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RESEARCH ARTICLE

High quality and purified DNA extraction protocol from transplastomic potato lines by cetyltrimethylammonium bromide (CTAB)-based method

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Abstract

For the advanced molecular techniques high quality and pure DNA is important requirement where a quick and cost-effective extraction method is a crucial factor. Commonly, prolong incubation period, multiple precipitations, and highly expensive commercial kits are remaining basic barrier to introduce swift and easiest method of DNA extraction from transgenic plants. Non-cellulose components present in the leaf and elevated level of starch content as well as protein content also makes this extraction procedure challenging and difficult. Present study was conducted to establish a unique DNA isolation method with some modification of existing CTAB method to ensure high-quality and purified genomic DNA from the potato plants. Through the implementation of solution based and selective DNA precipitation method, 100 mg of potato leaves from every transplastomic potato lines was applied to extract genomic DNA. Ethanol (70%) was used as elution buffer where maximum DNA yield was 3415.500 ng/µl and the ranges of DNA purity (A260/A280 nm) was recorded as 1.954-2.048, respectively. Compared to existing methods, this modified CTAB mediated procedure is swift (at least one hour can be saved) and proficient enough for high quality DNA yield, free from the selective precipitation, regents and laboratory equipment's are cost-effective and non-toxic to human health.

Keywords

CTAB; high quality DNA; non-toxic chemicals; cost effective

Introduction

Cetyltrimethylammonium Bromide (CTAB, $[(C_{16}H_{33})N(CH_3)_3]Br.$) is a detergent of cationic nature that consist of a long chain of hydrophobic property and the head is hydrophilic nature and compatible with the high concentrations of salt, often utilize for the extraction of DNA from chromosomal proteins (Fig.1). CTAB mediated plant DNA extraction protocol was initiated since 1980 and CTAB-isolated DNA is being routinely used (1) for different aspects of molecular genetics including mapping (2) and cloning (3) and it has opened a new horizon for the study of gene functions.

For the detect of small portion of a gene sequence (specific) from the complex mixture of sample by using PCR, to screen the transgenic events without selection process, for the detection of pathogenic availability (4) in the specimen

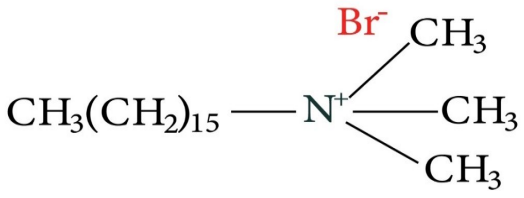

Figure 1. Molecular structure of CTAB (Cetyltrimethylammonium bromide).

and to identify the absence of certain transgene (5) this CTAB isolated DNA is crucially important.

Extra ordinary utility of CTAB mediated DNA extraction protocol is that besides the plant tissues it can be applied to diverse ranges of organisms including bacteria (6), fungi (7), and animal tissues (8). Application ranges of CTAB extracted DNA is diverse and it includes ecology (9), evolution (10, 11), and forensic sciences (12). As the potato leaf samples generally contain high amounts of polyphenols, tannins and polysaccharides and cell wall is strong enough against breakage, to isolate inner component especially DNA it is required to do enzymatic digestion and mechanical breakage of cell wall (Fig.2). The fundamental principle for an ideal DNA isolation protocol consists of the disruption of the cell wall as well as cell membrane and nuclear membrane that release the highly intact DNA into a solution followed by precipitation of DNA. The removal of contaminating biomolecules (the proteins and polysaccharides, secondary metabolites) by enzymatic or chemical methods is also a crucial part of standard DNA extraction protocol that ensure the purity of extracted DNA (13).

In our current endeavour, we frozen and broke potato tissues by in a cold mortar and pestle with liquid nitrogen. Besides the breakage of cell wall, the frozen powder of potato leaves should be thawed in preheated extraction buffer that is very crucial to protect DNA from degradation.

After the first release of original CTAB extraction of DNA several modifications have been done aimed to make it swift and financially feasible. Selective CTAB mediated DNA precipitation and a CsCl–EtBr mediated density gradient ultracentrifugation is important steps of original protocol those are essential for the removal of RNA, proteins, and polysaccharides. Therefore, centrifugation is laborious, time consuming and not cost effective.

