# An Efficient Protocol for Isolation of High Quality Genomic DNA from Seeds of Apple Cultivars (*Malus × Domestica*) for Random Amplified Polymorphic DNA (RAPD) Analysis

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**Abstract:** Genomic DNA represents the total genome of an organism and is valuable for different molecular studies. The isolation of high quality DNA is a prerequisite for these molecular techniques. The seeds of apple (*Malus × domestica*) contain high amount of polysaccharides, polyphenols and other secondary metabolites that can hamper DNA isolation, amplification, restriction digestion and subsequent molecular studies. Here, we report a simple, inexpensive and an efficient protocol for the isolation of high quality genomic DNA from seeds of different apple cultivars for random amplified polymorphic DNA (RAPD) analysis. This procedure purifies greater amounts of DNA which can be amplified via PCR or digested with endonucleases. The yield of DNA was 200-300 ng/$\mu$L (total 8-12 $\mu$g DNA from 1-2 apple seeds). The simplicity of the procedure makes it very practical for DNA extraction.

## INTRODUCTION

The domesticated apple (*Malus × domestica* Borkh., Rosaceae) is a common fruit crop in the temperate world. It has been an important part of the human diet for thousands of years; however, its consumption is linked to the prevention of various chronic diseases [1]. A number of pharmaceutical products from apples such as apple peel and seed extracts are available in the world market. Apple intake has been shown to reduce cancer incidence, cardiovascular disease, symptoms of chronic obstructive pulmonary disease, and the risk of thrombotic stroke [2-6]. Apples contain a large concentration of several classes of polyphenolic compounds such as flavonoids and phenolic acids. Some of the most well studied antioxidant compounds in apples include quercetin-3-galactoside, quercetin-3-glucoside, quercetin-3-rhamnoside, catechin, epicatechin, procyanidin, cyanidin-3-galactoside, coumaric acid, chlorogenic acid, gallic acid, and phloridzin [1, 4, 7-14]. The concentration of these phytochemicals may depend on many factors including cultivars. Thus, it is important to have genetic analysis of various apple cultivars to develop apples as pharmaceutical crops.

There are several molecular marker techniques being used to determine the genetic architecture of wide varieties of closely related individuals. Some of these methods have been successfully applied in revealing genetic identities and diversity in apple species [15-17]. With the ever growing requirements for environmental protection and food safety in the production of high quality apples, modern apple breeding has become more dependent on disease resistance genes [18]. Therefore, accurate characterization of the available genetic pool is important in breeding programs and essential for the protection of future property rights over new cultivars [19, 20].

The traditional methods for characterization and assessment of genetic variability based on morphological, physiological and agronomic traits are often not adequate, since these traits are developmentally regulated or influenced by the genotype × environment interaction and agronomic practices like selected rootstock or pruning. Molecular markers provide an opportunity for genetic characterization and allow direct comparison of different genetic material independent of environmental influences. DNA markers are also more abundant than morphological and biochemical markers and the whole genome can be assessed easily. Restriction fragment length polymorphisms (RFLPs) have been used with some success for apple cultivar identification and parentage analysis [21-23]. The random amplified polymorphic DNA (RAPD) analysis has already been proved to be valuable in apple genome analysis. These markers have been used to study relationships in the genus *Malus*, for identification of apple cultivars, apple rootstocks and for paternity analysis [15, 24-29]. Simple-sequence repeat (SSR, microstellites) markers have been successfully used on apple species for identification and positional comparison of major genes and quantitative trait loci (QTLs) for scab, powdery mildew, and fire blight resistance as well as morphological or physiological traits [30-38].

