The qPCR and loop mediated isothermal amplification for rapid detection of *Ustilago tritici*

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Loose smut of wheat caused by the basidiomycete fungus *Ustilago tritici*, a seed-borne disease, is difficult to control because of the expanse of wheat planting area and difficulty in pathogen detection. In this study, real-time fluorescence quantitative PCR (qPCR) and loop-mediated isothermal amplification (LAMP) assays are used to rapidly amplify the DNA of *U*. *tritici*. Five pairs of primers for qPCR and two series primers for LAMP were designed. Primarily, the specificity of the primer was assessed by using genomic DNA of *U*. *tritici*, *Fusarium graminearum*, *Blumeria graminis*, *Rhizoctonia cerealis*, *Puccinia striiformis*, *Bipolaris sorokiniana* and *Alternaria solani* as templates. Further, the amplification systems were optimized. Finally, the sensitivity of qPCR and LAMP assays were evaluated. The results showed that the primer Y-430 F/R, Y-307 F/R, Y-755 F/R and Y-139 F/R for qPCR and primers L-139 and L-988 for LAMP could be used for *U*. *tritici* detection. In the sensitivity test, the detection limit of qPCR assay was identified as 10 pg μL^{-1} of genomic DNA, the detection limit for LAMP assay was 100 fg μL^{-1} . We successfully performed qPCR and LAMP assays on wheat loose smut wheat samples. This paper establishes two methods for *U*. *tritici* detection, which can be used for diagnosis of wheat loose smut in the laboratory and in the field.

qPCR and loop-mediated isothermal amplification assays for rapid detection of *Ustilago tritici*

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ABSTRACT

Loose smut of wheat caused by the basidiomycete fungus *Ustilago tritici*, a seed-borne disease, is difficult to control because of the expanse of wheat planting area and difficulty in pathogen detection. In this study, real-time fluorescence quantitative PCR (qPCR) and loop-mediated isothermal amplification (LAMP) assays are used to rapidly amplify the DNA of *U. tritici*. Five pairs of primers for qPCR and two series primers for LAMP were designed. Primarily, the specificity of the primer was assessed by using genomic DNA of *U. tritici*, *Fusarium graminearum*, *Blumeria graminis*, *Rhizoctonia cerealis*, *Puccinia striiformis*, *Bipolaris sorokiniana* and *Alternaria solani* as templates. Further, the amplification systems were optimized. Finally, the sensitivity of qPCR and LAMP assays were evaluated. The results showed that the primer Y-430 F/R, Y-307 F/R, Y-755 F/R and Y-139 F/R for qPCR and primers L-139 and L-988 for LAMP could be used for *U. tritici* detection. In the sensitivity test, the detection limit of qPCR assay was identified as 10 pg μL^{-1} of genomic DNA, the detection limit for LAMP assay was 100 fg μL^{-1}. We successfully performed qPCR and LAMP assays on wheat loose smut wheat samples. This paper establishes two methods for *U. tritici* detection, which can be used for diagnosis of wheat loose smut in the laboratory and in the field.

Subjects Molecular biology, Biological detection

Keywords LAMP, qPCR, *Ustilago tritici*, wheat loose smut

INTRODUCTION

Wheat is one of the most important food crops in the world, and it is also the staple food for most of the world's population (Garg et al., 2014). Its global cultivation area is as high as 220 107 600 hectares (Zhao et al., 2018). Loose smut of wheat caused by the basidiomycete fungus *Ustilago tritici*, is one of the most serious diseases on wheat (*Triticum aestivum* L.) globally. The disease is favored by moist and cool climate during

anthesis (Quijano et al., 2016). This fungus converts the spike floral tissues to fungal teliospores, and the carrier seeds of loose smut are the only way of transmission (Singh et al., 2018). The mycelium of *U. tritici* is kept viable in the embryo of the infected seed and moves on the growth point of the tiller without any visible symptoms (Kumar et al., 2018), but will be revealed after the heading period of the next year. The yield losses of a single plant are nearly 100% after the onset of disease, and the general disease rate is 1% to 5%. When the incidence is serious, it is more than 10%, which can reduce the yield of wheat by 5% to 20% (Quijano et al., 2016). With the popularization of wheat planting area, the damage caused by loose smut in wheat has become more serious, and it has gradually become one of the most harmful diseases in wheat cultivation (Knox & Menzies, 2012).

At present, the most effective control method of loose smut in wheat is sterilizing before seeding (Singh et al., 2014; Duan et al., 2016). However, there are no suitable and effective control measures after sowing. The long-term use of chemical agents can easily pollute the environment, endanger the human health and livestock, and the excessive use of a single drug can easily lead to the emergence of drug resistance (Crane et al., 2013). Therefore, timely detection of the pathogen *U. tritici* becomes imperative. Host diversity often leads to diversity of pathogen species, and detection of pathogens requires homozygous strains. Separating and cultivating the strains on the diseased plants is a common effective method for obtaining pure strain. The standard detection method for *U. tritici* is serological identification, but it is time-consuming and needs specific expertise. Meanwhile, its accuracy and sensitivity are not satisfactory (Walcott, 2003; Munkvold, 2009). Currently, the common detection method for loose smut in wheat is PCR (Martínez-Espinoza et al., 2003). However, PCR cannot be used to perform accurate quantitative analysis, and cross-contamination can easily give false positive results (Bretagne, 2003). Quantitative PCR (qPCR) has quickly become an indispensable tool in scientific

research and clinical diagnosis (Kuypers et al., 2006; Yan et al., 2012). However, there are some shortcomings, such as the high instrument cost, the need for trained personnel for operation, and not applicable for field testing. Notomi et al. (2000) invented a novel method for rapid, efficient, and highly specific amplification of target DNA-loop mediated isothermal amplification (LAMP). The principle of the experiment is based on design of four different primers (F3, B3, FIP, BIP) for six different positions of the target sequence (F3c, F2c, F1c, B1, B2, B3) (Tomita et al., 2008; Notomi et al., 2015), acting under the action of *Bst* DNA polymerase, in a water bath instead of the PCR instrument. The reaction is performed at 60 - 65 °C for 60 - 90 min and the target DNA amplification is increased to 10^9 - 10^{10} (Dhama et al., 2014). Compared to PCR, LAMP shortens the reaction time, eliminates the gel electrophoresis step, does not require expensive instruments, and completes the experiment with the *Bst* DNA polymerase in constant temperature conditions. The results are determined by the color reaction of fluorescent dyes. Currently, the fluorescent dyes used are calcein (Rane et al., 2015), PicoGreen (Curtis, Rudolph & Owen, 2008), hydroxy naphthol blue (HNB) (Goto et al., 2010; Mohon et al., 2014), and SYBR Green (Balne et al., 2013; Zhou et al., 2014). SYBR Green Ⅰ and HNB have the highest detection sensitivity, 10 times that of calcein (Gao et al., 2009), and HNB and SYBR Green Ⅰ can produce long-term stable color changes with brightness in pipes and prevent cross-contamination (Almasi et al., 2013). Moreover, their high affinity with double-stranded DNA makes them the most commonly used fluorescent dyes. In this study, SYBR Green Ⅰ was used in both qPCR and LAMP assay. However, since it binds to any dsDNA molecule, confirming the specificity of the primers is essential for further identification. Therefore, in the qPCR assay, an extra melting curve step was performed to identify the specificity of the primers. As for the LAMP assay, the primers were screened by PCR to detect any primer dimer formation, and control bacteria DNA was used to determine the specificity of the primers. The combination of LAMP and fluorescent dyes makes bio-

detection simpler and more intuitive. At present, LAMP and qPCR have been used for detection of many bacteria, fungi, and viruses and in other microbial detection, such as *Verticillium albo-atrum* (Tian et al., 2016), *Listeria monocytogenes* strains (Wang et al., 2012), parasites (Abdul-Ghani, Al-Mekhlafi & Karanis, 2012), and *Candidatus Liberibacter asiaticus* (Rigano et al., 2014). There have been reports on the detection of *Rhizoctonia cerealis* (Sun et al., 2015) and *Tilletia controversa* Kühn (Nian et al., 2009) by qPCR assays, and the detection of Fusarium head blight (Niessen & Vogel, 2010) and wheat stripe rust (Huang et al., 2011) by LAMP assays. However, the detection of wheat loose smut by these two methods has not yet been reported. In this study, we used qPCR and LAMP assays to rapidly detect wheat loose smut (Kuboki et al., 2003; Poon L & L M, 2005; Kono et al., 2004).

MATERIALS AND METHODS

Materials

Fungal strains.

