



DNA based identification of medicinal materials in Chinese patent medicines

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Chinese patent medicines (CPM) are highly processed and easy to use Traditional Chinese Medicine (TCM). The market for CPM in China alone is tens of billions US dollars annually and some of the CPM are also used as dietary supplements for health augmentation in the western countries. But concerns continue to be raised about the legality, safety and efficacy of many popular CPM. Here we report a pioneer work of applying molecular biotechnology to the identification of CPM, particularly well refined oral liquids and injections. What's more, this PCR based method can also be developed to an easy to use and cost-effective visual chip by taking advantage of G-quadruplex based Hybridization Chain Reaction. This study demonstrates that DNA identification of specific Medicinal materials is an efficient and cost-effective way to audit highly processed CPM and will assist in monitoring their quality and legality.

Traditional Chinese Medicine (TCM) has been used for health care in China and other oriental countries like Korea and Japan for thousands of years. It also enjoys a growing popularity both in eastern and western countries nowadays rather than abandoned by the highly developed science and technology. According to different processing technology, TCM can be divided into Chinese medicinal raw materials, prepared slices and Chinese patent medicines (CPM). CPM are highly processed drugs which consist of several kinds of or even dozens kinds of plants or animals. Compared to the other two types of TCM, CPM are more convenient to use for both doctors and patients. The market for CPM in China alone is tens of billions US dollars annually and since the therapeutic benefits of some CPM have been validated scientifically, they are more widely used all over the world. For example, after validation studies, Diao Xin Xue Kang capsule has already been licensed for sale in EU member state in April 2012. Moreover, Compound Danshen Dripping Pills has already been approved to enter the Phase III trials by the US Food and Drug Administration (FDA) and hopefully will be marketed in the U.S. next year. The increasing demand for CPM not only promotes business opportunities with tremendous financial profit but also drives fraudulent companies to adulterate or even substitute the labeled medicinal materials with cheaper plants or animals which share a close resemblance in morphology, chemical composition or clinical usage. These kind of fake drugs are against the theory of Traditional Chinese Medicine and can reduce the efficacy of the drug sharply or even make them poisonous. For instance, in Belgium¹ during 1990–1992, the toxic herb *Aristolochia fangchi* was misused as *Stephania tetrandra* in a diet drug that resulted in over one hundred women's renal failure. So it is of great importance to establish an unequivocal identification system in the quality control of CPM for the safety of patients and benefits of other legal companies.

Traditionally, the authentication of TCM mainly relies on organoleptic parameters² which is an expert-dependent technique. But the product identification of highly-processed CPM in the form of tablet, capsule, oral liquid and injection are out of the scope of an experienced taxonomist. In the past few decades, the techniques for chemo-profiling such as TLC, HPLC³ and GC⁴ have a wide application in the quality assurance of CPM. These methods can complement the limitations of morphological identification to some extent but as the chemotype-driven fingerprints only addressed the detection of certain compounds, so can easily be misled. For example in CPM product of ginseng (*Panax ginseng* C.A. Meyer), some dealers substitute the ginseng root with that of codonopsis (*Codonopsis pilosula* (Franch.) Nannf.) (Supplementary Figure S7), which is less curative in effect and much cheaper than ginseng⁵. More than that, by adding ginsenosides extract alone rather than the whole ginseng root they can conveniently pass through the chromatographic detection because the ginsenosides was supposed to be the major pharmacologically active components and the chemical marker of ginseng.

