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Research Article

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Evaluation and application of an efficient plant DNA extraction protocol for laboratory and field testing

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Abstract: Nucleic acids in plant tissue lysates can be captured quickly by a cellulose filter paper and prepared for amplification after a quick purification. In this study, a published filter paper strip method was modified by sticking the filter paper on a polyvinyl chloride resin (PVC) sheet. This modified method is named EZ-D, for EASY DNA extraction. Compared with the original cetyl trimethylammonium bromide (CTAB) method, DNA extracted by EZ-D is more efficient in polymerase chain reaction (PCR) amplification due to the more stable performance of the EZ-D stick. The EZ-D method is also faster, easier, and cheaper. PCR analyses showed that DNA extracted from several types of plant tissues by EZ-D was appropriate for specific identification of biological samples. A regular PCR reaction can detect the EZ-D-extracted DNA template at concentration as low as 0.1 ng/μL. Evaluation of the EZ-D showed that DNA extracts could be successfully amplified by PCR reaction for DNA fragments up to 3000 bp in length and up to 80% in GC content. EZ-D was successfully used for DNA extraction from a variety of plant species and plant tissues. Moreover, when EZ-D was combined with the loop-mediated isothermal amplification (LAMP) method, DNA identification of biological samples could be achieved without the need for specialized equipment. As an optimized DNA purification method, EZ-D shows great advantages in application and can be used widely in laboratories where equipment is limited and rapid results are required.

Key words: DNA extraction; Cellulose filter paper; Field test; Genetically modified (GM) crops; Chinese medicine ultra-fine powder; Rapid molecular identification

1 Introduction

Nucleic acid-based molecular diagnosis assays have increased in use in many fields in recent years, due to their outstanding sensitivity, specificity, and speed (Liesenfeld et al., 2014). An essential first step for all subsequent analyses is obtaining DNA of high molecular weight and purity (Moeller et al., 2014). For DNA extraction from plants, the cetyl trimethylammonium bromide (CTAB) method is one standard way to isolate high-quality DNA (Murray and Thompson, 1980). The CTAB extraction protocol has been continuously optimized to reduce the polyphenols and polysaccharides present in plant tissues (Peterson et al., 1997; Hou et al., 2005; Moeller et al., 2014). However, it is still difficult to extract DNA of high quality from plant materials that are rich in secondary metabolites or have a high degree of DNA degradation (Chen et al., 2014). Moreover, the CTAB method requires high-end instruments, strict and time-consuming procedures, and well-trained personnel. It is time-consuming and cannot facilitate rapid molecular detection when results are needed urgently.

Current rapid extraction methods include alkaline lysis and direct-polymerase chain reaction (PCR), both of which shorten the DNA extraction to about 10 min. However, these methods still require controlled heating, centrifugation, and other operations (Jiang et al., 2013; Zhang et al., 2017). Another rapid method uses a disposable polymeric microneedle patch to puncture leaf tissue to extract intracellular DNA. This is suitable for rapid molecular diagnosis of plant diseases, but not for molecular identification of a plant's genetic

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material (Paul et al., 2019). A cellulose filter paper purification method was reported for the extraction of DNA from plant tissues for rapid molecular detection (Zou et al., 2017). The method uses a cellulose filter paper-based stick with a DNA-binding zone and a zone impervious to water, prepared by dipping half of the cellulose filter paper into molten wax. While these cellulose filter paper-based sticks (abbreviated to Z-Dipsticks in the following text) can absorb DNA efficiently, we have observed that when immersed in extraction buffer or a PCR reaction system, the filter paper at the DNA-binding zone readily becomes loose and soft. When this loosened filter paper is immersed in the PCR solution, a large amount of solution is sucked away, resulting in a decline in PCR efficiency. This situation is even more serious when a large number of samples need to be processed at the same time, requiring the test strips to be retained in the extraction or PCR system for a longer time.

Here, we present a modified Z-Dipstick plant DNA extraction method named EASY DNA (EZ-D). Instead of imbedding the handle of the test strip with paraplast, a polyvinyl chloride (PVC) resin material is used as the handle of the dipstick, as well as the support of the DNA-binding zone. EZ-D has been successfully used for DNA extraction from a variety of plant species and plant tissues and the DNA is suitable for a wide range of downstream applications including PCR and loop-mediated isothermal amplification (LAMP). The method has great prospects for application in the field or market for easy and fast molecular identification.

2 Materials and methods

2.1 Sample materials

Six different types of transgenic plants and their corresponding non-transgenic controls were used in this study (Table 1). Transgenic plants included: *Oryza sativa* T51, containing the *HPT* and *cry1Ab/Ac* transgenes (Tu et al., 2000); *Zea mays* ZC1, containing the *cry1Ab* and *g10-epsps* transgenes; *Glycine max* GC1, containing the *cry1Ab* and *cp4-epsps* transgenes; soybean SWEET15, containing the *bar* transgene (Wang et al., 2019); soybean ZUTS-33, containing the *g10-epsps* transgene (Lu et al., 2014); and *Larix gmelinii* BADH, containing the *35S* transgene (Zhang et al., 2013). Corresponding non-transgenic controls included the rice line MH63, the maize line HiII, the soybean lines Wandou28, Williams82 and HC-3, and the larch line WM. In addition, four kinds of Chinese medicines, prepared as ultrafine powders from *Fritillaria* spp. in the Lillaceae family, were used, namely Zhe Beimu (ZB, prepared from *Fritillaria thunbergii*), Chuan Beimu 1 (CB1, from *Fritillaria unibracteata*), CB2 (from *Fritillaria przewalskii*), and CB3 (from *Fritillaria delavayi*). According to industry standards, the sizes of the Chinese medicine ultrafine powders were from 25 to 48 μm.

