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Plant genomic DNA extraction protocol for genotyping by sequencing as policy issues & planning in agriculture in India

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Abstract
High quality DNA is essential for next generation sequencing, yet CTAB-based extractions from polysaccharide- and metabolite rich plant tissues often yield fragmented, contaminated DNA with high chaotropic agents. To address these limitations, we present an minimal-reagent optimized CTAB protocol incorporating three key refinements: extended gentle incubation with 3X CTAB buffer at 60–65 °C to improve cell lysis, enhanced salt precipitation using 0.5×6 M NaCl and 0.1×3 M sodium acetate and ethanol to increase DNA yield and remove polysaccharides and secondary metabolites, and dual 70% ethanol washes with low-volume pipette to eliminate residual salts.
The optimized CTAB method yielded genomic DNA in Indian bean (*Lablab purpureus*) at 1481.65±673.98 ng/µL with purity (A260/280 = 2.1±0.07; A260/230 = 2.09±0.27). RNase A treatment increased yield to 1965.33±803.58 ng/µL while maintaining purity during −20 °C storage. Genotyping by sequencing (GBS) using *MspI/PstI* digestion of gDNA from 145 parental and recombinant inbred lines generated 87.5 M and 86.0 M reads per Ion 540 chip with ~1.09±0.57 M raw reads per sample (Phred≥15) and ~0.68±0.36 M high-quality reads retained after preprocessing (Phred ≥25). The protocol requires minor adjustments for recalcitrant tissues but remains a reliable, cost-effective, and scalable solution for SNP discovery, particularly in resource-limited genomics laboratories.

Keywords: CTAB-based extraction, DNA, next generation sequencing, genotyping by sequencing, SNP discovery

Introduction

High molecular weight (HMW) genomic DNA (≥50 kb) is indispensable for next generation sequencing platforms, which generate long reads that facilitate accurate *de novo* assemblies and structural variant analyses (Amiteye, 2021) [5]. However, plant tissues pose unique challenges for DNA isolation due to rigid cell walls, abundant polysaccharides, and secondary metabolites that co-precipitate with nucleic acids, leading to reduced yield and compromised purity (Doyle & Doyle, 1987; Gautam, 2022) [8, 11]. Cetyltrimethylammonium bromide (CTAB) based plant DNA isolation is widely used because this cationic detergent effectively removes polysaccharides and secondary metabolites during genomic DNA extraction (Gautam, 2022) [11]. The earliest CTAB-based plant DNA isolation method was introduced by Doyle and Doyle (1987) [8], later refined by Doyle (1991) [7], laid the foundation for plant genomic DNA extraction protocols. Over the years, various researchers have introduced targeted optimizations to the CTAB-based DNA isolation method to improve yield and purity across diverse plant species (Abdel-Latif & Osman, 2017; Aboul-Maaty & Oraby, 2019; Agbagwa *et al*., 2012; Gill *et al*., 2025; Mavrodiev *et al*., 2021; Xie *et al*., 2023) [1, 3, 4, 12, 20, 26]. Prolonged, gentle incubation at 60 °C helps preserve long DNA fragments in recalcitrant tissues (Healey *et al*., 2014) [13]. A recent study reported that 1 hour incubation with CTAB buffer and dual salt (NaCl and potassium acetate) precipitation enhances DNA purity across different plant orders (Aboul-Maaty & Oraby, 2019) [3]. Studies on modified CTAB based DNA extraction methods showed that dual-salt systems (NaCl and sodium acetate) yield cleaner DNA pellets by removing polysaccharides and secondary metabolites (Abdel-Latif & Osman, 2017; Agbagwa *et al*., 2012) [1, 4]. These key studies have shown that incorporation of several optimizations in CTAB based protocol can improve DNA quality and quantity. Commercial DNA extraction kits have addressed these issues by incorporating bead or silica based purification for improved quality and quantity of DNA but are too expensive if intended for large number of samples. Silica columns cleanup can also be used with CTAB based DNA extraction methods (Mavrodiev *et al*., 2021) [20]. Despite these advancements, limitations such as inconsistent yield and residual contamination persist in diverse plant species due to their complex genetic and phenotypic structures. Native CTAB-based extraction methods often yield fragmented DNA contaminated with polysaccharides, phenolics, and other secondary metabolites, particularly in plant species with complex biochemical profiles. To overcome these limitations, researchers have explored additives such as polyvinylpyrrolidone (PVP), Proteinase K, RNase A, and alternative buffer systems in different gDNA isolation protocols. While use of these additives improve DNA purity, the use of multiple chaotropic agents require

more labor intensive cleanup steps to remove contaminants, otherwise residual contaminants can interfere with downstream applications such as PCR, enzymatic digestion, and sequencing. Moreover, the success of next-generation sequencing (NGS) depends on stringent DNA quality parameters, including purity, integrity, and fragment length. High-quality genomic DNA should be intact with minimal shearing, as fragmentation reduces library complexity and sequencing efficiency. Optimal purity is indicated by A260/280 (~1.8) and A260/230 (>2.0) ratios, reflecting low contamination from proteins, polysaccharides, and phenolics (Heikrujam *et al*., 2020) [14]. DNA integrity is particularly important for long-read platforms, which require high-molecular-weight fragments (>50 kb), while ultra-long reads (>100 kb) significantly improve genome assembly and structural variant detection (Jain *et al*., 2018) [15]. Accurate quantification using fluorometric methods further ensures reliable library preparation and downstream analysis (Lucena-Aguilar *et al*., 2016) [19].

Genotyping-by-sequencing (GBS), first described by Elshire et al. (2011) [9], is a reduced-representation sequencing approach that employs restriction enzymes to simultaneously discover and genotype genome-wide SNP markers in a cost-effective and high-throughput manner. In plant genomics, GBS is extensively used for applications such as genetic diversity analysis, QTL mapping, genome-wide association studies (GWAS), and marker-assisted selection, making it highly dependent on high-quality genomic DNA typically obtained through optimized CTAB extraction protocols. Currently, GBS remains a widely adopted and evolving platform, increasingly integrated with advanced bioinformatics tools and high-quality reference genomes to enhance precision in crop improvement and functional genomics studies. For genotyping-by-sequencing (GBS), sequencing depth and read coverage are equally critical for SNP discovery and genotyping accuracy. GBS utilizes restriction enzyme–based genome reduction to enable cost-effective genotyping across diverse plant genomes (Elshire *et al*., 2011) [9]. Typically, 0.5–2 million reads per sample provide a balance between cost and genome coverage, though ~1–2 million reads are recommended for high-diversity species (Elshire *et al*., 2011; Thomas *et al*., 2023) [9, 25]. Lower read counts (~200,000–300,000) may be sufficient for preliminary analyses, whereas >1 million reads significantly improve marker density and genome coverage, as demonstrated in barley studies (Chen *et al*., 2024) [6]. In terms of sequencing depth, GBS generally operates at 1×–5× coverage, which is adequate for population studies; however, higher depth (5×–10×) is preferred for heterozygous species and improved genotyping accuracy (Elshire *et al*., 2011; Thomas *et al*., 2023) [9, 25]. Therefore, for *Lablab purpureus*, ~1–2 million reads per sample with 3×–5× depth are recommended for reliable SNP detection, while ~500,000 (0.5 M) reads may suffice for exploratory studies. These considerations highlight the importance of integrating high-quality DNA extraction with optimized sequencing design for robust GBS-based genomic analyses.