Ustilago tritici, *Fusarium graminearum*, *Blumeria graminis*, *Rhizoctonia cerealis*, *Puccinia striiformis*, *Bipolaris sorokiniana*, *Alternaria solani*. All strains are provided by the Pathology Laboratory of the College of Agriculture, Yangtze University.

Culture environment.

The wheat variety used in the experiment was Mingxian169 provided by the Pathology Laboratory of the College of Agriculture, Yangtze University. After germination for 24 hours (h) in dark conditions, the seeds were planted in a pot and placed in a light incubator at 22 °C for the 12h of the day and 18 °C for the 12h of the night. *U. tritici* were collected from the spike tissues of diseased wheat. Wheat powdery mildew was derived from diseased

101 leaves. *F*. *graminearum*, *B*. *graminis*, *R*. *cerealis*, *B*. *sorokiniana*, *A*. *solani* were inoculated on the Potato
102 Dextrose Agar (PDA, It was provided by the Pathology Laboratory of the College of Agriculture, Yangtze
103 University) mediums covered with glass paper and cultured at 25 °C for 7 to 8 days. *P*. *striiformis* was collected
104 from the field.

105 **Genomic DNA extraction**.

106 DNA was extracted from wheat loose smut by modified Cetyltrimethylammonium Ammonium Bromide
107 (CTAB) (Allen et al., 2006). Briefly, lysis buffer (10 mM Tris-HCl [pH 8.0], 100 mM EDTA [Ethyl-
108 enediaminetetraacetic acid], 0.5 % sodium dodecyll sulfate, and 100 μg mL^{-1} proteinase) was added to the
109 sample, followed by incubation in a water bath at 55 °C for 1 h. The DNA was extracted with by phenol-
110 chloroform-isoamyl alcohol (25:24:1) method, precipitated with isopropanol, and washed with ethanol (70%).
111 After centrifugation, 30 μL of ddH_2O was added and the final elute was stored at -20 °C.

112 **Methods**

113 **The qPCR and Lamp primer design**.

114 Primers were designed by Primer Premier 5.0 based on the sequences of *U*. *tritici* published in National Center
115 for Biotechnology Information (NCBI). JN367334.1, AF135430.1 (Bakkeren et al., 2000), JN367307.1,
116 KP256755.1 (Hemetsberger et al., 2015) and AJ236139.1 were selected for qPCR assay (Table 1). After
117 selecting a large number of DNA sequences for loose smut from NCBI, the sequences were adopted by the
118 multiple sequence alignment to identify highly homologous sequences such as AJ236139.1 and DQ132988.1.
119 and designed primers for AJ236139.1 and DQ132988. by Primer Explorer V5
120 (http://primerexplorer.jp/lampv5e/index.html) for LAMP assay (Table 2). The length of the DNA used was less
121 than 300 bp, and the six parts of the primer were amplified separately for different sequences. Each set of primers

consists of two outer primers (F3/B3) and two inner primers (FIP/BIP), FIP containing F1c and F2, BIP containing B1c and B2 (Fig. 1). The primers were synthesized by The Beijing Genomics Institute (BGI, Beijing, China), dissolved in ddH_2O, and stored at -20 °C.

qPCR assays

Specificity of qPCR assays.

DNA of *U. tritici* was used to detect the specificity of the primers. The amplification conditions were initial denaturation at 95 °C for 3 min, followed by 34 cycles of denaturation at 95 ℃ for 30 s, annealing at 52 ℃ for 30 s, amplification at 72 ℃ for 40 s and a final amplification at 72 ℃ for 5 min. The amplification products were preserved at 16 ℃ (PCR Thermal Cyclers, Shanghai, China). The amplification products were detected by 1% agarose gel electrophoresis followed by sequencing (Fig. 2). Based on the preliminary screening results, primers were further screened by qPCR using the control strains as templates, and primer specificity was determined by the Ct value presented by the amplification curves (Fig. 3).

Optimization of qPCR assays.

Appropriate proportion of reaction contents affect the accuracy of the qPCR results, therefore, system optimization becomes indispensable. System optimization experiments were performed using gradients of different volumes of ChamQ^{TM}SYBR® qPCR Master Mix, such as 4 μL, 6 μL, 8 μL, 10 μL, 12 μL, 14 μL and 16 μL in qPCR assays (Fig. 4). the ideal temperature was determined after demonstrating the optimum system proportions. seven temperature gradients were designed, 52 °C, 54 °C, 56 °C, 58 °C, 60 °C, 62 °C and 64 °C (Fig. 5). Melting temperature and gel electrophoresis were combined to determine the optimum temperature.

qPCR for *U. tritici*.

142 For a total reaction volume of 20 μL, the reaction mix comprised the following 1 μL DNA, 1 μL of individual
143 forward and reverse primer, 10 μL Master Mix, and 7 μL ddH_2O. The reaction conditions were, initial
144 denaturation at 94 ℃ for 3 min followed by 40 cycles of denaturation at 94 ℃ for 20 s, primer annealing at 60
145 ℃ for 30 s, and amplification at 72 ℃ for 5 min. Each template DNA was diluted to six concentration gradients,
146 with triplicates in each group. We then analyzed melting curves to verify the specificity of the results (Fig. 6).

147 **Sensitivity detection of qPCR assays**.

148 100 ng μL^{-1} DNA of *U. tritici* was used to the template and diluted to seven concentration gradients, 100 ng μL^{-1},
149 10 ng μL^{-1}, 1 ng μL^{-1}, 100 pg μL^{-1}, 10 pg μL^{-1}, 1 pg μL^{-1}, 100 fg μL^{-1}. We performed qPCR on Y-430 with two
150 replicates per concentration (Fig. 7).

151 **LAMP assay**

152 **Specificity of LAMP assays**.

153 We analyzed whether the primers were normal and whether there was primer-dimer formation in PCR. The PCR
154 system for a volume of 25 μL was as follows: DNA 0.5 μL, dNTPs 1 μL, *Taq* DNA polymerase 1 μL, F3 1 μL,
155 B3 1 μL, $MgSO_4$ 1 μL, 2 x Phanta Max Buffer 12.5 μL, ddH_2O 7 μL, the two sets of primers F3-1F/B3-1R, F3-
156 2F/B3-2R, with three replicates for each set, ddH_2O was used as control. Fluorescent dye was added to observe
157 the amplification results (Fig. 8). Additionally, *F. graminearum*, *B. graminis*, *R. cerealis*, *P. striiformis*, *B.*
158 *sorokiniana*, and *A. solani* were used as the controls for LAMP assay (Fig. 9).

159 **Temperature optimization for LAMP assay**.

160 In order to determine the accuracy and sensitivity of the results, the system concentration and temperature for
161 the LAMP assay were optimized. The concentration presented here is the optimal concentration ratio. The
162 concentration ratio of the inner and outer primers used in this experiment was 8:1, the concentration of FIP and

BIP was 1.6 μmol L^{-1}, and of F3 and B3 was 0.2 μmol L^{-1}. The concentration of Mg^{2+}after referral to relevant literatures (Kubota et al., 2008; Abdelsalam et al., 2011) was established as 6 mmol L^{-1}. And nine temperature gradients were designed for LAMP to determine the optimum temperature based on the final color of the reaction (Fig. 10A).

Sensitivity detection of LAMP assays.

We diluted the extracted DNA by ten-fold and then used it as the template for further diluting the template DNA to obtain nine concentration gradients, which were 100 ng μL^{-1}, 10 ng μL^{-1}, 1 ng μL^{-1}, 100 pg μL^{-1}, 10 pg μL^{-1}, 1 pg μL^{-1}, 100 fg μL^{-1}, 10 fg μL^{-1}, and 1 fg μL^{-1}. The results were analyzed by observation under natural light and differentiated by color of the reaction (Fig. 10B).

LAMP assay for *Ustilago tritici*.

The final volumes of the components of the LAMP reaction system of 25 μL used in the experiment were as follows: 1 μL DNA, 2.5 μL 10 x *Thermopol* buffer, 2 μL $MgSO_4$, 2 μL dNTPs, 1 μL F3, 1 μL B3, 1 μL FIP, 1 μL BIP, 1 μL *Bst* DNA polymerase, 5 μL betaine, and 7.5 μL ddH_2O, with triplicates for each set of primers. ddH_2O was used as a template in control reactions (Fig. 11).