2.2 EZ-D stick

Whatman cellulose filter paper No. 1 (Whatman, UK) was cut into strips measuring 4 mm×20 cm. A commercial self-adhesive PVC sheet (YouLong Biotech, China) was cut into strips of 44 mm×20 cm. Four millimeters of tape on the bottom of the plastic sheet

Table 1 Plant materials used in this study

Plant species	Name of the material	Type of the material	Content of the transgene cassette
Oryza sativa	T51	Transgenic rice	*35S::cry1Ab/Ac*; *35S::HPT*
	MH63	Non-transgenic control	None
Zea mays	ZC1	Transgenic maize	*35S::cry1Ab*; *35S::g10-epsps*
	HiII	Non-transgenic control	None
Glycine max	GC1	Transgenic soybean	*35S::cry1Ab*; *35S::cp4-epsps*
	Wandou28	Non-transgenic control	None
Glycine max	SWEET15	Transgenic soybean	*35S::bar*
	Williams82	Non-transgenic control	None
Glycine max	ZUTS-33	Transgenic soybean	*35S::g10-epsps*
	HC-3	Non-transgenic control	None
Fritillaria unibracteata	CB1	Chinese medicine	Without *ZB1* gene
Fritillaria przewalskii	CB2	Chinese medicine	Without *ZB1* gene
Fritillaria delavayi	CB3	Chinese medicine	Without *ZB1* gene
Fritillaria thunbergii	ZB	Chinese medicine	With *ZB1* gene

was peeled off manually and then a filter paper strip was attached to the exposed adhesive area. The plastic sheet with attached filter paper was cut into strips of 2 mm width to form the final EZ-D sticks (Fig. 1a), which consisted of a 2 mm×44 mm PVC waterproof handle with a 2 mm×4 mm piece of Whatman No.1 filter paper on the end of it. After sticking to the PVC sheet, the filter paper was very stable during the experiments.

To avoid contamination, filter papers were sterilized prior to the preparation. Only the PVC handle side of EZ-D stick was touched during the preparation. The ready EZ-D sticks were further treated with ultraviolet (UV)-light for 30 min, and then packed.

2.3 DNA extraction using EZ-D

The protocol for rapid DNA extraction from biological samples using EZ-D is presented in Fig. 1b. Twenty milligrams of young plant tissues (roots, stems, leaves, or flowers) were added to a 2-mL centrifuge tube containing 300 μL of DNA extraction solution (100 mmol/L Tris (pH 8.0), 1 mol/L NaCl, 10 mmol/L ethylenediaminetetraacetic acid (EDTA)) along with two zirconia porcelain balls (Z2359-05, I-Quip, USA). Plant samples were disrupted by hand shaking the tube for 10 s. For ultrafine powder samples, about 10 mg of powder was transferred directly by the EZ-D stick into the DNA extraction solution. Using the stick, the solution was stirred for 10 s.

A TL2010S high throughput ball mill (DHS Life Science & Technology, China) was used for grinding a large number of samples or hard samples, such as the needle leaves of larch. In addition, we also tested drilling soybean seeds with a miniature electric drill fitted with a 1-mm diameter bit (P-500-3, Slite, USA)

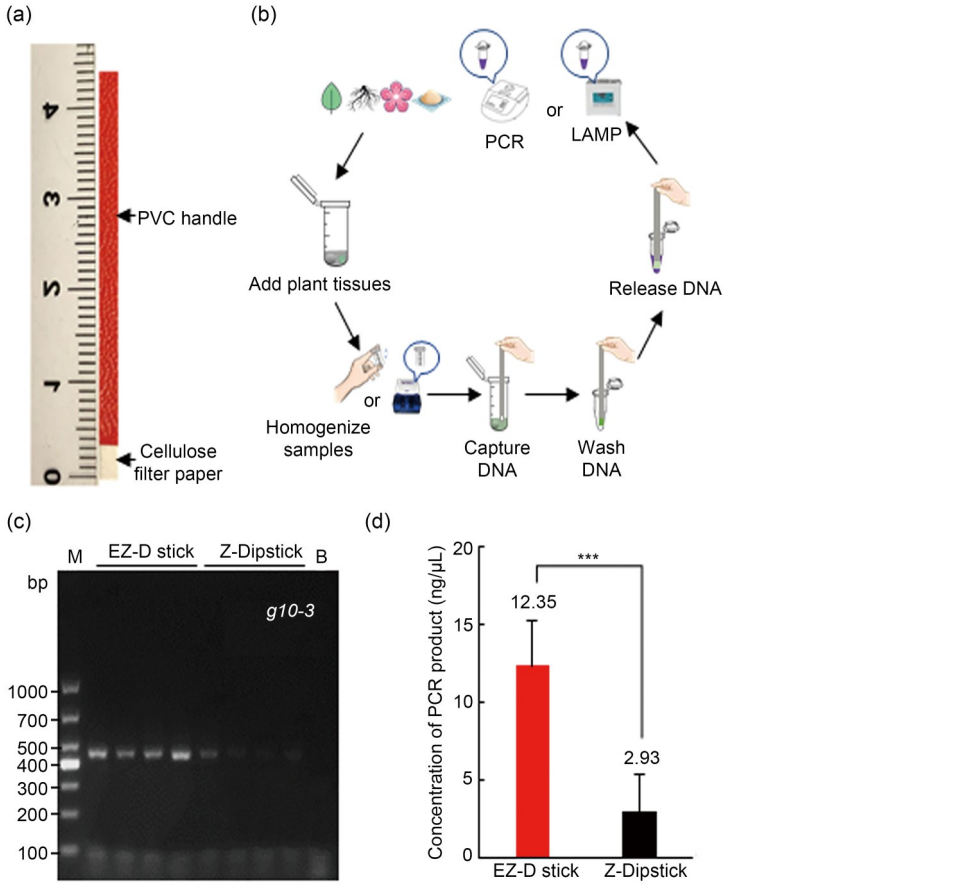

Fig. 1 EZ-D stick is more efficient for PCR amplification than the dipstick reported by Zou et al. (2017). (a) An EASY DNA (EZ-D) stick is made of a polyvinyl chloride (PVC) resin plastic handle with a Whatman No. 1 cellulose filter paper attached to the bottom; (b) A flowchart of the EZ-D method; (c, d) Comparison of PCR efficiency of the fragment 3 of transgene *g10-epsps* (*g10-3*) using DNA extracts from EZ-D and the Z-Dipstick (Zou et al., 2017). Four replicates for each extraction method, and date are expressed as mean±standard deviation. M: DL1000 marker; B: blank control; LAMP: loop-mediated isothermal amplification. * *P*<0.01.**

to obtain seed powder from the cotyledons, which allowed the seeds to retain their viability (Cheng et al., 2016).

After disruption, the EZ-D stick was dipped into the plant tissue lysate for 10 s and then transferred into 200 μL of wash buffer (10 mmol/L Tris (pH 8.0), 0.1% Tween-20) for 5 s. The stick was then dipped into the DNA amplification reaction system for 5 s, to elute the DNA. The whole protocol took about 30 s, after which the purified DNA was ready for amplification or other molecular identification.

