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

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Wheat loose smut caused by *Ustilago tritici* a seed-borne disease, is difficult to control due to the expansion of wheat planting area and difficulty of pathogen detection. In this study, real-time fluorescence quantitative PCR (qPCR) and loop-mediated isothermal amplification (LAMP) assays were used to rapidly amplify the DNA of *U*. *tritici*. Five pairs primers for qPCR and two series primers for LAMP were designed. Firstly, the specify of primers were carried out by using the DNAs of *U*. *tritici*, *Fusarium graminearum*, *Blumeria graminis*, *Rhizoctonia cerealis*, *Puccinia striiformis*, *Bipolaris sorokiniana*, and *Alternaria solani* as templates. Then the amplification systems are optimized. Finally, the sensitivity of qPCR and LAMP assays were quantified. The results show that using the primers pairs Y430F/R, Y307F/R, Y755F/R and Y139F/R for qPCR, primers L-139 and L-988 for LAMP assay could be used for *U*. *tritici* detection. In the sensitivity test, the detection limit of qPCR assay is 10 pg μL^{-1} of genomic DNA, the detection limit of LAMP assay is 100 fg μL^{-1} . We successfully performed qPCR and LAMP assays on two wheat loose smut wheat samples, and confirmed sequenced *U*. *tritici* infection by subsequently sequencing. This paper established two methods for *U*. *tritici* detection, which could be used for wheat loose smut diagnose in lab and field.

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ABSTRACT

Wheat loose smut caused by *Ustilago tritici* a seed-borne disease, is difficult to control due to the expansion of wheat planting area and difficulty of pathogen detection. In this study, real-time fluorescence quantitative PCR (qPCR) and loop-mediated isothermal amplification (LAMP) assays were used to rapidly amplify the DNA of *U. tritici*. Five pairs primers for qPCR and two series primers for LAMP were designed. Firstly, the specify of primers were carried out by using the DNAs of *U. tritici*, *Fusarium graminearum*, *Blumeria graminis*, *Rhizoctonia cerealis*, *Puccinia striiformis*, *Bipolaris sorokiniana*, and *Alternaria solani* as templates. Then the amplification systems are optimized. Finally, the sensitivity of qPCR and LAMP assays were quantified. The results show that using the primers pairs Y430F/R, Y307F/R, Y755F/R and Y139F/R for qPCR, primers L-139 and L-988 for LAMP assay could be used for *U. tritici* detection. In the sensitivity test, the detection limit of qPCR assay is 10 pg μL^{-1} of genomic DNA, the detection limit of LAMP assay is 100 fg μL^{-1}. We successfully performed qPCR and LAMP assays on two wheat loose smut wheat samples, and confirmed sequenced *U. tritici* infection by subsequently sequencing. This paper established two methods for *U. tritici* detection, which could be used for wheat loose smut diagnose in lab and 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 planting area is as high as 220 107 600 hectares (Zhao et al., 2018). With the popularization of wheat planting area and the immatureness seed detection technology, the damage of wheat loose smut has become more serious, and it has gradually become one of the most important diseases in wheat field (Knox & Menzies, 2012).

At present, the most effective control method of wheat loose smut disease is seed dressing sterilization before seeding (Singh et al., 2014; Duan et al., 2016). There are no suitable and effective control measures after sowing. The long-term use of chemical agents can easily pollute the environment, endanger the health of humans and livestock, and a large number of single used can easily lead to the emergence of drug resistance (Crane et al., 2013). Therefore, the detection of pathogen *Ustilago tritici* is very necessary. The original detection method of *U. tritici* is serological identification, but it is time-consuming and can only be concluded by professional. Meanwhile, its accuracy and sensitivity are not satisfactory (Walcott, 2003; Munkvold, 2009). Now, the common detection method for wheat loose smut is PCR (Martínez-Espinoza et al., 2003). However, the PCR cannot perform accurate quantitative analysis, and cross-contamination can easily cause false positive (Bretagne, 2003). Quantitative PCR (qPCR) has quickly become a hotspot in scientific research and clinical diagnosis (Kuypers et al., 2006; Yan et al., 2012). At the same time, there are some shortcomings of it, such as the expensive instruments, the need for professional personnel operation, poor practicality. 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 to design 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), under the action of *Bst* DNA polymerase, the water bath instead of the PCR instrument, the reaction is performed at 60-65 °C for 60-90 min and the target DNA is amplified 10^9-10^{10} times (Dhama et al., 2014). Compared to PCR, the LAMP assay shortens the reaction time, eliminates the gel electrophoresis, does not require expensive PCR instruments, and completes the experiment with the *Bst* DNA polymerase and reaction under constant temperature conditions. The reaction results are determined by the color reaction of fluorescent dyes. Currently, the fluorescent dyes are calcein (Rane et al., 2015), PicoGreen (Curtis, Rudolph & Owen, 2008), hydroxy naphthol blue (HNB) (Goto et al., 2010; Mohon et al., 2014), SYBR Green (Balne et al.,

2013; Zhou et al., 2014), etc. SYBR Green I and HNB have the highest detection sensitivity between them, which is 10 times of calcein (Gao et al., 2009). And HNB and SYBR Green I can produce long-term stable color changes and brightness in closed pipes and prevent cross-contamination (Almasi et al., 2013). Meanwhile, its high affinity with double-stranded DNA makes it one of the most commonly used fluorescent dyes. In this study, SYBR Green I was used in both qPCR assay and LAMP assay. However, it can bind to both specific and non-specific products so that the specificity of the primers should be further identified. Therefore, in qPCR assay, an extra melting curves were performed to identify the specificity of the primers. And in LAMP assay, the primers were screened by PCR to determine whether the primers form dimers, and the control bacteria DNA were used to determine the specificity of the primers. The combination of LAMP and fluorescent dyes makes biodetection simpler and more intuitive. At present, LAMP technology and qPCR has been used in many bacteria, fungi, viruses and other aspects detection, such as *Verticillium albo-atrum* (Tian et al., 2016), *Listeria monocytogenes* strains (Wang et al., 2012), parasites (Abdul-Ghani, Al-Mekhlafi & Karanis, 2012), *Candidatus Liberibacter asiaticus* (Rigano et al., 2014), etc. Now there have been reports on the detection of *Rhizoctonia cerealis* (Sun et al., 2015), *Tilletia controversa* Kühn (Nian et al., 2009) by qPCR assays. And the detection of Fusarium head blight (Niessen & Vogel, 2010), wheat stripe rust (Huang et al., 2011) by LAMP assays. However, the detection of wheat loose smut by two methods has not been reported. In this study, we used qPCR and LAMP assays to rapidly detect wheat loose smut.

