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(54) **COMPOSITION AND KIT FOR DETECTING MYCOPLASMA**

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C12Q 1/68 (2018.01)
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CPC ***C12Q 1/689*** (2013.01); *C12Q 2600/156* (2013.01)

(58) **Field of Classification Search**
None
See application file for complete search history.

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(57) **ABSTRACT**

A composition and a kit for detecting *mycoplasma* are provided. The composition for detecting *mycoplasma* is an aqueous solution including a primer M-F, a primer M-R, and a probe M-P. A sequence of the M-F is shown in SEQ ID NO: 1. A sequence of the M-R is shown in SEQ ID NO: 2. A nucleotide sequence of the probe M-P is shown in SEQ ID NO: 3, and includes a fluorophore FAM linked at a 5' terminus and a quencher BHQ1 linked at a 3' terminus. The composition exhibits high sensitivity, strong specificity, and a wide detection range when used in the detection of *mycoplasma*.

10 Claims, 6 Drawing Sheets

Specification includes a Sequence Listing.

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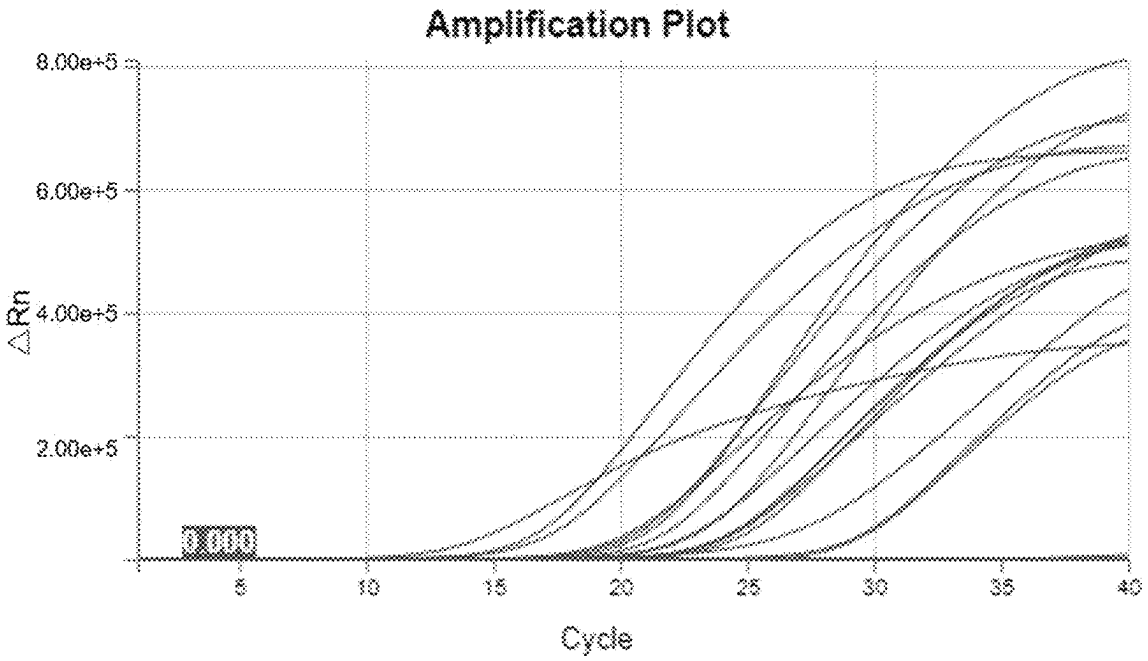
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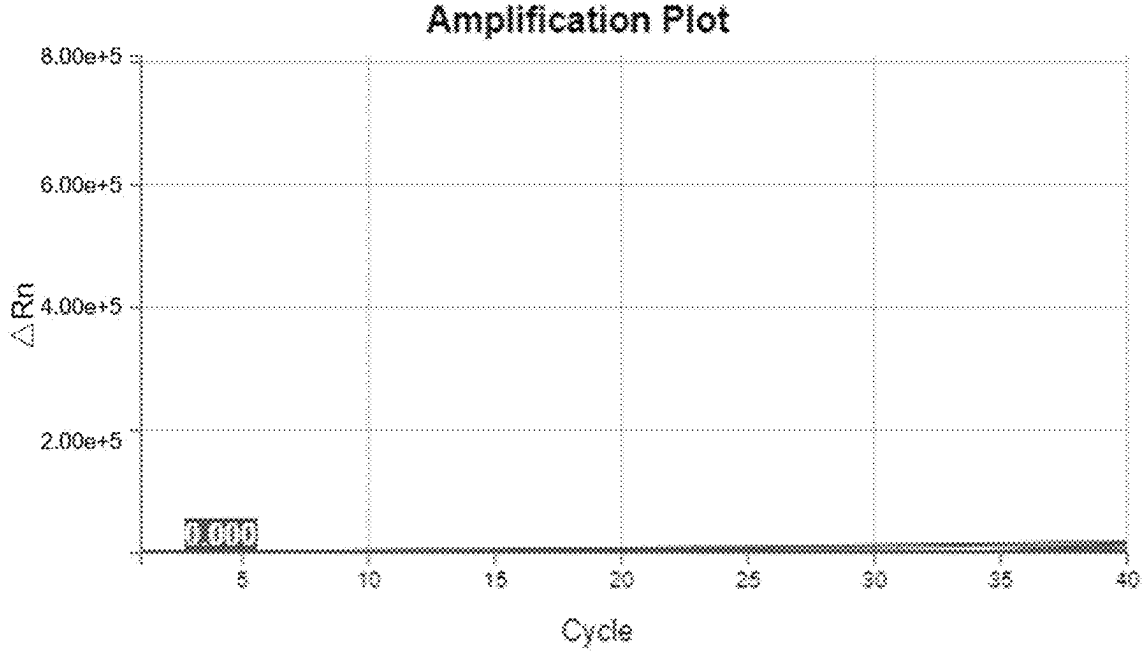
6.00e+5
4.00e+5
2.00e+5
ΔRn
5
10
15
20
25
30
35
40
Cycle

FIG. 1A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8.00e+5
6.00e+5
4.00e+5
2.00e+5
ΔRn
5
10
15
20
25
30
35
40
Cycle

FIG. 1B

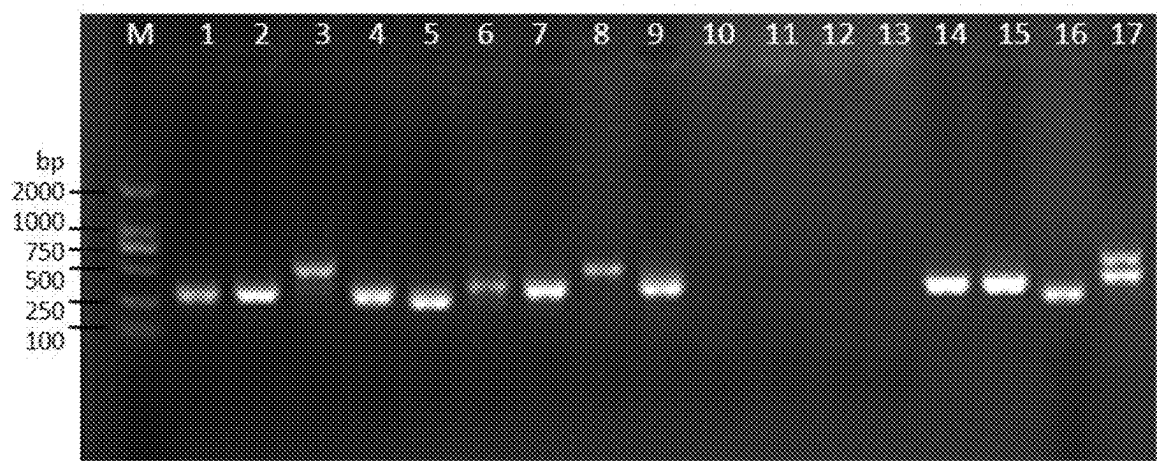
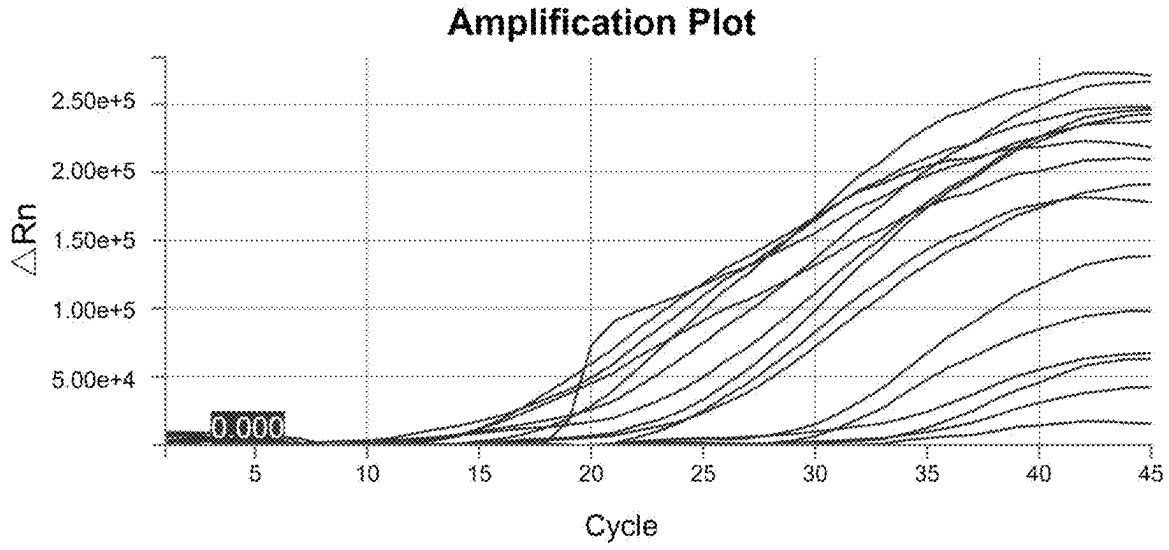

