



Ultrafast identification of *Pinelliae Rhizoma* using colorimetric direct-VPCR

Rong Chen¹ · Sheng Ding² · Yinghua Wei² · Jiawen Yu³ · Ruichao Xu³ · Xiao Luo⁴ · Gang Fan¹ · Hongxiang Yin¹ · Jinhui Bian¹

Received: 22 June 2021 / Accepted: 18 October 2021 / Published online: 15 November 2021
© King Abdulaziz City for Science and Technology 2021

Abstract

With the increasing growth of the herbal market, a rapid and easy-to-use system is highly desirable in the high-throughput identification of massive herbal medicine samples. Here, an ultrafast and colorimetric detection system was devised based on simplifying template preparation and a newly developed amplification technique, named colorimetric direct-VPCR. The system was successfully applied to the identification of *Pinelliae Rhizoma*. Compared to the traditional method, the whole test can be finished within 30 min from the sample treatment to the testing results. The method was evaluated by correctly identifying 72 samples obtained from 9 different habitats, demonstrating its high reliability. In summary, we present an ultrafast (less than 30 min) and colorimetric detection platform (under ultraviolet lamp) based on direct-VPCR for the identification of *Pinelliae Rhizoma*. The high practicability (100% accuracy) of this pipeline enables it to be a promising method in the routine detection of other herbal materials.

Keywords Ultrafast · VPCR · Colorimetric detection · Medicinal herb · *Pinelliae Rhizoma*

Introduction

Herbal plants are important raw materials for traditional drugs that are used as supplements or phytomedicines. According to an estimation in 2016, the global herbal market was worth 60 billion dollars and was expanding dramatically (Mishra et al. 2016). With the great need for herbal medicines and related products, the herbal industries are facing the challenge of adulteration and substitution, which is an increasingly severe problem worldwide. Great efforts have

been devoted to developing methods to authenticate herbal materials. Since the last decade, DNA-based molecular detection has been gradually prevailing in the identification of botanicals, showing great promise for complementing traditional methods of taxonomic and chemical analysis for species identification (Coutinho Moraes et al. 2015). Generally, DNA-based identification follows a standard pipeline: template preparation, amplification, and post-amplification analysis. However, the canonical methods used in the pipeline require complicated operations, which are labor intensive. Besides, the total time needed for a conventional test is about 3 h, which is inefficient to meet the demand for high-throughput detection of massive samples. Thus, a new platform that simplifies the testing procedure and minimizes the assay time could be of great significance to improve the detection efficacy.

In regular DNA-based detection, DNA amplification is the main speed-limiting step. Although there are many well-developed nucleic acid amplification methods so far, polymerase chain reaction (PCR) is the most used one that is recommended as the gold standard technique in many detection platforms (Yip et al. 2019; Zhang et al. 2018). The traditional PCR process requires three steps, including denaturation, annealing, and extension to complete the

✉ Hongxiang Yin
hongxiangy@126.com

✉ Jinhui Bian
bianjinhui@cduetcm.edu.cn

¹ Chengdu University of Traditional Chinese Medicine, Chengdu 611137, People's Republic of China

² Natural Products Research Center, Chengdu Institute of Biology, Chinese Academy of Sciences, Chengdu 610041, People's Republic of China

³ Taiji Group Chongqing Fuling Pharmaceutical Co, Ltd., Chongqing 408000, People's Republic of China

⁴ Chengdu Institute for Food and Drug Control, Chengdu 610000, People's Republic of China

amplification, meaning that much time is wasted in the thermal cycles. Recently, various efforts have been devoted to realizing faster DNA amplification (Chen et al. 2019; Farrar and Wittwer 2015; Li et al. 2016; Son et al. 2015). In our previous research, a new technique named “V” shape polymerase chain reaction (VPCR) was established to shorten the amplification time by using a dynamic heating and cooling process (Chen et al. 2019). Compared to the conventional PCR process, the VPCR saved about 2/3 of the amplification time, showing a great advantage in realizing fast molecular detection. Another step affecting the speed of a test is the pretreatment of samples. Typically, template preparation is a multi-step and laborious operation that comprises DNA extraction and purification, thus making it inconvenient when dealing with numerous samples. Recently, many direct DNA isolation solutions have been reported in the PCR-based detection of plant genomes (Choudhary et al. 2019), which would help improve pretreatment efficacy when coping with large-scale samples.