MATERIALS AND METHODS

Materials

Bacterial strain.

Ustilago tritici, *Fusarium graminearum*, *Blumeria graminis*, *Rhizoctonia cerealis*, *Puccinia striiformis*, *Bipolaris sorokiniana*, *Alternaria solani*.

Culture environment.

The wheat variety used in the experiment is Mingxian169. After germination for 24 h in dark conditions, the seeds were planted in a pot and placed in a light incubator at 22 °C, 12 h days and 18 °C, 12 h night. *U. tritici* were collected from the diseased wheat ears. Wheat powdery mildew were derived from diseased leaves. *F. graminearum*, *R. cerealis*, *B. sorokiniana*, *A. solani* were inoculated on the PDA mediums covered with glass paper, and cultured at 25 °C for 7 to 8 days. *P. striiformis* was collected in the experimental field.

Genomic DNA extraction.

DNA was extracted from wheat loose smut by modified CTAB. Briefly, lysis buffer (10 mM Tris-HCl [pH 8.0], 100 mM EDTA, 0.5% sodium dodecyl sulfate and 100 μg mL^{-1} proteinase) was added to the sample, followed by a water bath at 55 °C for 1 h. The DNA was extracted with phenol-chloroform-isoamyl alcohol (25:24:1), precipitated with isopropanol and washed with ethanol (70%). After centrifugation, 30 μL of ddH_2O was added and stored at -20 °C.

Methods

The qPCR and Lamp primer design.

Primers (Table 1) were designed by Primer Premier 5.0 according to the sequences of *U. tritici* sequences published in NCBI. We selected the AJ236139.1, DQ132988.1 nucleic acid sequence for LAMP assay, and designed primers for AJ236139.1 and DQ132988.1 by Primer Explorer V5 (http://primerexplorer.jp/lampv5e/index.html) (Table 2). The primers were synthesized by BGI, and dissolved in ddH_2O, stored at -20 °C.

QPCR assays

Specificity of qPCR assays.

DNA of *U. tritici* are used as template for primers specific detection, the amplification condition is 95 °C for 3 min, 95 ℃ for 30 s, 52 ℃ for 30 s, 72 ℃ for 40 s, 34 cycles, 72 ℃ for 5 min, preservation at 16 ℃. The amplification products are detected by 1% agarose gel electrophoresis and then sequenced (Fig. 1). Based on the preliminary screening results, primers are further screened by qPCR using the control strains as temples, and then determined whether the primers were specific by the Ct value reflected with the amplification curves (Fig. 2).

Optimization of qPCR assays.

Appropriate ratios have an impact on the accuracy of the results of qPCR so that system optimization is indispensable for it. System Optimization experiment was performed on the volume of ChamQ^{TM} SYBR qPCR Master Mix with 4 μL, 6 μL, 8 μL, 10 μL, 12 μL, 14 μL and 16 μL volume gradient for qPCR assays (Fig. 3). After demonstrating the optimum system ratio, the temperature was tested. We designed seven temperature gradients, 52 °C, 54 °C, 56 °C, 58 °C, 60 °C, 62 °C, 64 °C (Fig. 4). The melting temperature and gel electrophoresis can be combined to determine the optimum temperature.

QPCR for *U. tritici*.

The reaction system is 20 μL: DNA 1 μL, each of the forward and reverse primer is 1 μL, Master Mix 10 μL, ddH_2O 7 μL. The reaction condition is 94 ℃ for 3 min, 94 ℃ for 20 s, 60 ℃ for 30 s, 40 cycles, 72 ℃ for 5 min. Each template DNA is diluted to 6 concentration gradients, with 3 replicates in each group. Then took the melting curves to verify the specificity of the results (Fig. 5).

Sensitivity detection of qPCR assays.

We used 100 ng μL^{-1} DNA as the template and diluted to seven concentration gradients. These DNA concentrations are 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}. Then performed qPCR on Y430 with two replicates per concentration (Fig. 6).

LAMP assay

Specificity of LAMP assays.

Tested whether the primers were normal and whether there was primer-dimer formation by PCR assay. The PCR system is 25 μL: DNA 0.5 μL, dNTPs 1 μL, *Taq* DNA polymerase 1 μL, F3 1 μL, B3 1 μL, $MgSO_4$ 1 μL, 2 x Phanta Max Buffer 12.5 μL, ddH_2O 7 μL, the two sets of primers are F3-1F/B3-1R, F3-2F/B3-2R, and each set of primers had three replicates. The control template is complemented by ddH_2O. Added the fluorescent dye to the amplification results (Fig. 7). And then *F. graminearum*, *B. graminis*, *R. cerealis*, *P. striiformis*, *B. sorokiniana*, *A. solani* were used as the controls for LAMP assay (Fig. 8).

Temperature optimization of LAMP assay.

In order to determine the accuracy and sensitivity of the results, the system concentration and temperature of LAMP assay were optimized. Here, the concentration we used is already the optimal concentration ratio. The concentration ratio of the inner and outer primers used in this experiment are 8:1, the concentration of FIP and BIP are 1.6 μmol L^{-1}, F3 and B3 are 0.2 μmol L^{-1}. The concentration of Mg^{2+} referred to the relevant literatures (Kubota et al., 2008; Abdelsalam et al., 2011) is 6 mmol L^{-1}. We designed nine temperature gradients for LAMP assay to determine the optimum temperature based on the final color reaction (Fig. 9).

LAMP assays on *Ustilago tritici*.

The LAMP reaction system used in the experiment is 25 μL: DNA 1 μL, 10 x *Thermopol* Buffer 2.5 μL, $MgSO_4$ 2 μL, dNTPs 2 μL, F3 1 μL, B3 1 μL, FIP 1 μL, BIP 1 μL, *Bst* DNA polymerase 1 μL, Betaine 5 μL, ddH_2O 7.5 μL, and each set of primers is repeated three times. The control template is complemented by ddH_2O (Fig. 10).

Sensitivity detection of LAMP assays.

We diluted the extracted DNA 10 times and then used it as the template and then diluted the template DNA to 9

concentration gradients, and the concentrations are 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}, 1 fg μL^{-1}. Observing the experimental results with naked eyes under natural light, the experimental results can be judged according to the color (Fig. 11).