M 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17
bp
2000
1000
750
500
250
100

FIG. 2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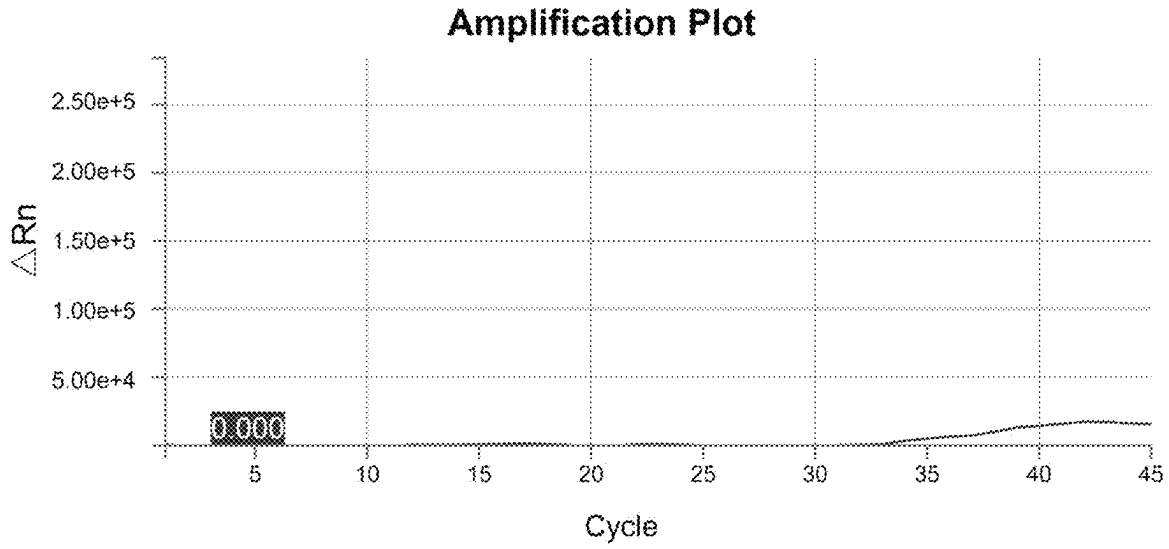
1.50e+5
1.00e+5
5.00e+4
△Rn
0.000
5
10
15
20
25
30
35
40
45
Cycle

FIG. 3A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2.00e+5
1.50e+5
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△Rn
5
10
15
20
25
30
35
40
45
Cycle

FIG. 3B

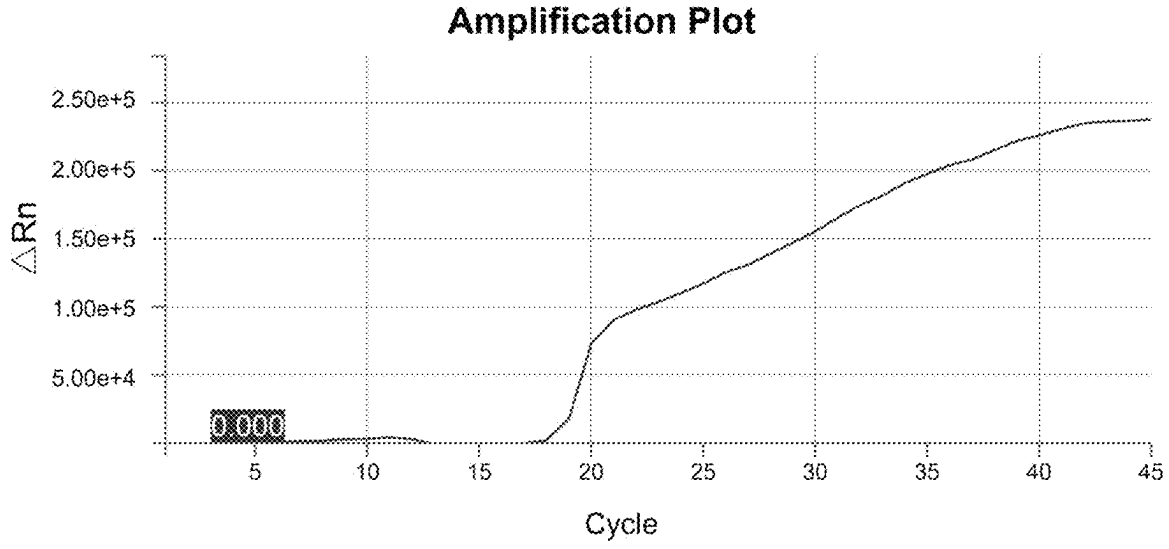
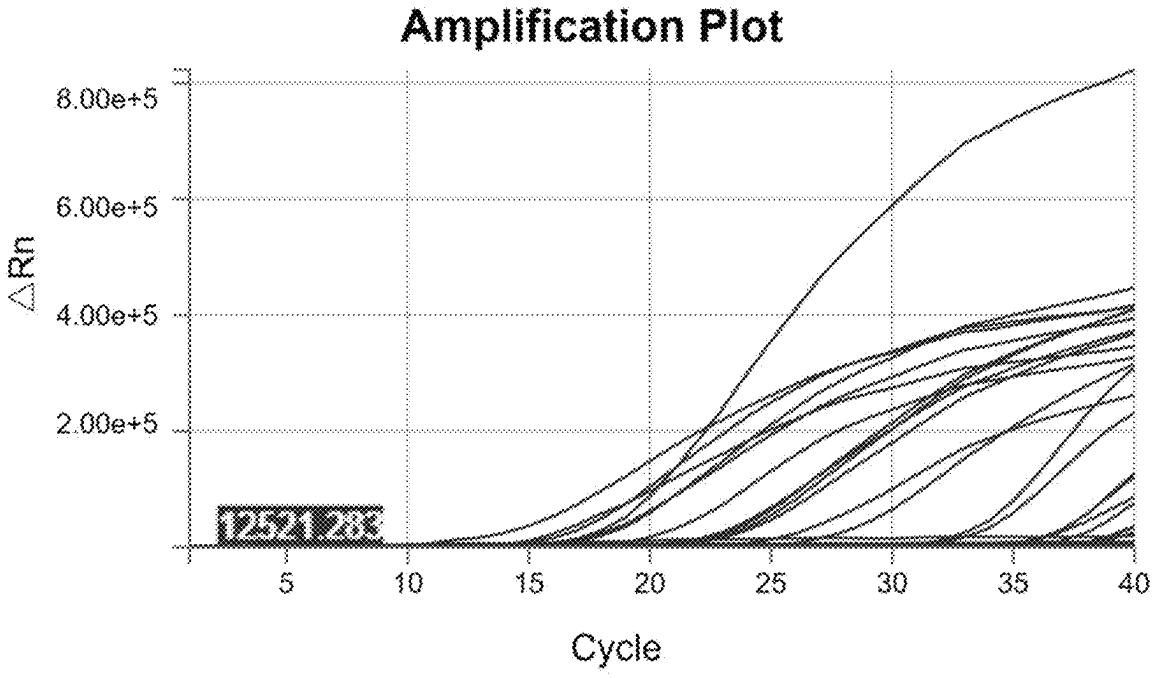