Besides these, to remove all remaining CTAB from the final product CsCl gradient and subsequent dialysis is also essential for enzymatic activations (e.g., restriction digestion and PCR). When CsCl–EtBr step is missing, removal can be done by several wash procedure with ethanol aided precipitation from the high salt buffers. In these steps, complete dissolve of DNA is required prior to each precipitation and those steps are time consuming.

To ensure shorter and faster extraction protocol it is better to avoid the selective precipitation steps. In our described protocol DNA was initially solubilized in extraction buffer harboring highly concentrated sodium chloride and then a series of centrifugation was applied along with ethanol precipitation. In a nutshell, this modified protocol is swift, cost effective and capable to yield high quality and purified DNA for different molecular analysis.

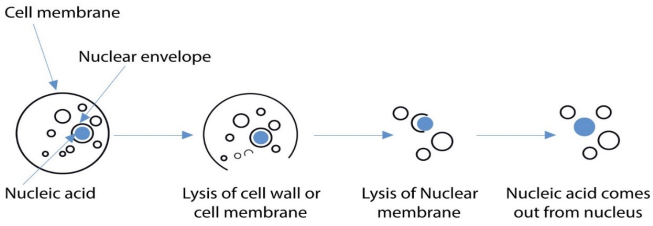

Figure 2. Enzymatic digestion and mechanical breakage of cell wall.

Materials and Methods

Materials

- Mortar and pestle (precooled to -70°C).
- Micropipettes (P-20, 200 and P-1000).
- Micropipette (Nuclease-free wide-bore).
- Eppendrop Tubes (1.0 ml and 2.0 ml).
- Digital vortex mixer to mix the samples
- Digital heating block, capable of maintaining 37°C and 65°C
- Fume hood.
- Laboratory microcentrifuge.

Buffers and Chemicals

Presented in Table-1.

Plant material and tissue grinding

Fresh leaves from transplastomic potato lines (Kuroda, Simply Red, Challenger and Sante) were used as plant materials. The entire *in-vitro* production process of transplastomic lines can be found in our previous article (14). Transplastomic potato leaves were cut into small pieces (Fig.3.1) and approximately 0.2 gm leaf tissues were placed into two ml Eppendrop tubes and cooled in liquid nitrogen. Before starting the grinding, the mortar and pestle was precooled to -70 degrees. Extraction buffers (Table-1) were measured according to the number of plant samples and preheated to 60 to 65°C. A small amount of liquid nitrogen was poured into a mortar and the frozen samples were placed into the mortar. The liquid nitrogen

Table 1. Amount required, preparation and functions of extraction buffers and chemicals used for DNA extraction

