

Research Article

Visual Differentiation of *Bupleurum marginatum* var. *stenophyllum* From *Bupleurum chinense* DC. Using a PCR-Coupled G-Quadruplex Colorimetric Assay

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Abstract

Background: Adulteration of official *Bupleurum chinense* DC. with the morphologically similar *Bupleurum marginatum* Wall. ex DC. var. *stenophyllum* (Wolff) Shan et Y. Li poses safety risks. This study aimed to develop a rapid and visual method for differentiating these two species using a polymerase chain reaction (PCR)-coupled G-quadruplex/DNA enzyme (DNAzyme) colorimetric assay. **Methods:** Using species-specific single nucleotide polymorphism (SNP) loci in the internal transcribed spacer 2 (ITS2) region of ribosomal DNA, we designed forward and reverse primers tagged with the reverse complement of a G-quadruplex-forming sequence at their 5' termini. Following PCR, the target sequences were specifically amplified, generating amplicons flanked by G-quadruplex-forming motifs at both termini. The amplicons were then subjected to thermal denaturation, after which the G-rich sequences folded into G-quadruplex structures in the presence of K⁺. These structures were subsequently bound to hemin to form a G-quadruplex/hemin DNAzyme with peroxidase-like activity. The DNAzyme catalyzes H₂O₂-mediated oxidation of 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS) to a blue-green product. The PCR amplification and colorimetric reaction conditions were optimized by single-factor gradient experiments. **Results:** The optimal conditions were determined as follows: for PCR, a final primer concentration of 0.32 μmol/L, an annealing temperature of 60 °C, and 35 amplification cycles; for the colorimetric reaction, final concentrations of 5 μmol/L hemin and 2 mmol/L ABTS, with denaturation at 95 °C for 8 min, and incubation at 4 °C for 20 min. Validation of this assay using 21 *Bupleurum* samples confirmed that it specifically identified *B. marginatum* var. *stenophyllum*, while showing no cross-reactivity with *B. chinense*, *Bupleurum scorzoniferifolium* Willd., or other congeneric *Bupleurum* species included in the study. The statistical LOD of the assay was determined to be 1 ng/μL, with a reliable naked-eye visual threshold at ≥2 ng/μL and a validated detection range extending to at least 50 ng/μL with no observable hook effect. **Conclusions:** This assay enables naked-eye readout with high specificity and sensitivity. It provides a promising technical platform for the rapid differentiation of *B. marginatum* var. *stenophyllum* from *B. chinense*, as well as a broadly adaptable research framework for the visual differentiation of congeneric adulterants in traditional Chinese medicinal materials.

Keywords: polymerase chain reaction; G-quadruplexes; colorimetry; *Bupleurum chinense* DC.; *Bupleurum marginatum* var. *stenophyllum*; drugs; Chinese herbal; species specificity

1. Introduction

Bupleuri Radix (Chaihu) is a widely used traditional Chinese medicinal material with a well-documented history of more than 2000 years. It was first described in the *Shennong Bencao Jing* (Divine Farmer's Classic of Materia Medica). Modern pharmacological studies have further confirmed its anti-inflammatory, lipid-regulating, anti-tumor, and immunomodulatory activities, establishing it as a core raw material in numerous Chinese patent medicines and clinical formulations [1]. According to the *Pharmacopoeia of the People's Republic of China* (2025 edition), the official botanical origins of *Bupleuri Radix* are *Bupleurum chinense* DC. and *Bupleurum scorzoniferifolium* Willd.

[2]. However, dwindling wild populations, long cultivation cycles, and rapidly growing market demand have created a persistent supply shortage of these two official species, driving the widespread entry of non-official *Bupleurum* species into the commercial medicinal material market.

Bupleurum marginatum Wall. ex DC. var. *stenophyllum* (Wolff) Shan et Y. Li [3] shows high similarity in macroscopic characteristics to *B. chinense*. It often contains comparable or even higher levels of key active constituents such as saikosaponins, yet offers the commercial advantages of a shorter cultivation period, higher yield, and lower cost. The frequent adulteration of *B. chinense* with this species in commercial supply chains and manufacturing processes has made it the most prevalent adul-



terant of official *Bupleuri Radix* [4]. However, pharmacological and toxicological investigations have identified potential hepatotoxicity risks associated with *B. marginatum* var. *stenophyllum*. Specifically, Gansu-produced samples of *B. marginatum* var. *stenophyllum* showed markedly stronger acute toxicity and higher cytotoxicity ($p < 0.01$) than *B. chinense* [5]. Therefore, the adulteration of *B. chinense* with *B. marginatum* var. *stenophyllum* not only compromises the quality of *Bupleuri Radix* but, more importantly, poses a serious threat to clinical safety and therapeutic efficacy. Accurate discrimination between the two species remains challenging. Traditional macroscopic and microscopic identification methods are often inconclusive due to their near-identical morphology. Chemical profiling methods, such as high-performance liquid chromatography (HPLC) fingerprinting, are limited by extensive peak overlap and similarity in chromatographic fingerprints (correlation coefficients: 0.50–0.80), preventing accurate authentication [6]. Species-specific PCR methods have been developed and are capable of detecting adulteration levels as low as 5% [7,8]. However, these techniques rely on agarose gel electrophoresis for result interpretation, necessitating specialized instrumentation and technical expertise. This reliance limits their practical deployment in grassroots regulatory settings and on-site manufacturing inspections. Hence, there is a clear need for a rapid, accurate, and visual differentiation method to ensure the quality and safe use of *Bupleuri Radix*.

G-quadruplexes are four-stranded helical structures assembled from guanine (G)-rich nucleic acid sequences via Hoogsteen hydrogen bonding [9]. In the presence of K^+ , these structures specifically bind to hemin to form a G-quadruplex/hemin DNAzyme with peroxidase-like activity, which efficiently catalyzes H_2O_2 -mediated oxidation of 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS) to a blue-green product, enabling visual detection with the naked eye [10]. DNAzymes exhibit major advantages compared to natural enzymes, including superior thermal stability, ease of synthesis, and facile modification, leading to wide adoption for the rapid detection of nucleic acids, metal ions, and biological macromolecules [11,12,13,14].

In recent years, researchers have integrated the specific amplification capability of PCR with the signal amplification properties of G-quadruplexes. Incorporation of the reverse complement of a G-quadruplex sequence at the 5' termini of primers yields amplicons that contain abundant G-quadruplex-forming units, enabling visual differentiation of the target sequence by a DNAzyme-catalyzed colorimetric reaction [15,16]. This strategy has been successfully applied to pathogen gene detection and the discrimination of closely related species. For instance, the combination of G-quadruplex with asymmetric PCR has enabled visual detection of *Salmonella* and *Mycobacterium* [17]. In addition, Wang et al. [18] established a visual method for differ-

entiating *Panax ginseng* from *Panax quinquefolius* based on this principle. Prior DNA barcoding investigations have confirmed the presence of stable, species-specific single nucleotide polymorphism (SNP) loci within the internal transcribed spacer 2 (ITS2) region, providing a reliable molecular target for distinguishing *B. marginatum* var. *stenophyllum* from *B. chinense* [19,20]. Using this known molecular target, we developed a species-specific PCR-coupled G-quadruplex colorimetric assay (PCR-G4 assay). We designed specific primers targeting these discriminatory SNP loci, incorporating the reverse complement of a G-quadruplex sequence at their 5' termini. Through systematic single-factor gradient experiments, we optimized both the PCR amplification and colorimetric reaction conditions, establishing a visual readout method without the need for gel electrophoresis or specialized imaging equipment. This approach provides technical support for rapid, on-site authentication of *Bupleuri Radix* and its derived products, and may be applied for the purposes of regulatory market surveillance and pre-production screening by manufacturers.