Pinelliae rhizoma, dried rhizoma of *Pinellia ternata* (Thunb.) Breit is one of the most commonly used medicinal herbs in China, Japan, and Korea. It has been used in over 1000 prescriptions and more than 100 herbal products (Sun et al. 2019). Among these herbal products, “Huo Xiang Zheng Qi Ye” is a famous one that has been sold in dozens of countries and districts, including the USA, Russia, Canada, and so on. As the main raw material of “Huo Xiang Zheng Qi Ye,” the annual demand for pinelliae rhizoma exceeds 1000 tons, generating millions of dollars in trade per year. The tremendous financial incentive also prompts dishonest merchants to adulterate pinelliae rhizoma with other cheaper materials. There are three main adulterants, including dried rhizoma of *Pinellia pedatisecta* Schott, *Typhonium flagelliforme* (Lodd.) Blume, and *Arisaema heterophyllum* Blume. Since these adulterates are highly similar in morphology to pinelliae rhizoma, the development of a robust detection platform is highly appreciated.

Herein, we developed a direct-VPCR platform for ultra-fast authentication of herbal medicinal plants by combining a one-step direct template preparation with the VPCR technique. The pinelliae rhizoma was used for the proof-of-concept assay. Additionally, the visualized detection was achieved by adding a universal fluorescent dye to the direct-VPCR platform. The protocol is highly simplified and the

test time is reduced from more than 3 h to less than 30 min. Furthermore, the practical application of the developed method was evaluated by identifying samples from different batches.

Materials and methods

Plant material

The positive samples of pinelliae rhizoma were purchased from a local pharmacy in Chengdu, People’s Republic of China. Three processed samples of pinelliae rhizoma and the adulterants used in our experiments were kind gifts from Taiji Group Chongqing Fuling Pharmaceutical Co., Ltd. A total of 72 samples used to verify the practicality of the method were collected from local farmers in 9 different areas, such as Gansu, Yunnan, Guizhou, Shaanxi, Shandong, and Hebei. Taq DNA polymerase and dNTPs were purchased from TransGen Biotech (Beijing, China). The DNA isolation kit (Plant DNA Isolation Kit) was purchased from Genesee Co., Ltd. (Chengdu, China). SYBR Green I was purchased from Invitrogen (Shanghai, China).

Sequences

The specific primer pairs (BP3 and BRP3) designed in previous study (Chen et al. 2012) were re-evaluated and a pair of universal primers (API and ARP1) were designed based on the internal transcribed spacer (ITS) sequences (Figure S1) of *P. ternata* (AF469036.1), *P. pedatisecta* (AF469040.1), *A. heterophyllum* (KT634029.1), and *T. flagelliforme* (OK103618.1), and synthesized by Sangon Biotech (Shanghai, China). The sequences and final concentrations used in PCR are listed in Table 1.

DNA extraction

The CTAB (Cetyltrimethylammonium Bromide) method and three different direct DNA isolation solutions were used in this study.

CTAB method: about 50 mg of samples was suspended in 750 μ L of CTAB buffer (2% CTAB; 100 mM Tris-HCl, pH 8.0; 20 mM EDTA (Ethylenediaminetetraacetic Acid); 2.5 M

Table 1 The primers used in this study

Descriptions	Primers	Sequence (5’-3’)	Final concentration (μ M)
Specific primers for <i>P. ternata</i>	BP3	GGGAGACTCCCCGACGATCGCA	0.3
Specific primers for <i>P. ternata</i>	BRP3	CCCGCACGGACGGATGGAT	0.3
Sequencing primer	API	GCCTGCCTGGGCGTC	0.2
Sequencing primer	ARP1	GCGTCGTCCACCACTC	0.2

NaCl) and then incubated at 65 °C for 2 h with occasional shaking. The lysate was extracted with 600 µL of chloroform: isoamyl alcohol (24:1). DNA was precipitated with an equal volume of 100% isopropanol (30 min at –20 °C, followed by centrifugation at 10,000 g for 15 min). DNA pellet was washed twice with cold 70% ethanol, vacuum dried, and resuspended in 100 µL of TE buffer (10 mM Tris–HCl, pH 8.0; 1 mM EDTA).

