
Plant Molecular Biology Reporter **21:** 459a–459h, December 2003

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A High-Throughput DNA Extraction Protocol for Tropical Molecular Breeding Programs

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Abstract. Liquid handling robotics and capillary electrophoresis genetic analyzers now offer high-throughput solutions for 2 of the 4 key steps in PCR-based DNA marker-assisted fingerprinting (DNA extraction, PCR amplification, electrophoresis, data analysis). Thus, DNA extraction remains the most significant bottleneck at the bench for large-scale applications in plant breeding and germplasm characterization. We report on a rapid and low-cost method for relatively high-throughput extraction of high-quality DNA from young and mature leaves of sorghum, pearl millet, chickpea, groundnut, and pigeonpea. The procedure uses a modified CTAB/β-mercaptoethanol method for DNA extraction in a 96-well plate. The quantity and quality of the DNA extracted per sample is adequate for more than 1000 PCR reactions. A relatively high throughput of 96-384 samples per person per day can be achieved, depending on the crop. A major timesaving aspect of the protocol is the absence of a manual sample-grinding step. Finally, the cost is a magnitude lower than commercial plate-based kits, and, as such, is likely to have substantial application in tropical molecular breeding programs.

Full text[‡]: This manuscript, in detail, is available only in the electronic version of the *Plant Molecular Biology Reporter.*

Key words: DNA isolation, high throughput, molecular breeding, PCR

Introduction

Molecular plant breeding requires the genotyping of a large number of individuals for diversity analysis, marker-assisted selection, and variety fingerprinting. For high-throughput genotyping programs, the DNA must be of sufficient quality to generate robust and easily scored data with minimal repeats. This is particularly important as programs scale up to robotic liquid handling systems and capillary

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[‡]Editor's note: Although the scientific content of this paper has been reviewed, the full text Web document has not been edited in detail.

electrophoresis, where quality control and manual interventions can quickly become a major bottleneck. The DNA extraction step is a time-consuming and expensive component of molecular breeding, constituting 30-60% (Ragot and Hoisington, 1993; Dilworth and Frey, 2000) of the total time required for sample processing (from leaf collection to RFLP/PCR genotyping, respectively). To overcome this bottleneck, several in-house DNA extraction protocols have been described for temperate crops, based on the use of 96-well microtitre plates (e.g., Dilworth and Frey, 2000, Paris and Carter, 2000) and the increasing number of relatively expensive commercial products that are now available (e.g., DNeasy 96 Plant Kit [QIAGEN], Wizard Magnetic 96 DNA Plant System [Promega]).

We have developed a CTAB-based DNA extraction protocol (modified from Doyle and Doyle, 1987; Saghai-Maroof et al., 1984) for 5 crops of critical importance to agriculture in the semiarid tropics: sorghum (*Sorghum bicolor* [L.] Moench), pearl millet (*Pennisetum glaucum* [L.] R. Br.), chickpea (*Cicer arietinum* L.), groundnut (*Arachis hypoaea* L.), and pigeonpea (*Cajanus cajan* [L.] Millspaugh). The optimized protocol described here combines low-cost DNA extraction at excellent throughput rates of 96-384 samples per person per day for each of the tested species. Most importantly, the resulting DNA is of excellent quality suitable for use in capillary electrophoresis PCR detection of PCR-based markers.

Materials and Methods

Recommended growth conditions

- Grow seedlings in a growth room in 8 × 12-well format in 30- or 38-mm-diameter[1] rehydrated Jiffy-7 pellets (P/N 31130100, Jiffy International, Norway) placed in plastic trays (P/N 33010430, Jiffy International, Norway) with illumination for 12 h/d at a minimum photosynthetic active radiation (PAR) level of 100 $\mu mol \cdot s^{-1} \cdot m^{-2}$ at the leaf surface.