2. Materials and Methods

2.1 Materials and Reagents

A total of 21 *Bupleurum* samples, including 1 certified reference material (CRM) and 20 test samples, were used in this study. The CRM of *Bupleuri Radix* (*B. chinense* DC., batch No. 120992-201510) was obtained from the National Institutes for Food and Drug Control (NIFDC, Beijing, China). The 20 test samples were authenticated by Xiaoxia Han (Senior Engineer, Department of Traditional Chinese Medicine, Lanzhou Institute for Food and Drug Control), with detailed sample information provided in Table 1. All primers were synthesized by Sangon Biotech Co., Ltd. (Shanghai, China). Premix Taq DNA polymerase, Agarose Regular, and Tris-Acetate-EDTA (TAE) buffer were purchased from Takara Biomedical Technology Co., Ltd. (Beijing, China). Hi-DNAsecure Plant Kit, TIAN-gel Midi Purification Kit, and GeneRed were purchased from Tiangen Biotech Co., Ltd. (Beijing, China). Hemin was purchased from Beijing Solarbio Science & Technology Co., Ltd. (Beijing, China), and Tris-HCl was purchased from Beijing Lanjiek Technology Co., Ltd. (Beijing, China). Triton X-100, Dimethyl sulfoxide (DMSO), and ABTS were purchased from Sigma-Aldrich (St. Louis, MO, USA).

2.2 Instruments and Equipment

PCR thermal cycler (Analytik Jena AG, Jena, Germany). Electrophoresis apparatus (Bio-Rad Laboratories, Inc., Hercules, CA, USA). Gel imaging system (Azure Biosystems, Inc., Dublin, CA, USA). Gel cutter (Tiangen Biotech Co., Ltd., Beijing, China). High-speed refrigerated centrifuge and ultra-micro UV-Vis spectrophotometer (Eppendorf China Co., Ltd., Shanghai, China). Microplate reader (Molecular Devices, LLC, San Jose, CA, USA).

Table 1. Information on the *Bupleurum* samples.

Sample No.	Name	Latin name	Source
1	<i>B. chinense</i> (CRM)	<i>Bupleurum chinense</i> DC.	National Institutes for Food and Drug Control
2	<i>B. chinense</i>	<i>Bupleurum chinense</i> DC.	Longxi County, Gansu Province
3	<i>B. chinense</i>	<i>Bupleurum chinense</i> DC.	Lintao County, Gansu Province
4	<i>B. chinense</i>	<i>Bupleurum chinense</i> DC.	Weiyuan County, Gansu Province
5	<i>B. chinense</i>	<i>Bupleurum chinense</i> DC.	Zhouqu County, Gansu Province
6	<i>B. chinense</i>	<i>Bupleurum chinense</i> DC.	Kangle County, Gansu Province
7	<i>B. chinense</i>	<i>Bupleurum chinense</i> DC.	Heyang County, Shaanxi Province
8	<i>B. chinense</i>	<i>Bupleurum chinense</i> DC.	Wanrong County, Shanxi Province
9	<i>B. scorzonerifolium</i>	<i>Bupleurum scorzonerifolium</i> Willd.	Shenmu County, Shaanxi Province
10	<i>B. marginatum</i> var. <i>stenophyllum</i>	<i>Bupleurum marginatum</i> Wall. ex DC. var. <i>stenophyllum</i> (Wolff) Shan et Y. Li	Kangle County, Gansu Province
11	<i>B. marginatum</i> var. <i>stenophyllum</i>	<i>Bupleurum marginatum</i> Wall. ex DC. var. <i>stenophyllum</i> (Wolff) Shan et Y. Li	Hezheng County, Gansu Province
12	<i>B. marginatum</i> var. <i>stenophyllum</i>	<i>Bupleurum marginatum</i> Wall. ex DC. var. <i>stenophyllum</i> (Wolff) Shan et Y. Li	Lintao County, Gansu Province
13	<i>B. marginatum</i> var. <i>stenophyllum</i>	<i>Bupleurum marginatum</i> Wall. ex DC. var. <i>stenophyllum</i> (Wolff) Shan et Y. Li	Weiyuan County, Gansu Province
14	<i>B. marginatum</i> var. <i>stenophyllum</i>	<i>Bupleurum marginatum</i> Wall. ex DC. var. <i>stenophyllum</i> (Wolff) Shan et Y. Li	Zhuoni County, Gansu Province
15	<i>B. marginatum</i> var. <i>stenophyllum</i>	<i>Bupleurum marginatum</i> Wall. ex DC. var. <i>stenophyllum</i> (Wolff) Shan et Y. Li	Longxi County, Gansu Province
16	<i>B. marginatum</i> var. <i>stenophyllum</i>	<i>Bupleurum marginatum</i> Wall. ex DC. var. <i>stenophyllum</i> (Wolff) Shan et Y. Li	Datong County, Qinghai Province
17	<i>B. smithii</i>	<i>Bupleurum smithii</i> H. Wolff	Tianzhu County, Gansu Province
18	<i>B. smithii</i>	<i>Bupleurum smithii</i> H. Wolff	Ningwu County, Shanxi Province
19	<i>B. bicaule</i>	<i>Bupleurum bicaule</i> Helm	Chifeng City, Inner Mongolia
20	<i>B. marginatum</i>	<i>Bupleurum marginatum</i> Wall. ex DC.	Rong County, Sichuan Province
21	<i>B. smithii</i> var. <i>parvifolium</i>	<i>Bupleurum smithii</i> H. Wolff var. <i>parvifolium</i> Shan et Y. Li	Tianzhu County, Gansu Province

2.3 Experimental Methods

2.3.1 Genomic DNA Extraction

Total genomic DNA was extracted from all samples according to the manufacturer's instructions for the Hi-DNAsecure Plant Kit. DNA concentration and purity (OD₂₆₀/OD₂₈₀) were assessed using an ultra-micro UV-Vis spectrophotometer.

2.3.2 Primer Design

The ITS2 sequences of *B. chinense* (GenBank accession number: HQ687914.1) and *B. marginatum* var. *stenophyllum* (GenBank accession number: HQ687952.1) were downloaded from the National Center for Biotechnology Information (NCBI) GenBank database. Multiple sequence alignment was performed using DNAMAN 9.0 (Lynnon Biosoft, Vaudreuil-Dorion, Canada), which identified several SNP loci and insertion-deletion (InDel) variants between the two species. Based on these discriminatory regions and in accordance with the general principles of primer design for PCR, the forward and reverse primer sequences were BMS1-F (5'-GGCGACATCCTTCTGATAA-3') and BMS1-R (5'-ATGGCAAGAGGTTAGTAGTG-3'),

respectively. Modified primers tagged with the reverse complement of the G-quadruplex sequence at their 5' termini were also designed as follows: [(G₃T)₃G₃] (5'-CCCACCCACCCACCC-3'). The final sequences are as follows:

BMS1-G-F:5'-CCCACCCACCCACCCGCGACA TCCTTCTGATAA-3';

BMS1-G-R:5'-CCCACCCACCCACCCATGGCAA GAGGTTAGTAGTG-3'.