Amplification Plot
2.50e+5
2.00e+5
1.50e+5
1.00e+5
5.00e+4
△Rn
5
10
15
20
25
30
35
40
45
Cycle

FIG. 3C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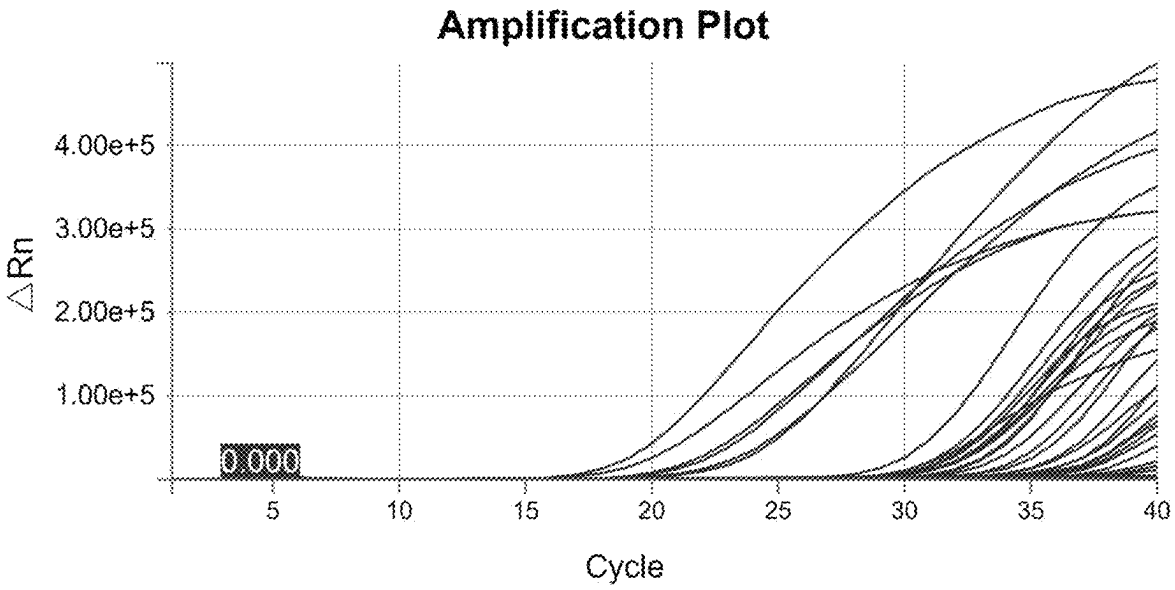
4.00e+5
2.00e+5
△Rn
12521.283
5
10
15
20
25
30
35
40
Cycle

FIG. 4A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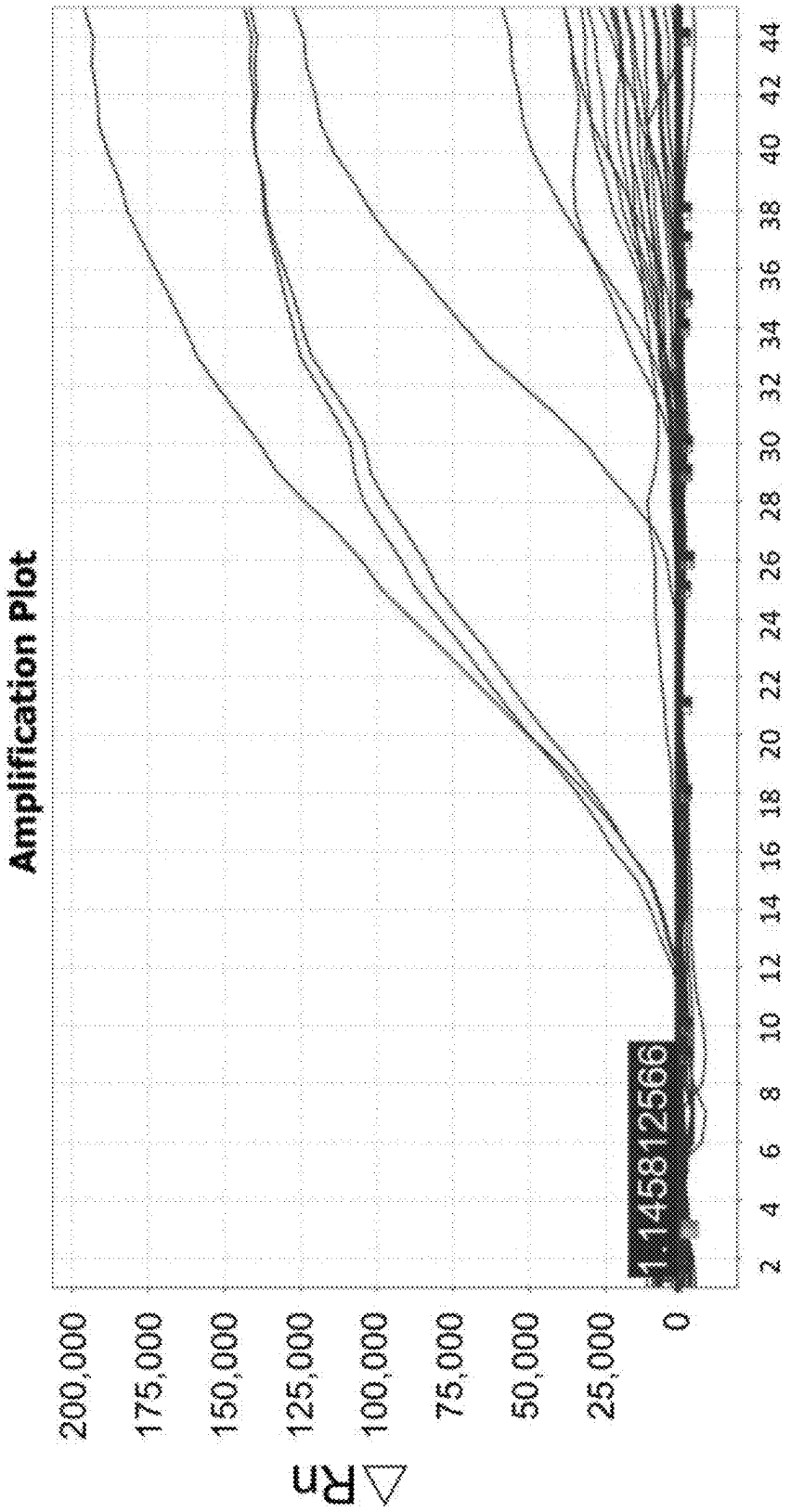
3.00e+5
2.00e+5
1.00e+5
△Rn
0.000
5
10
15
20
25
30
35
40
Cycle

FIG. 4B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ΔRn
200,000
175,000
150,000
125,000
100,000
75,000
50,000
25,000
0
2 4 6 8 10 12 14 16 18 20 22 24 26 28 30 32 34 36 38 40 42 44
Cycle
1.145812566

