A peer-reviewed version of this preprint was published in PeerJ on 30 September 2019.

View the peer-reviewed version (peerj.com/articles/7766), which is the preferred citable publication unless you specifically need to cite this preprint.

Yan H, Zhang J, Ma D, Yin J. 2019. qPCR and loop mediated isothermal amplification for rapid detection of *Ustilago tritici*. PeerJ 7:e7766
https://doi.org/10.7717/peerj.7766

PeerJ Preprints NOT PEER-REVIEWED

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

Hanwen Yan [1] , **Jian Zhang** [1] , **Dongfang Ma** [Corresp., 2] , **Junliang Yin** [Corresp. 1]

[1] Department of Agriculture, Yangtze University, JingZhou, HuBei, China

[2] Engineering Research Center of Ecology and Agricultural Use of Wetland, Ministry of Education/Hubei Collaborative Innovation Center for Grain Industry/College of Agriculture, Yangtze University, Jingzhou, Hubei, China

Corresponding Authors: Dongfang Ma, Junliang Yin
Email address: madf@yangtzeu.edu.cn, yinjunliang@yangtzeu.edu.cn

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.

PeerJ Preprints NOT PEER-REVIEWED

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

2 **Hanwen Yan[1], Jian Zhang[1], Dongfang Ma[1,2*], Junliang Yin[1*]**

3 [1] Engineering Research Center of Ecology and Agricultural Use of Wetland, Ministry of Education/Hubei

4 Collaborative Innovation Center for Grain Industry/College of Agricultural, Yangtze University, Jingzhou

5 434025, Hubei, China.

6 [2] Institute of Plant Protection and Soil science, Hubei Academy of Agricultural Sciences, Wuhan 430064,

7 Hubei, China.

8 *Corresponding author:

9 Dongfang Ma[1,2*]

10 Street Address, College of Agriculture, Yangtze University, Jingzhou 434000, Hubei, China

11 E-mail address: madf@yangtzeu.edu.cn (D. Ma)

12 **Corresponding author:

13 Junliang Yin[1*]

14 Street Address, College of Agriculture, Yangtze University, Jingzhou 434000, Hubei, China

15 E-mail address: yinjunliang@yangtzeu.edu.cn

16

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).

39 At present, the most effective control method of wheat loose smut disease is seed dressing sterilization
40 before seeding (Singh et al., 2014; Duan et al., 2016). There are no suitable and effective control measures after
41 sowing. The long-term use of chemical agents can easily pollute the environment, endanger the health of
42 humans and livestock, and a large number of single used can easily lead to the emergence of drug resistance
43 (Crane et al., 2013). Therefore, the detection of pathogen *Ustilago tritici* is very necessary. The original
44 detection method of *U. tritici* is serological identification, but it is time-consuming and can only be concluded
45 by professional. Meanwhile, its accuracy and sensitivity are not satisfactory (Walcott, 2003; Munkvold, 2009).
46 Now, the common detection method for wheat loose smut is PCR (Martínez-Espinoza et al., 2003). However,
47 the PCR cannot perform accurate quantitative analysis, and cross-contamination can easily cause false positive
48 (Bretagne, 2003). Quantitative PCR (qPCR) has quickly become a hotspot in scientific research and clinical
49 diagnosis (Kuypers et al., 2006; Yan et al., 2012). At the same time, there are some shortcomings of it, such as
50 the expensive instruments, the need for professional personnel operation, poor practicality. Notomi et al. (2000)
51 invented a novel method for rapid, efficient, and highly specific amplification of target DNA-loop mediated
52 isothermal amplification (LAMP). The principle of the experiment is to design four different primers (F3, B3,
53 FIP, BIP) for six different positions of the target sequence (F3C, F2C, F1C, B1, B2, B3) (Tomita et al., 2008;
54 Notomi et al., 2015), under the action of *Bst* DNA polymerase, the water bath instead of the PCR instrument,
55 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
56 al., 2014). Compared to PCR, the LAMP assay shortens the reaction time, eliminates the gel electrophoresis,
57 does not require expensive PCR instruments, and completes the experiment with the *Bst* DNA polymerase and
58 reaction under constant temperature conditions. The reaction results are determined by the color reaction of
59 fluorescent dyes. Currently, the fluorescent dyes are calcein (Rane et al., 2015), PicoGreen (Curtis, Rudolph &
60 Owen, 2008), hydroxy naphthol blue (HNB) (Goto et al., 2010; Mohon et al., 2014), SYBR Green (Balne et al.,

61 2013; Zhou et al., 2014), etc. SYBR Green I and HNB have the highest detection sensitivity between them,
62 which is 10 times of calcein (Gao et al., 2009). And HNB and SYBR Green I can produce long-term stable
63 color changes and brightness in closed pipes and prevent cross-contamination (Almasi et al., 2013).
64 Meanwhile, its high affinity with double-stranded DNA makes it one of the most commonly used fluorescent
65 dyes. In this study, SYBR Green I was used in both qPCR assay and LAMP assay. However, it can bind to both
66 specific and non-specific products so that the specificity of the primers should be further identified. Therefore,
67 in qPCR assay, an extra melting curves were performed to identify the specificity of the primers. And in LAMP
68 assay, the primers were screened by PCR to determine whether the primers form dimers, and the control
69 bacteria DNA were used to determine the specificity of the primers. The combination of LAMP and fluorescent
70 dyes makes biodetection simpler and more intuitive. At present, LAMP technology and qPCR has been used in
71 many bacteria, fungi, viruses and other aspects detection, such as *Verticillium albo-atrum* (Tian et al., 2016),
72 *Listeria monocytogenes* strains (Wang et al., 2012), parasites (Abdul-Ghani, Al-Mekhlafi & Karanis, 2012),
73 *Candidatus Liberibacter asiaticus* (Rigano et al., 2014), etc. Now there have been reports on the detection of
74 *Rhizoctonia cerealis* (Sun et al., 2015), *Tilletia controversa* Kühn (Nian et al., 2009) by qPCR assays. And the
75 detection of Fusarium head blight (Niessen & Vogel, 2010), wheat stripe rust (Huang et al., 2011) by LAMP
76 assays. However, the detection of wheat loose smut by two methods has not been reported. In this study, we
77 used qPCR and LAMP assays to rapidly detect wheat loose smut.
78
79 **MATERIALS AND METHODS**
80 **Materials**
81 **Bacterial strain**.

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

84 **Culture environment**.

85 The wheat variety used in the experiment is Mingxian169. After germination for 24 h in dark conditions, the
86 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*
87 were collected from the diseased wheat ears. Wheat powdery mildew were derived from diseased leaves. *F.*
88 *graminearum*, *R. cerealis*, *B. sorokiniana*, *A. solani* were inoculated on the PDA mediums covered with glass
89 paper, and cultured at 25 °C for 7 to 8 days. *P. striiformis* was collected in the experimental field.

90 **Genomic DNA extraction**.

91 DNA was extracted from wheat loose smut by modified CTAB. Briefly, lysis buffer (10 mM Tris-HCl [pH 8.0],
92 100 mM EDTA, 0.5% sodium dodecyl sulfate and 100 μg mL^{-1} proteinase) was added to the sample, followed
93 by a water bath at 55 °C for 1 h. The DNA was extracted with phenol-chloroform-isoamyl alcohol (25:24:1),
94 precipitated with isopropanol and washed with ethanol (70%). After centrifugation, 30 μL of ddH_2O was added
95 and stored at -20 °C.

96 **Methods**

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

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

103 **QPCR assays**

104 **Specificity of qPCR assays**.

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

111 **Optimization of qPCR assays**.

112 Appropriate ratios have an impact on the accuracy of the results of qPCR so that system optimization is
113 indispensable for it. System Optimization experiment was performed on the volume of ChamQ^{TM} SYBR qPCR
114 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).
115 After demonstrating the optimum system ratio, the temperature was tested. We designed seven temperature
116 gradients, 52 °C, 54 °C, 56 °C, 58 °C, 60 °C, 62 °C, 64 °C (Fig. 4). The melting temperature and gel
117 electrophoresis can be combined to determine the optimum temperature.

118 **QPCR for *U. tritici***.

119 The reaction system is 20 μL: DNA 1 μL, each of the forward and reverse primer is 1 μL, Master Mix 10 μL,
120 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
121 min. Each template DNA is diluted to 6 concentration gradients, with 3 replicates in each group. Then took the
122 melting curves to verify the specificity of the results (Fig. 5).

