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A high-throughput, high-quality plant genomic DNA extraction protocol

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ABSTRACT. The isolation of high-quality genomic DNA (gDNA) is a crucial technique in plant molecular biology. The quality of gDNA determines the reliability of real-time polymerase chain reaction (PCR) analysis. In this paper, we reported a high-quality gDNA extraction protocol optimized for real-time PCR in a variety of plant species. Performed in a 96-well block, our protocol provides high throughput. Without the need for phenol-chloroform and liquid nitrogen or dry ice, our protocol is safer and more cost-efficient than traditional DNA extraction methods. The method takes 10 mg leaf tissue to yield 5-10 µg high-quality gDNA. Spectral measurement and electrophoresis were used to demonstrate gDNA purity. The extracted DNA was qualified in a restriction enzyme digestion assay and conventional PCR. The real-time PCR amplification was sufficiently sensitive to detect gDNA at very low concentrations (3 pg/µL). The standard curve of gDNA dilutions from our phenol-chloroform-free protocol showed better linearity (R^2 = 0.9967) than the phenol-chloroform protocol (R^2 =

0.9876). The results indicate that the gDNA was of high quality and fit for real-time PCR. This safe, high-throughput plant gDNA extraction protocol could be used to isolate high-quality gDNA for real-time PCR and other downstream molecular applications.

Key words: Genomic DNA extraction; Liquid nitrogen-free protocol; High-throughput protocol; Phenol-chloroform-free protocol; High-quality protocol; Real-time PCR

INTRODUCTION

Isolation of high-quality genomic DNA (gDNA) is a crucial step in modern molecular biological techniques (e.g., Southern blotting, conventional PCR, and real-time PCR). Numerous methods have been developed for gDNA extraction in various plant species (Kang et al., 1998; Chen and Ronald, 1999; Paris and Carter, 2000). Many plants are very rich in secondary metabolites (such as polysaccharide and polyphenol). Most isolation methods are complex and limited to low throughputs due to the required removal of polysaccharide and polyphenol. Thus, these methods produce low gDNA yields (Marsal et al., 2011). Some rapid gDNA isolation methods have been developed, but the DNA quality they provide is suitable only for conventional PCR (Dilworth and Frey, 2000; Kamiya and Kiguchi, 2003).

Most conventional gDNA extraction methods are based on a 1.5- or 2.0-mL micro-centrifuge tube format (Moslem et al., 2010; Mohd-Hairul et al., 2011; Alatar et al., 2012; Mornkham et al., 2012; Ojeda et al., 2012; Souza et al., 2012). Although it is effective in low-throughput applications, these methods are inconvenient for a large number of samples. The 96-well format protocol is a better choice for isolating gDNA from a large number of plant samples (Clark and Krysan, 2007). The 96-well format is also more convenient for downstream applications as the gDNA is stored on the 96-well plate in which it is eluted.

Liquid nitrogen or dry ice is required for grinding in most traditional plant gDNA extraction methods (Dellaporta et al., 1983; Mohd-Hairul et al., 2011; Alatar et al., 2012; Mornkham et al., 2012; Souza et al., 2012). However, liquid nitrogen or dry ice cannot be obtained quickly in remote locations, especially in many developing countries. Safety is also a concern. Thus, a gDNA extraction method that does not require liquid or dry ice would be helpful and economical (Pervaiz et al., 2011). Phenol-chloroform isolation is another common step in numerous gDNA isolation protocols (Pervaiz et al., 2011). Phenol-chloroform is a commonly used organic reagent for removing protein from DNA samples. However, phenol-chloroform is harmful to human health and the environment. Special manipulation and personal protective equipment must be used in the disposal of phenol-chloroform. Moreover, phenol in gDNA inhibits enzymatic reactions in downstream applications; a protocol that does not require phenol-chloroform would be safer.

Real-time PCR has become a major analytical platform for nucleic acid quantification due to its better quantitative performance and greater sensitivity and accuracy in comparison to conventional PCR (Maciel et al., 2011). High-quality gDNA is important for real-time PCR because impurities in the gDNA affect PCR amplification and influence the reliability of real-time PCR (Bustin et al., 2009). Dauphin et al. (2010) reported that poor-quality gDNA reduces the positivity rate and limit-of-detection in real-time PCR analysis. Although real-time PCR pro-

vides increased speed and throughput, the isolation of high-quality gDNA has lagged. A cost-efficient, high-throughput extraction method to produce high-quality DNA is urgently needed.

