# Authenticity Identification of the Traditional Chinese Herb Glycyrrhiza uralensis by Real-Time PCR and Loop-Mediated Isothermal Amplification

**Tiantian Jia**
Minzu University of China

**Dan Han**
Minzu University of China

**Minmin Ma**
Minzu University of China

**Dan Jiang**
Dalian Ocean University

**Yaojiang Huang** ( ✉ yaojiangh@hotmail.com )
Beijing Engineering Research Center of Food Environment and Public Health, Minzu University of China
https://orcid.org/0000-0001-5409-5605

---

---

# Abstract

Background: Glycyrrhiza uralensis is a traditional Chinese herb, and sales volume of this herb is large. However, adulterant herbal materials threaten trade and consumer safety. Methods：A rapid detection system for the identification of G. uralensis was established using loop-mediated isothermal amplification (LAMP) and real-time fluorescence quantitative PCR (Real-Time PCR). The DNA was extracted and the G. uralensis primers were designed. LAMP and Real-Time PCR were performed to assessment the specificity of primers in species. The sensitivity of LAMP and Real-Time PCR was contrast by diluting DNA concentration gradient from 101 ng/µL to 10-5 ng/µL. Results: tThe results showed that LAMP and Real-Time PCR were specificity because G. uralensis were positive while similar species were not. The sensitivity of LAMP was similar to Real-Time PCR at DNA concentration of 10-4ng in 60 min. The results indicated that LAMP and Real-Time PCR are accurately, specificity and sensitivity in the Authenticity Identification of G. uralensis. Conclusions: LAMP is less time-consuming, convenient, and does not require expensive equipment. Thus, these findings suggested that a method system and standard of traditional Chinese herb market supervision should be established based on a DNA barcode sequence and LAMP for authenticity identification.

# Background

Traditional Chinese herbs (TCHs) play an important role in disease treatment and studies as indicated the clinical efficacy of TCHs against certain diseases[ 1 ]. *G. uralensis* is a well-known and well-regarded traditional Chinese medicine. *G. uralensis* is often used as a cough expectorant that effectively relieves sore throats due to its anti-inflammatory and anti-allergy effects.

The quality of TCHs is important for the effect and safety. Some defective goods and falsified herbs are entering the TCHs market due to resource depletion and profit-driven influences, which poses a serious risk to public health. However, it has been recently reported the adulterant drugs cause life-threatening poisonings[ 2-5 ]. Therefore, the problem of authenticity identification of Chinese medicinal materials has become an important issue in improving the quality of TCHs and guaranteeing import, export, and human health[ 6 ].

The traditional identification and shape classification method do not meet the needs for identification of TCHs, especially for broken up into small pieces after complicated processing, which have disadvantages, including insufficient effects and accuracy [ 7 ]. In addition, none of these methods can be used to identify the closely related species with obviously similar chemical and morphological characteristics[ 8 ].

LAMPand Real-Time PCR are rarely used in the identification of TCH because they are often use for the identification of microorganism[ 9-11 ][ 12-15 ]. In the present study, a real-time LAMP protocol was developed, and the reliability and sensitivity of this protocol was investigated for authenticity identification of traditional Chinese herbs using the ITS2 gene as the DNA barcoding gene.

# Methods

## Traditional Chinese herbs

Four Chinese herbs with similarity to *G. uralensis* were used as negative samples to test for specificity (Table 2), considering the following characteristics. First, the difficulty in distinguishing them from *G. uralensis* based on shape, smell and colour when TCHs are processed into small pieces or powder due to these herbs were similar to *G. uralensis*. Second, these herbs had similar therapeutic effects on disease. However, these herbs were less expensive than *G. uralensis* but also less effective. Lastly, detection of *G. uralensis* by the Entry-Exit Inspection and Quarantine Bureau has generally found these herbs adulterated.

No specific permits were required for these activities because the G. uralensis and negative samples used in this study were obtained from an orchard, which is a demonstration base of Minzu University of China. Liaoning Entry-Exit Inspection and Quarantine Bureau undertook the formal identification of the samples and provide details of any voucher specimens deposited by obtained.

## DNA extraction and primer design

DNA was extracted by an improved cetyltrimethylammonium ammonium bromide(CTAB) method [ 16 ]. The sensitivity of LAMP and Real-Time PCR was contrast by diluting DNA concentration gradient from $10^1$ ng/μL to $10^{-5}$ ng/μL. The barcoding gene was screened, and a *G. uralensis* gene was selected (Table 2) [ 8 ]. *ITS2* and Genbank accession number were used as keywords to search the NCBI database. The Blast N was utilized in the Nr database to identify similarly sequence. Analysis of these sequences was performed using MEGA 6.0 software to identify unique regions. Lastly, primers were designed using Primer Express 3.0 software.

## Real-time PCR and LAMP

Real-time PCR was performed using a Q-PCR Mix kit. The conditions were as follows: 95 °C for 30 s; 40 cycles at 95°C for 5 s and 55°C for 30 s; and fluorescence was detected at 72°C.

The Real-time PCR and LAMP [ 17 , 18 ] depends on DNA barcoding genes, which were screened to obtain a unique *G. uralensis* gene (GenBank No. AY065622). DNA extractions from *G. uralensis* mixed with those from relevant species has tested. And such a test be used to better address the specificity. LAMP Primers were designed using PrimerExplorer software[ 8 ]. LAMP was performed using a Loopamp kit with Fluorescent detection reagent (Eiken Chemical Co. Ltd., Japan), incubated at 65°C for 90 minutes and then at 80°C for 2 min in a Loopamp real-time turbidimeter (LA-500, Teramecs Co. Ltd., Japan).