123 **Sensitivity detection of qPCR assays**.

124 We used 100 ng μL^{-1} DNA as the template and diluted to seven concentration gradients. These DNA
125 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
126 performed qPCR on Y430 with two replicates per concentration (Fig. 6).

127 **LAMP assay**

128 **Specificity of LAMP assays**.

129 Tested whether the primers were normal and whether there was primer-dimer formation by PCR assay. The
130 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,
131 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
132 set of primers had three replicates. The control template is complemented by ddH_2O. Added the fluorescent dye
133 to the amplification results (Fig. 7). And then *F. graminearum*, *B. graminis*, *R. cerealis*, *P. striiformis*, *B.*
134 *sorokiniana*, *A. solani* were used as the controls for LAMP assay (Fig. 8).

135 **Temperature optimization of LAMP assay**.

136 In order to determine the accuracy and sensitivity of the results, the system concentration and temperature of
137 LAMP assay were optimized. Here, the concentration we used is already the optimal concentration ratio. The
138 concentration ratio of the inner and outer primers used in this experiment are 8:1, the concentration of FIP and
139 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
140 (Kubota et al., 2008; Abdelsalam et al., 2011) is 6 mmol L^{-1}. We designed nine temperature gradients for LAMP
141 assay to determine the optimum temperature based on the final color reaction (Fig. 9).

142 **LAMP assays on *Ustilago tritici***.

143 The LAMP reaction system used in the experiment is 25 μL: DNA 1 μL, 10 x *Thermopol* Buffer 2.5 μL,
144 $MgSO_4$ 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,
145 ddH_2O 7.5 μL, and each set of primers is repeated three times. The control template is complemented by ddH_2O
146 (Fig. 10).

147 **Sensitivity detection of LAMP assays**.

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

149 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
150 pg μL^{-1}, 100 fg μL^{-1}, 10 fg μL^{-1}, 1 fg μL^{-1}. Observing the experimental results with naked eyes under natural
151 light, the experimental results can be judged according to the color (Fig. 11).
152
153 **RESULTS**

154 In qPCR assays, in order to determine whether the designed primers are specific, we performed PCR
155 experiments on the DNA of the *Ustilago tritici* (Fig. 1), and it was very accurate to obtain five sets of sequence
156 bands by primers. Based on the preliminary screening results, primers are further screened by qPCR using the
157 control strains as temples, and then determined whether the primers were specific by the Ct value reflected with
158 the amplification curves (Fig. 2). The results show that Y334 can amplify *Fusarium graminearum* (Fig. 2A)
159 and there for it is not specific for *U. tritici*. The primers Y430, Y307, Y755, Y139 can specifically amplify the
160 target DNA. The four sets of primers were used to detect the *U. tritici*. By optimizing the system, we get the
161 most volume added to ChamQTM SYBRqPCR Master Mix. The results of gel electrophoresis on the
162 amplification are shown in Fig. 3. When the volume of ChamQ^{TM} SYBRqPCR Master Mix was added to 8-12
163 μL, the amplification results are the best. We took the intermediate value, the optimal volume of ChamQ^{TM}
164 SYBR qPCR Master Mix added in qPCR assay was 10 μL. At the same time, in the results of qPCR
165 temperature gradient test on *U. tritici*, the relative fluorescence unit can reach a higher value at 58 °C and 60
166 °C, and the Ct value is about thirty-three, which is in line with our expected results. In gel electrophoresis, the
167 band shown at 58 °C is the brightest. In combination with Fig. 4A and Fig. 4B, we finally chose 58 °C as the
168 temperature set in the final experiment. The amplification curves of the four pairs of primers show that the Ct
169 values ranged from twenty-nine to thirty-five for samples (Fig. 5). It indicated that the four sets of primers
170 could amplify the target DNA under certain concentration conditions. The melting curves corresponding to

171 each primer exhibit a single peak, which further ruled out non-specific amplification. Combining the
172 amplification curves and the melting curves, it can be known that the target DNA can be successfully amplified
173 by using the four pairs of primers designed to quantify the *U. tritici*. Finally, we used 100 ng μL^{-1} DNA as the
174 template and diluted to seven concentration gradients to verify the lowest concentration detectable by qPCR.
175 The results of seven concentrations of fluorescence show (Fig. 6) that the first five concentration gradients had
176 fluorescence signal accumulation so that the lowest DNA concentration detected by qPCR is 10 pg μL^{-1}.
177 In LAMP assays, the results of the PCR system under natural light and gel imager (Fig. 7) indicated that
178 primers designed for LAMP assay can be used for PCR amplification. Then pathogens such as *F. graminearum*
179 and so on (Table 3) as controls to verify the specificity of the primers. In order to determine the specificity of
180 the primers, five other fungal diseases commonly found in wheat were selected as controls. At the same time, a
181 group of non-wheat fungi were selected as controls. L-139 and L-988 were used to perform LAMP assays on
182 seven different bacteria (Fig. 8). The results show that L-139 and L-988 only amplified the DNA sequence of

183 *U. tritici*. As can be seen from Fig. 9, the optimum reaction temperature for LAMP assay is from 62 °C to 64
184 °C. This experiment is taken at 63 °C. In the optimal ratio and temperature, we carried out LAMP test on *U.*
185 *tritici*. Under the gel imaging system (Fig. 10A), the positive samples are white and the negative controls are
186 colorless. Under natural light (Fig. 10B), the three replicates of the two sets of primers show bright green, and
187 the negative controls are light orange. The light orange color of the negative control indicated that no primer-
188 dimer formed and no false positives caused by the external environment. Sensitivity testing of the LAMP test
189 indicates that the DNA concentration of the lowest *U. tritici* detectable by the LAMP assay is 100 fg μL^{-1}. We
190 performed multiple verifications for experimental accuracy in order to test seed carriers and compare the two
191 methods in terms of sensitivity and operation, so we did not distinguish between the different species.

192 Based on the successful amplification of DNA from *U. tritici* by qPCR and LAMP assays, we used Y334
193 to perform qPCR and L-139 and L-988 to perform LAMP assay on diseased seed (Fig. 12). In the qPCR
194 results, the Ct value tends to be flat at twenty-seven, and the melting curve also shows a single peak. Similarly,
195 in the LAMP experiment results, both samples show bright green color, and the expected slow band appeared in
196 agarose gel electrophoresis. Combining the results of the two methods, we conclude that we can use qPCR and
197 LAMP technology to efficiently and sensitively detect *U. tritici*.
198
199 **DISCUSSION**
200 Wheat loose smut is a systemic disease infested flower organ (Ngugi & Scherm, 2006). It currently occurs in
201 all wheat growing area of the world, particularly serious in Canada (Randhawa et al., 2009) and parts of Africa
202 (Zegeye, Dejene & Ayalew, 2015). Due to the expansion of the disease area and the single prevention method
203 that the detection of pathogenic fungi in seeds is particularly important. In this study, both of the qPCR and
204 LAMP assays can specifically, efficiently and accurately amplify the DNA of *Ustilago tritici*.
205 Our ultimate goal is to find a simple and high-speed detection method. Both of the two methods to detect
206 seeds can help to prevent the spread and occurrence of pathogens from the source. By comparing the two
207 methods, it is not difficult to find that the qPCR assay can accurately determine the initial amount and
208 amplification of the template, it can also be used to visually determine whether there was non-specific
209 amplification by melting curve peaks. Combining the amplification curves with melting curves, it was verified
210 that the primers Y430, Y307, Y755, Y139 can specifically, efficiently and accurately detect *U. tritici*. This
211 method avoids the influence of gel electrophoresis and other operations on the result. However, the
212 experimental requirements for personnel operations, equipment, and environment are much high. Therefore, it
213 has been widely used in the analysis of gene expression (Ma et al., 2013), virus detection (Albinana-Gimenez et

