SEQ ID NO: 68. The myco-IPC is preferably in the form of a double-stranded DNA molecule, more preferably in the form of a gBlock. According to a
35 particularly preferred embodiment, the myco-IPC is a gBlock comprising or consisting of SEQ ID NO: 68 and its reverse complement.

For detecting the myco-IPC amplicons, Probe 3 can be used. Thus, according to one embodiment, the reaction mixture further comprises Probe 3, which may be present in a pre-mix of the reaction mixture or added before the PCR is started. Probe 3 preferably has one or more of the specifics described above in the context of Probes 1 and 2 regarding their length, T_m and label, with the proviso that the label for Probe 3 is different from the label selected for Probe 1 and Probe 2, allowing to distinguish Probe 3 from Probes 1 and 2. Probe 3 may also comprise one or more of an LNA and/or MGB modification. Probe 3 preferably has a length of between 20 and 42 nucleotides, more preferably of between 21 and 30 nucleotides, and most preferably of between 22 and 24 nucleotides. According to one embodiment, Probe 3 has a T_m of between about 55°C and 70°C, preferably between about 58°C and 69°C, more preferably between about 59°C and 68°C, and most preferably of about 60°C. Probe 3 preferably comprises or consists of a nucleic acid sequence of SEQ ID NO: 69 or its reverse complement. More preferably, Probe 3 consists of a nucleic acid sequence of SEQ ID NO: 69 or its reverse complement. According to a preferred embodiment, Probe 3 comprises the fluorochrome/quencher pair Cy5 / IABkRQ. According to a particularly preferred embodiment, Probe 3 consists of a nucleic acid sequence of SEQ ID NO: 69 and comprises the fluorochrome/quencher pair Cy5 / IABkRQ.

If the myco-IPC is present during the PCR, Primers 1 and 2 also bind to the IPC and allow amplification thereof. The presence of IPC amplicons can be detected using for example Probe 3. Alternatively, the presence of IPC amplicons can be detected using the real time techniques or end point techniques described herein. If IPC amplification product is detected during and/or at the end of the PCR, the reaction parameters chosen were sufficient for enabling amplification of potential targets in a sample. If only myco-IPC amplicons are detected and no further PCR products, this means that the sample did not contain any mycoplasma.

In the embodiment for detecting MMV, the positive control nucleic acid sequence or mmv-IPC comprises at least 18 consecutive nucleic acids of the nucleic acid sequence of SEQ ID NO: 109, at least 18 consecutive nucleic acids of the nucleic acid sequence of SEQ ID NO: 110, and an intervening nucleic acid sequence of a length of at least 18 nucleotides between these two nucleic acid sequences. This intervening sequence preferably does not allow binding of Probe 3 (if present) or Probe 5 (if present) to the intervening nucleic acid sequence. According to a particularly preferred embodiment, Probe 4 anneals or binds to said intervening sequence or to its reverse complement. The intervening nucleic acid sequence preferably has a length of about 20 to 150 nucleotides, about 30 to 140 nucleotides, or about 40 to 120 nucleotides, such as 50, 60, 70, 80, 90, 100, 110 or about 120 nucleotides. According to a particularly preferred embodiment, the intervening nucleic acid sequence has a length of about 120 nucleotides.

In the embodiment for detecting MMV, the internal positive control nucleic acid sequence or mmv-IPC preferably comprises or consists of a nucleic acid sequence at least 90% identical to the nucleic acid sequence of SEQ ID NO: 112 or 116 or to the reverse complement of a nucleic acid

sequence at least 90% identical to the nucleic acid sequence of SEQ ID NO: 112 or 116. According to an alternative embodiment, the mmv-IPC comprises or consists of a nucleic acid sequence at least 90% identical to the nucleic acid sequence of 68 or 112 or 115, or to the reverse complement of a nucleic acid sequence at least 90% identical to the nucleic acid sequence of SEQ ID NO: 68 or 112 or 115. The myco-IPC is preferably in the form of a double-stranded DNA molecule, more preferably in the form of a gBlock. According to a particularly preferred embodiment, the myco-IPC is a gBlock comprising or consisting of SEQ ID NO: 68 and its reverse complement.

For detecting the mmv-IPC amplicons, Probe 4 can be used. Thus, according to one embodiment, the reaction mixture further comprises Probe 4, which may be present in a pre-mix of the reaction mixture or added before the PCR is started. Probe 4 as described herein above. Probe 4 may also comprise one or more of an LNA and/or MGB modification. Probe 4 preferably has a length of between 20 and 42 nucleotides, more preferably of between 21 and 30 nucleotides, and most preferably of between 22 and 24 nucleotides. According to one embodiment, Probe 4 has a T_m of between about 55°C and 70°C, preferably between about 58°C and 69°C, more preferably between about 59°C and 68°C, and most preferably of about 60°C. Probe 4 preferably comprises or consists of a nucleic acid sequence of SEQ ID NO: 69 or its reverse complement. More preferably, Probe 4 consists of a nucleic acid sequence of SEQ ID NO: 69 or its reverse complement. According to a preferred embodiment, Probe 4 comprises the fluorochrome/quencher pair Cy5 / IABkRQ. According to a particularly preferred embodiment, Probe 4 consists of a nucleic acid sequence of SEQ ID NO: 69 and comprises the fluorochrome/quencher pair Cy5 / IABkRQ.

If the mmv-IPC is present during the PCR, Primers 3 and 4 also bind to the IPC and allow amplification thereof. The presence of IPC amplicons can be detected using for example Probe 4. Alternatively, the presence of IPC amplicons can be detected using the real time techniques or end point techniques described herein. If IPC amplification product is detected during and/or at the end of the PCR, the reaction parameters chosen were sufficient for enabling amplification of potential targets in a sample. If only mmv-IPC amplicons are detected and no further PCR products, this means that the sample did not contain any mouse minute virus.

According to one embodiment, in the method of the present invention, the reaction mixture or the sample additionally comprises a discriminatory positive control nucleic acid sequence, which is also referred to as DPC. Alternatively, the DPC can be used in a separate control reaction using the same parameters as the PCR for mycoplasma or MMV detection but comprising the DPC instead of the sample.

In the embodiment for detecting mycoplasma, the discriminatory positive control nucleic acid sequence or myco-DPC comprises at least 18 consecutive nucleic acids of the nucleic acid sequence of SEQ ID NO: 1, at least 18 consecutive nucleic acids of the nucleic acid sequence of SEQ ID NO: 2, and between these two nucleic acid sequences an intervening nucleic acid sequence of a length of at least 18 nucleotides. This intervening sequence of at least 18 nucleotides preferably does not differ

from but rather comprises SEQ ID NO: 53 or a part therefrom. The length of the intervening nucleic acid sequence preferably allows annealing or binding of Probe 1 (if present) and preferably Probe 2 (if present). According to a preferred embodiment, the intervening sequence has a length allowing binding or annealing of a fourth probe (Probe 4). Thus, the intervening sequence of the DPC
5 preferably has a length of between 18 and about 200 nucleotides, such as between about 25 and 190, between about 30 and 180, between about 35 and 170, between about 40 and 160, between about 50 and 150, between about 60 and 140, between about 70 and 130, between about 80 and 120, or between about 90 and 110 nucleotides. Preferably, the sequence of the intervening nucleic acid sequence or to its reverse complement to which Probe 4 anneals to or binds to is different from the sequence of the
10 intervening nucleic acid sequence to which Probe 1 and/or Probe 2 anneals or binds to.

In the embodiment for detecting mycoplasma, the discriminatory positive control nucleic acid sequence or myco-DPC preferably comprises or consists of a nucleic acid sequence at least 90% identical to the nucleic acid sequence of SEQ ID NO: 70, or to the reverse complement of a nucleic acid sequence at least 90% identical to the nucleic acid sequence of SEQ ID NO: 70. The myco-DPC
15 is preferably in the form of a double-stranded DNA molecule, more preferably in the form of a gBlock. According to a particularly preferred embodiment, the myco-DPC is a gBlock comprising or consisting of SEQ ID NO: 70 and its reverse complement.

In the embodiment for detecting mycoplasma, for detecting the myco-DPC, a further Probe 4 can be used. Probe 4 preferably binds or anneals to the myco-DPC in a region distinct from the region
20 to which Probe 1 (if present) and Probe 2 (if present) bind or anneal to. Probe 4 preferably has one or more of the specifics described above in the context of Probes 1 and 2 regarding their length, T_m and label, with the proviso that the label for Probe 4 is different from the label selected for Probe 1, Probe 2 and Probe 3, allowing to distinguish Probe 4 from the other probes. Probe 4 may also comprise one or more of an LNA and/or MGB modification. According to one embodiment, Probe 4 has a length of
25 between 20 and 42 nucleotides, preferably of between 21 and 30 nucleotides, and most preferably of between 22 and 24 nucleotides. According to a preferred embodiment, Probe 4 has a T_m of between about 55°C and 70°C, preferably of between about 58°C and 69°C, more preferably of between about 59°C and 68°C, and most preferably of about 60°C. Probe 4 preferably comprises or consists of a nucleic acid sequence of SEQ ID NO: 71 or its reverse complement. More preferably, Probe 4 consists
30 of a nucleic acid sequence of SEQ ID NO: 71 or its reverse complement. According to a preferred embodiment, Probe 4 comprises the fluorochrome/quencher pair HEX/ IABkFQ. According to a particularly preferred embodiment, Probe 4 consists of a nucleic acid sequence of SEQ ID NO: 71 and comprises the fluorochrome/quencher pair HEX/ IABkFQ. According to one embodiment, the reaction mixture further comprises the myco-DPC and Probe 4, both of which may be present in a pre-mix of
35 the reaction mixture or added before the PCR is started. Alternatively, if the myco-DPC is used in a separate control reaction, the separate control reaction uses the same parameters as the PCR for mycoplasma detection but comprises the myco-DPC instead of the sample, and Probe 4.

A particularly preferred combination of primers and probes to be used in the method of the invention for detecting mycoplasma comprises primer P1 consisting of the nucleic acid sequence of SEQ ID NO: 9, primer P2 consisting of the nucleic acid sequence of SEQ ID NO: 50, a first probe (Probe 1) consisting of the nucleic acid sequence of SEQ ID NO: 60, and a second probe (Probe 2) consisting of the nucleic acid sequence of SEQ ID NO: 67. In this particularly preferred combination of primers and probes for detecting mycoplasma in a sample, Probe 1 preferably comprises the fluorochrome/quencher pair FAM / IABkFQ, and Probe 2 preferably comprises the fluorochrome/quencher pair FAM / IABkFQ.

In the embodiment for detecting MMV, the discriminatory positive control nucleic acid sequence or mmv-DPC comprises at least 18 consecutive nucleic acids of the nucleic acid sequence of SEQ ID NO: 109, at least 18 consecutive nucleic acids of the nucleic acid sequence of SEQ ID NO: 110, and between these two nucleic acid sequences an intervening nucleic acid sequence of a length of at least 18 nucleotides. The intervening nucleic acid sequence preferably allows annealing or binding of Probe 5 (if present) and preferably Probe 3 (if present). Thus, the intervening sequence of the mmv-DPC preferably has a length of between 18 and about 200 nucleotides, such as between about 25 and 190, between about 30 and 180, between about 35 and 170, between about 40 and 160, between about 50 and 150, between about 60 and 140, between about 70 and 130, between about 80 and 120, or between about 90 and 110 nucleotides. According to a preferred embodiment, the intervening nucleic acid sequence of the mmv-DPC has a length of at least 29 nucleotides. According to a particularly preferred embodiment, the intervening nucleic acid sequence of the mmv-DPC does not differ from SEQ ID NO: 111 so that Probe 5 is capable of annealing to said intervening nucleic acid sequence.

In the embodiment for detecting MMV, the discriminatory positive control nucleic acid sequence or mmv-DPC preferably comprises or consists of a nucleic acid sequence at least 90% identical to the nucleic acid sequence of SEQ ID NO: 113, or to the reverse complement of a nucleic acid sequence at least 90% identical to the nucleic acid sequence of SEQ ID NO: 113. According to one embodiment, the mmv-DPC comprises or consists of a nucleic acid sequence at least 90% identical to the nucleic acid sequence of SEQ ID NO: 114, or to the reverse complement of a nucleic acid sequence at least 90% identical to the nucleic acid sequence of SEQ ID NO: 114. The mmv-DPC is preferably in the form of a double-stranded DNA molecule, more preferably in the form of a gBlock. According to a particularly preferred embodiment, the mmv-DPC is a gBlock comprising or consisting of SEQ ID NO: 114 and its reverse complement.

In the embodiment for detecting MMV, Probe 5 preferably binds or anneals to the mmv-DPC in a region distinct from the region to which Probe 3 binds or anneals to. Probe 5 preferably has one or more of the specifics described herein above e.g. regarding its length, T_m and label, with the proviso that the label for Probe 5 is different from the label selected for Probe 3 and/or Probe 4, allowing to distinguish Probe 5 from the other probes.