The quality of the DNA extracted by EZ-D was evaluated by a NanoDrop 1000 Lite spectrophotometer (Thermo Scientific, USA). The DNA absorbed by the EZ-D stick was finally released by dipping the stick into 20 μL of double-distilled water (ddH_2O) for 5 s. In this way, we could evaluate the quality of DNA released into the PCR solution. The UV absorption peaks of DNA, proteins, and polysaccharides were at 260, 280, and 230 nm, respectively. The ratios of absorbance at 260 nm to 280 nm (A_{260}/A_{280}) and A_{260}/A_{230} were recorded to evaluate the purity of DNA against proteins and polysaccharides, respectively (Varma et al., 2007).

2.4 CTAB DNA extraction method

A modified CTAB method was used as the control, to represent a conventional DNA extraction method. One hundred milligrams of plant samples were finely ground using liquid nitrogen. The plant powder was mixed with 700 μL of extraction buffer (2% (20 g/L) CTAB, 0.7 mol/L NaCl, 10 mmol/L EDTA, 50 mmol/L Tris-HCl (pH 8.0), 0.5% β-mercaptoethanol, which was preheated at 65 ℃). After 1.5 h at 65 ℃, 700 μL of chloroform was added to the mixture which was rotated gently at room temperature, followed by centrifugation at 12 000 r/min for 10 min. Three hundred microliters of supernatant was transferred to a new tube and mixed gently with 210 μL of isopropanol. After the DNA precipitated, the flocculent DNA was removed and transferred to a new tube and washed twice with 1 mL 75% ethanol. After washing, the DNA was suspended in 100 μL of ddH_2O and quantified using the NanoDrop 1000 Lite spectrophotometer (Thermo Scientific).

2.5 Detecting PCR sensitivity of EZ-D

Genomic DNA samples purified by the CTAB method were diluted in series by a factor of 10, with final concentrations ranging from 100 to 0.01 ng/μL. For the detection of PCR sensitivity of EZ-D DNA, dilutions of DNA were prepared by adding 1 μL of genomic DNA with diluted concentrations onto the DNA-binding zone of the EZ-D sticks, followed by washing. The resulting sticks were immersed into a PCR reaction mixture for testing.

2.6 Primer design and PCR reaction conditions

Specific primers for each of the genes were designed using the Oligo software (Version 7.0, Molecular Biology Insights, USA). The genes, primer sequences, corresponding amplicon lengths, and GC contents of the amplicons used for the tests are listed in Table 2. Genes *bar*, *HPT*, *cry1Ab*, *cry1Ab/Ac*, *g10-epsps*, and *cp4-epsps* refer to the transgenes encoding phosphinothricin acetyltransferase, hygromycin B phosphotransferase, *Bacillus thuringiensis* cry1Ab protien, *B. thuringiensis* cry1Ab/Ac fusing protein, and 5-enolpyruvylshikimate 3-phosphate synthase, respectively (Lu et al., 2014). The primers for other genes were designed as follows: *OsbHLH156*, according to Wang SD et al. (2020); *trnL-trnF*, according to Zhao et al. (2019); *ZB1*, according to Li et al. (2014); *35S*, *cry1Ab*, and *cp4-epsps*, according to unpublished work on new transgenic plants.

PCR reactions were performed in a total volume of 20 μL in a reaction mix that contained 10 μL of 2× Taq Master Mix Kit (CWBiotech, China) or PrimeSTAR® Premix (TaKaRa Biotech, Japan), 0.4 μL of 10 μmol/L primers, 8.2 μL of ddH_2O, and 1 μL of template DNA (the volume of template DNA transferred from the EZ-D stick was defaulted at 1 μL). The PCR cycling parameters were as follows: initial denaturation at 95 ℃ for 2 min, and then 40 cycles of denaturation at 95 ℃ for 20 s, 55–60 ℃ for 20 s, 72 ℃ for 20 s, followed by final extension of 72 ℃ for 5 min or 98 ℃ for 2 min, 40 cycles of 98 ℃ for 20 s, 55–60 ℃ for 20 s, 72 ℃ for 1–3 min, followed by final extension of 72 ℃ for 5 min.

For DNA fragments rich in GC content, the reaction mix consisted of 10 μL 2× GC Buffer II, 3.2 μL 2.5 mmol/L deoxyribonucleoside triphosphates (dNTPs), 0.2 μL La Taq (TaKaRa Biotech), 0.2 μL of 10 μmol/L primers, 6.2 μL of ddH_2O, and 1 μL of template DNA (the volume of template DNA transferred from the EZ-D stick was defaulted at 1 μL). The PCR cycling parameters were initial denaturation at 94 ℃ for 5 min, followed by 40 cycles of denaturation at 94 ℃ for 30 s, 60 ℃ for 30 s, 72 ℃ for 20 s, and a final extension at 72 ℃ for 2 min.