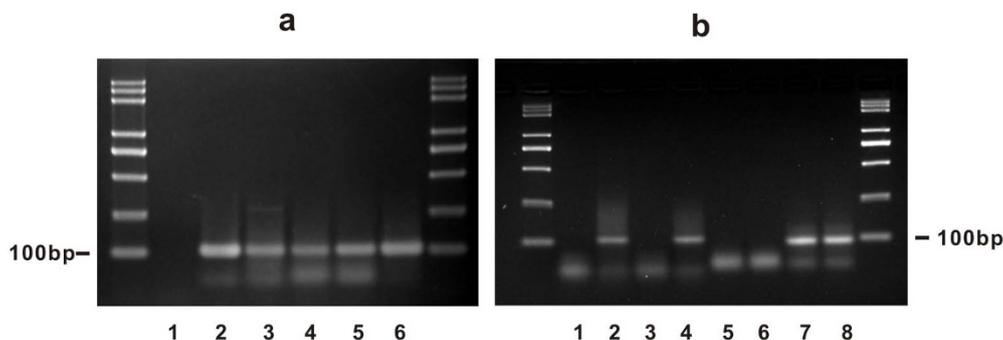


Figure 1 | (a) Agarose gel electrophoresis of PCR results from ginseng samples. Lane 1–6: negative control (water was used as sample), tablet, pill, oral liquid, injection, positive control (DNA extracted from reference medical material is used as sample). Tablet and pill samples are subjected to one-step simple PCR while oral liquid and injection samples are subjected to nested PCR. (b) Research on the specificity of PCR amplification. In the left four lanes ginseng primers are used. Lane 1–4: negative control (water was used as sample), ginseng oral liquid, codonopsis oral liquid, mixture of ginseng and codonopsis oral liquid. In the right four lanes codonopsis primers are used. Lane 5–8: negative control (water was used as sample), ginseng oral liquid, codonopsis oral liquid, mixture of ginseng and codonopsis oral liquid.

Recently, various studies like random amplified polymorphic DNA (RAPD)⁶, simple sequence repeats (SSRs)^{7,8}, restriction fragment length polymorphism (RFLP)^{9,10} and subtracted diversity array (SDA)¹¹ have been reported to authenticate medicinal herbs. Polymerase chain reaction (PCR) has given scientists a powerful arsenal of molecular tools as it can be used to exponentially amplify trace amounts of DNA. With the help of PCR, it becomes possible to detect DNA of specific natural products in processed drugs and audit the quality of CPM. But among those DNA-based methods of species identification, most of them deal with raw or unprocessed TCM. To our knowledge, this study represents the first application of PCR based technology to the identification of CPM especially the oral liquid and injection¹² in which the plant is used in the form of extracts and has undergone industrial extraction, filtration and sterilization. There is no doubt about the discriminatory power of DNA among different species. Numerous studies^{13,14} and sequences in Genbank have proved the internal transcribed spacer (ITS) region of nuclear ribosome DNA to be a perfect DNA marker in angiosperm species identification. Because they are much less conserved¹⁵ than the coding regions and can provide more genetic information, so we focused our study on the polymorphism of the ITS region of each plants.

The ginseng root is one of the best known herbal medicines which has been used as a natural tonic, anti-fatigue and anti-ageing agent traditionally. It has also been reported that ginseng root or CPM product ginseng contained are used to treat diabetes-like conditions¹⁶ and showed anti-proliferative effect on human prostate cancer cells¹⁷. There are hundreds of drugs which used ginseng as the mainly therapeutic plant sold in the market of China alone. As mentioned before, ginseng is often adulterated with codonopsis to reduce manufacturing costs. So firstly, we chose ginseng contained CPM and its possible adulterations as our targets to verify our idea.

Results

Primarily, the classical CTAB (Cetyl trimethylammonium Bromide) method¹⁸ was used for genomic DNA extraction from solid samples (root powder, tablet and pill) and ethanol precipitation for liquid samples (oral liquid and injection). Extracted DNA was analyzed through agarose gel electrophoresis. **Supplementary Figure S1** shows that moderately degraded genomic DNA can be extracted from root powder and some of the tablets in concentrations detectable by agarose gel electrophoresis. While no DNA from pill, oral liquid and injection was observed in agarose gel because most of the DNA could have been removed or degraded during the industrial processing.

While in the pill and oral liquid, the DNA extracted directly by CTAB or ethanol precipitation without purification is dark brown

and viscous (**Supplementary Figure S2**). Most of those samples couldn't be amplified by PCR. We speculate that the pigments or other substances in the pellet could prohibit the PCR amplification. So the commercial available silica-based spin column DNA purification kit was used. The DNA obtained by column purified shows a normal color and viscosity, which could be amplified by following PCR efficiently. For injection samples magnetic beads kit, ethanol precipitation and the column DNA purification kit were applied to isolate DNA. A similar amplification yield was observed with the DNA extracted by all these methods (**Supplementary Figure S3** and **Figure S4**). But considering magnetic beads kit is costly and ethanol precipitation requires large volume high-speed centrifugation, we prefer column DNA purification kit to concentrate DNA in oral liquid and injection samples.