Here we describe a high-throughput protocol for obtaining high-quality gDNA for real-time PCR. Without using phenol-chloroform and liquid nitrogen or dry ice, our protocol is safer and more cost-efficient. One person can accomplish 1600 (16 96-well blocks) gDNA extractions in a single day.

MATERIAL AND METHODS

Plant materials

Fresh leaves of rice (*Oryza sativa* L.), maize (*Zea mays* L.), wheat (*Triticum aestivum* L.), and rape (*Brassica napus* L.) were collected and used to test the following gDNA extraction method.

Consumables

- 1.3 mL 96-well deep-well block (Nunc, Roskilde, Denmark)
- Sealing mat for 96-well deep-well block (Nunc)
- 3-mm tungsten-carbide grinding bead (Qiagen, CA, USA)
- 96-well PCR plate and plate seals (Axygene, CA, USA)
- Multi-channel pipette reagent reservoir (Sangon, Shanghai, China)

Reagents

- Genomic DNA extraction buffer: 100 mM Tris, pH 8.0, 50 mM EDTA
- pH 8.0, 1 M NaCl, 1.25% SDS (w/v). Add 1% 2-mercaptoethanol before use
- 5 M potassium acetate, pH 5.2
- Isopropanol
- 70% ethanol
- 0.1X TE buffer: 1 mM Tris-HCl, pH 8.0; 0.1 mM EDTA, pH 8.0
- Restriction endonuclease *Hin*dIII (Thermo Scientific, DE, USA)
- DNA size markers (Takara, Dalian, China)

Equipment

- -80° and -20°C freezer, 4°C refrigerator (Zhongke-Meiling, Hefei, China)
- Fume cupboard for handling isopropanol and 2-mercaptoethanol (ZhiCheng, Shanghai, China)
- Clean bench for gDNA drying (ZhiCheng)
- Corning 3081 storage mat applicator (Corning, MA, USA)
- Spex Geno 2000 (Spex CertiPrep Group, NJ, USA)
- Vortex Genie 2 mixer fitted with a 96-well plate adapter (Scientific Industries, NY, USA)
- Eppendorf 5810R centrifuge fitted with an A-4-62-MTP plate rotor (Eppendorf,

Hamburg, Germany)
- Gel electrophoresis system (Bio-Rad, CA, USA)
- NanoDrop 2000 Spectrophotometer (Thermo Scientific)
- Suitable multi-channel pipettor (Eppendorf)

DNA extraction protocol

A summary of the gDNA extraction protocol is presented in Figure 1.

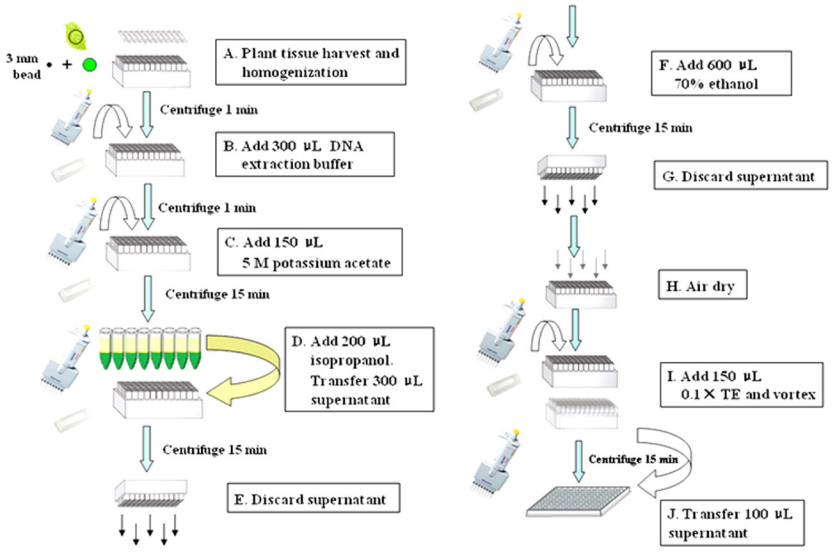

Figure 1. Summary of the gDNA extraction protocol.

Sample preparation

1. Prepare 1.3 mL 96-well deep-well blocks with 3 mm beads (1 bead per sample).
2. Using a hand-held punch (Φ 6 mm), harvest 10 mg (about 1-2 pieces) of fresh leaf tissue into each well. Firmly mat the blocks with the mat applicator. Store the blocks at -80°C at least 2 h before homogenizing.