# Results

## Real-Time PCR Specificity analysis

The PCR efficiency of ITS2 and *psbA-trnH* sequences has compared in 400 TCHs samples, and it showed that the success rate of ITS2 and *psbA-trnH* sequences is 92.8% [ 8 ]. Thus, ITS2 was selected after filtering the barcoding sequences (Table 1) based on the highest success rate of the ITS2 primer pair.

The Real-Time PCR Specificity was indicated by the Ct values of *G. uralensis*, which were 21.75, 21.76 and 21.80, while the Ct values of ZY27, ZY28, ZY29 and ZY30 were greater than 30 (Fig. 1), indicating no non-specific amplification reaction. Thus, The Real-Time PCR and specific primer can identify *G. uralensis* with higher specificity and less time-consuming contrast to conventional PCR, and it monitors the reaction by the curve observing in real-time. Nonetheless, real-time PCR requires expensive kits and equipment.

## Specificity of LAMP

Three primers groups of LAMP were designed and preliminarily selected, and the best group was selected according to the initial turbidity time. Four primers (B3, F3, BIP and FIP) required by LAMP and six different distinct sequences of target DNA identified were shown in figure 2 (Fig. 2). For primer group 2, turbidity occurred after 20 minutes, and the curve decreased after 50 minutes. LAMP pyrophosphate detection by white turbidity after the precipitation peak caused a reduction in the precipitation amount, which decreased the detection value. For primer group 1, turbidity occurred after 60 minutes, while a detection line did not show up for the other group until the end (Fig. 3). Thus, primer group 2 was selected as the optimal group for LAMP, and loop primers where then designed according to the reaction time. The LAMP was considered positive when lines appeared after 20 minutes for *G. uralensis* without lines appearing for the other similar species (Fig. 4). Therefore, these findings indicated that the LAMP is specific to *G. uralensis*. The early appearance in turbidity with the addition of loop primers indicated that the addition of loop primers accelerates the LAMP reaction. The turbidity appearance early with add loop primers indicated the loop primers accelerates the LAMP.

We perform the LAMP Specificity test of the mixing the DNA from *G. uralensis* with those from relevant species. And such a test to better address the specificity for authenticity identification of *G. uralensis* (Fig. 5).

## Sensitivity between Real-Time PCR and the LAMP

The DNA template was diluted in concentration gradient of $10^1$-$10^{-5}$ ng/μL. The detection concentration limit in the Real-Time PCR for herbal DNA was $10^{-4}$ ng/μL (Fig. 6). The Ct value was greater than 30 when the DNA concentration was $10^{-5}$ ng/μL and The Ct value increased with dilution of DNA. Because sensitivity of Real-Time PCR was higher which can detect a lower concentration of DNA than conventional PCR.

The sensitivity of Real-Time PCR was compared to that of LAMP using the same DNA template. Turbidity was lower with the reduction of DNA concentration. However, no turbidity was detected when DNA concentration was $10^{-5}$ ng/μL (Fig. 7a), which was no change when the amplification time varied 20~ 35 minutes. Moreover, no amplification was observed when the DNA concentration was lower than $10^{-4}$ ng/μL (Fig. 7b), which validated sensitivity of LAMP at $10^{-4}$ ng/μl.

# Discussion

LAMP is an isothermal nucleic acid amplification technique. Four different primers were used to identify 6 distinct regions on the target gene, which added to the specificity. An additional pair of loop primers can further accelerate the reaction [ 19 , 20 ]. Due to the specific nature of the action of these primers, the amount of DNA produced in LAMP is considerably higher than in PCR-based amplification. In contrast to PCR technology, in which the reaction is performed with a series of alternating temperature steps or cycles, isothermal amplification is performed at a constant temperature and does not require a thermal cycler.

LAMP allows observation of fluorescent signals without opening the tubes. The turbidity of the LAMP linearly increases with incubation time or DNA template concentration [ 9 ]. A minimal amount of DNA template can be amplified to visible turbidity after 60 minute incubation [ 9 , 21 ]. The by-product magnesium phosphate accumulated gradually as A large amount of DNA was amplified by LAM, which is visible as white turbidity [ 9 , 10 , 17 , 21 ]. LAMP is less time-consuming and does not require expensive equipment. Therefore, LAMP is an attractive method that is exploited in poor regions where facilities are not well equipped.

# Conclusions

LAMP and Real-Time PCR showed higher sensitivity compared to conventional PCR when detecting *G. uralensis* in the present study. LAMP and real-time PCR techniques are sensitive, less time-consuming, which make them useful tools for molecular authenticity of TCH[ 22 ], and These techniques are more practical than other molecular biology techniques and PCR where on-site diagnostics are required. LAMP is cost-effective, sensitive, less time-consuming, and expensive equipment is not necessary for the LAMP method. The present study reported for the first time the use of LAMP for authenticity identification of the traditional Chinese herb, *G. uralensis*. The present findings indicated that a method system and standard of TCH market supervision should be established based on DNA barcode sequence and LAMP for authenticity identification

## Abbreviations

TCHs: Traditional Chinese herbs

LAMP: Real-Time PCR; *Glycyrrhiza uralensis*

Real-Time PCR : Real-time fluorescence quantitative PCR

# Declarations

## Funding

## Availability of data and material

The datasets generated and analyzed in the current study are available from the corresponding author on reasonable request.

## Authors' contributions

DH, MM and TJ contributed equally to this work and conducted the experiments; YH and DJ conceived the experiments; and TJ and DH analysed the results. All authors reviewed the manuscript.

## Ethics approval and consent to participate

Not applicable. This article does not contain any studies with human participants or animals performed by any of the authors. No specific permits were required for these activities because the *G. uralensis* used in this study were obtained from an orchard, which is a demonstration base of Minzu University of China.