To evaluate and refine the native CTAB based DNA extraction protocol for isolating high quality, high molecular weight DNA, we selected Indian Bean (*Lablab purpureus*) recombinant inbred lines (RILs) as a model system due to their pronounced genetic diversity, complex genome architecture, and rich biochemical composition. High quality DNA isolation of those RILs is also important for studying genotyping by sequencing analysis (GBS) and genome wide association (GWAS) mapping of quantitative trait loci (QTL) in the field of molecular plant breeding. As a legume of significant agronomic value, *Lablab purpureus* is known to accumulate high levels of phenolic compounds, polysaccharides, and secondary metabolites, cellular components that commonly interfere with DNA extraction and purification. These biochemical challenges, coupled with the species' genomic complexity, make it an ideal candidate for testing the robustness and reproducibility of modified extraction protocols. The inclusion of six genetically diverse parental lines, RILs of Indian bean (*Lablab purpureus*) and thirteen different crop and fruit tree species enabled a comprehensive assessment of optimized CTAB based DNA isolation protocol performance across a wide genetic background. Despite its effectiveness, the CTAB-based method may still require minor modifications depending on tissue type, metabolite composition, and species-specific characteristics. The protocol can be time-consuming and involves multiple steps that may increase the risk of DNA shearing or contamination if not carefully executed. Additionally, the use of hazardous chemicals such as chloroform necessitates proper handling and safety precautions. In some cases, residual contaminants (e.g., polysaccharides or phenolic compounds) may still affect DNA purity, potentially impacting highly sensitive downstream applications. This experiment will enable researchers to isolate high quality, high molecular weight genomic DNA (gDNA) using an optimized CTAB based method using commonly available laboratory reagents. The approach enhances extraction efficiency from recalcitrant plant tissues and ensures the suitability of the recovered gDNA for downstream applications, including next-generation sequencing (NGS) and other molecular analyses.

Materials and Methods

Plant materials

This experiment consists of six genetically and phenotypically diverse parental lines, 139 RILs of Indian bean (*Lablab purpureus*) and thirteen different crop and fruit tree species representing a broad spectrum of genetic and phenotypic diversity for CTAB based DNA isolation. The Indian bean RILs and parental lines were grown in augmented block design in research farm and laboratory work was done in the Laboratory of Department of Genetics and Plant Breeding, BHU, Varanasi during my doctorate (2003-2007) as minor courses & DMR building IARI, New Delhi, India during 2008-09 with other students for genotyping and phenotyping. Young leaves were taken as sample for DNA isolation from Indian bean lines, however, green mature leaves from diverse crop and fruit trees were collected directly from farm because of their perennial nature. The diverse plant species used for gDNA isolation were thirteen cultivated and wild plant species, including rice, sugarcane, Indian bean, pigeon pea, cotton, turmeric, castor, *Anona*, *Sesbania*, finger millet, neem, banana, and money plant. These plant species were selected based on their agricultural, medicinal, and ecological relevance. The tested genotypes exhibited variation in pigmentation, growth habit, flowering behavior, fruit characteristics, and yield potential traits often associated with elevated levels of polysaccharides, phenolics, and other secondary metabolites

that complicate DNA extraction using conventional CTAB methods. The list of crops and samples are given in supplementary data.

Reagents and Instruments

The chemicals and reagents used in this study, along with their manufacturers, catalog numbers, EC (European Community) numbers, and CAS (Chemical Abstracts Service) numbers, are listed below (Table 1). All materials were sourced from certified suppliers to ensure consistency, purity, and reproducibility of the experimental procedures. The inclusion of EC and CAS numbers provides unique identifiers for each chemical substance, supporting accurate reference, regulatory compliance, and traceability.

Table 1: Chemical reagents required for the optimized CTAB based DNA isolation

S.N.	Chemicals required	Manufacturer	Catalog Number or Product Code	EC Number	CAS Number
1	Cetrimonium bromide (CTAB)	HIMEDIA, India	MB101	200-311-3	57-09-0
2	Sodium Chloride (NaCl)	HIMEDIA, India	MB023	231-598-3	7647-14-5
3	Tris, Free base	HIMEDIA, India	MB029	201-064-4	77-86-1
4	Ethylenediaminetetraacetic acid (EDTA), disodium salt dihydrate	HIMEDIA, India	MB011	205-358-3	6381-92-6
5	Sodium Acetate ($C_2H_3NaO_2$) anhydrous	SRL, India	1944117	204-823-8	127-09-3
6	Hydrochloric acid (HCl) (37%)	Merck, Germany	193001	231-595-7	7647-01-0
7	Sodium hydroxide (NaOH), low chloride pellets	Merck, Germany	193102	215-185-5	1310-73-2
8	Glacial Acetic Acid (99.5%), extrapure	SRL, India	90868	200-580-7	64-19-7
9	Chloroform (99.8%)	SRL, India	96764	200-663-8	67-66-3
10	Isoamyl Alcohol (98.5%)	HIMEDIA, India	MB091	204-633-5	123-51-3
11	2-β-Mercaptoethanol (≥98%)	HIMEDIA, India	MB041	200-464-6	60-24-2
12	Absolute ethanol (100%)	Merck, Germany	108543	200-578-6	64-17-5
13	Agarose, DNA grade for Molecular biology	HIMEDIA, India	MB080	232-731-8	9012-36-6
14	RNase A, DNase and protease-free (10 mg/mL)	Thermo Fisher Scientific, USA	EN0531		

Note: The chemicals used in this experiment for optimized CTAB based DNA isolation given above are commonly available in India, however, equivalent chemicals from different manufacturers based on CAS number can be conveniently used.

Summary of chemical reagents and laboratory equipments required per sample for this optimized CTAB based DNA isolation protocol are given in Table **2**. This summary helps to estimate the volume of reagents needed to isolate gDNA from any desired number of samples, making it easy to scale the optimized CTAB-based extraction protocol.