The isolation of high quality genomic DNA is a prerequisite for these molecular techniques. However, high amounts of polysaccharides, polyphenols and various secondary metabolites such as alkaloids, flavonoids and tannins in tree species usually interfere with DNA isolation [39-41]. Polyphenols released from the vacuoles during the cell lysis process are oxidized by cellular oxidases and undergo irreversible interactions with nucleic acids causing browning of the DNA [39, 42]. The presence of gelling polysaccharides

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prevents complete dissolution of nucleic acids and imparts a viscous constituency to the DNA making it stick to the wells during gel electrophoresis [39, 42-44]. These residual polyphenols, polysaccharides and secondary metabolites have also been reported to interfere with the activity of several biological enzymes like polymerases, ligases and restriction endonucleases [39, 40, 42, 44].

Researchers often modify a protocol or blend two or more different procedures to obtain DNA of the desired quality [42]. A good isolation protocol should be simple, rapid and efficient, yielding appreciable levels of high quality DNA suitable for molecular analysis. So far, there is no report of DNA isolation from seeds of apple. The seeds of *Malus* species contain high amount of polysaccharides, polyphenols, and other secondary metabolites that can hamper DNA isolation, amplification, restriction digestion and subsequent molecular cloning [40, 45]. So here, we report an efficient and new protocol for isolating high molecular weight PCR amplifiable DNA from seeds of different apple cultivars. We developed this protocol by thoroughly optimizing the existing protocols for plant DNA extraction [40, 41, 43, 44, 46]. While adapting the existing protocols for DNA isolation from apple seeds, it was observed that the final DNA preparation was having lot of mucilage with low yield and recalcitrant to restriction digestion and amplification. Hence, we performed several optimization steps to develop a new protocol with increased yield.

## MATERIALS AND METHODS

### Plant Materials

For DNA extraction, seeds from different varieties of *Malus × domestica* available in the region of Uttar Pradesh, India, were collected such as 'Red Delicious' (Jammu & Kashmir), 'Golden Delicious' (Jammu & Kashmir), 'Red Delicious' (Himachal Pradesh), 'Maharaji' (Jammu & Kashmir), 'Kali Devi', 'Chocolaty', 'Panchmukhi' and Chinese apple. The voucher specimens were deposited at Department of Botany, Deen Dayal Upadhyay Gorakhpur University, Gorakhpur, U.P., India.

### Reagents and Chemicals

The chemicals and reagents used in the isolation of DNA were: 1 M Tris-HCl pH 8.0, 0.5 MEDTA pH 8.0, 10% sodium dodecyl sulphate (SDS), 5 M NaCl solution, extraction buffer (100 mM Tris-HCl pH 8.0, 50 mM $Na_3EDTA$ pH 8.0; 1.5 M NaCl, 1% SDS, 1% β-mercaptoethanol), 7.5 M Ammonium acetate, TE buffer (10 mM Tris HCl, 1 mM EDTA pH 8.0), 10× TAE buffer pH 8.0, Ethidium bromide (10 mg/mL), RNase A (2 mg/mL), TE buffer for PCR (10 mM Tris HCl, 0.05 mM EDTA, pH 8.3), Chloroform: Isoamyl alcohol (24:1 v/v). Random decamer primers, dNTPs (10 mM), Taq DNA polymerase (3 U/μL), 10× PCR buffer, Restriction enzymes and 10× Restriction endonuclease buffer were from Bangalore Genei, India. All the chemicals used were of high quality grade purchased from Himedia Inc., Qualigens and Merck, India.

### Genomic DNA Isolation from Apple Seeds

DNA was isolated from fresh seeds of different apple cultivars by thoroughly optimizing the existing protocols for

plant DNA extraction [40, 41, 43, 44, 46]. This rapid extraction method of apple seed DNA consists of three steps: extraction of DNA by lysis buffer, precipitation by using high salt concentration and purification by chloroform: isoamyl alcohol. The whole protocol is as follows:

Fresh apple seeds (0.1 gm, 1-2 seeds) was collected and peeled off in sterile condition and homogenized in 25 μL of freshly prepared 1.0 % β-mercaptoethanol. After grinding, 750 μL of extraction buffer (100 mM Tris-HCl pH 8.0, 50 mM EDTA, 1% SDS, 1.5 M NaCl) is added to the homogenate. Transferred the homogenate into a fresh eppendorf tube and incubated at 37 °C for one hour. One half volumes of freshly prepared 7.5 M Ammonium acetate was added to the homogenate. The mixture was incubated on ice for 30 min. and centrifuged for 10 min (10,000 rpm, at 4 °C). The supernatant was transferred to fresh tube and added double volume of absolute ethanol. The tube was left on ice for 20 min then at -20°C for 30 min or over night to precipitate the DNA completely. After centrifugation at 10,000 rpm for 10 min, the supernatant was discarded and DNA pellet was vacuum dried. Resuspend the pellet in 500 μL of TE buffer (10 mM Tris-HCl pH 8.0, 1 mM EDTA pH 8.0). Two microliters of RNase A (2 mg/mL) was added to the solution and incubated at 37°C for 30 min. One volume of Chloroform: Isoamyl alcohol (24:1) was added and emulsified by inverted shaking to remove RNase and other impurities. Centrifuged the mixture at 10,000 rpm for 5 min and took the upper aqueous layer. This process of purification was repeated once again. In the aqueous layer, added double volume of absolute alcohol and left on ice for 20 min and then at -20 °C for 30 min or overnight. After centrifugation at 10,000 rpm for 1 min the pellet was washed with 1 mL 80% ethanol and vacuum dried. The DNA pellet was re-dissolved in 30 μL TE buffer.

### DNA Quantification

The quantification of genomic DNA was achieved using a spectrophotometer (Shimadzu UV-240). The yield was determined by measuring the absorbance at $A_{260}$ and $A_{280}$. The level of DNA purity was determined by the $A_{260}/A_{280}$ absorbance ratio. DNA purity was further tested by running the extracted genomic DNA samples on 0.8% agarose gel containing 0.5 μg/mL ethidium bromide. A molecular weight marker (Hind III digested lambda DNA) was included on each gel. The gels were visualized and photographed under UV light (Gel documentation system, Bio-Rad).

### Restriction Endonuclease Digestion

The suitability of the extracted genomic DNA for downstream molecular analysis was further determined by restriction digestion using EcoR V, Hind III, Dra I, Bam HI, Bgl II and Kpn I restriction enzymes. The digestion was performed at 37 °C for 5 h in a 25 μL reaction volume containing 1 μg genomic DNA, 2.5 μL of 10X restriction endonuclease buffer and 20 U of restriction endonuclease enzyme. The digested DNA was confirmed on 0.8% agarose gel with Hind III digested lambda DNA as a marker.

### PCR Amplification

To assess the quality of the isolated DNA for PCR amplification, RAPD profile of eight different apple cultivars was

**Table 1.** **Comparative Results of the Protocols Followed for DNA Extraction from Apple Seeds**

| Methods | DNA Yield (ng/$\mu$L) | $A_{260}/A_{280}$ |
|---|---|---|
| Mishra *et al.,* 2008 [41] | 120-140 | 1.54-1.60 |
| Diadema *et al.,* 2003 [44] | 110-125 | 1.47-1.56 |
| Pirttila *et al.,* 2001 [40] | 115-130 | 1.43-1.61 |
| Barnell *et al.,* 1998 [43] | 115-120 | 1.49-1.57 |
| Kim *et al.,* 1997 [46] | 140-180 | 1.58-1.64 |
| Protocol of the Current Study | 200-300 | 1.75–1.94 |