2.3.3 Optimization of PCR Reaction Conditions

Optimization of the PCR amplification and reaction conditions was performed using the genomic DNA of *B. marginatum* var. *stenophyllum* as the template and the BMS1-G-F/R primer pair. To assess the potential impact of primer-dimer formation on the G-quadruplex colorimetric reaction, PCR was first performed in a template-free system at primer working solution concentrations of 1, 2, 3, 4, 5, 6, 8, and 10 µmol/L. Next, the matching between primer working solution concentration and template mass concentration was optimized, with primer working solution concentration gradients set at 4, 6, 8, and 10 µmol/L and template con-

centration gradients set at 0.001, 0.01, 0.1, 1, and 10 ng/μL. All primer concentrations described in this section refer to the working solution concentrations added to the PCR mixture, not the final concentrations in the reaction. Finally, the optimal annealing temperature and number of amplification cycles were determined empirically, with annealing temperature gradients set at 50, 55, 57, 60, 65, and 70 °C and cycle number gradients set at 25, 28, 30, 35, and 40 cycles. PCR amplicons were analyzed by electrophoresis on a 2% agarose gel in 1 × TAE buffer (130 V, 45 min) and visualized using a gel imaging system.

2.3.4 Optimization of the PCR-G4 Assay

The colorimetric reaction mixture was prepared as follows: 10 μL of purified PCR product (template), 50 μL of binding buffer (20 mmol/L Tris-HCl, pH 7.4; 40 mmol/L KCl; 0.05% [w/v] Triton X-100; 1% [v/v] DMSO), 15 μL of hemin, and 85 μL of sterile nuclease-free water. After denaturation at 95 °C and incubation at 4 °C, 40 μL of a substrate mixture containing ABTS and H₂O₂ was added to initiate the colorimetric reaction. The colorimetric reaction conditions were optimized using a single-factor gradient approach, with the following parameters evaluated: final hemin concentration (0, 2, 5, 10, 20 μmol/L), final ABTS concentration (0.5, 1, 2, 5, 10 mmol/L), denaturation time at 95 °C (2, 4, 6, 8, 10 min), and incubation time at 4 °C (5, 10, 20, 30, 40 min). After incubation at room temperature in the dark for 8 min, the absorbance at 420 nm (A_{420}) was measured using a microplate reader. A blank control, where template DNA was replaced with an equal volume of sterile nuclease-free water during PCR amplification, was processed in parallel for each assay.

2.3.5 Specificity of the PCR-G4 Assay

To assess the specificity of the established method, genomic DNA was extracted from the 21 *Bupleurum* samples listed in Table 1. Each sample was subjected to PCR amplification and G-quadruplex-mediated visual differentiation under the optimized conditions. A_{420} was measured for each reaction, and the results were assessed to confirm the method's ability to discriminate the target species without cross-reactivity.

2.3.6 Sensitivity of the PCR-G4 Assay

To evaluate the analytical sensitivity and detection range of the established method, genomic DNA from *B. marginatum* var. *stenophyllum* was serially diluted to 0.1, 1, 2, 4, 6, 8, 10, 15, 30, and 50 ng/μL with respect to the initial template concentration added to the PCR pre-amplification mixture. Each dilution was subjected to PCR amplification and G-quadruplex-mediated visual differentiation under the optimized conditions, and A_{420} was measured for each reaction.

2.4 Data Analysis

Experimental data were processed and visualized as bar and line graphs in Microsoft Excel (Microsoft Corporation, Redmond, WA, USA), with all figures assembled in Adobe Illustrator (Adobe Inc., San Jose, CA, USA). All absorbance measurements were performed in triplicate technical reads, and the mean value of these three reads was used for subsequent calculations. All serial template dilutions and reported limit of detection (LOD) values are expressed as the initial genomic DNA concentration loaded into each PCR reaction system. Two LOD metrics were defined for separate detection workflows: (1) PCR LOD, which is the minimum initial template concentration yielding distinct specific bands on 2% agarose gel electrophoresis. This reflects only PCR amplification performance and does not involve subsequent G-quadruplex color development; (2) statistical LOD of the full PCR-G4 assay, calculated via the standard $Mean_{blank} + 3 \times SD$ threshold rule. Blank controls were PCR mixtures in which template DNA was replaced with an equal volume of nuclease-free water. The lowest initial template concentration with a mean A_{420} reading consistently above this absorbance cut-off was defined as the statistical LOD. A sample was judged positive if either of the following criteria was met: (i) a distinct blue-green color visible under standard indoor light, or (ii) the measured A_{420} value exceeded the precomputed cut-off value. In routine visual interpretation, the naked-eye criterion is sufficient; the A_{420} cut-off serves as an auxiliary criterion when the color is faint or ambiguous, or when an objective record is required. For the determination of the statistical LOD, only the A_{420} cut-off was applied. The signal-to-noise ratio (SNR) was calculated as $SNR = A_{420}(\text{template}) / A_{420}(\text{blank})$. SNR values were used merely as auxiliary quantitative indicators to screen optimal reaction conditions and evaluate background color interference. Sample positivity was not judged based on a fixed SNR threshold. All experiments for the specificity and sensitivity validations were conducted in three fully independent biological replicates ($n = 3$). Each replicate contained three microplate technical reads, whose average value was used for final analysis. Data for these validation experiments are presented as the mean $\pm SD$ ($n = 3$). Intergroup differences were analyzed by one-way ANOVA followed by Tukey's post hoc test, with $p < 0.05$ defined as statistically significant. For comparisons involving only two groups (e.g., 35-cycle vs. 40-cycle groups), an unpaired two-tailed t -test was used.

3. Results

3.1 Optimization of PCR Reaction Conditions

3.1.1 Effect of Primer Concentration on Colorimetric Performance in a Template-Free System

To rule out the potential confounding effect of primer-dimer formation on the PCR-G4 assay, primer working so-

lution concentrations of 1, 2, 3, 4, 5, 6, 8, and 10 $\mu\text{mol/L}$ were tested in a template-free PCR system, followed by visual evaluation of the G-quadruplex colorimetric reaction. Gel electrophoresis confirmed the absence of target amplicons across all primer concentrations tested in template-free reactions. The abundance of primer-dimers formed was positively correlated with increasing primer working solution concentration (Fig. 1A). However, the primer working solution concentration had no visible effect on the colorimetric reaction, and no substantial color development was observed visually (Fig. 1B).