Table 2 Primers and products used in the PCR amplification reactions

Gene	Primer name	Primer sequence (5'→3')	GC content (%)	Amplicon length (bp)
bar	bar-F	GTCAACCACTACATCGAGACAAGC	68	260
	bar-R	AGCAGGTGGGTGTAGAGCGT		
cry1Ab/Ac	cry1Ab/Ac-F	GGCCATACAACTGCTTGAGT	49	1000
	cry1Ab/Ac-R	GCGTTTCCCATAGTTCCATA		
cry1Ab	cry1Ab-F	GACAACAACCCCAACATCAACGAGTG	65	583
	cry1Ab-R	GGTCGTTGTAGCGGCTGTTGATGGT		
cp4-epsps	cp4-epsps-F	TGATCCTCACGCTTCAGGAGATGGGAGC	63	488
	cp4-epsps-R	CAGATCCATGAACTCTGGGAAGCTCGTC		
*g10-1**	g10-1-F	TCTAAGGTCATCGGAGGCAGTAGCT	54	604
	g10-1-R	TAGAGCACAGCCATCCAAGAACTAC		
*g10-2**	g10-2-F	TCTCACCTCTGGCACCACTTTCG	70	703
	g10-2-R	CCACGTTCTCCCAGGTGGTGT		
*g10-3**	g10-3-F	TTTGATGAGCCTTTGTACACAGC	39	468
	g10-3-R	AAGCAATAAAATAAAATGAAGACAAGGT		
g10-epsps	33-F	TAATTCGGGGGATCTGGATTTTAGTA		
	33-R1	CTCTTAGACTCAAGGAATGCGATAGA	44	500
	33-R2	AGTACGCTTCCGCCCTTATGTTCCTC	48	1000
	33-R3	CTCTTCCAGCTACCTTCGACGTTATC	50	1500
	33-R4	GATGTGATATCTCCACTGACGTAAGG	48	2000
	33-R5	TCAAAAGGACAGTAGAAAAGGAAGGT	48	2500
	33-R6	GGGTTTTCCCAGTCACGACGTTGTAA	48	3000
GmActin	Actin-F	TTTGATGAGCCTTTGTACACAGC		
	Actin-R1	AAGCAATAAAATAAAATGAAGACAAGGT	41	500
	Actin-R2	ACCATCCTTAACATTATTTATGT	37	1000
	Actin-R3	AGCATCGTCACCAGCAAATCCTG	37	1500
	Actin-R4	ATAGATTGGTACAGTGTGACTCA	39	2000
	Actin-R5	GGTGGAGCTACAACCTTAATCTT	40	2500
	Actin-R6	TACAAAAAGAAAATGGAGGAAAAAGAGAT	40	3000
	Actin-F7	TCAATGTGCCTGCCATGTATGTG	44	246
	Actin-R7	CCAGCGAGATCCAAACGAAG		
HPT	HPT-F	GAAGTGCTTGACATTGGGGAGT	58	472
	HPT-R	AGATGTTGGCGACCTCGTATT		
OsbHLH156	156-F	GGTCGCCGCGCTCGAGGAGGC	80	360
	156-R	GCCACGACGCGGTCCGGCGAG		
trnL-trnF	trnL-trnF-F	GGGGATAGAGGGACTTGAAC	38	540
	trnL-trnF-R	CGAAATCGGTAGACGCTACG		
ZB1	ZB1-F	GCCCTTGAAAGATTCTGGTA	43	207
	ZB1-R	AGTTGGTGGACTCACTGG		
35S	35S-F	GCTCCTACA AATGCCATCA	50	195
	35S-R	GATAGTGGG ATTGTGCGTCA		

* *g10-1*, *g10-2*, and *g10-3* are three different fragments of *g10-epsps*.

All the resulting DNA products were separated on a 1% (0.01 g/mL) agarose gel at 150 V for 30 min.

2.7 LAMP-HNB assay

Relevant LAMP primers (Table 3) were designed according to the sequences of the *g10-epsps* and *ZB1* genes using Primer Explorer V5 (http://primerexplorer.jp/lampv5e/index. html). LAMP reactions were performed using an LAMP-hydroxynaphthol blue (HNB) discoloration amplification kit (HaiGene, China), following the manufacturer's protocol. LAMP reactions were performed in a total volume of 25 μL in a reaction mix consisting of 12.5 μL of 2× LAMP-HNB reagent, 2.5 μL of 10× LAMP Primer Mix (16 μmol/L each of forward inner primer (FIP) and backward inner primer (BIP), 2 μmol/L each of forward outer primer (F3) and backward outer primer (B3), and 8 μmol/L of left loop primer (LF)), 3 μL of 5 mol/L betaine, 1 μL of *Bacillus stearothermophilus* 2.0 (Bst2.0) DNA polymerase, 5 μL of ddH_2O, and 1 μL of template DNA (the volume of

Table 3 LAMP species-specific primers

Gene	Primer type*	Gene location (nt)	Primer sequence (5'→3')	Sample for detection
g10-epsps	F3	718–735	AAGAGGAGCGTGGGACTT	Transgenic soybean ZUTS-33
	B3	926–944	GACCTCAGGGTGACCTTCT	
	FIP	742–761/TTTT/784–805	CGCTTCCGCCCTTATGTTCCTCTTTTAC CGGTGAGTCTAAGTTCGA	
	BIP	853–874/TTTT/896–915	TGGACCGGAAACGGAGATAGGGTTTT GAAAGACTTGGTGCTTGGGT	
	LF	763–863	GGTCCTCTTCTTCCTGACGGA	
ZB1	F3	446–465	GCCCTTGAAAGATTCTGGTA	Chinese medicine ZB1
	B3	635–652	AGTTGGTGGACTCACTGG	
	FIP	466–485/T/507–526	ATTGCTACACGGTGATGCCCTATCAAC CGTTGAAAAGTTGC	
	BIP	533–556/T/596–614	CTAAGGGAGTAGTGAAACTGGTGTTT TTTGGATGCCTGTTCAG	

* F3, B3, FIP, BIP, and LF were forward outer primer (F3) and backward outer primer (B3), forward inner primer (FIP), backward inner primer (BIP), and left loop primer (LF), respectively. LAMP: loop-mediated isothermal amplification.

template DNA transferred from the EZ-D stick was defaulted at 1 μL). The mixture was incubated at 63–65 ℃ for 60 min. The LAMP amplification products were examined by directly observing the color change of the reactions.

HNB in the LAMP amplification mixture is as a colorimetric indicator. A color change from violet to sky blue indicates that the tested sample contains the gene of interest (Goto et al., 2009).

3 Results

3.1 Comparison of DNA extraction with the EZ-D stick and the Z-Dipstick

Equipment-free nucleic acid extraction dipstick methodology using untreated cellulose-based paper to capture nucleic acids was reported by Zou et al. (2017). While the "Z-Dipstick"-based protocol had great advantages for its simplicity, speed, and low cost, we found that the filter paper very easily became loosened at its DNA-binding zone and the loose filter paper sucked up a large amount of PCR solution, which reduced the PCR efficiency (Fig. S1). Moreover, during the preparation of the sticks, the coated wax in the handle was easily broken and could contaminate the filter paper in the DNA-binding area. This could reduce the PCR efficiency when the binding area contacted the PCR system. To solve these problems, we replaced the wax-coated filter paper handle in the Z-Dipstick with a handle made of PVC with filter paper attached (described in Section 2; Fig. 1a).

To demonstrate the high efficiency of the EZ-D stick in rapid DNA extraction, we compared the PCR amplification efficiency of DNA extracted from the EZ-D stick and from the Z-Dipstick. The DNA of 20-mg leaves of ZUTS-33 was extracted using each of these two sticks. The concentration and quality of DNA extracted by the two types of sticks were the same. However, the results showed that significantly more PCR product was obtained by the DNA extracted from the EZ-D stick than from the Z-Dipstick (Figs. 1c and 1d). The concentration and quality of DNA extracted by EZ-D are shown in Table 4.