**Table 2.** **Optimization of Extraction Buffer Composition by Modifying the Concentration of Different Constituents (Shown in Bold)**

| No. | Extraction Buffer | DNA Yield (ng/$\mu$L) | $A_{260}/A_{280}$ |
|---|---|---|---|
| 1 | 200 mM Tris–HCl pH 8.0, 25 mM EDTA, 250 mM NaCl, 0.5% SDS (Kim *et al.,* 1997) | 140-180 | 1.58-1.64 |
| 2 | **100 mM Tris–HCl** pH 8.0, 25 mM EDTA, 250 mM NaCl, 0.5% SDS | 145-175 | 1.60-1.65 |
| 3 | 100 mM Tris–HCl pH 8.0, **50 mM EDTA**, 250 mM NaCl, 0.5% SDS | 142-178 | 1.59-1.63 |
| 4 | 100 mM Tris–HCl pH 8.0, 50 mM EDTA, **500 mM NaCl**, 0.5% SDS | 150-185 | 1.62-1.68 |
| 5 | 100 mM Tris–HCl pH 8.0, 50 mM EDTA, 500 mM NaCl, **1.0 % SDS** | 150-190 | 1.65-1.70 |
| 6 | 100 mM Tris–HCl pH 8.0, 50 mM EDTA, **1 M NaCl**, 1.0 % SDS | 190-225 | 1.73-1.87 |
| 7 | 100 mM Tris-HCl pH 8.0, 50 mM EDTA, **1.5 M NaCl**, 1% SDS (Current protocol) | 200-300 | 1.75–1.94 |

generated by using six different decamer primers (GTTTCGCTCC**,** AAGAGCCCGT, CCCGTCAGCA**,** TCCCGAACCG, AACGCGCAAC and CCCGTCAGCC) [47]. PCR amplification was performed in 25 $\mu$L reaction volume containing 1X PCR buffer, 2.5 mM $MgCl_2$, 0.25 mM each of dNTPs (dATP, dTTP, dCTP, dGTP), 0.2 $\mu$M primer, 50 ng genomic DNA and 0.75 units of Taq DNA polymerase. Amplification was performed in a thermal cycler (Master cycler, Eppendorff, Germany) programmed for 45 cycles of denaturation at 94 $^{\circ}$C for 1 min, annealing at 34 $^{\circ}$C for 1 min, extension at 72 $^{\circ}$C for 2 min with an initial denaturation at 94 $^{\circ}$C for 3 min and final extension at 72 $^{\circ}$C for 8 min. Amplified products were loaded in 1.5% agarose gel containing 0.5 $\mu$g/mL of ethidium bromide and documented by a gel documentation system (Bio-Rad, USA).

**RESULTS AND DISCUSSION**

Seeds are storage organs and hence rich in proteins, lipids, polysaccharides, alkaloids and other secondary metabolites. These compounds can interfere with DNA isolation and successive amplification. In the present study, we have successfully isolated DNA from fresh seeds of apple cultivars.

The comparative results of the protocols followed for DNA isolation from apple seeds and optimization of extraction buffer composition by modifying the concentration of different constituents have been shown in Tables **1** and **2**. In this study, we followed different protocols for genomic DNA isolation out of which method of Kim *et al.,* (1997) was found to give satisfactory results in terms of DNA yield and purity ($A_{260}/A_{280}$ ratio) (Table **1**). Then, we optimized the concentration of extraction buffer constituents by modifying them one by one and finally, developed a new protocol (Table **2**).

The DNA extraction from apple seeds employing existing protocols was found to contain lot of mucilage and low yield. Thus the critical change in our modified DNA isolation procedure turned out to be the combined use of high concentration of NaCl (1.5 M) and ammonium acetate (7.5 M) that significantly increased the efficiency of proteins, secondary metabolites and polysaccharide removal. Further the use of ethanol in contrast to isopropanol was found to be better in precipitating the final DNA yield in the solution. The yield of DNA was 200-300 ng/$\mu$L (total 8-12 $\mu$g DNA from 1-2 apple seeds).