FIG. 5A

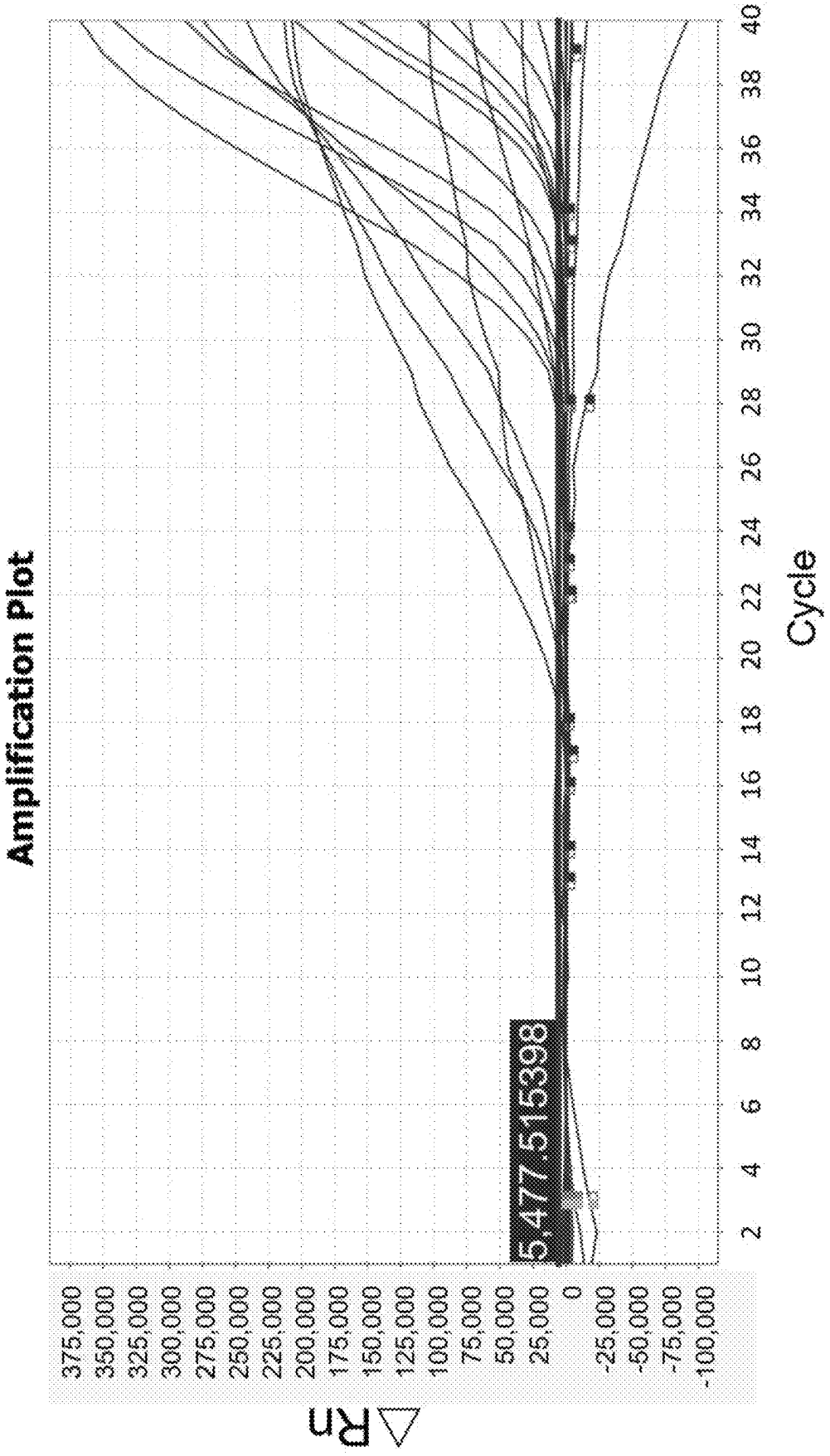
Amplification Plot
ΔRn
Cycle
5,477,515398
375,000
350,000
325,000
300,000
275,000
250,000
225,000
200,000
175,000
150,000
125,000
100,000
75,000
50,000
25,000
0
-25,000
-50,000
-75,000
-100,000
2
4
6
8
10
12
14
16
18
20
22
24
26
28
30
32
34
36
38
40

FIG. 5B

COMPOSITION AND KIT FOR DETECTING MYCOPLASMA

CROSS REFERENCE TO THE RELATED APPLICATIONS

This application is a continuation application of International Application No. PCT/CN2023/109885, filed on Sep. 6, 2023, which is based upon and claims priority to Chinese Patent Application No. 202310719274.5, filed on Jun. 16, 2023, the entire contents of which are incorporated herein by reference.

SEQUENCE LISTING

The instant application contains a Sequence Listing which has been submitted in XML format via EFS-Web and is hereby incorporated by reference in its entirety. Said XML copy is named GBHS014-PKG_Sequence_Listing_20241023.xml, created on Oct. 23, 2024, and is 8,906 bytes in size.

TECHNICAL FIELD

The present disclosure belongs to the field of biotechnologies, and specifically relates to a composition and kit for detecting *mycoplasma*.

BACKGROUND

Mycoplasma contamination is one of the major challenges for cell culture. In 1956, researchers at Johns Hopkins reported the *mycoplasma* contamination of HeLa cells used in the laboratory, and it was the first time *mycoplasma* was detected in a cell culture. *Mycoplasma*-contaminated cells can undergo weakened metabolism and slowed proliferation. However, due to the non-lethality of *mycoplasma* contamination for cells, *mycoplasma* often coexists with cells for a long time and generally does not cause a significant morphological change in cells. At an early stage of *mycoplasma* contamination, the medium does not become turbid, which makes it difficult to determine whether the cell culture undergoes *mycoplasma* contamination with naked eyes. However, *mycoplasma*-contaminated cells may undergo a series of biological changes, such as a change in composition of the cell membrane, chromosomal abnormalities, a change in the enzyme system, and a change in the viral load, which can mislead scientific research tremendously and seriously interfere with experimental results.

The main sources of *mycoplasma* as a contaminant for cell culture are animal serum, trypsin, and aerosols. *Acholeplasma laidlawii* (*A. laidlawii*) (one of the most common contaminants) can also come from soil and other inanimate sources. Since the trypsin commonly on the market is acquired from commercially available porcine pancreases, *Mycoplasma* hyorhinis (*M. hyorhinis*) can also enter the cell culture through this reagent. As early as 1960, Pollock et al. found that 57% of 166 mammalian cell lines and sublines were contaminated with *mycoplasma*. Studies have shown that, in terms of the in vitro growth of mammalian cells, a *mycoplasma*-contaminated cell culture undergoes slowed growth and a shortened logarithmic growth phase.

The "Veterinary Pharmacopoeia of the People's Republic of China" stipulates the following two methods for detecting *mycoplasma*: the cultivation method and the DNA fluorescent staining method. However, when the conventional cultivation method is used to detect *mycoplasma*, there are disadvantages such as a heavy workload and a long cycle time. Some *mycoplasma* individuals with strict nutritional requirements may be missed, and there may be false positives of contamination due to the large time span during cultivation. The DNA fluorescent staining method has high sensitivity, but the result is not easy to determine and is easily affected by the subjective determination of the detector. The DNA fluorescent staining method takes about 1 week, which is slightly shorter than the time required by the cultivation method. There are many other limiting factors for the application of the DNA fluorescent staining method in scientific research. There is a lack of *mycoplasma* detection methods with high sensitivity, strong specificity, and a wide detection range in the art.

SUMMARY

An objective of the present disclosure is to provide a composition for detecting *mycoplasma*, with high sensitivity, strong specificity, and wide detection range.

The objective of the present disclosure is allowed through the following technical solutions:

The present disclosure provides a composition for detecting *mycoplasma*, where the composition is an aqueous solution including a primer M-F, a primer M-R, and a probe M-P; a sequence of the M-F is shown in SEQ ID NO: 1; a sequence of the M-R is shown in SEQ ID NO: 2; and a nucleotide sequence of the probe M-P is shown in SEQ ID NO: 3, and includes a fluorophore carboxyfluorescein (FAM) linked at a 5' terminus and a quencher black hole quencher 1 (BHQ1) linked at a 3' terminus.

In the present disclosure, the primer M-F, the primer M-R, and the probe M-P are in a molar concentration ratio of 1:(0.8-1.2): (0.8-1.2).

The present disclosure also provides a kit for detecting *mycoplasma*, including the composition.

In the present disclosure, the primer M-F, the primer M-R, and the probe M-P in the kit are in a molar concentration ratio of 1:(0.8-1.2): (0.8-1.2).

In the present disclosure, the kit further includes a positive plasmid, and the positive plasmid is obtained by inserting a fragment with a sequence shown in SEQ ID NO: 4 into a pUC57 plasmid vector.

The present disclosure also provides a method for detecting *mycoplasma* using the composition for a non-diagnostic purpose, including the following steps:

(1) extracting DNA from a sample;

(2) with the DNA of the sample as a template, conducting quantitative polymerase chain reaction (qPCR) detection using the primer M-F, the primer M-R, and the probe M-P; and

(3) when a cycle threshold (Ct) value of the qPCR detection for the DNA of the sample is smaller than 38 and there is a typical S-type amplification curve, determining as positive, indicating that there is *mycoplasma* in the sample; and when the Ct value of the qPCR detection for the DNA of the sample is larger than or equal to 38 or there is no Ct value or there is no typical S-type amplification curve, determining as negative, indicating that there is no *mycoplasma* in the sample.

In the present disclosure, a reaction system for the qPCR detection includes: 12.5 μL of a fluorescent polymerase chain reaction (PCR) solution, 1 μL of the DNA of the sample, 3 μL of the composition, and 8.5 μL of double distilled water (ddH_2O).