RESULTS

In qPCR assays, in order to determine whether the designed primers are specific, we performed PCR experiments on the DNA of the *Ustilago tritici* (Fig. 1), and it was very accurate to obtain five sets of sequence bands by primers. Based on the preliminary screening results, primers are further screened by qPCR using the control strains as temples, and then determined whether the primers were specific by the Ct value reflected with the amplification curves (Fig. 2). The results show that Y334 can amplify *Fusarium graminearum* (Fig. 2A) and there for it is not specific for *U. tritici*. The primers Y430, Y307, Y755, Y139 can specifically amplify the target DNA. The four sets of primers were used to detect the *U. tritici*. By optimizing the system, we get the most volume added to ChamQTM SYBRqPCR Master Mix. The results of gel electrophoresis on the amplification are shown in Fig. 3. When the volume of ChamQ^{TM} SYBRqPCR Master Mix was added to 8-12 μL, the amplification results are the best. We took the intermediate value, the optimal volume of ChamQ^{TM} SYBR qPCR Master Mix added in qPCR assay was 10 μL. At the same time, in the results of qPCR temperature gradient test on *U. tritici*, the relative fluorescence unit can reach a higher value at 58 °C and 60 °C, and the Ct value is about thirty-three, which is in line with our expected results. In gel electrophoresis, the band shown at 58 °C is the brightest. In combination with Fig. 4A and Fig. 4B, we finally chose 58 °C as the temperature set in the final experiment. The amplification curves of the four pairs of primers show that the Ct values ranged from twenty-nine to thirty-five for samples (Fig. 5). It indicated that the four sets of primers could amplify the target DNA under certain concentration conditions. The melting curves corresponding to

each primer exhibit a single peak, which further ruled out non-specific amplification. Combining the amplification curves and the melting curves, it can be known 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 as the template and diluted to seven concentration gradients to verify the lowest concentration detectable by qPCR. The results of seven concentrations of fluorescence show (Fig. 6) that the first five concentration gradients had fluorescence signal accumulation so that the lowest DNA concentration detected by qPCR is 10 pg μL^{-1}.

In LAMP assays, the results of the PCR system under natural light and gel imager (Fig. 7) indicated that primers designed for LAMP assay can be used for PCR amplification. Then pathogens such as *F. graminearum* and so on (Table 3) as controls to verify the specificity of the primers. In order to determine the specificity of the primers, five other fungal diseases commonly found in wheat were selected as controls. At the same time, a group of non-wheat fungi were selected as controls. L-139 and L-988 were used to perform LAMP assays on seven different bacteria (Fig. 8). The results show that L-139 and L-988 only amplified the DNA sequence of *U. tritici*. As can be seen from Fig. 9, the optimum reaction temperature for LAMP assay is from 62 °C to 64 °C. This experiment is taken at 63 °C. In the optimal ratio and temperature, we carried out LAMP test on *U. tritici*. Under the gel imaging system (Fig. 10A), the positive samples are white and the negative controls are colorless. Under natural light (Fig. 10B), the three replicates of the two sets of primers show bright green, and the negative controls are light orange. The light orange color of the negative control indicated that no primer-dimer formed and no false positives caused by the external environment. Sensitivity testing of the LAMP test indicates that the DNA concentration of the lowest *U. tritici* detectable by the LAMP assay 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, we used Y334 to perform qPCR and L-139 and L-988 to perform LAMP assay on diseased seed (Fig. 12). In the qPCR results, the Ct value tends to be flat at twenty-seven, and the melting curve also shows a single peak. Similarly, in the LAMP experiment results, both samples show bright green color, and the expected slow band appeared in agarose gel electrophoresis. Combining the results of the two methods, we conclude that we can use qPCR and LAMP technology to efficiently and sensitively detect *U. tritici*.

DISCUSSION

Wheat loose smut is a systemic disease infested flower organ (Ngugi & Scherm, 2006). It currently occurs in all wheat growing area of the world, particularly serious in Canada (Randhawa et al., 2009) and parts of Africa (Zegeye, Dejene & Ayalew, 2015). Due to the expansion of the disease area and the single prevention method that the detection of pathogenic fungi in seeds is particularly important. In this study, both of the qPCR and LAMP assays can specifically, efficiently and accurately amplify the DNA of *Ustilago tritici*.

Our ultimate goal is to find a simple and high-speed detection method. Both of the two methods to detect seeds can help to prevent the spread and occurrence of pathogens from the source. By comparing the two methods, it is not difficult to find 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 melting curve peaks. Combining the amplification curves with melting curves, it was verified that the primers Y430, Y307, Y755, Y139 can specifically, efficiently and accurately detect *U. tritici*. This method avoids the influence of gel electrophoresis and other operations on the result. However, the experimental requirements for personnel operations, equipment, and environment are much high. 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 advantages of high sensitivity, short reaction time, easy operation, and low equipment requirements (Kiddle et al., 2012). After its combination with fluorescent dyes, the color reaction is more favorable for our observation of the amplification results so that it is widely used 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 concentrations of them are higher than PCR. However, there are still many problems in the basic application of the two methods. For example, 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 that the LAMP assay has a high false positive rate. And the field situation is complicated, the reaction system and concentration ratio are difficult to optimize, etc.

At present, the qPCR and LAMP assays are being improved. On the basis of qPCR, multiplex PCR have emerged to make up for the shortcomings of detecting only one disease at a time. The various conditions of qPCR assay limit its ability to be applied to field-based assays. Accurate analysis of the data and good reproducibility of the experiment make it become the ubiquitous mainstay of molecular biology. With the deepening of molecular biology, fluorescence quantification will become an indispensable part of it. At the same time, the Multiple LAMP assay have also been proposed to accelerate the efficiency of detection (Chen et al., 2016; Lodh et al., 2017). And kits for LAMP assay have been developed (Marti, Stalder & González, 2015), which eliminates system optimization and matching, thus simplifying the procedure. Its advantages make it well suited for use in resource-poor areas. With the advancement of technology and humanity, chemical control will be gradually replaced by early prevention. The LAMP method is a very valuable diagnostic alternative with a potential of being used also in endemic settings. The improvement of LAMP technology will make it

more promising in disease prevention and control.

Conflicts of interest

Compliance with Ethical Standards: The authors declare no conflicts of interest.

