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# A Method for Isolation and Purification of Peanut Genomic DNA Suitable for Analytical Applications

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**Abstract.** Numerous methods are available for isolation of plant genomic DNA, but in practice these procedures are empirical due to variability in plant tissue composition. Consistent isolation of quality DNA from peanut (*Arachis hypogaea* L.) is particularly problematic due to the presence of phenolic compounds and polysaccharides. Inconsistencies in extraction results can be attributed to the age and growth stage of the plant material analyzed. Mature leaves have higher quantities of polyphenols, tannins, and polysaccharides that can contaminate DNA during isolation. We show that four published protocols could not be used to isolate peanut DNA of sufficient quality for PCR amplification or Southern hybridization. We have devised a new protocol that uses DEAE-cellulose purification to isolate peanut DNA useful for downstream applications.

**Key words:** DNA isolation, PCR amplification, peanut, Southern hybridization, transgenic plants

**Abbreviations:** CTAB, hexadecyltrimethylammonium bromide; IPCVcp, coat protein gene of Indian peanut clump virus; *npt* II, neomycin phosphotransferase II; RPC, reverse phase chromatography

## Introduction

Many standard methods are available for isolation of plant genomic DNA, however problems are invariably encountered when DNA is first isolated from a plant species. Polysaccharides are particularly problematic. For example, the acidic polysaccharides are inhibitory for *Hin*d III enzyme restrictions of lambda DNA, whereas neutral plant polysaccharides are not inhibitory (Do and Adams, 1991). In addition, acidic polysaccharides can inhibit classical two primer PCR (Demeke and Adams, 1992; Pandey et al., 1996) by inhibiting *Taq* DNA polymerase activity (Fang et al., 1992). But neutral polysaccharides are not inhibitory to PCR amplification of spinach DNA (Pandey et al., 1996). The addition of Tween 20, DMSO, or PEG 400 to the PCR reaction mixture can partially restore the ability to generate RAPDs. The most effective way to eliminate the effects of polysaccharide inhibition is by diluting the DNA extracts, and thereby diluting the

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polysaccharide inhibitors (Pandey et al., 1996). However, excessive dilution of the DNA solution makes it unsuitable for analysis by Southern hybridization.

Several methods have been used to overcome these problems including sedimentation in cesium chloride gradients or extracton with CTAB (Couch and Fritz, 1990; Rogers and Benedich, 1985; Scott and Benedich, 1985), selectively binding the DNA by using a RPC-5 column (Pearson et al., 1971; Guillemaut and Marechal-Drouard, 1992) or by selective binding to an ion exchanger to avoid their co-precipitation with DNA after alcohol addition (Marechal-Drouard and Guillemaut, 1995). Rether et al. (1993) subjected the total nucleic acids to a mixture of RNases and glycoside hydrolases that degrade RNA and polysaccharides without affecting DNA integrity. However, no single method can be used to obtain large quantities of reasonably pure DNA from all plant species.

After several attempts, we were unable to obtain DNA of consistent quality from glasshouse-grown peanut (*Arachis hypogaea* L.) plants. Some inconsistencies were due to the stage of leaf growth and development. Mature leaves have high quantities of polyphenols, tannins, and polysaccharides. Dealing with such components in mature leaves becomes necessary when younger expanding leaves and shoots are not available during the time of collection (Porebski et al., 1997). We were unable to amplify DNA by PCR and identify transgenes using plant DNA extracted by reported methods (Dellaporta et al., 1983; Guillemaut and Marechal-Drouard, 1992; Marechal-Drouard and Guillemaut, 1995; Rogers and Bendich, 1985; Porebski et al., 1997; Nucleon PhytoPure™ Kit, Amersham; Plant DNAzol™, Life Technologies Inc.). These methods either gave large quantities of impure DNA consistently under alkaline pH (Dellaporta et al., 1983), or lower quantities of reasonably pure DNA inconsistently under acidic pH (Guillemaut and Marechal-Drouard, 1992). The Nucleon PhytoPure kit gave highly unsuitable DNA; the Plant DNAzol gave very low quantities of DNA sufficient only for PCR reactions. Thus, we found it necessary to devise a new protocol for genomic DNA extraction from fully developed expanded leaf tissue. The protocol described here is relatively simple and provides clean DNA of high molecular weight from different genotypes of peanut. The DNA is consistently amplifiable by PCR and restrictable for Southern blot hybridisation with known probes.