Table 4 DNA concentration and quality of different samples extracted by the EASY DNA (EZ-D) method

Type	DNA concentration (ng/μL)	A_{260}/A_{280}	A_{260}/A_{230}
Root	20.59±4.13	1.73±0.27	0.77±0.01
Stem	21.85±1.68	1.50±0.08	0.36±0.01
Leaf	24.10±6.05	1.41±0.16	0.43±0.08
Flower	22.13±5.16	1.47±0.15	0.58±0.06
Seed powder	9.38±1.86	1.50±0.31	0.40±0.09
Ultrafine powder	5.82±1.38	1.41±0.58	0.30±0.05

Data are expressed as mean±standard deviation (n=5).

3.2 Comparison of EZ-D and CTAB DNA extraction methods

The advantages of the EZ-D method over a conventional CTAB method are listed in Table 5.

Like the Z-Dipstick, EZ-D takes 30–60 s to prepare amplification-ready DNA, while CTAB takes 1.5–3.0 h. The cost of the EZ-D, including the cost of the stick, reagent, and other materials, is only 0.2 CNY (equivalent to 0.03 US dollar) per sample, while CTAB costs 0.9 CNY per sample. Thus, EZ-D is faster, simpler, and cheaper than the CTAB method for the purification of DNA from plant samples.

3.3 Amplifiability of the EZ-D-extracted DNA

To assess the quality of DNA extracted by EZ-D, the DNA samples were used to amplify eight DNA fragments of six different genes by PCR. DNA was extracted from leaves of transgenic plants or the corresponding non-transgenic control plants using the EZ-D method as described in Fig. 1b. Genomic DNA of the transgenic plants extracted by CTAB was diluted to 10 ng/μL as the positive controls. The amplified DNA from EZ-D DNA templates was as clear and bright as the bands obtained from the CTAB-extracted genomic DNA samples (Fig. 2a), indicating that the DNA purified by EZ-D was sufficient for PCR detection.

To determine whether the DNA captured by the EZ-D sticks could be used for multiple PCR assays, a single EZ-D stick was placed in the lysate of the transgenic soybean ZUTS-33 for 10 s, immersed in the wash buffer for 5 s, and finally dipped sequentially into each of 16 PCR tubes containing only water, for 5 s each tube. The 16 tubes were divided into two groups which were used to amplify the transgene *g10-epsps* and the housekeeping gene *GmActin*, respectively, by adding the rest of the corresponding components of the PCR reactions. The results showed that the DNA content in a single EZ-D stick could be used for at least 16 PCR reactions (Fig. S2).

To further examine the amplifiability of the EZ-D-extracted DNA and take the GC content of the amplified fragment into consideration, a DNA fragment of *OsbHLH156* (Wang SD et al., 2020) with an average GC content of 80% was tested. Results showed that the target fragment was successfully amplified (Figs. S3a and S3b). Sequencing of the PCR product showed that the target gene was amplified precisely (Fig. S3c). Thus, the EZ-D-based PCR method can be used for gene amplification of fragments with a wide GC content (Table 2). The concentration and quality of DNA extracted by EZ-D are shown in Table 4.

3.4 Sensitivity of EZ-D stick

To evaluate the sensitivity of EZ-D sticks, genomic DNA samples extracted by the CTAB method from four different transgenic plants were to be tested. One microgram of the diluted DNA, with final concentrations ranging from 100 to 0.01 ng/μL, was added into the EZ-D sticks and then applied to the PCR system. The first four concentrations of DNA could be amplified, while the 0.01 ng/μL DNA samples from either protocol could not be amplified. Therefore, the PCR detection limit for the DNA samples prepared by the EZ-D method is considered the same as that of samples prepared by the CTAB method, i.e., 0.1 ng/μL(Fig. 2b).

3.5 Integrity of DNA from the EZ-D

To evaluate the integrity of DNA extracted by EZ-D, the DNA extracted from the transgenic soybean plant ZUTS-33 using EZ-D was amplified by PCR using primer pairs targeting the soybean *GmActin* and *g10-epsps* genes (Table 2). Six DNA fragments of the *GmActin* gene and *g10-epsps* gene, ranging from 500 to 3000 bp, were successfully amplified using the DNA extracted by EZ-D (Fig. 2c). This indicated that EZ-D can extract DNA with good integrity and that the length of amplification products can be as long as 3000 bp (Fig. 2c).

3.6 Application of EZ-D to a variety of plant samples

To determine if EZ-D is suitable for DNA extraction from various herbaceous plant tissues, samples of roots, stems, leaves, flowers at flowering stage, and dry seeds were collected from transgenic soybean line ZUTS-33 and subjected to the EZ-D and CTAB DNA extraction protocols. The fragments of transgene *g10-epsps* and the housekeeping gene *GmActin* were amplified. Results showed that both fragments were successfully amplified

Table 5 Comparison of EZ-D and CTAB DNA extraction methods

Method	Total time cost	Personal skill	Organic reagent	Electrical device	Cost per sample (CNY)
EZ-D	30–60 s	Low	No	No	0.2
CTAB	1.5–3.0 h	High	Yes	Yes	0.9

EZ-D: EASY DNA; CTAB: cetyl trimethylammonium bromide.