RESULTS

In order to determine the specificity of the designed primers, we performed PCR experiments on the DNA of the *U*. *tritici* (Fig. 2). Accordingly, five sets of sequence bands corresponding to the primers were obtained. Based on the preliminary screening results, primers were further screened by qPCR using the control strains as templates, and specificity was determined by the Ct value of the amplification curves (Fig. 3). The results showed that Y-334 amplified *F*. *graminearum* (Fig. 3A) and therefore it was non-specific for *U*. *tritici*. The primers Y-

430, Y-307, Y-755, and Y-139 specifically amplified the target DNA. Four sets of primers were used to detect *U. tritici*. By optimizing the system, the optimal volume for ChamQ^{TM}SYBR$^{®}$qPCR Master Mix could be formulated. The results of gel electrophoresis after amplification are shown in Fig. 3. The amplification results were the best for a master mix volume of 8 μL - 12 μL. We considered the intermediate value is 10 μL, as the optimal volume for ChamQ^{TM}SYBR$^{®}$ qPCR Master Mix in qPCR assay. Additionally, the results of qPCR temperature gradient test in *U. tritici*, revealed that the relative fluorescence units reached a high detectable levels at 58 °C and 60 °C, and the Ct value was about thirty-three, which was in line with our expected results. The bands at 58 °C were the brightest as shown by gel electrophoresis. In accordance with these results (Fig. 5A, Fig. 5B), we chosen 58 °C as the temperature for the final experiment. The amplification curves for the four pairs of primers show that the Ct values ranged from twenty-nine to thirty-five in the samples (Fig. 6). It indicated that the four sets of primers could amplify the target DNA under certain concentration conditions. The melting curves corresponding to each primer exhibited a single peak, which further ruled out non-specific amplification. Combining the amplification curves and the melting curves, it can be stated that the target DNA can be successfully amplified by using the four pairs of primers designed to quantify the *U. tritici*. Finally, we used 100 ng μL^{-1} DNA by diluting it to give seven concentration gradients for verification of lowest concentration detectable by qPCR. The fluorescence results of seven gradients showed (Fig. 7) that the first five gradients gave high signal noise so that the lowest detectable DNA concentration by qPCR was 10 pg μL^{-1}.

In LAMP assays, the results of the PCR system analyzed under natural light and gel imager (Fig. 8) indicated that primers designed for LAMP assay can be used for PCR amplification. The pathogens such as *F. graminearum* (Table 3) were used as controls to verify the specificity of the primers. Additionally, five other fungal diseases commonly found in wheat were selected as controls. At the same time, a group of non-wheat

fungi were also selected. L-139 and L-988 were used to perform LAMP assays on seven different bacteria (Fig. 9). The results showed that L-139 and L-988 only amplified the DNA sequence of *U. tritici*. As can be seen from Fig. 10A, the optimum reaction temperature for LAMP assay was from 62 °C to 64 °C. This experiment was performed at 63 °C. The LAMP assay was performed under the optimal reaction proportions and temperature, for *U. tritici*. Through the gel imaging system (Fig. 11A), the positive samples were white and the negative were colorless. Under natural light (Fig. 11B), the three replicates of the two sets of primers were bright green, and the negative controls were light orange. The light orange color of the negative controls indicated the absence of primer-dimers and false positives due to external contamination. Sensitivity testing of the LAMP test indicated that the DNA concentration of the lowest *U. tritici* detectable by the LAMP is 100 fg μL^{-1}. We performed multiple verifications for experimental accuracy in order to test seed carriers and compare the two methods in terms of sensitivity and operation, so we did not distinguish between the different species.

Based on the successful amplification of DNA from *U. tritici* by qPCR and LAMP assays, Y-430 was used to perform qPCR and L-139 and L-988 were used to perform LAMP assay on diseased seeds (Fig. 12). The qPCR results give a consistent Ct value at twenty-seven, and the melting curve also showed a single peak. Similarly, in the LAMP experiment, both samples showed bright green color, and the expected ladder band appeared in agarose gel electrophoresis. Combining the results of the two methods, we conclude that qPCR and LAMP technology can be used for efficient and sensitive detection of *U. tritici*.

DISCUSSION

Loose smut of wheat is a systemic disease infesting flower organs (Ngugi & Scherm, 2006). It currently occurs in all wheat-growing regions of the world, particularly prevalent in Canada (Randhawa et al., 2009) and parts of

Africa (Zegeye, Dejene & Ayalew, 2015). The detection of loose smut in seeds is particularly important due to the increased area of the disease and a single method of prevention. both qPCR and LAMP assays can specifically and efficiently amplify the DNA of *U. tritici* in this study.

Our ultimate goal was to develop a simple and high-speed detection method. Both the qPCR and LAMP are beneficial to detect pathogens from the source. By comparing the two methods, it is not difficult to conclude that the qPCR assay can accurately determine the initial amount and amplification of the template; it can also be used to visually determine whether there was non-specific amplification by comparison of the melting curves. Combining the amplification curves with melting curves, it was concluded that the primers Y-430, Y-307, Y-755 and Y-139 can specifically, efficiently, and accurately detect *U. tritici*. This method avoids the use of gel electrophoresis and other supplementary operations. Therefore, it has been widely used in the analysis of gene expression (Ma et al., 2013), virus detection (Albinana-Gimenez et al., 2009), disease diagnosis (Moreira et al., 2013). Compared with qPCR, the LAMP assay has the advantage of sensitivity, time, ease of operation, and equipment requirement (Kiddle et al., 2012). In combination with fluorescent dyes, the colored reaction is more favorable for observation of the amplification results and can be used widely in biological disease detection (Jung et al., 2015), medical diagnosis (Hopkins and Bell, 2013), food testing (Sun et al., 2015) and other aspects. Compared with PCR, both the qPCR and LAMP assays can effectively avoid the influence of agarose gel electrophoresis and the minimum detectable concentration is lower than that by PCR. However, there are still many problems in the application of the two methods. Primarily, the instruments for performing qPCR assays are expensive. The LAMP assay can only detect one disease at a time. The combination of fluorescent dye with dsDNA is not specific, so the LAMP assay has a high false positive rate. Meanwhile the field environment is complex, the reaction system and concentration ratio are difficult to optimize, etc.

247 At present, the qPCR and LAMP assays are being improved up. Based on qPCR, multiplex PCR has emerged
248 to compensate for the shortcomings of detecting only one disease at a time. The various conditions of qPCR
249 assay limits its ability for application in field-based assays. Accurate analysis of the data and good reproducibility
250 of the experiment make it the ubiquitous mainstay of molecular biology. With the progress of molecular biology,
251 fluorescence quantification has become an indispensable tool. Meanwhile, multiple LAMP assays have also been
252 proposed to accelerate the efficiency of detection (Chen et al., 2016; Lodh et al., 2017). Kits for LAMP assays
253 have been developed (Marti, Stalder & González, 2015), that eliminate the need for system optimization, thus
254 simplifying the procedure. Its advantages make it suitable for use in resource-poor areas. With advancements in
255 technology and human knowledge, chemical control methods of disease resistance will be gradually replaced by
256 early prevention. The LAMP methodology is a very valuable diagnostic alternative with a potential for use in
257 endemic diseases. The improvements in LAMP technology will make it more effective in disease prevention and
258 control.
259

260 **CONCLUSIONS**

261 The methods of qPCR and LAMP for the detection of *U. tritici* have been developed in this study, both of them
262 are great significance for the diagnosis of *U. tritici*. The qPCR with better sensitivity and comparable specificity
263 to current diagnostic tests. The method of LAMP has better availability in laboratories of various standards, and
264 has the potential to be utilized on a large scale.
265

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Table 1(on next page)

Primers designed for qPCR

1 **Table 1:**

2 **Primers designed for qPCR.**

GenBank	Primer name	Type	Primer sequence（5’-3’）	Production length(nt)
JN367334.1	Y-334	Forward	CACGGACCAAGGAGTCTAACAT	199
		Reverse	CCTCTGGCTTCACCCTATTCA	
AF135430.1	Y-430	Forward	CCATTTATCGTGGCTCCCTT	134
		Reverse	TACCCATCTCAACCTCTCCG	
JN367307.1	Y-307	Forward	CATTTATCGTGGCTCCCTT	138
		Reverse	TCCTACCCATCTCAACCTCTCC	
KP256755.1	Y-755	Forward	CTGCTTCTAACAATGCTGACG	162
		Reverse	CAACCATCTTACCTAACCCGC	
AJ236139.1	Y-139	Forward	GGGTAGGAGGTCAGAGATGC	211
		Reverse	CGTAAAGGTGCCCGAAGG	

3

Table 2(on next page)

Primers designed for LAMP

1 **Table 2:**

2 **Primers designed for LAMP.**

GenBank	Primer name	Type	Sequence（5’-3’）	Length(nt)
AJ236139.1	L-139	F3	GGGTAGGAGGTCAGAGATGC	20
		B3	CGTAAAGGTGCCCGAAGG	18
		FIP (F1c+F2)	CCGACGTTGGCCTGCAATCT-GGTCTGGGATTCAGCCTTG	39
		BIP (B1c+B2)	GTGGAAGGAATGTGGCACCTCT-AGTACGCTGCTGTCCTCG	40
DQ132988.1	L-988	F3	AAGGGAGCCACGATAAATGG	20
		B3	GGCAACGGATCTCTTGGTT	19
		FIP (F1c+F2)	CCTGTTTGAGGGCCGCGAATT-CCGATCCGTCAACCTTTTCC	41
		BIP (B1c+B2)	GAGCGCAAGGTGCGTTCAAAG-CGATGAAGAACGCAGCGAA	40

3

Table 3(on next page)

Experimental and control strains

“+” means the amplification result is positive, “-” means the amplification result is negative.