## Materials and Methods

### *Plant materials*

Leaves of peanut (*Arachis hypogaea* L.) cultivar JL 24 were harvested from transformed plants growing in a containment glasshouse. The transformants containing the *npt* II and IPCVcp genes were obtained as reported elsewhere (Sharma and Anjaiah, 2000). The T3 progeny (six plants) of a transformed plant that was confirmed positive and having single insertions of *npt* II and IPCVcp genes was selected for this work. Fully expanded green leaves from the glasshouse-grown plants were harvested immediately prior to use and frozen in liquid nitrogen. Unless mentioned otherwise, the DNA was isolated and purified according to the following protocol designated as ICRISAT DNA Extraction procedure (ICE).

*Solutions and reagents*

- DNA extraction medium (Dellaporta et al., 1983):
  100 mM tris, pH 8.0; 50 mM EDTA, pH 8.0; 500 mM NaCl; 10 mM β-mercaptoethanol
- 5 M potassium acetate buffer: pH 4.8
- RNase A (10 mg/mL; Sigma)
- DNA elution medium: 2 M NaCl, 10 mM tris, pH 7.5, 1 mM EDTA
- DNA wash medium: 400 mM NaCl, 10 mM tris pH 7.5, 1 mM EDTA
- Pre-swollen DEAE-cellulose (Whatman DE 52): Stir 15 g of DEAE-cellulose with 200 mL of elution medium for 10 min. Decant and remove the supernatant containing any fine particles. Resuspend DEAE-cellulose in 200 mL of wash medium, decant and remove the supernatant. Repeat the final step once, and finally resuspend DEAE-cellulose in 40 mL of wash medium. The suspension can be stored up to one week at 4°C.

*DNA extraction protocol*

1. Freeze 1 g freshly harvested leaf tissue in liquid nitrogen and grind to a fine powder. Add 15 mL of extraction buffer. Transfer the contents into 25 mL polypropylene centrifuge tubes and vortex briefly to mix the contents well.
2. Add 1 mL of 20% SDS and shake the tube gently once (vortexing at this stage can cause shearing of the DNA) and incubate the tubes at 65°C for 10 min.
3. Add 5 mL of 5 M potassium acetate, gently mix the contents and incubate on ice for 20 min.
4. Centrifuge at 19,150 *g* for 20 min and transfer the supernatant to a fresh tube and add 10 mL isopropanol. Mix and incubate at -20°C for 30 min.
5. Centrifuge at 19,150 *g* for 15 min to pellet the nucleic acids. Wash the pellet with ice-cold 70% ethanol and transfer the pellet to a 2 mL microcentrifuge tube. Dry the pellet and dissolve in 2 to 3 mL of 10 mM tris containing 1 mM EDTA (pH 8.0).
6. Treat the nucleic acid solution with RNase (10 mg/mL) and incubate for 1 h at 37°C to remove the RNA.

*DNA purification protocol*

1. To 800 µL of the DNA solution add 1 mL of DEAE-cellulose suspension and mix gently for 3 min to keep the DEAE-cellulose suspended thereby maximizing its interaction with DNA.
2. Centrifuge for 30 s at 2,627 *g* to sediment the DEAE-cellulose to which the DNA has been bound.
3. Pour off the supernatant and carefully resuspend the DEAE-cellulose in 1-2 mL of wash medium to eliminate proteins, polysaccharides and secondary metabolites that are not bound to DEAE-cellulose.
4. Centrifuge for 30 s at 2,627 *g* and pour off the supernatant. Repeat this washing step at least once.