Extracted DNA from all the samples was subjected to PCR amplification with specific primers for ITS regions. The successful PCR amplification of different kinds of CPM samples which contain ginseng root as starting materials was evidenced by the specific and clear EtBr-stained bands in agarose gel electrophoresis (**Figure 1a**). 111 bp specific amplification bands were observed only in the positive control, tablet and pill samples (**Figure 1a** lane 2 and 3) but no amplification was observed in oral liquid and injection samples (data not shown). Considering the low yield of DNA in oral liquid and injection samples, nested PCR was used to improve the efficacy of amplification (**Figure 1a** lane 4, 5 and **Supplementary Figure S3**). It should be noted that all of the samples were composed of several kinds of medicinal plants or animals rather than ginseng alone. Selected ginseng containing pills “Wu Ji Bai Feng Wan” is a famous and effective gynecological medicine which consists of 20 kinds of medicinal materials including plants and animals (Lane 3, **Figure 1a**). The specificity of PCR products were further identified by molecular cloning, DNA sequencing and alignment with the already known sequence in genbank and it was found 100% (98.9% codonopsis samples) same as the reported sequence.

To check the general applicability of DNA based detection of medicinal materials in CPM. Different types of CPM products containing *Codonopsis pilosula* (Franch.) Nannf have been investigated with similar DNA extraction method and specific PCR amplification. All the samples including tablet, pill and oral liquid gave the same desired PCR products based on the gel analysis (**Supplementary Figure S5**). Those PCR products have been further identified with molecular clone, DNA sequencing too. The cross test of oral liquid CPM samples which contain different materials (ginseng, codonopsis or both) was carried out by using the separate PCR detections for ginseng and codonopsis to verify the specificity of the PCR based assay (**Figure 1b**). The successful amplification happened in the orthogonal

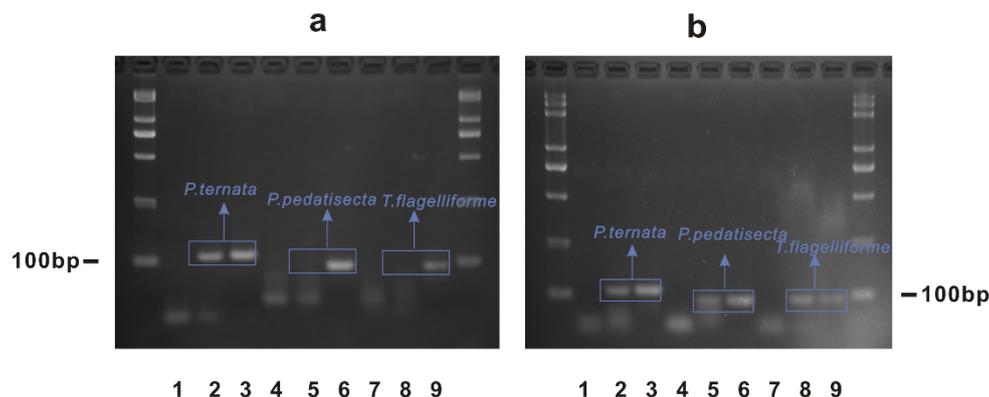


Figure 2 | Agarose gel electrophoresis of PCR results from sample a and b. Lane 1–3: isolation negative control (water is used as sample when extracted DNA), sample, positive control (DNA extracted from *P. ternata* is used as template), 4–6: isolation negative control (water is used as sample when extracted DNA), sample, positive control (DNA extracted from *P. pedatisecta* is used as template), 7–9: isolation negative control (water is used as sample when extracted DNA), sample, positive control (DNA extracted from *T. flagelliforme* is used as template).

PCR reactions where the specific PCR primers were matching the medical materials (Lane 2 and 7, **Figure 1b**). The arbitrary mixture of two different oral liquids containing gensing and codonopsis resulted in positive PCR amplification for both gensing and codonopsis detections (Lane 4 and 8, **Figure 1b**). Those results indicate the possible application of PCR based assay in the detection of the adulteration in CPM.