Alternative growth conditions

- For etiolated seedlings, pretreat seeds with fungicide (e.g., Capton and Thirum) and place on filter paper prewetted with sterile distilled water. Carefully fold the filter paper and place in a beaker containing sterile distilled water. Place the beaker in a 37°C incubator in the dark.
- For glasshouse-grown seedlings, grow seedlings in pots of 3-in diameter arranged in 8 × 12 format.

Sample preparation

- Harvest leaves from seedlings 5-10 days after sowing.
- Collect 20-30 mg of leaf tissue and place in 12 × 8-well strip tubes with strip caps (Marsh Biomarket, USA) in a 96 deep-well plate together with two 4-mm stainless steel grinding balls (Spex CertiPrep, USA).

CTAB extraction

- Add 450 µL of preheated (65°C) extraction buffer (100 mM Tris-HCl [pH 8], 1.4 M NaCl, 20 mM EDTA, CTAB [2-3% w/v], β-mercaptoethanol [0.03-3% v/v])[2] to each sample and secure with 8-strip caps (Marsh Biomarket).
- Process samples in a GenoGrinder 2000 (Spex CertiPrep, USA), following the manufacturer's instructions, at 500 strokes/min for 2 min.[3]
- Incubate the samples for 10 min in a 65°C water bath with occasional mixing.

Solvent extraction

- Add 450 µL of chloroform-isoamylalcohol (24:1) to each sample and invert twice to mix.
- Centrifuge plate at 6200 *g* for 10 min (Sigma centrifuge model 4K15C with QIAGEN rotor model NR09100: 2 × 1120 g SW).
- Transfer fixed volume (400 µL) of aqueous layer to fresh strip tubes (Marsh Biomarket).

Crude DNA pellet precipitation and RNase treatment

- Add 0.7 vol isopropanol (stored at –20°C) to each sample and invert once to mix.
- Centrifuge plate at 6200 *g* for 15 min.
- Decant supernatant from each sample and air-dry pellet for 30 min.
- Add 200 µL low-salt TE (10 mM Tris, 0.1 mM EDTA [pH 8]) to each sample.
- Optional step: Add 3 µL RNase A (10 mg/mL) to each sample and incubate at 37°C for 30 min.

Solvent extraction

- Add 200 µL phenol-chloroform-isoamylalcohol (PCI, 25:24:1) to each sample and invert twice to mix.
- Centrifuge plate at 4000 *g* for 5 min.
- Transfer fixed volume of aqueous layer to a fresh 96 deep-well plate (Marsh Biomarket).
- Add 200 µL chloroform-isoamylalcohol (24:1) to each sample and invert twice to mix.
- Centrifuge plate at 4000 *g* for 5 min.
- Transfer fixed volume of aqueous layer to a fresh 96 deep-well plate (Marsh Biomarket).

Purification

- Add 315 µL ethanol-acetate solution (30mL EtOH, 1.5mL 3 M NaOAc [pH 5.2]) to each sample and place in –20°C for 5 min.
- Centrifuge plate at 6200 *g* for 5 min.
- Decant supernatant from each sample and wash pellets with 70% EtOH.
- Centrifuge plate at 6200 *g* for 5 min.
- Optional step: Repeat 70% EtOH wash.

- Decant supernatant from each sample and air dry for approximately 1 h.
- Resuspend pellet in 100 μL low-salt TE and store at 4°C.

Evaluation of resultant DNA

The quality of genomic DNA was examined by using agarose (0.8%) gel electrophoresis and accurately quantified by using spectrophotometric analysis. The extracted DNA was further evaluated by means of enzymatic restriction with *Hin*d III restriction endonuclease, in addition to PCR amplification and polyacrylamide gel electrophoresis and/or capillary electrophoresis (ABI 3100, Applied Biosystems) detection of PCR products.

Notes

1. The optimum size of the Jiffy-7 pellet depends on the species. For the 5 species under study, the smaller sized Jiffy-7 pellet of 30-mm diameter was suitable for pearl millet, chickpea, and pigeonpea. For groundnut and sorghum, seedlings grown in the 38-mm-diameter Jiffy-7 pellet grew faster and yielded more leaf tissue.