Components of extraction buffer	Required Amount (for 250 ml)	Preparation	Functions
CTAB (10%)	50 ml	In a 50 mL polypropylene tube, combine 50 mL 0.7 M NaCl with 2.5 g CTAB. Dissolve the powder slowly at 60°C for several hours. Store up to 6 months at room temperature.	Under the watery condition, CTAB enters into the contact of biological membrane, captures lipids and causes nucleus release. The effects of polysaccharides and secondary metabolites are minimized using a CTAB and certain other chemical substances (PVP) (42). At a low ionic strength, nuclear acid and acidic polysaccharides precipitate while at a high ionic strength, binds to and forms complex polysaccharides, also inhibits protein and enzyme activation (43).
1.0 M Tris HCl stock, pH 8.0	25 ml	Dissolve 121.1 grams of Trizma in 700 ml of H_2O. Use concentrated HCl to adjust pH to 8.0. Concentrated HCl of approximately 50 ml is necessary. Add H2O to reach the total volume up to 1.0 litre.	Cellular decompartmentalization is achieved during tissue grinding and cytoplasmic materials are released, which changes the p^H. The pH of the solution maintained by Tris buffer (43).
5 M NaCl	70 ml	Add 292.2 g NaCl into 700 ml of H_2O and Adjust the volume to 1L with H_2O.	It helps remove proteins that are bound to DNA and keep proteins in the aqueous layer so that the negative charges do not precipitate into the alcohol in addition to the DNA (43). Discard the polysaccharides and increase DNA output and prevent DNA-polysaccharide interaction (44).
0.5 M EDTA	10 ml	Dissolve 186.12 g of EDTA (disodium dihydrate salt) in 750 ml of H_2O. Add approximately 20 g of NaOH pellets and use 5 M NaOH solution to adjust the final pH to 8.0.	Dilute 186.12 g of EDTA in 750 ml of H2O. Add approximately 20 g NaOH pellets and apply the 5 M NaOH solution to adjust final pH to 8.0.
H_2O	105 ml	Ready to use	When cells and membranes are softened, DNA can be released. The hot water bath also denatures (deactivates) DNA degrading enzymes in the mixture.
10% PVP	50 ml	10 g of PVP is added into 100 mL (Commercial 10% PVP also available, ready to use).	Browning of DNA samples is caused by the removal of phenolic compounds from plant DNA extracts through hydrogen bonding (45).
βME (2-Mercaptoethanol)	Instantly add 0.5–1 percent (2-Mercaptoethanol) to the extraction buffer.		Assist in the prevention of oxidation Because it is an extremely potent reducing agent, it can remove tannins and other polyphenols that are commonly found in crude plant extracts (43).
	Chemicals /Reagents (for one sample)		
Liquid nitrogen	Adequate	Ready to use	Initially, the nuclear material must be mechanically ground to gain access to it, liquid nitrogen ensures it.
Phenol: chloroform: isoamyl alcohol	500µl	Ready to use	With its greater density, chloroform helps separate the two liquid phases (organic-lipid, proteins, and other impurities; and aqueous-DNA), making it easier to remove the organic phase with minimal cross-contamination. Isoamyl alcohol inhibits RNase activity and prevents the solubilization of long RNA molecules with long poly (A) portions in the phenol phase of the reaction. As a result, the purity of DNA will be improved (43).
Isopropanol	500µl	Ready to use	When we incubate at low temperatures for long periods of time, isopropanol is able to precipitate larger species and lower concentrations of nucleic acids than ethanol does.
70% Ethanol	500µl	Absolute ethanol was mixed with H_2O at a ratio of 70 ethanol: 30 H_2O.	To remove the pellet's salt. Due to the fact that sodium salts are poorly soluble in ethanol, the salt would not wash off. Dehydration and removal of water molecules from DNA during precipitation in 100% ethanol renders the DNA insoluble. A 70 % ethanol wash allows the DNA to retain some water molecules, making it soluble.
TE buffer	50-100 µl	To prepare the solution, combine 10ml of 1M Tris (pH 8.2) with 2ml of 0.5% sodium EDTA, and add water until it is 1.0 liter.	DNA is protected from degradation by dissolving in the TE buffer. In DNA extraction buffer, it aids in the lysis of cell walls and nuclear membranes. Protects nucleic acid from degradation by DNase or RNase.

was allowed to evaporate slowly, meanwhile grinding the sample with a pestle was continued. Although the grinding intensity was started slowly but it was increased when the liquid nitrogen evaporated completely. Based on the consequences, additional liquid nitrogen was added, and grinding was continued until the sample turned to a fine powder (Fig.3.2). The frozen and grinded sample powders were transferred carefully into the 2 ml Eppendrop tube with a precooled spatula. Evaporation of liquid nitrogen from sample powders was ensured before sealing the tubes to prevent pop open and loss of the samples. Promptly, the Eppendrop tubes containing ground powder was place into liquid nitrogen.