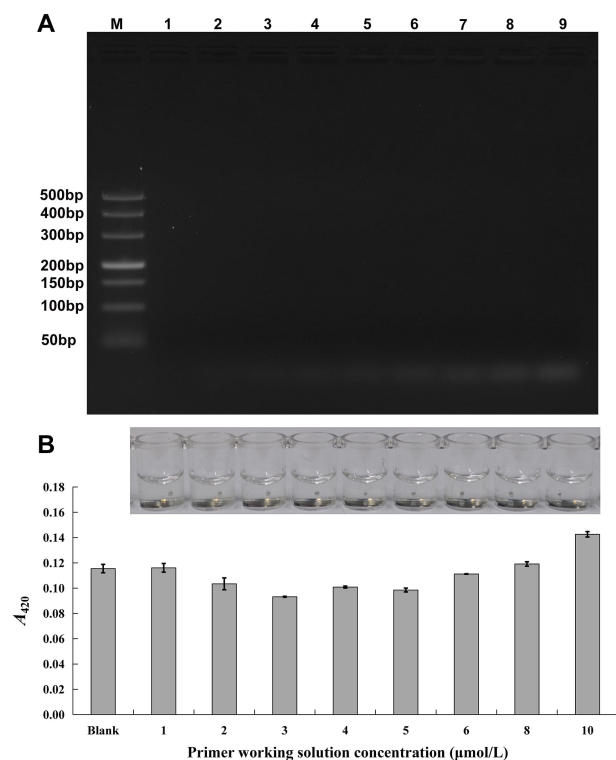


Fig. 1. Effect of different primer concentrations on the G-quadruplex colorimetric reaction in a template-free PCR system. (A) Agarose gel electrophoresis image of PCR products under different primer working solution concentrations. M: DL500 DNA Marker; 1: Blank; 2–9: 1, 2, 3, 4, 5, 6, 8, and 10 $\mu\text{mol/L}$. (B) Colorimetric results and A_{420} of the visual differentiation assay under different primer working solution concentrations. Data are shown as mean $\pm$ SD ($n = 3$ independent replicates). PCR, polymerase chain reaction.

3.1.2 Optimization of Primer and Template DNA Concentrations in the PCR System

PCR amplification efficiency is directly influenced by primer and template DNA concentrations. Accordingly, we tested a concentration matrix consisting of four primer working solution concentration gradients (4, 6, 8, and 10 $\mu\text{mol/L}$) and five template DNA gradients (0.001,

0.01, 0.1, 1, and 10 $\text{ng}/\mu\text{L}$). The amplification performance of each combination was then assessed by agarose gel electrophoresis. At a primer working solution concentration of 8 $\mu\text{mol/L}$, the PCR LOD for the template reached 0.01 $\text{ng}/\mu\text{L}$, and no further improvement was observed with higher primer working solution concentrations. Under these conditions, a template concentration of 10 $\text{ng}/\mu\text{L}$ yielded a bright and distinct target band with no evidence of non-specific amplification (Fig. 2). Sequencing of the gel-purified amplicon confirmed its identity as the expected target sequence.

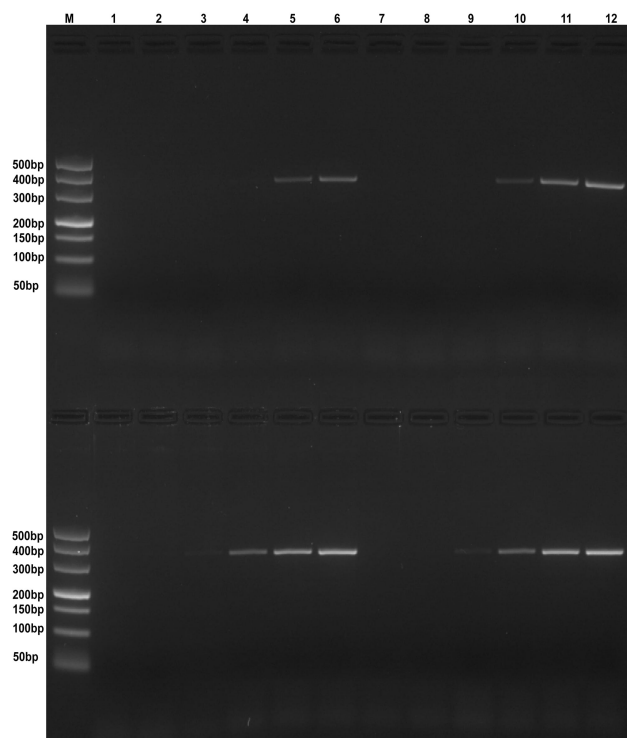


Fig. 2. Optimization of primer-template concentration ratio for PCR. (Upper panel) M: DL500 DNA Marker; 1, 7: Blank; 2–6: 0.001, 0.01, 0.1, 1, and 10 $\text{ng}/\mu\text{L}$ (primer working solution concentration: 4 $\mu\text{mol/L}$); 8–12: 0.001, 0.01, 0.1, 1, and 10 $\text{ng}/\mu\text{L}$ (primer working solution concentration: 6 $\mu\text{mol/L}$). (Lower panel) M: DL500 DNA Marker; 1, 7: Blank; 2–6: 0.001, 0.01, 0.1, 1, and 10 $\text{ng}/\mu\text{L}$ (primer working solution concentration: 8 $\mu\text{mol/L}$); 8–12: 0.001, 0.01, 0.1, 1, and 10 $\text{ng}/\mu\text{L}$ (primer working solution concentration: 10 $\mu\text{mol/L}$).

3.1.3 Optimization of Annealing Temperature in the PCR System

The annealing temperature is a critical parameter governing the efficiency and specificity of primer-template hybridization. We therefore tested annealing temperature gradients of 50, 55, 57, 60, 65, and 70 $^{\circ}\text{C}$ under the previously optimized primer and template concentration conditions. Increasing the annealing temperature led to a reduc-

tion in primer-template mismatches, resulting in a progressive decrease in non-specific amplification. At an annealing temperature of 60 °C, the non-specific bands observed in the *B. chinense* lanes were completely eliminated, indicating optimal discrimination specificity for the SNP loci characteristic of *B. marginatum* var. *stenophyllum*. Further increases in annealing temperature resulted in a noticeable decline in the intensity of the target band for *B. marginatum* var. *stenophyllum*, suggesting a marked reduction in the amplification efficiency (Fig. 3).

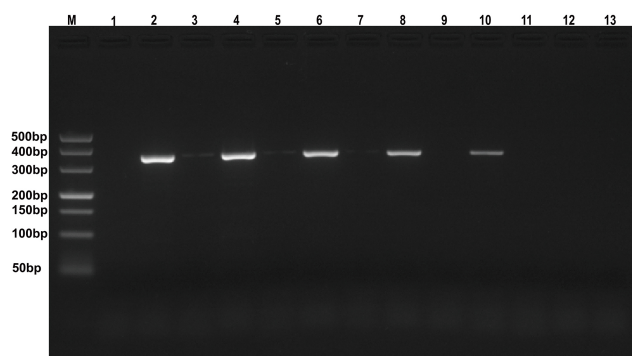


Fig. 3. Optimization of PCR annealing temperature. M: DL500 DNA Marker; 1: Blank; 2, 4, 6, 8, 10, 12: amplification products of *B. marginatum* var. *stenophyllum* (annealing temperatures: 50, 55, 57, 60, 65, and 70 °C, respectively); 3, 5, 7, 9, 11, 13: amplification products of *B. chinense* (annealing temperatures: 50, 55, 57, 60, 65, and 70 °C, respectively).

3.1.4 Optimization of Amplification Cycle Number in the PCR System

A gradient of cycle numbers was evaluated to identify the optimal parameter for this system. The A_{420} values of the samples increased progressively with the number of amplification cycles. Both the 35- and 40-cycle groups produced substantially higher colorimetric signals compared to the other tested groups. An unpaired two-tailed *t*-test revealed no statistically significant difference between the 35- and 40-cycle groups ($p = 0.082$, $n = 3$), confirming that increasing the cycle number from 35 to 40 did not provide a meaningful gain in colorimetric signal intensity. Considering amplification efficiency, reaction time, and the increased risk of non-specific amplification associated with excessive cycles, 35 cycles were selected as optimal for subsequent experiments (Fig. 4).