1 **Table 3:**

2 **Experimental and control strains.**

Species	No. of strains	Host plants	PCR		LAMP	
			L-139	L-988	L-139	L-988
Ustilago tritici	2	Wheat	+	+	+	+
Fusarium graminearum	2	Wheat	-	-	-	-
Blumeria graminis	2	Wheat	-	-	-	-
Rhizoctonia cerealis	3	Wheat	-	-	-	-
Puccinia striiformis	3	Wheat	-	-	-	-
Bipolaris sorokiniana	3	Wheat	-	-	-	-
Alternaria solani	1	Tomato	-	-	-	-

3

Figure 1

Distribution of primers L-139 and L-988 for LAMP assays on DNA sequences.

(A) The position of L-139 in AJ236139.1. (B) The position of L-988 in DQ132988.1.

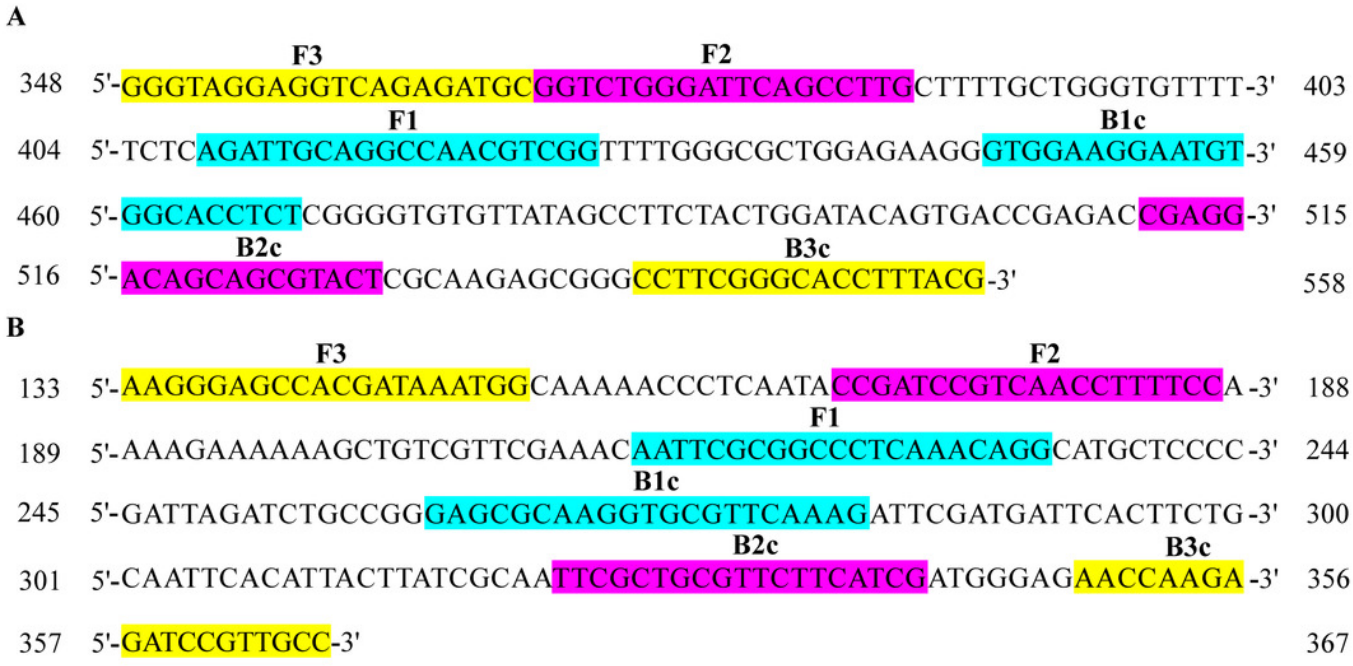

Figure 2

PCR amplification of template DNA with different primers.

M: DNA maker, 1-6: ITS-4/ITS-5, JN367334.1, AF135430.1, JN367307.1, KP256755.1, AJ236139.1.

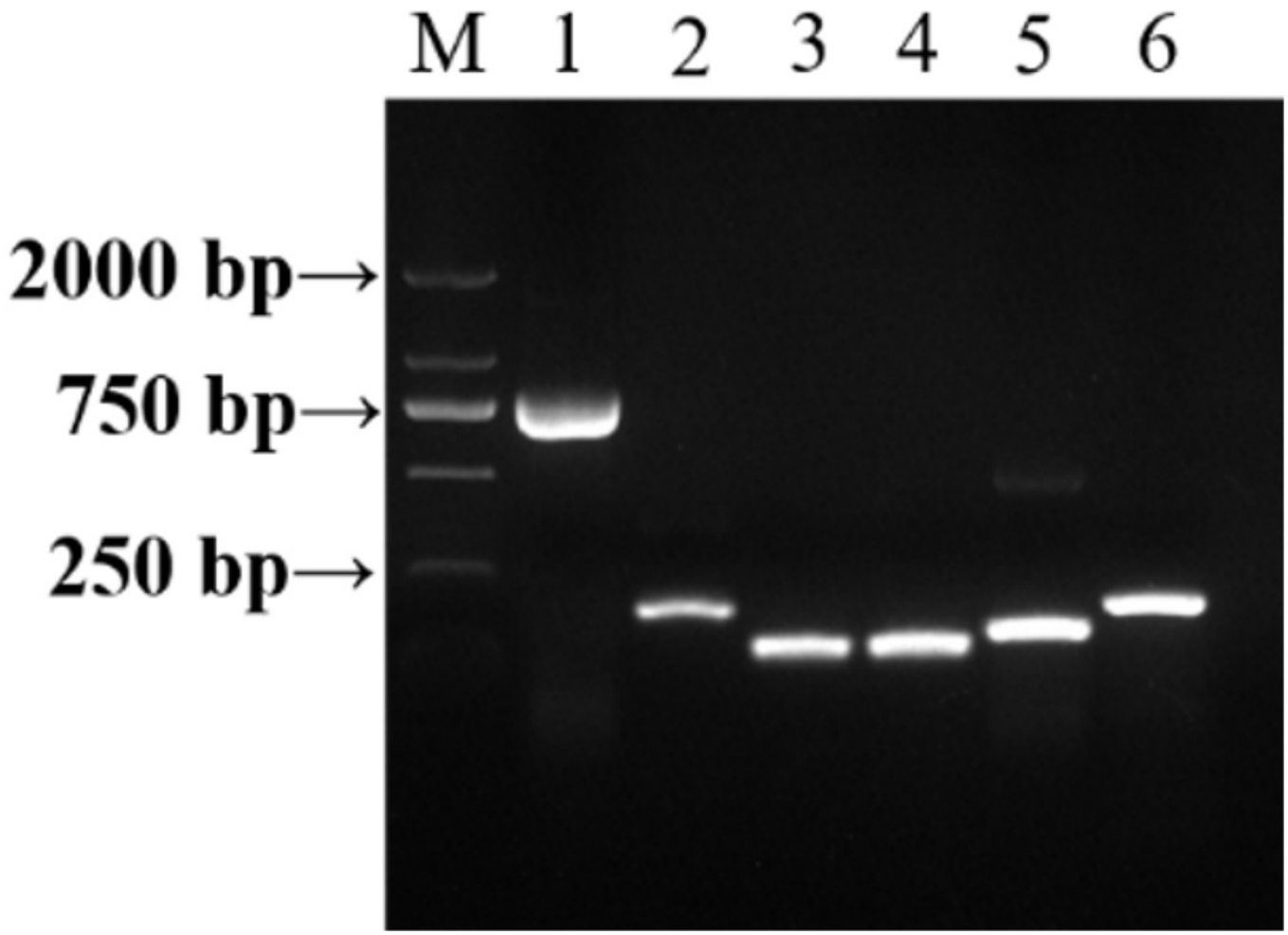

Figure 3

. Amplification curves of qPCR for control fungi using five sets of primers.