DNA extraction from grinded tissues

About 1.2 ml of the preheated extraction buffer was added to the frozen and grinded tissue powder and vortex for 5–10 seconds to ensure the proper mixing. Thirty minutes long incubation was done at 60 to 65°C and inversion of tubes after every 5–10 mins intervals was maintained to allow the proper mixing (Fig.3.3). To remove the non-soluble debris, centrifugation was done at 14000g for 10 min at room temperature (Fig.3.4). Supernatant was transfer to a new 2 ml Eppendrop tube and 800 ml of phenol: chloroform: isoamyl alcohol (25:24:1) was added with these supernatant. Proper mixture of supernatant and chemicals were confirmed by inversion tubes and incubated for 20 min at 20–22 °C. Phase separation was conducted by centrifuging that mixture at 14000g for 10 min at room temperature. Supernatant / aqueous (upper) layer was transferred to a 1.5 ml Eppendrop tube (Fig. 3.5) and precooled (stored at -20°C) isopropanol/ ethanol (0.6 ml isopropanol × amount of supernatant) was added (Fig.3.6). Inversion of mixture tube ensured the proper mixing of isopropanol and supernatant. For adequate DNA precipitation, incubation of mixture for 10 minutes was ensured at room temperature. The mixture was centrifuged at 14000g for 10 mins and the supernatant was removed (Fig. 3.7). Pellets adhered to the lower wall of test tube was slightly dry on tissue paper carefully without disturbing it (Fig.3.8). To wash the pellets, 500µl of precooled (stored at -20°C) 70% ethanol was added to the pellets and centrifuged for 10 minutes at 14800 rpm at 4^0C (Fig. 3.9 and 3.10). The removal of supernatant was done, and pellets were allowed to proper dry for 1 hour at room temperature (Although, the dried samples are stable overnight at room temperature, overnight dissolve of DNA at 4°C is recommended). About 50-100 µL of TE buffer (Tris-EDTA) was added to dissolve the dried pellets and mixed properly (for the instant use of extracted DNA for PCR, dilution can be done with H_2O, however, for the prolong storage, TE is recommended contains a chelator and affect the molecular reactions like PCR or restriction digestion and DNA stored with TE required to be diluted before using) (Fig. 3.11). After that, 2 µL of RNAse enzyme was added to the mixture tube and mixed by inverting or tapping. Gradual incubation at 37°C for 45 minutes and 5 minutes at 65^0C with slight shaking was ensured up to the