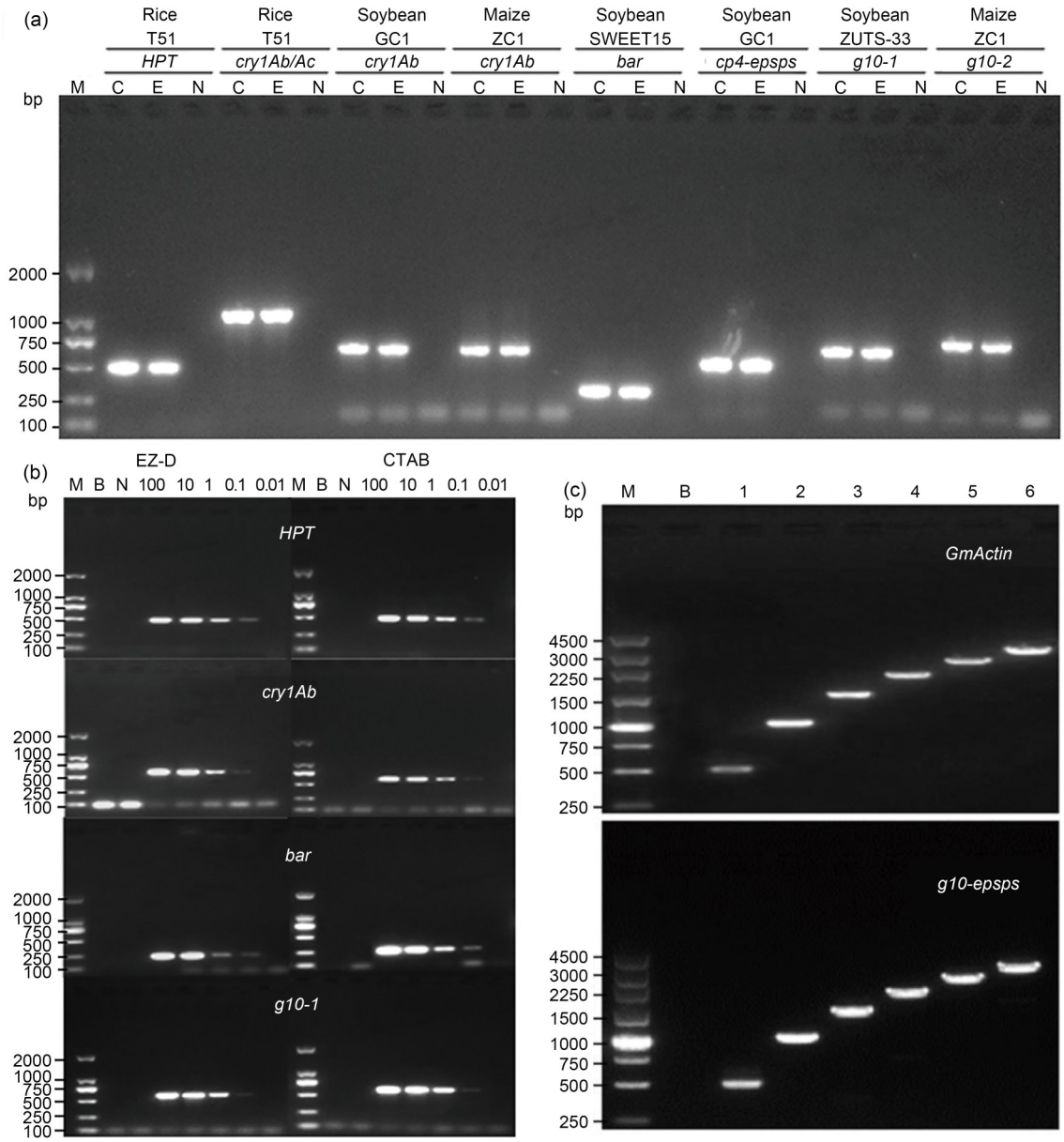

Fig. 2 Molecular identification of DNA components using EZ-D DNA. PCR efficiency (a) and sensitivity (b) were tested using DNA extracted by the CTAB and the EZ-D methods. Plant samples were from several independent transgenic plants including rice line T51, maize line ZC1, soybean lines GC1, SWEET15, and ZUTS-33 with their corresponding non-transgenic controls of MH63 rice, HiII maize, and soybean lines Wandou 28, Williams 82, and HC-3; *g10-1* and *g10-2* are two fragments of the *g10-epsps* gene. Details of transgenic genes are listed in the Table 1. The labels of 100, 10, 1, 0.1, and 0.01 at the top of the electropherogram of (b) are the DNA concentrations (ng/μL). (c) DNA extracted by EZ-D was amplified for DNA fragments ranging from 500 to 3000 bp in length. 1–6, PCR products for amplification of DNA fragments of 500, 1000, 1500, 2000, 2500, and 3000 bp, respectively. M: DL2000 marker; B: blank control; C and E: DNA extracted by CTAB and EZ-D, respectively; N: non-transgenic control. CTAB: cetyl trimethylammonium bromide; EZ-D: EASY DNA.

from the EZ-D DNA from all five soybean tissues with amplification efficiency similar to those from using CTAB DNA (Fig. 3a). To test if the EZ-D method can be used for DNA extraction from woody plants, needles of 11 putative transgenic larch accessions, possibly containing the transgene driven by the *35S* promoter, were sampled. A chloroplast intergenic region, *trnL-trnF*, was chosen as an internal control (Zhao et al., 2019). The PCR results using DNA extracted by EZ-D showed that none of the tested plants contained the