Homogenization of plant tissue (*Timing: 5 min/8 blocks*)

3. Using a Geno 2000, homogenize samples at 1200 strokes/min for 30 s. NOTE: avoid excessive homogenization to prevent thawing of tissue powder.
4. Centrifuge at 4000 rpm (3220 *g*) for 1 min at room temperature.

DNA extraction (*Timing: 90 min/8 blocks*)

5. Carefully remove the mat from the block. Add 300 µL DNA extraction buffer to each well. Firmly mat the blocks with the mat applicator and grind again at 1200 strokes/min for 30 s.

6. Centrifuge at 4000 rpm (3220 *g*) for 1 min at room temperature. Carefully remove the mat from block. Add 150 µL 5 M potassium acetate to each well, firmly mat the blocks with the mat applicator. Mix the contents vigorously for 2 min on the plate mixer.

7. Centrifuge at 4000 rpm (3220 *g*) for 15 min at room temperature.

8. (During step 7) prepare fresh mats and fresh blocks, each well containing 200 µL isopropanol.

9. Carefully remove the mat from the block. Carefully transfer 300 µL supernatant from each well to the freshly prepared blocks. NOTE: transfer the supernatant carefully, avoiding disturbance of the bottom layer.

10. Firmly mat the blocks with the mat applicator. Mix the contents vigorously for 2 min on the plate mixer.

11. Centrifuge at 4000 rpm (3220 *g*) for 15 min at room temperature. Carefully remove the mat from block and discard the supernatant. NOTE: discard the supernatant carefully, to avoid losing the DNA pellets.

DNA washing (*Timing: 60 min/8 blocks*)

12. Wash the DNA pellets with 600 µL 70% ethanol to remove SDS, EDTA, and other contaminants. Firmly mat the blocks with the mat applicator. Mix the contents vigorously by inverting the blocks 5 times.

13. Centrifuge at 4000 rpm (3220 *g*) for 15 min at room temperature. Carefully remove the mat from block and discard the ethanol. NOTE: discard the ethanol carefully, not to lose the DNA pellets.

14. Remove any remaining traces of ethanol by inverting the blocks onto absorbent paper. Put the block on a clean bench and air dry the DNA pellets for 30 min (wind speed 0.5 m/s).

DNA dissolving and storing (*Timing: 60 min/8 blocks*)

15. Dissolve the DNA pellet with 150 µL 0.1X TE. Firmly mat the blocks with the mat applicator. Mix the contents vigorously for 20 to 30 min on the plate mixer at room temperature. NOTE: to dissolve the pellet more quickly, pre-warm 0.1X TE at 65°C.

16. Centrifuge at 4000 rpm (3220 *g*) for 15 min at room temperature. Carefully remove the mat from block. Carefully transfer 100 µL supernatant from each well to the new 96-well PCR plate. NOTE: transfer the supernatant carefully, avoiding disturbance of the bottom layer.

17. Seal PCR plates with sealing film and store at 4°C for immediate use or -20°C for long-term storage.

This is a phenol-chloroform-free protocol. In order to evaluate the influence of phenol-chloroform on real-time PCR and restriction endonuclease digestion, some gDNA samples were isolated by the phenol-chloroform protocol. In the phenol-chloroform protocol, after step 7, transfer 350 µL supernatant from each well to the freshly prepared blocks, then add 200 µL phenol-chloroform-isoamylalcohol (25:24:1). Mix the contents vigorously for 2 min on the plate mixer. Centrifuge at 4000 rpm (3220 *g*) for 15 min at room temperature. The next procedure (from step 8) is the same as the phenol-chloroform-free protocol.

DNA purity and concentration detection

The absorbance of gDNA at 260 and 280 nm was measured in a NanoDrop 2000 spectrophotometer according to the manufacturer protocol. The concentration of DNA was calculated from the absorbance at 260 nm. The ratio of nucleic acids to proteins in the gDNA sample was evaluated by the ratio of absorbance at 260 and 280 nm (A_{260}/A_{280} ratio) (Sambrook and Russell, 2001).

The presence and quality of gDNA were also evaluated by electrophoresis on 1% agarose gel at 100 V for 120 min in 1X TAE (Tris-base, glacial acetic acid, EDTA) gel buffer. The gel was stained with 0.25 μg/mL ethidium bromide for DNA visualization.

Restriction digestion analysis

To determine the suitability of gDNA for restriction digestion, 1 μg gDNA was digested with *Hin*dIII. DNA was incubated for 8 h at 37°C with 5 U enzyme. The digested DNA was separated on 1% agarose gel stained with ethidium bromide.