The concentrations of the individual components in the reaction mixture can be readily selected by the skilled person. According to a particularly preferred embodiment, preferably in combination with the particularly preferred combination of primers and probes described herein above, the PCR reaction mixture comprises primers such as P1 and P2 or P3 and P4 in a concentration of about 400 nM; and Probe 1 and Probe 2 or Probe 5 in a concentration of about 200 nM. In 5 embodiments comprising the internal positive control (IPC), the PCR reaction mixture preferably further comprises the control such as Probe 3 and optionally Probe 4 in a concentration of about 200 nM. The IPC is preferably present in an amount of about 200 copies in the PCR reaction mixture. It is to be noted that the indicated concentrations refer to the respective concentrations in the final PCR 10 reaction mixture used in the actual reaction. The skilled person readily understands that master mixes or pre-mixes of the reaction mixture or parts thereof can be prepared, in which the concentrations deviate from the above cited final concentrations. Such master mixes or pre-mixes are commonly diluted with H₂O and/or buffer solutions, giving rise to the final reaction mixture.

According to a preferred embodiment, the reaction mixture further comprises an enzyme for 15 degrading unwanted DNA such as remnant and/or contaminating DNA. A preferred enzyme for degradation is UNGase. Thus, according to a particularly preferred embodiment, the reaction mixture in addition to the particularly preferred combination of primers, probes and IPC described herein above comprises UNGase, preferably in a final concentration of 1U per 100 µL of reaction mixture.

A most preferred combination of primers, probes and other components for the polymerase 20 chain reaction is set forth in the following table 3.

Table 3.1: preferred concentrations in reaction mixture for mycoplasma detection

Component	[C] _{final}
qPCR Master Mix	1x
UNGase	1 u/100µL
Primer 1	400 / 800 nM
Primer 2	400 / 800 nM
Probe 1	200 nM
Probe 2	200 nM
optionally Probe 3	200 nM
optionally Probe 4	200 nM
optionally myco-IPC gBlock	200 cp

Table 3.2: preferred concentrations in reaction mixture for MMV detection

Component	[C] _{final}
qPCR Master Mix	1x

Component	[C] _{final}
UNGase	1 u/100µL
Primer 1	400 / 800 nM
Primer 2	400 / 800 nM
Probe 5	200 nM
optionally Probe 3	200 nM
optionally mmv-IPC gBlock	200 cp

The primer concentration for mycoplasma detection or for MMV detection may be either 400 nm for both primers or alternatively 800 nm for both primers.

5 According to a particularly preferred embodiment of the present invention relating to the detection of mycoplasma, the concentration of primers and probes is as set forth in the above table 3.1, with Primer 1 being Fw Myco7, Primer 2 being Rev Myco1*, Probe 1 being Myco5 bis, Probe 2 being Acho1, Probe 3 (if present) being SAtPSY, Probe 4 (if present) being StraA, and IPC gBlock (if present) comprising SEQ ID NO: 68. Thus, according to a particularly preferred embodiment, the
10 present invention comprises the combination set forth in the following table 4.1.

Table 4.1: Preferred components and their preferred concentrations for mycoplasma detection

Component	[C] _{final}
qPCR Master Mix (e.g. Luna Universal Probe)	1x
UNGase	1 u/100µL
Primer 1 (Fw Myco7)	400 nM
Primer 2 (Rev Myco1*)	400 nM
Probe 1 (Myco5 bis)	200 nM
Probe 2 (Acho1)	200 nM
optionally Probe 3 (SAtPSY)	200 nM
optionally Probe 4 (StraA)	200 nM
optionally IPC gBlock (SEQ ID NO: 68)	200 cp

15 According to a particularly preferred embodiment of the present invention relating to the detection of MMV, the concentration of primers and probes is as set forth in the above table 3.2, with Primer 3 being Fw MMV, Primer 4 being Rev MMV, Probe 5 being Probe MMV, Probe 3 (if present) being SAtPSY, Probe 4 (if present) being StraA, and IPC gBlock (if present) comprising SEQ ID NO: 68. Thus, according to a particularly preferred embodiment, the present invention comprises the combination set forth in the following table 4.2.

Table 4.2: Preferred components and their preferred concentrations for MMV detection

Component	[C] _{final}
qPCR Master Mix (e.g. Luna Universal Probe)	1x
UNGase	1 u/100µL
Primer 3 (Fw MMV)	800 nM
Primer 4 (Rev MMV)	800 nM
Probe 5 (Myco5 bis)	200 nM
optionally Probe 3 (SAtPSY)	200 nM
optionally Probe 4 (StraA)	200 nM
optionally IPC gBlock (SEQ ID NO: 68)	200 cp

In accordance with a particularly preferred embodiment for detecting MMV, the reaction mixture comprises the following: Primer 3 having SEQ ID NO: 104, Primer 4 having SEQ ID NO: 105, Probe 5 having SEQ ID NO: 106 and comprising the fluorochrome/quencher pair FAM / IABkFQ, and mmv-IPC having SEQ ID NO: 68. This reaction mixture preferably further comprises Probe 4 having SEQ ID NO: 71 and comprising the fluorochrome/quencher pair HEX/ IABkFQ. This reaction mixture optionally further comprises mmv-DPC comprising SEQ ID NO: 113 and preferably Probe 3 having SEQ ID NO: 69 and comprising the fluorochrome/quencher pair Cy5 / IABkRQ.

It will be appreciated that IPC and DPC can be present in the same reaction mixture as the PCR reaction mixture comprising Primers 1 and 2 or 3 and 4. Alternatively, IPC and DPC can be present combined in a separate reaction mixture or separated in separate reaction mixtures.

The present invention further provides a pair of primers comprising a primer P1 annealing to the reverse complement of a nucleic acid sequence of SEQ ID NO: 1, and a primer P2 annealing to a nucleic acid sequence of SEQ ID NO: 2. These primers can be used for detecting mycoplasma in a sample, and in particular in respective PCR assays for detecting mycoplasma as described herein. According to one embodiment, primer P1 and primer P2 have one or more of the characteristics described for P1 and P2 above. P1 preferably comprises or consists of a nucleic acid sequence of any one of SEQ ID NOs: 3 to 49. More preferably, primer P1 comprises or consists of a nucleic acid sequence according to SEQ ID NO: 9. Most preferably, P1 consists of the nucleic acid sequence according to SEQ ID NO: 9. Primer P2 preferably comprises or consists of a nucleic acid sequence of any one of SEQ ID NOs: 50 to 52. More preferably, primer P2 comprises or consists of a nucleic acid sequence according to SEQ ID NO: 50. Most preferably, P2 consists of the nucleic acid sequence according to SEQ ID NO: 50.

A particularly preferred primer pair according to the present invention comprises primer P1 consisting of SEQ ID NO: 9, and primer P2 consisting of SEQ ID NO: 50.

The present invention also provides a pair of primers comprising a primer P3 annealing to the reverse complement of a nucleic acid sequence of SEQ ID NO: 109, and a primer P4 annealing to a

nucleic acid sequence of SEQ ID NO: 110. These primers can be used for detecting MMV in a sample, and in particular in respective PCR assays for detecting virus and in particular MMV as described herein. According to one embodiment, primer P3 and primer P4 have one or more of the characteristics described for P3 and P4 above. P3 preferably comprises or consists of a nucleic acid sequence of SEQ ID NO: 104. More preferably, P3 consists of the nucleic acid sequence according to SEQ ID NO: 104. Primer P4 preferably comprises or consists of a nucleic acid sequence of SEQ ID NO: 105. More preferably, primer P4 consists of the nucleic acid sequence according to SEQ ID NO: 105.

The present invention further provides a kit for detecting mycoplasma in a sample. The kit comprises the pair of primers of the present invention as described herein above, and a first nucleic acid probe (Probe 1) annealing to or binding to a nucleic acid sequence of SEQ ID NO: 53 or to its reverse complement. According to one embodiment, the kit further comprises a second nucleic acid probe (Probe 2) annealing to or binding to SEQ ID NO: 53 or to its reverse complement. Probe 1 and/or Probe 2 preferably have one or more of the characteristics of Probe 1 and Probe 2, respectively, as described herein above. Probe 1 preferably comprises or consists of a nucleic acid sequence of SEQ ID NOs: 54 to 65. More preferably, Probe 1 comprises or consists of a nucleic acid sequence according to SEQ ID NO: 62, most preferably Probe 1 consists of a nucleic acid sequence according to SEQ ID NO: 62. Probe 1 may comprise one or more locked nucleic acids (LNA). In addition or as an alternative to LNAs, Probe 1 may comprise one or more of a minor groove binding (MGB) moiety. According to one particularly preferred embodiment, Probe 1 consists of a nucleic acid sequence according to SEQ ID NO: 62 and comprises LNAs, preferably five LNAs as shown in SEQ ID NO: 60 (underlined nucleotides represent LNA modifications). Probe 2, if present, preferably comprises or consists of a nucleic acid sequence of SEQ ID NO: 66. More preferably, Probe 2 consists of a nucleic acid sequence according to SEQ ID NO: 66. Probe 2 may comprise one or more LNAs. In addition or as an alternative to LNAs, Probe 2 may comprise one or more of a minor groove binding (MGB) moiety. According to one particularly preferred embodiment, Probe 2 consists of a nucleic acid sequence according to SEQ ID NO: 66 and comprises LNAs, preferably three LNAs as shown in SEQ ID NO: 67 (underlined nucleotides represent LNA modifications).

According to a particularly preferred embodiment, the kit comprises primer P1, primer P2, Probe 1 and Probe 2 as described herein, more preferably primer P1 consisting of the nucleic acid sequence of SEQ ID NO: 9, primer P2 consisting of the nucleic acid sequence of SEQ ID NO: 50, Probe 1 consisting of the nucleic acid sequence of SEQ ID NO: 60, and Probe 2 consisting of the nucleic acid sequence of SEQ ID NO: 67. In this particularly preferred combination of primers and probes of the kit, Probe 1 preferably comprises the fluorochrome/quencher pair FAM / IABkFQ, and Probe 2 preferably comprises the fluorochrome/quencher pair FAM / IABkFQ.

The kit may further comprise one or more of a PCR reaction mixture as described herein above, an internal positive control nucleic acid sequence (IPC) as described herein above, a

discriminatory positive control nucleic acid sequence (DPC) as described herein above, means for extracting and/or purifying DNA from the sample as described herein above, and instructions for performing PCR with the primers. If an IPC and/or a DPC is part of the kit of the invention, the kit preferably further comprises a Probe 3 and Probe 4, respectively, as described herein, for aligning to
5 or binding to the IPC and DPC, respectively.

Using the present invention, that is the method of the invention, the set of primers of the present invention, or the kit of the present invention, it is possible to detect mycoplasma in a sample. The mycoplasma that can be detected is preferably one or more of the *Mycoplasma* species *Mycoplasma arginini*, *Mycoplasma buccale*, *Mycoplasma hominis*, *Mycoplasma orale*, *Mycoplasma salivarium*, *Mycoplasma fermentans*, *Mycoplasma hyorhinis*, *Mycoplasma synoviae*, *Mycoplasma pneumoniae*, *Acholeplasma laidlawii*, *Mycoplasma gallisepticum* and *Spiroplasma citri*.
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The present invention further provides a kit for detecting MMV in a sample. The kit comprises the pair of primers of the invention as described herein above, and a nucleic acid probe (Probe 5) annealing to a nucleic acid sequence of SEQ ID NO: 111 or its reverse complement.

Probe 5 preferably has one or more of the characteristics of Probe 5 as described herein above. Probe 5 preferably comprises or consists of a nucleic acid sequence of SEQ ID NO: 106. Probe 5 may comprise one or more locked nucleic acids (LNA). In addition or as an alternative to LNAs, Probe 5 may comprise one or more of a minor groove binding (MGB) moiety. According to one particularly preferred embodiment, Probe 5 consists of a nucleic acid sequence according to SEQ ID NO: 106 and
20 comprises LNAs. In addition or as an alternative to LNAs, Probe 5 may comprise one or more of a minor groove binding (MGB) moiety.

According to a particularly preferred embodiment, the kit comprises primer P3, primer P4, and Probe 5 as described herein, more preferably primer P3 consisting of the nucleic acid sequence of SEQ ID NO: 104, primer P4 consisting of the nucleic acid sequence of SEQ ID NO: 105, and Probe 5
25 consisting of the nucleic acid sequence of SEQ ID NO: 106. In this particularly preferred combination of primers and probes of the kit, Probe 5 preferably comprises the fluorochrome/quencher pair FAM / IABkFQ.

According to one embodiment, the kit further comprises one or more of a PCR reaction mixture as described herein above, an internal positive control nucleic acid sequence as described
30 herein above, a discriminatory positive control nucleic acid sequence as described herein above, means for extracting and/or purifying DNA from the sample as described herein above, and instructions for performing PCR with the primers. If an IPC and/or a DPC (such as mmv-IPC and/or mmv-DPC as described herein above) is part of the kit of the invention, the kit preferably further comprises Probe 5, Probe 3 and/or Probe 4 as described herein.

According to one embodiment, in the method according to the invention, in the pair of primers according to the invention or in the kit according to the invention, the virus is selected from the group consisting of Anelloviridae, Inoviridae, Parvoviridae, preferably Parvovirus, Erythrovirus,
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Dependovirus, Amdovirus and Bocavirus, more preferably Parvovirus, and most preferably Mouse Minute Virus.

Also disclosed herein is a combination of the set of primers or the kit for detecting mycoplasma and of the set of primers or the kit for detecting virus. In other words, the present invention also provides a method, primers and a kit for the detection of mycoplasma and virus in a sample. According to one embodiment, the detection of mycoplasma and virus is performed simultaneously, preferably in the same reaction vessel such as a PCR tube. According to an alternative embodiment, the detection of mycoplasma and virus is performed subsequently, preferably in different reaction vessels such as a PCR tubes.