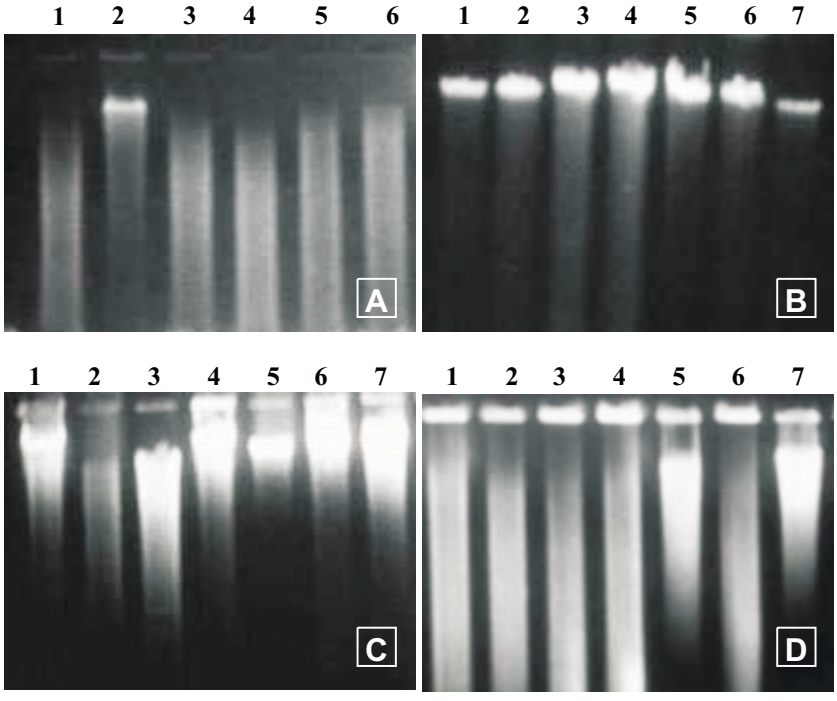


*Figure 1.* Electrophoretic analysis of total DNA from independent transformants (Lanes 1 to 7) of peanut transgenic plants isolated by the following four different methods: **A.** RB (Rogers and Bendich, 1985); **B.** ICE (Sharma et al., this work); **C.** DP (Dellaporta et al., 1983); **D.** GMD (Guillemaut and Marechal-Drouard, 1992). DNA was resolved on 0.8% agarose gels containing ethidium bromide.

5. Add 500 µL of elution medium to the DEAE-cellulose pellet and gently mix to elute the DNA. Centrifuge briefly and collect the supernatant.
6. Add 300 µL of elution medium to DEAE-cellulose, mix, centrifuge, and pool supernatants.
7. Add 0.8 vol of isopropanol to the supernatant, mix and centrifuge at 7,100 *g* for 10 min at room temperature.
8. Wash the pellet with 70% ethanol and dry the pellet. Dissolve in 100 µL of 10 mM tris containing 1 mM EDTA and use for analytical applications.

### *DNA isolation by other methods*

DNA from identical leaf samples was also isolated by three other methods (Dellaporta et al., 1983; Roger and Benedich, 1985; Guillemaut and Marechal-Drouard, 1992). These were designated DP, RB, and GMD respectively. In addition, the DNA isolated by the DP method was subjected to purification by DEAE-cellulose. The DNA obtained by these methods was subjected to analysis by agarose gel electrophoresis, restriction digestion, PCR and Southern hybridisation as follows.