In the present disclosure, a procedure for the qPCR is as follows: 95° C. for 3 min; 95° C. for 15 sec, and 60° C. for 30 sec, with 40 amplification cycles in total.

The composition of the present disclosure exhibits high sensitivity, strong specificity, and a wide detection range when used in the detection of *mycoplasma*. A total of 106 random cell samples from different laboratories in different regions are collected for testing. Positive samples detected by the composition of the present disclosure have a coincidence rate of 100% with position samples detected by the cultivation method, and a detection time is significantly shortened.

BRIEF DESCRIPTION OF THE DRAWINGS

FIGS. **1**A-**1**B show detection results of the qPCR method in Example 1, where FIG. **1**A shows qPCR detection results of 15 *mycoplasma* species and FIG. **1**B shows qPCR detection results of cells, bacteria, and viruses.

FIG. **2** is an electropherogram illustrating detection results of *mycoplasma* by a commercial nested *mycoplasma* detection PCR kit, where M: DL2000 DNA Marker; 1: *Mycoplasma gallisepticum* (MG); 2: *Mycoplasma hyosynoviae* (Mhs); 3: *Mycoplasma pneumoniae* (Mp); 4: *Mycoplasma orale* (*M. orale*); 5*: M. hyorhinis; 6: A. laidlawii;* 7: *Mycoplasma fermentans* (*M. fermentans*); 8: *Mycoplasma synoviae* (MS); 9*: Spiroplasma citri* (*S. citri*); 10: *Mycoplasma flocculare* (Mf); 11: *Mycoplasma ovipneumoniae* (MO); 12: *Mycoplasma hominis* (Mh); 13: negative control; 14: positive control; 15: *Mycoplasma bovis* (Mb); 16: *Mycoplasma arginini* (*M. arginini*); and 17: *Mycoplasma pirum* (*M. pirum*);

FIGS. **3**A-**3**C show detection results of *mycoplasma* by a commercial qPCR kit, where FIG. **3**A shows amplification curves of 15 *mycoplasma* samples, FIG. **3**B shows an amplification curve of *M. pirum*, and FIG. **3**C shows an amplification curve of *A. laidlawii;*

FIGS. **4**A-**4**B show amplification curves of 106 cell samples detected by the qPCR method in Example 1; and

FIGS. **5**A-**5**B show amplification curves of 106 cell samples detected by a commercial qPCR kit.

DETAILED DESCRIPTION OF THE EMBODIMENTS

Example 1 Composition, Kit, and Method for Detecting *Mycoplasma*

1. Composition for Detecting *Mycoplasma*

In order to find a highly-sensitive and universal qPCR method for detecting *mycoplasma*, the applicants conducted genome-wide alignment analysis for 143 *mycoplasma* sequences published in an NCBI database, and designed dozens of pairs of primers and probes. It was found that only one pair of primers (M-F and M-R) and a probe M-P could detect the tested 15 *mycoplasma* species with high sensitivity.

A sequence (SEQ ID NO: 1) of the M-F was as follows: 5'-ATCCATCCCCACGTTCTCGT-3'. A sequence (SEQ ID NO: 2) of the M-R was as follows: 5'-TGCGGTGAATACGTTCTCGGG-3'. A nucleotide sequence (SEQ ID NO: 3) of the probe M-P was as follows: 5'-ACGGGCGGTGTGTACA-3', with a fluorophore FAM (carboxyfluorescein) linked at a 5' terminus and a quencher BHQ1 (succinimide ester) linked at a 3' terminus.

The composition for detecting *mycoplasma* was an aqueous solution including 10 μM of the M-F, 10 μM of the M-R, and 10 μM of the probe M-P.

2. qPCR Method for Detecting *Mycoplasma*

The qPCR method for detecting *mycoplasma* included the following steps:

(1) DNA was extracted from a sample.

(2) qPCR detection:

With the DNA of the sample as a template, qPCR was conducted. A total reaction system for the qPCR was of 25 μL, including: 12.5 μL of a fluorescent PCR solution (Vazyme, Item No. Q112-AA), 1 μL of the DNA of the sample, 3 μL of the composition for detecting *mycoplasma*, and 8.5 μL of ddH_2O. The reaction system was specifically shown in Table 1. A PCR tube with the total reaction system for qPCR was placed in a detection hole of an ABI fluorescence PCR instrument. An FAM channel was selected for detection (quencher: BHQ-1), a reaction system was set to 25 μL, and cycle parameters were set as follows: 95° C. for 3 min, 95° C. for 15 sec, and 60° C. for 30 sec, with 40 amplification cycles in total. At the end of annealing in each cycle, an FAM fluorescence signal was acquired.

In addition, a negative control and a positive control were set. The negative control and the positive control were the same as the qPCR detection method except that the DNA of the sample was replaced with ddH_2O in the negative control and the DNA of the sample was replaced with a positive plasmid DNA in the positive control. The positive plasmid DNA used in the positive control was a positive plasmid obtained by ligating a gene fragment Spiroplasma (with a sequence shown in SEQ ID NO: 4) from *S. citri* to a pUC57 plasmid vector through two enzyme cleavage sites of BamHI and Xhol. The positive plasmid was chemically transformed into a competent *Escherichia coli* (*E. coli*) strain XL10 for proliferation.

TABLE 1

qPCR system

Component	System (μL)
Fluorescent PCR solution	12.5
Composition for detecting mycoplasma	3
Sterile nuclease-free water (ddH_2O)	8.5
DNA of the sample (10 ng/μL)	1
Total	25

(3) Result Determination

When a Ct value of the qPCR detection for the DNA of the sample was smaller than 38 and there was a typical S-type amplification curve, it was determined as positive, indicating that there was *mycoplasma* in the sample. When the Ct value of the qPCR detection for the DNA of the sample was larger than or equal to 38 or there was no Ct value or there was no typical S-type amplification curve, it was determined as negative, indicating that there was no *mycoplasma* in the sample.

Example 2 Specificity and Sensitivity of qPCR

1. Specificity

(1) 15 *mycoplasma* species, various bacteria, viruses, and different cells each were detected by the qPCR method in Example 1. The 15 *mycoplasma* species were *A. laidlawii, M. fermentans, M. hyorhinis, M. orale, M. arginini*, Mp, MG, MS, *S. citri*, Mhs, Mh, *M. pirum*, Mf, Mb, and MO,

respectively. The various bacteria, viral nucleic acids, and different cells included *Salmonella pullorum* (*S. pullorum*), *E. coli, Staphylococcus aureus* (*S. aureus*), *Pseudomonas fragi* (*P. fragi*), Yeast, porcine circovirus type 2 (PCV-2), pseudorabies virus (PRV), porcine reproductive and respiratory syndrome virus (PRRSV), African green monkey kidney cells (Vero), porcine kidney cells (PK-15), canine kidney cells (MDCK), human laryngeal epidermoid carcinoma cells (Hep-2), mouse mononuclear macrophage leukemia cells (RAW264.7), or the like.

When the qPCR method in Example 1 was used to detect the above-mentioned common cells, viruses, and bacteria, no peak appeared. When the qPCR method in Example 1 was used to detect DNA of the above 15 *mycoplasma* species, a Ct value was smaller than 38 (Table 2) and there was a typical S-type amplification curve (FIGS. **1A-1B**). The above results show that the qPCR method in Example 1 exhibits excellent broad-spectrum activity and specificity when used in the detection of *mycoplasma*.

TABLE 2

CT values of qPCR detection for the 15 mycoplasma species

No.	Sample name	CT
1	Neg	Undet
2	*M. orale*	21.753
3	MS	11.681
4	Mf	27.571
5	Mp	14.406
6	Mb	27.371
7	*M. fermentans*	21.468
8	Mh	19.990
9	*A. laidlawii*	17.394
10	MO	19.585
11	Mhs	14.090
12	*M. arginini*	17.749
13	*S. citri*	19.131
14	MG	22.151
15	*M. pirum*	18.373
16	*M. hyorhinis*	21.559
17	Pos	21.922

Notes: In Table 2, Undet indicates that no CT value is detected, Pos indicates a positive control, and Neg indicates a negative control, the same below.

(2) Commercial Nested PCR Method

The above 15 *mycoplasma* samples in (1) of Title 1 of this example were detected by a commercial nested *mycoplasma* detection PCR kit, GMyc-PCR *Mycoplasma* Test Kit (Yeasen BioTechnologies co., Ltd.).

Operation steps: For a first round of PCR, a reaction system was shown in Table 3 and a reaction procedure was shown in Table 4. After the first round of PCR was completed, an amplification product was collected, diluted 1,000-fold, and then used as a template for a second round of PCR. For the second round of PCR, a reaction system was shown in Table 5 and a reaction procedure was the same as the reaction procedure for the first round of PCR.