Acknowledgments

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Figure 1

PCR amplification of template DNA with different primers

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

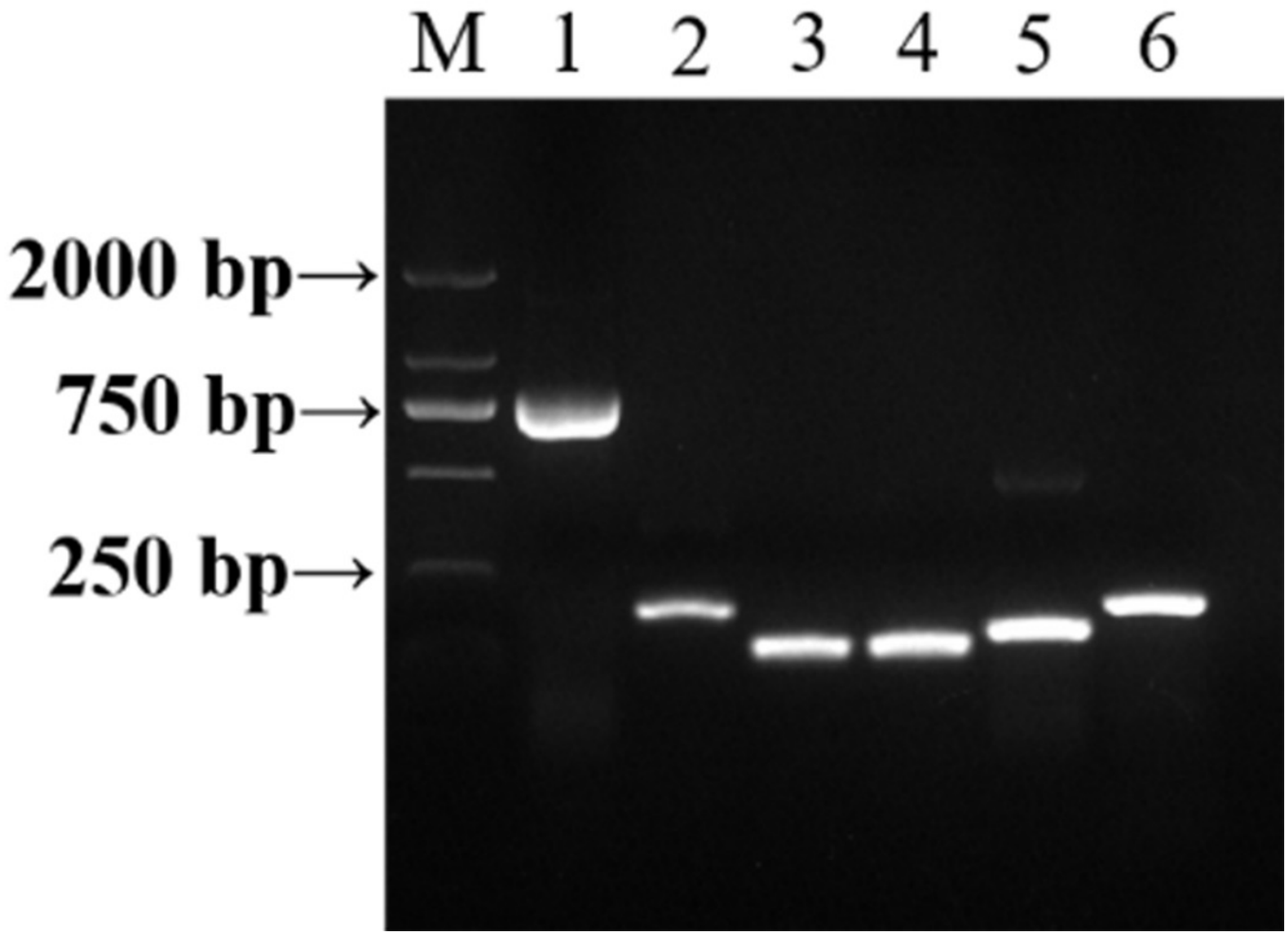


Figure 2(on next page)

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

(A): primers Y334. (B): primers Y430. (C): primers Y307. (D): primers Y755. (E): primers Y139. 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.

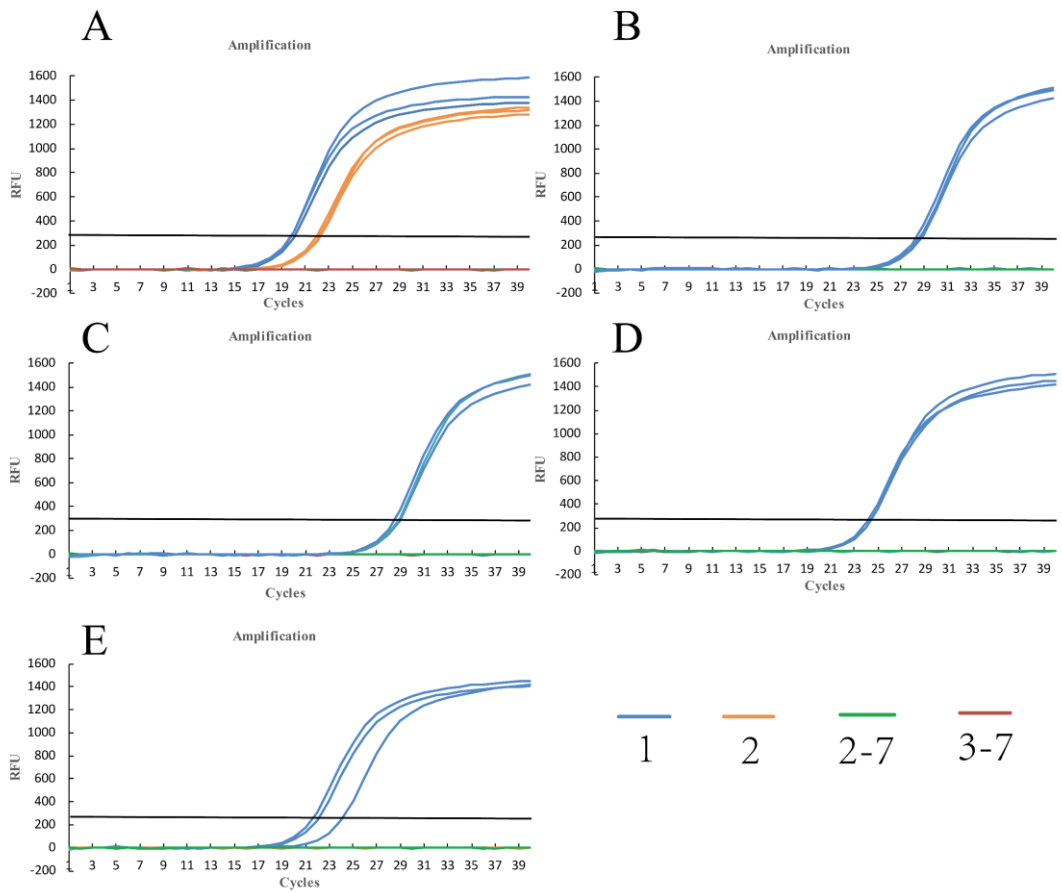

A
Amplification
RFU
Cycles
B
Amplification
RFU
Cycles
C
Amplification
RFU
Cycles
D
Amplification
RFU
Cycles
E
Amplification
RFU
Cycles
1
2
2-7
3-7

Figure 3

Volume Optimization of qPCR for ChamQTM SYBR qPCR Master Mix.