Examples

Materials and Methods

All sequences referred to herein were retrieved from SILVA and NCBI websites (www.arb-silva.de and www.ncbi.nlm.nih.gov). Sequence analysis was performed with Vector NTI, Snap Gene and Geneious softwares. For primer thermodynamics calculation (T_m, primer dimer formation and hairpin T_m), PrimerExpress® 3.0 (Life Technologies Corporation), Geneious (Geneious Prime 2023.2; Dotmatics) and the IDT (Integrated DNA Technologies, Inc., USA) online software were used. Settings were as follows

- PrimerExpress: standard settings
- Geneious: Monovalent ions 50 mM; Divalent ions 3 mM; Primer 200 nM; dNTP 0.8 mM
- IDT: Parameter set, qPCR; Monovalent ions 50 mM; Divalent ions 3 mM; Primer 200 nM; dNTP 0.8 mM.

Similar parameters were used for probe thermodynamics calculation.

Example 1: Identification of regions for primer design

Organisms used for the identification of regions for primer design are listed in following Table 5. These include the Mycoplasma species and other germs which presence should be investigated in various cell substrates taken from the production workflow (Master Cell Bank, Working Cell Bank, Unprocessed Bulk, End Of Production Cells) as indicated in various guidelines.

Table 5: Organisms used in the identification of regions for primer design

Organism	Strain	ATCC	NCTC	Genbank Accession No.	Total length (base pairs)
<i>Acholeplasma laidlawii</i> *	PG-8A	ATCC 23206	NCTC10116	NZ_LS483439.1	1514
<i>Mycoplasma arginini</i> *	G230	ATCC 23838	NCTC10129	NZ_LR215044.1	1525
<i>Mycoplasma buccale</i>	CH20247	ATCC 23636	NTCC10136	AF_125586.1	1463

<i>Mycoplasma fermentans</i> *	PG18	ATCC 19989	NCTC10117	LR_214962.1	1502
<i>Mycoplasma gallisepticum</i> *	PG31	ATCC 19610	NCTC10115	NZ_LS991952.1	1520
<i>Mycoplasma hominis</i>		ATCC 23114	NCTC10111	NC_013511.1	1522
<i>Mycoplasma hyorhinis</i> *	BTS7	ATCC 17981		NZ_KB911485.1	1536
<i>Mycoplasma hyorhinis</i> *		ATCC 17981	NCTC10130	NZ_LS991950.1	1528
<i>Mycoplasma hyorhinis</i> *	DBS1050	ATCC 29052	NCTC10130	NC_022807.1	1528
<i>Mycoplasma orale</i> *		ATCC 23714	NCTC10112	NZ_LR214940.1	1522
<i>Mycoplasma pneumoniae</i> *		ATCC 15531	NCTC10119	NZ_LR214945.1	1520
<i>Mycoplasma salivarium</i>		ATCC 23064	NCTC10113	NZ_LR214938.1	1531
<i>Mycoplasma synoviae</i> *		ATCC 25204	NCTC10124	NZ_LS991953.1	1506
<i>Spiroplasma citri</i> *	R8-A2	ATCC 27556	NCTC10164	NZ_CP013197.1	1532
<i>Bacillus cereus</i>		ATCC 14579		NR_074540.1	1512
<i>Bacillus subtilis</i>	IAM 1211	ATCC 6051		NR_112116.2	1550
<i>Clostridium* perfringens</i>		ATCC 13124		NR_121697.2	1513
<i>Clostridium* sporogenes</i>		ATCC 15579		ABKW02000002	1500
<i>Escherichia coli</i>	A99			MK506978.1	1370
<i>Lactobacillus* acidophilus</i>		ATCC 4796		ACHN01000039.1	1552
<i>Lactobacillus* plantarum</i>	NRRL B-14768			NR_042394.1	1474
<i>Micrococcus luteus</i>		ATCC 4698		NR_114673.1	1325
<i>Staphylococcus aureus</i>		ATCC 12600		NR_118997.2	1552
<i>Staphylococcus saprophyticus</i>		ATCC 15305		NR_074999.2	1553
<i>Streptococcus* bovis</i>		ATCC 33317		AB002482.1	1457
<i>Streptococcus* pneumoniae</i>		ATCC 33400		MW407059.1	1515
<i>Homo sapiens</i>				EU545435.1	1558
<i>Cricetulus griseus</i>	CCL-14			DQ334846.1	1032
<i>Cricetulus griseus</i>				AY011148.1	808

* Required by PhEu 2.6.7

In order to identify genomic regions to be targeted by PCR and to ensure specific mycoplasma detection while excluding signals with other organisms, 16s and 23s ribosomal RNA sequences from the organisms listed in Table 5 were retrieved from SILVA and NCBI websites (www.arb-silva.de and www.ncbi.nlm.nih.gov) and compared. Sequences from 23s rRNA were excluded for being too unique and thus not suitable for identifying essentially all mycoplasma species with only a limited number of primers and probes. On the other hand, 16s RNA displays some specificity but retains enough homology across mycoplasma species. It was therefore decided to focus on the 16s rRNA sequences to identify regions that are common to all mycoplasma species which primer and probe sets should recognize, and which are further significantly different from all other species. Using such sequence stretches ensures specific mycoplasma detection without cross reactions with DNA from other species.

A first analysis focused on mycoplasma 16s rRNA sequences aimed at identifying 100 bp stretches with 80% homology across species, where primers and probes of 20 and 23 nucleotides, respectively, could be designed for mycoplasma detection by PCR. Domains with high homology to *E.*

coli 16s rRNA were disregarded, since *E. coli* is the organism closest to *Mycoplasma sp.*, thereby ensuring no cross-reactions with *E. coli* and the other organisms listed in Table 5. As shown in Figure 2, three sequence stretches were identified that met the criteria mentioned above.

In a second analysis, sequences were analyzed within the identified three regions. Surprisingly, no primers and probes could be designed in the first two regions highlighted in Figure 1, *i.e.* in the region between 500 and 600 bp, and in the region at around 800 bp, although all three regions showed homology between mycoplasma species. Instead, primers and probes were designed in the sequence stretch on the 16s rRNA of the third region at around 1100 bp, which further required a widening of the region to accommodate all three of the forward primer, the probe, and the reverse primer. Details of this region are presented in Figure 2.

Example 2: Primer design

Several primers were designed in the regions identified and fitness for PCR was determined. Regarding the forward primers, no sequence strictly identical across species could be identified, and primers that could accommodate mismatches with target DNA had to be selected. As shown in Table 6 below, several primers were selected, with one to three mismatches depending on the mycoplasma target sequence. A selection filter based on the elimination of primers with unfavorable primer dimer formation and primers with temperature resistant hairpin structures ($T_m > 50^\circ\text{C}$) led to the selection of a limited number of primers for subsequent experiments.

Table 6: Characteristics of forward primers

Type	Primer name	Sequence	SEQ ID NO	Bp	GC%	Dimer	Tm1	Tm2	TmHP
8 CGGT	FwMyco1	CCGCACAAGCGGTGGAGCATGTGG	3	24	67	ok	74,6	72,7	71
	FwMyco2	ACCCGCACAAGCGGTGG	4	17	71	ok	62,7	67,4	71
	FwMyco3	ACCCGCACAAGCGGTG	5	16	69	ok	58,7	65,0	71
	FwMyco4	CCCGCACAAGCGGTG	6	15	73	ok	57,6	62,9	71
	FwMyco5	CCCGCACAAGCGGTGG	7	16	75	ok	61,9	65,5	71
	FwMyco6	CCGCACAAGCGGTGGA	8	16	69	ok	59,9	64,6	71
	FwMyco7	CACAAGCGGTGGAGCATGT	9	19	58	ok	59,3	65,0	43
	FwMyco8	ACAAGCGGTGGAGCATGTG	10	19	58	ok	59,3	65,0	43
	FwMyco9	ACAAGCGGTGGAGCATGTGG	11	20	60	KO	62,9	67,1	43
	FwMyco10	CAAGCGGTGGAGCATGTG	12	18	61	ok	58,3	63,2	41
	FwMyco11	AAGCGGTGGAGCATGTGG	13	18	61	ok	59,2	64,7	41
	FwMyco12	CGGTGGAGCATGTGGTTTAATT	14	22	46	KO	59,7	63,5	13
	FwMyco13	CACAAGTGGTGGAGCATGTG	15	20	55	ok	57,1	63,3	44
	FwMyco14	CACAAGTGGTGGAGCATGTGG	16	21	57	ok	61,0	65,3	41
1 TGGT	FwMyco15	CACAAGTGGTGGAGCATGTGGT	17	22	55	ok	61,8	66,9	41
	FwMyco16	ACAAGTGGTGGAGCATGTG	18	19	53	ok	53,8	62,4	33
	FwMyco17	ACAAGTGGTGGAGCATGTGG	19	20	55	ok	58,0	64,7	33
	FwMyco18	ACAAGTGGTGGAGCATGTGGT	20	21	52	ok	58,9	66,3	33
	FwMyco19	ACAAGTGGTGGAGCATGTGGTT	21	22	50	ok	60,3	66,7	33
	FwMyco20	CAAGTGGTGGAGCATGTGG	22	19	58	ok	57,0	62,9	14
	FwMyco21	CAAGTGGTGGAGCATGTGGT	23	20	55	ok	58,0	64,7	14
	FwMyco22	CAAGTGGTGGAGCATGTGGTT	24	21	52	ok	59,5	65,0	14
	FwMyco23	CAAGTGGTGGAGCATGTGGTTT	25	22	50	ok	60,8	65,4	14
	FwMyco24	GCACAAGCGGTGGATCATG	26	19	58	ok-	59,6	63,4	50
1 CTTT	FwMyco25	GCACAAGCGGTGGATCATGT	27	20	55	ok	60,5	65,2	50

	FwMyco26	CACAAGCGGTGGATCATGT	28	19	53	ok	56,4	62,5	44
	FwMyco27	CACAAGCGGTGGATCATGTT	29	20	50	ok	58,0	63,0	44
	FwMyco28	CACAAGCGGTGGATCATGTTG	30	21	52	ok	60,9	63,7	44
	FwMyco29	ACAAGCGGTGGATCATGTTG	31	20	50	ok	58,0	63,0	33
	FwMyco30	ACAAGCGGTGGATCATGTTGT	32	21	48	ok	58,9	64,7	33
	FwMyco31	ACAAGCGGTGGATCATGTTGTT	33	22	46	ok	60,2	65,1	33
	FwMyco32	CAAGCGGTGGATCATGTTG	34	19	53	ok	57,0	61,1	14
	FwMyco33	CAAGCGGTGGATCATGTTGT	35	20	50	ok	58,0	63,0	14
3 TGTC	FwMyco34	GCACAAGTGGTGGAGCATGT	36	20	55	ok	58,2	65,2	50
	FwMyco35	GCACAAGTGGTGGAGCATGTT	37	21	52	ok	59,6	65,5	50
	FwMyco36	GCACAAGTGGTGGAGCATGTTG	38	22	55	ok	62,4	66,1	50
	FwMyco37	CACAAGTGGTGGAGCATGT	39	19	53	ok	53,8	62,4	44
	FwMyco38	CACAAGTGGTGGAGCATGTT	40	20	50	ok	55,6	62,9	44
	FwMyco39	CACAAGTGGTGGAGCATGTTG	41	21	52	ok	58,6	63,7	44
	FwMyco40	CACAAGTGGTGGAGCATGTTGC	42	22	55	ok	62,4	66,1	43
	FwMyco41	ACAAGTGGTGGAGCATGTT	43	19	47	ok	52,3	62,0	33
	FwMyco42	ACAAGTGGTGGAGCATGTTG	44	20	50	ok	55,6	62,9	33
	FwMyco43	ACAAGTGGTGGAGCATGTTGC	45	21	52	ok	59,6	65,5	37
	FwMyco44	ACAAGTGGTGGAGCATGTTGCT	46	22	50	ok	60,6	66,9	43
	FwMyco45	CAAGTGGTGGAGCATGTTG	47	19	53	ok	54,5	61,1	14
	FwMyco46	CAAGTGGTGGAGCATGTTGC	48	20	55	ok	58,7	63,8	42
	FwMyco47	CAAGTGGTGGAGCATGTTGCT	49	21	52	ok	59,8	65,3	57

Tm1 calculated using PrimerExpress3.0

Tm2, GC% and TmHP calculated with IDT web site program with qPCR parameters selected on batch analysis. TmHP is melting temperature of Hairpin. Tm was calculated assuming no mismatch with targets. Parameters highlighted in grey led to primer counterselection. Primers in bold were selected for further assays.

A similar approach led to the identification of reverse primers with sequences and characteristics presented in Table 7 below. Primer RevMyco1* was chosen for showing the best Tm criteria.

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Table 7: Characteristics of reverse primers

Primer name	Sequence	SEQ ID NO	bp	GC%	Dimer	Tm1	Tm2	TmHP
RevMyco1*	CTGACGACAACCATGCACCA	50	20	55	OK	60,1	64,8	29
RevMyco2*	ACGACAACCATGCACCA	51	17	53	Ok	53,3	61,6	29
RevMyco3*	TGACGACAACCATGCACCA	52	19	53	Ok	59,1	64,3	29

Tm1 from PrimerExpress3.0

Tm2, GC%, TmHP from IDT web site with qPCR parameters selected on batch analysis.

Parameters highlighted in gray led to primer counterselection.

Primer in bold was selected for further assays.

The forward and reverse primers identified to fulfil all criteria are presented in Table 8 below. For clarity, Tm was calculated assuming no mismatch with targets.