The quality of DNA was assessed by spectrophotometry, gel electrophoresis, restriction endonuclease digestion and PCR amplification. A ratio of absorbance ($A_{260}/A_{280}$) in the range 1.8-2.0 indicates a high level of purity [48, 49]. Spectrophotometer measurements (at 260 nm and 280 nm) of isolated DNA samples of all apple verities gave an absorbance ratio ($A_{260}/A_{280}$) of 1.75-1.94 indicating high purity.
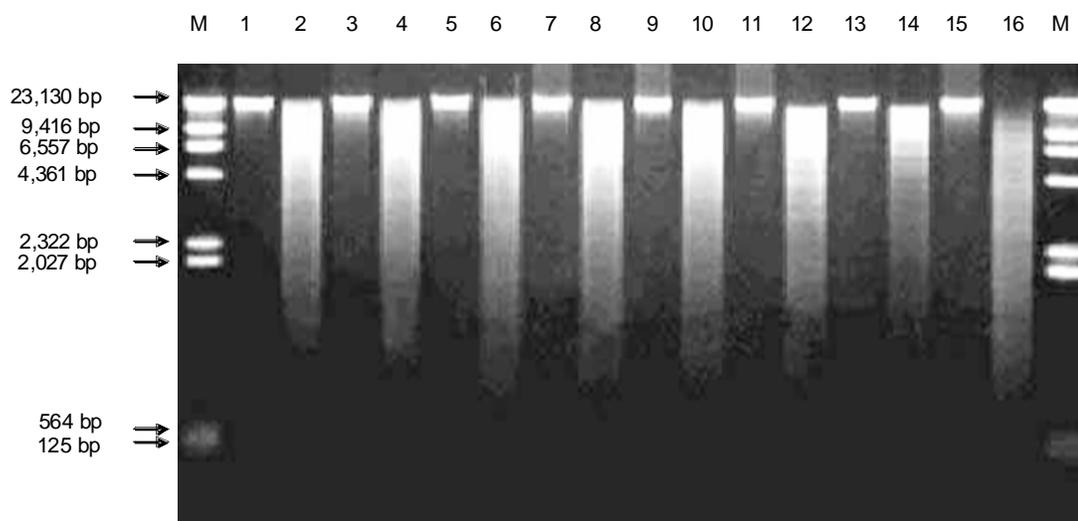
**Fig. (1).** Genomic DNA prepared from seeds of 8 different cultivars of apple (*Malus sp.*). M: DNA size marker (Hind III digested Lambda DNA). Lanes 1–16, 8 different apple cultivars and their respective restriction digests with EcoR V in adjacent lanes. 1-2: 'Chocolaty'; 3-4: 'Golden Delicious' HP; 5-6: 'Golden Delicious' JK; 7-8: 'Kali Devi'; 9-10: 'Maharaji'; 11-12: 'Punchmukhi'; 13-14: 'Red Delicious' JK; 15-16: Chinese.
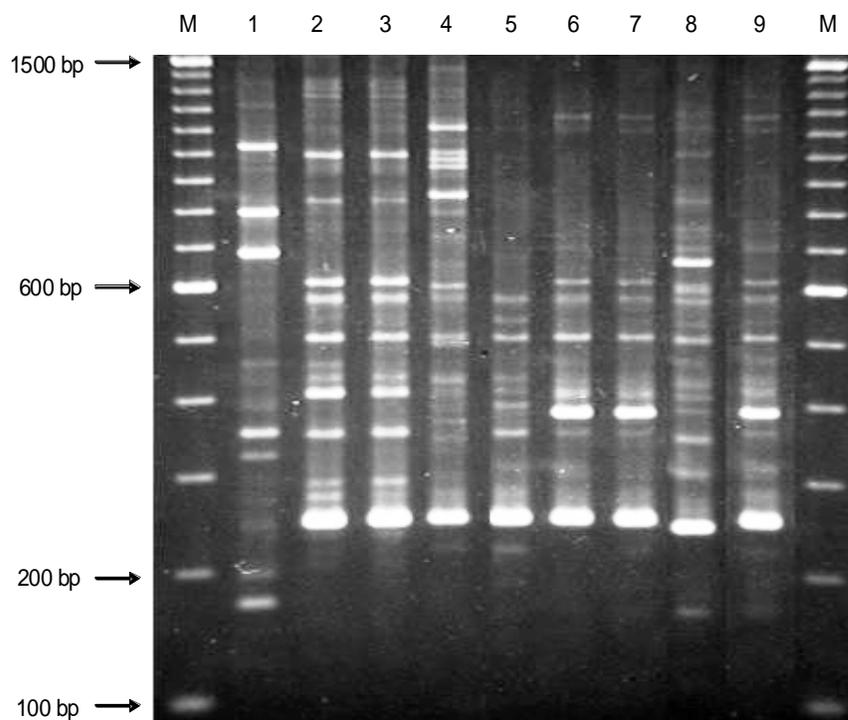


