Rapid and reliable distinguish of *Morinda officinalis How* by loop-mediated isothermal amplification (LAMP) assay

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Abstract

The medicinal plant *Morinda officinalis How* (MO), especially the root, has been frequently used in traditional medicines around the world as an herbal drug for treating variable human disorders and diseases. Various adulterations of MO were found for economic or production limitations. However, authentication of MO from its adulterants by LAMP has not yet been established. The present study introduces a commercially available nucleic acid amplification method, loop-mediated isothermal amplification (LAMP) assay for the distinguish of MO from its adulterants. In this method, we combined DNA barcodes technology to design 2 pairs independent LAMP primers, which based on the internal transcribed spacer 2 (ITS2) sequence of MO's nuclear ribosomal DNA. Our results showed that the LAMP could amplify the samples as expected and successfully identify target MO, and the limit for DNA template preciseness was verified as 1×10^{-1} pg/μl. All the visual or real-time turbidity detection was performed within 60 min at approximately 63 °C. The result showed that the LAMP assay and primers we designed have high accuracy and efficiency for the differentiation of MO and its adulterants. Our results illustrated that the proposed low-cost, fast and reliable LAMP assay without the need for expensive equipment or specialized techniques could be a good way for MO rapid authentication.

1. Introduction

Human cultivation and uses of *Morinda officinalis How* (MO) date to ancient Chinese, Japanese, and Indian cultures and now is widely spread in tropical, subtropical and temperate regions of Asia [1]. Belonging to the family *Rubiaceae*, tasted sweet root with special brown color, MO has biological functions on liver, kidney, bone and muscle [2]. It is also known to be beneficial to osteoporosis, rheumatoid arthritis, menstrual disorders, diabetes, rheumatoid and Alzheimer disease [3,4]. More than 400 active chemical compounds have been isolated from this plant, and the major constituents have been found to be anthraquinones, iridoid glycosides, saccharides, fatty acids and triterpenoids [5]. Crude extracts and pure compounds of the plant are used as effective agents in the treatment of many human biological diseases. In the market, many types of plants materials that resemble MO in appearance have been used to imitate MO because of unsustainable cultivation, over-harvesting, or economic reasons [6]. Although the morphological characteristics of these imitations are similar to high-quality MO, they are cheaper and their pharmacodynamic effects are remarkably different. According to Chinese Pharmacopoeia (2015 edition), currently methods used to identify MO include macroscopic characterization, phytochemical methods [7] or molecular level analysis [8], such as TLC(Thin-Layer Chromatography), genetic markers[9] and UHPLC-MS/MS[10]. These technologies regarded as benchmark assay because of authoritative, sensitive and stable, but would spend long run time with professional operation personnel and corresponding analysis infrastructure. It is very hard to identify them by the classical identification methods for consumers to immediately distinguish *Morinda* species, then causing confusion in market of medicinal materials. More seriously, MO adulterants obviously influence the clinical therapeutic effects and cause serious side effects [11]. Therefore, a useful, fast and accuracy assay method for screening adulterated MO is urgently required.

The loop-mediated isothermal amplification (LAMP) method, established by Notomi in 2000, is a simple nucleic acid amplification technology with high efficiency and specificity [12]. The whole work is able to completed in 40 ~ 60 min, with similar test limit to PCR (Polymerase chain reaction) technology[13]. Furthermore, compared with PCR-based methods, LAMP is several orders of magnitude more sensitive that can overcome the specificity limitations [14]. The method requires 4 ~ 6 suitable primers that are specifically designed for recognizing and able to bind 6 ~ 8 precise gene regions, the whole process can be performed at 60 ~ 65 °C and only required bacillus stearothermophilus DNA (Bst DNA) polymerase [15]. Figure 1 describes the DNA loop amplification process in detail. The amplification obtained single-stranded DNA by Bst DNA to achieve polymerase-mediated strand-displacement synthesis, thus obviating the step of thermal denaturation and the thermal cycler equipment [16]. Since temperature changes are not required, the LAMP technology are promising approaches that allows the reaction to carry out applicably in a shorter time [17]. Various types of DNA dyes can use directly and then observe with the naked eye without opening tubes because of large output of the amplification products [18]. To avoid potentially subjective, relatively simple quantitative detection methods (turbidity or fluorescence), can revealed the results because of the high specificity. Additionally, there is no need for complicated operation and specific laboratory with highly sophisticated instrumentation during application, such as a thermal cycler and other imaging devices. Based on its rapidity, simplicity, and high specificity, LAMP assay give satisfactory results in widely fields, for example pathogen and genetic detection in clinical diagnosis such as global pandemic COVID-19[19], fast microbe and harmful insect detection in agriculture products and environmental samples [20,21]. However, authentication of MO from its adulterants by LAMP has not yet been established.

DNA barcode technology is an emerging technology that uses a conserved segment of biological DNA to quickly and accurately identify species [22]. DNA barcodes is an efficacious molecular tool that can provide species-level resolution to distinguish various creatures differentiation, currently has been widely used in plant identification [23]. The ITS2 sequence has been recommended as an effective core molecular marker of the DNA barcode [24], which can be used to verify a wider range of herbal species groups with high variability and species discrimination [25]. These advantages make ITS2 sequence becomes a prospective barcode gene target to increase the success rate of LAMP assay. This study sought to establish a rapid and reliable LAMP assay method to distinguish MO from its adulterants based on the ITS2 sequence.

2. Results

2.1 Primer Design and Multiple Alignment for LAMP Detection

Some researchers have shown that the ITS2 sequence has a high conservation rate, low mutation rate, moderate parsimony information rate, and moderate intra- and inter-species differences. Through nucleic acid sequence information, genetic distance analysis, DNA Barcoding Gap evaluation and analysis of

phylogenetic trees, all test species can be successfully identified. In this study, we therefore used the ITS2 sequences to design LAMP-specific primers. Then based on the comparison results, we use the online software Primer Explorer 4.0 (http://primerexplorer.jp/elamp4.0.0/index.html) to design LAMP-specific primers for MO and its adulterants. Table 1 and Fig. 2 showed the primers and target positions, among them the parameters of primers include the length of F1c / F1c, F3 / B3 is 16–20 bp, and the melting temperature (Tm) is 55–63℃.