(A) primer Y-334. (B) primer Y-430. (C) primer Y-307. (D) primer Y-755. (E) primer Y-139. Color 1: Ustilago tritici. Color 2: Fusarium graminearum. Color 2-7: Fusarium graminearum, Blumeria graminis, Rhizoctonia cerealis, Puccinia striiformis, Bipolaris sorokiniana, Alternaria solani. Color 3-7: Blumeria graminis, Rhizoctonia cerealis, Puccinia striiformis, Bipolaris sorokiniana, Alternaria solani.

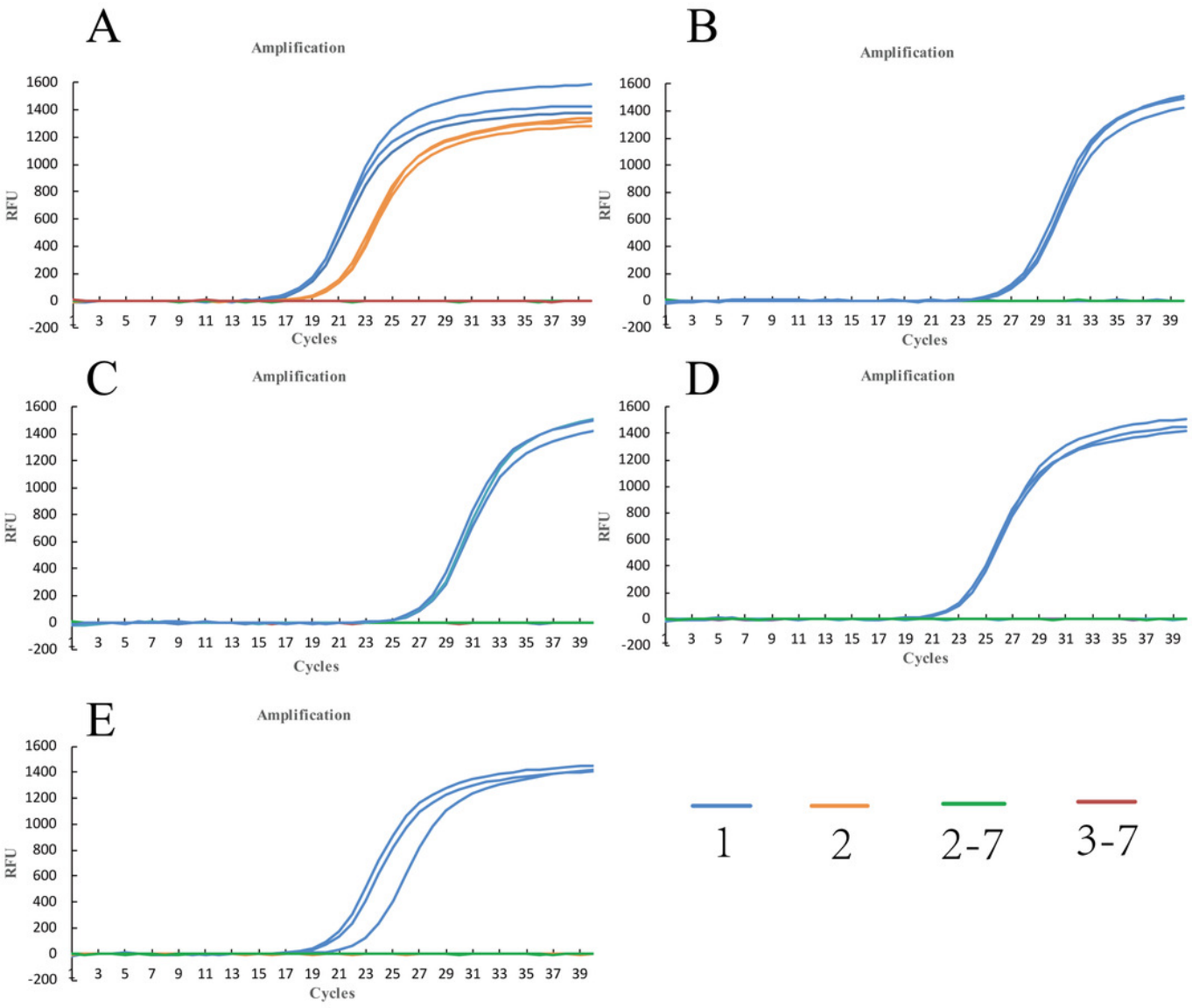
A
Amplification
RFU
Cycles
B
Amplification
RFU
Cycles
C
Amplification
RFU
Cycles
D
Amplification
RFU
Cycles
E
Amplification
RFU
Cycles
1
2
2-7
3-7

Figure 4

Volume Optimization of qPCR for ChamQTM SYBRR qPCR Master Mix.

M: Maker, 1-7: 4 μL, 6 μL, 8 μL, 10 μL, 12 μL, 14 μL, 16 μL.

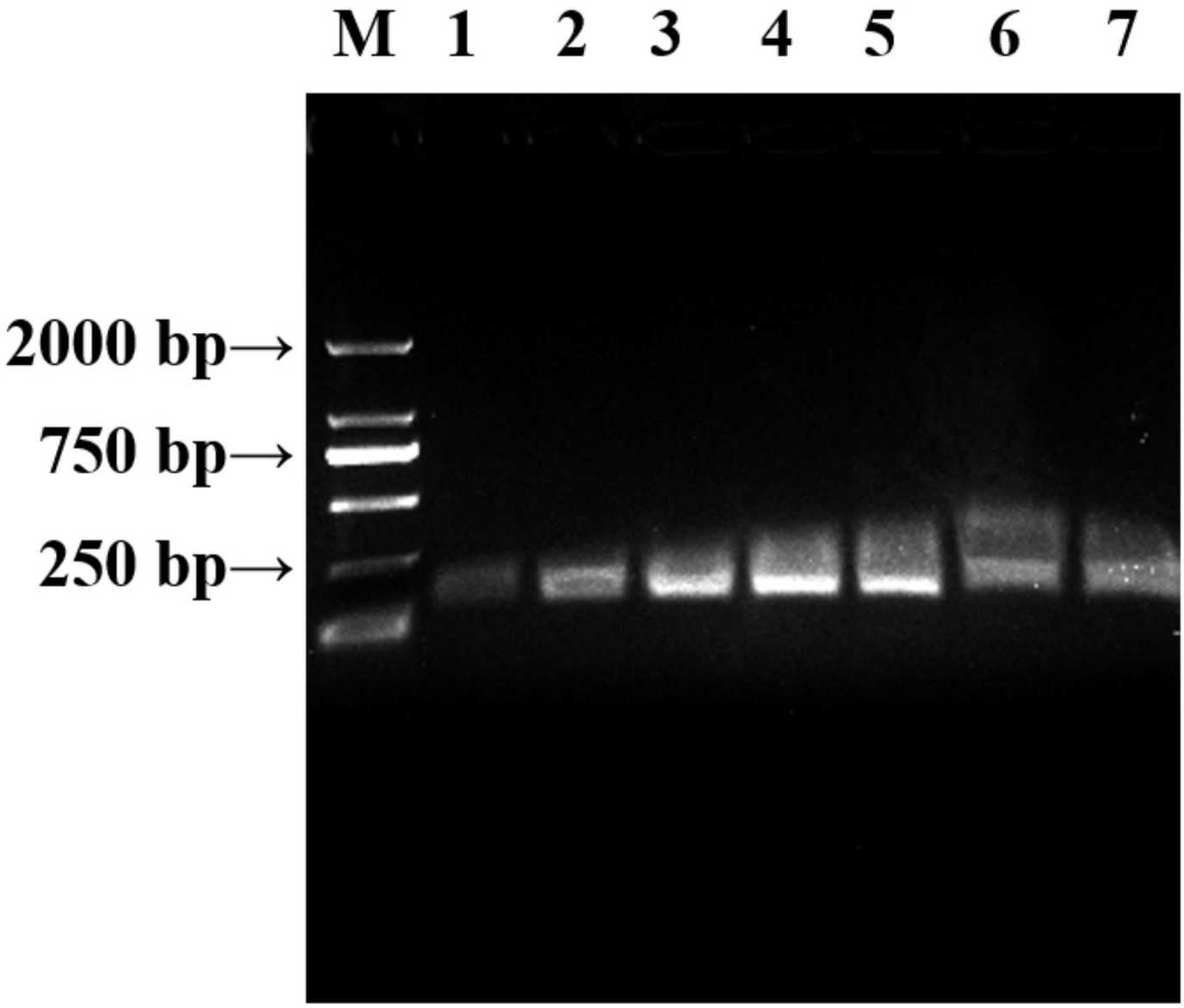

Figure 5

Temperature optimization of qPCR.