PCR analysis

Rice gDNA from different lines were extracted using our gDNA extraction protocol. The genome carried an inserted *phosphor mannose isomerase* (*pmi*) gene by *Agrobacterium*-mediated transformation (Duan et al., 2012). The PCR primers were 5'-CCGCCGGAGATAT CGTTTCACTG-3' (forward primer) and 5'-CACGGTTCACCCTGCTGGCTATC-3' (reverse primer), generating a target fragment of 490 bp in length. PCR amplification was carried as described by Duan et al. (2012).

Real-time PCR analysis

Eight rice gDNA samples from two protocols (phenol-chloroform-free protocol and phenol-chloroform protocol) were randomly selected to mix as 2-test gDNA samples. The 2-test gDNA samples were diluted by 0.1X TE. Their concentrations were 30 ng/μL (measured by a NanoDrop 2000 spectrophotometer). A standard curve based on threshold cycles (C_T) for a 10-fold dilution series of test gDNA (30, 3, 3 x 10^{-1}, 3 x 10^{-2}, 3 x 10^{-3} ng/μL) was constructed in triplicate real-time PCRs.

The internal *sucrose phosphate synthase* (*sps*) rice gene was selected as the target (Ding et al., 2004). The following primers and probe were synthesized: forward primer: 5'-TCTCCTCGTCCAGTGCTTCTC-3'; reverse primer: 5'-TTGGTGGACGCGCTTCTAG-3'; probe: 5'-TCCTCGCAACCGAAC-3' (Life Technologies, CA, USA). The probe labeled the 5'-terminus with fluorescein (FAM) and near the 3'-terminus with a dark quencher dye (TAMRA). The primers and probe were designed in Primer Express 3.0 (Life Technologies). The primer set amplified a product of 61 bp in length.

Real-time PCR was performed on an Applied Biosystems 7500 Real-Time PCR System (Life Technologies). Amplification was carried out in a total volume of 25 μL containing 500 nM of each primer, 200 nM probe, 12.5 μL ABGene Absolute QPCR Rox Mix (Thermo Scientific), and 5 μL DNA templates (different dilution samples of test gDNA), brought up to

volume with nuclease-free water. Amplification was carried out using the following program: 5 min at 95°C for pre-denaturation, then 50 cycles of 10 s at 95°C and 30 s at 60°C.

The C_T values were automatically calculated with the 7500 software version 2.0.5 (Life Technologies). A standard curve was obtained by plotting the C_T value versus the logarithm of the concentration of each dilution of the test gDNA.

In order to determine the influence of phenol-chloroform on restriction endonuclease activity, 1 µg gDNA from 2 protocols were digested with *Bst*UI. The product of 61 bp in length contains a *Bst*UI restriction site. DNA was incubated for 8 h at 37°C with 5 U enzyme. Then, real-time PCR was performed as described.

RESULTS AND DISCUSSION

Measuring gDNA concentration, purity, and yield by spectrophotometry

The A_{260}/A_{280} and A_{260}/A_{230} ratios were used to estimate DNA purity (Figure 2). The A_{260}/A_{280} ratio indicates protein contamination, and the A_{260}/A_{230} ratio indicates polysaccharide and polyphenol contamination. Typical DNA samples have an A_{260}/A_{280} >1.8 and A_{260}/A_{230} >2.0, meaning that most absorption is by the nucleic acids and the purity of the extracted DNA is acceptable. The DNA is suitable for immediate use in downstream applications without further purification (Sambrook and Russell, 2001).