Table 1
Primer information used in this study.

label	length	Sequence	5'dG	3'dG
F3	20	CCTCGCCTTAGGAATTCGGA	-6.87	-5.55
B3	16	AAGGCTCGCGGCTCCT	-5.58	-5.93
F2	16	CGGGGGTGACGGATGT	-7.57	-4.9
F1c	19	ACTCGCATTTAGGCCGGCC	-6.42	-7.97
B2	20	GCCCTAGGGTCGTTAGAGTT	-5.77	-4.24
B1c	22	TCACGACGAGTGGTGGTTGAAC	-5.6	-4.18
FIP	35	ACTCGCATTTAGGCCGGCCCGGGGGTGACGGATGT		
BIP	42	TCACGACGAGTGGTGGTTGAACGCCCTAGGGTCGTTAGAGTT		

2.2 Specificity of LAMP detection

A total of 16 normally plants samples were detected by LAMP method in this work. The products were directly visualized as shown in Fig. 3a, target MO DNA samples changed unequivocally from orange to green, while other test tubes with adulterated DNA remained orange after 60 minutes of amplification. Therefore, using the specific LAMP primer designed in this study and adding calcein within 60 minutes, MO and its common adulterants could be quickly identified. Figure 3b shows that the LAMP product changes in turbidity during detection (lines 2 ~ 7) was initially detected at about the first 15 minutes of the process, and the turbidity was subsequently increased within 50 minutes. At the end of the reaction, the turbidity of the products from 2 to 4 tubes reached 0.5 ~ 0.6, and none of the adulteration products (lines 8 ~ 16) produced an amplification curve. This result indicated that the LAMP primers designed in this study are applicable for rapid amplification and capable of the next step of sensitivity detection.

2.3 Sensitivity and repeatability of LAMP detection

The ITS2 gene template concentration was diluted with the serial dilution (1×10^{-3} pg/μl; 1×10^{-2} pg/μl; 1×10^{-1} pg/μl; 1 pg/μl; 1×10^{1} pg/μl; 1×10^{2} pg/μl; 1×10^{3} pg/μl) of the MO DNA. A visual comparison of calcein (Fig. 4a) shows that no color change was observed for LAMP reactions containing less than 1×10^{-1} pg/μl of template DNA. The results shown in Fig. 4b indicate that the template DNA at a concentration of 1×10^{-1} pg/μl started to be amplified from 20 minutes and reached a turbidity of 0.49, whereas no curve was generated at a concentration of 1×10^{-2} pg/μl. Therefore, the minimum amount of template used to detect MO in the LAMP process is about 1×10^{-1} pg/μl. Nucleic acid gel electrophoresis results in Fig. 4c showed only bands at concentrations above 1 pg/μl, which indicates that LAMP assay is about 10-fold higher than conventional PCR method.

3. Discussion

The aim of this study was to establish method for distinguish MO (*Morinda officinalis How*) from the three adulterants MU (*Morinda umbellata L.*), Huci (*Damnacanthus indicus C.F.Gaertn*) and schisantherin F (*Schisandra propinqua subsp. Sinensis*). At present, some studies have reported various of novel methods of medicinal plants identification, such as barcode-based melting curve analysis, DNA labeling or ^{1} HNMR metabolite fingerprinting and so on [26,27]. Although these methods can accurately identify species, they require specialized knowledge, statistical analysis, and complex instruments. LAMP assay is a sensitive method as an alternative to existing technologies, and the diagnosis results can be easily interpreted by researchers without understanding the complicated theoretical knowledge [28]. The method uses Bst DNA polymerase and 4 or 6 primers to amplify circular DNA, and the amplification time is usually less than 1 hour. Adding some fluorescent dye after the LAMP reaction can directly visualize the test results. At present, this detection technology has been widely used in field disease diagnosis [29]. In our study, a set of loop-mediated isothermal amplification (LAMP) primers and detection methods were designed for MO ITS2 sequences, which can quickly identify MO and adulterants. Using bioinformatics methods combined with plant DNA barcode technology, we selected the ITS2 sequence with the smallest sequence divergence, the largest genetic variation coefficient, and a very conservative sequence from the commonly used matK, psbA-trnH, rbcL, and ITS2 sequences as amplification primers. The high specificity of the primers is guaranteed after a detailed comparison of ITS2 sequences between species with high gene coincidence between genus, the optimal amplification region and sequence were selected, so the detection results have high accuracy and reliability. The gene to be tested must be highly matched with the designed primers, otherwise it will not be able to perform amplification and show negative results[30]. Therefore, the LAMP technology can successfully detect the template at 1×10^{-1} pg/μl, which is extremely sensitive, and the detection limit is 10 times that of PCR technology. After the amplification is completed, a large amount of nucleic acid is generated, and the reaction with magnesium ions in the reaction system produces a visible white precipitate, which is $Mg_2P_2O_7$ [31]. Adding fluorescent dyes such as SYBR Green I, calcein, and HNB can produce color changes, and the results are objective and simple to

judge, reducing the more complicated detection steps such as agarose gel electrophoresis [32]. The dye calcein selected for this experiment can exist in the reaction system before amplification, therefore the color change can be observed without opening the lid to obtain the detection result, which avoids aerosol pollution [33]. While reducing the detection time, it also avoids contact with toxic reagents, which can be more effectively applied to more places. Compare with other technologies about identify MO, our results indicate that LAMP assay may be a better choice.