TABLE 3

System for the first round of PCR

Reagent	Experimental group	Positive control	Negative control
GMyc-1st PCR Mix	25 μL	25 μL	25 μL
Template DNA	4 μL	4 μL	
ddH_2O	21 μL	20 μL	25 μL
Positive quality control template		1 μL	
Total volume	50 μL	50 μL	50 μL

TABLE 4

Conditions for the first round of PCR

PCR conditions	Temperature	Time	Number of reaction cycles
Pre-denaturation	94° C.	5 min	
Denaturation	94° C.	30 sec	30
Annealing	58° C.	30 sec	
Extension	72° C.	30 sec	
Re-extension	72° C.	7 min	

TABLE 5

System for the second round of PCR

Reagent	Experimental group	Positive control	Negative control
GMyc-2nd PCR Mix	25 μL	25 μL	25 μL
ddH_2O	24 μL	24 μL	24 μL
Product of the first round of amplification that is diluted 1,000-fold	1 μL	1 μL	1 μL
Total volume	50 μL	50 μL	50 μL

The commercial nested *mycoplasma* detection PCR kit was used to detect the 15 *mycoplasma* species, and results were shown in FIG. **2**. Only 12 *mycoplasma* species were detected by the commercial nested *mycoplasma* detection PCR kit. This method required two rounds of PCR and agarose gel electrophoresis, resulting in cumbersome operations. A detection rate of this method was 20% lower than a detection rate of the qPCR method of the present disclosure.

(3) Commercial qPCR Method

The 15 *mycoplasma* samples in (1) of Title 1 of this example were detected by the commercial qPCR kit, Myc-Away™ *Mycoplasma* Real-time qPCR Detection Kit (Yeasen BioTechnologies co., Ltd.).

Components for the commercial qPCR included 4×qPCR Reaction Buffer, Primer & Probe MIX, positive and negative controls, and sterile nuclease-free water. A qPCR system was shown in Table 6.

FAM was selected as a reporter fluorophore, and MGB was selected as a quenching fluorophore. A reaction system was set to 40 μL. Cycle parameters were set as follows: 95° C. for 5 min, 95° C. for 15 sec, and 62° C. for 30 sec, with 45 amplification cycles in total. At the end of annealing in each cycle, an FAM fluorescence signal was acquired. When Ct was smaller than 40 and there was a clear amplification curve, it was determined as positive. When Ct was greater than or equal to 40 or there was no obvious peak, it was determined as negative.

TABLE 6

qPCR system	
Component	System (μL)
4 × qPCR Reaction Buffer	10
Primer & Probe MIX	1
Template (10 ng/μL)	20
Sterile nuclease-free water	Making up to 40 μL
Total	40

Detection results of the commercial qPCR kit: CT values are shown in Table 7. It can be seen from FIGS. **3**A-**3**C that S-type amplification curves of *A. laidlawii* and *M. pirum* are atypical and negative. The qPCR method in Example 1 of the present disclosure has significant advantages over the commercial qPCR kit. The commercial qPCR kit requires 20 μL of a template (10 ng/μL), but the method of the present disclosure only requires 1 μL of sample DNA as a template during detection. The commercial qPCR kit requires 45 cycles, but the method of the present disclosure only requires 40 cycles. The method of the present disclosure can amplify a typical S-type amplification curve for all of the 15 *mycoplasma* species, and allows a stronger fluorescence intensity and a smoother curve than the commercial qPCR kit, making it not prone to mis-determination.

TABLE 7

CT values of 15 mycoplasma species detected by the commercial qPCR kit		
No.	Sample	CT
1	Neg	Undet
2	*M. orale*	22.229
3	MS	13.693
4	Mf	22.27
5	Mp	29.855
6	Mb	28.997
7	*M. fermentans*	19.764
8	Mh	21.833
9	*A. laidlawii*	17.9
10	MO	11.621
11	Mhs	13.263
12	*M. arginini*	17.43
13	*S. citri*	11.27
14	MG	33.484
15	*M. pirum*	33.904
16	*M. hyorhinis*	20.428
17	Pos	11.483

2. Sensitivity

The *E. coli* carrying the positive plasmid in Example 1 was allowed to proliferate, the positive plasmid was extracted, and a concentration of the positive plasmid was determined by a spectrophotometer. The plasmid was diluted 10-fold serially to produce plasmid concentrations of 10^9 copies/μL, 10^8 copies/μL, 10^7 copies/μL, 10^6 copies/μL, 10^5 copies/μL, 10^4 copies/μL, 10^3 copies/μL, 10^2 copies/μL, 10^1 copies/μL, 10^0 copies/μL, and 10^{-1} copies/μL, respectively. 1 μL of the positive plasmid at each concentration was taken as a template and used for analysis by the qPCR method in Example 1 to investigate the sensitivity of the method. Ten parallel tests were conducted for each concentration.

According to results of the qPCR detection in Example 1 (Table 8): When a concentration of the positive plasmid was 10^{-1} copies/μL, a Ct value could not be stably detected in 3 of 10 reactions. When a concentration of the positive plasmid was 10^0 copies/μL, a Ct value could be stably detected, and the Ct value was smaller than 38. When a concentration of the positive plasmid was 10^{-1} copies/μL, a Ct value could not stably appear. Therefore, the sensitivity of the qPCR method was determined to be 10^0 copies/μL, and a Ct threshold was 38.

TABLE 8

Ct values for the positive plasmid at each concentration detected by the qPCR method											
	Plasmid concentration,	Ct value									
Sample No.	copies/ μL	Replicate 1	Replicate 2	Replicate 3	Replicate 4	Replicate 5	Replicate 6	Replicate 7	Replicate 8	Replicate 9	Replicate 10
1	10^9	7.416	7.646	6.917	7.181	6.922	6.815	6.556	6.459	6.166	6.033
2	10^8	10.043	10.219	10.224	10.652	10.438	10.055	10.089	10.174	10.356	9.917
3	10^7	13.589	13.893	13.928	14.406	13.567	13.699	13.913	13.839	13.750	13.469
4	10^6	17.123	17.048	16.856	17.651	17.216	16.797	17.376	17.221	17.452	17.249
5	10^5	20.982	20.836	21.013	21.814	20.808	21.170	20.442	20.654	20.826	20.489
6	10^4	24.245	23.758	23.759	24.866	24.428	24.446	23.974	23.801	24.213	24.229
7	10^3	27.756	27.071	27.317	27.401	27.636	27.794	27.653	27.598	27.771	27.598
8	10^2	31.096	30.850	31.443	31.425	31.050	30.961	30.795	31.027	30.732	31.038
9	10^1	34.070	33.923	34.237	35.380	33.048	33.617	33.814	35.409	34.259	33.539
10	10^0	36.585	36.109	36.813	37.786	36.030	37.271	37.633	37.500	36.098	36.604
11	10^{-1}	Undet	38.289	38.254	39.541	38.572	39.894	Undet	Undet	38.572	38.672

When other primers and probes were used to detect *mycoplasma*, such as a primer MP03-F: 5'-GGTCGTC-TACGTCAAAACTTGC-3' (SEQ ID NO: 5), a primer MP03-R: 5'-GCCATTTGGTCCCCGTCAAAG-3' (SEQ ID NO: 6), and a probe MP03-P: FAM-TACCTTGT-TACGACTT-BHQ1 (SEQ ID NO: 7), there was a poor broad-spectrum activity, a typical S-type curve could not be provided for 2 of the 15 tested *mycoplasma* species, and a sensitivity was 102 copies/μL.

Example 3 Detection of *Mycoplasma* Contamination in a Cell Culture by the qPCR Method

A total of 106 cell samples from various laboratories were detected by the qPCR method in Example 1, the cultivation method in the 2020 edition of the "*Veterinary Pharmacopoeia of the People's Republic of China*", and the commercial qPCR method for *mycoplasma* to investigate a coincidence rate of the qPCR method in Example 1 with the cultivation method in the 2020 edition of the "*Veterinary Pharmacopoeia of the People's Republic of China*".

1. The qPCR Method in Example 1

A supernatant from each cell sample was taken to prepare a template through boiling. Specific steps were as follows: A supernatant was collected from a cell culture to be tested, added to a centrifuge tube, heated to 100° C. and boiled for 10 min, and cooled. A resulting supernatant was collected and centrifuged for 5 s to 6 s. A resulting supernatant was collected (or subjected to DNA extraction by a kit) as sample DNA for the qPCR detection method.