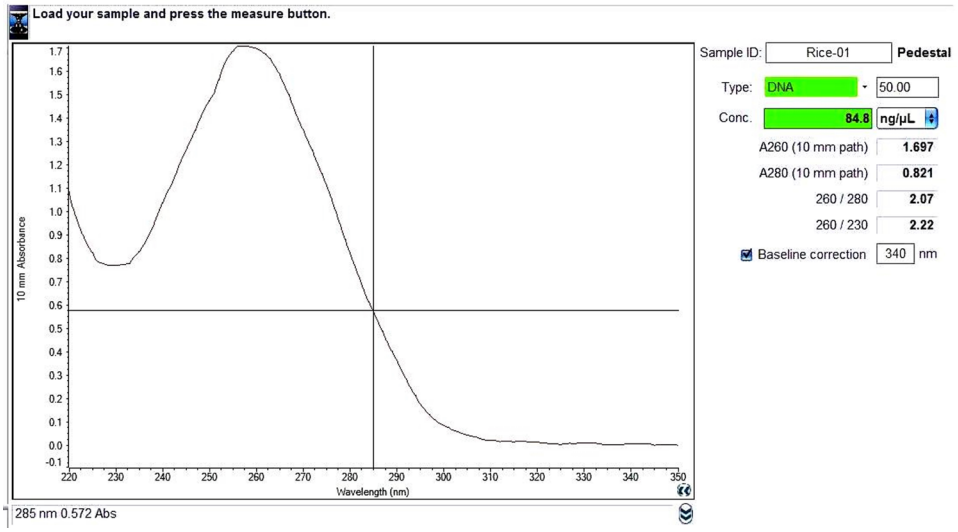

Figure 2. Nanodrop spectrophotometry measurements of gDNA.

The A_{260}/A_{280} ratio of 4 crop samples of gDNA ranged from 1.89 to 2.09, indicating that the isolated gDNA is free from protein contamination. The A_{260}/A_{230} ratio ranged from 2.05 to 2.24, showing that the gDNA has little polysaccharide and polyphenol contamination. The gDNA yield (as measured by A_{260}) ranged from 5 to 10 µg per 10 mg starting tissue (Table 1). Thus, our protocol provides high-yield, high-quality gDNA from very little starting material.

Table 1. Concentration, purity, and yield of gDNA.

Plant	gDNA concentration (ng/µL)	A_{260}/A_{280}	A_{260}/A_{230}	gDNA yield (ng/mg tissue)
Rice	90.08 ± 15.1	2.09 ± 0.06	2.24 ± 0.07	900.8 ± 151
Wheat	100.2 ± 22.7	1.94 ± 0.04	2.14 ± 0.08	1002 ± 227
Maize	70.8 ± 12.2	1.99 ± 0.04	2.19 ± 0.05	708 ± 122
Rape	50.2 ± 8.8	1.89 ± 0.09	2.05 ± 0.05	502 ± 88

Data are reported as means ± standard deviation for N = 8 in each grup.

Analysis of gDNA quality by electrophoresis

Genomic DNA from different crop samples was electrophoresed on 1% agarose gel to analyze the quality of the DNA preparation (Sambrook and Russell, 2001). High molecular weight DNA was obtained and the wells of the gel were very clear, indicating the absence of polysaccharide and protein contamination (Figure 3). These results indicate that our method provides high-quality gDNA without degradation.

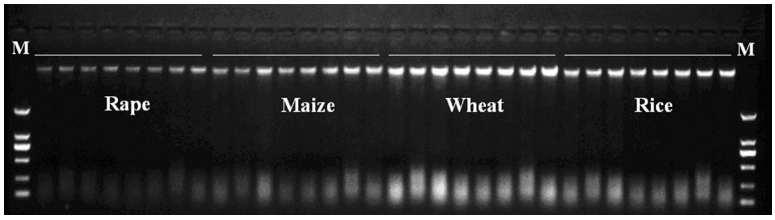

Figure 3. Electrophoresis analysis of gDNA. *Lane M* = 2-kb marker.

The gDNA of 4 crops was isolated and checked by a restriction digestion using *Hin*dIII and displayed on 1% agarose gel. Extensive smears were obtained, showing that the gDNA was completely digested (Figure 4). Therefore, the purity and quality of the gDNA is sufficient for use in downstream restriction enzymatic reactions, such as restriction fragment length polymorphism (RFLP) analysis.

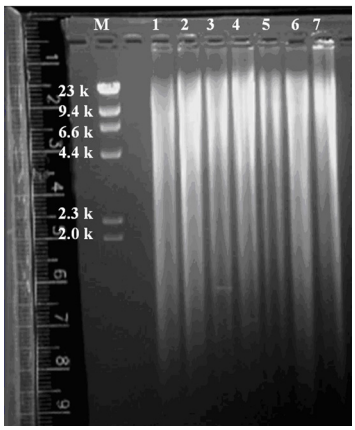

Figure 4. Electrophoresis analysis of gDNA restriction digestion. *Lanes 1* and *2* = rice gDNA; *lanes 3* and *4* = wheat gDNA; *lanes 5* and *6* = maize gDNA; *lane 7* = rape gDNA; *lane M* = λ DNA/*Hin*dIII marker.

Analysis of gDNA quality by PCR and real-time PCR

PCR amplification showed the target *pmi* gene fragment with a clear and sharp band (Figure 5). Successful PCR amplification indicated that the gDNA was of high quality and amplifiable.