Huoxiangzhengqi water is one of the best selling oral liquid in China especially in summer, because it can effectively help relieve the discomfort caused by the hot weather. It consists of ten medicinal materials, but among these materials Banxia (*Pinellia ternata*

(Thunb.) Breit.) rhizoma is the most expensive herbal materials. People have suspected that there could be adulteration in some product for years because the selling price of some manufacturers was less than the cost according to the price of the medicinal materials in the market. The possible adulterations could be the rhizomes of Zhangye banxia (*Pinellia pedatisecta* Schott) and Shui banxia (*Typhonium flagelliforme* (Lodd.) Blume.). They are ten times cheaper than the Banxia and are highly similar in morphology with each other (**Supplementary Figure S7**). To prove the PCR could be applied in the detection of adulteration in CPM. We prepared two kinds of Huoxiangzhengqi water as the description of Chinese Pharmacopoeia. In

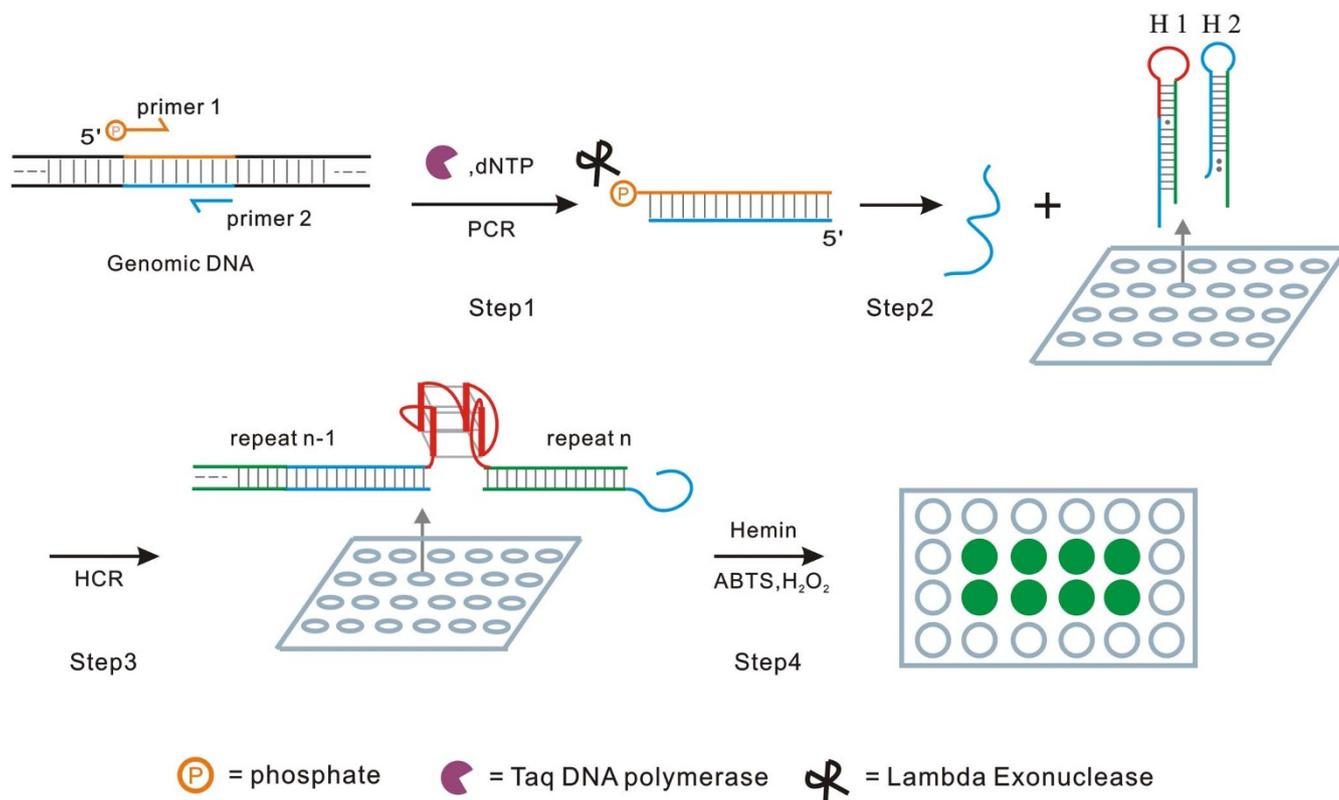


Figure 3 | The principle of the chip-based detection strategy. Step 1: a 5'-phosphorylated primer was used in PCR to get duplex product with all antisense sequences containing phosphate groups at 5' ends. Step 2: the 5'-phosphorylated stand of duplex DNA was selectively digested by Lambda Exonuclease. Step 