Direct DNA isolation solutions: about 50 mg of samples was suspended in 200 µL of DNA extraction solution. The mixture was incubated at 95 °C for 5 min and this 200 µL of DNA extracts was used as DNA templates in the PCR.

PCR reaction

All PCR reactions were performed in a 30 µL mixture containing 1 µL of DNA extracts, 1 × Taq buffer (20 mM Tris–HCl (pH 8.4), 20 mM KCl, 10 mM (NH₄)₂SO₄ and 2 mM MgSO₄), 0.2 mM dNTPs, 3 units of EasyTaq polymerase, and a certain amount of primers (see Table 1). All PCR amplifications were carried out under the following cycling conditions: initial template denaturation at 94 °C for 2 min, 35 cycles of thermal cycling (94 °C for 30 s, 62 °C for 30 s and 72 °C for 30 s). The PCR reaction was incubated in a C1000TM Thermal Cycler PCR (Bio-Rad Laboratories, USA).

VPCR reaction

The VPCR reaction was carried out in the same conditions as the PCR reaction, except that the thermal cycle was changed as a dynamic change of two temperatures (35 cycles of 94 °C for 0 s, 62 °C for 0 s). The VPCR reaction was incubated in a C1000TM Thermal Cycler PCR (Bio-Rad Laboratories, USA).

Amplification analysis

A 3% agarose gel was used to validate the amplification products in the traditional analytical method. For the real-time assay, 0.6 µL of 20 × SYBR Green I was preadded to the reaction condition and the fluorescence was monitored by the real-time fluorescence quantitative analyzer (PiKo-Real, Thermo Fisher Scientific Corporation, USA). As for colorimetric detection, 0.5 µL of SYBR Green I (1000 ×) was added to the described VPCR products, and the color change was observed under the ultraviolet lamp (365 nm).

Sequencing

The PCR products of the positive samples were directly sequenced using the specific primer BP3. The DNA extraction of the negative samples was amplified by a pair of

primers, AP1 and ARP1, which can amplify ITS2 partial sequences of *P. ternata*, *P. pedatisecta*, *T. flagelliforme*, and *A. heterophyllum*. The corresponding amplification products were directly sequenced using the primer AP1.

Results and discussions

Establishment of VPCR-based amplification

The newly developed VPCR is a simplified version of conventional PCR which contains three steps of thermal cycling. As shown in Fig. 1a, VPCR uses a dynamic repeating of thermal change. Thus, its thermal curve presents a continuous fluctuation. However, conventional PCR uses a three-step temperature change, which displays three plateau phases in its thermal curve. To establish the VPCR-based detection of herbal medicinal materials, pinelliae rhizoma was used as the model in this study. Firstly, the traditional PCR-based detection method was successfully used to detect pinelliae rhizoma according to a previous report (Figure S2) (Chen et al. 2012). Then the original PCR cycling parameters “95 °C for 3 min, 35 cycles of 94 °C for 30 s, 62 °C for 30 s and 72 °C for 30 s” were directly changed to the VPCR program “35 cycles of 94 °C for 0 s, 62 °C for 0 s” to verify its feasibility. As presented in Fig. 1b, the amplified products of VPCR showed a clear band on the agarose gel, and the band size was the same as the PCR amplicons, indicating the VPCR could effectively amplify the pinelliae rhizoma genomic template. Notably, the amplification time of VPCR was about 20 min, which was greatly shortened compared to that of conventional PCR (77 min), demonstrating that VPCR could be applied to molecular detection of pinelliae rhizoma. Furthermore, the low temperature (TL) in the newly developed VPCR was investigated following the VPCR guidelines. A real-time assay was conducted to monitor the whole process of VPCR and the corresponding amplification products were also analyzed by end-point agarose gel. As shown in Fig. 1c, d, the amplification could be achieved when the TL was set from 60 °C to 74 °C. However, the optimal result of VPCR in terms of time and amplification efficiency was obtained when the TL was set at 72 °C. The corresponding reaction time is further reduced from 20 to 16 min when the TL is increased from 62 °C to 72 °C. These results indicated that the VPCR technique performed better than traditional PCR as regards reaction time, showing great potential in the fast detection of pinelliae rhizoma.