Table 2: Summary of reagents and instruments required for optimized CTAB based DNA isolation

S.N.	Name of reagents and instruments	Quantity required per sample and its use
1	Plant sample (leaves)	1-2 g green young leaves for easy grinding in liquid nitrogen and sufficient to collect powder (1-2 small freshly developed trifoliate leaves in case of Indian bean)
2	3X CTAB Extraction Buffer	800 µL
3	2 mL centrifuge tube	1 per sample, for powdered sample collection and 3X CTAB extraction buffer digestion in water bath
4	Chloroform:Isoamyl Alcohol (v/v)	800 µL
5	1.5 mL centrifuge tube	600 µL aqueous phase DNA collection
6	6M NaCl	300 µL (1/2 aqueous phase DNA)
7	3M Sodium Acetate	60 µL (1/10 aqueous phase DNA)
8	Icecold 100% Ethyl Alcohol (Ethanol)	500 µL (for DNA precipitation)
9	70% Ethanol (freshly prepared)	500 µL for wash steps
10	1X TE Buffer	100 µL for resuspension of gDNA from pellet
11	Liquid Nitrogen	100 mL per sample
12	Mortar & pestle	1 set per sample
13	Water bath	65 °C for 1-2 hour
14	Centrifuge machine	≥13000 at RT (24 °C)
15	Nanodrop for pure DNA	A260/280 and A260/230 ratios ≥1.8 (recommended)
16	Pipette and tips	10µL, 20 µL, 100 µL, 1000 µL size range

Note: The required concentration and volume of reagents need to be prepared first from stock solutions.

The stock reagents required for the this optimized CTAB based DNA extraction method can be prepared at low cost even in resource-poor laboratory with ease for large number of samples. The list of reagents used in this experiment are given in Table 1. The stock reagents with desired molarity and volume for CTAB based DNA isolation were prepared using calculations given in Table 3. Following formulas can be used for calculation of stock solution preparations and dilutions:

a. Formula to calculate the required weight of solute for a desired stock molarity:

Required Weight of Solute (g) =

$$\frac{Weight\ for\ 1\ M\ solution\ in\ g}{Volume\ for\ 1\ M\ solution\ in\ mL} \times (Desired\ Volume\ in\ mL \times Desired\ Stock\ Molarity\ in\ M) \quad (2.1)$$

b. Formula to dilute the stock concentration in to working solution:

$$Stock\ Concentration\ (C1) * Stock\ Volume\ (V1) = Final\ Concentration\ Required\ (C2) * Final\ Volume\ Required\ (V2) \quad (2.2)$$

Table 3: Stock reagents preparations with desired molarity and volume

S.N.	Reagents required [use equation (2.1)]	Molecular Weight_g (for 1M)	Volume for 1M in ml	Desired Molarity in M	Desired Volume in ml	Quantity required for Desired Molarity in g or ml	Remarks
A.	**Primary reagent preparation at Laboratory**						
1	10% CTAB (*w/v*)	364.45	1000	10%	100	10	10% Stock
2	NaCl	58.44	1000	6	250	87.66	6M Stock
3	Tris-HCl, pH 8.0 (HCl adjusted pH)	121.14	1000	1	100	12.114	1M Stock
4	EDTA, pH 8.0 (NaOH adjusted pH)	372.24	1000	0.5	100	18.612	0.5M Stock
5	Sodium Acetate (Glacial Acetic Acid adjusted pH)	82.03	1000	3	100	24.609	3M Stock
B.	**Reagents for pH adjustment**						
6	HCl (11.97M) 37% (w/w) 1M=1N, density=1.18g/mL for balancing Tris pH at 8.0	0.4366	1000	1	500	41.754	1N HCl Dilution Stock
7	NaOH 1M for balancing EDTA pH at 8.0	40	1000	1	500	20	1M NaOH Stock
8	Glacial Acetic Acid (>99% purity) for balancing Sodium Acetate pH at 5.2	Use pure glacial acetic acid for balancing pH of Sodium Acetate solution at pH 5.2					Stock
C.	**Other reagents**						
9	Chloroform: Isoamyl Alcohol (v/v)	24 mL Chloroform:1 mL Isoamyl Alcohol (24:1 v/v)					Fresh use
10	2-β-Mercaptoethanol (37%)	Add 0.3% (v/v) of 3X CTAB Extraction Buffer volume just before use					Fresh use
11	Ice cold 100% ethanol	Use chilled ethanol and fresh each time					Fresh use
12	70% Ethanol	Freshly prepared chilled ethanol					
13	Agarose (Molecular Biology Grade)	0.8% agarose gel electrophoresis in 1X TAE or 1X TBE at 80V for 90 minutes					Gel electrophoresis
14	Double distilled or millipore water	Autoclaved water for making dilutions and working solutions					Dilution preparations

After preparation of stock solutions and adjusting pH, the stock solutions and double distilled water are autoclaved and can be stored at room temperature (20-30 °C) for use within six months to one year without any problem in DNA extraction and without compromising quality. While using stock solution, these need to be mixed well and incubated at 65 °C water bath before use if salt is precipitated at the bottom of the bottle. These stock solutions prepared above in Table 3 can be used for 1X TE and 3X CTAB Extraction buffer preparations. The 1X TE buffer can be prepared using Table 4 from the stock solutions prepared above in Table 3.

Table 4: Preparing 1X TE (low TE) buffer from stock solutions

1X TE Buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0) for 100 mL [use equation (2.2)]	Stock Concentration (C1) in M	Final Concentration (C2) in M	Final Volume (V2) in mL	Stock Volume Required (V1) in mL	Volume in µL
10mM Tris=0.01M Tris	1	0.01	100	1	1000
1mM EDTA=0.001M EDTA	0.5	0.001	100	0.2	200
H_2O				98.8	98800
Total				100	

The 3X CTAB extraction buffer need to be prepared fresh each time for DNA isolation because of precipitation of buffer salts and evaporation of β-mercaptoethanol in long storage at low temperature. The 3X CTAB extraction buffer can be prepared fresh using Table 5 from the stock solutions prepared above in Table 3.