3.2 Optimization of the PCR-G4 Assay

3.2.1 Optimization of the Final Hemin Concentration

Hemin binds to G-quadruplex structures to form the peroxidase-mimicking DNAzyme complex. Its concentration directly determines the catalytic efficiency of the DNAzyme, signal intensity, and overall signal-to-noise ratio (SNR) of the assay. A series of final hemin concentra-

tions of 0, 2, 5, 10, and 20 $\mu\text{mol/L}$ were evaluated based on changes in A_{420} , visual color differences, and the calculated SNR. A_{420} of the +Template groups showed a continuous upward trend with increasing hemin concentration, with no discernible plateau within the tested range of 0–20 $\mu\text{mol/L}$. However, concurrent observation of the blank control groups revealed that background A_{420} also rose steadily with increasing hemin concentration. This increase became markedly steeper at concentrations $>5 \mu\text{mol/L}$. Calculation of the SNR (A_{420} of experimental group / A_{420} of blank group) indicated that the ratio peaked at a hemin concentration of 5 $\mu\text{mol/L}$, with the SNR values at 0, 2, 5, 10, and 20 $\mu\text{mol/L}$ being 1.08, 2.49, 3.64, 3.42, and 2.79, respectively. Under this condition, a strong positive signal suitable for visual differentiation was achieved, while non-specific background interference was effectively minimized. Although further increases in hemin concentration yielded a modest enhancement of the positive signal, the accompanying increase in background noise was more pronounced, leading to a progressive decline in the SNR and consequently a reduction in discrimination and sensitivity (Fig. 5A).

3.2.2 Optimization of the Final ABTS Concentration

ABTS serves as the primary colorimetric substrate in the assay, with its concentration directly determining the colorimetric sensitivity, sample discrimination, and overall stability of the assay. Five final ABTS concentrations of 0.5, 1, 2, 5, and 10 mmol/L were evaluated by analyzing A_{420} , visual color differences, and the SNR. A_{420} of the +Template groups generally increased with higher ABTS concentrations. The SNR reached its maximum at an ABTS concentration of 2 mmol/L, with the SNR values at 0.5, 1, 2, 5, and 10 mmol/L being 1.64, 1.78, 3.26, 2.20, and 2.65, respectively, at which point the positive signal was unambiguous and the visual color differentiation between target samples and controls was most pronounced. Although A_{420} of the experimental groups continued to rise at ABTS concentrations $>2 \text{ mmol/L}$, the blank background signal also increased substantially, leading to a marked deterioration in the SNR and a consequent reduction in discrimination power (Fig. 5B). To maintain a balanced substrate ratio and ensure optimal reaction stability, the final concentration of H_2O_2 in the reaction mixture was set equal to that of ABTS.

3.2.3 Optimization of the Denaturation Time at 95 °C

To identify the optimal denaturation conditions, a time gradient of 2, 4, 6, 8, and 10 min was tested at 95 °C, with A_{420} and visual color development as the primary indicators. A_{420} increased initially, then declined as the denaturation time was prolonged. A_{420} peaked at a denaturation time of 8 min, which corresponded to the most intense color development. When the denaturation time exceeded 8 min, a noticeable decrease in absorbance was observed (Fig. 6A).

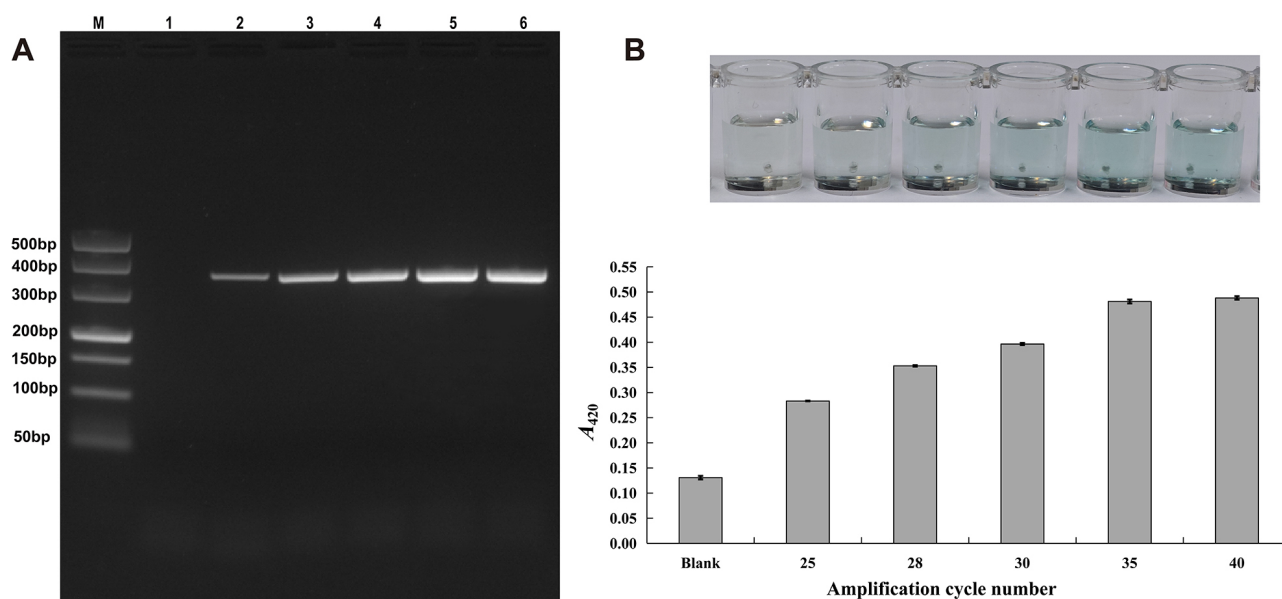


Fig. 4. Optimization of amplification cycle number in the PCR system. (A) M: DL500 DNA Marker; 1: Blank; lanes 2–6: PCR products amplified with 25, 28, 30, 35, and 40 cycles, respectively. (B) Colorimetric results and A_{420} of the visual differentiation assay under the corresponding amplification cycles. Data are shown as mean ± SD ($n = 3$ independent replicates). No statistically significant difference was observed between the 35- and 40-cycle groups (unpaired two-tailed t -test, $p = 0.082$).

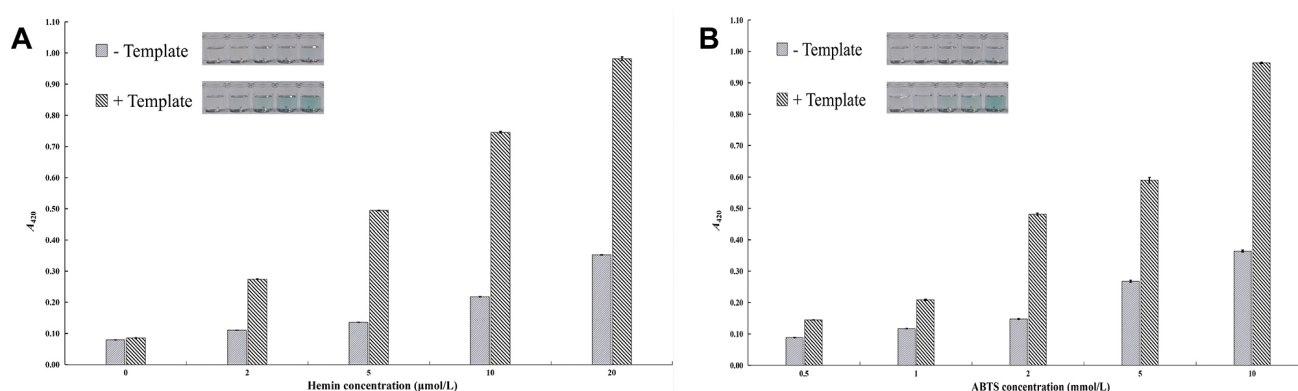


Fig. 5. Optimization of final hemin and ABTS concentrations for the colorimetric reaction. (A) Effect of final hemin concentration on colorimetric signal development. (B) Effect of final ABTS concentration on colorimetric signal development. Data are shown as mean ± SD ($n = 3$ independent replicates). ABTS, 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt.