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

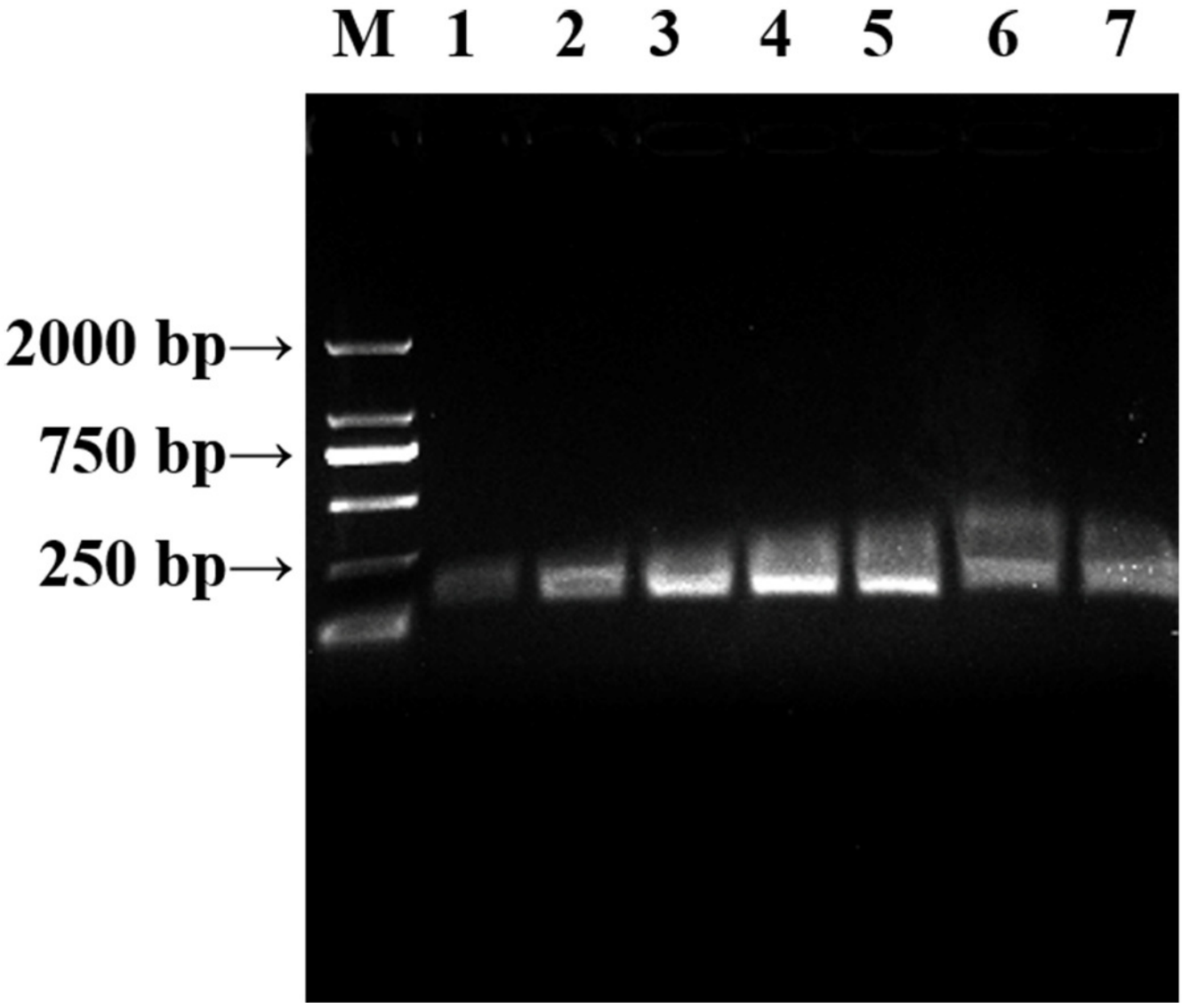


Figure 4(on next page)

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 ℃.