Table 5: Preparation of 3X CTAB extraction buffer from stock solutions

CTAB Extraction Buffer (10 Samples) – for 8 mL [use equation (2.2)]	Stock Concentration (C1) M	Final Concentration (C2) M	Final Volume (V2) mL	Stock Volume Required (V1) mL	Volume in µL
3% CTAB (w/v)	10	3	8	2.4	2400
1.4M NaCl	6	1.4	8	1.867	1866.67
0.1MTris-HCl, pH 8.0 (HCl adjusted pH)	1	0.1	8	0.8	800

0.02M EDTA, pH 8.0 (NaOH adjusted pH)	0.5	0.02	8	0.32	320
0.3% (v/v) 2-β-Mercaptoethanol (37%)	100	0.3	8	0.024	24
Subtotal				5.411	5410.67
H_2O added				2.589	2589.33
Final CTAB Extraction Buffer Volume (V2)				8	8000

Optimized CTAB based DNA Isolation Protocol

Sample preparation and cell lysis

1. Take 1-2 grams of fresh leaf tissue (1-2 young meristematic trifoliate leaves) for grinding by mortar & pestle in liquid nitrogen at -196 °C. Collect crushed tissue powder up to 0.5 mL label in 2 mL Eppendorf tube which is approximately 100-500 mg powder.
2. Add 800 µL of preheated (65 °C) 3X CTAB extraction buffer per sample with freshly added 0.3% volume of β-mercaptoethanol just before use.
3. Incubate sample mixed with extraction buffer at 60–65 °C for 1-2 hours with gentle mixing by inverting 15-20 times at 15 minutes interval. If the cell lysate is viscus or jelly-like, increase the time for incubation up to 4 hrs at 65 °C for proper digestion of cell debris. This will improve the transparency of the lysate and improve DNA yield and purity.

Phase separation and DNA collection

1. Cool down lysate from cell lysis step to room temperature (20-25 °C).
2. Add equal volume (800 µL per sample) of Chloroform:Isoamyl alcohol (24:1 v/v) to the incubated and cooled cell lysate in 2 mL centrifuge tube.
3. Centrifuge at 13,000 rpm for 20 min at room temperature (RT) and carefully collect the upper clear aqueous phase (~600 µL). The aqueous phase (DNA solution) is mostly clear or maybe with pigmented solution based on the tissue type, however, don't disturb the cell debris pellet floating in between solution and a thin layer at the top of upper aqueous phase.
4. Carefully transfer the clear upper aqueous phase (~600 µL) to 1.5 mL tube which contains the DNA. Set the pipette to 600 µL and then aspirate clear upper aqueous phase in one-time pipetting for consistency in all sample. For high quality DNA, leave remaining aqueous phase to prevent any trace of leaf debris and metabolites.
5. Now the collected aqueous phase in 1.5 mL tube is used further for DNA precipitation step.

DNA Precipitation

1. The DNA solution collected in new 1.5 ml centrifuge tube is now subjected to high salt ion concentration with addition of NaCl and sodium acetate ions along with ethanol which neutralizes the negative ions on DNA thus precipitating it from the solution to get the pellet form of DNA after centrifuge.
2. Half (1/2) the volume of aqueous phase collected above, i.e. 300 µL of 6M NaCl is added to the phase collected in 1.5 ml centrifuge tube above.
3. Add one tenth (1/10) the volume of aqueous phase collected above, i.e. 60 µL 3M sodium acetate to the same tube.
4. Add two thirds (2/3) or above the volume of aqueous phase collected above, i.e. 500 µL of ice cold 100% chilled Ethanol and mix well by gentle inversion or tap mixing with finger tips.
5. Invert gently 3-5 times to precipitate DNA until the formation of fine DNA threads.
6. Incubate at – 20 °C for 1-2 hr (quick protocol) or overnight (>24 hr) for higher DNA yield (full length protocol). Quick protocol is suitable for getting HMW long DNA fragments, however, full length protocol yields higher concentration of short DNA fragments.
7. After incubation at -20 °C, centrifuge at 13,000 rpm for 5 min, discard supernatant.
8. Carefully decant all supernatants, ensuring the translucent, watery-white DNA pellet remains undisturbed at the bottom of the tube.
9. Immediately remove any residual supernatant by gently tilting the tube and using a 20 µL pipette to aspirate or pull out the remaining liquid (precision pipetting) without disrupting the pellet. This step is critical for eliminating residual salts, proteins, and polysaccharides, which can interfere with downstream applications.

DNA Purification

1. Add 500 µL of freshly prepared 70% ethanol to DNA pellet obtained from precipitation step and invert 3-5 times to dissolve residual salts attached to the pellet as well as tube wall to increase purity of the DNA.
2. Centrifuge at 13,000 rpm for 10 min, discard 70% alcohol from tubes after centrifugation
3. Add 500 µL of freshly prepared 70% ethanol again to DNA pellet for second ethanol wash. Centrifuge at 13,000 rpm for 10 min, DNA pellet at this stage must be clear white.
4. Remove all the supernatants by pouring them off and give one gentle dry spin of 13000 rpm for 1 minute to drain all remaining salts and impurities at the bottom of tube with supernatant.
5. Use 20 µL pipette to drain out all supernatants with salts and impurities at the bottom of the tube without disturbing the DNA pellet. The lower the pipette volume capacity higher will be the precision of low volume aspirated or taken through pipette tips, thus precisely removing the traces of residues in the supernatant.
6. Allow tubes containing pellets to air dry at room temperature for 15-20 minutes (excessive air drying will make it difficult to resuspend DNA pellet into solution).

DNA Resuspension

1. After air drying the pure DNA pellet from dual wash purification step, the DNA pellet is now dissolved in low TE buffer i.e. 1X TE buffer for long term storage at -20 °C without degradation of DNA. Either nuclease free water or low TE can be used for resuspension, however, EDTA chelates the divalent Mg^{2+} and Ca^{2+} ions which are essential cofactors of nucleases (DNase, RNase) and ultimately inhibits the nuclease activity of

nucleases (Jones *et al*. 2021) [16] which protects DNA from degradation in 1X TE buffer for long time.
2. Resuspend the DNA pellet in 50-100 µL 1X TE buffer, pH 8.0 or nuclease free water.
3. Incubate the DNA at 50 °C for 1 to 2 hr to ensure complete resuspension.
4. Check DNA quality and quantity in 0.8-1% agarose gel electrophoresis, check quantity and quality in Nanodrop spectrophotometry and store at − 20 °C till further use.

Note: We have successfully applied that if previously isolated gDNA sample need to be repurified or resuspended at higher concentration, steps from DNA precipitation can be done successfully with satisfactory results by adding half (1/2) 6M NaCl, one tenth (1/10) 3M sodium acetate, and two thirds (2/3 or more) ice cold 100% chilled Ethanol of the volume of gDNA in tube. Further purification and resuspension steps can be followed following this same protocol. Higher concentration of sample can be obtained by resuspending the DNA pellet in lower volume of low TE buffer or nuclease free water (30-50µL) for getting concentrated DNA.

DNA Quantity and Quality Assessment

Gel electrophoresis

After DNA resuspension in low TE buffer, the integrity and purity of the genomic DNA (gDNA) was evaluated using agarose gel electrophoresis. A volume of 1 µL of DNA at a concentration of 500 ng/µL was mixed with loading dye and nuclease-free water to make a final loading volume of 5 µL per sample. Samples were loaded onto a 0.8% agarose gel prepared with ethidium bromide as DNA binding or staining dye. Electrophoresis was performed under standard conditions of 80V for 90 minutes in TAE (Tris-Acetate-EDTA) buffer, and DNA bands were visualized using the Bio-Rad Gel Doc XR imaging system (Bio-Rad, USA) following the manufacturer's guideline. The presence of intact, high molecular weight bands with minimal smearing was used as an indicator of good DNA integrity. Absence of smearing at the end of well in agarose gel was taken as an indicator of pure DNA with no RNA contamination.