3.2.4 Optimization of the Incubation Time at 4 °C

To identify the optimal incubation conditions, a time gradient of 5, 10, 20, 30, and 40 min was tested at 4 °C, with A_{420} and visual color development as the primary indicators. Visible color development was observed after just 5 min of incubation. A_{420} increased markedly with increasing incubation time, peaking at 20 min, which corresponded to the strongest colorimetric signal. The absorbance decreased progressively when incubation was extended beyond 20 min (Fig. 6B).

3.3 Specificity Assay

To validate the specificity of the established PCR-G4 assay, genomic DNA from various *Bupleurum* species (Ta-

ble 1) was used as a template for PCR amplification under the fully optimized conditions. The resulting amplicons were subjected to denaturation and incubation, followed by the G-quadruplex-mediated colorimetric reaction. The specificity of the method was comprehensively evaluated through a combination of agarose gel electrophoresis and measurement of A_{420} .

Agarose gel electrophoresis confirmed that a specific target amplicon was generated exclusively from samples No. 10–16 (*B. marginatum* var. *stenophyllum*). In contrast, no specific amplicons were detected for *B. chinense*, *B. scorzonrifolium*, or any other congeneric *Bupleurum* species, fully consistent with the expected results (Fig. 7A). Both visual inspection and quantitative A_{420} measurements

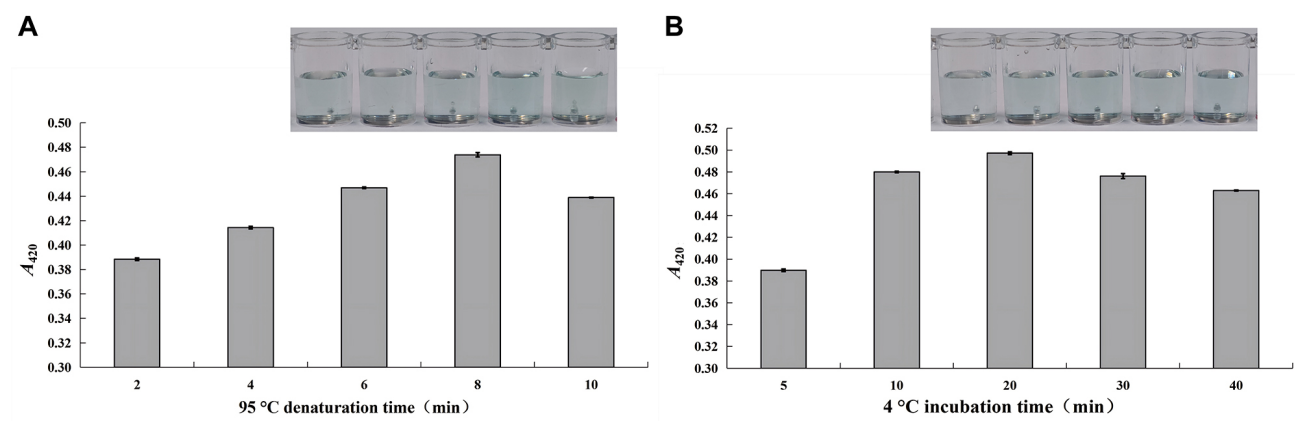


Fig. 6. Optimization of denaturation time at 95 °C and incubation time at 4 °C for the colorimetric reaction. (A) Effect of denaturation time at 95 °C on colorimetric signal development. (B) Effect of the incubation time at 4 °C on colorimetric signal development. Data are shown as mean $\pm$ SD (n = 3 independent replicates).

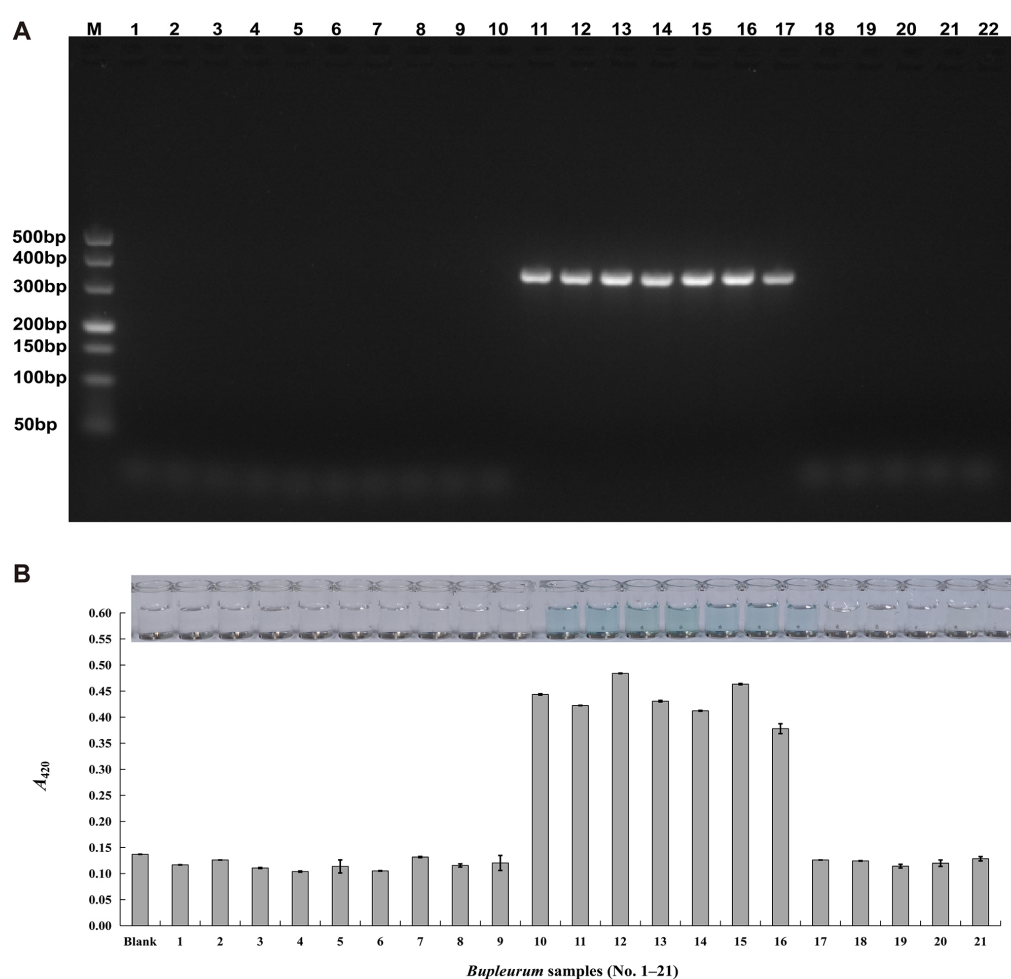


Fig. 7. Specificity evaluation of the established visual differentiation method. (A) M: DL500 DNA Marker; 1: Blank; lanes 2–22: PCR products from Bupleurum samples No. 1–21 listed in Table 1, respectively. (B) Color development results and A_{420} of the visual differentiation assay for the corresponding Bupleurum samples. Data are shown as mean $\pm$ SD (n = 3 independent replicates).