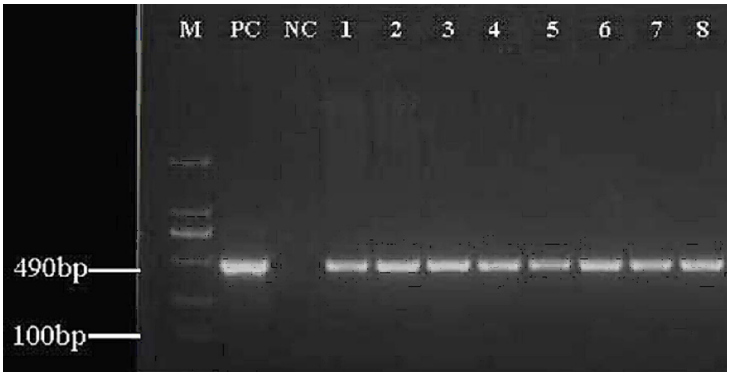

Figure 5. Electrophoresis analysis of PCR amplification of gDNA. *Lane M* = 2-kb marker; *PC* = positive control; *NC* = negative control; *lanes 1-8* = trangenic rice samples.

Figure 6 showed the internal *sps* gene real-time PCR amplification plot of dilutions from 30 to 3 x 10^{-3} ng/µL rice gDNA. The amplification plot was divided into exponential, linear, and plateau phases. The amplification curves of different dilution series showed a significant gradient in C_T (Figure 6). The gDNA at very low concentration, about 3 pg/µL (3 x 10^{-3} ng/µL), could be quantified.

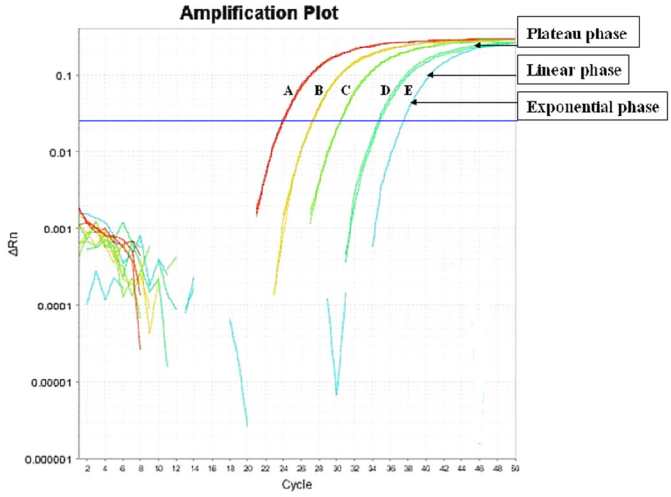

Figure 6. Real-time PCR analysis on gDNA. A = 30 ng/µL; B = 3 ng/µL; C = 3 x 10^{-1} ng/µL; D = 3 x 10^{-2} ng/µL; E = 3 x 10^{-3} ng/µL.

Figure 7 shows the influence of phenol-chloroform on real-time PCR. At the same gDNA concentration (30, 3, 3 x 10^{-1} ng/µL), the amplification plot of gDNA extracted by the phenol-chloroform-free protocol has lower C_T values, indicating that phenol-chloroform inhibits the enzymatic activity of DNA polymerase and affects PCR amplification efficiency.

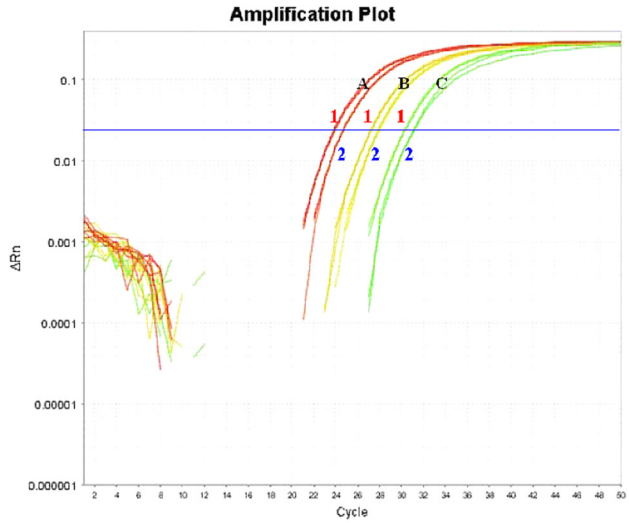

Figure 7. Real-time PCR analysis of gDNA extracted by two protocols. 1 = gDNA extracted by phenol-chloroform-free protocol; 2 = gDNA extracted by phenol-chloroform protocol; A = 30 ng/µL; B = 3 ng/µL; C = 3 x 10^{-1} ng/µL.