106 cell samples were detected by the qPCR method in Example 1. Results showed that 49 cell samples had a CT value of smaller than 38 (Table 9) and a typical amplification curve, and were positive for *mycoplasma*, as shown in FIGS. **4**A-**4**B. Thus, a positive detection rate was 46.23%.

TABLE 9

CT values of the 106 cell samples detected by the qPCR method in Example 1

No.	CT
1	19.488
2	Undet
3	16.9171
4	Undet
5	35.608
6	37.0916
7	12.060
8	Undet
9	Undet
10	Undet
11	15.107
12	Undet
13	32.115
14	17.542
15	Undet
16	Undet
17	Undet
18	32.582
19	15.626
20	Undet
21	35.800
22	21.760
23	22.583
24	35.458
25	Undet
26	26.516
27	Undet
28	37.493
29	Undet
30	16.7138
31	19.488
32	Undet
33	16.917
34	34.753
35	35.608
36	Undet
37	Undet
38	30.927
39	Undet
40	30.852
41	Undet
42	36.566
43	Undet
44	Undet
45	Undet
46	24.987
47	Undet
48	33.207
49	Undet
50	Undet
51	29.805
52	Undet
53	Undet
54	Undet
55	Undet
56	Undet
57	21.937
58	Undet
59	37.089
60	35.917
61	Undet
62	Undet
63	36.080
64	Undet
65	33.638
66	Undet
67	Undet
68	Undet
69	30.617
70	Undet
71	34.184
72	Undet
73	32.004
74	30.830
75	20.887
76	Undet
77	Undet
78	Undet
79	Undet
80	30.945
81	37.245
82	33.399
83	Undet
84	23.304
85	Undet
86	32.201
87	25.145
88	Undet
89	Undet
90	34.495
91	31.295
92	35.264
93	Undet
94	Undet
95	Undet
96	Undet
97	Undet
98	32.169
99	Undet
100	Undet
101	Undet

TABLE 9-continued

CT values of the 106 cell samples detected by the qPCR method in Example 1	
No.	CT
102	28.480
103	Undet
104	20.488
105	Undet
106	Undet
Positive control	21.011
Negative control	Undet

2. Detection of *Mycoplasma* in Cell Samples by the Isolation and Cultivation Method

According to the cultivation method in the 2020 edition of the "*Veterinary Pharmacopoeia of the People's Republic of China*", each cell sample was subjected to liquid and solid cultivation. At the end of cultivation, if no *mycoplasma* grew in a medium into which a cell sample was inoculated, the cell sample was qualified, otherwise, the cell sample was unqualified.

If *mycoplasma* grew, a color of a liquid medium would also change (pink or yellow). After aerobic cultivation in a solid medium at 37° C. for 30 d, if *mycoplasma* grew, pinpoint-like colonies could be observed by naked eyes in a medium and fried egg-like colonies could be observed under a microscope. In this experiment, known negative and positive samples were taken as negative and positive controls, respectively.

After about 21 d of cultivation, results showed that liquid media of 24 cell samples turned yellow (a pH decreased), and media of 7 cell samples turned pink (a pH increased). As a result, it was determined that these 31 cell samples were contaminated with *mycoplasma*. Cultures undergoing a color change each were inoculated into a solid medium and cultivated for about 30 d, and then fried egg-like colonies were formed on the solid medium. Thus, a positive detection rate was 29.25%.

3. Detection of *Mycoplasma* in Cell Samples by the Commercial qPCR Kit

Each cell sample was detected with the commercial qPCR kit, MycAway™ *Mycoplasma* Real-time qPCR Detection Kit (Yeasen BioTechnologies co., Ltd.). A specific method was implemented according to instructions.

106 cell samples were detected by the commercial qPCR kit. Results showed that 41 cell samples had a CT value of smaller than 40 (Table 10) and a typical amplification curve, and were positive for *mycoplasma*, as shown in FIGS. **5**A-**5**B. Thus, a positive detection rate was 38.68%.

TABLE 10

CT values of 106 cell samples detected by the commercial qPCR kit	
No.	CT
1	Undet
2	37.423
3	Undet
4	Undet
5	30.938
6	Undet
7	11.151
8	Undet
9	32.281
10	Undet
11	28.043

TABLE 10-continued

CT values of 106 cell samples detected by the commercial qPCR kit	
No.	CT
12	Undet
13	23.739
14	Undet
15	Undet
16	14.656
17	Undet
18	Undet
19	27.768
20	Undet
21	Undet
22	38.322
23	36.643
24	Undet
25	37.423
26	Undet
27	Undet
28	30.938
29	33.146
30	Undet
31	Undet
32	32.281
33	Undet
34	28.043
35	Undet
36	23.739
37	31.751
38	Undet
39	Undet
40	Undet
41	25.991
42	23.770
43	Undet
44	11.607
45	Undet
46	Undet
47	27.221
48	Undet
49	11.800
50	Undet
51	Undet
52	Undet
53	Undet
54	Undet
55	22.229
56	Undet
57	13.693
58	Undet
59	35.904
60	Undet
61	Undet
62	Undet
63	Undet
64	35.029
65	Undet
66	33.146
67	Undet
68	Undet
69	Undet
70	30.731
71	Undet
72	Undet
73	Undet
74	Undet
75	Undet
76	29.715
77	22.427
78	Undet
79	22.270
80	17.900
81	Undet
82	Undet
83	20.428
84	Undet
85	Undet
86	35.029

TABLE 10-continued

CT values of 106 cell samples detected by the commercial qPCR kit

No.	CT
87	Undet
88	Undet
89	Undet
90	Undet
91	31.751
92	Undet
93	Undet
94	Undet
95	Undet
96	30.731
97	Undet
98	31.068
99	Undet
100	Undet
101	Undet
102	16.515
103	29.855
104	Undet
105	13.274
106	27.545
Pos	11.27
Neg	Undet

4. Comparison of the Three Methods

The qPCR method in Example 1 completed the detection within 1 h, the commercial qPCR kit completed the detection in about 3 h, and the isolation and cultivation method took 21 d to 29 d to complete the detection of all cell samples.

TABLE 11

Comparison of detection performance of different methods

Method	Time consumption	Detection rate	Coincidence rate with the gold standard (isolation and cultivation method)
qPCR in Example 1	1 h	46.23%	100%
Isolation and cultivation method	21-29 d	29.25%	100%
Commercial qPCR	3 h	38.68%	87.10%

The collected 106 random cell samples were detected for *mycoplasma*, and results were shown in Table 11. 49 samples were detected as positive for *mycoplasma* contamination by the qPCR method in Example 1, which had a coincidence rate of 100% with the detection results of the classical cultivation method (a number of cell samples detected as positive by both methods/a number of cell samples detected as positive by the classical cultivation method * 100%). 41 samples were detected as positive by the commercial qPCR kit, and 4 samples were missed compared with the classical cultivation method. A coincidence rate of the commercial qPCR kit with the cultivation method was only 87.10%.

Therefore, when used in the detection of *mycoplasma*, the qPCR method in Example 1 is significantly superior to the prior art in terms of broad-spectrum activity, sensitivity, and accuracy.

A sequence of the gene fragment Spiroplasma (SEQ ID NO: 4) was as follows:

AACATAACAACAAAAGATAATCATTTAATCAATGAATATCCGTCATTAAAGCTAGGAACAAA

AACGATATTTTTTAATGAGAGTTTGATCCTGGCTCAGGATGAACGCTGGCGGCATGCCTAAT

ACATGCAAGTCGAACGGGGTGCTTGCACCCAGTGGCGAACGGGTGAGTAACACGTATCTAA

TCTACCCATTAGCGGGGGATAACAGTTGGAAACGACTGATAATACCGCATACGACATTTTCT

GGCATCAGAGAATGTTAAAAGGTCCGTTTGGATCACTAATGGATGAGGATGCGGCGTATTAG

TTAGTTGGTGGGGTAATGGCCTACCAAGACAATGATACGTAGCCGAACTGAGAGGTTGATC

GGCCACATCGGGACTGAGACACGGCCCGAACTCCTACGGGAGGCAGCAGTAGGGAATTTT

TCACAATGGGCGAAAGCCTGATGGAGCAATGCCGCGTGACTGAAGACGGTCTTCGGATTGT

AAAAGTCTGTTGTAAGGGAAGAACAGTAAGTATAGGAAATGATACTTATTTGACGGTACCTT

ACCAGAAAGCCACGGCTAACTATGTGCCAGCAGCCGCGGTAATACATAGGTGGCAAGCGTT

ATCCGGATTTATTGGGCGTAAAGCGTGCGCAGACGGTTTAACAAGTTTGGGGTCAAATCCT

GGAGCTCAACTCCAGTTCGCCTTGAAAACTGTTAAGCTAGAGTGTAGGAAAGGTCGATGG

AATTCCATGTGTAGCGGTGAAATGCGTAGATATATGGAGGAACACCAGTGGCGAAGGCGGT

CGACTGGCCTATCACTGACGTTTAGGCACGAAAGCGTAGGGAGCAAATAGGATTAGATACC

CTAGTAGTCTACGCCGTAAACGATGAGTACTAAGTGTCGGACTAAGTTCGGTGCTGCAGCT

AACGCATTAAGTACTCCGCCTGAGTAGTATGCTCGCAAGAGTGAAACTCAAAGGAATTGAC

GGGGACCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGAAGCAACGCGAAGAACCTTAC