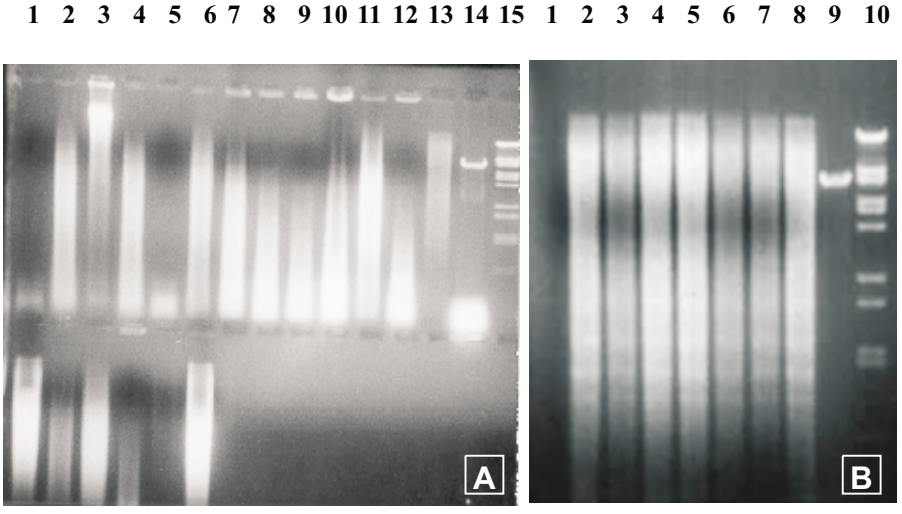


*Figure 2.* Restriction analysis of total genomic DNA of peanut transgenic plants isolated by different methods and resolved on 0.8% agarose gels after restriction with *Hin*d III. **A**. Lanes 1 to 6, RB; Lanes 6 to 12, GMD; Lane 13, negative control DNA; Lane 14, plasmid with *npt* II gene; lane 15, lambda-DNA restricted with *Bst*E II. Bottom: Lanes 16 to 21, DP. **B**. Lane 1, no DNA; Lanes 2 to 8, ICE; Lane 9, plasmid DNA having npt II gene; Lane 10, lambda-DNA restricted with *Bst*E II. Note the complete restriction of genomic DNA with ICE method in Figure 2B.

*DNA analysis*

Restriction analysis: The susceptibility of genomic DNA to cleavage by *Hin*d III was determined by overnight restriction with the enzyme.

Polymerase chain reaction: The genomic DNA was analyzed for the presence of *npt* II by PCR using oligonucleotide primers that amplify the 700 bp fragment of *npt* II gene as described elsewhere (Sharma and Anjaiah, 2000). The amplified products were assayed by electrophoresis on 1.2% agarose gels and the amplicons were confirmed in Southern hybridisation by probing blots with the *npt* II gene fragment (Sharma and Anjaiah, 2000) by using non-radioactive AlkPhos direct labeling and detection system (Amersham).

Southern hybridisation: Southern hybridisation was carried out after digesting the DNA with *Hin*d III to provide a single restriction within the T-DNA region (Sharma and Anjaiah, 2000). The blotted DNA on the nylon membrane was detected by using a non-radioactive Alkphos direct labeling and detection system.

## Results and Discussion

The method described here can be used to obtain consistently high quality genomic DNA from peanut. Although large quantities of DNA were obtained by the DP method, the DNA was contaminated with high levels of polysaccharides making it opaque and difficult to dissolve. Upon purification with DEAE-cellulose, good