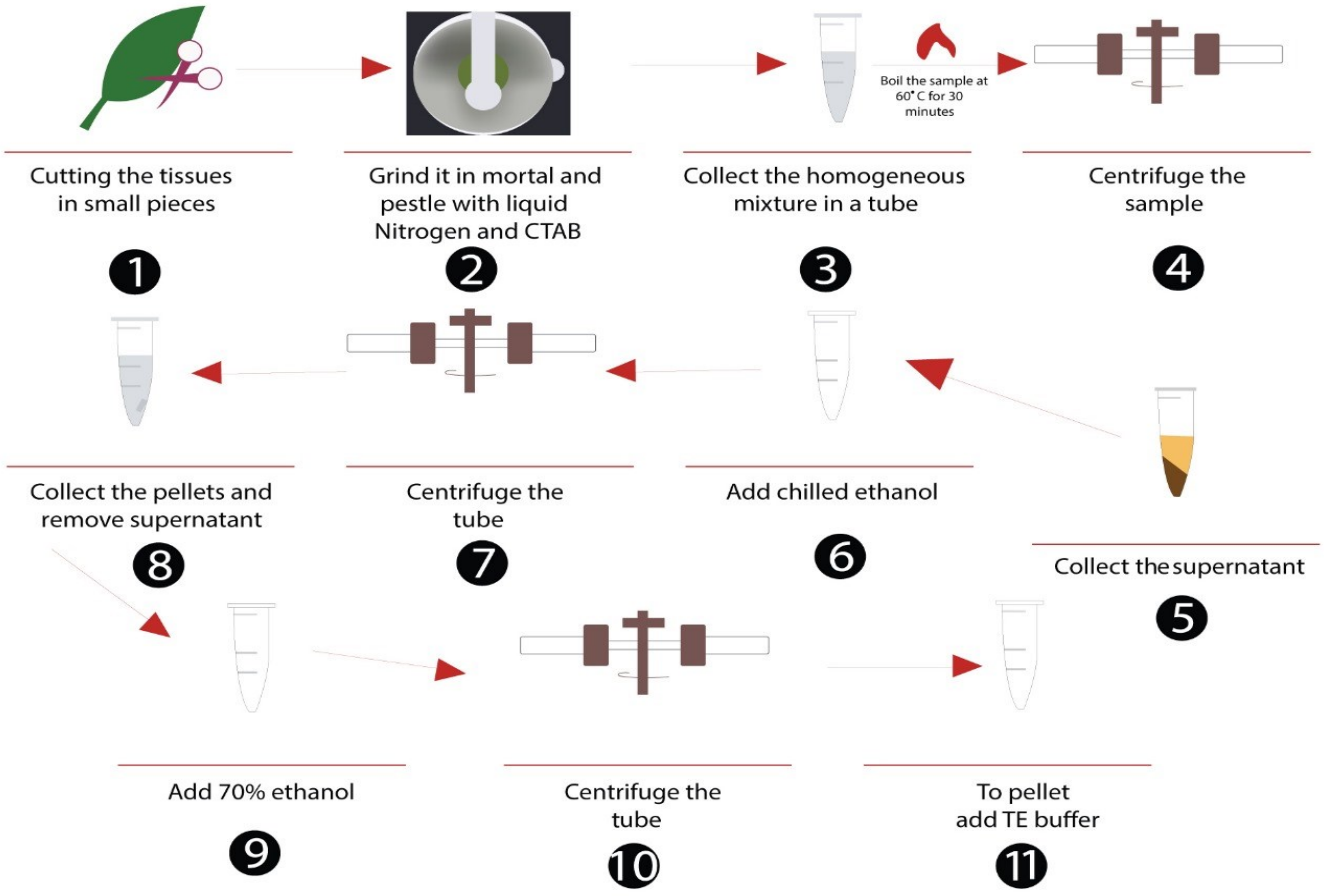

Figure 3. Successive steps of DNA extraction from potato leaf tissues by CTAB methods.

Table 2. At a glance method summary used in our study

Extraction method	Basic overview of the method	Initial materials	Buffers used in the study	Buffer used for elution	Highest DNA yield (ng /µl)	Purity of DNA (A260nm/ A280nm ratio)
Cetyltrimethylammonium Bromide (CTAB)	Based on solution; DNA precipitation (selective)	100 mg potato leaf tissues	1 ml of extraction buffer (Components are mentioned in table 1)	70% Ethanol (500 µl)	3415.500	1.954 - 2.048

complete dissolve of pellets (pellets having DNA with elevated molecular weight usually take an extended time to dissolve). Incubated tubes were centrifuged for 10 minutes at 14800 rpm at 4^{0}C. Supernatant (DNA) from centrifuged tubes were collected into a cleaned and autoclaved 1.5 ml Eppendrop tube. The summary of utilized method has been presented in Table-2.

Quantification of extracted DNA (Qualitative and Quantitative)

Spectrophotometric analyses of DNA

The DNA yield was measured at 260 nm by using a UV-spectrophotometer (Quawell-Q5000) whereas the purity (protein contamination) was quantified by the calculation of the A260/280 absorbance ratio. Polysaccharide / guanidine contamination was assessed by the calculation of the absorbance ratio A260/230 (15). The criteria for the DNA quality assessment with higher values associated with better DNA purity were both the spectrophotometric measurements.

Agarose gel electrophoresis

The integrity (quality and yield) of all DNA samples (3µl from each samples) were assessed by electrophoresis in 1% agarose gel and Ethidium Bromide (0.5%) was used to stain the DNA and bands were observed after 60 minutes while running at 90 voltages. Visualization of DNA bands (from 3µl of DNA) were performed by Gel Doc XR+ Imaging system (Bio-Rad Laboratories Inc., Germany) (16). Extracted DNA samples were digested with HindIII restriction enzyme and digested samples were also stained with Ethidium Bromide and electrophoresis was conducted with 1% agarose gel along with DNA samples to compare.

Results and discussion

Since the beginning, CTAB-mediated DNA extraction method has been modified several times aimed to save time and cost and also for the minimization of polyphenols and polysaccharides contaminants (17, 18). Existing CTAB mediated DNA extraction protocols are suitable enough to produce required level of DNA for the PCR amplification or restriction digestion but because of prolong incubation period, several precipitation steps as well as ethanol wash to ensure the genomic DNA with high purity and free from RNA contamination have risen the demand of swift and economically feasible methods over the time. Besides these, most of cases these additional steps reduce total yield and quality of DNA. While using commercial DNA extraction kit, the presence of polysaccharides in pellets and sheared band in an agarose gel prevents the quality and purity of genomic DNA (18). We encountered several difficulties while using existing conventional CTAB based DNA extraction methods specially pellets were very viscous and sticky those were difficult to handle and, contamination of phenolic compounds manifested by the brownish pellets and these same difficulties were reported earlier (19).