CAAGGCTTGACATCCAGTGCAAAGCTGTAGAAATACAGTGGAGGTTAACATTGAGACAGGT

GGTGCATGGTTGTCGTCAGCTCGTGCCGTGAGGTGTTTGGTTAAGTCCAGTAACGAGCGCA

ACCCTTGCCGTTAGTTACTCCATTAAGTTGAGATACTCTAACAGGACTGCTAGTGTAAGCTA

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ACAATGGTCGGTACAAACAGTTGCGATCTCGTAAGAGGGAGCTAATCTGAAAAAGCCGATC

TCAGTTCGGATTGAGGGCTGCAACTCGCCCTCATGAAGCCGGAATCGCTAGTAATCGCGAA

TCAGCAATGTCGCGGTGAATACGTTCTCGGGTCTTGTACACACCGCCCGTCACACCATGAG

AGTTGATAATACCAGAAGTCGGTATTCTAACCGCAAGGAGGAAGCCGCCCAAGGTAGGATT

GATGATTAGGGTGAAGTCGTAACAAGGTATCCGTACGAGAACGTGCGGATGGATCACCTCC

TTTCTATGGAGTTAATACTTTATAGTAATTAACTAGTTTTAATGACCGTTATGTTTAGTTTTCA

GAGATTAGTTTCTCTGAAAATAACAAGTAAATGTTATTGGAATTGTTCTTTGAAAACTGGAT

AATAGACATCTAGTTATTTTAATCACATGATTAAAATAACAATAATTCAAAATTTCTGTTATTT

TTAAAAAATAACTAAAATTTCACAGTTATATTTGTAAATGATTCTCAAAAAACTGATTTAAAA

TCAGGTCAAATAATTTATAAAACTTTGAAGTTACAAAGGGCGTATGGTGAATGCCTTGG.

SEQUENCE LISTING

Sequence total quantity: 7

SEQ ID NO: 1 moltype = DNA length = 20
FEATURE Location/Qualifiers
source 1..20
mol_type = other DNA
organism = synthetic construct
SEQUENCE: 1
atccatcccc acgttctcgt 20

SEQ ID NO: 2 moltype = DNA length = 21
FEATURE Location/Qualifiers
source 1..21
mol_type = other DNA
organism = synthetic construct
SEQUENCE: 2
tgcggtgaat acgttctcgg g 21

SEQ ID NO: 3 moltype = DNA length = 16
FEATURE Location/Qualifiers
source 1..16
mol_type = other DNA
organism = synthetic construct
SEQUENCE: 3
acgggcggtg tgtaca 16

SEQ ID NO: 4 moltype = DNA length = 1901
FEATURE Location/Qualifiers
source 1..1901
mol_type = genomic DNA
organism = Spiroplasma sp.
SEQUENCE: 4
aacataacaa caaaagataa tcatttaatc aatgaatatc cgtcattaaa gctaggaaca 60
aaaacgatat tttttaatga gagtttgatc ctggctcagg atgaacgctg gcggcatgcc 120
taatacatgc aagtcgaacg gggtgcttgc acccagtggc gaacgggtga gtaacacgta 180
tctaatctac ccattagcgg gggataacag ttggaaacga ctgataatac cgcatacgac 240
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gaggttgatc ggccacatcg ggactgagac acggcccgaa ctcctacggg aggcagcagt 420
agggaatttt tcacaatggg cgaaagcctg atggagcaat gccgcgtgac tgaagacggt 480
cttcggattg taaaagtctg ttgtaaggga agaacagtaa gtataggaaa tgatacttat 540
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-continued

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aagccggaat cgctagtaat cgcgaatcag caatgtcgcg gtgaatacgt tctcgggtct  1440
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caaggaggaa gccgcccaag gtaggattga tgattagggt gaagtcgtaa caaggtatcc  1560
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actagtttta atgaccgtta tgtttagttt tcagagatta gtttctctga aaataacaag  1680
taaatgttat tggaattgtt ctttgaaaac tggataatag acatctagtt attttaatca  1740
catgattaaa ataacaataa ttcaaaattt ctgttatttt taaaaaataa ctaaaatttc  1800
acagttatat ttgtaaatga ttctcaaaaa actgatttaa aatcaggtca aataatttat  1860
aaaactttga agttacaaag ggcgtatggt gaatgccttg g                      1901

SEQ ID NO: 5          moltype = DNA  length = 22
FEATURE               Location/Qualifiers
source                1..22
                      mol_type = other DNA
                      organism = synthetic construct
SEQUENCE: 5
ggtcgtctac gtcaaaactt gc                                           22

SEQ ID NO: 6          moltype = DNA  length = 21
FEATURE               Location/Qualifiers
source                1..21
                      mol_type = other DNA
                      organism = synthetic construct
SEQUENCE: 6
gccatttggt ccccgtcaaa g                                            21

SEQ ID NO: 7          moltype = DNA  length = 16
FEATURE               Location/Qualifiers
source                1..16
                      mol_type = other DNA
                      organism = synthetic construct
SEQUENCE: 7
taccttgtta cgactt                                                  16
```

What is claimed is:

1. A composition for detecting mycoplasma, wherein the composition is an aqueous solution comprising

a primer M-F consisting of SEQ ID NO: 1;

a primer M-R consisting of SEQ ID NO: 2; and

a probe M-P consisting of SEQ ID NO: 3, wherein the probe M-P further comprises a fluorophore carboxyfluorescein (FAM) linked at a 5' terminus and a quencher black hole quencher 1 (BHQ1) linked at a 3' terminus.

2. The composition according to claim **1**, wherein the primer M-F, the primer M-R, and the probe M-P are in a molar concentration ratio of 1:(0.8-1.2):(0.8-1.2).

3. A kit for detecting mycoplasma, comprising the composition according to claim **1**.

4. The kit according to claim **3**, wherein the primer M-F, the primer M-R, and the probe M-P in the kit are in a molar concentration ratio of 1:(0.8-1.2):(0.8-1.2).

5. The kit according to claim **4**, wherein the kit further comprises a positive plasmid, and the positive plasmid is obtained by inserting a fragment consisting of SEQ ID NO: 4 into a pUC57 plasmid vector.

6. A method for detecting mycoplasma using the composition according to claim **1** for a non-diagnostic purpose, comprising the following steps:

(1) extracting DNA from a sample;

(2) with the DNA of the sample as a template, conducting a quantitative polymerase chain reaction (qPCR) detection using the primer M-F, the primer M-R, and the probe M-P; and

(3) when a cycle threshold (Ct) value of the qPCR detection for the DNA of the sample is smaller than 38 and there is a typical S-type amplification curve, determining the sample as positive, indicating that there is the mycoplasma in the sample; and when the Ct value of the qPCR detection for the DNA of the sample is larger than or equal to 38 or there is no Ct value or there is no typical S-type amplification curve, determining the sample as negative, indicating that there is no mycoplasma in the sample.

7. The method according to claim **6**, wherein a reaction system for the qPCR detection comprises: 12.5 μL of a fluorescent polymerase chain reaction (PCR) solution, 1 μL of the DNA of the sample, 3 μL of the composition, and 8.5 μL of double distilled water (ddH_2O).

8. The method according to claim **7**, wherein a procedure for the qPCR detection is as follows: 95° C. for 3 min; 95° C. for 15 sec, and 60° C. for 30 sec, with 40 amplification cycles in total.

9. The method according to claim **6**, wherein the sample is a biological product.

10. The method according to claim **9**, wherein the biological product is a cell, a serum, or a vaccine.

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