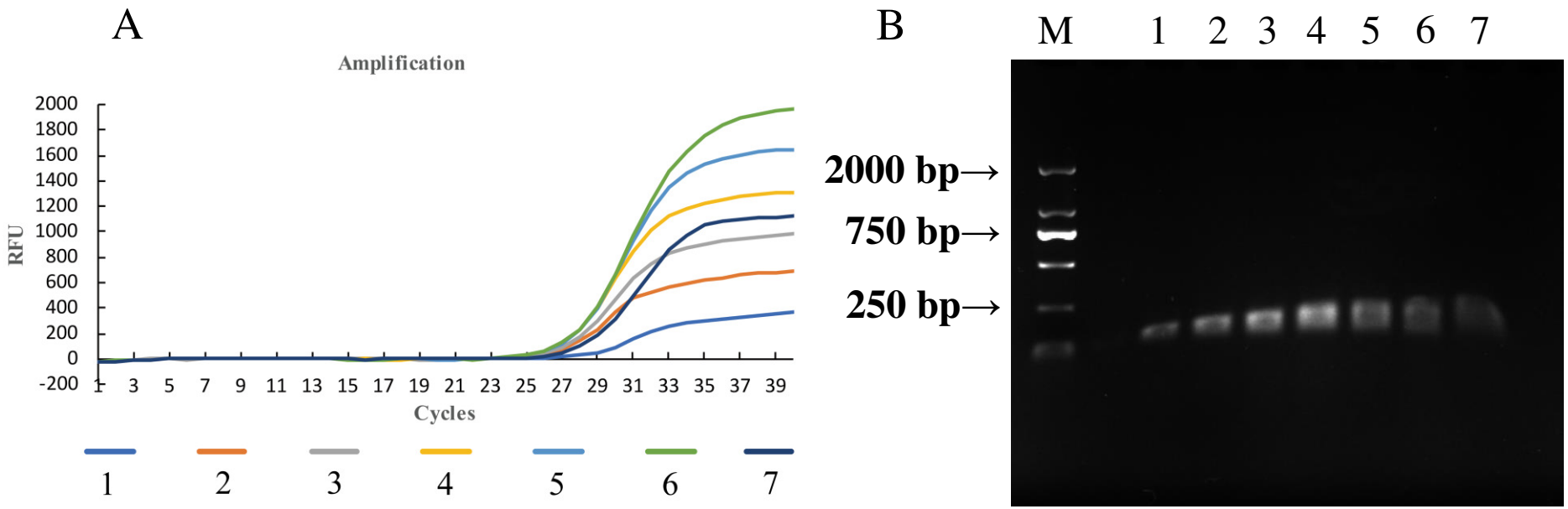

A
Amplification
RFU
2000
1800
1600
1400
1200
1000
800
600
400
200
0
-200
1 3 5 7 9 11 13 15 17 19 21 23 25 27 29 31 33 35 37 39
Cycles
1 2 3 4 5 6 7
B
M 1 2 3 4 5 6 7
2000 bp→
750 bp→
250 bp→

Figure 5(on next page)

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).

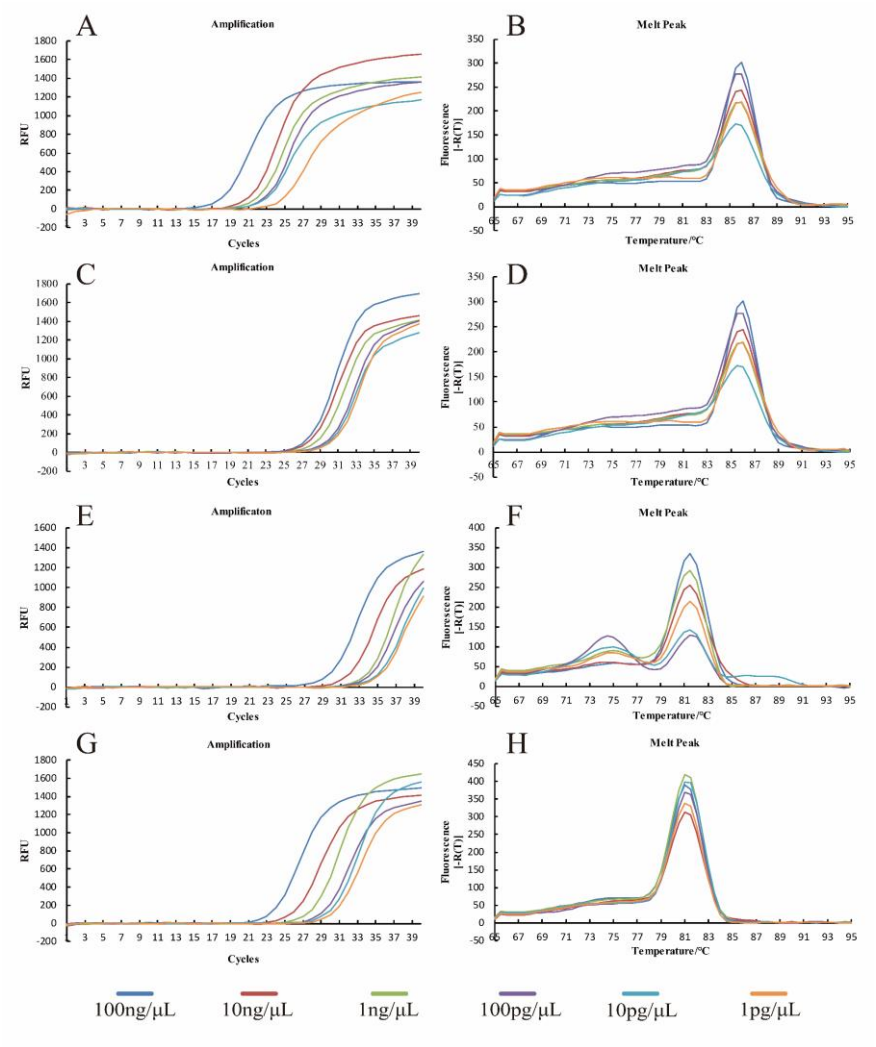

A
Amplification
RFU
Cycles
B
Melt Peak
Fluorescence [-R(T)']
Temperature/°C
C
Amplification
RFU
Cycles
D
Melt Peak
Fluorescence [-R(T)']
Temperature/°C
E
Amplificaton
RFU
Cycles
F
Melt Peak
Fluorescence [-R(T)']
Temperature/°C
G
Amplification
RFU
Cycles
H
Melt Peak
Fluorescence [-R(T)']
Temperature/°C
100ng/μL
10ng/μL
1ng/μL
100pg/μL
10pg/μL
1pg/μL

Figure 6(on next page)

Sensitivity detection of qPCR assays by primer Y430.