Nanodrop spectrophotometry

Spectrophotometry is a widely used method for assessing the concentration and purity of nucleic acids. In this technique, DNA samples are typically dissolved in 1X TE buffer, which is also used as the blank during measurement to account for background absorbance. Following reading were observed in Nanodrop One (Thermo Fisher Scientific) following manufacturer's protocol (Thermo Fisher Scientific, 2017a) by loading 2 µL of DNA for quantity and quality check:

1. **260 nm:** Absorbance of nucleic acids (DNA and RNA).
2. **280 nm:** Absorbance of proteins, particularly aromatic amino acids.
3. **230 nm:** Absorbance of contaminants such as salts, polysaccharides, and organic compounds.
4. **A260/280 ratio:** 1.8-2.0 for pure DNA and ≥2.1 for pure RNA free from proteins
5. **A260/230 ratio:** >1.8 free from chaotropic salt contaminants

RNA removal with RNase A treatment

RNA contamination can interfere with DNA quantification and downstream applications like NGS. To digest RNA from CTAB isolated gDNA, 4 µL (40 µg) of RNase A (10 µg/µL; Thermo Fisher Scientific, Catalogue Number EN0531) (Thermo Fisher Scientific, 2017b) was added to 100 µL of (~1000 ng/µL) gDNA per sample. The mixture was incubated at 37 °C for 1 hour with gentle mixing by tapping with finger tips every 15 minutes. After the enzymatic digestion of DNA sample, RNase A was inactivated by incubating the sample at 65 °C for 20 minutes. These digestion and inactivation steps finally yield highly pure DNA (A260/280, A260/230 ≥ 1.8) suitable for gel electrophoresis, Nanodrop, Qubit quantification, and NGS library preparation without further cleanup steps. In this experiment, we have tested several concentrations of RNase A (10 µg to 100 µg per sample) and observed that use of excess RNase A (>40 µg per sample) increases salt contamination, lowering A260/280 and A260/230 ratios. RNase A concentrations above 40 µg per 100 µL DNA reduce purity due to salt contamination because of high concentration of RNase A. Optimal results are achieved with 30–40 µg RNase A treatment to 100 µL of CTAB isolated DNA without repurification.

Qubit fluorometric assay

Qubit assay uses DNA-specific fluorescent dyes that bind selectively to double-stranded DNA. Unlike Nanodrop, Qubit fluorometric assay is highly sensitive and accurate for quantifying double-stranded DNA or single stranded RNA, with a detection range typically between 1–1000 ng/µL. It is especially reliable for low-concentration samples and is not affected by contaminants such as RNA, proteins, or salts. This makes Qubit the preferred method for DNA quantification in sensitive downstream applications, including next-generation sequencing (NGS). In this experiment, Qubit measurements were taken by loading 2 µL of DNA sample following manufacturer's protocol (Qubit™ dsDNA HS Assay Kit, Thermo Fisher Scientific, Catalog No. Q32854) (Thermo Fisher Scientific, 2022) [24]. Quantification results from Qubit are more reliable for NGS applications (accurate quantification of nucleic acids), however, gel electrophoresis and/or Nanodrop results are pre-requisite for quality assurance in any applications.

Validation Of CTAB Extracted DNA Quality in Genotyping By Sequencing (GBS)

Genomic DNA from *Lablab purpureus* accessions was processed for genotyping by sequencing (GBS) using the method protocol described by Abed *et al*. in 2019 [2] (Abed *et al*., 2019) [2], adapted for the Ion Torrent platform. All the required reagents and commercial kits essential for the GBS experiment were purchased from the same manufacturers and the methodology was followed exactly as described in the published protocol (Abed *et al*., 2019) [2]. DNA was extracted using a CTAB-based method, treated with RNase A, and normalized to 200 ng in 20 µL volume per sample. Libraries were prepared by ligating barcoded and common adapters (designed oligonucleotides) to fragments generated with *PstI* and *MspI* (New England Biolabs, USA). After ligation, fragments were pooled and cleaned using the QIAquick PCR Purification Kit (Qiagen, Germany), size-selected using 2% E-gel systems (Thermo Fisher Scientific), and enriched by PCR amplification with Ion Express primers and Q5 high-fidelity polymerase (New England

Biolabs). The enriched libraries were then purified using AMPure magnetic beads (Qiagen, Germany) prior to sequencing. Sequencing was performed on the Ion Torrent S5 system with 540v1 chips using the Ion 540 Kit-Chef (Thermo Fisher Scientific). Sequencing of GBS library was performed on the Ion S5 GeneStudio system using the Ion 540 Kit-Chef templating workflow and two Ion 540 chips. Single-end sequencing was carried out according to the manufacturer's instructions on Ion 540 Kit-Chef (Thermo Fisher Scientific), using the Ion S5 sequencing chemistry. Base calling and demultiplexing of barcoded samples were performed with the inbuilt Torrent Suite software (v5.18.1) plugins, following default parameters. The details of inputs and protocol used for GBS are given in Table 6.

Table 6: Genotyping by sequencing of Indian bean parental lines and RILs in IonTorrent

Particulars	Chip 1	Chip 2
Genomic DNA Sample (barcoded and multiplexed)	73 samples	72 samples
gDNA Qubit quantity per sample before library preparation	10 ng/µL per sample	10 ng/µL per sample
Template gDNA quantity used for library preparation	200 ng in 20 µL	200 ng in 20 µL
Final library used for Ion S5 Sequencing	100 pM	100 pM
Library size selection	150-200 bp	150-200 bp
Kits and protocol used	Published genotyping by sequencing protocol by (Abed *et al*., 2019).	

Note: gDNA Qubit quantity was different in different samples, however, were diluted to similar concentrations of 10 ng/µL per sample in nuclease free water before starting sequencing library preparation.

Validation Of CTAB Extracted DNA Quality in Diverse Crop Species

Additionally, genomic DNA was successfully isolated from thirteen cultivated and wild plant species, including rice, sugarcane, Indian bean, pigeon pea, cotton, turmeric, castor, *Anona, Sesbania*, finger millet, neem, banana, and money plant. These species were selected based on their agricultural, medicinal, and ecological relevance and are commonly used in molecular experiments. The tested genotypes exhibited variation in pigmentation, growth habit, flowering behavior, fruit characteristics, and yield potential often associated with elevated levels of polysaccharides, phenolics, and other secondary metabolites that complicate DNA extraction using conventional CTAB methods.