## Consent for publication

The authors agree for publication.

## Competing interests

No conflict of interest exits in the submission of this manuscript, and no any competing financial and/or non-financial interests in relation to the work described. At the same time, manuscript is approved by all authors for publication.

## Acknowledgements

# References

1. Busse W: The Significance of Quality for Efficacy and Safety of Herbal Medicinal Products. *Therapeutic Innovation & Regulatory Science* 2000, 34(1):15-23.

2. Dondorp AM, Newton PN, Mayxay M, Van Damme W, Smithuis FM, Yeung S, Petit A, Lynam AJ, Johnson A, Hien TT *et al*: Fake antimalarials in Southeast Asia are a major impediment to malaria control: multinational cross-sectional survey on the prevalence of fake antimalarials. *Tropical medicine & international health : TM & IH* 2004, 9(12):1241-1246.

3. Lee S, Lee T, Lee B, Choi H, Yang M, Ihm CG, Kim M: Fanconi's syndrome and subsequent progressive renal failure caused by a Chinese herb containing aristolochic acid. *Nephrology* 2004, 9(3):126-129.

4. Lo SH, Mo KL, Wong KS, Poon SP, Chan CK, Lai CK, Chan A: Aristolochic acid nephropathy complicating a patient with focal segmental glomerulosclerosis. *Nephrology, dialysis, transplantation : official publication of the European Dialysis and Transplant Association - European Renal Association* 2004, 19(7):1913-1915.

5. Vanherweghem JL, Tielemans C, Abramowicz D, Depierreux M, Vanhaelen-Fastre R, Vanhaelen M, Dratwa M, Richard C, Vandervelde D, Verbeelen D *et al*: Rapidly progressive interstitial renal fibrosis in young women: association with slimming regimen including Chinese herbs. *The Lancet* 1993, 341(8842):387-391.

6. Degardin K, Roggo Y, Margot P: Understanding and fighting the medicine counterfeit market. *J Pharm Biomed Anal* 2014, 87:167-175.

7. Chen S, Yao H, Han J, Liu C, Song J, Shi L, Zhu Y, Ma X, Gao T, Pang X *et al*: Validation of the ITS2 region as a novel DNA barcode for identifying medicinal plant species. *PLoS One* 2010, 5(1):e8613.

8. Han J, Pang X, Liao B, Yao H, Song J, Chen S: An authenticity survey of herbal medicines from markets in China using DNA barcoding. *Scientific reports* 2016, 6:18723.

9. Mori Y, Notomi T: Loop-mediated isothermal amplification (LAMP): a rapid, accurate, and cost-effective diagnostic method for infectious diseases. *Journal of infection and chemotherapy : official journal of the Japan Society of Chemotherapy* 2009, 15(2):62-69.

10. Basuni M, Muhi J, Othman N, Verweij JJ, Ahmad M, Miswan N, Rahumatullah A, Aziz FA, Zainudin NS, Noordin R: A pentaplex real-time polymerase chain reaction assay for detection of four species of soil-transmitted helminths. *The American journal of tropical medicine and hygiene* 2011, 84(2):338-343.

11. Song T, Toma C, Nakasone N, Iwanaga M: Sensitive and rapid detection of Shigella and enteroinvasive Escherichia coli by a loop-mediated isothermal amplification method. *FEMS microbiology letters* 2005, 243(1):259-263.

12. Yasuhara-Bell J, Kubota R, Jenkins DM, Alvarez AM: Loop-mediated amplification of the Clavibacter michiganensis subsp. michiganensis micA gene is highly specific. *Phytopathology* 2013, 103(12):1220-1226.

13. Perandin F, Manca N, Calderaro A, Piccolo G, Galati L, Ricci L, Medici MC, Arcangeletti MC, Snounou G, Dettori G *et al*: Development of a Real-Time PCR Assay for Detection of Plasmodium falciparum, Plasmodium vivax, and Plasmodium ovale for Routine Clinical Diagnosis. *Journal of Clinical Microbiology* 2004, 42(3):1214-1219.

14. Harper SJ, Ward LI, Clover GR: Development of LAMP and real-time PCR methods for the rapid detection of Xylella fastidiosa for quarantine and field applications. *Phytopathology* 2010, 100(12):1282-1288.

15. Yang C, Jiang Y, Huang K, Zhu C, Yin Y: Application of real-time PCR for quantitative detection ofCampylobacter jejuniin poultry, milk and environmental water. *FEMS Immunology & Medical Microbiology* 2003, 38(3):265-271.

16. Zhonggang Duan QH, Jinfen Yang: The optimum DNA extraction methods research in DNA barcoding indentification of traditional Chinese medicine. *Traditional Chinese Drug Research & Clinical* 2009, 20(5):480-484.

17. Notomi T: Loop-mediated isothermal amplification of DNA. *Nucleic Acids Research* 2000, 28(12):63e-63.

18. Savan R, Igarashi A, Matsuoka S, Sakai M: Sensitive and Rapid Detection of Edwardsiellosis in Fish by a Loop-Mediated Isothermal Amplification Method. *Applied and Environmental Microbiology* 2004, 70(1):621-624.

19. Annaka T: [Rapid and simple detection of Legionella species by LAMP, a mew DNA amplification method]. *Rinsho Biseibutsu Jinsoku Shindan Kenkyukai shi = JARMAM : Journal of the Association for Rapid Method and Automation in Microbiology* 2003, 14(1):25-30.

20. Nagamine K, Hase T, Notomi T: Accelerated reaction by loop-mediated isothermal amplification using loop primers. *Molecular and cellular probes* 2002, 16(3):223-229.