Although this research has obtained beneficial results, there are still some problems with this experimental method that need to be addressed. On the one hand, the process of plant DNA extraction is sometimes difficult, in the process of LAMP assay, problems such as failure of DNA extraction may lead to detection failure. On the other hand, although MO and its adulterants can be quickly identified through visual inspection, the type of adulteration cannot be identified, and quantitative estimation of adulterants cannot be performed.

4. Materials And Methods

4.1 Herbal sample collection and DNA extraction

According to our surveys, we choose the herbal materials that most commonly used to confuse MO include *Morinda umbellata L.* (MU), *Damnacanthus indicus C.F.Gaertn* (Huci), *Schisandra propinqua subsp. Sinensis* (schisantherin F) [34–36]. For the next DNA extraction and then LAMP test, we selected 25 plant material, of which 10 target MO were obtained from Guangzhou, Fujian and Guangxi, and 15 nontarget species were obtained from Guangzhou, Fujian and other regions. These above sample sources were displayed in Table 2. We performed DNA extraction of the above plant materials according to the instructions of the Sangon Biotechs™ Ezup columnar plant genomic DNA extraction kit. The extracted DNA was quantified using A260/280 ratio by QuickDrop™ Spectrophotometer, and all the values were greater than 1.80. To evaluate the sensitivity of the LAMP method, we diluted MO genomic DNA by a 10-fold dilution method, with a concentration range of 1×10^{-3} pg/μl to 1×10^{3} pg/μl [26].

Table 2
The species origin, collection area of MO and its common adulterants in this study.

	Species	portion	Origin
1	*Morinda officinalis How.*	root	Guangdong China
2	*Morinda officinalis How.*	root	Guangdong China
3	*Morinda officinalis How.*	root	Guangxi China
4	*Morinda officinalis How.*	root	Guangxi China
5	*Morinda officinalis How.*	root	Fujian China
6	*Morinda officinalis How.*	root	Fujian China
7	*Morinda officinalis How.*	leaf	Guangdong China
8	*Morinda officinalis How.*	leaf	Guangdong China
9	*Morinda officinalis How.*	leaf	Guangxi China
10	*Morinda officinalis How.*	leaf	Fujian China
11	*Morinda umbellata* L.	root	Guangdong China
12	*Morinda umbellata* L.	root	Guangdong China
13	*Morinda umbellata* L.	root	Fujian China
14	*Morinda umbellata* L.	leaf	Guangdong China
15	*Schisandra propinqua* subsp. *sinensis*	root	Henan China
16	*Schisandra propinqua* subsp. *sinensis*	root	Henan China
17	*Schisandra propinqua* subsp. *sinensis*	root	Henan China
18	*Schisandra propinqua* subsp. *sinensis*	leaf	Henan China
19	*Schisandra propinqua* subsp. *sinensis*	leaf	Henan China
20	*Damnacanthus indicus C.F.Gaertn.*	root	Guangdong China
21	*Damnacanthus indicus C.F.Gaertn.*	root	Guangdong China
22	*Damnacanthus indicus C.F.Gaertn.*	root	Guangdong China
23	*Damnacanthus indicus C.F.Gaertn.*	root	Guangxi China
24	*Damnacanthus indicus C.F.Gaertn.*	leaf	Guangdong China
25	*Damnacanthus indicus C.F.Gaertn.*	leaf	Guangxi China

4.2 Candidate sequence analysis

According to the commonly used genes encoding ITS, ITS2, psbA-trnH, matK, and rbcl published by GenBank, we selected the ITS2 gene sequence (No: AB715224.1) as the target gene for the primer sequence after referenced articles and used Blast analysis in NCBI (http://www.ncbi.nlm.nih.gov/BLAST). Then comparing with different closely related plants, it was found that more than 98% of the plants can be distinguished based on the sequence. The nucleotide sequences multiple alignments results are shown in Fig. 2 (made by clustalX, v1.83). Primer Explorer 5.0 (http://primerexplorer.jp/elamp4.0.0/index.html) software was used to design 4 sets of specific LAMP primers, each set 2 external primers F3 (forward prime), B3 (backward prime) and 2 pairs of inner primers FIP, BIP. Among them, the inner primers contain the whole F2. B2 and FIP, BIP sequences on the target region. According to the characteristics of the gene sequence itself and the primer parameters provided by the software, 4 primers were selected and sent to Sangon Biotechs™ for synthesis.

4.3 LAMP assay and product detection

In the fluorescent visual inspection method, we chose calcein as the nucleic acid dye. First, prepare 25 μl reaction mixture on ice in 200 μl test tube, including 10 mM dNTPs 3.5 μl, 2.5 M betaine 1 μl, 75 mM MgSO4 1 μl, 12.5 mM MnCl2 1 μl, 10 × Bst buffer 2.5 μl, 8000 U / ml BstDNA Polymerase 1 μl. Then add 4 μl of outer primers ITS2-FIP and ITS2-BIP, 2 μl of inner primers ITS2-F3 and ITS2-B3, 1 μl of DNA template, and 2 μl of ddH2O. 1 μl of calcein (a fluorescent detection reagent) was added to 25 μl of the LAMP reaction mixture before the LAMP reaction. Among them, the positive control is a reaction mixture containing genuine MO's DNA, and the negative control is a reaction mixture containing no genuine MO's DNA. Next, isothermal reaction after mixing the mixed solution and centrifuge, put it into a water bath and incubated it at constant 63℃ for 40 minutes to carried out the loop-mediated isothermal amplification of DNA molecules. After amplification, the reaction was stopped at 80℃ for 5 minutes [37]. Last, after the LAMP amplification of genuine MO and 3 kinds of mixed adulterants (MU, Huci, schisantherin F), we observed the color changes of the LAMP reaction solution. In order to perform real-time quantitative analysis of the LAMP method, the turbidity was monitored on a turbidimeter and followed the instructions (Loopamp®DNA Amplification kit, Loopamp®320, Eiken Chemical Co., Japan), then observed if the derivative "S" type amplification curves derivative. If the "S" type amplification curve appears, the amplification is successful, and the test is positive, which is a genuine MO; if the "S" type amplification curve doesn't appear, there is no amplification, and the test is a negative result, adulterations will be possible.