During our experiment we obtained white DNA pellets with no visible discoloration. We hope that high level of β-mercaptoethanol successfully removed (20). The presence of optimum concentration of β-mercaptoethanol facilitated to develop an outstanding DNA extraction protocol for high-quality DNA. Higher concentration of NaCl (5M) along with CTAB ensured the removal of polysaccharides during DNA extraction and same findings was reported by (19). Based on the explants or plant species NaCl concentration varied and ranges between 0.7 M (21) to 6M (22). Isolated high quality and quantity of DNA from roots, leaves, and seeds using modified CTAB protocol with the presence of 2.56 M (23). On the other hand, it was not able to isolate good quality and usable DNA from maize with 2.56 M NaCl in another study (24).

Quality and quantity assessment of extracted DNA

For the quantification of extracted DNAs both spectrophotometer (Nano-Drop) and agarose gel electrophoresis was utilized where the NanoDrop absorbance profile was useful for the detection of contaminants (protein, salts, and polysaccharides). The amount of DNA obtained from existing protocols was very low, and many samples were yielded low quality of DNA. For instant, A260/A280 ratio was recorded as 1.6 that actually lesser than the optimal limit 1.8 (25) and these less amount and low-quality DNA was not suitable for sensitive and precious molecular analyses. A better findings was enlisted from an experiment conducted earlier (26) where both quality and quantity of DNA was up to the mark, but that experiment was conducted to standardization of various concentration of Tris-HCL, β-mercaptoethanol, NaCl, and PVP.

In our current extraction protocol, higher level of NaCl (5 M) further improved the DNA quality and this high quality of DNA may be attributed to the higher concentration of PVP (10%) with lower molecular weight (10,000). Several research groups experienced phenolic problem while using low concentration of PVP (2%) at molecular weight 10,000 (27, 28). Using PVP with low molecular weight (10,000) favor the less tendency of precipitating with the nucleic acids, considering this we avoid using PVP with high molecular weight (40,000) that remarkably influenced adequate amount of polyphenol-free DNA (29). Previous studies (18, 30) was used 1-2% PVP

in CTAB method and their extracted DNA renders DNA unusable for downstream application. In a same manner, it was reported failure to visualize any band in 2% agarose when 1% PVP was used (24).

High purity DNA extracted from mentioned potato cultivars using the proposed modified CTAB extraction methods is shown in figure-5 and the findings are summarized in Table 3. The A260/A280 manifested the excellent purity of availed DNA ranges between 1.93 to 2.048 (with a single absorbance peak at 260 nm, shown in fig.5). A260/A230 ratio ranges between 1.95 to 2.080 denoted that the preparations free from proteins and polyphenolics/polysaccharide contamination. This findings correlates the findings of previous studies (18, 26). Extracted DNA from 11 blood samples where the purity of extracted DNA was evaluated based on 260/280 nm ratio that was recorded more than 1.8 (31) and those findings were compared to the findings of other reports (31, 32). It was mentioned that a low 260/280 nm ratio indicates the proteins contamination that inhibit the downstream applications and create disturbance for DNA-banking (33). On the other hand, a low 260/230 nm ratio indicator of phenol contamination. Besides these, other phenols or salt based organic compounds are also remaining potent inhibitors of downstream applications (34). In our current study, high absorbance ration from both 260/280 nm and 260/230 nm indicates the contamination free or purity of our extracted DNA.

An experiment was conducted to extract the maize DNA without liquid nitrogen where 260/280 ratio was recorded ranges between 1.2–2.07 and 260/280 ratio was found between 1.93 and 2.27 that indicates ignorable levels of contamination (24). This finding dramatically matched to our findings as well as few studies conducted earlier (35, 36) in cereals and cotton, respectively. But among the findings of (24), the purity ratio of >1.9 and <1.7 denotes the presence of RNA and proteins, respectively. Contamination free DNA was extracted (37) where 260/280 absorbance ratio was ranges between 1.6 to 1.8 and maximum absorbance ratio was enlisted as 2.7.