214 al., 2009), disease diagnosis (Moreira et al., 2013). Compared with qPCR, the LAMP assay has the advantages
215 of high sensitivity, short reaction time, easy operation, and low equipment requirements (Kiddle et al., 2012).
216 After its combination with fluorescent dyes, the color reaction is more favorable for our observation of the
217 amplification results so that it is widely used in biological disease detection (Jung et al., 2015), medical
218 diagnosis (Hopkins and Bell, 2013), food testing (Sun et al., 2015) and other aspects. Compared with PCR,
219 both the qPCR and LAMP assays can effectively avoid the influence of agarose gel electrophoresis and the
220 minimum detectable concentrations of them are higher than PCR. However, there are still many problems in the
221 basic application of the two methods. For example, the instruments for performing qPCR assays are expensive.
222 The LAMP assay can only detect one disease at a time. The combination of fluorescent dye with dsDNA is not
223 specific so that the LAMP assay has a high false positive rate. And the field situation is complicated, the
224 reaction system and concentration ratio are difficult to optimize, etc.
225 At present, the qPCR and LAMP assays are being improved. On the basis of qPCR, multiplex PCR have
226 emerged to make up for the shortcomings of detecting only one disease at a time. The various conditions of
227 qPCR assay limit its ability to be applied to field-based assays. Accurate analysis of the data and good
228 reproducibility of the experiment make it become the ubiquitous mainstay of molecular biology. With the
229 deepening of molecular biology, fluorescence quantification will become an indispensable part of it. At the
230 same time, the Multiple LAMP assay have also been proposed to accelerate the efficiency of detection (Chen et
231 al., 2016; Lodh et al., 2017). And kits for LAMP assay have been developed (Marti, Stalder & González, 2015),
232 which eliminates system optimization and matching, thus simplifying the procedure. Its advantages make it
233 well suited for use in resource-poor areas. With the advancement of technology and humanity, chemical control
234 will be gradually replaced by early prevention. The LAMP method is a very valuable diagnostic alternative
235 with a potential of being used also in endemic settings. The improvement of LAMP technology will make it

236 more promising in disease prevention and control.

237

238 **Conflicts of interest**

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

240 **Acknowledgments**

241 This work was funded by the "National Key R and D Program of China (2018YFD0200500)", "Open Project
242 Program of Key Laboratory of Integrated Pest Management Crops in Central China, Ministry of Agriculture. P.
243 R. China/Hubei Key Laboratory of Crop Disease, Insect Pests and Weeds Control (2017ZTSJJ7)".

244

245 **REFERENCES**

246 **Abd-Elsalam K, Bahkali A, Moslem M, Amin OE, Niessen L. 2011**. An optimized protocol for DNA extraction
247 from wheat seeds and loop-mediated isothermal amplification (LAMP) to detect *Fusarium graminearum*
248 contamination of wheat grain. Int. J. Mol. Sci. **12**(6), 3459-3472 DOI: 10.3390/ijms12063459

249 **Abdul-Ghani R, Al-Mekhlafi AM, Karanis P. 2012**. Loop-mediated isothermal amplification (LAMP) for malarial
250 parasites of humans: would it come to clinical reality as a point-of-care test ?. Acta. Trop. **122**(3), 233-240
251 DOI: 10.1016/j.actatropica.2012.02.004

252 **Albinana-Gimenez N, Miagostovich MP, Calgua B, Huguet JM, Matia L, Girones R. 2009**. Analysis of
253 adenoviruses and polyomaviruses quantified by qPCR as indicators of water quality in source and
254 drinking-water treatment plants. Water Res. **43**(7), 2011-2019 DOI: 10.1016/j.watres.2009.01.025

255 **Almasi MA, Dehabadi SH, Moradi A, Eftekhari Z, Ojaghkandi MA, Aghaei S. 2013**. Development and
256 application of loop-mediated isothermal amplification assay for rapid detection of *Fusarium oxysporum* f.
257 sp. lycopersici. J. Plant Pathol. Microbiol. **4**(5), 177 DOI: 10.4172/2157-7471.1000177

258 **Balne PK, Barik MR, Sharma S, Basu S. 2013**. Development of a loop-mediated isothermal amplification

259 assay targeting the mpb64 gene for diagnosis of intraocular tuberculosis. J. Clin Microbiol. **51**(11), 3839-
260 3840 DOI: 10.1128/JCM.01386-13
261 **Bretagne S. 2003**. Molecular diagnostics in clinical parasitology and mycology: limits of the current
262 polymerase chain reaction (PCR) assays and interest of the real time PCR assays. Clin. Microbiol. Infec.
263 **9**(6), 505-511 DOI: 10.1046/j.1469-0691.2003.00677
264 **Chen Y, Cheng N, Xu Y, Huang K, Luo Y, Xu W. 2016**. Point-of-care and visual detection of P. aeruginosa
265 and its toxin genes by multiple LAMP and lateral flow nucleic acid biosensor. Biosensors and
266 Bioelectronics. 81, 317-323 DOI: 10.1016/j.bios.2016.03.006
267 **Crane JM, Gibson DM, Vaughan RH, Bergstrom GC. 2013**. Iturin levels on wheat spikes linked to
268 biological control of *Fusarium* head blight by *Bacillus amyloliquefaciens*. Phytopathology. **103**(2), 146-
269 155 DOI: 10.1094/PHYTO-07-12-0154-R
270 **Curtis KA, Rudolph DL, Owen SM. 2008**. Rapid detection of HIV-1 by reverse-transcription, loop-mediated
271 isothermal amplification (RT-LAMP). J. Virol. Methods. **151**(2), 264-270 DOI:
272 10.1016/j.jviromet.2008.04.011
273 **Dhama K, Karthik K, Chakraborty S, Tiwari R, Kapoor S, Kumar A, Thomas P. 2014**. Loop-mediated
274 isothermal amplification of DNA (LAMP): a new diagnostic tool lights the world of diagnosis of animal
275 and human pathogens: a review. Pak. J. Biol. Sci. **17**(2), 151-166 DOI: 10.3923/pjbs.2014.151.166
276 **Duan M, Yan J, Guo Q, University Q. 2016**. Effects of seed dressing with five kinds of fungicides on loose smut
277 and yield of wheat. Journal of Qinghai University. **34**(6), 34 DOI: 10. 13901/j. cnki. qhwxxbzk
278 **Fa HW, Dong YH, Xiao LH, Xin Z, Wen JC. 2009**. Comparing Study on several Methods for DNA
279 Extraction from endophytic fungi. J. Chinese Agricultural Science Bulletin. 25(08), 62-64.
280 **Gao H, Lei Z, Jia J, Wang S, Chen Y, Sun M, Liang C. 2009**. Application of loop-mediated isothermal

281 amplification for detection of *Yersinia enterocolitica* in pork meat. J. Microbiol. Methods. **77**(2), 198-201
282 DOI: 10.1016/j.mimet.2009.02.001
283 **Garg S, Pandey D, Taj G, Goel A, Kumar A. 2014**. TRIPATH: A Biological Genetic and Genomic Database
284 of Three Economically Important Fungal Pathogen of Wheat–Rust: Smut: Bunt. Bioinformation, **10**(7),
285 466. DOI: 10.6026/97320630010466
286 **Goto M, Shimada K, Sato A, Takahashi E, Fukasawa T, Takahashi T, Ogura A. 2010**. Rapid detection of
287 *Pseudomonas aeruginosa* in mouse feces by colorimetric loop-mediated isothermal amplification. J.
288 Microbiol. Methods. **81**(3), 247-252 DOI: 10.1016/j.mimet.2010.03.008
289 **Hopkins H, González IJ, Polley SD, Angutoko P, Ategeka J, Asiimwe C, Bell D. 2013**. Highly sensitive
290 detection of malaria parasitemia in a malaria-endemic setting: performance of a new loop-mediated
291 isothermal amplification kit in a remote clinic in Uganda. J. Infect. Dis. **208**(4), 645-652 DOI:
292 10.1093/infdis/jit184
293 **Huang C, Sun Z, Yan J, Luo Y, Wang H, Ma Z. 2011**. Rapid and Precise Detection of Latent Infections of
294 Wheat Stripe Rust in Wheat Leaves using Loop Mediated Isothermal Amplification. J. Phytopathol.
295 **159**(7 8), 582-584 DOI: 10.1111/j.1439-0434.2011.01806
296 **Jung JH, Oh SJ, Kim, YT, Kim SY, Kim WJ, Jung J, Seo TS. 2015**. Combination of multiplex reverse-
297 transcription loop-mediated isothermal amplification with an immunochromatographic strip for subtyping
298 influenza A virus. Anal. Chim. Acta. 853, 541-547 DOI: 10.1016/j.aca.2014.10.020
299 **Kiddle G, Hardinge P, Buttigieg N, Gandelman O, Pereira C, McElgunn CJ, Tisi LC. 2012**. GMO
300 detection using a bioluminescent real time reporter (BART) of loop mediated isothermal amplification
301 (LAMP) suitable for field use. Bmc. Biotechnol. **12**(1), 15 DOI: 10.1186/1472-6750-12-15
302 **Knox R, Menzies J. 2012**. Resistance in wheat to loose smut. Disease resistance in wheat/ed. I. Sharma. CABI