(A) Amplification curves of qPCR for Ustilago tritici under temperature gradients. 1-7: 52 ℃, 54 ℃, 56 ℃, 58 ℃, 60 ℃, 62 ℃, 64 ℃.(B) Gel electrophoresis of qPCR on Ustilago tritici under temperature gradients M: Maker, 1-7: 52 ℃, 54 ℃, 56 ℃, 58 ℃, 60 ℃, 62 ℃, 64 ℃.

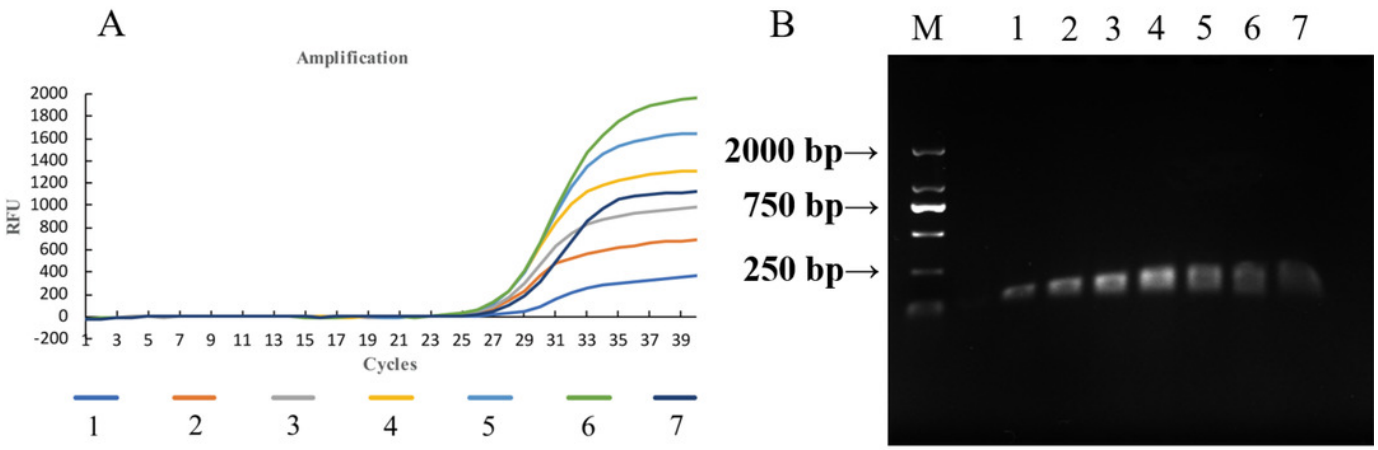

Figure 6

qPCR for Ustilago tritici.

The amplification curves of qPCR for AJ236139.1(A), KP256755.1(C), JN367307.1(E), AF135430.1(G). The melting curves of qPCR for AJ236139.1(B), KP256755.1(D), JN367307.1(F) and AF135430.1(H).

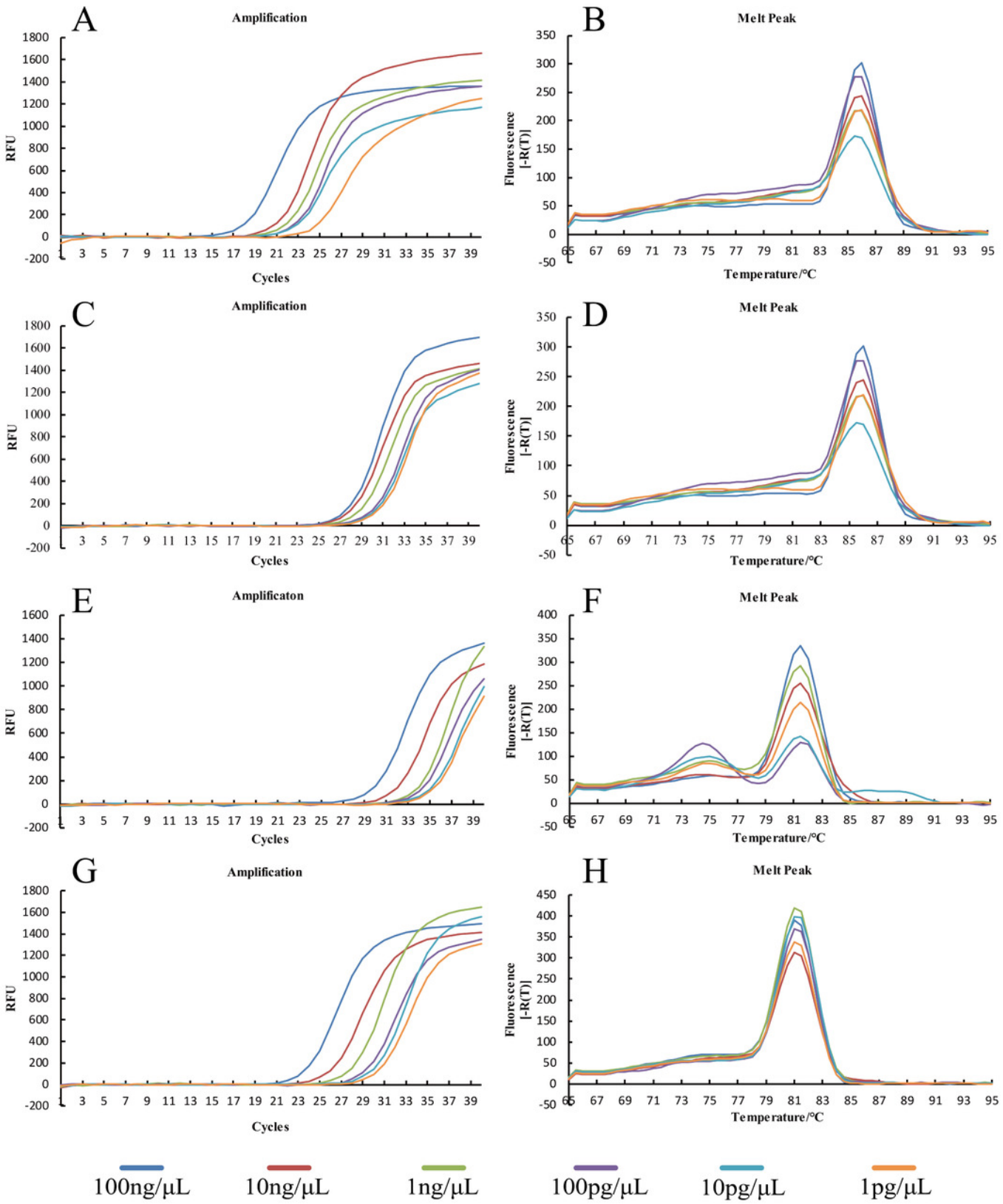
A
Amplification
RFU
Cycles
B
Melt Peak
Fluorescence [-R(T)]
Temperature/°C
C
Amplification
RFU
Cycles
D
Melt Peak
Fluorescence [-R(T)]
Temperature/°C
E
Amplificaton
RFU
Cycles
F
Melt Peak
Fluorescence [-R(T)]
Temperature/°C
G
Amplification
RFU
Cycles
H
Melt Peak
Fluorescence [-R(T)]
Temperature/°C
100ng/μL
10ng/μL
1ng/μL
100pg/μL
10pg/μL
1pg/μL

Figure 7

Sensitivity detection of qPCR assays by primer Y-430.