Direct template preparation

After the establishment of the VPCR amplification method, we sought to further improve the efficiency of

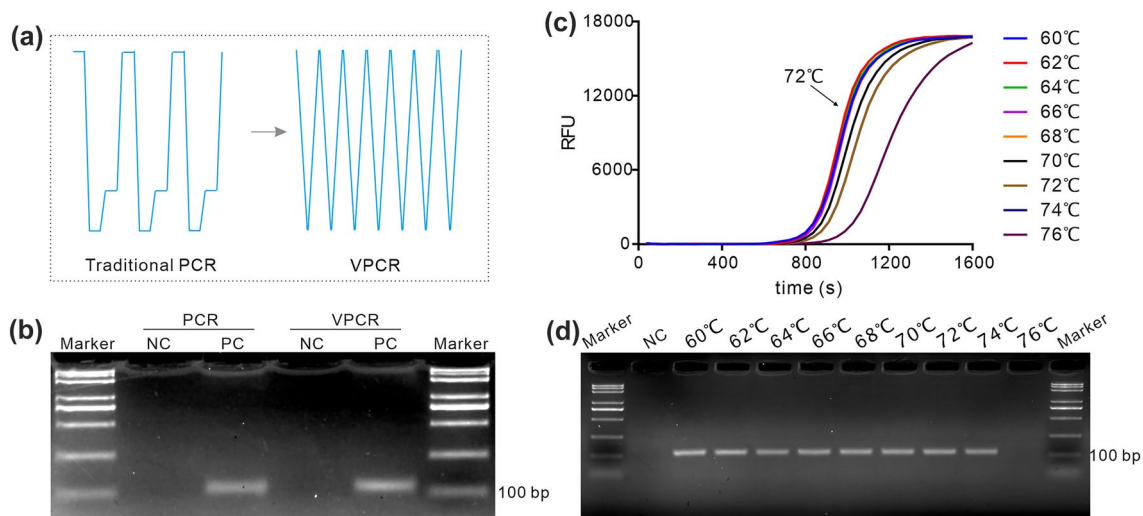


Fig. 1 Establishment of VPCR-based amplification of pinelliae rhizoma. **(a)** Comparison of traditional PCR with VPCR. The VPCR has a dynamic thermal change, while the traditional PCR shows a three-step thermal curve. **(b)** The gel image of the amplicon of PCR

and VPCR. *NC* No template control, *PC* positive control with pinelliae rhizoma genome. **(c)** Investigation of the low temperature (TL) of VPCR with real-time assay. **(d)** Gel image of the relative amplicons in **(c)**

pinelliae rhizoma detection. Traditional DNA extraction methods, like CTAB, usually involve multiple procedures and will use hazardous chemicals, like phenol and chloroform, to purify the DNA from a complex mixture. Meanwhile, the most widely used DNA extraction tool, the column-based DNA isolation kit, also comprises laborious steps, including cell lysis, centrifugation, washing, and elution. These standard methods always require 1.5 to 2 h (Moreira and Oliveira 2011; Wang et al. 2011) to obtain the DNA templates, thus the detection speed is highly impeded, especially when confronting massive samples. Recently, direct DNA isolation solutions (Svec et al. 2013) that require less than 5 min have been reported in molecular diagnostics, which could be an ideal strategy to shorten the test time. To simplify the DNA template presentation process, three different direct DNA extraction solutions, including NaOH solution, SDS (Sodium Dodecyl Sulfate) solution, and commercial solution, were evaluated by treating pinelliae rhizoma samples. As shown in Fig. 2, samples treated by three solutions could be efficiently amplified by the VPCR. Among three solutions, the commercial solution showed a similar extraction effect to the standard CTAB method as their amplification profile presented no significant difference (Fig. 2 lane 1 and lane 2). In contrast, the samples treated either by NaOH solution or SDS solution showed weaker amplification bands (Fig. 2 lane 3 and lane 4). Thus, the following experiments were carried out with the commercially available direct plant DNA extraction solution. Taken together, the direct template preparation was successfully combined with