303 Publishing. 160-190.

304 **Kubota R, Vine BG, Alvarez AM, Jenkins DM. 2008**. Detection of *Ralstonia solanacearum* by loop-
305 mediated isothermal amplification. Phytopathology. **98**(9), 1045-1051 DOI: 10.1094/PHYTO-98-9-1045

306 **Kuypers J, Wright N, Ferrenberg J, Huang M L, Cent A, Corey L, Morrow R. 2006**. Comparison of real-
307 time PCR assays with fluorescent-antibody assays for diagnosis of respiratory virus infections in children.
308 J. Clin. Microbiol. **44**(7), 2382-238 DOI: 10.1128/JCM.00216-06

309 **Lodh N, Mikita K, Bosompem KM, Anyan WK, Quartey JK, Otchere J, Shiff CJ. 2017**. Point of care
310 diagnosis of multiple schistosome parasites: Species-specific DNA detection in urine by loop-mediated
311 isothermal amplification (LAMP). Acta. Trop. 173, 125-129 DOI: 10.1016/j.actatropica.2017.06.015

312 **Ma S, Niu H, Liu C, Zhang J, Hou C, Wang D. 2013**. Expression stabilities of candidate reference genes for
313 RT-qPCR under different stress conditions in soybean. Plos One. **8**(10), e75271 DOI:
314 10.1371/journal.pone.0075271

315 **Marti H, Stalder C, González IJ. 2015**. Diagnostic accuracy of a LAMP kit for diagnosis of imported malaria
316 in Switzerland. Travel Med. Infect. Di. 13(2), 167-171 DOI: 10.1016/j.tmaid.2014.12.016

317 **Martínez-Espinoza AD, León-Ramírez CG, Singh N, Ruiz-Herrera J. 2003**. Use of PCR to detect infection
318 of differentially susceptible maize cultivars using *Ustilago maydis* strains of variable virul ence. Int.
319 Microbiol. **6**(2), 117-120 DOI: 10.1007/s10123-003-0117-0

320 **Mohon AN, Elahi R, Khan WA, Haque R, Sullivan Jr DJ, Alam MS. 2014**. A new visually improved and
321 sensitive loop mediated isothermal amplification (LAMP) for diagnosis of symptomatic falciparum
322 malaria. Acta. Trop. 134, 52-57 DOI: 10.1016/j.actatropica.2014.02.016

323 **Moreira OC, Ramírez JD, Velázquez E, Melo MFD, Lima-Ferreira C, Guhl F, Britto C. 2013**. Towards
324 the establishment of a consensus real-time qPCR to monitor *Trypanosoma cruzi* parasitemia in patients

325 with chronic Chagas disease cardiomyopathy: a substudy from the BENEFIT trial. Acta. Trop. **125**(1), 23-
326 31 DOI: 10.1016/j.actatropica
327 **Munkvold GP. 2009**. Seed pathology progress in academia and industry. Annu. Rev. Phytopathol. 47, 285-311
328 DOI: 10.1146/annurev-phyto-080508-081916
329 **Ngugi HK, Scherm H. 2006**. Biology of flower-infecting fungi. Annu. Rev. Phytopathol. 44, 261-282 DOI:
330 10.1146/annurev.phyto.44.070505.143405
331 **Nian S, Yuan Q, Yin Y, Cai J, Wang Z. 2009**. Detection of *Tilletia controversa* Kühn by real time quantitative
332 PCR. Scientia Agricultura Sinica. **42**(12), 4403-4410 DOI: 10.3864/j.issn.0578-1752
333 **Niessen L, Vogel RF. 2010**. Detection of *Fusarium graminearum* DNA using a loop-mediated isothermal
334 amplification (LAMP) assay. Int. J. Food Microbiol. **140**(2-3), 183-191 DOI: 10.1016/j.ijfoodmicro
335 **Notomi T, Mori Y, Tomita N, Kanda H. 2015**. Loop-mediated isothermal amplification (LAMP): principle,
336 features, and future prospects. J. Microbiol. **53**(1), 1-5 DOI: 10.1007/s12275-015-4656-9
337 **Notomi T, Okayama H, Masubuchi H, Yonekawa T, Watanabe K, Amino N, Hase T. 2000**. Loop-mediated
338 isothermal amplification of DNA. Nucleic acids Res. **28**(12), e63-e63 DOI: 10.1093/nar/28.12.e63
339 **Randhawa HS, Matheson F, Menzies JG, Fox SL. 2009**. Molecular and virulence relationships among races
340 of *Ustilago tritici* collected from durum and bread wheat. Can. J. Plant Pathol. **31**(2), 220-231 DOI:
341 10.1080/07060660909507595
342 **Rane TD, Chen L, Zec HC, Wang TH. 2015**. Microfluidic continuous flow digital loop-mediated isothermal
343 amplification (LAMP). Lab. Chip. **15**(3), 776-782 DOI: 10.1039/C4LC01158A
344 **Rigano LA, Malamud F, Orce IG, Filippone MP, Marano MR, Do Amaral AM, Vojnov AA. 2014**. Rapid
345 and sensitive detection of Candidatus *Liberibacter asiaticus* by loop mediated isothermal amplification
346 combined with a lateral flow dipstick. BMC. Microbiol. **14**(1), 86 DOI: 10.1186/1471-2180-14-86
347 **Rigano LA, Marano MR, Castagnaro AP, Do Amaral AM, Vojnov AA. 2010**. Rapid and sensitive detection