21. Mori Y, Nagamine K, Tomita N, Notomi T: Detection of loop-mediated isothermal amplification reaction by turbidity derived from magnesium pyrophosphate formation. *Biochemical and biophysical research communications* 2001, 289(1):150-154.

22. Lin Z, Zhang Y, Zhang H, Zhou Y, Cao J, Zhou J: Comparison of loop-mediated isothermal amplification (LAMP) and real-time PCR method targeting a 529-bp repeat element for diagnosis of toxoplasmosis. *Veterinary parasitology* 2012, 185(2-4):296-300.

# Tables

Due to technical limitations the tables could not be inserted and can be found as a PDF file in the supplemental section.
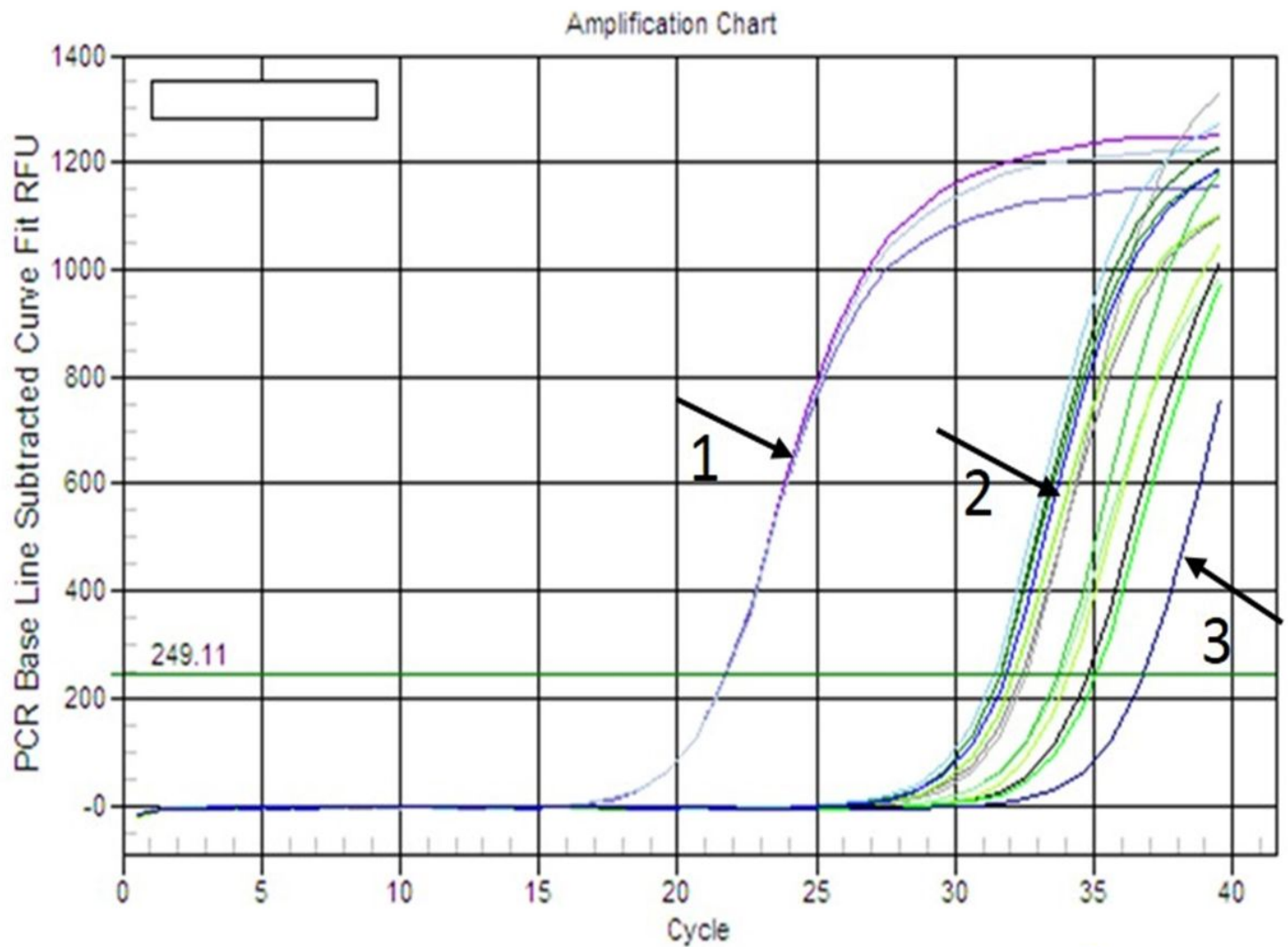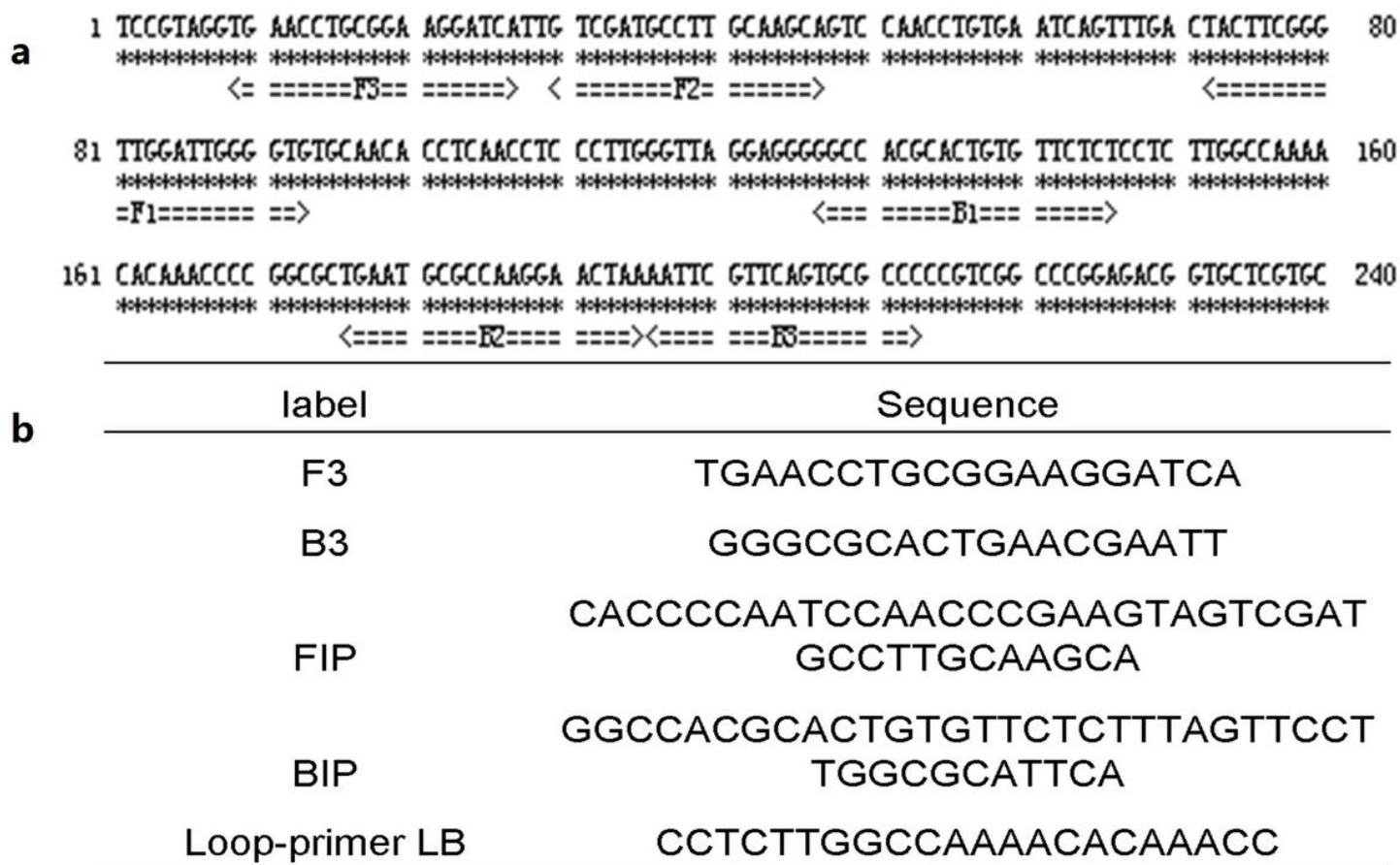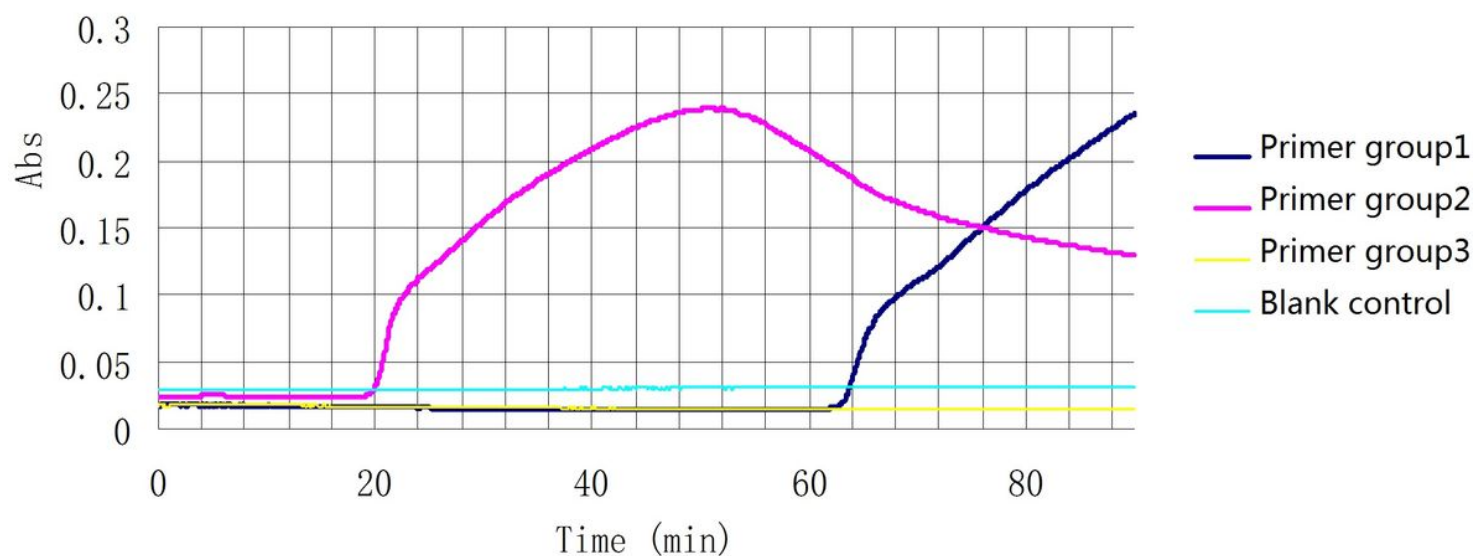
# Figures



**Figure 1**

Specific amplification of G. uralensis by real-time PCR. DNA was from 1) G. uralensis, 2) Chinese herb Nos. ZY27-ZY30 and 3) blank control.