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Table 8: Forward and reverse primers used in the experimental assessment.

Primer name	Sequence	SEQ ID NO	bp	GC%	Dimer	Tm1	Tm2	TmHP
FwMyco7	CACAAGCGGTGGAGCATGT	9	19	58	ok	59,3	65,0	43
FwMyco11	AAGCGGTGGAGCATGTGG	13	18	61	ok	59,2	64,7	41
FwMyco14	CACAAGTGGTGGAGCATGTGG	16	21	57	ok	61,0	65,3	41
FwMyco17	ACAAGTGGTGGAGCATGTGG	19	20	55	ok	58,0	64,7	33
FwMyco27	CACAAGCGGTGGATCATGTT	29	20	50	ok	58,0	63,0	44
FwMyco30	ACAAGCGGTGGATCATGTTGT	32	21	48	ok	58,9	64,7	33
FwMyco39	CACAAGTGGTGGAGCATGTTG	41	21	52	ok	58,6	63,7	44
FwMyco43	ACAAGTGGTGGAGCATGTTGC	45	21	52	ok	59,6	65,5	37
FwMyco46	CAAGTGGTGGAGCATGTTGC	48	20	55	ok	58,7	63,8	42
RevMyco1*	CTGACGACAACCATGCACCA	50	20	55	ok	60,1	64,8	29

Tm1 from PrimerExpress3.0.

Tm2, GC% and TmHP from IDT web site with qPCR parameters selected on batch analysis.

Example 3: Primer assessment by dye incorporation-based PCR on various organisms

Primers were first tested in SYBR green-based PCR in order to select the best sets, prior to designing, selecting and optimizing probes to be used in more specific TaqMan PCR.

- 5 DNA samples from different organisms were acquired as described in Table 9 below. For some DNA samples, lyophilized genomic DNA was acquired at the concentration indicated by the manufacturer (10 ng per tube). Alternatively, lyophilized qPCR standards provided as 108 copies/tube were used. For *E coli*, *Homo sapiens* and CHO samples, lyophilized genomic DNA was purchased and resuspend at 7×10^{10} , 7×10^9 and 1.6×10^6 genome copies/ μ L. In all cases, DNA solutions were diluted to reach a concentration of 3×10^3 gene copies/ μ L and used for primer characterization.
- 10

Table 9: Organisms and DNA sources used for primer assessment

Organism	Strain	ATCC	NCTC	Cat. No.	Supplier
<i>Acholeplasma laidlawii</i> *	PG-8A	ATCC 23206	NCTC10116	52-0116	Minerva Biolabs
<i>Mycoplasma arginini</i> *	G230	ATCC 23838	NCTC10129	52-0129	Minerva Biolabs
<i>Mycoplasma fermentans</i> *	PG18	ATCC 19989	NCTC10117	52-0117	Minerva Biolabs
<i>Mycoplasma gallisepticum</i> *	PG31	ATCC 19610	NCTC10115	52-0115	Minerva Biolabs
<i>Mycoplasma hominis</i>	PG21	ATCC 23114	NCTC10111	51-0111	Minerva Biolabs
<i>Mycoplasma hyorhinis</i> *	DBS1050	ATCC 29052	NCTC10130	52-0130	Minerva Biolabs
<i>Mycoplasma orale</i> *	CH19299	ATCC 23714	NCTC10112	52-0112	Minerva Biolabs
<i>Mycoplasma pneumoniae</i> *	FH	ATCC 15531	NCTC10119	52-0119	Minerva Biolabs
<i>Mycoplasma salivarium</i>	PG20	ATCC 23064	NCTC10113	51-0113	Minerva Biolabs
<i>Mycoplasma synoviae</i> *	WVU1835	ATCC 25204	NCTC10124	51-0124	Minerva Biolabs
<i>Spiroplasma citri</i> *	R8-A2	ATCC 27556	NCTC10164	52-0164	Minerva Biolabs
<i>Bacillus cereus</i>		ATCC 14579		51-0031	Minerva Biolabs
<i>Bacillus subtilis</i>		ATCC 6051		51-0010	Minerva Biolabs
<i>Clostridium* perfringens</i>		ATCC 13124		2108-004756	Minerva Biolabs
<i>Escherichia coli</i>		ATCC 11303	NCTC14380	J14380	Thermo Fisher
<i>Lactobacillus* acidophilus</i>		ATCC 4796		51-1723	Minerva Biolabs
<i>Micrococcus luteus</i>		ATCC 4698		51-0030	Minerva Biolabs
<i>Staphylococcus aureus</i>		ATCC 12600		51-0231	Minerva Biolabs
<i>Staphylococcus saprophyticus</i>		ATCC 15305		2120-20229	Minerva Biolabs
<i>Streptococcus* bovis</i>		ATCC 33317		2122-20480	Minerva Biolabs
<i>Streptococcus* pneumoniae</i>		ATCC 33400		51-0566	Minerva Biolabs
<i>Homo sapiens</i>				11691112001	Roche
<i>Cricetulus griseus</i>	9A9wt	NA	NA	NA	Sanofi

* Required by PhEu 2.6.7

All forward primers were used in PCR reactions with the unique reverse primer RevMyco1* as shown in Table 8. For each reaction, 20,000 copies of genomic DNA were used. The PCR C_q values obtained are presented in Table 10 below.

5 Table 10: C_q values of primer combinations used in qPCR experiments on the indicated organisms' reference DNA samples.

Couple Name	hyorhinis	arginini	fermentans	orale	pneumoniae	salivarium	gallisepticum	synoviae	hominis	laidlawii	citri
Rev1*/Fwd7	17,44	17,66	16,56	17,13	14,86	16,08	17,24	19,33	25,09	17,52	16,75
Rev1*/Fwd11	17,41	17,69	16,52	17,21	26,08	15,93	27,39	19,31	24,61	27,66	16,65
Rev1*/Fwd14	17,45	17,75	16,62	17,22	21,94	16,17	24,49	19,41	25,03	27,53	16,85
Rev1*/Fwd17	17,34	17,57	16,42	17,18	23,97	15,92	26,16	19,32	24,73	28,65	16,64
Rev1*/Fwd27	34,16	33,40	31,62	32,68	23,25	31,30	25,70	33,02	N.D.	17,44	33,21
Rev1*/Fwd30	30,19	34,06	31,26	32,31	32,82	29,39	34,99	35,22	35,89	19,75	33,06
Rev1*/Fwd39	24,19	25,40	24,01	24,67	16,63	23,43	19,43	26,41	31,37	20,29	24,56
Rev1*/Fwd46	31,43	31,73	31,37	31,88	17,28	31,43	20,21	32,41	32,32	32,23	33,55

DNA was loaded at approximately 20,000 genomes/well.

C_q>20 are highlighted in gray and led to primer set counterselection.

N.D.: Not detected.

Forward primers 27, 30, 39 and 46 produced the highest C_q values across all the species investigated. The best set was therefore chosen among forward primers 7, 11, 14 and 17. qPCR performed with Forward Primer 7 (Fwd7) displayed C_q values significantly below 20 for all organisms except for *Mycoplasma hominis*. The analysis of the PCR reactions by PAGE together with the melting curves further confirms the presence of a single amplicon per reaction of the expected size and T_m (Fig. 3).

15 Example 4: Hydrolysis probe design

The target sequence for the probes was chosen in the region shown in Figure 2. The task was to encompass a homologous sequence among mycoplasma species with significant mismatch to other organisms. Given the nature of the DNA sequences, it was not possible to design a 23-nucleotide probe with a T_m of about 70°C. It was therefore attempted to design longer probes of up to 40 nucleotides but such probes invariably encompassed non-homologous sequences in the 5' region. In order to overcome these limitations, two strategies were followed: 1) generating long probes with internal quenchers (ZEN probes), and 2) using short probes (about 23 nucleotides) and increasing T_m by the use of chemical modifications such as Locked Nucleic Acids (LNA) or minor groove binding (MGB) moieties to tighten the probe/target interaction. Plain long probes, probes with high temperature-resistant hairpin structures (T_m > about 45°C), and unfavourable primer dimer formation were excluded. Table 11 below lists the designed probes.

Table 11: Hydrolysis probes designed for the experimental assessment

Probe name	Sequence	SEQ ID NO	Bp	GC%	Dimer	Tm1	Tm2	TmHP
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SMycol ZEN	GCAAAGCTATAGAGATATAGTGGAGGTT AACA	54	32	38	KO	60,7	65,8	50
SMycol2 ZEN	GCAAAGCTATAGAGATATAGTGGAGGTT AAC	55	31	39	KO	58,9	64,6	50
SMycol3 ZEN	CAAAGCTATAGAGATATAGTGGAGGTTA ACA	56	31	36	KO	58,2	64,1	50
SMycol4 ZEN	CAAAGCTATAGAGATATAGTGGAGGTTA ACAGAATGACAG	57	40	38	ok	66,0	68,3	34
SMycol4bis ZEN	CAAAGCTATAGAGATATAGTGGAGGTTA ACAGAGTGACAG	58	40	40	ok	66,2	68,9	27
SMycol5 LNA	TATAGTGGAGGTTA <u>ACAGAA</u> TG	59	22	36	ok	47,9	68,7	46
SMycol5bis LNA	TATAGTGGAGGTTA <u>ACAGAG</u> TG	60	22	41	ok	47,8	68,6	22
SMycol5 MGB	TATAGTGGAGGTTAACAGAAATG	61	22	36	ok	70,0	57,4	46
SMycol5bis MGB	TATAGTGGAGGTTAACAGAGTG	62	22	41	ok	68,0	58,8	22
SMycol6 MGB	TATAGAGATATAGTGGAGGTTAACAGAG TG	63	30	37	ok	72,0	63,1	41
SMycol7 MGB	TATAGAGATATAGTGGAGGTTAACAGA	64	27	33	ok	70,0	60,6	41
SMycol8 MGB	TATAGAGATATAGTGGAGGTTAACAG	65	26	35	ok	69,0	59,3	41
SAcho1 LNA	TAAGTTCGGAGGCTAACAGATGT	67	23	44	ok	56,0	68,6	39

Tm1 from PrimerExpres3.0, adapted for MGB probe Tm calculation.

Tm2, GC%, TmIP from IDT web site with qPCR parameters selected on batch analysis, adapted for LNA probe Tm calculation.

Underlined bases were LNA modified.

Bold sequences were selected for evaluation.

Example 5: Probe assessment by hydrolysis probe-based PCR on various organisms

- Probes selected from Table 11 were tested with the unique Fwd7/Rev1* primer set (Table 10) in order to select the best primer/probe combination. As shown in Tables 12 and 13 below, besides *Acholeplasma laidlawii*, all other mycoplasma species were detected by LNA and MGB probes, although the Smyco5 MGB probes failed to detect *Mycoplasma gallisepticum* DNA. No or only limited background signal (Cq >37) was generated on DNA samples from other species.

Table 12: Evaluation of LNA and MGB probes on Mycoplasma DNA samples

Probe name	hyorhi nis	argini ni	fermenta ns	orale	pneumon iae	salivariu m	gallisepticu m	synovia e	homini s	laidlaw ii	citri
Smyco5 LNA	24,41	24,17	23,16	23,48	22,17	21,29	23,09	26,76	31,43	N.D.	23,11
Smyco5bis LNA	23,20	23,28	22,43	23,16	20,03	21,08	24,14	25,49	30,54	N.D.	23,42
Smyco5 MGB	22,64	29,89	23,08	29,01	27,25	23,41	N.D.	25,02	30,49	N.D.	25,85
Smyco5bis MGB	23,77	29,37	23,83	24,36	28,39	23,03	30,77	25,81	30,45	N.D.	26,48

Primers Fwd7/Rev1 used in addition with probes listed here.*

N.D.: Not detected.

Cq highlighted in gray are unwanted results.

10

Table 13: Evaluation of LNA and MGB probes on DNA samples of other organisms

Probe name	pneumoni ae	bovi s	subtili s	cereu s	perfringe ns	acidophil us	saprophytic us	aureu s	Luteu s	coli	sapien s	CHO
Smyco5 LNA	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
Smyco5bis LNA	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
Smyco5 MGB	N.D.	N.D.	N.D.	N.D.	40,17	N.D.	N.D.	N.D.	N.D.	37,7	N.D.	N.D.

Smyco5bis	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
MGB												

Primers Fwd7/Rev1* used in addition with probes listed here.

N.D.: Not detected.

Cq highlighted in gray are unwanted results.

The SAcho1LNA probe which is more specific to *Acholeplasma laidlawii* by design was also assessed in a similar assay. As shown in Tables 14 and 15 below, this probe successfully detected the amplification of *Acholeplasm lindlawii* DNA, and did not detect any other DNA sample, with no background detection on DNA from other species. Furthermore, when used in a single mix, the Smyco5bis LNA and SAcho1LNA probes detected the DNA of all mycoplasma species with no background detection on DNA from other species.

Table 14: Evaluation of LNA probes on Mycoplasma DNA samples

Probe name	hyorhi nis	argini ni	fermenta ns	orale	pneumon iae	salivariu m	Gallisepticu m	synovia e	homini s	laidlaw ii	citri
Smyco5bis LNA	24,88	24,78	24,09	24,66	21,46	22,54	26,11	27,39	32,03	N.D.	25,06
SAcho1 LNA	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	23,69	N.D.
Smyco5bis LNA + SAcho1LNA	24,61	24,38	24,12	24,57	21,73	22,44	26,46	27,26	32,11	24,04	25,31

Primers Fwd7/Rev1* used in addition with probes listed here.