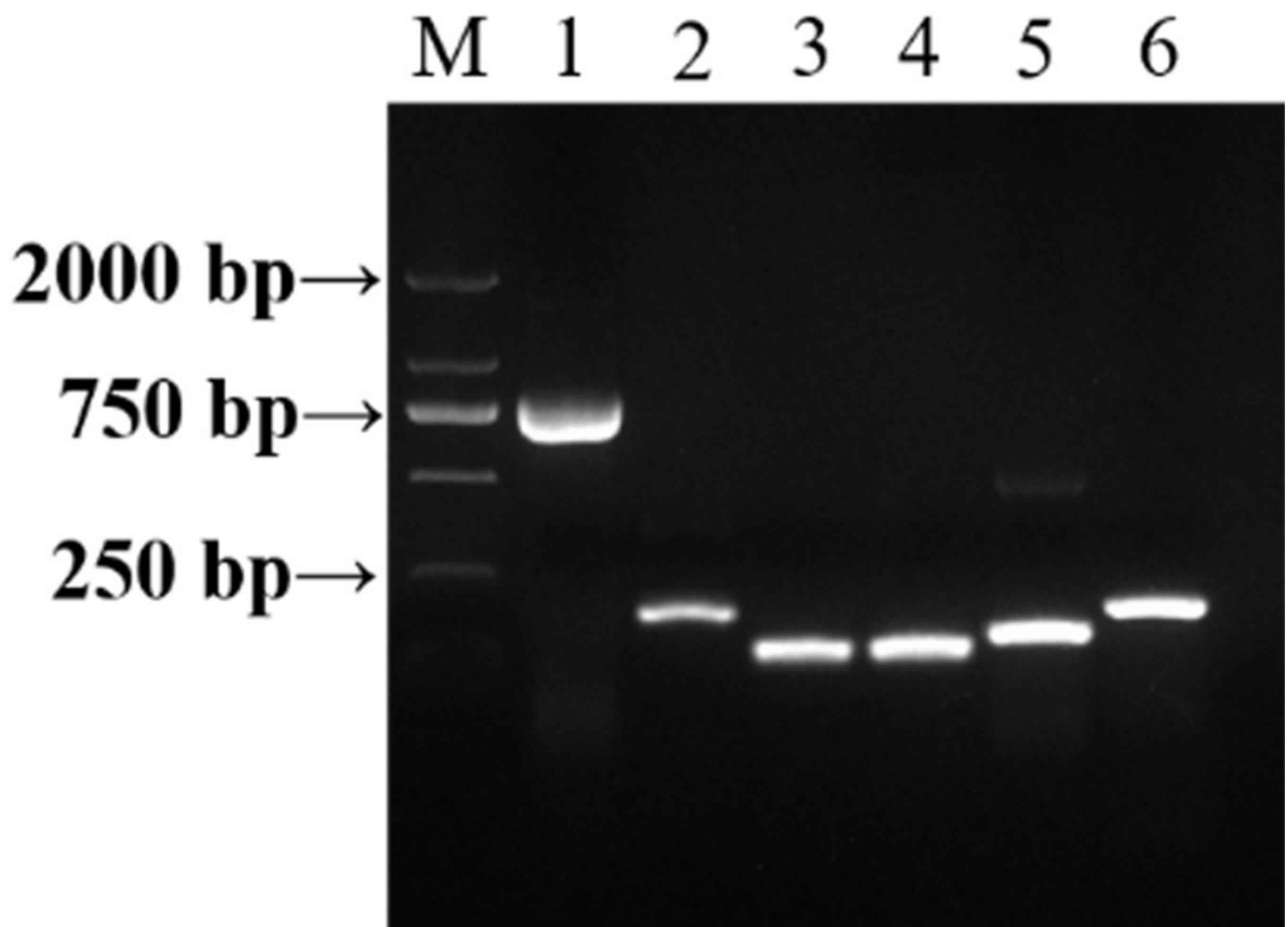
348 of Citrus Bacterial Canker by loop-mediated isothermal amplification combined with simple visual
349 evaluation methods. BMC. Microbiol. **10**(1), 176 DOI: 10.1186/1471-2180-10-176
350 **Singh DP, Saharan MS, Selvakumar R, Sharma AK, Sharma I. 2014**. Bio-efficacy of triticonazole 8%+
351 pyraclostrobin 4% FS, pyraclostrobin 20% FS, triticonazole 2.5% against loose smut of wheat. African
352 Journal of Crop Protection and Rural Sociology ISSN: Vol. **2**(1), 054-056.
353 **Sun BJ, Chen QQ, Yuan HX, Shi Y, Li HL. 2015**. Establishment of SYBR green I real-time PCR for
354 quantitatively detecting *Rhizoctonia cerealis* in winter wheat. Sci. Agric. Sin. 48, 55-62 DOI:
355 10.3864/j.issn.0578-1752
356 **Sun Y, Quyen TL, Hung TQ, Chin WH, Wolff A, Bang DD. 2015**. A lab-on-a-chip system with integrated
357 sample preparation and loop-mediated isothermal amplification for rapid and quantitative detection of
358 *Salmonella spp.* in food samples. Lab. Chip. **15**(8). 1898-1904 DOI: 10.1039/C4LC01459F
359 **Tian Q, Zeng D, Li B, Zhang H, Wang Y, Zheng X. 2016**. Rapid detection of *Verticillium albo-atrum* using a
360 loop-mediated isothermal amplification assay. JNAU. **39**(4), 582-588 DOI: 10.7685/jnau.201510022
361 **Tomita N, Mori Y, Kanda H, Notomi T. 2008**. Loop-mediated isothermal amplification (LAMP) of gene
362 sequences and simple visual detection of products. Nat. Protoc. **3**(5), 877 DOI: 10.1038/nprot.2008.57
363 **Walcott RR. 2003**. Detection of seedborne pathogens. HortTechnology. **13**(1), 40-47 DOI:
364 10.21273/HORTTECH.13.1.0040
365 **Wang L, Li Y, Chu J, Xu Z, Zhong Q. 2012**. Development and application of a simple loop-mediated
366 isothermal amplification method on rapid detection of *Listeria monocytogenes* strains. Mol. Biol. Rep.
367 **39**(1), 445-449 DOI: 10.1007/s11033-011-0757-7
368 **Yan J, Yuan F, Long G, Qin L, Deng Z. 2012**. Selection of reference genes for quantitative real-time RT-PCR
369 analysis in citrus. Mol. Biol. Rep. **39**(2), 1831-1838 DOI: 10.1007/s11033-011-0925-9

370 **Zegeye W, Dejene M, Ayalew D. 2015**. Importance of Loose Smut [*Ustilago nuda* (Jensen) Rostrup] of Barley
371 (Hordeum vulgare L.) in Western Amhara Region, Ethiopia. EAJS. **9**(1), 31-40.
372 **Zhao GC, Chang XH, Wang DM, Tao ZQ, Wang YJ, Yang YS, Zhu YJ. 2018**. General Situation and
373 Development of Wheat Production. Crops. **18**(4), 1-7.
374 **Zhou D, Guo J, Xu L, Gao S, Lin Q, Wu Q, Que Y. 2014**. Establishment and application of a loop-mediated
375 isothermal amplification (LAMP) system for detection of cry1Ac transgenic sugarcane. Sci. Rep-UK. 4,
376 4912 DOI: 10.1038/srep04912

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.



PeerJ Preprints | https://doi.org/10.7287/peerj.preprints.27751v1 | CC BY 4.0 Open Access | rec: 23 May 2019, publ: 23 May 2019

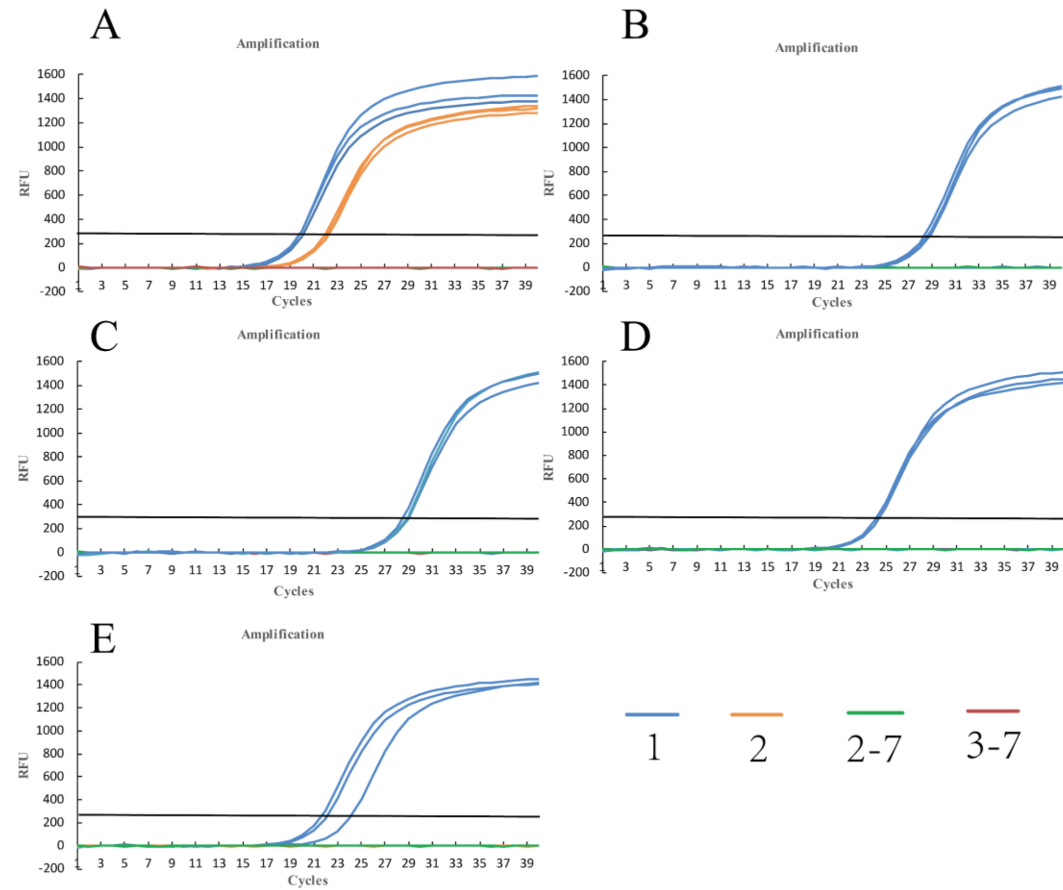
Figure 2(on next page)

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.