4.4 Specificity and sensitivity analysis

To determine the specificity of the LAMP assay, we selected MO and MU, Huci, schisantherin F samples with similar DNA concentration after extraction. To verify the sensitivity and the limit of detection of the LAMP assay, the MO gene template concentration was diluted with 5 gradients of 1×10^{-3} pg/μl; 1×10^{-2} pg/μl; 1×10^{-1} pg/μl; 1 pg/μl; 1×10^{1} pg/μl; 1×10^{2} pg/μl; 1×10^{3} pg/μl. All of the results were tested using the same LAMP assay method as described in 2.3 section and the results were detected by

observing color and turbidity changes. Then LAMP amplification products were compared to the products by PCR methods. PCR was carried out with the TaKaRa Ex Taq® DNA Polymerase and inner primers ITS2-F3 4 μl and ITS2-B3 4 μl. Reactions were performed with 2 μL of templete DNA in a final volume of 25 μL containing: Ex Taq (5 U/μl) 0.125 μl, 10 × Ex Taq Buffer (Mg^{2+} plus) (20 mM) 2.5 μl, dNTP Mixture 2 μl. Above mixture was incubated based on the TaKaRa Ex Taq® DNA Polymerase instructions: warm up at 98℃ for 30 s, 30 cycles of at 98℃ for 10 s, 55℃ for 30 s and 72℃ for 1 min, followed by a final extension step at 72 ℃ for 5 min. This reaction process is carried out in a Bio-rad T100™ Thermal Cycler. The PCR amplified products were detected by electrophoresis in 2% agarose gel in 1 × TAE buffer with 0.5 × GelRed™ at 120V for 30 min. All examinations were confirmed three replicates using at least two independent DNA samples from each sample by the same operator.

Declarations

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Authors contributions

Yao ruyong managed the project and Sui Aihua designed the study. Wang Shuhan, Han Yafei and Xu Hang performed the experiments. Zhou Quan analyzed the data. Wang Shuhan wrote the first draft of the manuscript. All authors read and approved the final manuscript for publication.

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Competing interests

The authors declare that there is no conflict of interest.

Additional information

All data generated or analysed during this study are included in the supplementary Information files.

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Figures

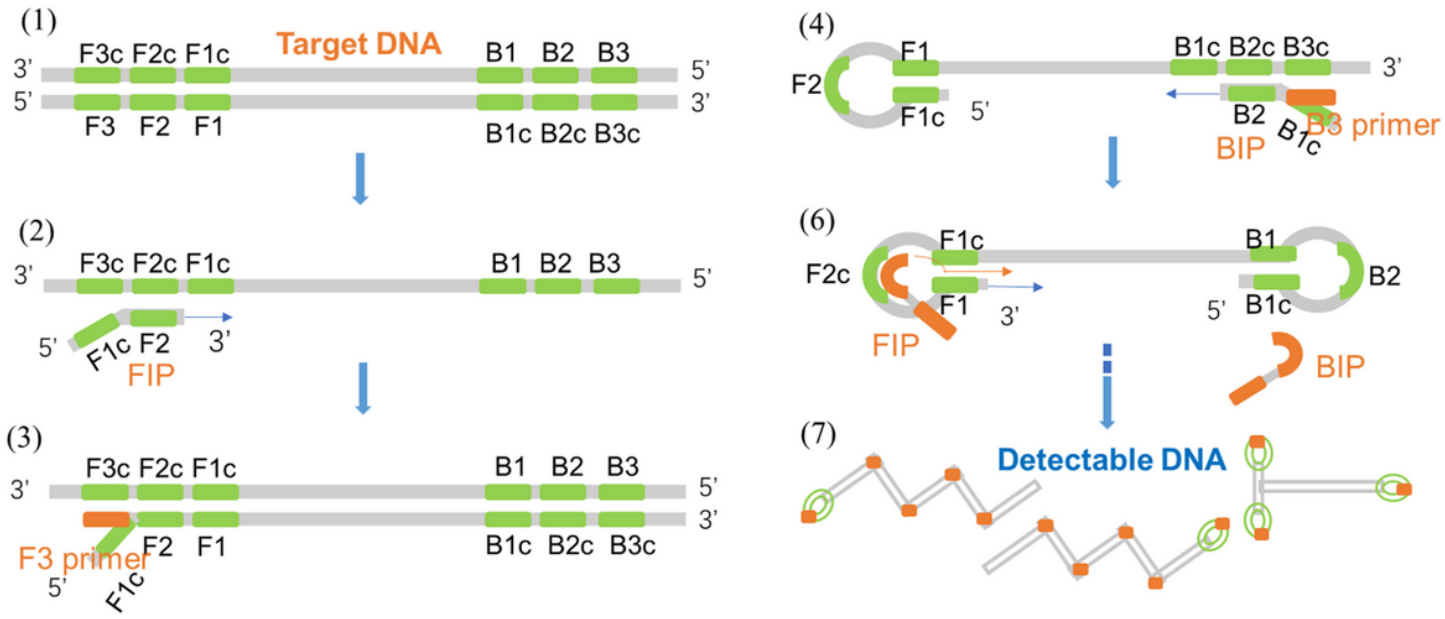
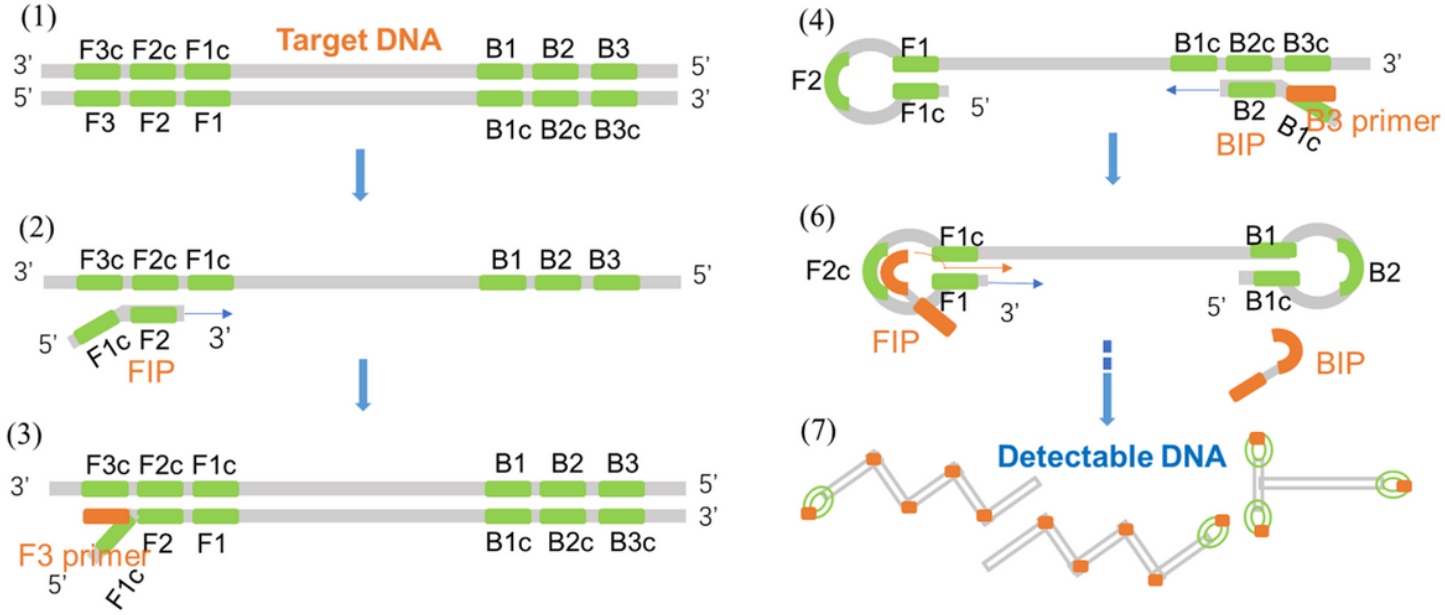