Crop	CTAB, %	β-Mercaptoethanol, %	Optimum Amount of Sampled Leaf Tissue
Sorghum	3	0.17	6-cm leaf lamina strip
Pearl Millet	3	0.17	6-cm leaf lamina strip
Chickpea	2	0.03	4-5 pinnules
Groundnut	3	3.00	1-3 leaf disks (0.6-cm diameter)
Pigeonpea	3	2.5	5 leaf disks (0.6-cm diameter)

2. The CTAB and β-mercaptoethanol concentrations in the CTAB extraction buffer were optimized for the 5 crops, as indicated below, together with the optimum starting material:
3. The 2-min grinding procedure was repeated twice, on average, in order to sufficiently disrupt leaf material; however, the leguminous crops may require the grinding procedure to be repeated 3 times in order to obtain sufficiently homogenized leaf material.

Results and Discussion

A number of parameters were varied in order to identify the optimum protocol. Three parameters were investigated for the sample collection step: weight of leaf sample (10-50 mg), age of leaf tissue (5 d to 3 mo), and growth conditions of sample plants (glasshouse, Jiffy-7 pellet under controlled photoperiod and etiolated seedling). We observed that 20-30 mg of leaf material from 5- to 10-day-old seedlings was ideal (Table 1) and suited growth in Jiffy-7 pellets in the growth room. Because the most widely used format for genotyping is the 96-well microtitre plate, plants grown in rehydrated Jiffy-7 pellets in an 8 × 12 format in a growth room offer a significant reduction in sampling time and errors.

Table 1. Mean total DNA yield (µg) for chickpea genotypes (based on a total of 48 samples), comparing 2 different sample weights, 2 different seedling age groups, and 3 different seedling growth conditions (glasshouse, incubated etiolated seedlings, Jiffy-7 pellets in a growth room), all based on the complete DNA extraction protocol (2 solvent extraction steps and 2 final EtOH washes).

	5- to 10-Day-Old Seedlings			15- to 25-Day-Old Seedlings	
Sample Weight	Glasshouse	Etiolated	Jiffy-7	Glasshouse	Jiffy-7
20 mg	2.75 µg	15.5 µg	…[1]	2.0 µg	…
30 mg	6.1 µg	23.9 µg	18.7 µg	9.4 µg	6.8 µg

[1]Ellipses (…) indicate data not recorded.

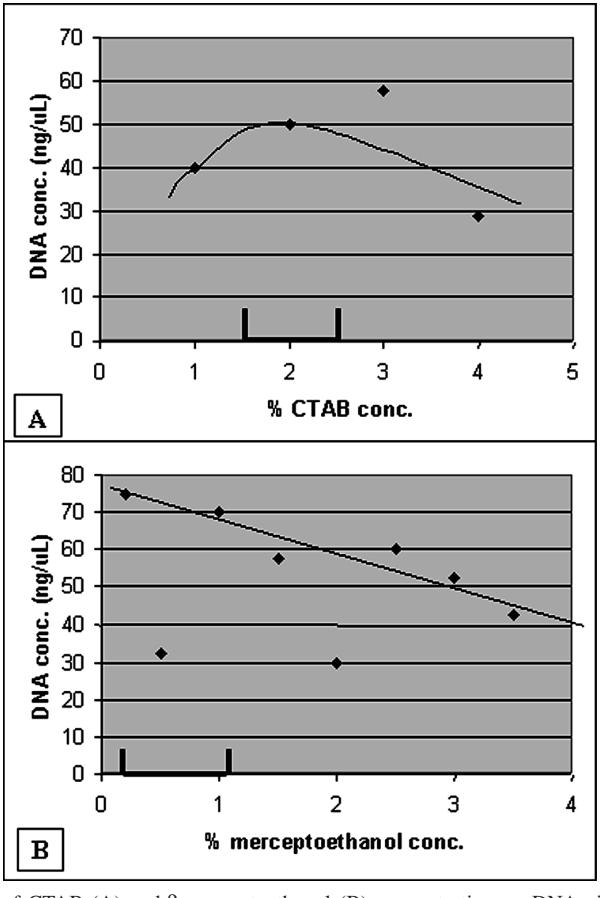

Figure 1. Effect of CTAB (A) and β-mercaptoethanol (B) concentration on DNA yield for chickpea accessions. Range bar on x-axis indicates optimum concentration chosen on the basis of various factors discussed in the text.