**a**

```
  1 TCCGTAGGTG AACCTGCGGA AGGATCATTG TCGATGCCTT GCAAGCAGTC CAACCTGTGA ATCAGTTTGA CTACTTCGGG  80
    ********** ********** ********** ********** ********** ********** ********** **********
             <= ======F3== ======>  < =======F2= ======>                          <========

 81 TTGGATTGGG GTGTGCAACA CCTCAACCTC CCTTGGGTTA GGAGGGGGCC ACGCACTGTG TTCTCTCCTC TTGGCCAAAA  160
    ********** ********** ********** ********** ********** ********** ********** **********
    =F1======= ==>                                        <=== =====B1=== =====>

161 CACAAACCCC GGCGCTGAAT GCGCCAAGGA ACTAAAATTC GTTCAGTGCG CCCCCGTCGG CCCGGAGACG GTGCTCGTGC  240
    ********** ********** ********** ********** ********** ********** ********** **********
             <==== ====B2==== ====><==== ===B3===== ==>
```

**b**

| label | Sequence |
|---|---|
| F3 | TGAACCTGCGGAAGGATCA |
| B3 | GGGCGCACTGAACGAATT |
| FIP | CACCCCAATCCAACCCGAAGTAGTCGATGCCTTGCAAGCA |
| BIP | GGCCACGCACTGTGTTCTCTTTAGTTCCTTGGCGCATTCA |
| Loop-primer LB | CCTCTTGGCCAAAACACAAACC |

**Figure 2**

Nucleotide sequence of LAMP primers. a Nucleotide sequence used to LAMP primers design from G. uralensis. The targeting sequences positions was indicated by Underlines. b Nucleotide sequences of the best LAMP primer group of G. uralensis.