**Fig. (2).** RAPD profiles of 8 different apple cultivars: PCR reaction was performed as described in Materials and Methods. DNAs were amplified using primer 5'-GTTTCGCTCC-3'. M: 1.5 kb DNA ladder as marker. Lanes 1–9, 8 different apple cultivars. 1: 'Chocolaty'; 2: 'Golden Delicious' HP; 3: 'Golden Delicious' JK; 4: 'Kali Devi'; 5: 'Maharaji'; 6-7: 'Punchmukhi' (duplicate) 8: 'Red Delicious' JK; 9: Chinese.

The quality of DNA was also checked by agarose gel electrophoresis. We observed conspicuous intact bands of high molecular weight DNA (Fig. **1**). Several reports have indicated that the presence of RNA can suppress PCR amplification and lead to non-reproducible and unreliable DNA amplification patterns in RAPD analysis [50]. The treatment of

RNase A degrades RNA into small ribonucleosides that are not detectable by gel electrophoresis.

Plant molecular applications such as RAPD, SSR and AFLP necessitate the successful isolation of high quality DNA, devoid of contaminants [39, 51, 52]. Without high quality DNA such downstream molecular manipulations are not feasible. The suitability of extracted DNA for down-

stream molecular processes was further verified by restriction endonuclease digestion and RAPD-PCR amplification. We successfully digested 1 $\mu$g of DNA of all eight different cultivars of apple with 20 U of every restriction enzyme such as EcoR V (Fig. **1**), Hind III, Dra I, Bam HI, Bgl II and Kpn I (results not shown). The genomic DNA of all eight varieties of apple was highly amplifiable by PCR as indicated by the amplification products resolved on 1.5 % agarose gel (Fig. **2**). Although, the RAPD profiles were generated with six primers as reported by Stark-Uranau [47], here we have shown the result with one primer only (5'-GTTTCGCTCC-3'). These results further confirmed the purity of the DNA, free of polysaccharide and polyphenol contamination, which would otherwise inhibit the activity of Taq DNA polymerase and restriction endonucleases [39, 52].

Although the experiments were carried out exclusively with eight different apple cultivars, we expect that this protocol might be applicable to all woody plants of Rosaceae family. In addition, this procedure is affordable and does not require sophisticated equipment, making it a superior choice relative to expensive commercial kits for DNA extraction.

## CONCLUSION

In summary, PCR amplifiable high quality DNA extracted from the protocol reported in the current study have the potential to play a very important role in developing strategies for further improvement of *Malus* species through DNA polymorphism, genome mapping, identification of the QTLs and other plant breeding approaches such as marker assisted breeding.

## CONFLICT OF INTEREST

None declared.

## ACKNOWLDGEMENTS

## ABBREVIATIONS

QTLs      =    Quantitative trait loci

RAPD      =    Random amplified polymorphic DNA

RFLPs     =    Restriction fragment length polymorphisms

SDS       =    Sodium dodecyl sulphate

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