Table 2. Mean total DNA yield, mean A_{260}/A_{280} purity ratio ranges, and mean successful PCR amplification frequency for chickpea genotypes (based on a total of 144 samples with 30 mg starting material), comparing 3 different treatments: (1) crude pellet, (2) 2 solvent extractions and 1 EtOH wash, and (3) 2 solvent extractions and 2 EtOH washes.

Treatment	Mean Total DNA Yield, µg	A_{260}/A_{280} Ratios	Amplification Frequency
Crude pellet	62.00	1.79	0.16
2 solvent extractions + 1 EtOH wash	8.25	1.89	0.95
2 solvent extractions + 2 EtOH washes	9.00	1.83	0.95

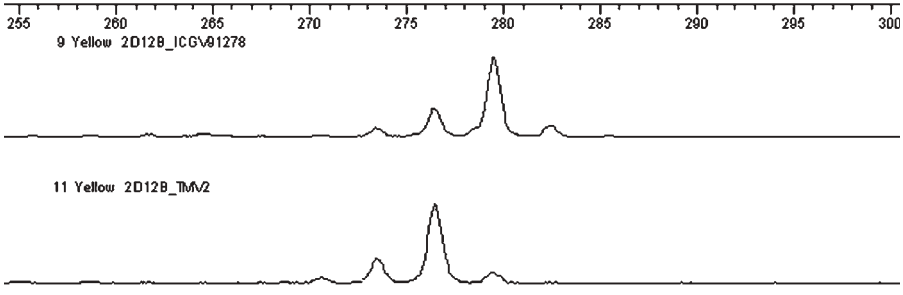

Figure 2. DNA amplification of 2 groundnut accessions, TMV2 and ICGV91278, as revealed by SSR-PCR with primer 2D12B (labeled with fluorescent dye, NED) and detected via capillary electrophoresis.

The DNA extraction steps included the following variations: CTAB (1-10%) and β-mercaptoethanol (0.03-3.5%) concentrations in extraction buffer, inclusion and exclusion of PCI solvent extraction step, and number of EtOH washes (1 vs. 2). A parabolic effect of increasing CTAB concentration on overall yield of DNA was observed (Figure 1A), whereupon past the optimum, higher CTAB concentrations caused a reduction in DNA yield. Thus, 3% CTAB buffer for glasshouse/Jiffy-7 pellet–grown groundnut and pigeonpea with high concentrations of β-mercaptoethanol, 3% and 2.5% respectively, was selected to maximize yield and purity. The concentration of β-mercaptoethanol commonly used in DNA extraction protocols is 0.2%. However, our experimentation showed that as little as 0.03% β-mercaptoethanol was sufficient to decrease polyphenol oxidation in young glasshouse/Jiffy-7 pellet–grown plants of chickpea (Figure 1B) and 0.17% β-mercaptoethanol for the cereals.

PCR amplification with a crude DNA pellet template, prior to the second solvent extraction and excluding the RNase step, resulted in nonreproducible PCR products (Table 2). This result was most probably due to the presence of contaminants in the DNA differentially inhibiting *Taq* polymerase activity (Pandey et al., 1996); therefore, for efficient high-throughput sample flow, the inclusion of 2 organic solvent extraction steps is recommended to ensure generation of consistent data and to avoid the unwelcome and inefficient re-extraction of poor-quality samples. Finally, comparing the use of 1 and 2 EtOH washes during the final

Table 3. Mean total DNA yield, mean DNA yield per mg of starting leaf material (30 mg total), and A_{260}/A_{280} purity ratio ranges obtained across ICRISAT's 5 mandate crops, all based on the complete DNA extraction protocol (2 solvent extraction steps and 2 final EtOH washes).