Statistical analysis

All data entry and simple analysis of CTAB isolated gDNA data measured from Nanodrop and Qubit were done in MS Office 365 Excel (Microsoft Corporation, USA) and summary statistics and graphs were generated with SAS Studio 3.8 (SAS on Demand for Academics, SAS Inc, USA). The specialized graphing and visualization procedures PROC SGPLOT, PROC FREQ and PROC GLM were used in SAS Studio following task reference guide (SAS Institute Inc, 2019) [21] with some manual modification for clear visualization. The sequencing data obtained in FASTQ reads were visualized and analyzed in Torrent Suite software (v5.18.1) in Ion Torrent platform (Thermo Fisher Scientific, USA) following default manufacturer's parameters.

Results

DNA Quantity and Quality Assessment

Gel electrophoresis results

High molecular weight DNA should appear as a distinct, high-intensity band with minimal smearing, indicating intact genomic DNA. The absence of RNA contamination is confirmed by the lack of low-molecular-weight smears or bands. This visual assessment complements spectrophotometric and fluorometric analyses, ensuring the DNA is suitable for downstream applications such as NGS. shows the gDNA isolated using CTAB based method before RNase A treatment.

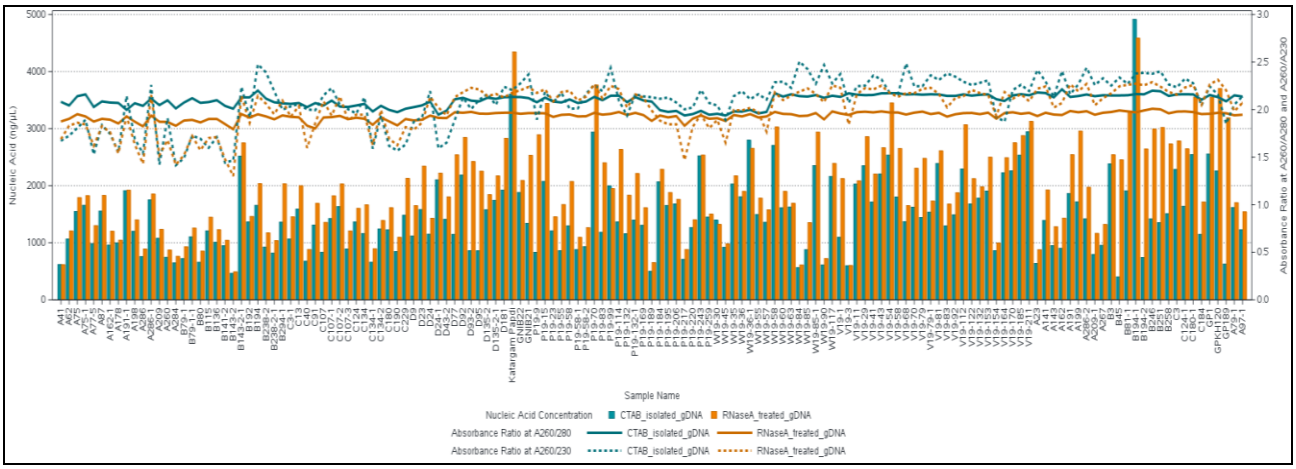

Fig: Nucleic acid concentration and purity ratios in Indian bean population

All the samples have acceptable nucleic acid concentration (≥ 200 ng/µL for most applications) and A260/280 and A260/230 purity ratios ≥1.8 in CTAB isolated gDNA suitable for next generation sequencing.

Qubit Fluorometry results of Indian bean RILs and parental lines

Qubit plus (Thermo Fisher Scientific, USA) was used for the precise quantification of double stranded DNA in each

sample before sequencing. All the Qubit readings and Nanodrop readings after RNase A treatment is given in
Fig: . Generally, we need total of 150-200 ng in 10-20 µL Qubit measured gDNA per sample for whole genome sequencing as well as genotyping by sequencing i.e. ≥10 ng/µL gDNA is recommended per sample. In this experiment, we also found that our sample meets the basic requirements for WGS and genotyping by sequencing (
Fig:). Based on the Qubit measurements, almost all samples have pure double stranded gDNA ≥10 ng/µL except few samples with ~4 ng/µL. The measurements recorded in Qubit are given in Supplementary Information.

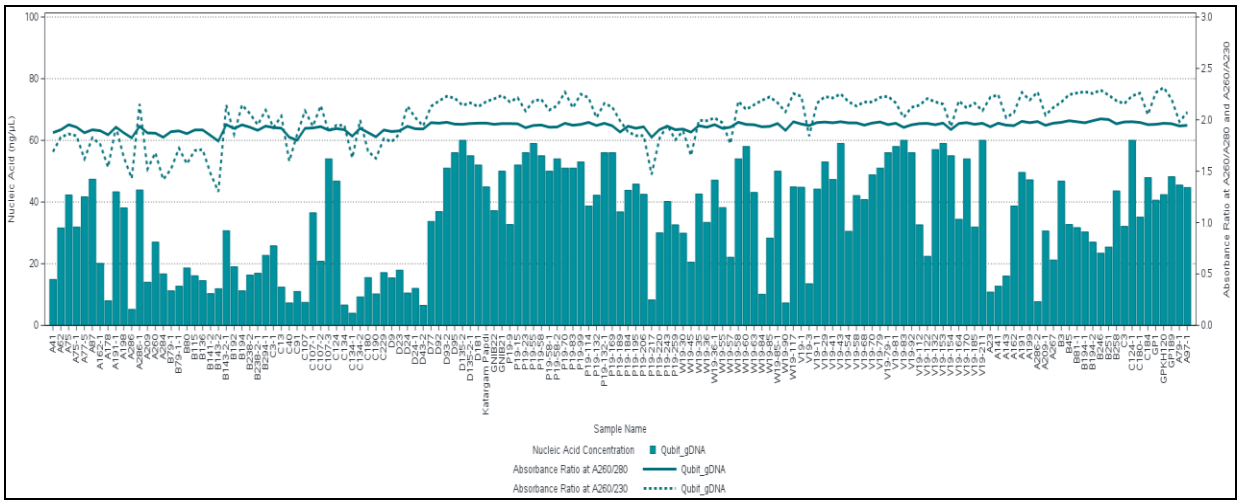

Fig: Nucleic acid concentration from Qubit and purity ratios from Nanodrop

Almost all the samples have acceptable Qubit measured quantity (≥10 ng/µL) and Nanodrop measured purity ratios A260/280 and A260/230 ≥ 1.8 recommended for next generation sequencing.