The standard curves of the gDNA dilutions from both protocols are shown in Figure 8. The curves of phenol-chloroform-free and phenol-chloroform gDNA curves were described by y = -3.2369x + 28.886 (*y* is the C_T, and *x* is the logarithm of the starting gDNA concentration) and y = -2.8758x + 29.409. R^2 (coefficient of determination) was 0.9967 for the phenol-chloroform-free protocol and 0.9876 for the phenol-chloroform protocol. In other words, the standard curve from the phenol-chloroform-free protocol showed better linearity (R^2 = 0.9967).

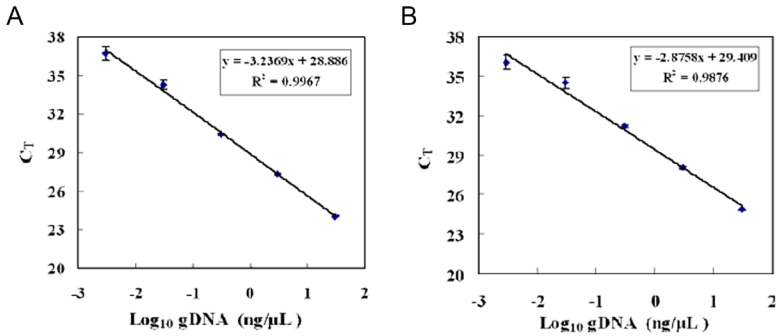

Figure 8. Standard curve for gDNA in real-time PCR assay. **A.** gDNA extracted by phenol-chloroform-free protocol. **B.** gDNA extracted by phenol-chloroform protocol. Each point represents the mean of three replicates. Error bars represent the standard deviation.

Figure 9 shows the influence of phenol-chloroform on restriction endonuclease (*Bst*UI) activity. At the same gDNA concentration and incubation time (8 h at 37°C), the amplification plot of gDNA extracted by the phenol-chloroform-free protocol reveals a higher C_T. The target fragment for real-time PCR has a *Bst*UI restriction site. If gDNA was more efficiently digested, there would be less available template for real-time PCR amplification and the C_T would be higher. In other words, phenol-chloroform inhibits restriction endonuclease activity.

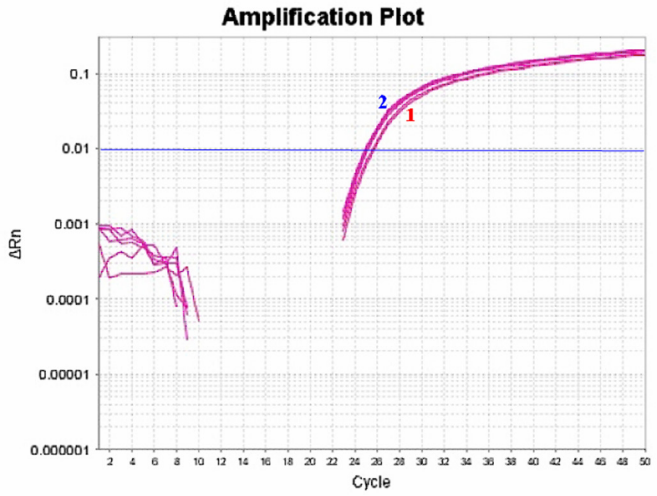

Figure 9. Analysis of phenol-chloroform on restriction endonuclease activity by real-time PCR. 1 = gDNA extracted by phenol-chloroform-free protocol; 2 = gDNA extracted by phenol-chloroform protocol.

These results indicated that gDNA isolated by the phenol-chloroform-free protocol was of high quality and suitable for real-time PCR with no interferents of PCR amplification.

Evaluation of the gDNA extraction protocol

The isolation of high-quality gDNA is an important and basic technique in plant molecular biology. Conventional plant gDNA extraction protocols require liquid nitrogen to homogenize the plant tissue and phenol-chloroform to remove protein. Commercial plant gDNA extraction kits cannot easily extract the gDNA of numerous samples in micro-centrifuge tubes. Our protocol does not require phenol-chloroform and liquid nitrogen or dry ice and is thus safer and more cost-efficient. Our protocol provided high yields of gDNA, ranging from 5 to 10 µg from only 10 mg fresh leaf tissue. Thus, little starting tissue is required.