**Figure 3**

Screening the best group of LAMP primers. Screening for the best group of LAMP primers for G. uralensis by real-time turbidity.
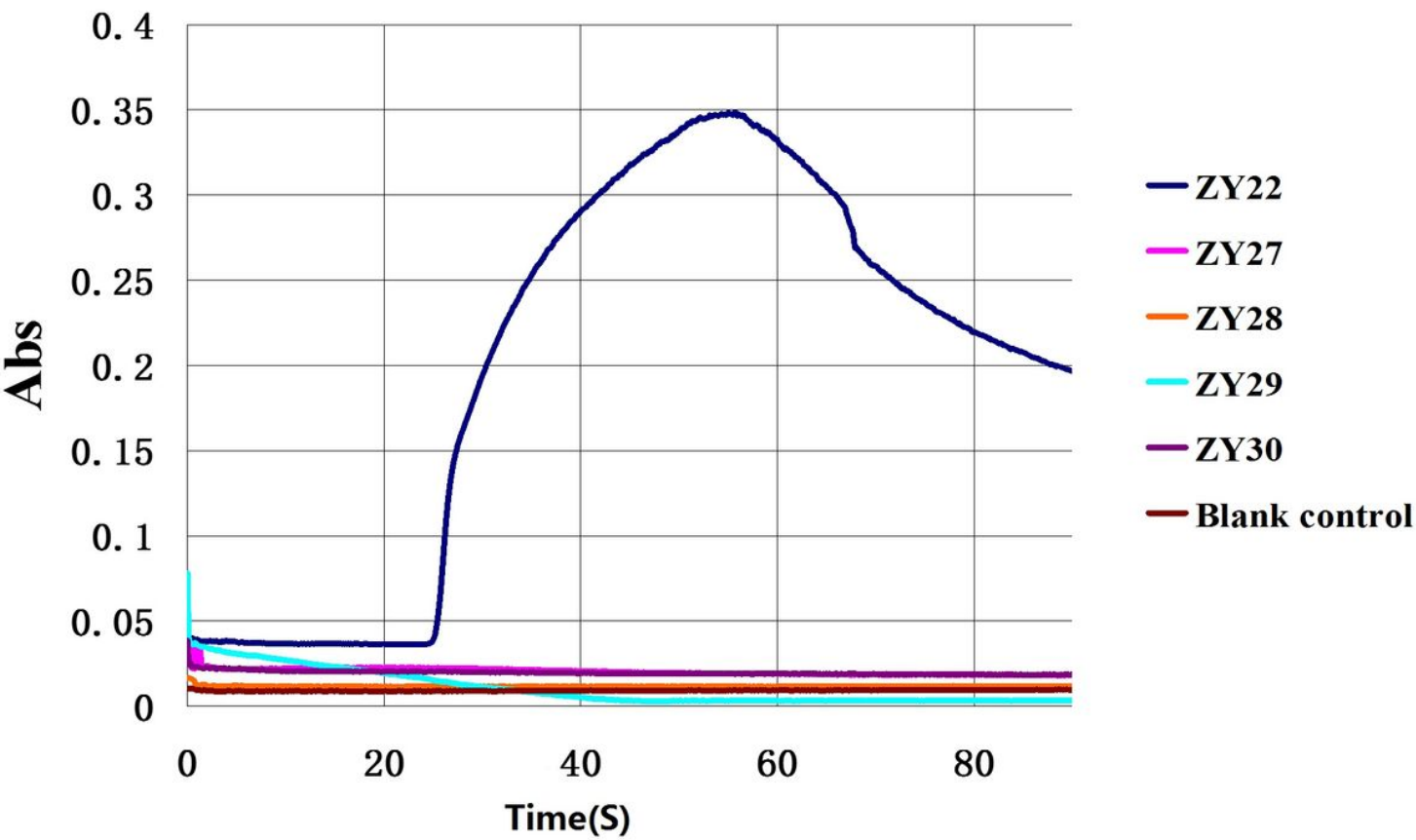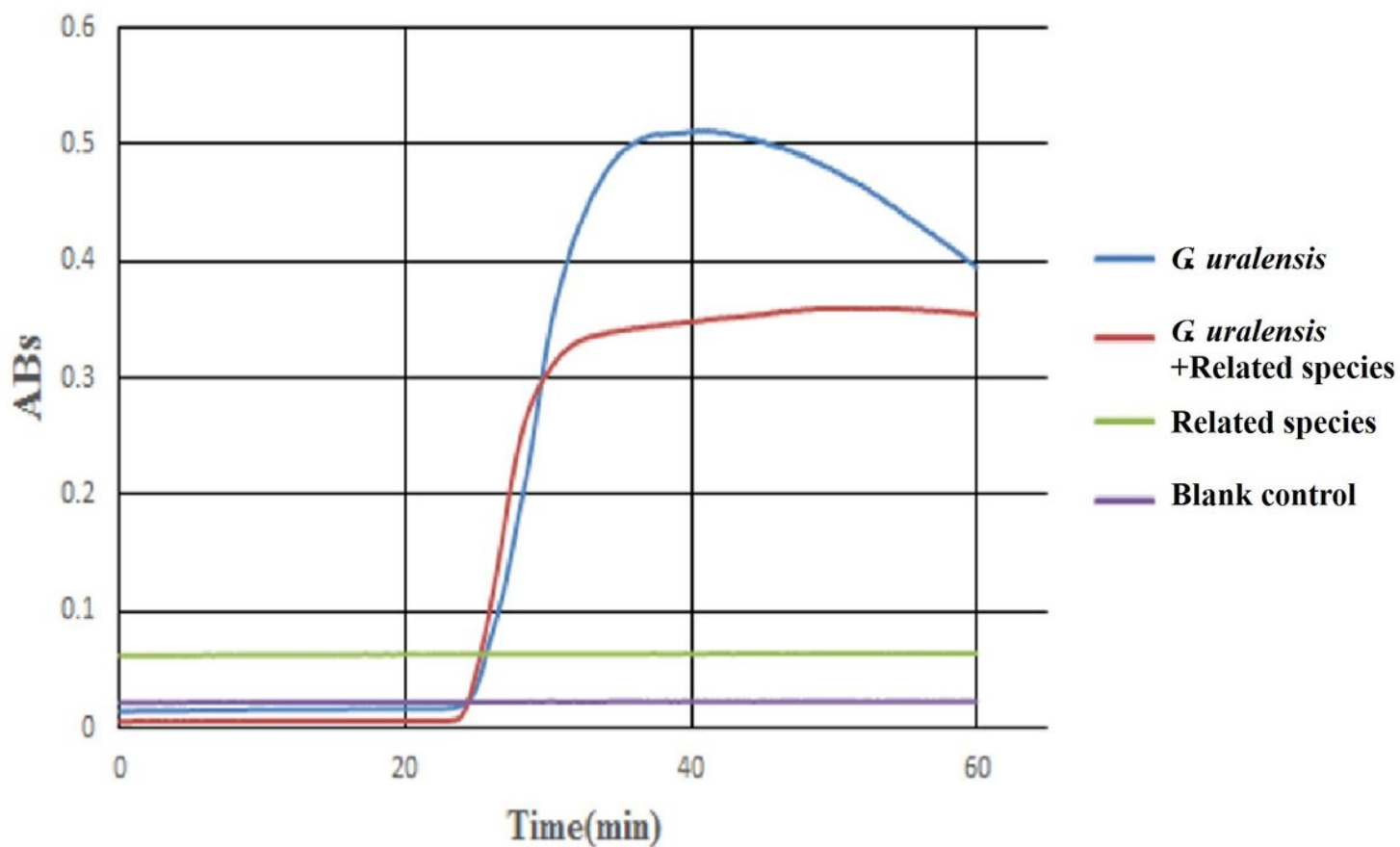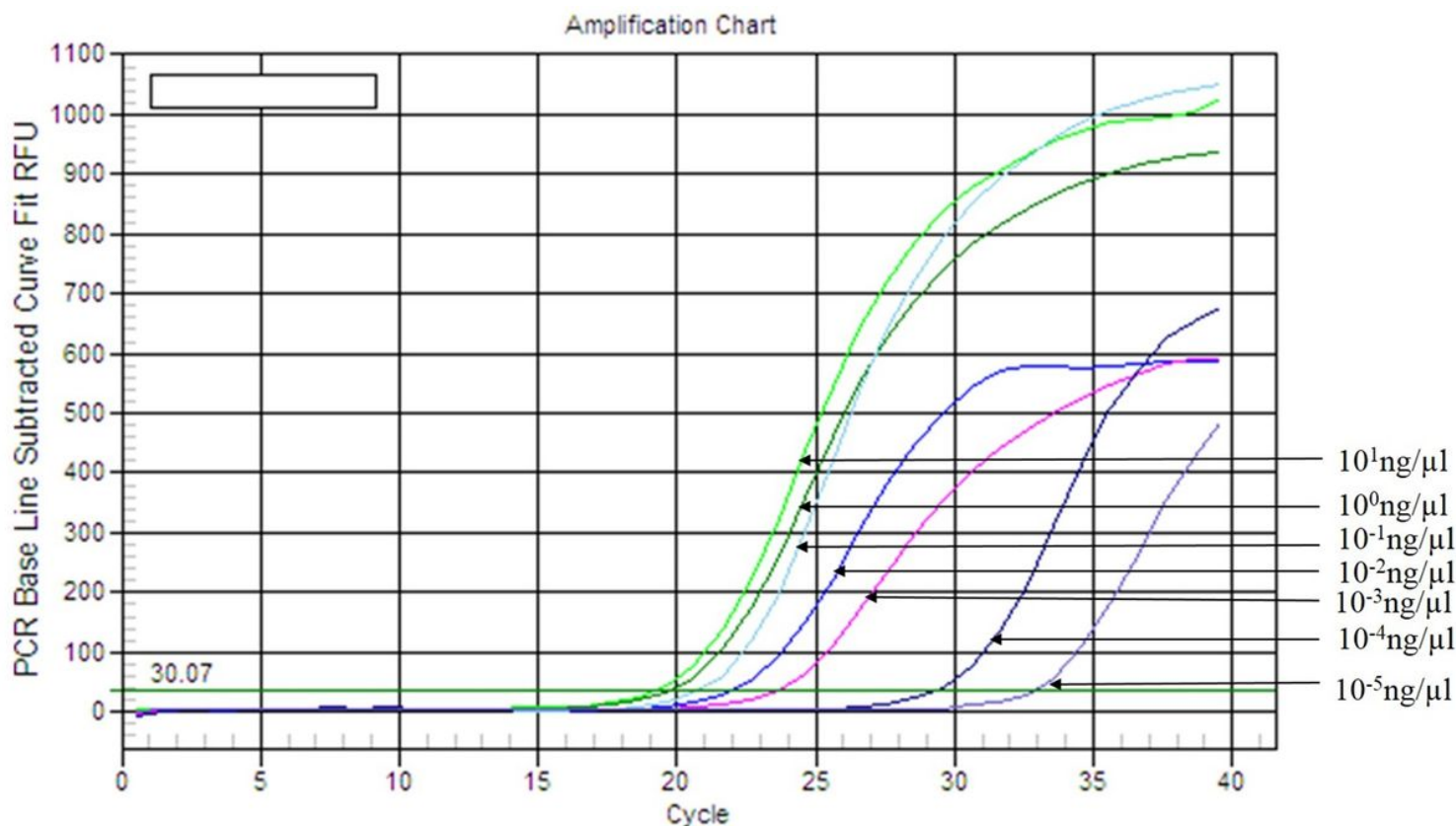