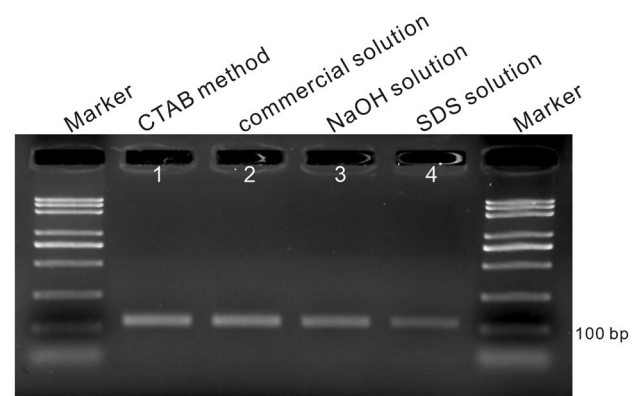


Fig. 2 Amplification results of pinelliae rhizoma templates prepared by the CTAB method and three different direct DNA isolation solutions. From lanes 1–4, DNA templates were prepared by the CTAB method; commercially available direct plant DNA extraction solution, NaOH solution, and SDS solution, respectively

VPCR detection, thereby forming direct-VPCR, which could further reduce the test time.

Colorimetric detection of direct-VPCR products

Now that the direct VPCR method has greatly enhanced the efficacy of detection; it is tempting to establish an ultrafast detection platform for massive identification. Since agarose gel electrophoresis is a main post-amplification reporting approach that is tedious and time consuming (about 15 min), one-tube colorimetric detection method could be a suitable choice to realize our aim. SYBR Green I is a fluorescent dye

that can bind to double-strand DNA and emit green light under the excitation of ultraviolet light. Based on that, a certain amount of SYBR Green I was added to the VPCR products to examine the feasibility of colorimetric reporting under the ultraviolet lamp. As depicted in Fig. 3a, only the tube with pinelliae rhizoma genomic template produced

a bright green fluorescence, while other tubes containing adulterants or non-template control remained unchanged in fluorescence. This result was completely consistent with that obtained by gel electrophoresis (Fig. 3b), indicating that the colorimetric report of direct-VPCR could be realized, thus promising in enhancing the testing efficacy of massive detection.

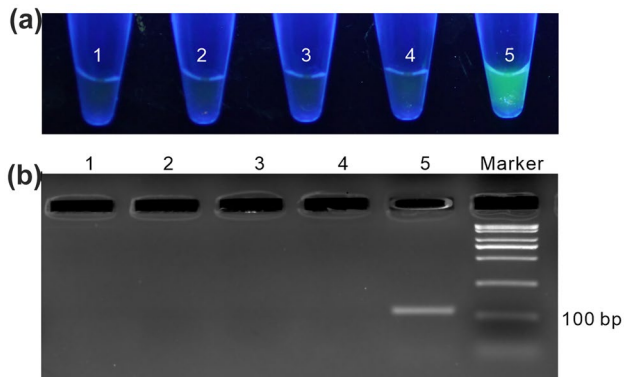
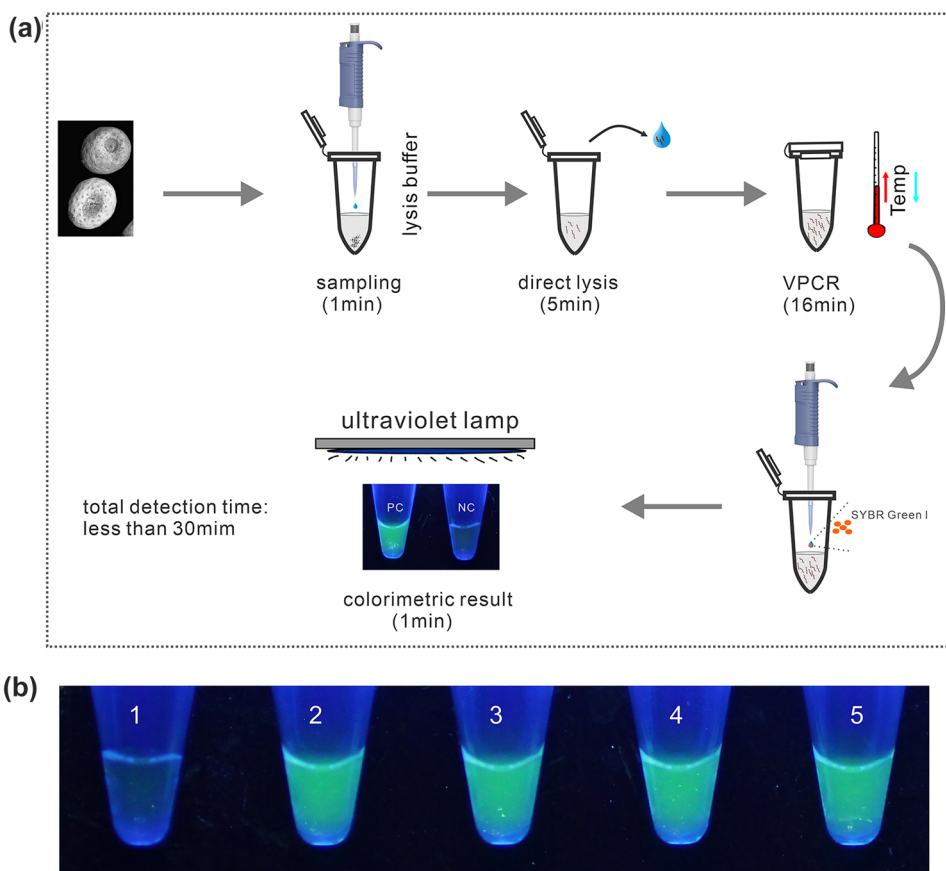