Enzymatic digestion, adapter ligation and GBS

GBS libraries were generated by MspI/PstI digestion followed by ligation of barcoded and common adapters to each sample, chip-based pooling and cleanup. Electropherogram profiles from Agilent TapeStation after size selection and PCR amplification show dominant peaks at ~174 bp (Chip 1, B1) and ~163 bp (Chip 2, C1), within the expected 150–200 bp range. Lower (~25 bp) and upper (~1500 bp) markers served as internal controls (
Fig:). Consistent peak profiles across both samples confirm efficient digestion, adapter ligation, successful amplification, and accurate size selection with minimal off-target fragments. This size selected and PCR amplified library was used for template preparation using Ion Chef and sequenced in Ion Torrent Gene Studio S5 Plus instrument.

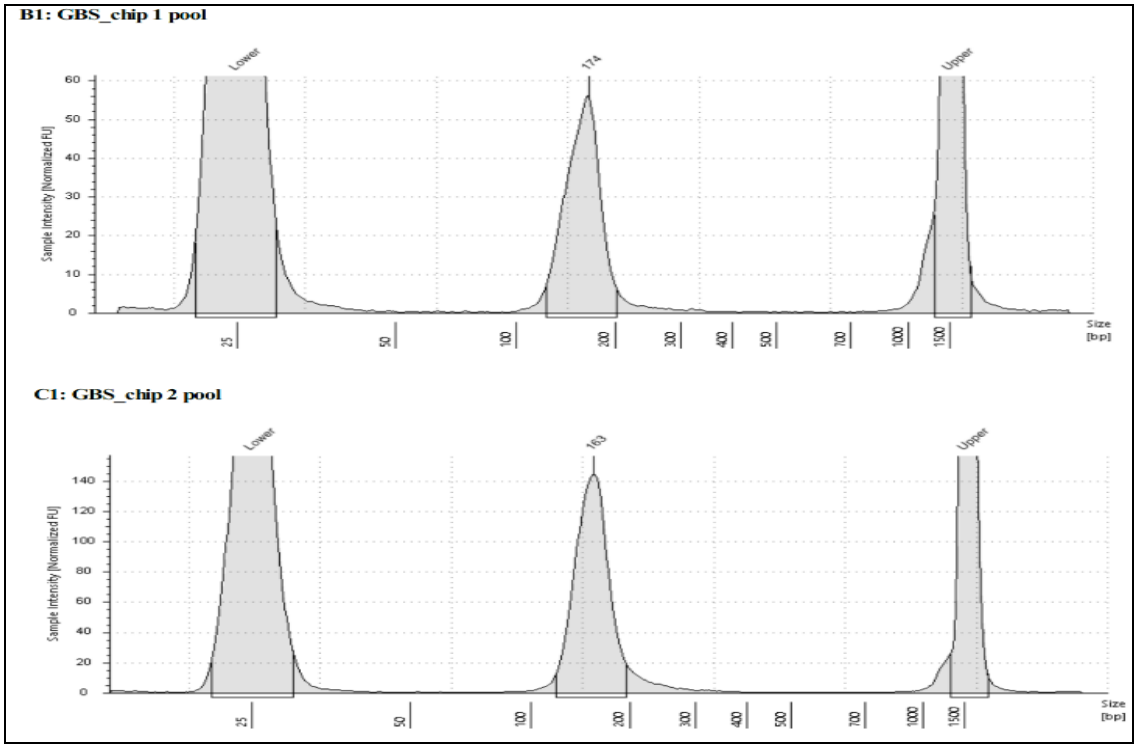

Fig: Size selected sequencing library for genotyping by sequencing (GBS) following MspI/PstI digestion and adapter ligation

TapeStation analysis after size selection and PCR amplification showed dominant peaks at ~174 bp (Chip 1, B1) and ~163 bp (Chip 2, C1), within the expected 150–200 bp range. Internal markers (~25 bp and ~1500 bp) confirmed accurate sizing. Consistent peak profiles indicate efficient digestion, adapter ligation, amplification, and precise size selection with minimal off-target fragments.

Discussion

Optimization in CTAB based DNA isolation protocol for complex plant tissues

Relative to commercial kits, the optimized CTAB protocol presented in this study offers a highly cost-effective and scalable alternative for plant genomic DNA extraction. Strategic modifications in CTAB based methods in terms of reagent concentrations, buffer composition, and purification steps allow researchers to precisely control the process, ensuring both flexibility and reproducibility across diverse plant genotypes. This reinforces the continued relevance of the CTAB method as a gold standard in plant molecular biology, particularly for laboratories operating at small to large scales. The protocol was validated across six parental lines and 139 recombinant inbred lines (RILs) of Indian bean (*Lablab purpureus*), representing a broad spectrum of genetic and phenotypic diversity within Indian bean. Additionally, genomic DNA was successfully isolated from thirteen cultivated and wild plant species, including rice, sugarcane, Indian bean (Lablab bean), pigeon pea, cotton, turmeric, castor, Anona, Sesbania, finger millet, neem (*Azadirachta indica*), banana, and money plant. These species were selected based on their agricultural, medicinal, and ecological relevance. The tested genotypes exhibited variation in pigmentation, growth habit, flowering behavior, fruit characteristics, and yield potential which are often associated with elevated levels of polysaccharides, phenolics, and other secondary metabolites that complicate DNA extraction using conventional CTAB methods.

In this experiment, we present an optimized cetyltrimethylammonium bromide (CTAB)-based DNA isolation protocol for extracting high-molecular-weight (HMW), high quality DNA from plant species with complex and biochemically diverse genomes. This protocol has demonstrated robust reproducibility across genetically and phenotypically diverse Indian bean parental lines, RILs and diverse plant species which have recalcitrant leaf tissue composition because of high secondary metabolites accumulation. Under the canonical CTAB protocol (Doyle, 1991; Doyle & Doyle, 1987) [7, 8] in diverse plant species, several genotypes yielded very low DNA and salt contamination persists in Nanodrop purity ratios. Lysates often became viscous or gelatinous during incubation, indicating incomplete lysis and contamination with polysaccharides. To overcome these limitations, the CTAB based protocol was optimized with minor modifications based on the key previous studies (Abdel-Latif & Osman, 2017; Aboul-Maaty & Oraby, 2019; Agbagwa *et al*., 2012; Fu *et al*., 2017) [1, 3, 4] including the canonical CTAB procedure (Doyle, 1991; Doyle & Doyle, 1987) [7, 8] as the basis. In this experiment, three key steps are optimized and are recommended for high quality DNA isolation using CTAB based gDNA extraction process as follows:

Celly lysis with 3X CTAB buffer and extended gentle-mix incubation at 60–65 °C for 1–2 hr