3: the ssDNA initiates the two probes open and polymerize to DNA polymers bearing a lot of G-quadruplexes. Step 4: the G-quadruplex bind to hemin and catalyze the oxidation of ABTS to a green product in the presence of H_2O_2 . Sequences with same color are the same or complementary to each other.

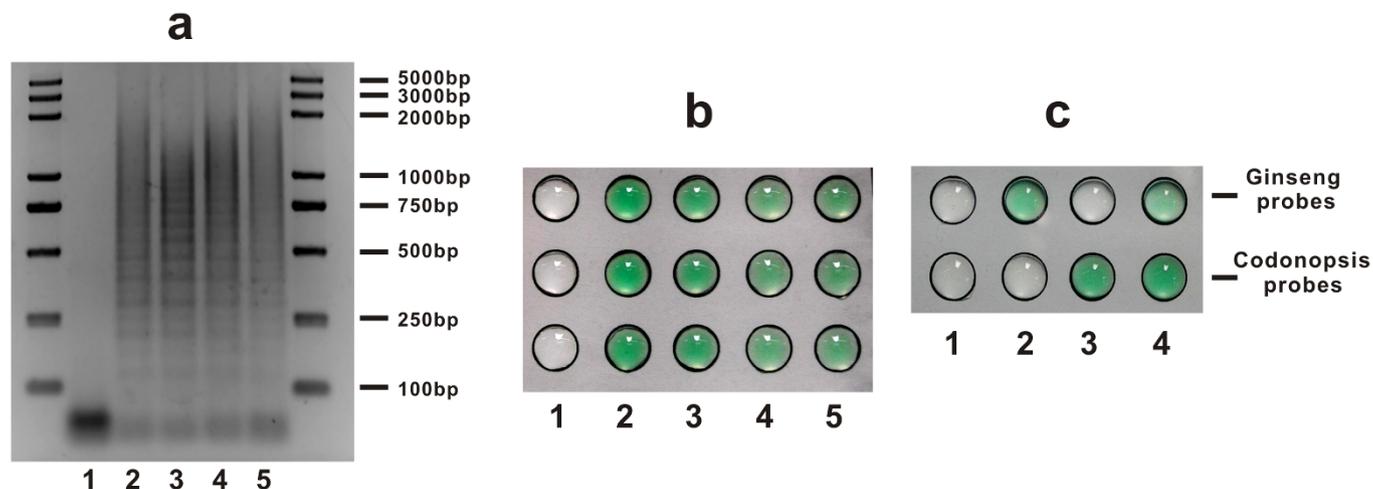


Figure 4 | (a) Agarose gel electrophoresis of G-quadruplex integrated hybridization chain reactions (GQ-HCR) which were initiated by the digested PCR product from different kinds of ginseng CPM samples. Lane 1–5: negative control (water is used as sample), tablet, pill, oral liquid, injection. (b) Research on the reproducibility of color reaction: the ginseng DNA samples were subjected to three parallel detections (the samples on the same column are the same kind of CPM). All the circles on the chip were pre-printed with special probes for ginseng. From 1–5: digested PCR products from negative control (water is used as sample), tablet, pill, oral liquid, injection samples. (c) Research on the specificity of chip-based detection: the two lines were pre-printed with ginseng probes and Codonopsis probes respectively. From 1–4: digested PCR products from negative control (water is used as sample), ginseng oral liquid, Codonopsis oral liquid, mixture of ginseng and Codonopsis oral liquid were added.