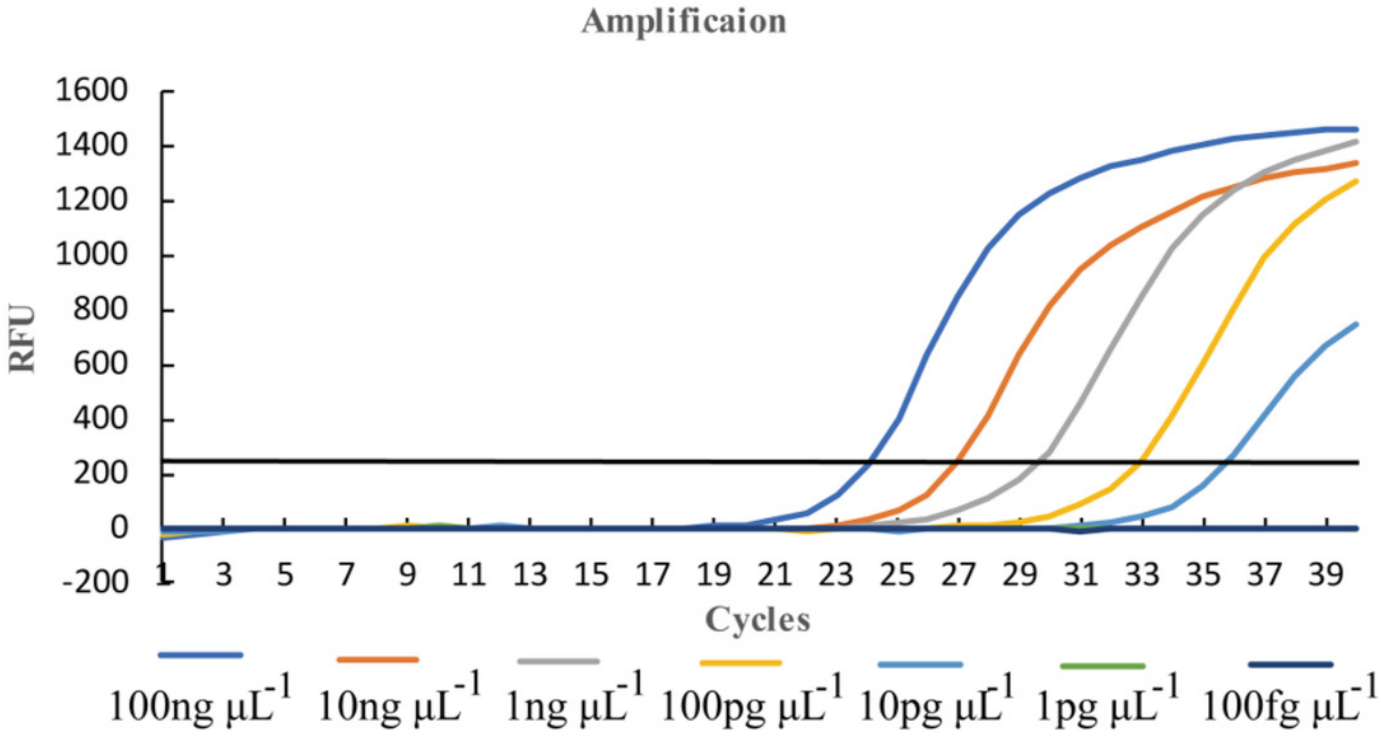

Figure 8

The results of PCR.

(A) Observing the amplification results in the gel imaging system. (B) Observing the amplification results by the naked eye. 1-3: L-139. 4-6: L-988. 7: Negative control.

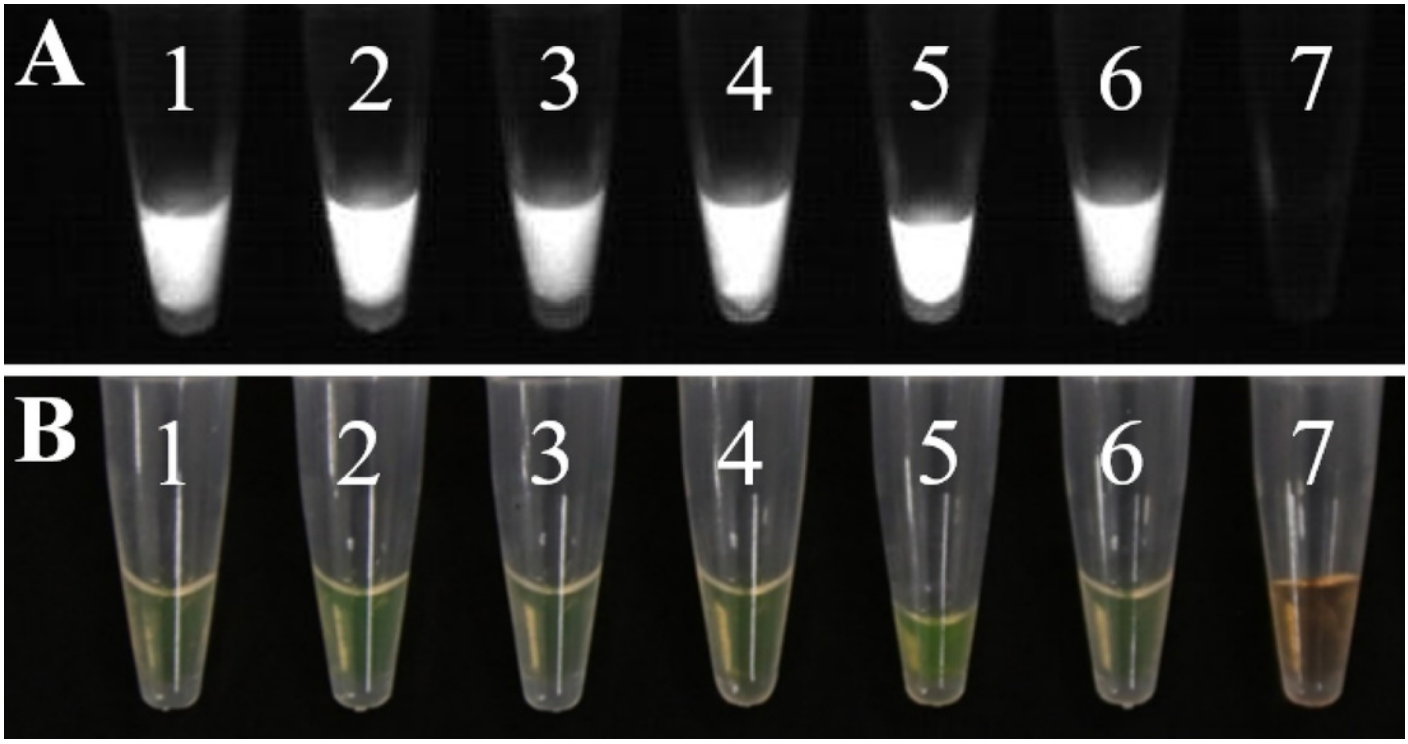

Figure 9

Specific detection of LAMP assays.

1-7: The results of LAMP assay with L-139. DNA from 1-7: Ustilago tritici, Fusarium graminearum, Blumeria graminis, Rhizoctonia cerealis, Puccinia striiformis, Bipolaris sorokiniana, Alternaria solani. 8-14: The results of LAMP assay with L-988. DNA from 8-14: Ustilago tritici, Fusarium graminearum, Blumeria graminis, Rhizoctonia cerealis, Puccinia striiformis, Bipolaris sorokiniana, Alternaria solani.