N.D.: Not detected.

Cq highlighted in gray are unwanted values.

Table 15: Evaluation of LNA probes on DNA samples of other organisms

Probe name	pneumoni ae	bovi s	subtili s	cereu s	perfringe ns	acidophil us	saprophytic us	aureu s	Luteu s	coli	sapien s	CHO
Smyco5bis LNA	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
SAcho1 LNA	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
Smyco5bis LNA + SAcho1LNA	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.

Primers Fwd7/Rev1* used in addition with probes listed here.

N.D.: Not detected.

Cq highlighted in gray are unwanted values.

A particularly preferred combination of primers and probes for mycoplasma detection is shown in Table 16 below.

Table 16: Preferred primer/probe combination

Name	Sequence	SEQ ID NO	Bp	GC%	Dimer	Tm1	Tm2	TmHP
FwMyc7	CACAAGCGGTGGAGCATGT	9	19	58	Ok	59,3	65,0	43
RevMyc1*	CTGACGACAACCATGCACCA	50	20	55	Ok	60,1	64,8	46
SMyc5bis LNA	TATAGTGGAGGTTAACAGAGTG	60	22	41	Ok	47,8	68,6	22

SAcho1 LNA	TAAGTTCGGAGGCTAACAGATGT	67	23	44	Ok	56,0	68,6	39
Tm1 from PrimerExpres3.0, adapted for MGB probe Tm calculation.								
Tm2, GC%, TmHP from IDT web site with qPCR parameters selected on batch analysis, adapted for LNA probe Tm calculation.								
Underlined bases were LNA modified.								

Example 6: Control probes

Two gBlocks (double stranded DNA fragments) were synthesized with structures described in Figure 4 and Table 17. The IPC (internal positive control) is added to all samples and contains the sequences that allow the annealing of the Mycoplasma PCR primers, and the annealing of a unique probe (TraA). This construct generates positive signals in the StraA channel in all samples and serves as positive PCR control.

The DPC (discriminatory positive control) construct is used instead of sample DNA in a separate reaction and contains the sequences that allows the annealing of the Mycoplasma PCR primers, the annealing of the Mycoplasma specific probes and the annealing of another unique probe (AtPSY). This construct generates positive signal in the Mycoplasma probes channel and confirms the presence of mycoplasma specific probes in the PCR mix. In addition, fluorescence in the AtPSY probe channel should be detected in this sample only. Samples positive in the AtPSY probe channel demonstrate sample contamination with this construct.

All control constructs are purchased from IDT as double strand gBlocks.

Table 17: IPC and DPC sequences

Construct name	Sequence	SEQ ID NO	Size (bp)
MycD DPC - gBlock	CGTCATCGGATCTCGAGGACCTGGCTTTAGAGCCTTGGAGCACACC AAATACTCCTGTTGCGGGCACTGCAGAAACCCAGAACACTGGGGA AGCTGGTTCCAAAGCCTGCCAAGATGGTCAACTGAGCCCAACTTGG TTTACAAGCGGTGGAGCATGTGGTTTAATTTGAAGATACGCGTA GAACCTTACCCACTCTTGACATCTTCTGCAAAGCTATAGAGATA TAGTGGAGGTTAAACAGAATGATTTAAGTTCGGAGGCTAACAGA TGTATTCCGACTCGCAGAACCGGAACGATTGATGGTGCATGGTTG TCGTACAGTTTGAACCTTGGACTAAGGTACGATGGCGCCTCCAGCTAA AAGAGCTAAAAGAGGTAAGGGTTTAAGGGATGGTTGGTTGGTGGG GTATTAATGTTTAATTACCTGTTTACAGGCCTGAAATCACTTGTTT TTAGGTTGGGTGCCTCCTGGCTACAAGTACCTGGGACCACGT	70	494
	CGTCATCGGATCTCGAGGACCTGGCTTTAGAGCCTTGGAGCACACC AAATACTCCTGTTGCGGGCACTGCAGAAACCCAGAACACTGGGGA AGCTGGTTCCAAAGCCTGCCAAGATGGTCAACTGAGCCCAACTTGG TTTACAAGCGGTGGAGCATGTGGTTTAATTTGAAGATACGCGTA AGGCGCCCGTCAAAAAGAAGTCCGTCACAAGCAGTGATCCCGGCT GCTGCGTCACATCTGATGATGTTCTTCCCGCTAAGACTTCAGCGA GCCGCTTGGTGCATGGTTGTCGTACAGTTTGAACCTTGGACTAAGGTA CGATGGCGCCTCCAGCTAAAAGAGCTAAAAGAGGTAAGGGTTTAA GGGATGGTTGGTTGGTGGGTATTAATGTTTAATTACCTGTTTACA GGCCTGAAATCACTTGTTTAGGTTGGGTGCCTCCTGGCTACAAG TACCTGGGACCACGT		
MycD IPC - gBlock	CGTCATCGGATCTCGAGGACCTGGCTTTAGAGCCTTGGAGCACACC AAATACTCCTGTTGCGGGCACTGCAGAAACCCAGAACACTGGGGA AGCTGGTTCCAAAGCCTGCCAAGATGGTCAACTGAGCCCAACTTGG TTTACAAGCGGTGGAGCATGTGGTTTAATTTGAAGATACGCGTA AGGCGCCCGTCAAAAAGAAGTCCGTCACAAGCAGTGATCCCGGCT GCTGCGTCACATCTGATGATGTTCTTCCCGCTAAGACTTCAGCGA GCCGCTTGGTGCATGGTTGTCGTACAGTTTGAACCTTGGACTAAGGTA CGATGGCGCCTCCAGCTAAAAGAGCTAAAAGAGGTAAGGGTTTAA GGGATGGTTGGTTGGTGGGTATTAATGTTTAATTACCTGTTTACA GGCCTGAAATCACTTGTTTAGGTTGGGTGCCTCCTGGCTACAAG TACCTGGGACCACGT	68	473

Bold sequences correspond to Mycoplasma/Acholeplasma reference sequences.
Underlined and italicized sequences correspond to Mycoplasma/Acholeplasma-specific probes and primers, respectively.
Gray highlighted sequences correspond to sequences of AtPSY (DPC) and TraA (IPC).

Example 7: Probe modifications and PCR reaction parameters

All primers and probes are purchased from IDT. Probes were linked to fluorochromes and quenchers as shown in Table 18.

5

Table 18: Probe sequences and chemical modification

Name	Sequence	SEQ ID NO	Fluorochrome/quencher
FwMyco7	CACAAGCGGTGGAGCATGT	9	N.A.
RevMyco1*	CTGACGACAACCATGCACCA	50	N.A.
SMyco5bis LNA	TATAGTGGAGGTAAACAGAGTG	60	FAM / IABkFQ
SAcho1 LNA	TAAGTTCGGAGGCTAACAGATGT	67	FAM/ IABkFQ
SAtPSY	CCGACTCGCAGAACCGGAACGA	71	HEX/ IABkFQ
StraA	CGCAGTGATCCCGGCTGCTG	69	Cy5/ IABkRQ

Underlined nucleotides are LNA modifications

The PCR premix was prepared and PCR reactions were performed as described in Table 19.
5µL of unknown DNA sample or DPC were added. Code UNGase is purchased from ArcticZymes.

Table 19: PCR reaction mixture

Premix	[C] _{initial}	[C] _{final}	Amount (µL) for 1 reaction
Luna Universal Probe qPCR Master Mix (2x)	2x	1x	10.0
Code UNGase	1 u/µL	1 u/100µL	0.2
Water	/	Qsp 15µL	3.48
Fw Myco7	100 µM	400 nM	0.08
Rev Myco1*	100 µM	400 nM	0.08
S Myco5 bis	100 µM	200 nM	0.04
S Acho1	100 µM	200 nM	0.04
SAtPSY	100 µM	200 nM	0.04
StraA	100 µM	200 nM	0.04
<i>gBlock IPC</i>	<i>200 cp/µL</i>	<i>200 cp</i>	<i>1</i>
Final volume			15.0

The cycling conditions used are described in Table 20 below.

15

Table 20: Cycling conditions for PCR

Step	Temperature	Time	Cycle
UNG	40°C	120s	1
Activation	90°C	60s	1
Denaturation	90°C	15s	45
Amplification	60°C	30s	

Example 8: Determining detection and quantification limits

As described above, probes and primers were developed with purified genomic DNA used at known concentrations in PCR reactions (15,000 to 20,000 genome copies per reaction). However, guidelines ask for assays capable of detecting a defined number of colony forming unit (CFU)/mL. Lyophilized mycoplasma samples of known CFU contents were therefore purchased from vendors as shown in Table 21 below, and tested together with various amounts of reference DNA (see Table 6) in order to link CFU to genome copies and to determine the limit of quantification (LOQ) and the limit of detection (LOD) of the method of the present invention, expressed in CFU/mL.

Table 21: Mycoplasma colony reference standards for confirming LOD

Organism	Strain	ATCC	NCTC	Cat. No.	Supplier
<i>Acholeplasma laidlawii</i> *	PG-8A	ATCC 23206	NCTC10116	102-8003	Minerva Biolabs
<i>Mycoplasma arginini</i> *	G230	ATCC 23838	NCTC10129	102-1003	Minerva Biolabs
<i>Mycoplasma fermentans</i> *	PG18	ATCC 19989	NCTC10117	102-6003	Minerva Biolabs
<i>Mycoplasma gallisepticum</i> *	PG31	ATCC 19610	NCTC10115	102-3003	Minerva Biolabs
<i>Mycoplasma hyorhinis</i> *	DBS1050	ATCC 29052	NCTC10130	102-7003	Minerva Biolabs
<i>Mycoplasma orale</i> *	CH19299	ATCC 23714	NCTC10112	102-2003	Minerva Biolabs
<i>Mycoplasma pneumoniae</i> *	FH	ATCC 15531	NCTC10119	102-4003	Minerva Biolabs
<i>Mycoplasma salivarium</i>	PG20	ATCC 23064	NCTC10113	102-1103	Minerva Biolabs
<i>Mycoplasma synoviae</i> *	WVU1835	ATCC 25204	NCTC10124	102-5003	Minerva Biolabs
<i>Spiroplasma citri</i> *	R8-A2	ATCC 27556	NCTC10164	102-9003	Minerva Biolabs

* Required by PhEu 2.6.7

10

In a first step, the LOQ of the method was determined on each reference DNA samples listed in Table 9. Briefly, reference DNA of known concentrations were diluted and PCR was performed as described herein to correlate Cq to DNA copies. The results obtained for each mycoplasma DNA sample are shown in Table 22 below. All samples were positive in the IPC channel at the expected Cq value (data not shown). The LOQ for all mycoplasma was set to the second to last reference DNA dilution for which the qPCR software was able to calculate a Cq. All LOQs were therefore set to 100 mycoplasma genome copies per reaction, except for *M. synoviae* for which the LOQ was set to 1,000 mycoplasma genome copies per reaction.

20

Table 22: Calibration curves performed on mycoplasma reference DNA

Genome Copy	<i>M. hyorhinis</i>	<i>A. laidlawii</i>	<i>S. citri</i>	<i>M. synoviae</i>	<i>M. pneumoniae</i>	<i>M. arginini</i>	<i>M. gallisepticum</i>	<i>M. orale</i>	<i>M. fermentans</i>
10000	27,42	26,43	26,77	29,46	24,52	27,39	27,78	26,61	26,31
10000	27,44	26,30	26,67	28,85	24,26	27,16	27,71	26,52	26,18
10000	27,67	26,32	26,69	29,96	24,63	27,03	27,80	26,33	25,78
1000	31,49	29,30	30,18	33,60	27,90	30,42	31,33	29,88	29,39
1000	31,49	29,31	30,14	33,02	27,63	30,61	31,10	29,61	29,28

1000	31,24	29,28	30,15	32,73	27,65	30,56	31,14	29,95	29,60
100	35,34	32,58	33,50	38,44	31,11	34,09	34,81	33,65	33,19
100	34,96	32,67	33,13	36,81	31,06	33,21	34,74	32,76	32,77
100	34,63	32,32	33,43	40,25	30,87	33,72	34,99	33,00	33,07
10	40,98	37,52	36,74	N.D.	34,64	37,19	N/A	N/A	40,45
10	N/A	34,61	38,33	N.D.	34,24	N/A	N/A	39,17	37,07
10	N/A	37,52	36,86	N.D.	34,11	39,79	N/A	37,90	37,33
1	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
1	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
1	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
R ²	0,995	0,998	0,998	0,936	0,998	0,992	0,999	0,992	0,995
Slope	-3,735	-3,086	-3,324	-4,538	-3,272	-3,239	-3,541	-3,323	-3,460
Intercept	42,502	38,648	40,045	47,293	37,552	40,183	41,890	39,781	39,888
E (%)	85%	111%	100%	66%	102%	104%	92%	100%	95%

Values highlighted in gray are those of the dilution that should be used to determine the LOQ for each calibration curve.

N.A.: Not applicable, an amplification is visible, but software did not provide Cq value.

N.D.: Not detectable, no amplification visible.

In a second step, the method was applied on the colony reference standards listed in Table 21 and all Cq values were determined. The equations calculated from the calibration curves (Table 22) were then used to determine the equivalence between CFU and genome copies. The number of CFU corresponding to the LOQ of the method determined on reference DNA standards was then calculated as the LOQ expressed in CFU/mL, taking into account a maximum sample volume 5µL. As is shown in Table 23 below, LOQ and LOD were <100 and <10 CFU/mL, respectively. The LOD < 10 CFU/mL matches the requirements set forth in Chapter 2.6.7 of the European Pharmacopoeia.