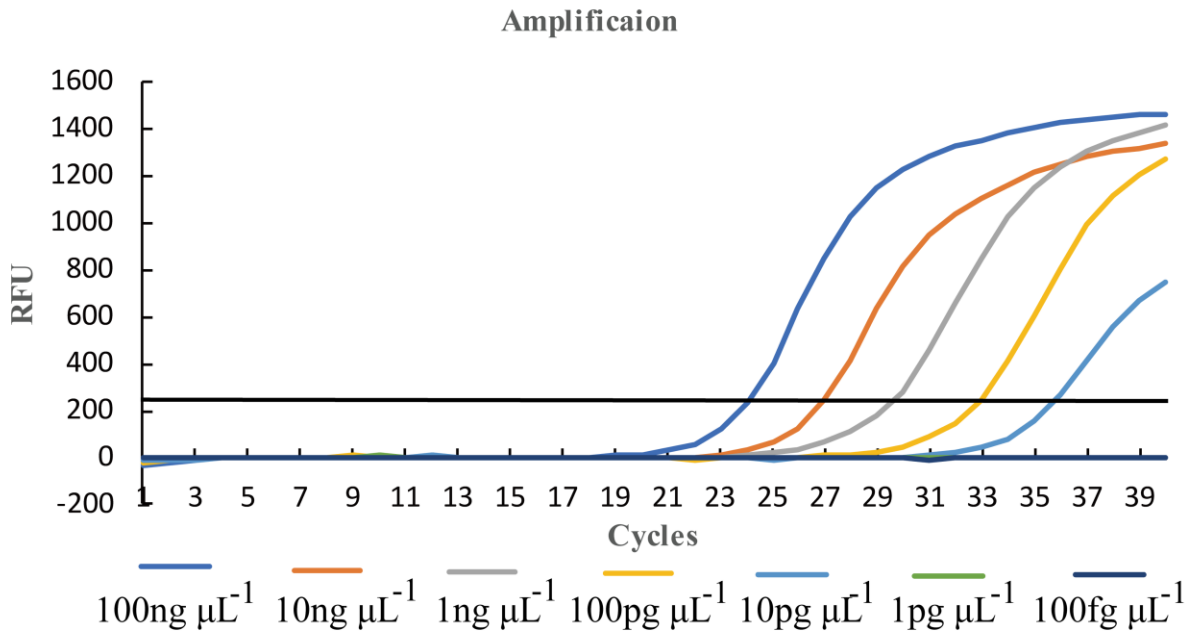

Amplificaion
RFU
1600
1400
1200
1000
800
600
400
200
0
-200
1 3 5 7 9 11 13 15 17 19 21 23 25 27 29 31 33 35 37 39
Cycles
100ng μL-1
10ng μL-1
1ng μL-1
100pg μL-1
10pg μL-1
1pg μL-1
100fg μL-1

Figure 7

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.

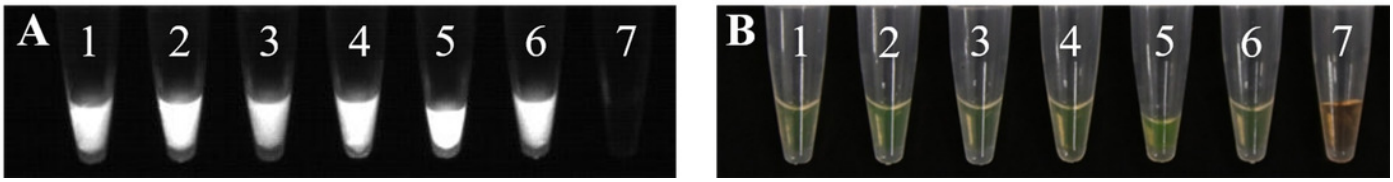


Figure 8

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.

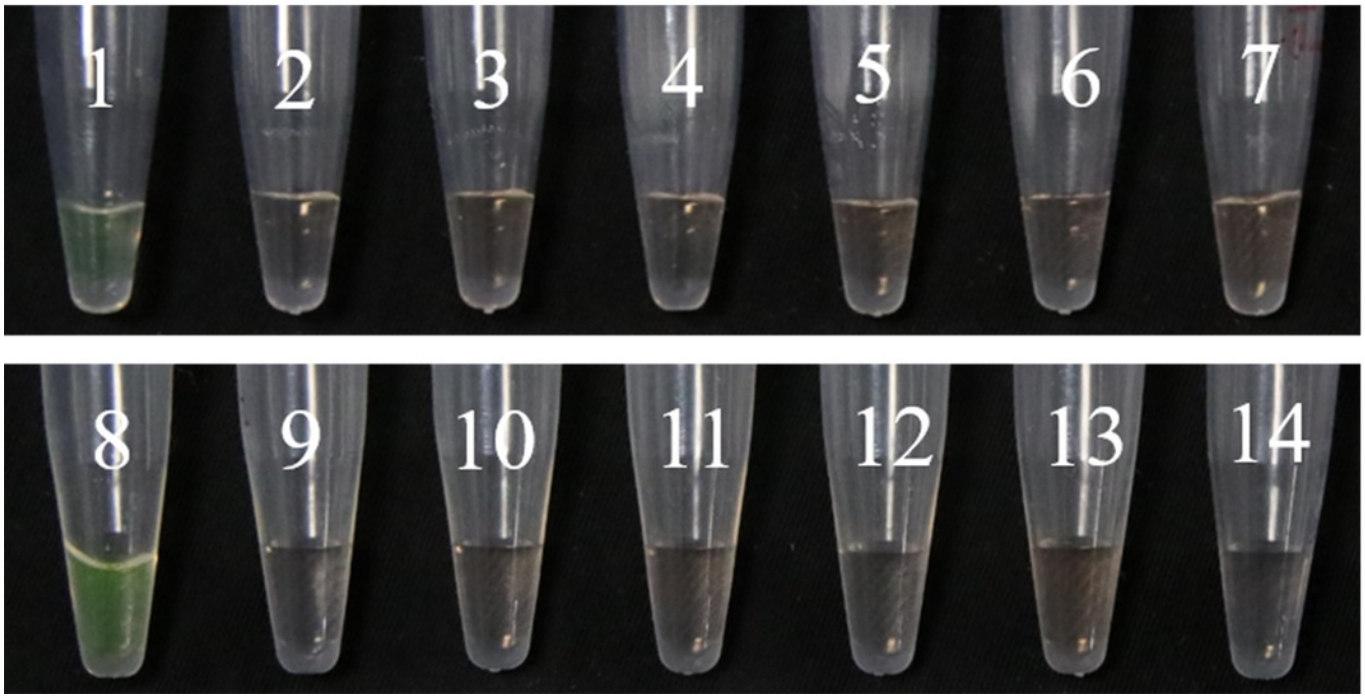


Figure 9

Temperature optimization of LAMP assay.

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

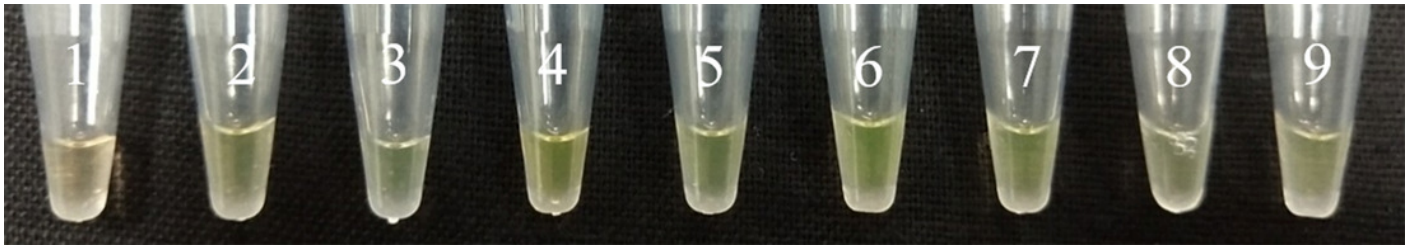


Figure 10

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.