**Figure 4**

Specificity detection of the LAMP for G. uralensis. Controlled test between G. uralensis and similar species (ZY27-ZY30) with the best primers group and addition of loop primers.
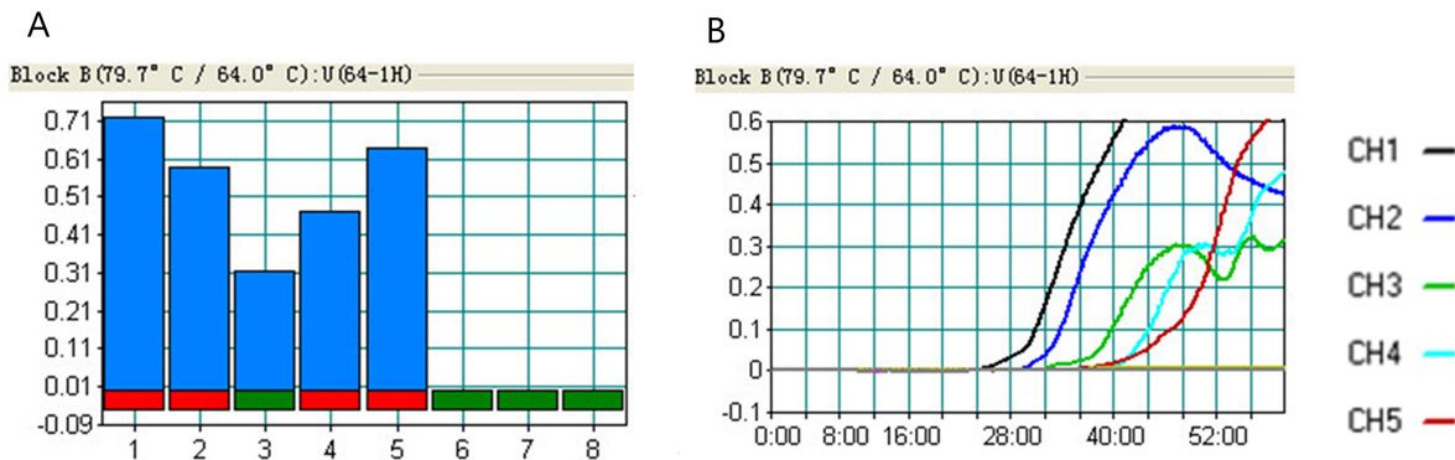
**Figure 5**

Specificity test of the mixing the DNA from G. uralensis with that from related species. The results of the test were obtained by mixing the DNA from G. uralensis with that from related species. Blue line, G. uralensis; Red line, mixing the DNA from G. uralensis with that from related species (ZY27-ZY30); green line, mixing the DNA from related species (ZY27-ZY30).

**Figure 6**

Sensitivity test of the real-time PCR for G. uralensis. Real-time PCR assays with different DNA concentrations of G. uralensis. Lanes 1–7: 101 ng/μL, 100 ng/μL, 10-1 ng/μL, 10-2 ng/μL, 10-3 ng/μL, 10-4 ng/μL, and 10-5 ng/μL.



**Figure 7**

Sensitivity test of the LAMP for G. uralensis. a Histogram of G. uralensis primer sensitivity detection. The Y-axis is turbidity (OD). b Graph of G. uralensis primer sensitivity detection. CH1-CH5: Different DNA concentrations ranging from 101 to 10-5 ng/μL.

# Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- supplement1.pdf