Figure 1

DNA loop amplification schematic depiction



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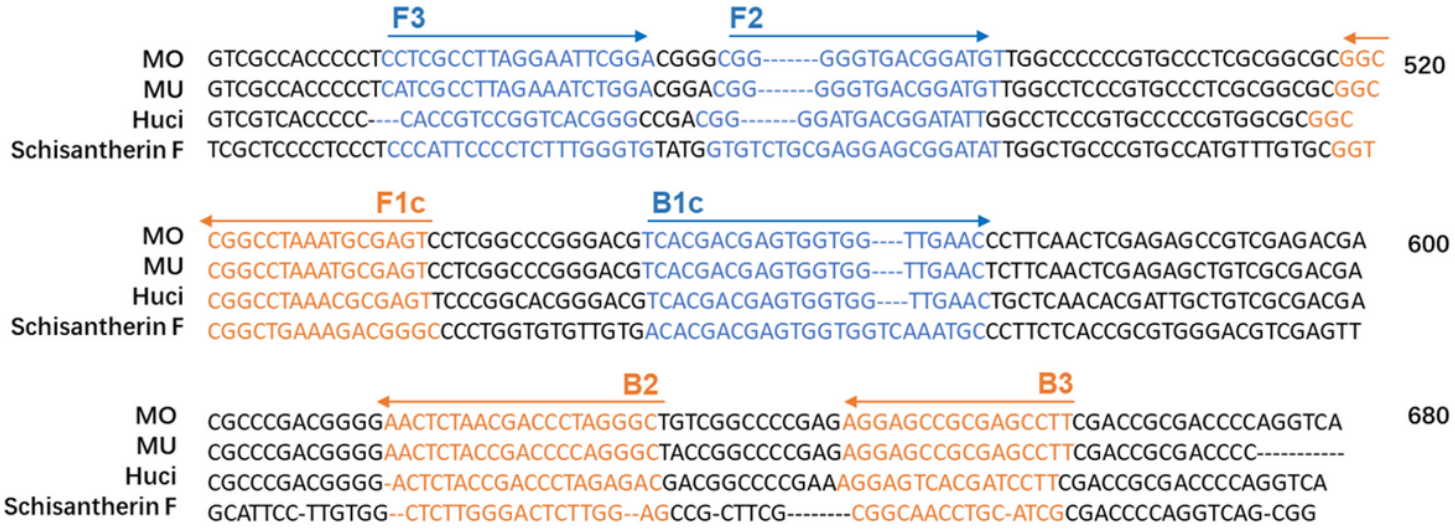
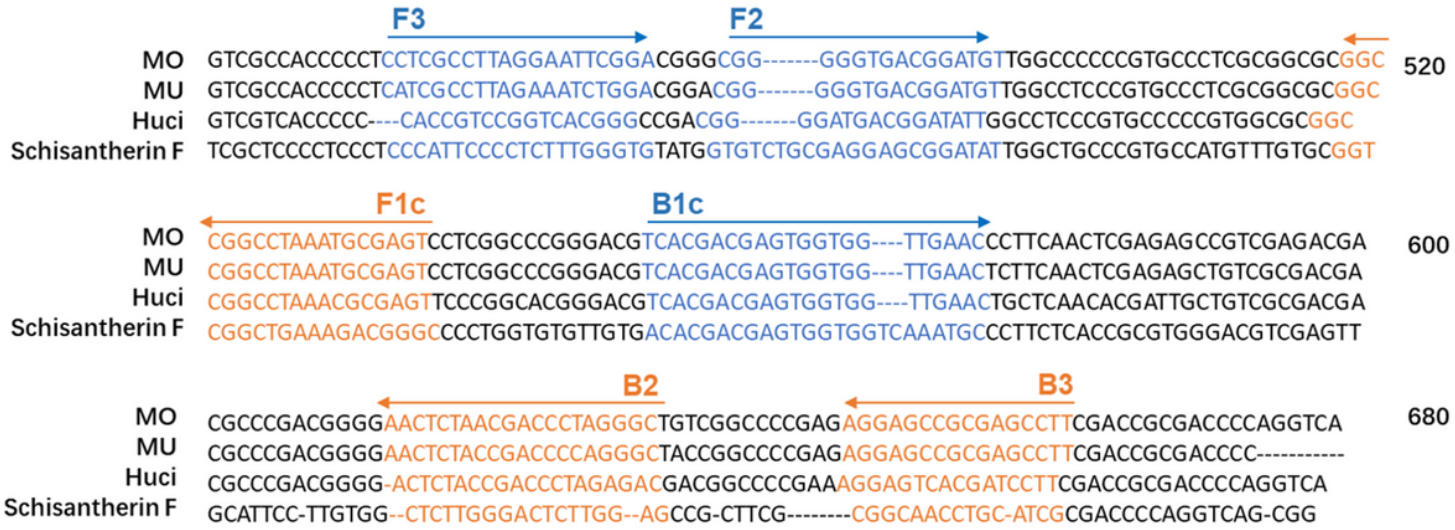