Fig. 3 Colorimetric detection of VPCR products from pinelliae rhizoma and relevant adulterants. **(a)** Colorimetric detection of direct-VPCR products. **(b)** Gel image of the corresponding tube in **(a)**. The PCR template used in tubes 1–5: no template negative control, genomic DNA isolated from dried rhizoma of *P. pedatisecta*, *T. flagelliforme*, *A. heterophyllum*, and *P. ternata*

Evaluation of the method in practical detection

So far, the ultrafast detection based on colorimetric direct-VPCR was successfully established, which could complete a test in less than 30 min (Fig. 4a). As raw pinelliae rhizoma is toxic to humans, crude pinelliae rhizoma should be processed before clinical practice. It is challenging to detect the treated pinelliae rhizoma preparations due to their different processing ways. To examine the feasibility of the proposed method in detecting processed pinelliae rhizoma preparations, three kinds of pinelliae rhizoma preparations, including pinelliae rhizoma praeparatum cum zingibere et alumine, pinelliae rhizoma praeparatum cum alumine, and rhizoma pinelliae preparatum, were analyzed. As illustrated in Fig. 4b, both three tubes containing treated pinelliae rhizoma preparations showed unambiguous green fluorescence, which was in agreement with the

Fig. 4 Evaluation of the ultrafast direct colorimetric VPCR. **(a)** The workflow of the proposed method. The sample was firstly treated with lysis buffer (5 min). Then, the lysed sample was added to the PCR mix. After a short period of running (16 min), the SYBR Green I was added to enable the visual readout under the ultraviolet lamp. The whole test could be done within 30 min. **(b)** Colorimetric detection of processed pinelliae rhizoma. Tubes 1–5: no template negative control, pinelliae rhizoma praeparatum cum zingibere et alumine, pinelliae rhizoma praeparatum cum alumine, rhizoma pinelliae preparatum, and positive control



gel electrophoresis assay (Figure S3), suggesting that the colorimetric direct-VPCR could be a robust method in the detection of processed pinelliae rhizoma samples.

The original plant of pinelliae rhizoma is widely distributed across East Asia; thus, pinelliae rhizoma samples grown in different areas with different climates come in various shapes and sizes. To verify the availability of high-throughput detection and universality of the developed method, 72 pinelliae rhizoma samples (Table S1 and Fig. 5a) from 9 different producing areas were identified accordingly. As presented in Fig. 5b, all 72 samples were identified through the colorimetric detection and results showed that there were 56 positive and 16 negative cases. To verify the results, all these samples were identified by sequencing (Figure S4). It turned out that the results of the colorimetric assay were completely in accordance with those of sequencing results, demonstrating 100% sensitivity and 100% accuracy of the proposed ultrafast identification system. These results strongly demonstrated the practicability of the colorimetric direct-VPCR, providing an alternative platform for ultrafast molecular detection of pinelliae rhizoma. Having demonstrated its robustness, we compared several current methods with direct-VPCR. When compared with these methods (Table S2), direct-VPCR showed some advantages in operation and measurement speed. Although an auxiliary ultraviolet lamp is needed in our method, the method is still a portable and cost-effective one in on-site identification. In contrast, the

instruments used in those chromatographic techniques may limit their broad use.