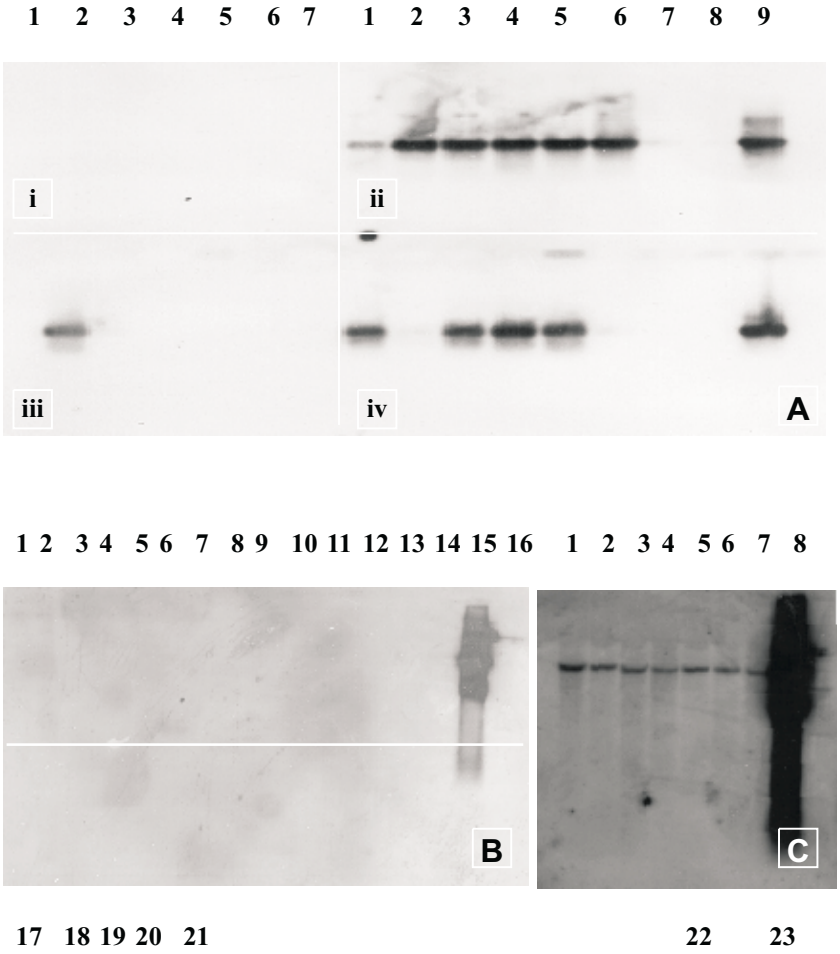


*Figure 3.* Molecular analysis of genomic DNA of peanut transgenic plants isolated by different methods: **A**. PCR amplification of 700 bp fragment of *npt* II coding region in DNA from i, DP; ii, ICE; iii, RB; iv, GMD. The PCR fragments were resolved on 1.2% agarose gels and probed with non-radioactive Alkphos-labeled *npt* II gene by Southern hybridization. [Lanes 1-6 of each panel: Genomic DNA; Lanes 7 and 8 of panels ii and iv contain untransformed DNA; Lane 9 of panels ii and iv contain plasmid with *npt* II gene]. **B, C**. Southern blot analysis of genomic DNA for the detection of *npt* II gene. The DNA was digested with *Hin*d III and the blot probed with non-radioactive Alkphos-labeled 700 bp PCR amplified *npt* II gene fragment. **B**. DNA isolated by DP (lanes 1 to 7), RB (lanes 8 to 14), and GMD (lanes 17 to 23) methods. None of the DNAs showed hybridization except the positive control (lane 15), and control DNA (lane 16). **C**. DNA isolated by ICE method showing positive hybridization to the probe. Lane 8 carries the plasmid containing *npt* II gene.

quality DNA was recovered that could be satisfactorily restricted and used in analytical applications such as restriction digests, PCR and Southern hybridisation (Figures 1, 2 and 3). The results in Figure 1 show that the total genomic DNA isolated by both the DP and ICE methods provided intact DNA while the RB and GMD methods produced large quantities of sheared DNA. The DNA isolated

with the ICE method produced good restriction patterns when compared to equal quantities isolated by the DP, RB, and GMD methods (Figures 2A, B).

PCR analysis of *npt* II in the genomic DNA obtained by the ICE protocol resulted in good amplification as indicated by ethidium bromide staining (data not shown) and Southern hybridisation (Figure 3A ii). Amplification from the GMD method was however not consistent and the DNA was invisible after ethidium bromide staining. The amplicons were visible only in Southern blots indicating a very low concentration of amplified products. DNA from the DP and RB protocols resulted in no amplification of the *npt* II gene fragment (Figure 3A). Such inhibition of PCR could be due to acidic polysaccharides (Demeke and Adams, 1992; Pandey et al., 1996).

In Southern hybridisation, positive signals for the *npt* II gene were obtained only in DNA from the ICE protocol while no signal was visible in the other three protocols (Figure 3B, C). Southern hybridisation requires high quality, intact DNA free from any polysaccharides, proteins, or other inhibitors. Nucleic acids form complexes with secondary compounds, such as polysaccharides or polyphenols, released by cell disruption, leading either to the embedding of DNA in a sticky gelatinous matrix or to brown-coloured products (Guillemaut and Marachel-Drouard, 1992). Polysaccharides also co-precipitate with DNA after alcohol addition during DNA isolation and finally lead to highly viscous solutions (Demeke and Adams, 1992; Do and Adams, 1991). Such DNA is usually neither restrictable nor suitable for Southern hybridisation. It often remains in the wells during electrophoretic separation (Figure 2A). The presence of contaminants or inhibitors in DNA might have lead to the sheared unrestrictable DNA obtained by isolation with the RB and GMD protocols. Moreover, contaminants could interfere with the DNA transfer onto the nylon membrane thus leading to no detectable signals in the Southern hybridisation (Figure 3B, C). These contaminants were likely eliminated by purification with DEAE-Celulose.

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