Figure 2

Sequence alignment of the ITS2 of MO and its adulterants. The colored sequence (blue and orange) indicates the regions that share the specifically designed LAMP primer sequences. The green arrow symbols indicate the direction of DNA polymerization from the LAMP primers



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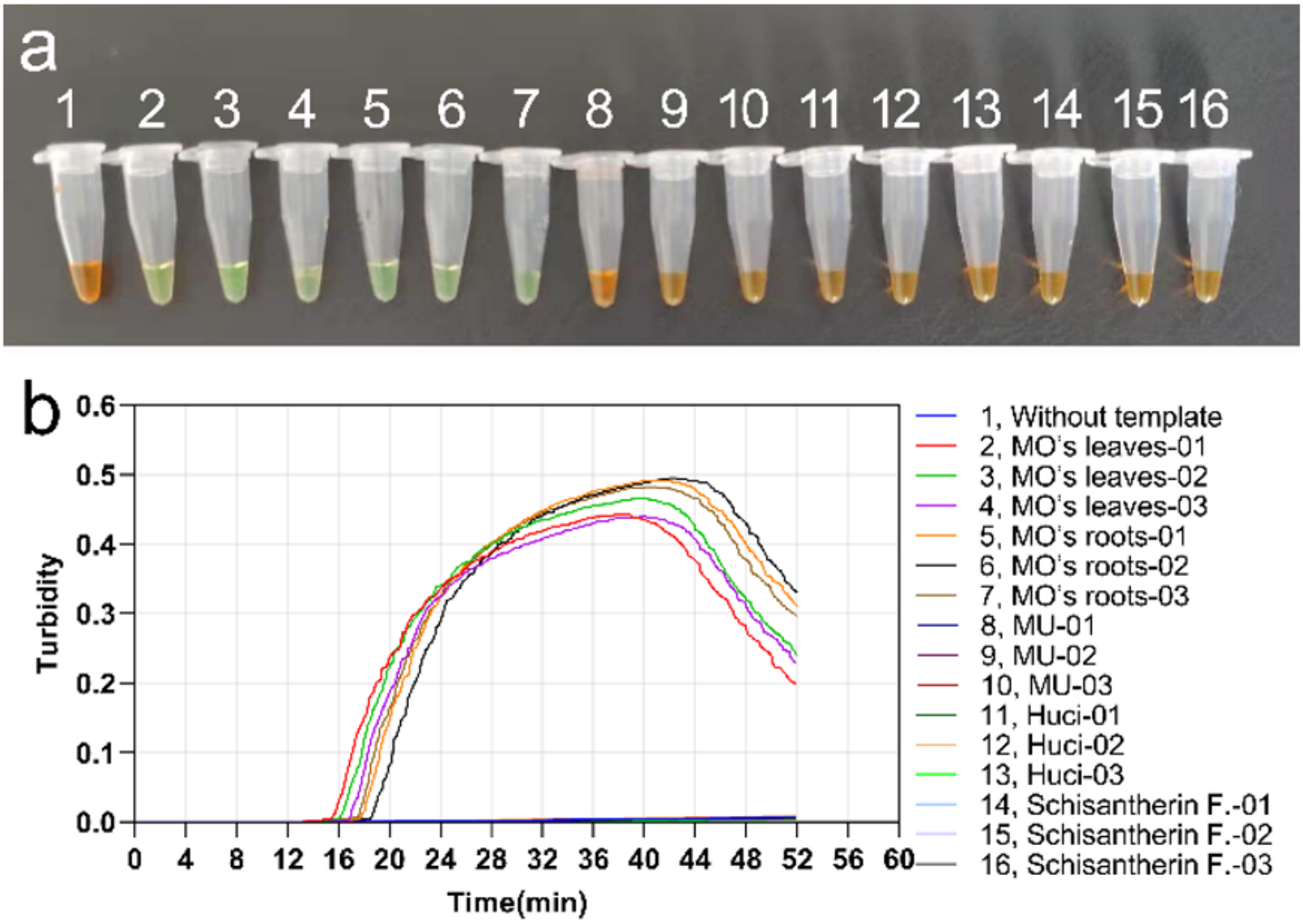
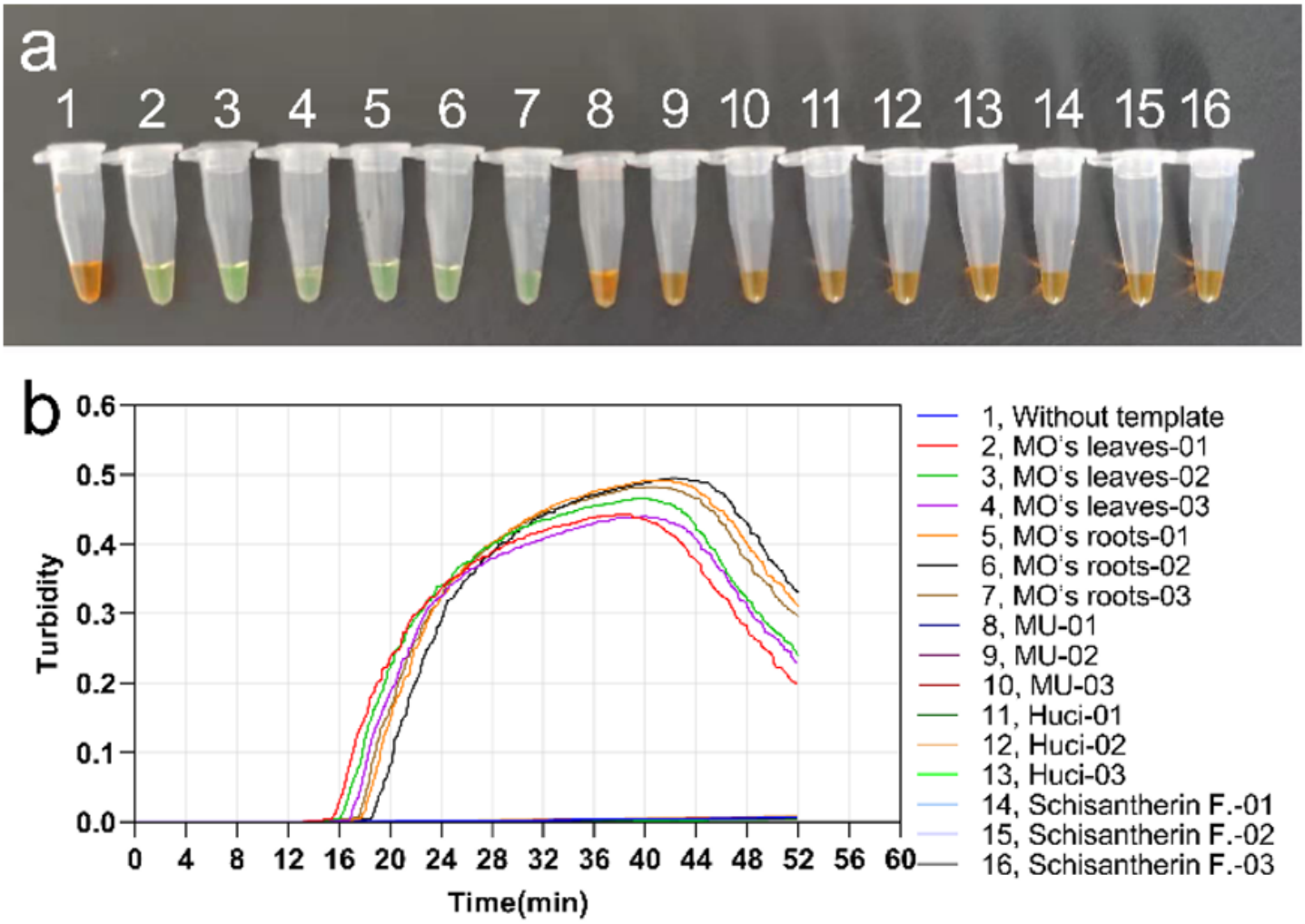