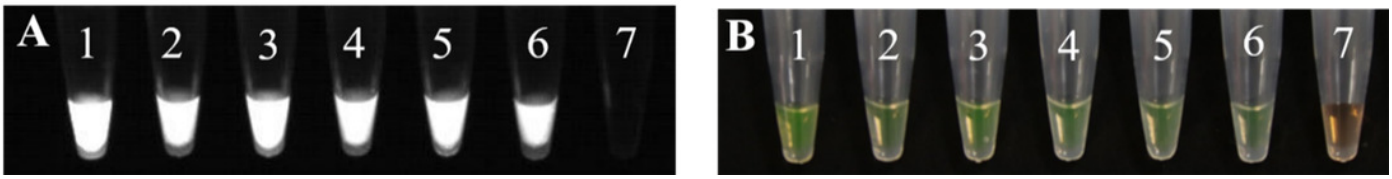


Figure 11

Sensitivity detection of LAMP assays.

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.

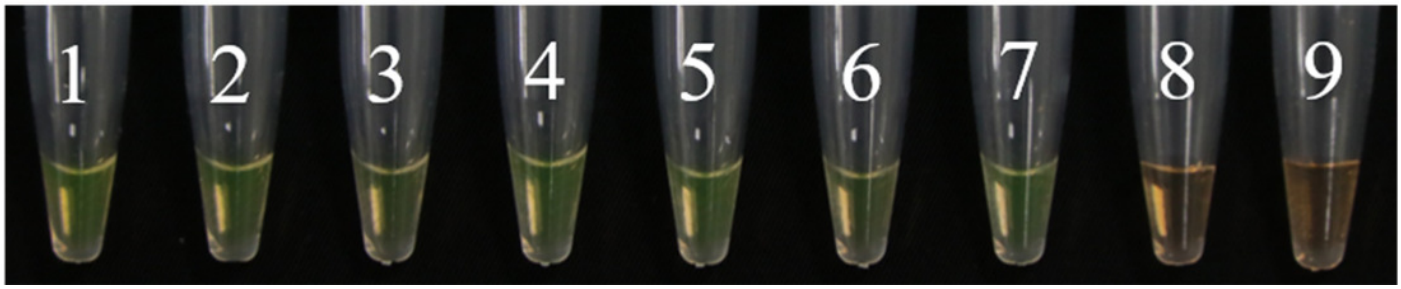
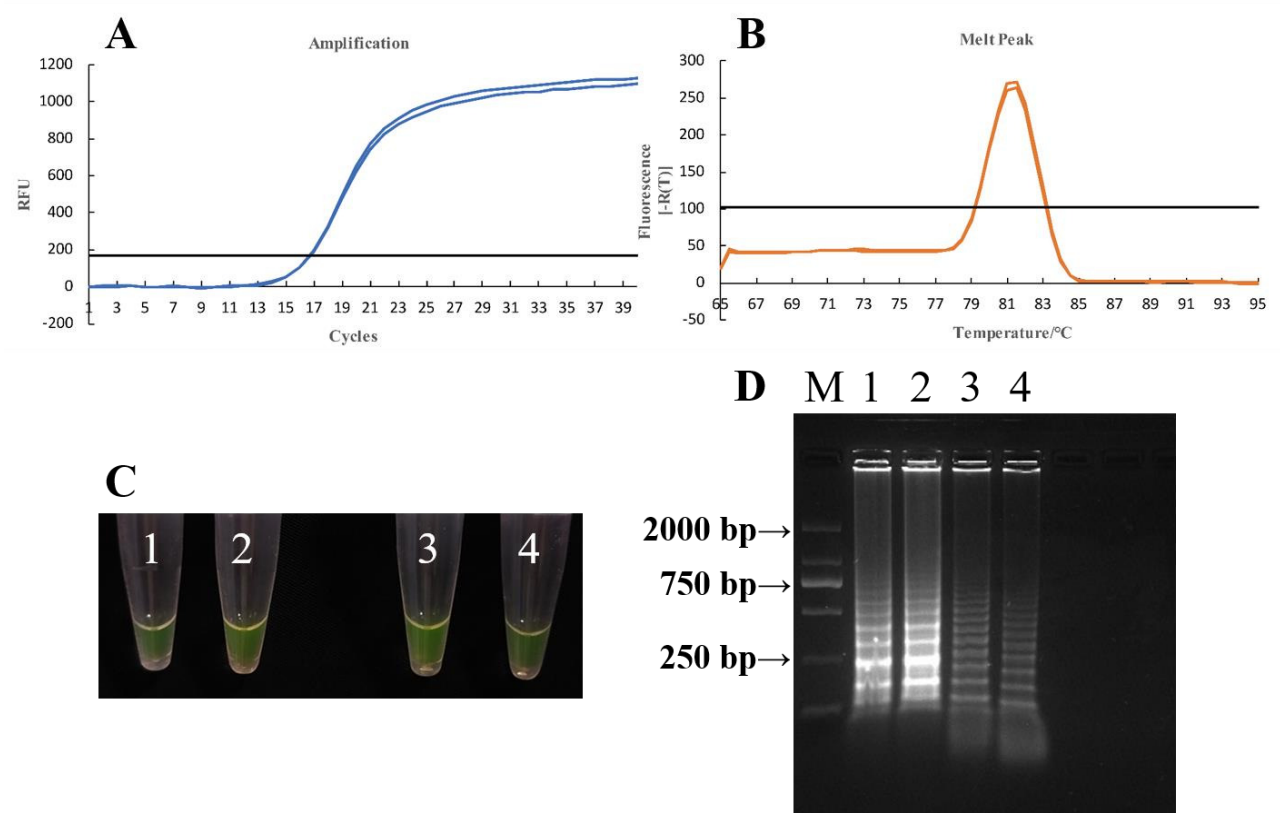


Figure 12(on next page)

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. (B). Melting curves of qPCR for detection of wheat loose smut in field. (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.


A
Amplification
RFU
Cycles
B
Melt Peak
Fluorescence [-R(T)]
Temperature/°C
C
1
2
3
4
D
M 1 2 3 4
2000 bp→
750 bp→
250 bp→