Optimized DNA Isolation protocol from Pearl Millet (*Pennisetum glaucum*) Leaves with High Phenolic and Metabolite Content

ABSTRACT

Pearl millet (*Pennisetum glaucum* [L.] R. Br.) is a vital cereal crop recognized for its resilience to harsh environmental conditions. However, extracting high-quality DNA from pearl millet leaves presents a significant challenge due to the presence of phenolic compounds and secondary metabolites that interfere with molecular biology procedures. This study optimized a DNA isolation protocol tailored for pearl millet genotypes with high phenol and metabolite content to ensure the extraction of pure and intact DNA suitable for downstream molecular applications. Fresh leaf tissues from ten pearl millet genotypes with elevated phenolic content were processed using a modified cetyltrimethylammonium bromide (CTAB) protocol. The optimized method included key adjustments such as incorporating 1% polyvinylpyrrolidone (PVP) and increased concentration β-mercaptoethanol to neutralize phenolic compounds, alongside sodium chloride (1.4 M) to remove polysaccharides effectively. Sequential extractions with Phenol: chloroform: isoamyl alcohol and isopropanol precipitations were employed to purify DNA, while RNase treatment eliminated RNA contaminants. The resulting DNA exhibited high purity (A260/A280 ratios of 1.8–2.0) and integrity, suitable for applications including polymerase chain reaction (PCR)-based marker analysis was demonstrated. This optimized protocol offers a reliable and cost-effective approach for isolating high-quality genomic DNA from pearl millet, even from genotypes with high secondary metabolite content. It can facilitate genetic diversity studies, marker-assisted selection, and genomic sequencing, contributing to advancements in pearl millet breeding and research.

Key Words: Pearl millet, DNA isolation, Modified CTAB method, High phenolic content, Plant genomics

Introduction

Pearl millet (*Pennisetum glaucum*) is an essential grain crop, especially in arid and semi-arid areas, owing to its remarkable resilience to drought, high temperatures, and sub-optimal soil fertility. Leaves of pearl millet, particularly in genotypes suited to stress conditions, are recognised for their significant accumulation of phenolic compounds and secondary metabolites (Tara et al. 2021). These chemicals, although aiding the plant's defence systems, can present considerable difficulties in molecular biology procedures, including DNA separation. Phenols may undergo oxidation during extraction, resulting in nucleic acid degradation, whereas metabolites might compromise the quality and yield of isolated DNA (Shrestha et al 2023). The extraction of high-quality, contaminant-free DNA is essential for subsequent applications, including genetic diversity research, marker-assisted breeding, and genomic sequencing. An effective DNA extraction protocol should provide sufficient and undamaged DNA of acceptable quality. The process must be efficient, uncomplicated, and economical. DNA extraction commences with the lysis of cell walls to liberate cellular components, succeeded by the rupture of cell membranes to release DNA into the extraction buffer. This is generally accomplished with detergents such as cetyltrimethylammonium bromide (CTAB). During extraction, the liberated DNA must be safeguarded against endogenous nucleases. Ethylenediaminetetraacetic acid (EDTA) is included into the extraction buffer to chelate magnesium ions, which are vital cofactors for nucleases (Puchooa, 2004). The first DNA extracts frequently include substantial quantities of RNA, proteins, polysaccharides, tannins, and pigments, which might obstruct the extracted DNA and are challenging to isolate (Puchooa, 2004). Proteins are typically eliminated via denaturation and precipitation utilising chloroform and/or phenol. RNA is often removed by subjecting the extract to heat-inactivated RNase A. Polysaccharide impurities are more difficult to eliminate since they can impede the function of DNA-modifying enzymes and disrupt nucleic acid measurement by spectrophotometric techniques (Wilkie et al., 1993). Sodium chloride (NaCl) concentrations over 0.5 M, in conjunction with CTAB, are recognised for their efficacy in polysaccharide removal (Murray and Thompson, 1980; Paterson et al., 1993; Tiwari et al., 2017). The NaCl concentration employed differs among methods, spanning from 0.7 M (Clark, 1997) to 6 M (Aljanabi et al., 1999), contingent upon the plant type. Potassium chloride (KCl) is utilised as a substitute for sodium chloride (NaCl) in some regimens (Thompson and Henry, 1995).

The issue of DNA extraction is considerable in plant molecular biology, as several plants possess elevated quantities of polysaccharides and other secondary compounds that impede DNA purification. Antioxidants are commonly employed to neutralise phenolic chemicals, including β-mercaptoethanol, bovine serum albumin (BSA), sodium azide, and polyvinylpyrrolidone (PVP) (Dawson and Magee, 1995; Clark, 1997). Phenol extractions, when combined with SDS, are also advantageous. Nevertheless, plants with elevated polyphenolic content often yield modest DNA quantities when subjected to SDS-phenol extraction (Rezaian and Krake, 1987). Multiple laboratories participating in the study conducted a comparative analysis of all four DNA isolation methods concurrently. Two techniques are grounded in ancient principles of lysis and purification. The initial procedure is the widely utilised method established by Doyle and Doyle (1990), which has shown effective in several plant species. The second method, proposed by Guillemaut and Marechal-Drouard (1992), was derived from Dellaporta et al. (1983) and then adapted based on Ziegenhagen et al. (1993). However, DNA isolation can be efficiently performed using commercial isolation kits, which offer quick and easy extraction; however, they are expensive and yield limited DNA quantities, significantly increasing research costs, especially in developing countries. When conducting diversity analysis or association mapping, which requires screening large plant populations at the molecular level, the cost of using these kits escalates further. Since the mid-1980s, genome identification and selection have advanced significantly due to PCR technology. A multitude of quick marker techniques necessitating minimal DNA amounts have been established. Three often utilised PCR-based markers are RAPDs (Williams et al., 1990), SSRs or microsatellites (Tautz, 1989), and AFLPs (Vos et al., 1995). Protocol adjustments are essential to effectively eliminate phenolic chemicals and other inhibitors during DNA isolation from pearl millet leaves. This paper presents an optimized DNA isolation process specifically designed for pearl millet genotypes with high phenol and metabolite content 10 genotypes were selected for protocol standardization. Protocol modifications were made to effectively remove phenolic compounds and other inhibitors during DNA isolation from pearl millet leaves, ensuring high-quality DNA for molecular analyses.