Conclusions

In this study, an ultra-fast and colorimetric detection method was developed to detect herbal medicinal materials. The ultrafast detection was achieved through three aspects: direct genomic DNA extraction (5 min), VPCR amplification (20 min), and a colorimetric reporter (less than 1 min). Therefore, the whole detection process can be finished within 30 min. As a proof-of-concept assay, the described procedure proved to be ultrafast and specific for identifying pinelliae rhizoma from other adulterants. To exemplify the capacity of this platform, 72 samples from 9 different areas were tested. The results demonstrated 100% sensitivity and specificity, demonstrating its robustness in practical application. Although chemical and chromatographic techniques are still dominating the field, the DNA-based method is being widely accepted because of its high specificity and simple operation. Besides, the method provided here could be regarded as an improved version of canonical DNA-based methods, which comprised all the merits of conventional PCR but showed faster speed. With the increasing requirement for high-throughput identification in the industrial herbal medicine market, we believe that our direct-VPCR could be an appealing choice in realizing highly efficient

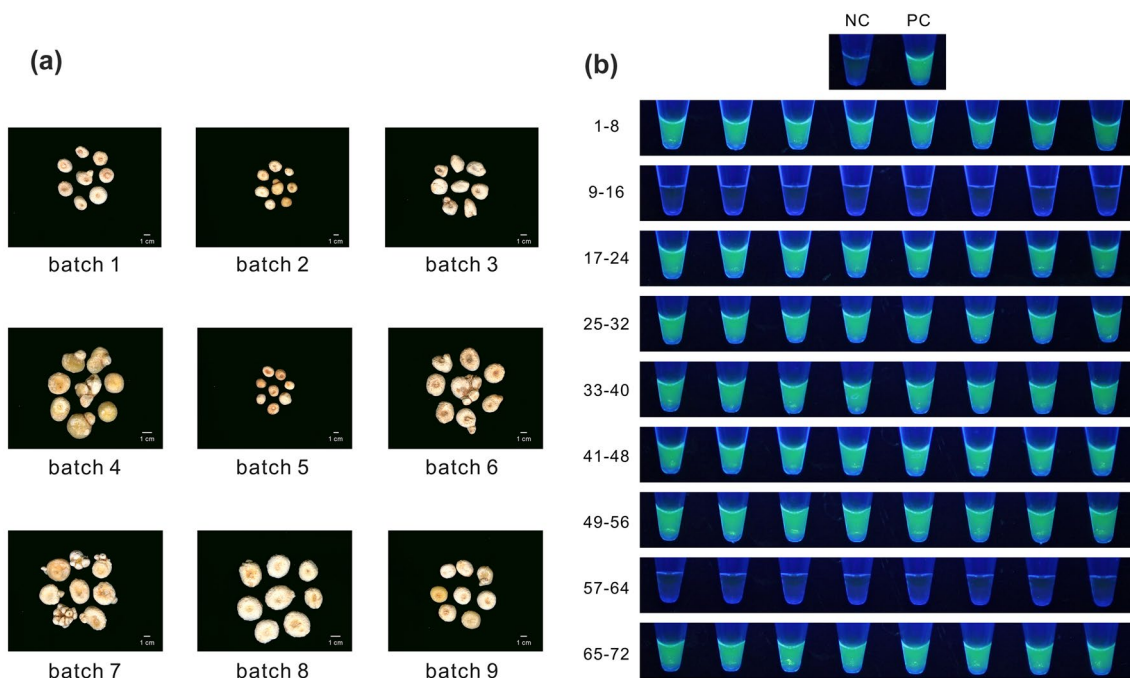


Fig. 5 Evaluation of the ultrafast direct colorimetric VPCR. **(a)** Different batches of pinelliae rhizoma from 9 producing areas. **(b)** Colorimetric identification of 72 samples from 9 different batches collected from different areas

on-site detection. In summary, the proposed method could be an ultrafast and easy-to-use platform for on-site identification of pinelliae rhizoma, which has great potential in the quality surveillance of other medicinal materials.