sample a, only *P. ternata* was used. While in **sample b**, *P. ternata* was adulterated with *P. pedatisecta* and *T. flagelliforme* at the ratio of 1 : 1 : 1. Specific primers of *P. ternata*, *P. pedatisecta* and *T. flagelliforme* were designed on the basis of the ITS regions. DNA of the two samples were extracted and amplified with the corresponding specific primers. Only the PCR amplification product for *P. ternata* was detected in **sample a** (Figure 2a) comparing to the successful amplifications for all three materials in **sample b** (Figure 2b). As our expectation, the adulterated *P. pedatisecta* and *T. flagelliforme* in the Huoxiangzhengqi water can be easily detected based on the agarose gel electrophoresis assay of PCR products. Three commercial available Huoxiangzhengqi water products from different companies have been analyzed with the same method. Two of them were found the adulteration with *P. pedatisecta* and *T. flagelliforme* (data doesn't show to protect the manufactures' identities).

As agarose gel electrophoresis requires a labour-intensive work, we developed a chip-based colorimetric detection approach to distinguish the PCR products of ginseng CPM from its possible adulterants conveniently by taking advantage of a GQ-HCR reaction which is also developed by our group¹⁹. Figure 3 shows the principle of the detection strategy. One of the PCR primers was 5'-phosphorylated to obtain the duplex products in which all antisense sequences of target DNA would contain phosphate groups at the 5'-ends after PCR amplification. Lambda exonuclease²⁰ is a highly processive 5'-3' exodeoxyribonuclease that can selectively digest the 5'-phosphorylated strand of duplex DNA. After digestion of the undesired strand, the PCR product becomes single stranded which could trigger the following G-quadruplex^{21,22} integrated hybridization chain reaction (GQ-HCR)^{23,24}. Two hairpin probes H1 and H2 of GQ-HCR amplification has been printed on a plastic sheet in advance. The ssDNA obtained with Lambda exonuclease digestion was pipetted on the sheet to initiate the two probes open successively to obtain DNA polymers bearing a lot of G-quadruplexes. The G-quadruplex exhibited peroxidase-like activity in the presence of hemin, which could catalyze the oxidation of ABTS (2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid)) with aid of H₂O₂ to obtain green product.

The digested PCR products from ginseng CPM were added to initiate the two specific hairpins probes to polymerize. It is shown in the agarose gel (Figure 4a) that the hairpin monomers have assembled to DNA polymers with different molecular weight and

the efficiency of the polymerization is proportional to the initial concentration of PCR products. As shown in the picture, there is more substrate left in injection compared to the tablet since it has less amplification product in injection sample (Line 2–5, Figure 4a).

DNA from different ginseng containing CPM was extracted and subjected to following detection in three parallel experiments. All kinds of CPM samples gave positive response (the bright green color in ginseng containing drugs samples, column 2–5, Figure 4b) comparing the negligible background of negative control (water is used as template in PCR amplification) (column 1, Figure 4b). There is no obvious difference between the colorimetric results from the same kind of drug, which indicate the good reproducibility of this method. The color density is also consistent with the GQ-HCR results (Figure 4a): since the tablet and pill have yielded more HCR product, so intensity of their color is somehow greener on the visual chip compared to oral liquid and injection.

The visual-chip based assay should be ideal method to detection of adulteration in CPM. As shown in Figure 4c, the first row was printed with ginseng probes and the second was printed with Codonopsis probes respectively. Lambda exonuclease Digested PCR products from ginseng oral liquid, Codonopsis oral liquid and the mixture of ginseng with Codonopsis oral liquid were pipetted onto the corresponding circles on the chip in both rows. Green color on the chip indicated that Ginseng and Codonopsis could be detected successfully with their respective probes on the chip with negligible background in negative control (column 2 and 3, Figure 4c). Additionally, Ginseng and Codonopsis could be detected simultaneously in artificially mixed sample (column 4, Figure 4c), which revealed the adulteration in the detected sample. What's more, the visual results can also be detected by the spectrophotometer or other similar instruments, more details please see Supplementary Figure S7.

Discussion

Here we report a new approach for the authentication of CPM especially the oral liquid and injection samples by applying Molecular Biotechnological tools. Described procedure is sensitive enough to detect trace amounts of DNA in CPM which has undergone intense industrial processing. Further it has been developed to an easy to use and cost-effective visual-chip based assay that needs no expensive sequence or Gene chip scanner. With a high reproducibility and



specificity, similar approach can also be rationalized to authenticate other medicinal plants or animals in CPM. Therefore, with TCM globalization, a potential increase in the export of authenticated CPM as drugs or as botanical dietary supplements for health enhancement will be possible all over the world. In this regard proposed method could be used to minimize health risk of adulterants, protect the commercial interests of quality suppliers and may finally become a powerful tool in the quality assurance of CPM not only in China but also around the world.