Age and type of leaf explants also an important determinant of good quality DNA and young and fresh leaf samples are the best choice for DNA extraction because mature leaves harbour higher level of polyphenols as well as polysaccharides that hindrance the quality of DNA (38). Some of cases it is required to extract DNA from mature or dried leaves, this optimized protocol can apply to solve this problem without interfering the quality of DNA.

While extracting, Shearing of DNA protected DNA fragmentation and this lack of smears demonstrated the elevated level of DNA purity. It is previously established that the shearing of DNA can interrupt the enzymatic reactions directly and indirectly (39) but *Taq* DNA

Table 3. Ranges of DNA yield and purity achieved from samples extracted with liquid nitrogen by CTAB method

Sample ID	Sample name	Nucleic Acid Concentration (ng /µl)	Unit	A260 (10mm)	A280 (10mm)	260/280	260/230	Sample Type	Factor
K-01	Kuroda-1	2592.500	ng/µL	51.850	25.536	2.031	2.035	dsDNA	50
K-02	Kuroda-2	2372.050	ng/µL	47.441	23.166	2.048	2.080	dsDNA	50
SR-01	Simply Red-1	3308.750	ng/µL	66.175	33.873	1.954	1.950	dsDNA	50
SR-02	Simply Red-2	1634.750	ng/µL	32.695	16.010	2.042	1.998	dsDNA	50
Ch-01	Challenger-1	3415.500	ng/µL	68.310	35.259	1.937	1.959	dsDNA	50
Ch -02	Challenger-2	3086.350	ng/µL	61.727	30.888	1.998	2.030	dsDNA	50
Sa-01	Sante-1	3288.750	ng/µL	65.773	33.077	1.988	1.980	dsDNA	50
Sa -02	Sante-2	3282.644	ng/µL	65.644	33.115	1.982	1.962	dsDNA	50