Using a 96-well block to isolate gDNA, our procedure provides high throughput. In one day, one person can isolate gDNA from about 1600 samples (16 96-well blocks). Moreover, the 96-well block is more convenient for quality control of the gDNA extraction process. Quality control is very important when handling large numbers of plant samples. Using barcodes, every sample block or gDNA plate is traceable in our extraction process. Figure 10 shows an example of our gDNA extraction process. We have extracted about 40,000 gDNA samples without a single case of error or confusion (Duan et al., 2012).

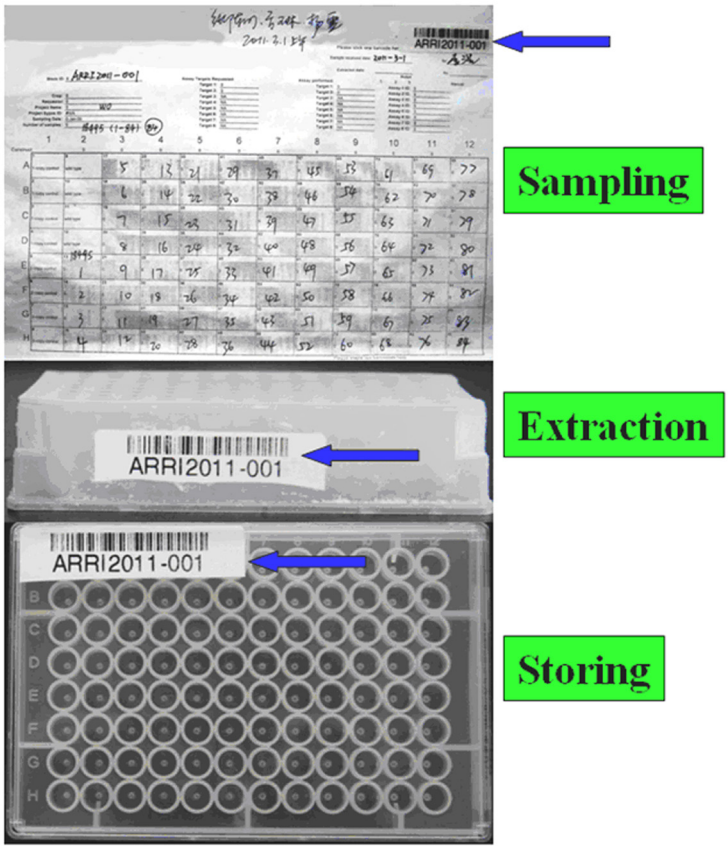

Figure 10. Quality control in gDNA extraction process with barcode. Blue arrow indicates a barcode.

Real-time PCR permits monitoring of the progression of DNA amplification after each cycle through fluorescence probes (Ginzinger, 2002). Real-time PCR has been applied to copy number variations, allelic discrimination, gene expression, and molecular diagnostics (Hindson et al., 2011). The purity of the nucleic acid is critical to obtaining reproducible results in real-time PCR (Cankar et al., 2006). Sensitive and reproducible real-time PCR amplification indicated that the gDNA isolated by our procedure is of high quality and contains no contamination by polysaccharide and polyphenol. Polysaccharide and polyphenol are difficult to separate from DNA as they co-precipitate with nucleic acids (Murray and Thompson, 1980). Polysaccharide and polyphenol inhibit several enzymatic reactions, such as PCR, RFLP, and restriction endonuclease digestion (Shioda and Marakami-Murofushi, 1987). One molar NaCl in the gDNA extraction buffer removes polysaccharides by increasing the solubility in ethanol (Fang et al., 1992). Polyphenol can be eliminated effectively by 2-mercaptoethanol in the gDNA extraction buffer (Niu et al., 2008). Thus far, we have obtained copy number variation data from about 30,000 gDNA samples by real-time PCR (Duan et al., 2012). These data also proved that our gDNA extraction is a high-throughput protocol and the gDNA purity is sufficiently high to meet the requirements of real-time PCR.

In conclusion, we have developed a high-throughput protocol for obtaining high-quality plant gDNA. The gDNA can be extracted from several plants without the need for phenol-chloroform and liquid nitrogen or dry ice. About 1600 samples (16 96-well blocks) can be processed by one person in a single day. The extracted gDNA is sufficient in quantity and purity for real-time PCR and other downstream molecular applications.

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