Figure 3

Specificity of the LAMP assay for the authentication of MO and its adulterants. Tubes and lines: 1, the LAMP product without template DNA; 2~4, DNA from MO's leaves; 5~7, DNA from MO's roots; 8~10, MU; 11~13, Huci; 14~16, Schisantherin F. a Visual color change detection method was compared. b Turbidity curves was monitored at 400nm every 6s



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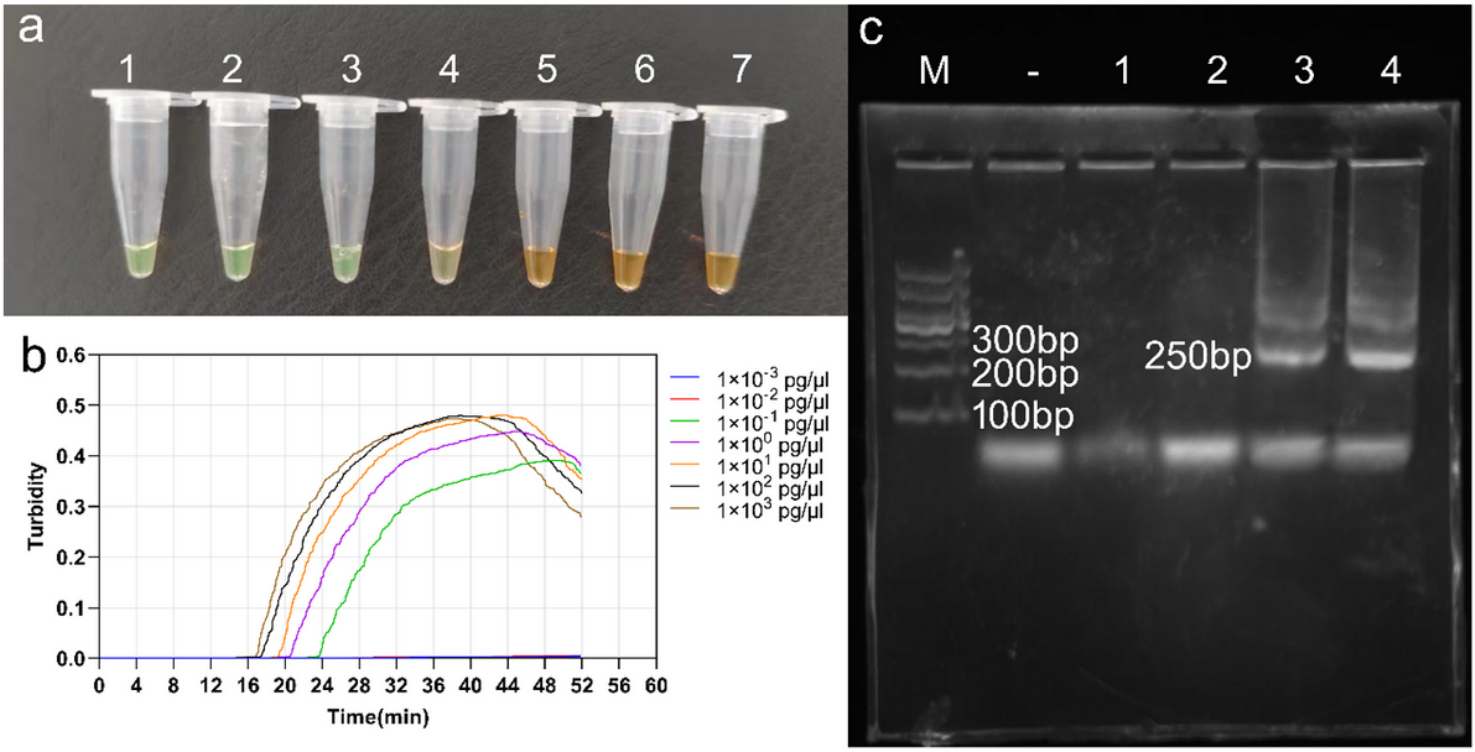
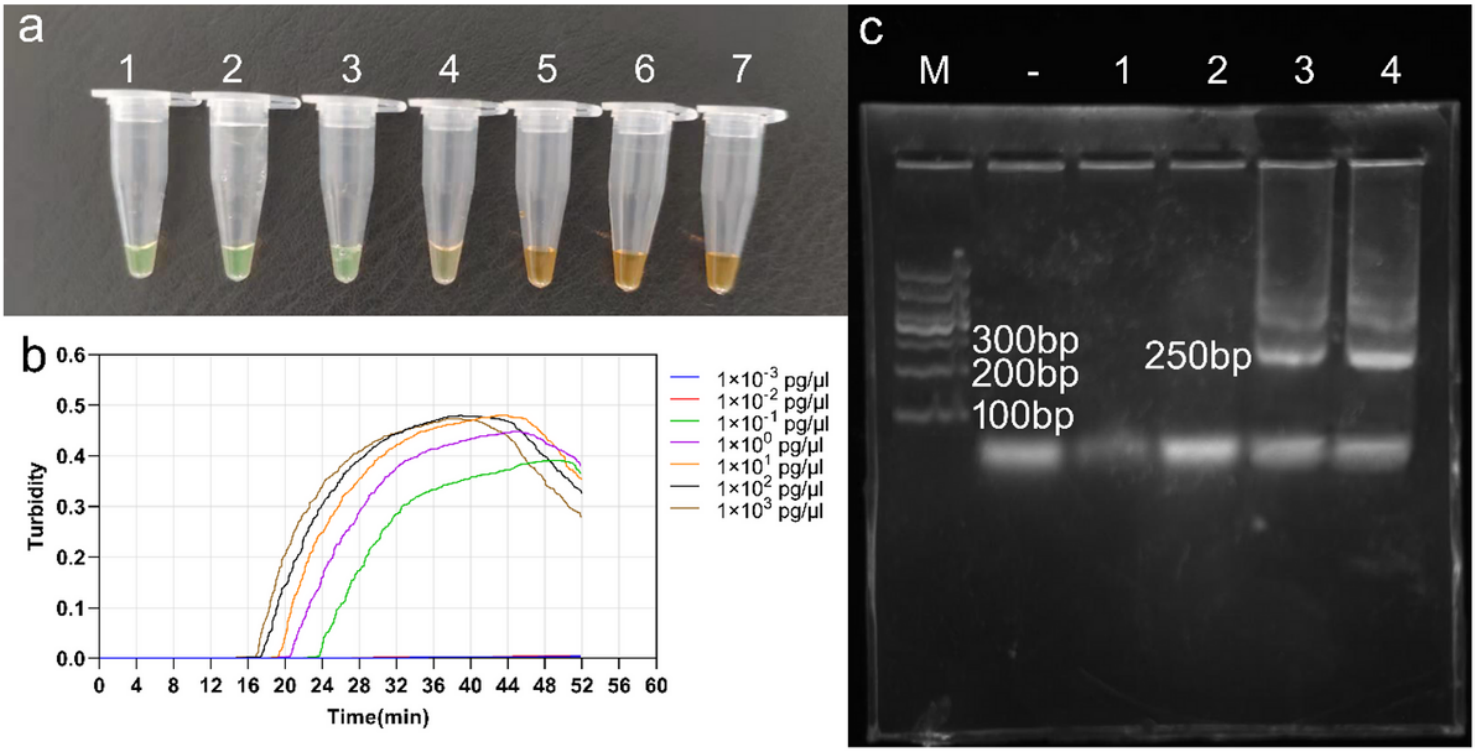


Figure 4

Sensitivity of the LAMP reaction for the detection of MO. The pure genomic DNA extracted from saffron was diluted in a serial 10-fold dilution. a Visual color change detection method was compared. Tubes: 1, 1×10-3 pg/μl; 2, 1×10-2 pg/μl; 3, 1×10-1 pg/μl; 4, 1 pg/μl; 5, 1×101 pg/μl; 6, 1×102 pg/μl; 7,1×103 pg/μl. b Turbidity was monitored at 400nm every 6s; c PCR results are displayed in 2% agarose gel staining with ethidium bromide visualized under UV light. The band is constituted by: M, Molecular marker of 1000 bp; –, Control negative; 1, 1×10-2 pg/μl; 2, 1×10-1 pg/μl; 3, 1 pg/μl; 4, 1×101 pg/μl.



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