Methods

Drugs used in this study. *Panax* Ginseng drugs (tablet, pill, oral liquid and injection) and *Codonopsis pilosula* drugs (tablet, pill, oral liquid) were purchased from local pharmacy in Chengdu, People's Republic of China. To protect the manufacturer's identities, sample sources are described as its preparation name. Root powder of *Panax ginseng* C. A. Meyer, *Codonopsis pilosula* (Franch.) Nannf and *Pinellia ternata* (Thunb.) Breit were purchased from National institute for food and drug control as positive control.

DNA extraction. The classic CTAB (Cetyl trimethylammonium Bromide) method is used in the DNA extraction of the root powder with a slight modification. 100 mg material was suspended in 750 μ l of CTAB buffer (2% CTAB; 100 mM Tris-HCl, pH 8.0; 20 mM EDTA; 2.5 M NaCl) and then incubated at 65°C for 2 h with occasional shaking. The lysate was extracted with 600 μ l of chloroform:isoamyl alcohol (24:1). DNA was precipitated with 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 40 μ l of TE buffer (10 mM Tris-HCl, pH 8.0; 1 mM EDTA)²⁵. As the tablets and pills contain some pharmaceutical excipients, column DNA purification kit was used as described in the manufacturer's instructions after isopropanol precipitation. This kit was also used to concentrate and purify DNA in oral liquid and injection.

PCR amplification. *Simple PCR.* PCR was performed in a 50 μ l reaction mixture containing 1–2 μ l of DNA template from the root powder, tablets or pills, 10 \times Taq buffer (200 mM Tris-HCl, pH 8.4, 200 mM KCl, 100 mM (NH₄)₂SO₄, 20 mM MgSO₄), 0.2 mM dNTPs, 1 μ M of each primer and 5 units of *EasyTaq* polymerase. Initial template denaturation was done at 95°C for 3 min, followed by 35 cycles of 94°C for 30 s, 55°C (50°C for *Codonopsis* samples) for 30 s & 72°C for 30 s with final extension of 72°C for 5 min.

Nested PCR. 1st round PCR was carried out in a 50 μ l reaction mixture containing 5–10 μ l of DNA, 10 \times Taq buffer (200 mM Tris-HCl, pH 8.4, 200 mM KCl, 100 mM (NH₄)₂SO₄, 20 mM MgSO₄), 0.2 mM dNTPs, 0.25 μ M of each external primers and 5 units of *EasyTaq* polymerase. Initial template denaturation was done at 95°C for 3 min, followed by 30 cycles of 94°C for 30 s, 60°C (54°C for *Codonopsis* samples) for 30 s & 72°C for 30 s with final extension of 72°C for 5 min.

1 μ l PCR product of the first round PCR was used as template for 2nd round PCR and other details of the second round PCR was the same as described for simple PCR.

GQ-HCR and color reaction. The PCR product was incubated with 10 units of Lambda exonuclease enzyme at 37°C for 1 h to digest one strand of double stranded DNA. The GQ-HCR was carried out in a 10 μ l mixture containing 8 μ l digested products, 0.5 μ M probes, 400 mM NaCl. For visual chip, 8 μ l of digested PCR products were pipetted on a plastic film which was pre-printed 5 pmol of DNA probes. The film was incubated at 37°C for 1 h and subjected to detection reagents (1.5 μ l of 60 mM ABTS, 0.5 μ l of 60 μ M Hemin & 0.5 μ l of 60 mM H₂O₂) afterwards, color change was observed by naked eyes on the visual DNA chip.

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Author contributions

X.C., Y.D. and Z.T. conceived and designed the research. R.C., X.C., W.W. and Y.Z. performed experiments. R.C., A.Y. and Z.T. analyzed the data and wrote the manuscript.

Additional information

Supplementary information accompanies this paper at <http://www.nature.com/scientificreports>

Competing financial interests: The authors declare no competing financial interests.

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