PeerJ Preprints | https://doi.org/10.7287/peerj.preprints.27751v1 | CC BY 4.0 Open Access | rec: 23 May 2019, publ: 23 May 2019

PeerJ Preprints NOT PEER-REVIEWED



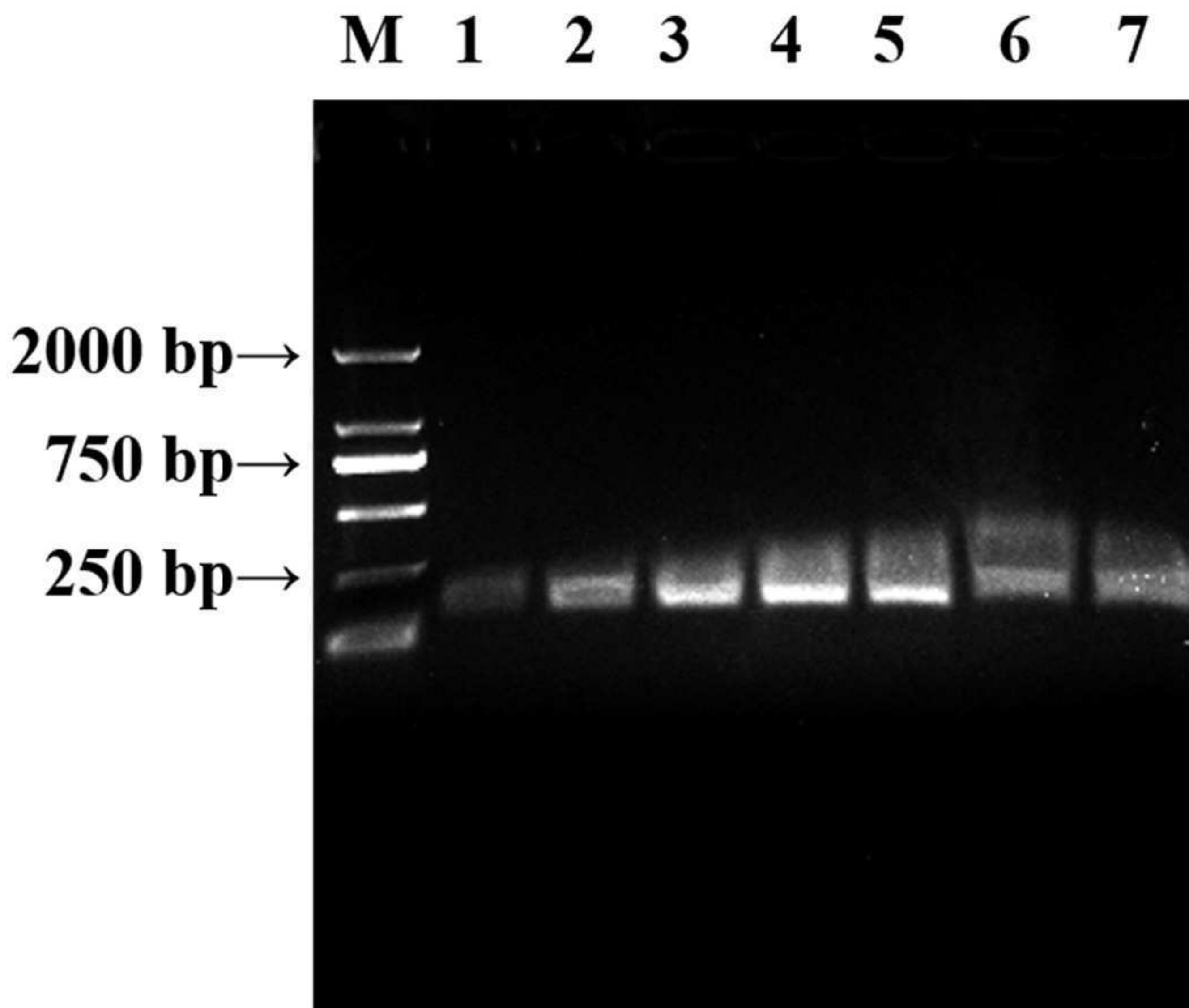
PeerJ Preprints | https://doi.org/10.7287/peerj.preprints.27751v1 | CC BY 4.0 Open Access | rec: 23 May 2019, publ: 23 May 2019

PeerJ Preprints NOT PEER-REVIEWED

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.



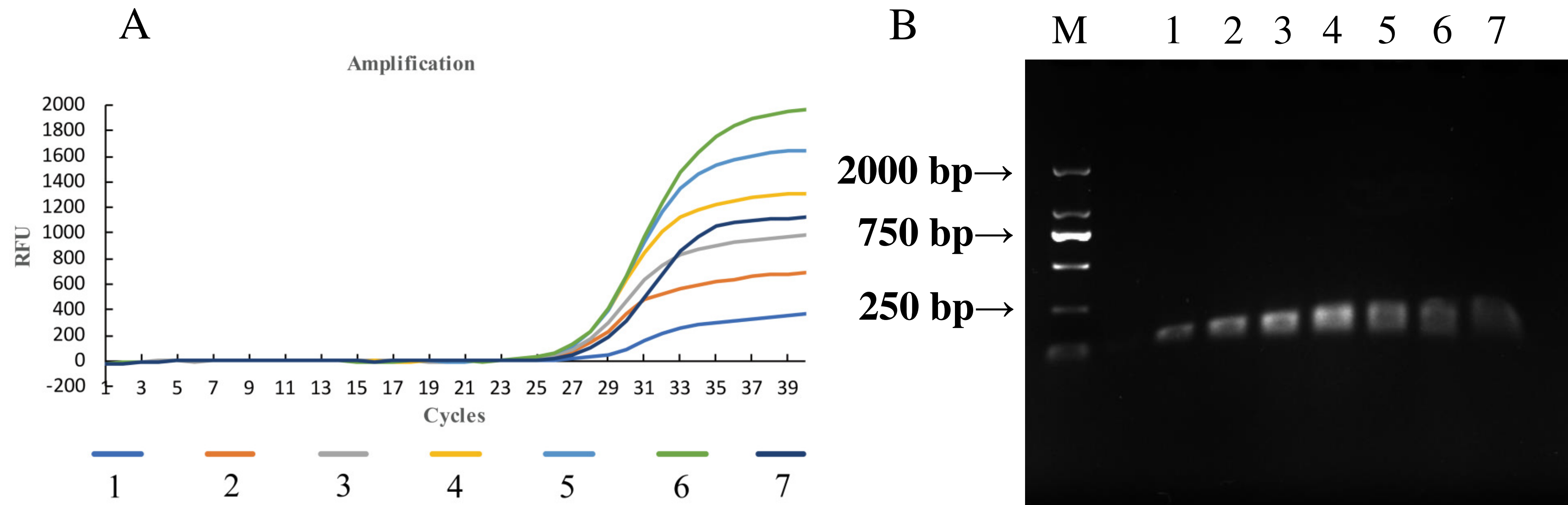
PeerJ Preprints | https://doi.org/10.7287/peerj.preprints.27751v1 | CC BY 4.0 Open Access | rec: 23 May 2019, publ: 23 May 2019

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 ℃.