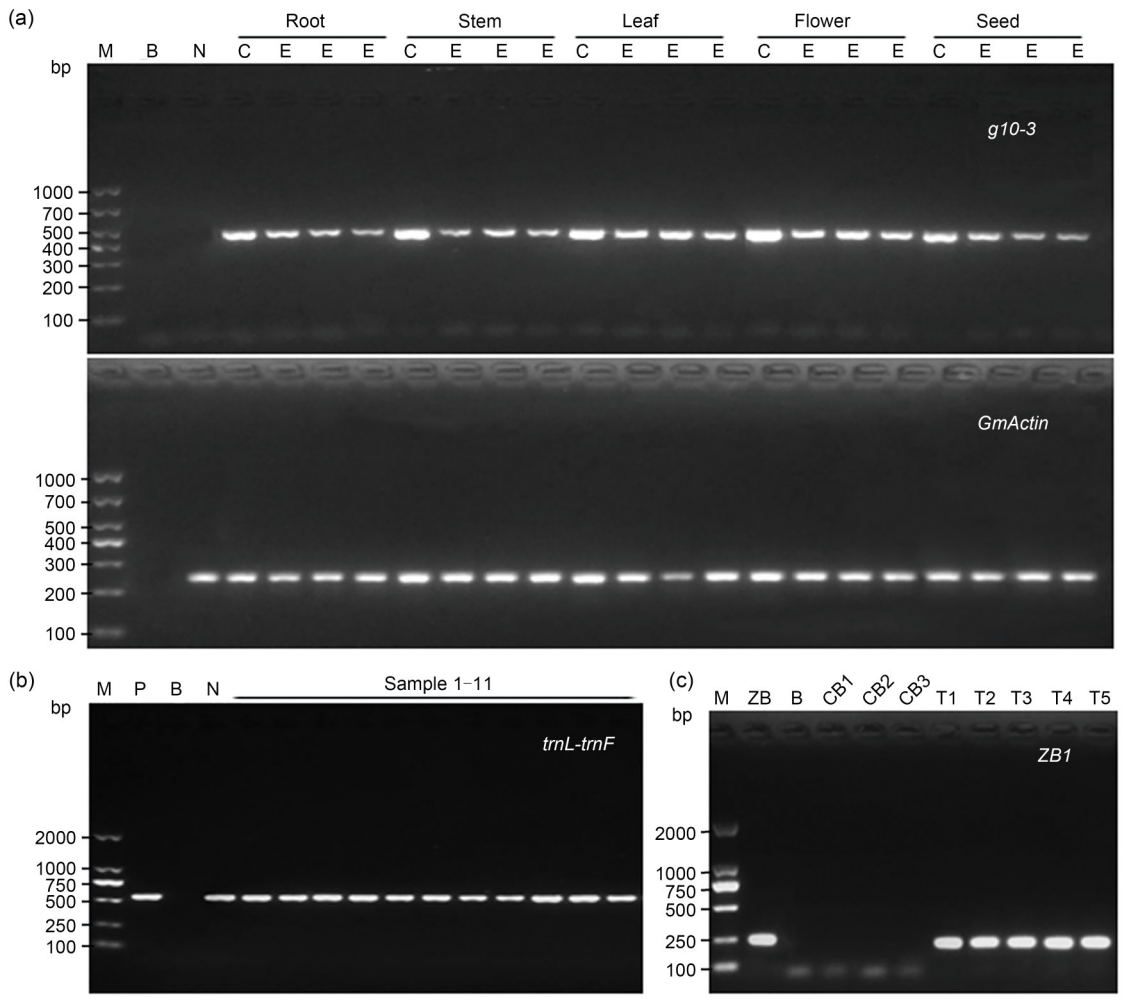

Fig. 3 Application of EASY DNA (EZ-D) to a variety of plant samples. (a) Electropherogram of PCR using DNA extracted from different soybean tissues by EZ-D or cetyl trimethylammonium bromide (CTAB) methods. *g10-epsps* and *GmActin* are two genes used for the assay; *g10-3* is fragment 3 of *g10-epsps* (Table 2). M, DL1000 marker; N, non-transgenic control; B, blank control; C, DNA extracted by CTAB; E, DNA extracted by EZ-D with three replicates. (b) Electropherogram of PCR using DNA extracted from larch needles by EZ-D. P, positive control; Samples 1−11, 11 larch leaf samples. (c) Application of EZ-D for molecular identification of ultrafine powdered Chinese medicine. ZB, Zhe Beimu, *Fritillaria thunbergii*; CB1, Chuan Beimu 1, *Fritillaria unibracteata*; CB2, Chuan Beimu 2, *Fritillaria przewalskii*; CB3, Chuan Beimu 3, *Fritillaria delavayi*; T1−T5, ultrafine powders of CB containing 5%, 10%, 15%, 20%, and 25% of ZB, respectively; ZB1, a molecular marker of ZB.

transgene region (data not shown), while the *trnL-trnF* internal control fragment was successfully amplified (Fig. 3b). Thus, EZ-D can be used for DNA detection in woody plants and for detecting DNA from organelles.

Many Chinese medicines produced from plant materials are prepared as an ultrafine powder (Fan et al., 2016). Rapid and accurate molecular identification of these ultrafine powders is important for quality control (Li et al., 2017). The EZ-D protocol was assessed for its ability to extract DNA from ultrafine powder of the Chinese medicine Beimu. Beimu is derived from various *Fritillaria* spp. The market value of CB is about 300–500 times higher than that of ZB. ZB is often used as a counterfeit CB (Liu et al., 2019). Species within the genus *Fritillaria* used for various Beimu preparations include ZB, CB1, CB2, and CB3. The ultrafine powder samples of four medicinal preparations (ZB, CB1, CB2, and CB3) were subjected to the EZ-D method for DNA extraction. For DNA molecular identification, PCR primers were designed to amplify a DNA fragment that was present only in ZB (Table 2). The DNA fragment, named *ZB1*, is a molecular marker of

ZB (Li and Zhao, 2012; Li et al., 2014). Results showed that the amplicon was only obtained from the DNA extracted from ZB, but not from the other three species (Fig. 3c), indicating that the EZ-D protocol could be used for molecular identification of ultrafine powder samples. To further determine the sensitivity of the PCR reaction using the DNA extracted by the EZ-D protocol, CB powder mixtures with different percentages of ZB ultrafine powder were prepared. ZB could be detected when it was as low as 5% of the mixture (Fig. 3c). The concentration and quality of DNA extracted by EZ-D are shown in Table 4.

3.7 Combination of EZ-D with LAMP for detecting DNA in the field

LAMP is one of the most powerful isothermal amplification techniques. It uses four to six primers and *Bst* DNA polymerase. The LAMP method does not require thermocycling since the amplification is performed at a constant temperature between 60 and 65 °C (Notomi et al., 2000; Tomita et al., 2008; Zhang et al., 2014). It has been increasingly used outside of the laboratory for the molecular determination of transgenic crops (Kiddle et al., 2012; Li et al., 2016; Lan et al., 2019). To test whether the combination of the EZ-D DNA extraction method and the LAMP-HNB assay could rapidly obtain reliable results for genetic analysis, tissues from the transgenic soybean line ZUTS-33 and its non-transgenic control were tested. Results showed that the negative sample appeared violet, while the samples containing the transgene appeared sky blue (Fig. 4a). Thus, the combination of EZ-D with the LAMP-HNB protocol can be used for the molecular identification in the field where instrumentation is not available.

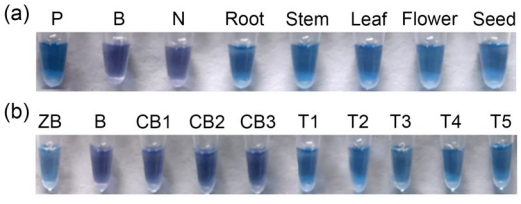

Fig. 4 Combination of the EZ-D and LAMP methods for DNA detection of transgenic soybean (a) and ultrafine powder samples of Chinese medicine (b) sampled in field conditions. P: positive control; B: blank control; N: non-transgenic control; Root, stem, leaf, flower, and seed samples were taken from transgenic soybean line ZUTS-33; ZB: Zhe Beimu, *Fritillaria thunbergii*; CB1: Chuan Beimu 1, *Fritillaria. unibracteata*; CB2: Chuan Beimu 2, *Fritillaria przewalskii*; CB3: Chuan Beimu 3, *Fritillaria delavayi*; T1−T5, ultrafine powders of CB containing 5%, 10%, 15%, 20%, and 25% of ZB, respectively.