Supplementary Information The online version contains supplementary material available at <https://doi.org/10.1007/s13205-021-03035-9>.

Funding This work was supported by the National Natural Science Foundation of China [Grant number 81973434], Health Commission of Sichuan Province [Grant number 20PJ165], Xinglin Scholar Research Promotion Project of Chengdu University of TCM [Grant number 18TD0017].

Declarations

Conflicts of interest The authors declare no conflict of interest.

Data availability All data generated or analyzed during this study are included in this published article [and its supplementary information files].

References

- Chen R et al (2012) DNA based identification of medicinal materials in Chinese patent medicines. *Sci Rep* 2:958. <https://doi.org/10.1038/srep00958>
- Chen R et al (2019) Polymerase chain reaction using “V” shape thermal cycling program. *Theranostics* 9:1572–1579. <https://doi.org/10.7150/thno.31986>
- Choudhary P, Das S, Chakdar H, Singh A, Goswami SK, Saxena AK (2019) Rapid high throughput template preparation (rHTTP) method: a novel cost effective method of direct PCR for a wide range of plants. *BMC Biotechnol* 19:69. <https://doi.org/10.1186/s12896-019-0560-4>
- Coutinho Moraes DF, Still DW, Lum MR, Hirsch AM (2015) DNA-based authentication of botanicals and plant-derived dietary supplements: where have we been and where are we going? *Planta Med* 81:687–695. <https://doi.org/10.1055/s-0035-1545843>
- Farrar JS, Wittwer CT (2015) Extreme PCR: efficient and specific DNA amplification in 15–60 seconds. *Clin Chem* 61:145–153. <https://doi.org/10.1373/clinchem.2014.228304>
- Li T-J, Chang C-M, Chang P-Y, Chuang Y-C, Huang C-C, Su W-C, Shieh D-B (2016) Handheld energy-efficient magneto-optical real-time quantitative PCR device for target DNA enrichment and quantification. *Npg asla Mater*. <https://doi.org/10.1038/am.2016.70>
- Mishra P, Kumar A, Nagireddy A, Mani DN, Shukla AK, Tiwari R, Sundaresan V (2016) DNA barcoding: an efficient tool to overcome authentication challenges in the herbal market. *Plant Biotechnol J* 14:8–21. <https://doi.org/10.1111/pbi.12419>
- Moreira PA, Oliveira DA (2011) Leaf age affects the quality of DNA extracted from *Dimorphandra mollis* (Fabaceae), a tropical tree species from the Cerrado region of Brazil. *Gen Mol Res* 10:353–358. <https://doi.org/10.4238/vol10-1gmr1030>
- Son JH, Cho B, Hong S, Lee SH, Hoxha O, Haack AJ, Lee LP (2015) Ultrafast photonic PCR. *Light Sci Appl* 4:e280. <https://doi.org/10.1038/lsa.2015.53>
- Sun F, Chen Y, Wang K-Y, Wang S-M, Liang S-W (2019) Identification of genuine and adulterated pinellia ternata by mid-infrared (MIR) and near-infrared (NIR) spectroscopy with partial least squares - discriminant analysis (PLS-DA). *Anal Lett*. <https://doi.org/10.1080/00032719.2019.1687507>
- Svec D, Andersson D, Pekny M, Sjoback R, Kubista M, Stahlberg A (2013) Direct cell lysis for single-cell gene expression profiling. *Front Oncol* 3:274–274. <https://doi.org/10.3389/fonc.2013.00274>
- Wang XH, Xiao HL, Chen GX, Zhao X, Huang CH, Chen CY, Wang F (2011) Isolation of high-quality RNA from *Reaumuria soongorica*, a desert plant rich in secondary metabolites. *Mol Biotechnol* 48:165–172. <https://doi.org/10.1007/s12033-010-9357-3>
- Yip CCY et al (2019) Evaluation of RealStar (R) alpha herpesvirus PCR kit for detection of HSV-1, HSV-2, and VZV in clinical specimens. *Biomed Res Int*. <https://doi.org/10.1155/2019/5715180>
- Zhang XM et al (2018) Development and evaluation of a PCR-based assay kit for authentication of *Zaocys dhumnades* in traditional Chinese medicine. *Mitochondrial DNA Part A* 29:102–106. <https://doi.org/10.1080/24701394.2016.1248429>