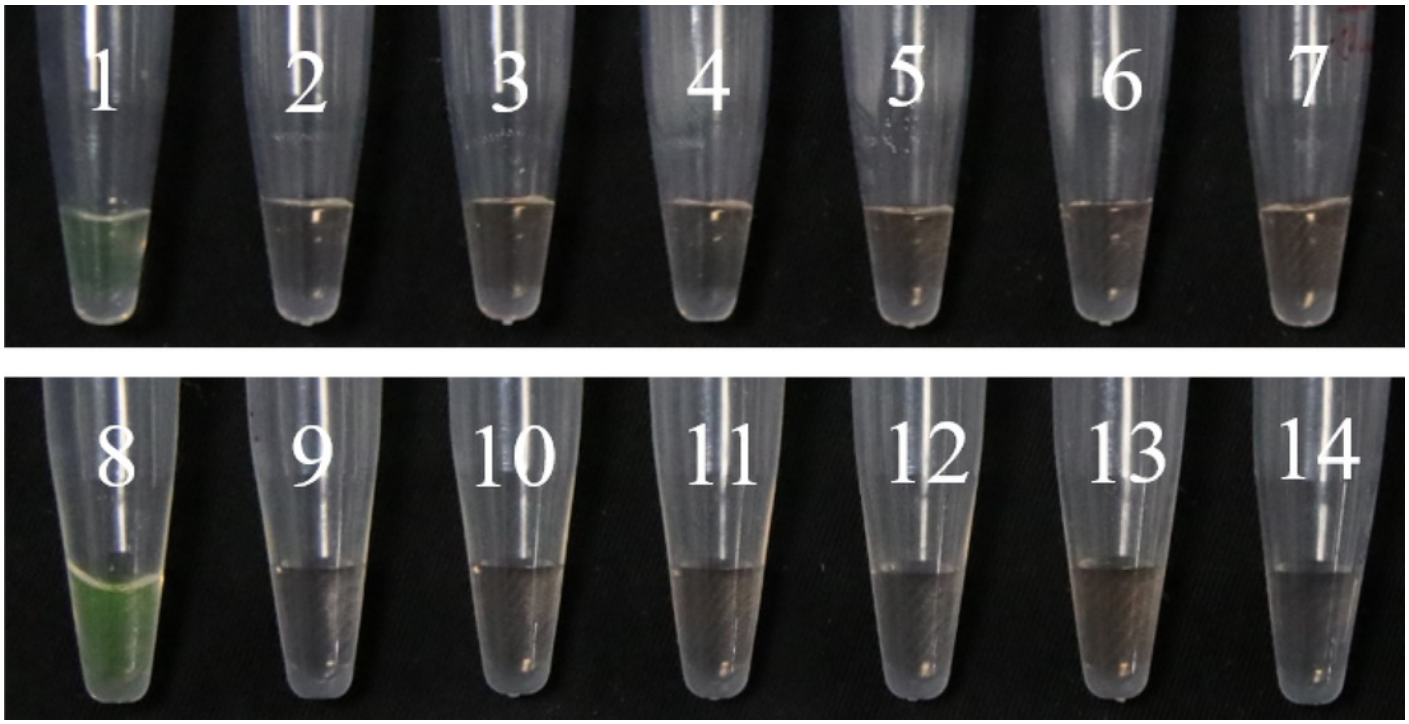

Figure 10

Temperature optimization and sensitivity detection of LAMP assays

(A) 1-9: 58 ℃, 59 ℃, 60 ℃, 61 ℃, 62 ℃, 63 ℃, 64 ℃, 65 ℃, 66 ℃.

(B) DNA concentration from 1-9: 100 ng uL-1, 10 ng uL-1, 1 ng uL-1, 100 pg uL-1, 10 pg uL-1, 1 pg uL-1, 100 fg uL-1, 10 fg uL-1, 1 fg uL-1.

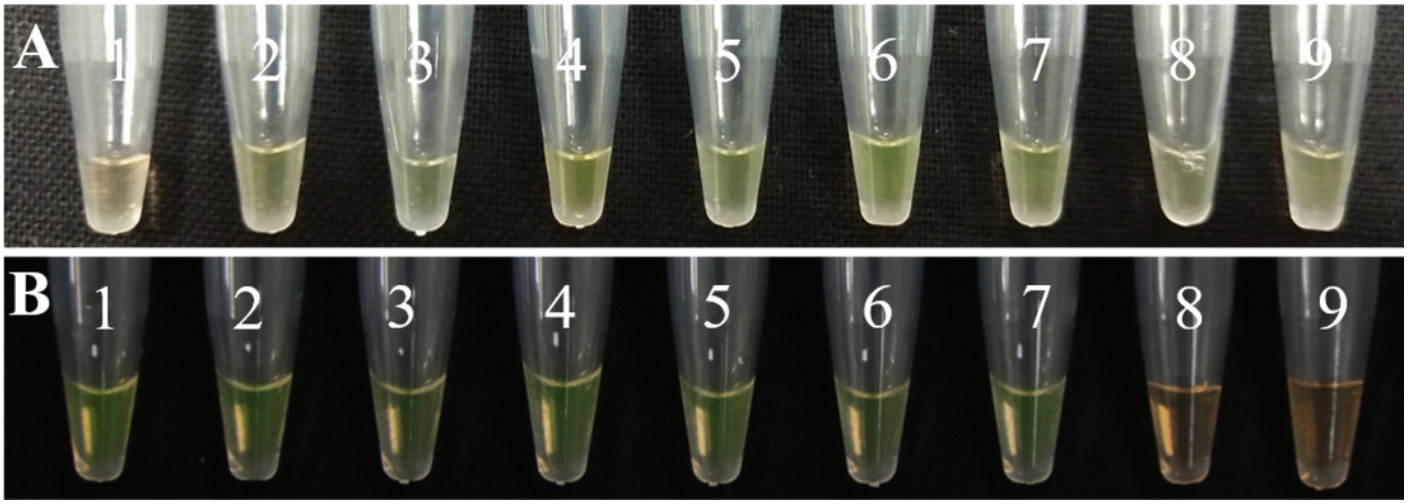

Figure 11

The results of LAMP assay.

(A) Observing the amplification results in the gel imaging system. (B) Observing the amplification results of the LAMP assays by the naked eye. 1-3: L-139. 4-6: L-988. 7:Negative control.

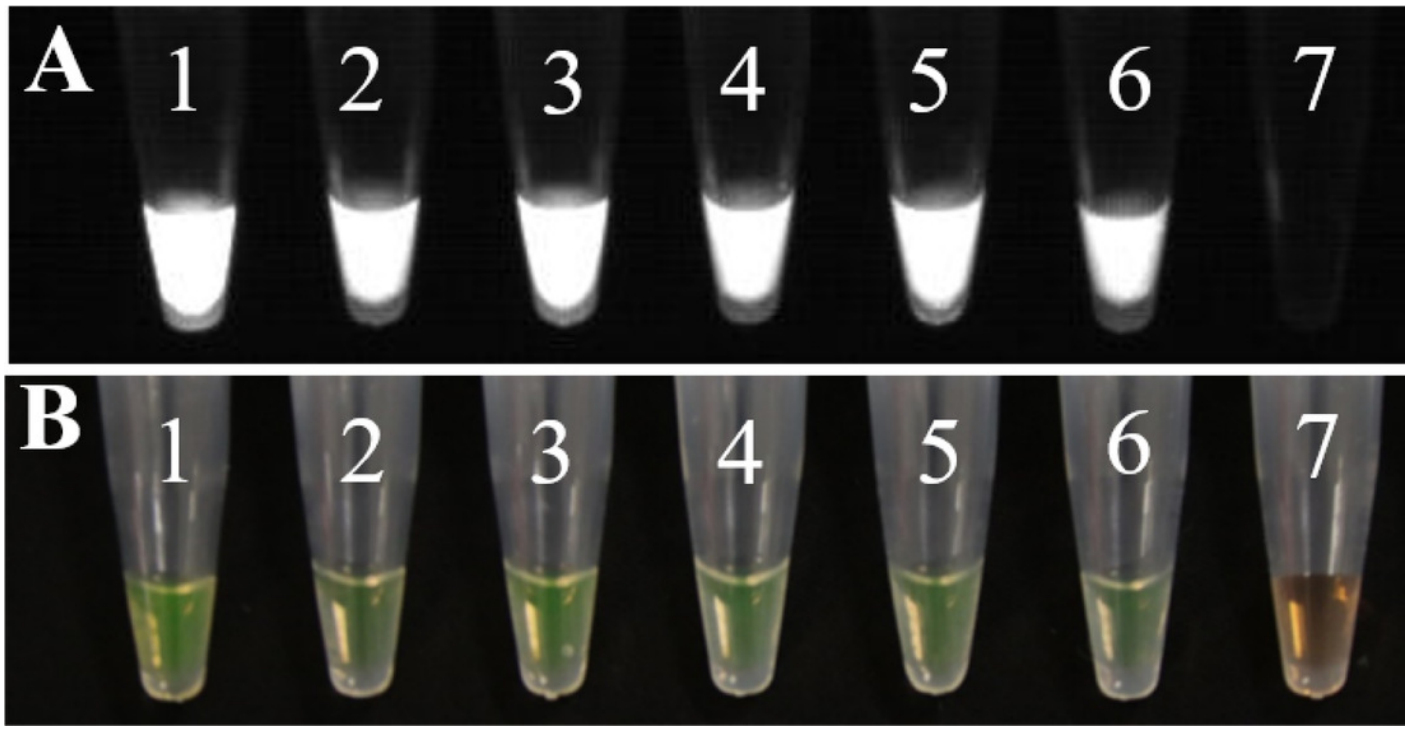

Figure 12

Detection of diseased seed in the field by qPCR and LAMP assays.

(A) Amplification curves of qPCR for detection of wheat loose smut in field by primer Y-430. (B) Melting curves of qPCR for detection of wheat loose smut in field by primer Y-430. (C) LAMP detection of wheat loose smut in the field under natural light. Tube 1–2: The results of LAMP assay with L-139. Tube 3–4: The results of LAMP assay with L-988. D. Detection by agarose electrophoresis after LAMP assay. Strip 1-2: The results of LAMP assay with L-139. Strip 3-4: The results of LAMP assay with L-988.