Table 23: Method LOQ and LOD determined on colony reference standards

Organisms	M.hyorhinis	A.laidlawii	S.citri	M.synoviae	M.pneumoniae	M.arginini	M.gallicepticum	M.orale	M.fermentans
Samples mean Cq	30.8	27.5	24.4	32.0	29.9	30.9	31.7	30.2	31.7
Corresponding genome copies	1328	3968	51306	2363	218	721	757	782	234
Method LOQ (CFU/mL)	7.5	2.5	0.19	42.3	45.9	13.9	13.2	12.8	42.7
LOD (CFU/mL)	0.8	0.3	0.02	4.2	4.6	1.4	1.3	1.3	4.3

The DNA samples used for the PCR contained 0.5 CFU.

The LOQ was calculated based on a maximum sample volume of 5µL per reaction

Example 9: Identification of genome regions to be targeted by PCR

Organisms investigated are listed in Table 24 below. These include the 4 MMV serotypes (p, i, m & c) and parvoviruses from close species which presence should be investigated in various cell substrates taken from the production workflow: Upstream & Down Stream Processing (for viral clearance), Master Cell Bank, Working Cell Bank, Unprocessed Bulk, End Of Production Cells for viral safety, as indicated in various guidelines.

Table 24 - Sequences of organisms addressed in this study.

Organism	Strain	RefSeq	Genome	Genbank Accession No.	Total length (base pairs)
<i>Mouse Minute Virus p</i>	<i>Prototype</i>	GCF_000838465.1	Complete	NC_001510.1	5149
<i>Mouse Minute Virus p</i>	<i>Prototype</i>		Complete	J02275.1	5149
<i>Mouse Minute Virus p</i>	<i>Prototype</i>		Partial	JB292193.1	5149
<i>Mouse Minute Virus p</i>	<i>Prototype</i>		Partial	V01115.1	5081
<i>Mouse Minute Virus i</i>	<i>Immunosuppressive</i>		Complete	M12032.1	5085
<i>Mouse Minute Virus i</i>	<i>Immunosuppressive</i>		Partial	X02481.1	5087
<i>Mouse Minute Virus m</i>	<i>M</i>		Complete	DQ196317.1	4760
<i>Mouse Minute Virus c</i>	<i>Cutter</i>		Partial	U34256.1	4761
<i>Hamster Parvovirus</i>	HPV		Complete	NC_001358.1	5176
<i>Hamster Parvovirus</i>	H1		Complete	X01457.1	5176
<i>Hamster orphan Parvovirus</i>	HaPV		Partial	U34255.1	4773
<i>Canine Parvovirus</i>	CPV-N		Complete	NC_001539.1	5323
<i>Porcine Parvovirus</i>	NADL-2		Complete	NC_001718.1	5075
<i>Human parvovirus</i>	B19 (or HuPV)		Complete	KM393169	5596
<i>Bovine Parvovirus</i>	BPV		Complete	NC_001540.1	5517
<i>Homo sapiens</i> *	-	GCF_000001405.40	Complete	GRCh38.p14	3,1Gb
<i>Cricetulus griseus</i> *	17A/GY	GCF_003668045.3	Complete	CriGri-PICRH-1.0	2,4Gb
<i>Escherichia coli</i> *	K-12	GCF_000005845.2	Comple	ASM584v2	4,6Mb

* Not parvovirus sequence, used for primer off-target analysis.

In order to identify the best genomic region to be targeted by PCR and ensure specific MMV detection, full genome of parvovirus listed in Table 24 were retrieved from NCBI website (www.ncbi.nlm.nih.gov) and compared.

5 A first pass analysis focused on MMV genomes aimed at identifying 300 bp stretches with 80% homology across genomes where primers and hydrolysis probes of 20 and 23 nucleotides, respectively, could be designed. As shown in Figure 5, only one stretch was identified that met the criteria mentioned above. A highly conserved region of around 315 bp identified at position 2002-2316 (NC_001510.1). Coding sequences for NS1, NS2, VP1, VP2, VP3 and VP4 proteins are present
10 in this strategic position and it is just before a “VP intron” where various primers for a putative future RT-qPCR could be designed.

In a second filtering approach, sequences were analyzed within this 315 bp domains where primers and probes could be designed for all serotypes, with T_m of 60°C and 70°C, respectively. Sequences that would generate primer dimers and hairpin structures >50°C and amplicon >100 bp
15 where disregarded. Once selected, zones of interest were again checked against sequences of parvoviruses listed in Table 24. Details of this region are presented in Figure 6.

Example 10: Primer design

Primer sets and probes were designed and tested as listed in Table 25 below.

Table 25: List of primers and probe with their characteristics

Name	Sequence	SEQ ID NO	bp	GC%	Dimer	Tm1	Tm2	TmHP
FwMMV	CAGAGATCGAGGAGGATTTGAGA	104	23	48	Ok	58,5	62,9	25
RevMMV	GCGGCTCGCTGAAGTCT	105	17	65	OK	56,3	64,1	50
ProbeMMV	CGTGCCTCGGTGCGGAACCG	106	20	70	Ok	69,7	70,0	65

Tm1 from PrimerExpres3.0, adapted for MGB probe Tm calculation.
Tm2, GC%, TmHP from IDT web site with qPCR parameters selected on batch analysis, adapted for LNA probe Tm calculation.

Hairpin Tm is higher than in the usual selection (>50°C) for probe design, but it was decided to keep this sequence to be close to the targeted end of *ns1* gene. Table 26 displays the probe sequences and chemical modifications if they were introduced.

Table 26: Probe sequences and chemical modifications

Name	Sequence	SEQ ID NO	Fluorochrome/quencher
FwMMV	CAGAGATCGAGGAGGATTTGAGA	104	N.A.
RevMMV	GCGGCTCGCTGAAGTCT	105	N.A.
ProbeMMV	CGTGCCTCGGTGCGGAACCG	106	FAM / IABkFQ
FtraA12*	GCGCCCGTCAAAAAGAAGT	107	N.A.
RtraA11*	GCGGGAAGAACATCATCAGAA	108	N.A.
FwMyco7	CACAAGCGGTGGAGCATGT	9	N.A.
RevMyco1*	CTGACGACAACCATGCACCA	50	N.A.
SMyco5bis LNA	TATAGTGGAGGTTAAACAGAGTG	60	FAM / IABkFQ
SAcho1 LNA	TAAGTTTCGGAGGCTAACAGATGT	67	FAM/ IABkFQ
SAtPSY	CCGACTCGCAGAACCGGAACGA	71	HEX/ IABkFQ
StraA	CGCAGTGATCCCGGCTGCTG	69	Cy5/ IABkRQ

*traA primers are purely optional
Underlined nucleotides are LNA modifications.

Sequences used for both, the MMV assay and for the mycoplasma assay are shown in Table 27 below.

Table 27: List sequences for the MMV assay and common with Mycoplasma Assay

Name	Sequence	bp	Remark
DPC (for MMV)	AAAGGTGCCAACTCCTATAAAATTTACTAGGTTGCGGCACGCTCACCATTCA CGACACCGAAAAGTACGCCTCTCAGCCAGAACTATGCACTAACTCCACTT GCATCGGATCTCGAGGACCTGGCTTTAGAGCCTTGGAGCACACCAAATAC TCCTGTTGCGGGCACTGCAGAAACCCAGAACTGGGGAAGCTGGTTCCA AAGCCTGCCAAGATGGTCAACTGAGCCCACTTGGT CAGAGATCGAGGAG GATTTGAGA CGTGCTTCGGTTCGGGAACCG TTG CGCACTCGCGAACC AACGAGAAAGACTTCAGCGAGCCGC TGAACCTGGACTAAGGTACGATGG CGCCTCCAGCTAAAAGAGCTAAAAGAGGTAAGGGTTAAGGGATGGTTGG TTGGTGGGGTATTAATGTTTAATTACCTGTTTACAGGCCTGAAATCACT TGGTTTATAGTTGGGTGCCTCCTGGCTACAAGTACCTGGGACCAGGG	497	SEQ ID NO: 114. Used as a positive control and to discriminate false positive signals. Contains MMV primers and probe, plus a discriminatory SAtPSY probe sequence
DPC (for MMV)	CAGAGATCGAGGAGGATTTGAGAGCGTGCTTCGGTTCGGGAACCGTTGCCG ACTCGCAGAACCGGAACGAAGAAAGACTTCAGCGAGCCGC	90	SEQ ID NO: 113. Sequences containing primers and probes sequences of interest for MMV detection.
IPCv2 (for MMV)	AAAGGTGCCAACTCCTATAAAATTTACTAGGTTGCGGCACGCTCACCATTCA CGACACCGAAAAGTACGCCTCTCAGCCAGAACTATGCACTAACTCCACTT GCATCGGATCTCGAGGACCTGGCTTTAGAGCCTTGGAGCACACCAAATAC TCCTGTTGCGGGCACTGCAGAAACCCAGAACTGGGGAAGCTGGTTCCA AAGCCTGCCAAGATGGTCAACTGAGCCCACTTGGT CAGAGATCGAGGAG GATTTGAGA GCGCCCGTCAAAAAGAAGT CGTTTTTTTCCAAATTCACCTC GTCTGAATATGCTTCGCCTGGCTCG CGCAGTGATCCCGGCTGGCTGTTCTG ATGATGTTCTTCCCGCT TGAAGAA AGACTTCAGCGAGCCGC TGAACTTGG ACTAAGGTACGATGGCGCCTCCAGCTAAAAGAGCTAAAAGAGGTAAGGGT TTAAGGGATGGTTGGTTGGTGGGGTATTAATGTTTAATTACCTGTTTTT	498	SEQ ID NO: 115. Used as an internal control to discriminate false negative signals. Contains MMV primers and Episome F' TraA primers and probe (StraA) sequences.
IPCv2 (for MMV)	CAGAGATCGAGGAGGATTTGAGA GCGCCCGTCAAAAAGAAGT CGTTTTT TTCCAAATTCACCTCGTCTGAATATGCTTCGCCTGGCTCG CGCAGTGATCC	155	SEQ ID NO: 112. Sequences containing primers and probe

IPCv3 (for Myco & MMV)	<u>CGGCTGCTG</u> <u>TTCTGATGATGTTCTTCCCGCT</u> TTGAAGAAAGACTTCAGCGA GCCGC	sequences of interest for internal control.
	CGTCATCGGATCTCGAGGACCTGGCTTTAGAGCCTTGGAGCACACCAAAT ACTCCTGTTGCGGGCACTGCAGAAACCCAGAACACTGGGGAAGCTGGTTC CAAAGCCTGCCAAGATGGTCAACTGAGCCCACTTGGTTT <u>CACAAGCGGT</u> <u>GGAGCATGT</u> <u>GACAGAGATCGAGGAGGATTGAGA</u> <u>GCGCCGTCAAAAAG</u> <u>AAGTCCGT</u> CACAC <u>CGCAGT</u> GATCCGGCTGCTGCGTCACATTTCTGATGATG TTCTTCCCGCTAAGACTTCAGCGAGCCGCCTGGTGTCATGGTTGTCGTCAG TTTGAACCTGGACTAAGGTACGATGGCGCCTCCAGCTAAAAGAGCTAAAA GAGGTAAGGGTTTAAGGGATGGTTGGTTGGTGGGGTATTAATGTTTAATT ACCTGTTTTACAGGCCTGAAATCACTTGGTTTTAGGTTGGGTGCCTCCTG GCTACAAGTACCTGGGACCACGT	
IPCv3 (for Myco & MMV)	<u>CACAAGCGGTGGAGCATGT</u> <u>GACAGAGATCGAGGAGGATTGAGA</u> <u>GCGCC</u> <u>CGTCAAAAAGAAGT</u> CCGTACAC <u>CGCAGT</u> GATCCGGCTGCTGCGTCACAT <u>TCTGATGATGTTCTTCCCGCTA</u> <u>AGACTTCAGCGAGCCGC</u> <u>TGGTGTCATGG</u> <u>TTGTCGTCAG</u>	473 Contains Mycoplasma and MMV primers, and Episome F' TraA primers and probe (StraA) sequences 160 SEQ ID NO: 116. Sequences containing primers and probe sequences of interest for internal control

Bolt sequences are primers
Highlighted sequences are probes
Underlined sequences are MMV specific
Double underlined sequences are Mycoplasma specific
Italicized sequences are common to MMV and Mycoplasma assay.

Figure 7 shows the different positions of the primers and probes on control constructs.

Example 11: MMV detection method qualification

To demonstrate the specificity of the method, various gDNA extracts were used as matrix for amplification in parallel of MMV vDNA (see Table 28 below). A specific triplex assay was developed to allow for the detection of these gDNA in samples tested for MMV method specificity. MMV and gDNA quantification was performed at the same time and in the same plate using two PCR mixes (Mix 1 targeting MMV and Mix 2 targeting human, E. coli and CHO gDNA concomitantly). To be comparable, 1,500 to 2,000 copies of each target were added to the matrix, taking in account the type of genome of each species (dsDNA ssDNA, haploid or diploid). Amplification results are shown in Figures 8 and 9. Amplification curves in Fig. 8 are as expected: MMV Mix1 amplifies neither human, nor E. coli or CHO gDNA. It amplifies only the desired target at a specific channel. For IPC (Cy5 channel), not all amplifications reach the same plateau. This is due to heterogeneous Probe and Primer consumption attributable to the design of the method. This is unproblematic because all Cq are similar and within SD < 0,5 as expected. Similarly, amplification curves in Figure 9 are as expected: gDNA Mix 2 amplifies neither MMV nor DPC nor IPC. It amplifies only gDNAs and amplification is seen in the specific channel. In conclusion, the method is specific for MMV.