PeerJ Preprints NOT PEER-REVIEWED



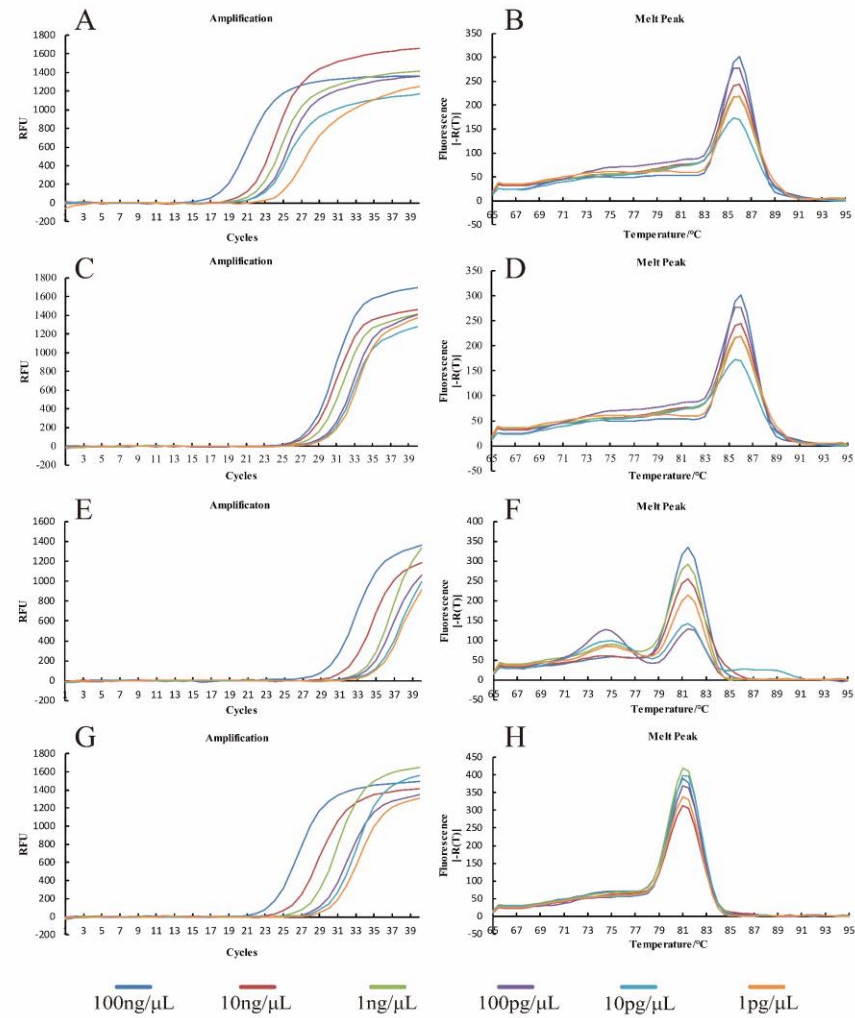
PeerJ Preprints | https://doi.org/10.7287/peerj.preprints.27751v1 | CC BY 4.0 Open Access | rec: 23 May 2019, publ: 23 May 2019

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).

PeerJ Preprints | https://doi.org/10.7287/peerj.preprints.27751v1 | CC BY 4.0 Open Access | rec: 23 May 2019, publ: 23 May 2019



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

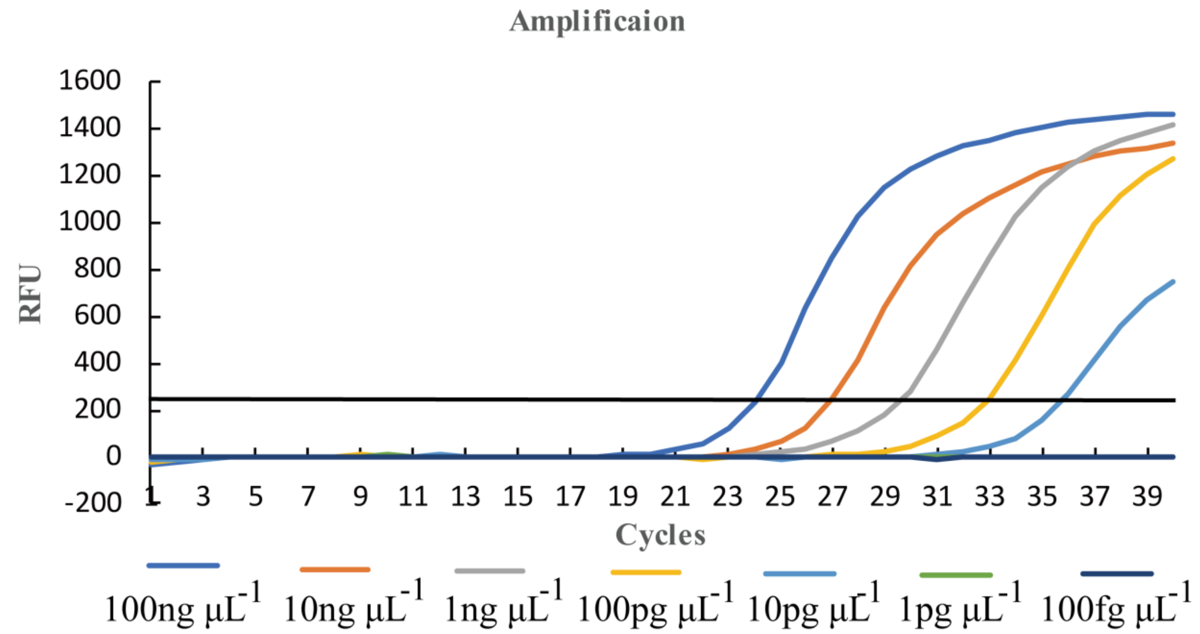
PeerJ Preprints NOT PEER-REVIEWED

Figure 6
(on next page)

Sensitivity detection of qPCR assays by primer Y430.

PeerJ Preprints | https://doi.org/10.7287/peerj.preprints.27751v1 | CC BY 4.0 Open Access | rec: 23 May 2019, publ: 23 May 2019

PeerJ Preprints NOT PEER-REVIEWED



PeerJ Preprints | https://doi.org/10.7287/peerj.preprints.27751v1 | CC BY 4.0 Open Access | rec: 23 May 2019, publ: 23 May 2019

PeerJ Preprints NOT PEER-REVIEWED

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.

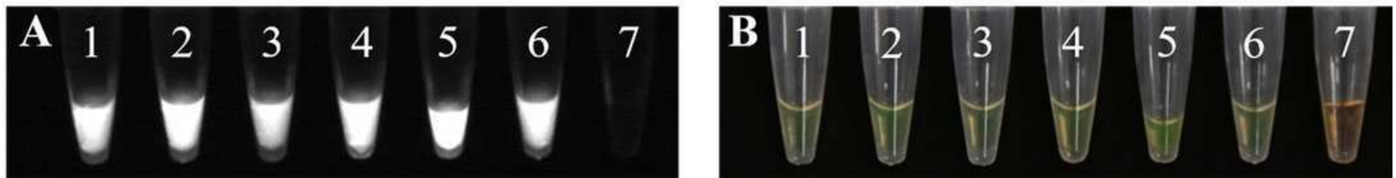
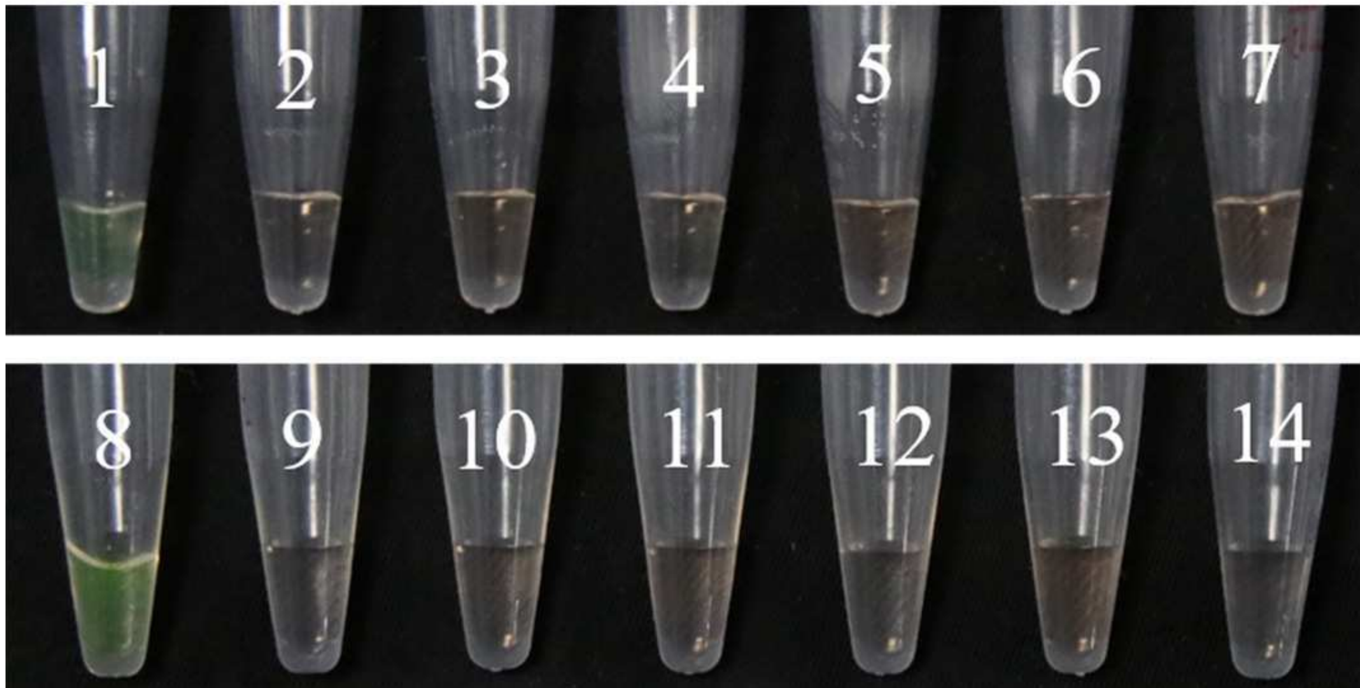


Figure 8

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.