Cell lysis was found better when preheated 3X CTAB buffer at 60-65 °C (freshly added with 0.3% β-mercaptoethanol to neutralize polyphenols) was mixed immediately with tissue powder in centrifuge tube before incubation. Extended incubation at 60–65 °C for 1–2 hours with gentle mixing facilitates the complete solubilization of polysaccharides and phenolic compounds, which are abundant in recalcitrant plant tissues. This step minimizes mechanical shearing and preserves DNA integrity. If the lysate remains viscous or gelatinous, increasing the incubation time improves digestion efficiency. In this experiment, extending incubation to 2 hours yielded a transparent aqueous phase during chloroform:isoamyl alcohol (C:I) extraction and a clear, translucent DNA pellet during ethanol washes, indicating improved DNA quality and integrity. Our approach in this experiment is also supported by the previous works (Aboul-Maaty & Oraby, 2019; Healey *et al*., 2014) [3, 13] that demonstrated that prolonged incubation enhances DNA yield and quality in difficult plant species. A study has shown that prolonged incubation at 60 °C with gentle mixing preserves long DNA fragments in recalcitrant tissues (Healey *et al*., 2014) [13]. Similarly, it was reported that 1–2 hr incubation with higher concentration of CTAB buffer and β-mercaptoethanol enhances purity in diverse plant orders and confirmed that use of sodium chloride and potassium acetate improve DNA yield and purity (Aboul-Maaty & Oraby, 2019) [3]. In this experiment, we have used sodium chloride and sodium acetate to precipitate and extract high quality gDNA suggesting that either sodium acetate or potassium acetate along with sodium chloride can be equivalently used in CTAB based high quality gDNA isolation process.

DNA precipitation with sodium chloride, sodium acetate and chilled ethanol

After aqueous DNA phase collection, we used sodium acetate (0.1× volume of 3 M CH_3COONa solution) in combination with sodium chloride (0.5× volume of 6 M NaCl solution) along with 2.5x volume of 100% chilled Ethanol to enhance precipitation and effective polysaccharide and protein removal. Sodium ions neutralize the negative phosphate backbone of DNA, reducing its solubility and promoting efficient precipitation upon alcohol addition. Sodium acetate also maintains a slightly acidic pH (~5.2), which is optimal for DNA precipitation and minimizes RNA and polysaccharides contamination (Kalendar *et al*., 2021) [17], which is effective for isolating high molecular weight DNA from complex plant tissues. Previous research works on CTAB based DNA isolation also reported that dual-salt systems (NaCl and acetate) yield cleaner pellets (Abdel-Latif & Osman, 2017; Aboul-Maaty & Oraby, 2019; Agbagwa *et al*., 2012) [1, 3, 4]. Our findings suggest that sodium acetate or potassium acetate can be used equivalently for effective gDNA precipitation. In addition, duration of incubation at -20 °C for gDNA precipitation can be of 2 hr (quick) for high molecular weight longer fragments precipitation or can be prolonged to >24 hr (overnight) for precipitation of longer and smaller fragments of gDNA based on our purpose. This flexibility in CTAB makes it more useful and popular for easy handling of large numbers of samples with minimum resources.

DNA purification with dual 70% Ethanol washes and precision pipetting

To eliminate residual CTAB and salts, we performed two sequential washes with freshly prepared 70% ethanol. During the final wash, we used a 20 µL pipette to precisely remove residual supernatant without disturbing the DNA pellet. This step is critical, as residual salts absorb at 230 nm and can lower the A260/230 ratio, a key indicator of DNA purity. Presence of phenolic compounds, proteins and chaotropic salts in DNA solutions alters the A260/280 as well as A260/230 ratios. Dual washes of freshly prepared 70% ethanol are critical for long-read NGS platforms (Jones *et al*., 2021) [16].

To remove RNA contamination, RNase A treatment was also done. A higher A260/280 and A260/230 ratios >2.0 was observed in almost all Indian bean samples (Fig. 6) and >1.8 in diverse plant species (Fig. 12), reflecting low contamination from salts and organic compounds, ensuring compatibility with downstream applications such as polymerase chain reaction and next-generation sequencing (Abed *et al*., 2019; Koetsier & Cantor, 2025) [2, 18]. In this experiment, acceptable range of nucleic acid concentrations and purity ratios were observed (Table 7). In common laboratory practice, DNA and RNA samples with A260/A280 A260/A230 and >1.8 are considered to be "clean", and suitable for use in most downstream applications (Koetsier & Cantor, 2025; Xie *et al*., 2023) [18, 26]. Nanodrop results showed that average gDNA concentration of 1481.65 ±673.98 ng/µL was obtained per sample in Indian bean parental lines and RILs with purity ratios A260/280 of 2.1±0.07 and A260/230 of 2.09±0.27 before RNase A treatment. Even after RNase A treatment of those CTAB isolated gDNA samples, both the purity ratios were >1.8 which is also recommended (Koetsier & Cantor, 2025) [18]. The RNase A treated samples also exhibited A260/280 of 1.94±0.05 and A260/230 of 2.02±0.23, indicating suitability of CTAB extracted gDNA free from impurities without further cleanup steps after RNase A treatment. In case of thirteen diverse plant species used for optimized CTAB based protocol, the gDNA concentration on Nanodrop was ranging from 271.15 to 899.85 ng/µL concentration with purity ratios A260/280 of 2.1±0.05 and A260/230 of 2.24±0.6, thus meeting the quality criteria as recommended for downstream applications (Koetsier & Cantor, 2025) [18]. The lower concentration of gDNA in diverse plant species as compared to Indian bean population was because of mature leaves taken for DNA extraction for diverse species sample because of perennial nature, whereas in case of Indian bean the leaves were very young and rich with meristematic tissue thus yielding higher gDNA concentration in Nanodrop. Therefore, this optimized CTAB method can be successfully used for high quality high molecular weight genomic DNA isolation for high throughput next generation sequencing using wide range of plant species and tissue types.

Conclusion

The optimized CTAB-based protocol produced genomic DNA of acceptable quantity and purity from Indian bean parental lines, recombinant inbred lines, and selected diverse plant species. The main refinements, including extended incubation in 3X CTAB buffer, dual-salt precipitation with sodium chloride and sodium acetate, and repeated ethanol washing, improved DNA recovery and reduced common contaminants associated with plant tissues rich in polysaccharides and secondary metabolites. The extracted DNA was suitable for agarose gel assessment, gene-specific PCR amplification, Qubit quantification, and genotyping-by-sequencing library preparation. The GBS results from Indian bean samples further indicated that DNA obtained through this protocol can support reduced-representation sequencing workflows. The method is based on commonly available laboratory reagents and may be useful for plant molecular breeding and genomics laboratories where commercial kits are costly or impractical. However, the protocol may require adjustment for highly recalcitrant tissues, mature leaves, or species with high concentrations of interfering metabolites.

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