Table 28: Material used as DNA matrix for method evaluation

Organism	Reference	Provider	Batch	Concentration
Mouse Minute Virus	ATCC VR-1346	ATCC	3382456	1.6 x 10 ³ TCID50/μL
Homo sapiens	11691112001	ROCHE	N.A.	200 ng/μL
Escherichia coli	14380	ThermoFisher	4229216	2 μg/μL
Cricetulus griseus	CHO rWCB	Sanofi	N.A.	4.47 x 10 ³ cells/μL

Subsequently, relevant amplification products were analyzed by 15% PAGE to assess the quality of the amplification. Results are shown in Figure 10. Visualization of amplification products shows clear results for both MMV and gDNA mixes. Number of bands and sizes observed comply with the expected results. For PCR Mix 1, the IPC band (about 150 bp) is present in all wells (01 to 07), not in negative control well 08 (MMV mix with no DNA nor IPC). Well 02 contains in addition to IPC band the viral band at about 70 bp, and well 03 contains the discriminatory band at about 90bp. Wells 05, 06 and 07 which are gDNA do not generate other significative bands. The primer dimer band is observed on all wells (01 to 08). For PCR Mix 2, several primer dimer bands are observable as no primer optimization was performed. This assay was designed to check for presence of gDNAs in the assay. There is no significative bands on wells 09 to 12 which are reagents involved in MMV assay. Expected bands at different sizes are observed in well 13 (human) at about 59 bp, in well 14 (E. coli) at about 62 bp, and in well 15 (CHO) at about 81 bp. The 15% PAGE analysis confirms that qPCR amplification products are specific for MMV.

The limit of detection of a qPCR method is by definition one copy per reaction of the targeted DNA. For determining detection limits of the present method, a 10-fold serial dilution of DPC and IPC was performed down to about 0,08 copy/reaction. The last 7 points (called G4 to G10) were analyzed in hexaplicates. For each dilution point, at least 3 replicates must give a signal to be considered. Results are summarized in Tables 29 and 30 below.

Table 29: Mean Cq values for DPC serial dilutions

Name	Copy	FAM			HEX			Rem
		Cq	SD	RSD	Cq	SD	RSD	
NTC	0	NA	NA	NA	NA	NA	NA	
G10	0,08	ND	ND	ND	ND	ND	ND	< LOD
G9*	0,8	38,5	1,0*	2,7%	38,2	1,0*	2,6%	= LOD
G8	8,0	36,5	0,3	0,9%	36,3	0,3	0,9%	= LOQ
G7	79,5	33,0	0,1	0,4%	32,8	0,1	0,4%	
G6	795	29,7	0,1	0,2%	29,5	0,1	0,3%	
G5	7 950	26,2	0,0	0,0%	26,1	0,0	0,1%	
G4	79 500	22,9	0,0	0,1%	22,7	0,0	0,1%	

* 3 of 6 replicates were detected
ND: No detection

Table 30: Mean Cq values for IPC serial dilutions

Name	Copy / reaction	Cy5			Rem
		Cq	SD	RSD	
NTC	0	NA	NA	NA	

Name	Copy / reaction	Cy5			Rem
		Cq	SD	RSD	
G10	0,06	ND	ND	ND	< LOD
G9**	0,6	39,2	1,0**	2,6%	= LOD
G8	5,8	36,8	0,6***	1,7%	= LOQ
G7	57,5	33,6	0,1	0,3%	
G6	575,0	30,1	0,1	0,2%	
G5	5 750,0	26,7	0,0	0,0%	
G4	57 500,0	23,3	0,0	0,1%	

** 4 of 6 replicates were detected, 2 of 6 are out of range
*** Accepted SD for calibration
ND: No detection

In summary, the detection limit for both samples is fixed at one copy per reaction.

For establishing the limit of quantification, data from the sample preparation as described in Example 11 above was used. A calibration curve was made for points with a SD < 0.5 (G4 to G8).

5 Intercepts for all targets are in the same range (mean 39.43) with an SD of 0,08 (<0.5). While keeping the last point G8 for Cy5 target that has a SD >0.5, all the parameters comply with acceptance criteria for all the targets:

Slope -3.32 (±0,3) and r² > 0.98. As shown in Fig. 11, all curves were perfectly superposed with a slight SD increase at lower concentration. This observation is mostly observed for the Cy5 reporter as

10 expected. Table 31 below shows the variability of the lower point at eight copies. Taken together, the limit of quantification was estimated at ten copies per reaction.

Table 31: Variability of lower point: 8 copies

SampleName	FAM	HEX
G8_1	36,00	35,86
G8_2	36,55	36,40
G8_3	36,16	36,00
G8_4	36,77	36,60
G8_5	36,74	36,59
G8_6	36,70	36,49
SD	0,33	0,32

15

Robustness of the method was evaluated by performing a 10-fold viral sample dilution. Followed by DNA extraction added with about 4x10⁵ cells or TE (10; 0,1) pH8,0. Extracts were

quantified by droplet digital PCR to link TCID₅₀/mL value to copy/μL unit. Each extracted DNA sample was then cycled in triplicate. To comply, no significative difference should be observable between Cq values of MMV extracted with cells or TE (10;0,1) pH8,0. Plus, ΔCq should be < 1. Results are summarized in Tables 32 to 35 below.

5

Table 32: Extraction serial dilution

Name	TCID ₅₀ /mL	Log (Q)	MMV + Cells		MMV + TE		ΔCq
			Mean Cq	SD	Mean Cq	SD	
F	0,8	0,2	29,16	0,08	28,78	0,11	0,38
E	8	1,2	25,79	0,38	25,80	0,05	0,01
D	80	2,2	22,61	0,06	22,19	0,03	0,42
C	800	3,2	19,29	0,27	18,56	0,06	0,74
B	8000	4,2	15,65	0,07	15,43	0,03	0,23
A	80000	5,2	12,32	0,45	11,94	0,03	0,38

At each dilution point, extraction of MMV added with cells or TE returned similar Cq values. ΔCq is < 0.5 for 5 of 6 dilution points and < 1 for all. This means that there is no significative impact on MMV detection and quantification when the viral genetic material is extracted from the buffer (TE) or from a complex medium (cell suspension).

10

Table 33: Dilution curve parameters

Name	MMV + Cells	MMV + TE
Slope	-3,37	-3,40
Intercept	30,93	30,66
r ²	0,998	0,999
E	98%	97%

Table 33 shows that although it is an extraction, these parameters perfectly meet the specification of a calibration curve as described above. These observations lead to the conclusion that the method is robust.

15

Intercept value will depend of the unit used for the variable x of the curve, in that case the infectivity "TCID₅₀/mL". This unit is not commonly used for qPCR, this is why it is preferable to reformat Tables 32 and 33 with a genome equivalent "copy" unit determined with ddPCR absolute quantification as shown in the following Tables 34 and 35.

20

Table 34: Extraction serial dilution reformatted

Dilution	Copy	Log (cp)	MMV + Cells		MMV + TE		ΔCq
			Mean Cq	SD	Mean Cq	SD	
F	2237	3,3	29,16	0,08	28,78	0,11	0,38
E	22369	4,3	25,79	0,38	25,80	0,05	0,01
D	223688	5,3	22,61	0,06	22,19	0,03	0,42
C	2236875	6,3	19,29	0,27	18,56	0,06	0,74
B	22368750	7,3	15,65	0,07	15,43	0,03	0,23
A	223687500	8,3	12,32	0,45	11,94	0,03	0,38

Table 35: Dilution curve parameters reformatted ("copy" means copy per reaction)

Name	MMV + Cells	MMV + TE
Slope	-3,37	-3,40
Intercept	40,51	40,33
r ²	0,998	0,999
E	98%	97%

In the reformatted tables, no changes in Cq, SD, Slope, r² and E are shown. As expected, only Intercept values were modified in calculation and they are now very close to those shown in Table 33.

5 The calibration curves for two different data units are shown in Fig. 11. After data reformatting, a 10 Cq drift is observed meaning about 3Log10 difference in absolute value.

 Taking MMV CoA and the quantification into account, a relationship between infectivity TCID₅₀/mL and Genome Copy/μL was established, which is specific to the viral sample that was analyzed. MMV virus stock solution was titrated at 1.6x10⁶ TCID₅₀/mL. The same solution was
10 quantified by ddPCR at 2.24x10¹¹ copy/mL. This means that one TCID₅₀/mL equals 139,805 copies/mL (about 140 copies/μL).

Claims

1. A method for detecting microorganisms in a sample, comprising:
 - (i) performing PCR on a sample suspected of comprising microorganism DNA with a PCR reaction mixture comprising
 - 5 (1) a first primer (P1) annealing to the reverse complement of a nucleic acid sequence of SEQ ID NO: 1 and a second primer (P2) annealing to a nucleic acid sequence of SEQ ID NO: 2; and/or
 - 2) a third primer (P3) annealing to the reverse complement of a nucleic acid sequence of SEQ ID NO: 109 and a fourth primer (P4) annealing to a nucleic acid sequence of SEQ
 10 ID NO: 110, and
 - (ii) detecting a PCR product,

wherein the detection of a PCR product indicates that the sample comprises microorganisms, and the absence of a PCR product indicates that the sample does not comprise microorganisms.
- 15 2. A method for detecting mycoplasma in a sample, comprising:
 - (i) performing PCR on a sample suspected of comprising mycoplasma DNA with a PCR reaction mixture comprising a first primer (P1) annealing to the reverse complement of a nucleic acid sequence of SEQ ID NO: 1 and a second primer (P2) annealing to a nucleic acid sequence of SEQ ID NO: 2; and
 - 20 (ii) detecting a PCR product,

wherein the detection of a PCR product indicates that the sample comprises mycoplasma, and the absence of a PCR product indicates that the sample does not comprise mycoplasma.
3. The method according to claim 2, wherein:
 - 25 (i) P1 has a length between 18 and 24 nucleotides, preferably between 19 and 21 nucleotides and most preferably 19 nucleotides, and/or P2 has a length between 17 and 22 nucleotides preferably between 18 and 20 nucleotides, most preferably 20 nucleotides; and/or
 - (ii) P1 has a T_m of between 60°C and 70°C, preferably between 63°C and 69°C, more preferably between 66°C and 68°C, and/or P2 has a T_m of between 60°C and 67°C,
 30 preferably of between 62 to 66°C and most preferably between 64°C and 65°C; and/or
 - (iii) P1 comprises or consists of a nucleic acid sequence of SEQ ID NO: 3 to 49, and/or P2 comprises or consists of a nucleic acid sequence of SEQ ID NO: 50 to 52.

4. The method according to claim 2 or 3, wherein the reaction mixture further comprises at least a first nucleic acid probe (Probe 1) annealing to a nucleic acid sequence of SEQ ID NO: 53 or its reverse complement.
5. The method of claim 4, wherein:
 - (i) the reaction mixture further comprises at least a second nucleic acid probe (Probe 2) annealing to SEQ ID NO: 53; and/or
 - (ii) Probe 1 and/or Probe 2 each has a length between 20 and 42 nucleotides, preferably between 21 and 30 nucleotides and most preferably 22 and 24 nucleotides; and/or
 - 10 (iii) Probe 1 and/or Probe 2 each has a T_m of between 55°C and 70°C, preferably between 58°C and 69°C, more preferably between 59°C and 68°C; and/or
 - (iv) Probe 1 comprises or consists of a nucleic acid sequence of SEQ ID NO: 54 to 65 and/or Probe 2 comprises or consists of a nucleic acid sequence of SEQ ID NO: 66; and/or
 - (v) Probe 1 and/or Probe 2 each comprises one or more locked nucleic acids (LNA) and/or a
15 minor groove binding (MGB) moiety; and/or
 - (vi) Probe 1 and/or Probe 2 each comprises a detectable label, preferably wherein Probe 1 and Probe 2 comprise the same detectable label.
6. The method according to any one of claims 1 to 5, wherein:
 - 20 (i) the PCR is qPCR, dPCR, ddPCR or cdPCR; and/or
 - (ii) the PCR uses an annealing temperature of between 55°C and 65°C, preferably between 58°C and 60°C; and/or
 - (iii) the PCR comprises an activation step and/or a degradation step; and/or
 - (iv) annealing and elongation are performed at the same temperature; and/or
 - 25 (v) the PCR comprises between 40 and 50 cycles of denaturation and annealing/elongation, preferably 45 cycles.
7. The method according to any one of claims 1 to 6, wherein the reaction mixture further comprises an internal positive control nucleic acid sequence comprising at least 18 consecutive
30 nucleic acids of the nucleic acid sequence of SEQ ID NO: 1 and at least 18 consecutive nucleic acids of the nucleic acid sequence of SEQ ID NO: 2 and between these two nucleic acid sequences an intervening nucleic acid sequence of a length of at least 18 nucleotides, wherein the intervening nucleic acid sequence preferably differs from SEQ ID NO: 53 in that neither Probe 1 nor Probe 2 anneals to the intervening nucleic acid sequence, wherein the reaction
35 mixture preferably further comprises at least a third nucleic acid probe (Probe 3) annealing to the intervening nucleic acid sequence or its reverse complement.