confirmed that a distinct blue-green color developed exclusively in reaction mixtures from samples No. 10–16. A_{420} values for these samples were consistently >0.37 , markedly

higher than those for the blank control, *B. chinense*, *B. scorzonrifolium*, and all other congeneric *Bupleurum* species. Conversely, reaction mixtures for all non-target samples

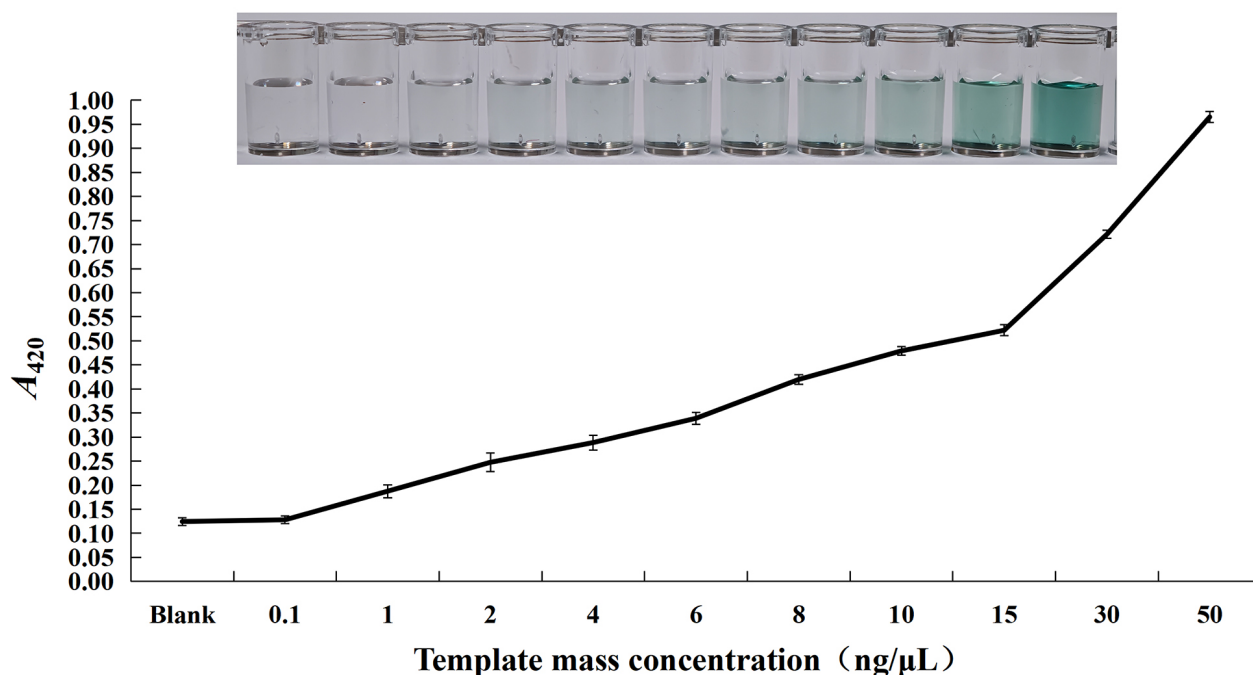


Fig. 8. Sensitivity evaluation of the established visual differentiation method. Representative photographs of color development (upper panel) and quantitative A_{420} measurements (lower panel) for template concentrations ranging from 0.1 to 50 ng/μL. Data are shown as mean ± SD ($n = 3$ independent replicates).

(No. 1–9 and 17–21) showed no visible color change, with A_{420} values remaining <0.14 and not appreciably different from the blank control (Fig. 7B).

3.4 Sensitivity Assay

To determine the analytical sensitivity and detection range of the developed PCR-G4 assay, serially diluted genomic DNA from *B. marginatum* var. *stenophyllum* was used as a template at concentrations of 0.1, 1, 2, 4, 6, 8, 10, 15, 30, and 50 ng/μL, alongside a blank control and under the fully optimized conditions. The absorbance cut-off threshold for positive identification was calculated as 0.1489 ($Mean_{blank} = 0.1245$, $SD = 0.00814$, $n = 3$). The concentration of 1 ng/μL was the lowest initial template concentration whose A_{420} (0.1877) exceeded this cut-off. The statistical LOD for this assay was therefore confirmed as 1 ng/μL. Visual inspection revealed that the color change at 1 ng/μL was too faint for reliable judgment by the naked eye, whereas a consistently distinguishable blue-green color was achieved at template concentrations of ≥ 2 ng/μL ($A_{420} = 0.2477$), which we defined as the practical visual threshold. When the template concentration was extended to 50 ng/μL, the A_{420} continued to rise to 0.9652 without any signal suppression, confirming the absence of a hook effect within the tested range. Thus, the effective detection range of this assay was defined as 1 ng/μL (statistical LOD) to at least 50 ng/μL (validated upper limit), with a reliable naked-eye positive call at ≥ 2 ng/μL (Fig. 8).

4. Discussion

This study successfully applied the developed PCR-G4 assay to achieve specific discrimination between *B. marginatum* var. *stenophyllum* and *B. chinense*. Notably, primer-dimer formation observed in template-free controls did not produce a detectable colorimetric background signal, even at a primer working solution concentration of 10 μmol/L (Fig. 1). We speculate that 3'-terminal complementarity can promote primer-dimer formation [21]. The lack of background signal may be attributed to the fact that the G-rich tail is located at the 5' terminus, whereas primer-dimer formation is driven by 3'-terminal complementarity. Consequently, any G-quadruplex structures that might form from primer-dimers would likely lack the appropriate spatial configuration for effective hemin binding and catalytic activity. However, we did not perform any direct structural characterization such as native polyacrylamide gel electrophoresis (PAGE) or circular dichroism spectroscopy to confirm this hypothesis. The proposed explanation is therefore a reasonable speculation based on our indirect observations, and further structural studies are required to definitively resolve the underlying mechanism. This finding has important implications for the design of similar G-quadruplex-based primer systems, and suggests that primer-dimer formation may not necessarily introduce uncontrollable background signal in such assays. Nevertheless, some researchers have incorporated additional steps, such as magnetic separation, into analogous methods to

remove primer-dimers and eliminate potential interference [22]. Therefore, depending on the primer sequences and amplicon detection formats, this factor may still warrant careful consideration.

During system optimization, we found that the PCR LOD reached 0.01 ng/μL at a primer working solution concentration of 8 μmol/L, which was two orders of magnitude lower than the statistical LOD of 1 ng/μL for the full PCR-G4 assay. This discrepancy can be rationalized by considering that the PCR LOD reflects the detectability of the amplification product itself (which relies on gel electrophoresis), whereas the statistical LOD is constrained by the cumulative signal generation efficiency of the downstream colorimetric reaction. Prior research has indicated that the catalytic efficiency of the G-quadruplex/hemin DNAzyme is not yet equivalent to that of its natural enzyme counterparts [23]. This relative catalytic limitation necessitates the production of a substantially larger quantity of amplicon to generate a colorimetric signal that is visible to the naked eye. Notably, when the template concentration was increased to 50 ng/μL, the A_{420} value continued to rise to 0.9652 without any sign of signal suppression, confirming the absence of a detectable hook effect within the tested range (1–50 ng/μL). This finding supports the reliability of the assay for samples with a high DNA concentration.