We also tested whether the LAMP-HNB technique can be used for identification of Chinese medicines as described in last section. While no visible color change was observed in the negative control or pure CB samples, CB powders mixed with as little as 5% ZB turned sky blue in the test tube and could be identified (Fig. 4b). Therefore, combining the EZ-D method and the LAMP technique offers a novel and swift means for the authentication of Chinese medicine and can be used for quality control in the market.

4 Discussion

In this study, we optimized the cellulose filter paper DNA extraction method based on the protocol of Zou et al. (2017). The main modification included in our EZ-D protocol is the use of a PVC waterproof handle to replace the wax-coated handle in the protocol of Zou et al. (2017). Using the original Z-Dipstick, the DNA-binding area is easily loosened and the paraplast used to seal the filter paper in the handle can drop and contaminate the DNA sample. Our modified EZ-D stick overcomes these shortcomings. It is easier to handle and yield DNA with better amplifiability (Fig. 1). In addition, we found that the extraction buffer #1 used in Zou et al. (2017)'s work was not efficient enough for DNA extraction from soybean. When we increased the NaCl concentration of the extraction buffer from 150 mmol/L, bright amplified bands gradually became visible (data not shown). Thus, we developed a new DNA extraction buffer (100 mmol/L Tris (pH 8.0), 1 mol/L NaCl, 10 mmol/L EDTA), with which DNA of soybean was stably extracted (Fig. 3a).

Although the concentration of DNA extracted with EZ-D was relatively low, it could still be sensitively detected by PCR for at least 16 times (Fig. S2). The standard values of A_{260}/A_{280} and A_{260}/A_{230} of pure DNA are expected to 1.8−2.0 and >1.8, respectively (Varma et al., 2007). However, the ratios of A_{260}/A_{280} and A_{260}/A_{230} of DNA extracted by EZ-D indicated that the DNA extracts may contain proteins and polysaccharides (Table 4). Paul et al. (2019) compared the quality of DNA extracted from tomato leaves by sodium dodecyl sulfate (SDS) extraction and NaOH rapid extraction methods, finding that the ranges of A_{260}/A_{280} and

A_{260}/A_{230} of isolated DNA were 1.4–1.6 and 0.4–0.8, respectively. The DNA extracted by EZ-D was similar to the results of the above two conventional methods in both ratios. Thus, EZ-D can isolate DNA with a quality comparable to that of conventional methods.

In recent years, there have been a few reports on the method of rapid DNA purification using cellulose filter paper (Zou et al., 2017; Yang et al., 2019; Wang SS et al., 2020). However, these reports focused only on the use of rapidly extracted DNA to amplify multiple copies of genes up to 500 bp in length. It is unclear whether single-copy genes or long-segment DNA sequences can be successfully amplified with the DNA. Moreover, whether the extracted DNA can be used to amplify fragments with different GC contents is another key point that has not been explored. Solving these two problems will benefit the application of this DNA extraction method. Thus, the quality of DNA extracted by EZ-D for PCR amplification was evaluated. Results showed that the EZ-D-based genomic DNA could be amplified for the sequence of a transgenic gene with a single copy and also for DNA fragments up to at least 3000 bp with high sensitivity (Fig. 2). DNA extracted by EZ-D could be used for PCR of amplicons with high GC content (Fig. S3). The protocol can be employed for different tissues of herbaceous plant species, leaves of woody plants, and ultrafine powders of Chinese medicines (Fig. 3). Among the samples tested, soybean seed contains many polysaccharides, polyphenols, proteins, and esters, which hinder DNA purification (Ren et al., 2012). Thus, applying a fast protocol to extract DNA from soybean seed is considered to be difficult. The SDS extraction method is often used to extract DNA from soybean seeds, and the process is complicated (Wu et al., 2015; Yu et al., 2016). In our study, using the optimized EZ-D method, we realized a rapid DNA extraction for soybean seeds (Fig. 3a). In woody plant larch leaves, secondary metabolites present in high amounts, such as polysaccharides, polyphenols, and turpentine, often bind with their genomic DNA. These compounds are not readily removed, which inhibits the PCR amplification reaction (Wang et al., 2012). The EZ-D method uses filter paper to absorb DNA. After washing, the inhibitors of the PCR reaction in samples from woody plants are greatly reduced so that the resulting DNA can be amplified efficiently (Fig. 3b). Thus, EZ-D is suitable for DNA extraction to identify of wide range of plant species and tissue samples.

The safety assessment of genetically modified (GM) plants in the field and products in the food market is one of the most important applications of molecular identification (Liu et al., 2020). Our data demonstrated that EZ-D could simplify molecular diagnosis of the existence of transgenes. By combining EZ-D with the LAMP method, GM products can be identified in the field or market where specialized instruments are not available (Fig. 4a). Similarly, the combination of EZ-D and LAMP can be used for the quality control of high-value market products, such as ultrafine powders of Chinese medicines (Fig. 4b).

5 Conclusions

In this study, we have developed a modified filter paper stick, the EZ-D stick, for rapid DNA extraction from transgenic plants and Chinese medicine ultrafine powder, based on a published method. The EZ-D stick overcomes the three main disadvantages of the published Z-Dipstick, including the complicated production process, ease of contamination, and instability resulting from the loss of PCR compounds from deformed cellulose filter paper. The EZ-D method can extract DNA from a variety of plants and their tissues. The sensitivity of PCR based on the EZ-D extraction method can reach 0.1 ng/μL. Fragments of up to 3000 bp and with a GC content of up to 80% can be amplified. In terms of practicability, EZ-D has realized the application and popularization of cellulose filter paper for rapid DNA purification in laboratories and markets.

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Author contributions

Qi WANG and Huixia SHOU designed the study and wrote the manuscript. Qi WANG, Xiaoxia SHEN, Tian QIU, Wei WU, Lin LI, and Zhi'an WANG performed the experiments and interpreted the results. All authors have read and approved the final manuscript and, therefore, have full access

to all the data in the study and take responsibility for the integrity and security of the data.

Compliance with ethics guidelines

Qi WANG, Xiaoxia SHEN, Tian QIU, Wei WU, Lin LI, Zhi'an WANG, and Huixia SHOU declare that they have no conflict of interest.

This article does not contain any studies with human or animal subjects performed by any of the authors.

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Supplementary information

Figs. S1–S3