8. The method according to claim 7, wherein
- i) Probe 3 has a length between 20 and 42 nucleotides, preferably between 21 and 30 nucleotides and most preferably 22 and 24 nucleotides; and/or
 - (ii) Probe 3 has a T_m of between 55°C and 70°C, preferably between 58°C and 69°C, more preferably between 59°C and 68°C; and/or
 - (iii) Probe 3 comprises or consists of a nucleic acid sequence of SEQ ID NO: 69 or its reverse complement; and/or
 - (iv) Probe 3 comprises one or more locked nucleic acids (LNA) and/or a minor groove binding (MGB) moiety; and/or
 - (v) Probe 3 comprises a detectable label different from the label of Probe 1 and Probe 2.
9. The method according to claim 7 or 8, wherein the internal positive control nucleic acid sequence comprises or consists of a nucleic acid sequence at least 90% identical to the nucleic acid sequence of SEQ ID NO: 68 or its reverse complement.
10. The method according to any one of claims 1 to 9, wherein the reaction mixture further comprises a discriminatory positive control nucleic acid sequence comprising at least 18 consecutive nucleic acids of the nucleic acid sequence of SEQ ID NO: 1 and at least 18 consecutive nucleic acids of the nucleic acid sequence of SEQ ID NO: 2 and between these two nucleic acid sequences an intervening nucleic acid sequence of a length of at least 18 nucleotides, wherein the intervening nucleic acid sequence preferably does not differ from SEQ ID NO: 53 so that Probe 1 and Probe 2 are capable of annealing to the intervening nucleic acid sequence, wherein the reaction mixture preferably further comprises at least a fourth nucleic acid probe (Probe 4) annealing to the intervening nucleic acid sequence or to its reverse complement at a sequence that is different from the sequence to which Probe 1 and/or Probe 2 anneals.
11. The method according to claim 10, wherein
- i) Probe 4 has a length between 20 and 42 nucleotides, preferably between 21 and 30 nucleotides and most preferably between 22 and 24 nucleotides; and/or
 - (ii) Probe 4 has a T_m of between 55°C and 70°C, preferably between 58°C and 69°C, more preferably between 59°C and 68°C; and/or
 - (iii) Probe 4 comprises or consists of a nucleic acid sequence of SEQ ID NO: 71 or its reverse complement; and/or
 - (iv) Probe 4 comprises one or more locked nucleic acids (LNA) and/or a minor groove binding (MGB) moiety; and/or

- (v) Probe 4 comprises a detectable label different from the label of Probe 1, Probe 2, and Probe 3.
12. The method according to claim 10 or 11, wherein the discriminatory positive control nucleic acid sequence comprises or consists of a nucleic acid sequence at least 90% identical to the nucleic acid sequence of SEQ ID NO: 70 or its reverse complement.
13. The method according to any one of claims 1 to 12, wherein the sample is a biological sample preferably selected from the group consisting of a cell bank, a culture medium, a cell culture, a cell culture supernatant, a medicinal product, a vaccine preparation, blood, saliva, and sputum.
14. A pair of primers for detecting mycoplasma in a sample comprising: a primer P1 annealing to the reverse complement of a nucleic acid sequence of SEQ ID NO: 1, and a primer P2 annealing to a nucleic acid sequence of SEQ ID NO: 2.
15. A kit for detecting mycoplasma in a sample, wherein the kit comprises the pair of primers of claim 9 and a first nucleic acid probe (Probe 1) annealing to a nucleic acid sequence of SEQ ID NO: 53 or its reverse complement, and optionally a second nucleic acid probe (Probe 2) annealing to SEQ ID NO: 53 or its reverse complement, preferably wherein the kit further comprises one or more of:
- (i) a PCR reaction mixture;
 - (ii) an internal positive control nucleic acid sequence comprising at least 18 consecutive nucleic acids of the nucleic acid sequence of SEQ ID NO: 1 and at least 18 consecutive nucleic acids of the nucleic acid sequence of SEQ ID NO: 2 and between these two nucleic acid sequences an intervening nucleic acid sequence of a length of at least 18 nucleotides, wherein the intervening nucleic acid sequence preferably differs from SEQ ID NO: 53 in that neither Probe 1 nor Probe 2 anneals to the intervening nucleic acid sequence, wherein the kit preferably further comprises at least a third nucleic acid probe (Probe 3) annealing to the intervening nucleic acid sequence or to its reverse complement;
 - (iii) a discriminatory positive control nucleic acid sequence comprising at least 18 consecutive nucleic acids of the nucleic acid sequence of SEQ ID NO: 1 and at least 18 consecutive nucleic acids of the nucleic acid sequence of SEQ ID NO: 2 and between these two nucleic acid sequences an intervening nucleic acid sequence of a length of at least 60 nucleotides, wherein the intervening nucleic acid sequence preferably does not differ from SEQ ID NO: 53 so that Probe 1 and Probe 2 are capable of annealing to the intervening nucleic acid sequence, wherein the kit preferably further comprises at least a fourth nucleic acid probe

- (Probe 4) annealing to the intervening nucleic acid sequence or to its reverse complement at a location different from the annealing of Probe 1 and/or Probe 2;
- (iv) means for extracting and/or purifying DNA from the sample; and
- (v) instructions for performing PCR with the primers.
- 5
16. The method according to any one of claims 1 to 13, the pair of primers according to claim 14 or the kit according to claim 15, wherein the mycoplasma is one or more *Mycoplasma* species selected from the group consisting of *Mycoplasma arginini*, *Mycoplasma buccale*, *Mycoplasma hominis*, *Mycoplasma orale*, *Mycoplasma salivarium*, *Mycoplasma fermentans*, *Mycoplasma hyorhinis*, *Mycoplasma synoviae*, *Mycoplasma pneumoniae*, *Acholeplasma laidlawii*, *Mycoplasma gallisepticum* and *Spiroplasma citri*.
- 10
17. A method for detecting virus in a sample, comprising:
- (i) performing PCR on a sample suspected of comprising virus DNA with a PCR reaction mixture comprising a third primer (P3) annealing to the reverse complement of a nucleic acid sequence of SEQ ID NO: 109 and a fourth primer (P4) annealing to a nucleic acid sequence of SEQ ID NO: 110; and
- 15
- (ii) detecting a PCR product,
- wherein the detection of a PCR product indicates that the sample comprises virus, and the absence of a PCR product indicates that the sample does not comprise virus.
- 20
18. The method according to claim 17, wherein:
- (i) P3 has a length between 20 and 25 nucleotides, preferably between 21 and 24 nucleotides and most preferably 23 nucleotides, and/or P4 has a length between 15 and 21 nucleotides preferably between 16 and 20 nucleotides, most preferably 23 nucleotides; and/or
- 25
- (ii) P3 has a T_m of between 60°C and 70°C, preferably between 63°C and 69°C, more preferably between 66°C and 68°C, and/or P4 has a T_m of between 60°C and 67°C, preferably of between 62 to 66°C and most preferably between 64°C and 65°C; and/or
- (iii) P3 comprises or consists of a nucleic acid sequence of SEQ ID NO: 104, and/or P4 comprises or consists of a nucleic acid sequence of SEQ ID NO: 105.
- 30
19. The method according to claim 17 or 18, wherein the reaction mixture further comprises at least one nucleic acid probe (Probe 5) annealing to a nucleic acid sequence of SEQ ID NO: 111 or its reverse complement.
- 35
20. The method of claim 19, wherein:

- (i) Probe 5 has a length between 15 and 30 nucleotides, preferably between 18 and 22 nucleotides and most preferably 19 and 21 nucleotides; and/or
 - (ii) Probe 5 has a T_m of between 59°C and 71°C, preferably between 60°C and 70°C, more preferably between 63°C and 67°C; and/or
 - 5 (iii) Probe 5 comprises or consists of a nucleic acid sequence of SEQ ID NO: 106; and/or
 - (iv) Probe 5 comprises one or more locked nucleic acids (LNA) and/or a minor groove binding (MGB) moiety; and/or
 - (v) Probe 5 comprises a detectable label.
- 10 21. The method according to any one of claims 17 to 20, wherein:
- (i) the PCR is qPCR, dPCR, ddPCR or cdPCR; and/or
 - (ii) the PCR uses an annealing temperature of between 55°C and 65°C, preferably between 58°C and 60°C; and/or
 - (iii) the PCR comprises an activation step and/or a degradation step; and/or
 - 15 (iv) annealing and elongation are performed at the same temperature; and/or
 - (v) the PCR comprises between 40 and 50 cycles of denaturation and annealing/elongation, preferably 45 cycles.
- 20 22. The method according to any one of claims 17 to 21, wherein the reaction mixture further comprises an internal positive control nucleic acid sequence comprising at least 18 consecutive nucleic acids of the nucleic acid sequence of SEQ ID NO: 68 or 112 or 115.
- 25 23. The method according to claim 22, wherein the internal positive control nucleic acid sequence comprises or consists of a nucleic acid sequence at least 90% identical to the nucleic acid sequence of SEQ ID NO: 68 or 112 or 115, or its reverse complement.
- 30 24. The method according to any one of claims 17 to 23, wherein the reaction mixture further comprises a discriminatory positive control nucleic acid sequence comprising at least 18 consecutive nucleic acids of the nucleic acid sequence of SEQ ID NO: 109 and at least 18 consecutive nucleic acids of the nucleic acid sequence of SEQ ID NO: 110 and between these two nucleic acid sequences an intervening nucleic acid sequence of a length of at least 18 nucleotides, wherein the reaction mixture preferably further comprises at least a third nucleic acid probe (Probe 3) and/or a fourth nucleic acid probe (Probe 4) annealing to the intervening nucleic acid sequence or to its reverse complement at a sequence that is different from the
- 35 sequence to which Probe 5 anneals.
25. The method according to claim 24, wherein

- a) Probe 3:
- (ai) has a length between 20 and 42 nucleotides, preferably between 21 and 30 nucleotides and most preferably 22 and 24 nucleotides; and/or
 - (aii) has a T_m of between 55°C and 70°C, preferably between 58°C and 69°C, more preferably between 59°C and 68°C; and/or
 - (aiii) comprises or consists of a nucleic acid sequence of SEQ ID NO: 69 or its reverse complement; and/or
 - (aiv) comprises one or more locked nucleic acids (LNA) and/or a minor groove binding (MGB) moiety; and/or
 - (av) comprises a detectable label different from the label of Probe 4 and Probe 5; and/or
- b) Probe 4:
- (bi) has a length between 20 and 42 nucleotides, preferably between 21 and 30 nucleotides and most preferably between 22 and 24 nucleotides; and/or
 - (bii) has a T_m of between 55°C and 70°C, preferably between 58°C and 69°C, more preferably between 59°C and 68°C; and/or
 - (biii) comprises or consists of a nucleic acid sequence of SEQ ID NO: 71 or its reverse complement; and/or
 - (biv) comprises one or more locked nucleic acids (LNA) and/or a minor groove binding (MGB) moiety; and/or
 - (bv) comprises a detectable label different from the label of Probe 3 and Probe 5.
26. The method according to claim 24 or 25, wherein the discriminatory positive control nucleic acid sequence comprises or consists of a nucleic acid sequence at least 90% identical to the nucleic acid sequence of SEQ ID NO: 113 or its reverse complement.
27. The method according to any one of claims 17 to 26, wherein the sample is a biological sample preferably selected from the group consisting of a cell bank, a culture medium, a cell culture, a cell culture supernatant, a medicinal product, a vaccine preparation, blood, saliva, and sputum.
28. A pair of primers for detecting MMV in a sample comprising: a primer P3 annealing to the reverse complement of a nucleic acid sequence of SEQ ID NO: 109, and a primer P4 annealing to a nucleic acid sequence of SEQ ID NO: 110.
29. A kit for detecting MMV in a sample, wherein the kit comprises the pair of primers of claim 28 and a nucleic acid probe (Probe 5) annealing to a nucleic acid sequence of SEQ ID NO: 111 or its reverse complement, preferably wherein the kit further comprises one or more of:

- (i) a PCR reaction mixture;
 - (ii) an internal positive control nucleic acid sequence comprising at least 18 consecutive nucleic acids of the nucleic acid sequence of SEQ ID NO: 109 and at least 18 consecutive nucleic acids of the nucleic acid sequence of SEQ ID NO: 110 and between these two
5 nucleic acid sequences an intervening nucleic acid sequence of a length of at least 18 nucleotides;
 - (iii) a discriminatory positive control nucleic acid sequence comprising at least 18 consecutive nucleic acids of the nucleic acid sequence of SEQ ID NO: 109 and at least 18 consecutive nucleic acids of the nucleic acid sequence of SEQ ID NO: 110 and between these two
10 nucleic acid sequences an intervening nucleic acid sequence of a length of at least 29 nucleotides, wherein the intervening nucleic acid sequence preferably does not differ from SEQ ID NO: 111 so that Probe 5 is capable of annealing to the intervening nucleic acid sequence;
 - (iv) means for extracting and/or purifying DNA from the sample; and
 - 15 (v) instructions for performing PCR with the primers.
30. The method according to any one of claims 17 to 27, the pair of primers according to claim 28 or the kit according to claim 29, wherein the virus selected from the group consisting of Anelloviridae, Inoviridae, Parvoviridae, preferably Parvovirus, Erythrovirus, Dependovirus,
20 Amdovirus and Bocavirus, more preferably Parvovirus, and most preferably Mouse Minute Virus.