Crop	Mean Total Yield, µg	Mean Yield Per mg Leaf Material, ng	A_{260}/A_{280} Ratios
Sorghum	7.5	250	1.65-1.80
Pearl millet	10.5	350	1.65-1.80
Groundnut	18.0	600	1.70-1.80
Chickpea	13.5	450	1.70-1.80
Pigeonpea	7.5	250	1.65-1.90

Table 4. Cost breakdown of reagents and plastics required for HTP DNA extraction protocol.

Reagent/Plastics	Bulk Cost*	Cost Per Sample, USD	Cost Per Sample, Euro Dollar
CTAB†	$132/1 kg	0.0023	0.0020
Tris	$85/1 kg	0.0245	0.0216
NaCl	$8.7/5 kg	0.0007	0.0006
EDTA‡	$95/1 kg	0.0053	0.0047
β-mercaptoethanol	$19.8/100 mL	0.0038	0.0033
Chloroform	$18/2.5 L	0.0048	0.0042
Isoamylalcohol	$5/500 mL	0.0032	0.0028
Isopropanol	$11/2.5 L	0.0009	0.0008
Ribonuclease A	$642.4/5 g	0.0065	0.0057
Phenol	$21.25/100 mL	0.0220	0.0194
Ethanol	$12.9/500 mL	0.0050	0.0044
Total reagent cost		0.0790	0.069
96 deep-well plate with 12 × 8-well strip tubes	$30/10 96-well rack	0.1300	0.1145
8-well strip caps	$10/120 strips	0.0400	0.035
Total plastics cost		0.1700	0.1495

*Prices valid as of 05-08-03 in India and including 10% freight charges. 1 USD = 0.881 Euro dollar (as of 05-08-03).

†CTAB indicates hexadecyltrimethylammonium bromide.

‡EDTA indicates ethylenediaminetetraacetic acid, disodium salt.

cleaning step revealed that a second EtOH wash was unnecessary for successful PCR amplification across species (Table 2). The DNA resulting from the optimized protocol was observed to be largely free from polyphenolics and secondary metabolites, as determined by successful digestion with restriction endonucleases and amplification in PCR (Figure 2) as revealed through capillary electrophoresis.

The average total yield of the DNA extractions (across all 5 crops) was 11.4 mg, i.e., 380 ng of DNA for every 1 mg of leaf tissue used (Table 3), with A_{260}/A_{280} ratios in the range of 1.65-1.9 (Table 3). This DNA yield and quality is sufficient for approximately 1500 PCR reactions in all crops.

The protocol is cost-effective, with a reagent cost per sample as low as $0.08 and a total cost (excluding labor) of $0.25 per sample (Table 4). Although the costs of labor, reagents, and plastic consumables vary dramatically through time and between countries, we believe that a comprehensive list of materials and costs is a prerequisite for decision-making in any molecular plant breeding strategy.

The protocol presented here offers a quick, simple, and truly cost-effective approach to large-scale DNA extraction for high-throughput genotyping in marker-assisted breeding of tropical crops. The extracted DNA is high quality and even suitable for capillary electrophoresis analysis systems.

Acknowledgments

The research work at ICRISAT described in this publication was supported by restricted grants from the Government of Japan, UK's Department for International Development (DFID), the Commission of the European Communities (CEC), and the Asian Development Bank (ADB).

The authors wish to thank all those colleagues in the Applied Genomics Lab who have contributed to this work, particularly Dr Rolf Folkerstma, Ms Santosh Gurtu, and Mr Brian Moss for their assistance with developing the protocol and to Dr L. Krishnamurthy, Mr A. Gaffoor, Mr Eshwar, Ms Seetha Kannan, and Mr Luke for technical assistance.

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