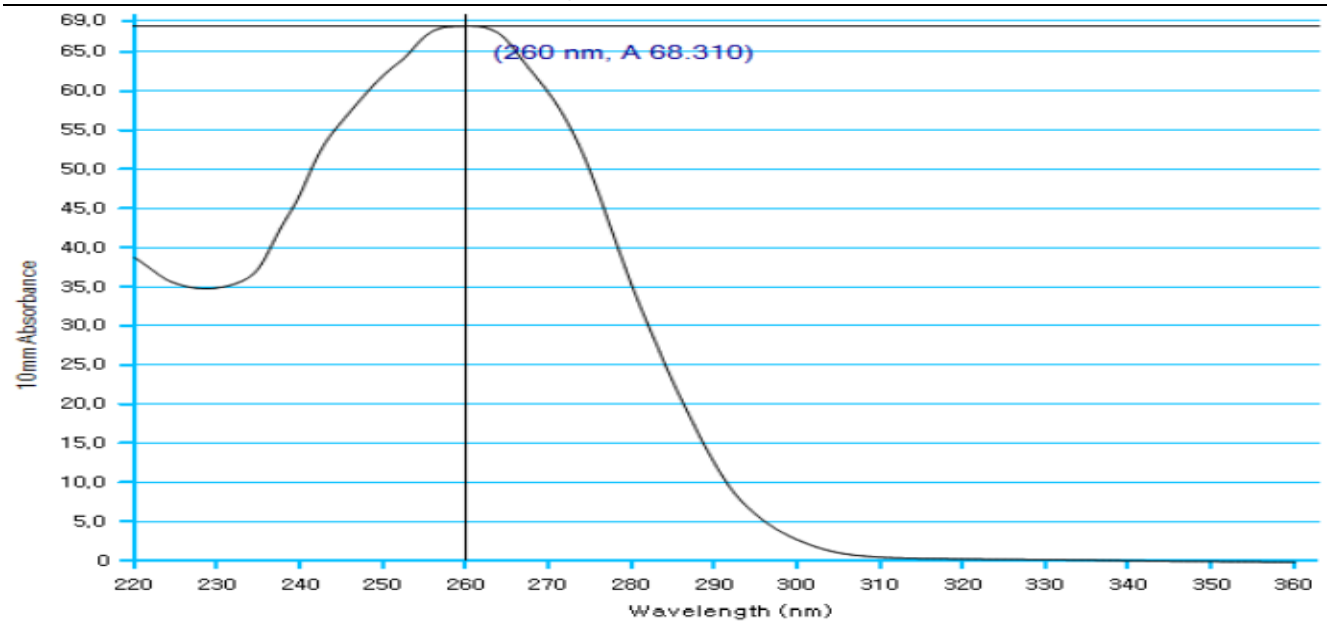

Figure 5. Genomic DNA profile of transplastomic potato cultivars measured by Nano-Drop

polymerase activity was not inhibited. Constant treatment with chloroform: isoamyl alcohol ensured removal of chlorophyll, pigments, and dyes. Besides the potato, this optimized protocol can be utilized to extract high quality and purified DNA from another plant species harboring high level of secondary metabolites in a cost effective and time saving manner.

Agarose gel electrophoresis aided visualization of extracted DNA

As our findings, gel electrophoresis depicted a single DNA band with high molecular weight and the absence of RNA contamination was ensured. Figure 4 shows the distinct bands separated at their corresponding high molecular weight and none of the DNA samples showed significant

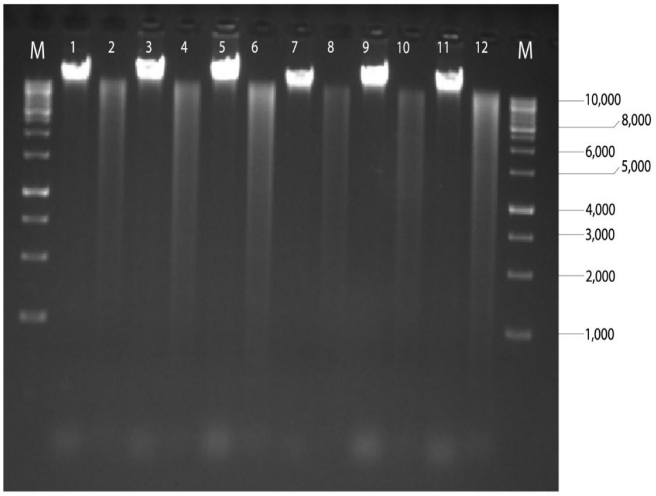

Figure 4. Restriction digestion and electrophoretic analyses and of DNA extracted from transplastomic potato leaves. First and last lane represent 1kb DNA ruler mix, Lane 1, 3,5,7,9,11 represent the image of 3 µl of undigested DNA, Lanes 2, 4, 6,8, 10 and 12 represent HindIII-digested DNA that was analysed on a 1% agarose gel. Electrophoresis was carried out for 4 h at 40 V. Lane 1-12 DNA from transplastomic potato lines (lanes 1 and 2: Kuroda; lanes 3 - 6: Simply Red; lanes 5 - 8: Challenger, Lane 9-12: Sante).

smearing. To remove the secondary metabolites (carbohydrates and proteins) attached to the DNA and to increase the solubility of polysaccharides in ethanol presence of high concentrated NaCl in extraction buffer is remaining very crucial. Besides this, NaCl proficiently inhibit the polysaccharides co-precipitation together with the DNA (36, 40, 41).

Conclusion

This article summed up a simple, quick, reliable and cost-effective DNA extraction method, which ensures the efficient removal of polysaccharide and polyphenolic compounds to ensure highly quality and purified DNA from transplastomic potato lines. In low technology labs for high-performance preparation of samples conducive to different molecular investigations, this proposed optimized CTAB Protocol permits the high-end genomic DNA isolation (whole-genome sequencing, bioinformatic tools, and advanced sequencing technologies etc.).

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Authors' contributions

The research was planned and designed by AB and FAJ. JH did all experiments, collected, and analyzed data. FAJ, EA, NZOG and MSK participated in project activities, critically reviewed, and revised the manuscript. AB supervised the overall research activities of the project.

Compliance with ethical standards

Conflict of interest: Authors do not have any conflict of interests to declare.

Ethical issues: None.

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