In this assay, the double-stranded structure of the PCR amplicons physically restricts G-quadruplex folding. Adequate thermal denaturation is therefore a prerequisite for generating a detectable colorimetric signal. During optimization of the denaturation time at 95 °C, A_{420} peaked at 8 min and decreased when the duration was extended (Fig. 6A). This decline likely reflects heat-induced degradation of the target DNA fragments, which reduces the yield of functional G-quadruplex structures. Following denaturation, rapid cooling to 4 °C and incubation at this temperature favors G-quadruplex folding while reducing immediate re-annealing, allowing time for hemin binding. A_{420} reached its maximum at 20 min and then gradually decreased with further incubation (Fig. 6B). The exact cause of this signal decline remains unclear, as we did not perform direct biophysical characterization such as native PAGE to monitor the single-stranded versus re-annealed duplex DNA population, or circular dichroism spectroscopy to track G-quadruplex folding integrity over time. Based on our observations and previous literature, we propose three plausible explanations that warrant further investigation: (i) gradual re-annealing of single strands into double-stranded DNA, thereby reducing the population of G-quadruplexes available for catalysis; (ii) diminished stability of the pre-formed G-quadruplex/hemin complex over an extended incubation period; and (iii) hemin precipitation or inactivation. These hypotheses remain to be verified, and future studies employing biophysical approaches are required to definitively distinguish among them. The present findings underscore that the kinetic balance between denaturation and reanu-

ration in the colorimetric reaction is a critical determinant of detection sensitivity, requiring careful optimization to achieve sufficient strand separation while mitigating the risk of DNA degradation and re-annealing.

The specificity of this method was rigorously validated against a panel of 21 *Bupleurum* samples including *B. chinense*, *B. scorzonrifolium*, *B. marginatum* var. *stenophyllum*, *B. smithii*, *B. bicaule*, *B. marginatum*, *B. smithii* var. *parvifolium*, and other closely related species. Our results demonstrated that only samples of *B. marginatum* var. *stenophyllum* produced a specific amplification band and a strong colorimetric response ($A_{420} > 0.37$), whereas all non-target species yielded A_{420} consistently < 0.14 (Fig. 7).

5. Limitations

Several limitations of this study should be acknowledged. First, the current assay is qualitative. While it can confirm the presence of *Bupleurum marginatum* var. *stenophyllum*, it cannot quantify its proportion in mixed samples. This qualitative feature still supports basic binary screening tasks, such as rapid preliminary screening and the elimination of adulterated batches during factory material inspection and primary market supervision. Nevertheless, this shortcoming restricts its wider practical application in herbal quality control. If quantitative evaluation of adulteration ratios is required for quality grading and market standard management, the current assay is unable to provide such data. To overcome this limitation, multiple quantitative transformation strategies could be explored in future research, including real-time qPCR based on the same SNP-specific primers, droplet digital PCR (ddPCR), and competitive amplification. These methods could be applied to accurately calculate the content ratio of target adulterants in mixed medicinal samples and to expand the application scope of this visual detection system. Beyond these approaches, a recent study has also demonstrated the feasibility of quantitative detection using G-quadruplex-based platforms without relying on expensive equipment, thus offering alternative strategies for quantitative adaptation [24]. Second, PCR thermal cyclers are required to perform target gene amplification before colorimetric readout. Integrating isothermal amplification could further extend its applicability in field settings. Third, the specificity validation relied on 21 sample batches covering seven common *Bupleurum* species collected from various genuine producing areas. Although the sample panel included official pharmacopoeia species and the primary adulterant was investigated here, the sample size was still relatively limited. For successive validation, follow-up research should expand the sample library and introduce specimens harvested in different years, as well as commercially available crude herb slices and compound herbal preparations. This will allow comprehensive assessment of the stability and practical performance of our method under real market supervision conditions. Importantly, the findings presented here are based on

a defined sample panel, and further validation using larger quantities of real commercial mixed medicinal materials is necessary to support broader practical deployment of this method.

6. Conclusions

Using species-specific SNP loci in the ITS2 barcode region, this study developed a visual detection method based on PCR coupled with a G-quadruplex/hemin DNAzyme for the rapid and accurate differentiation of *B. chinense* from its common adulterant *B. marginatum* var. *stenophyllum*. Based on amplification efficiency and signal intensity, the final optimized PCR reaction mixture (25 μ L) contained: 12.5 μ L Premix Taq DNA polymerase, 4 μ L template DNA (10 ng/ μ L), 1 μ L of each forward and reverse primer (8 μ mol/L working solution, final concentration in the 25 μ L reaction: 0.32 μ mol/L), and sterile nuclease-free water (6.5 μ L). The optimized thermal cycling protocol was as follows: initial denaturation at 95 °C for 5 min; followed by 35 cycles of denaturation at 95 °C for 30 s, annealing at 60 °C for 30 s, and extension at 72 °C for 50 s; with a final extension at 72 °C for 5 min. Specificity validation using 21 *Bupleurum* samples confirmed that only *B. marginatum* var. *stenophyllum* generated specific amplification and produced a discernible colorimetric reaction. No visual color change was observed for *B. chinense* or any other closely related *Bupleurum* species, demonstrating excellent specificity and no cross-reactivity among the tested *Bupleurum* species. Sensitivity testing established a statistical LOD of 1 ng/ μ L and a reliable detection range of 1–50 ng/ μ L, with a practical naked-eye positive threshold at ≥ 2 ng/ μ L.

This assay provides a rapid, visual readout for the authentication of *Bupleuri Radix* without the need for gel electrophoresis or specialized imaging equipment. With further validation using authentic commercial samples, this approach may offer practical utility in the regulation of the herbal medicine market, on-site pre-processing inspection, and routine testing in primary drug control laboratories. Furthermore, this work provides a promising and adaptable paradigm for developing visual molecular authentication assays for other closely related medicinal plant species.

Availability of Data and Materials

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

Author Contributions

Conceptualization, SY and JY; methodology, SY; software, SY; validation, WC and ZS; formal analysis, JL and WB; investigation, ZT, XH and LF; resources, XH; data curation, FW and BZ; writing—original draft preparation, SY; writing—review and editing, BZ; data analysis, FW; literature search, XH; project administration, ZT and FW.

All authors read and approved the final manuscript. All authors contributed to editorial changes in the manuscript. All authors have participated sufficiently in the work and agreed to be accountable for all aspects of the work.

Ethics Approval and Consent to Participate

All experimental research on plants, including the handling of plant material, complied with relevant institutional, national, and international guidelines. The plant samples used in this study were obtained from routine regulatory sampling and retained by the Lanzhou Institute for Food and Drug Control. No wild plant materials were collected, and no specific permits were required.

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Conflicts of Interest

The authors declare no conflicts of interest.

Declaration of AI and AI-Assisted Technologies in the Writing Process

During the preparation of this work, the authors used DeepSeek in order to check spelling and grammar. After using this tool, the authors reviewed and edited the content as needed, and took full and sole responsibility for the entire content of the publication.

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