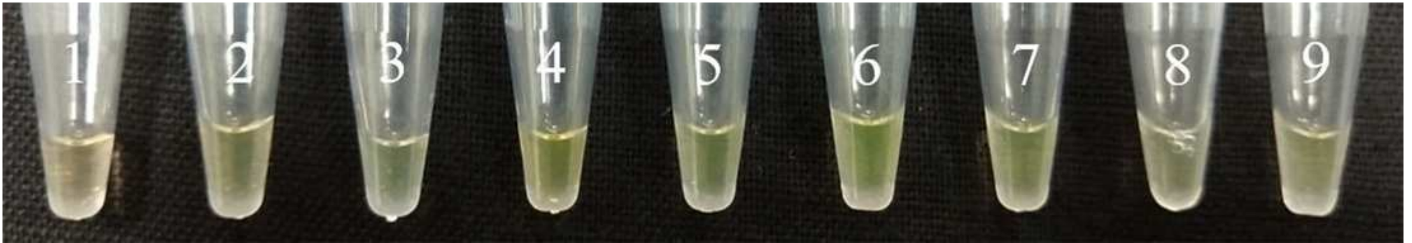


PeerJ Preprints | https://doi.org/10.7287/peerj.preprints.27751v1 | CC BY 4.0 Open Access | rec: 23 May 2019, publ: 23 May 2019

Figure 9

Temperature optimization of LAMP assay.

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



PeerJ Preprints NOT PEER-REVIEWED

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.

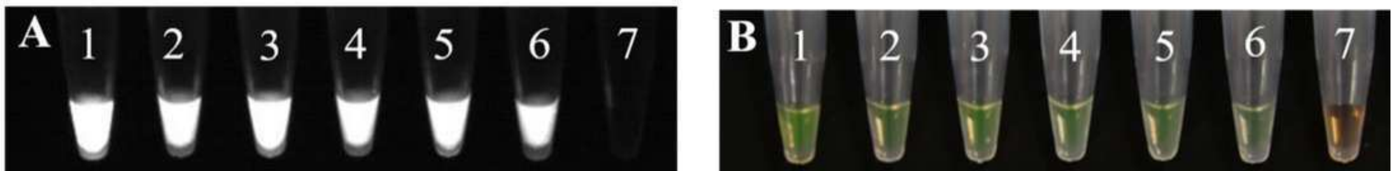
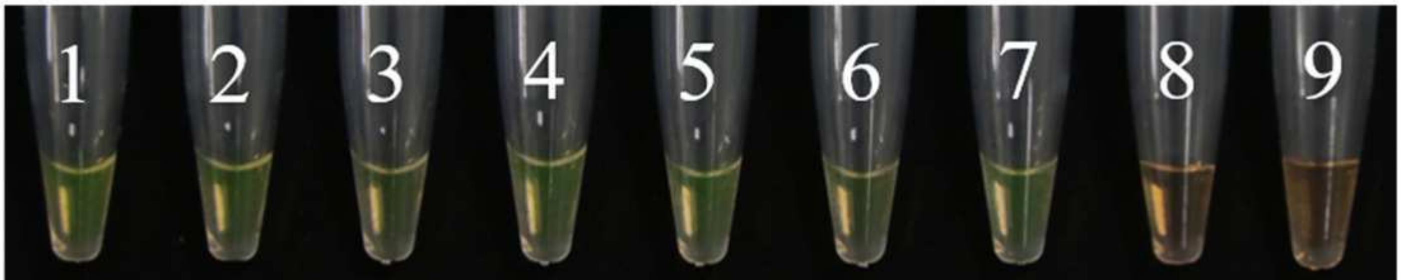


Figure 11

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.



PeerJ Preprints | https://doi.org/10.7287/peerj.preprints.27751v1 | CC BY 4.0 Open Access | rec: 23 May 2019, publ: 23 May 2019

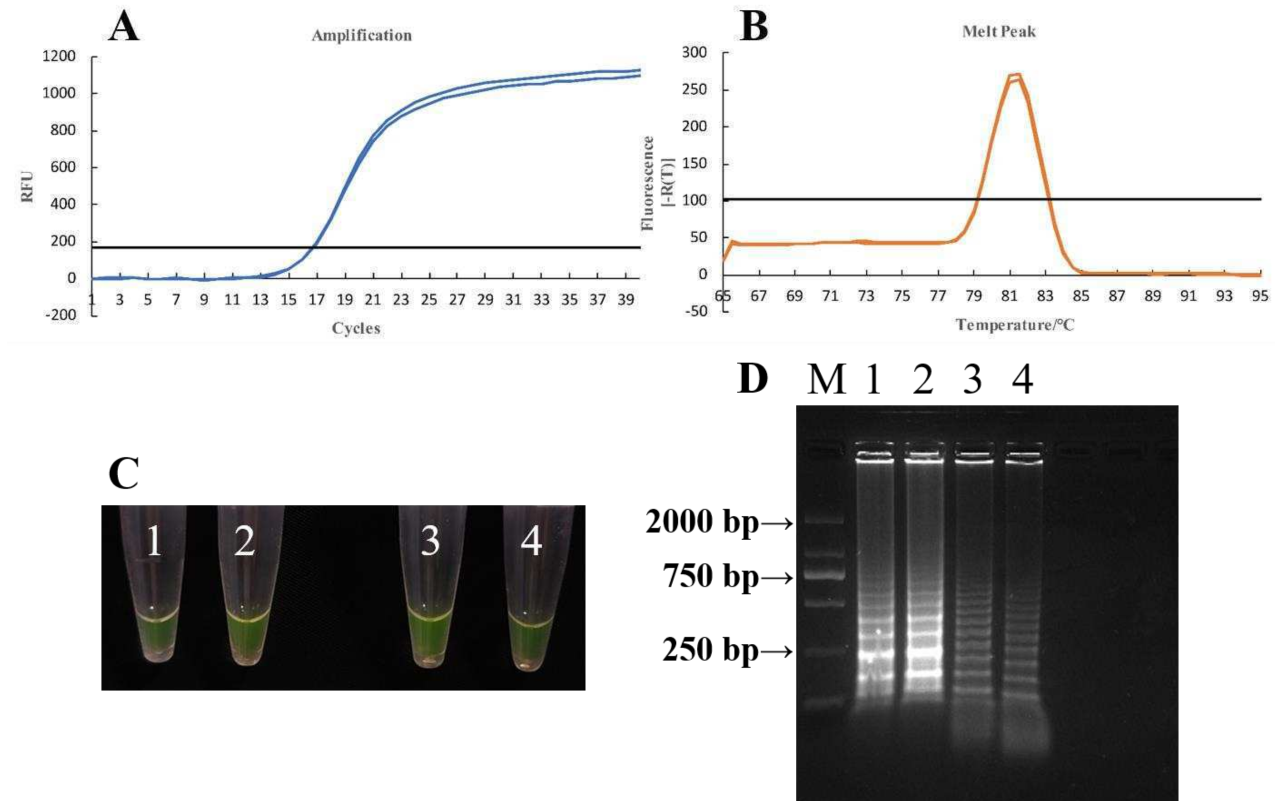
Figure 12(on next page)

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.

PeerJ Preprints | https://doi.org/10.7287/peerj.preprints.27751v1 | CC BY 4.0 Open Access | rec: 23 May 2019, publ: 23 May 2019

PeerJ Preprints NOT PEER-REVIEWED



PeerJ Preprints | https://doi.org/10.7287/peerj.preprints.27751v1 | CC BY 4.0 Open Access | rec: 23 May 2019, publ: 23 May 2019