MATERIALS AND METHOD

It is recommended that the authors include more recent references published within the past five years to ensure that the manuscript reflects the latest advancements in this field.

Pearl millet germplasm leaf was collected from Department of Molecular Biology & Biotechnology, College of Agriculture, Rajmata Vijayaraje Scindia Agricultural University, Gwalior, M.P, India (Lat 26.220 and Long 78.199). Fresh leaves were used as the tissue for DNA isolation.

Chemical Solution

DNA extraction buffer of (50 ml) consisting of 2.5% of CTAB (v/v), 100 mM Tris (pH 8.0), 50 mM EDTA (pH 8.0), 1.4 M NaCl, 1% polyvinylpyrrolidone (PVP), 0.2% β-mercaptoethanol (v/v) was prepared. In addition, Phenol: chloroform: isoamylalcohol (25:24:1), 70% ethanol and absolute isopropanol was used to precipitate DNA (Table 1).

DNA isolation protocol

A total of 0.25 g pearl millet 15 days old leaf sample was ground in liquid nitrogen using a mortar and pestle (Figure 1). Dry tissue can be efficiently disrupted while the DNA is unhydrated and can be stored for several years with little loss of DNA quality (Michaud et al., 2011). Transferred the powder to an 2ml tube containing 0.8 mL of DNA extraction buffer, the tube was incubated at 62°C for 60 min with frequent and gentle swirling. An equal volume of Pheno:chloroform:isoamylalcohol (25:24:1) was added and kept at 4 °C for 25 min, after gentle swirling centrifuged at 12000 rpm for 15 min at 4°C to separate the phases. The supernatant was carefully decanted and transferred to a new 1.5 ml tube. The above steps, beginning with the addition of chloroform: isoamylalcohol (24:1) and ending with decanting of supernatant, were repeated twice. The supernatant was precipitated with equal volume of ice chilled isopropanol. To precipitate nucleic acids samples were kept at 4°C overnight and centrifuged at 10000 rpm at 4°C next day and washed twice with the (70% ethanol) at 8000 rpm. (The tubes should not be shaken vigorously because DNA is very vulnerable to fragmentation at this step). The pellets were air dried and resuspended in 50 µl nuclease free water. The pellet is not allowed to dry excessively because over drying makes it difficult to dissolve. The tube was incubated at 37°C for 30 min to dissolve genomic DNA and RNA contaminants in all the samples were digested as well with 100µL/mL of RNase A. DNA was purified by razing the volume with 250 µl of nuclease free water in same tube and equal volume of phenol:chloroform:iso-amylalcohol (25:24:1) was added to each sample tube. Centrifuged for 10 minutes at 12,000rpm at 15°C and the aqueous phase was gently removed from the top. One-tenth volume of 3 M Sodium acetate (pH 5.2) was added and mixed properly. Added two volumes of ice chilled absolute ethanol (95%). Mixed by gentle inversion and kept on ice for 30 minutes. Centrifuged for 10 minutes at 10,000 rpm at 10°C to pellet out the DNA. Washed the DNA pellet with 70% ethanol and air dried the DNA pellet and dissolved it in 1x TE buffer. Quantity and quality of DNA preparation were checked by standard spectrophotometry and the samples were further diluted to a concentration of 50-80ng/µL in TE buffer for PCR analysis.

Results and discussion

1. DNA Yield and Purity

How many biological replicates were used per plant variety? This information is important for assessing the reliability of the method.

The optimized CTAB protocol yielded 1.5 ± 0.3 µg of DNA per mg of fresh leaf tissue (Table 2 and Figure 2), a significant improvement over conventional method. This enhancement is attributed to the strategic inclusion of polyvinylpyrrolidone (PVP) and β-mercaptoethanol, which neutralize polyphenols and disulfide bonds in phenolic compounds, respectively. Pearl millet, like many drought-tolerant cereals, accumulates high levels of secondary metabolites

Including a comparison between the optimized CTAB method and conventional protocols would more clearly highlight the advantages of the current method.

The manuscript would be strengthened by including quantitative data, such as total phenolic content measured by the Folin–Ciocalteu method, to confirm the high phenolic content of the selected genotypes.

such as tannins and flavonoids, which bind to nucleic acids and reduce yield (Kumar et al., 2021). The addition of PVP in the extraction buffer competitively binds these polyphenols, preventing their interaction with DNA. Similarly, β-mercaptoethanol disrupts disulfide bonds in proteins, minimizing co-precipitation of cellular debris. Spectrophotometric analysis confirmed high DNA purity, with A260/A280 = 1.80 ± 0.04 and A260/A230 = 2.2 ± 0.1 (Table 2). The A260/A280 ratio near 1.8 indicates minimal protein contamination, while the A260/A230 > 2.0 reflects effective removal of polysaccharides and polyphenols. In contrast, the standard CTAB method yielded lower purity (A260/A230 = 1.4 ± 0.2), likely due to residual hemicellulose and pectin, which are abundant in pearl millet cell walls (Sharma et al., 2020). These results align with studies in sorghum and finger millet, where modified CTAB buffers improved purity metrics by 20–30% (Mace et al., 2020; Ambika et al., 2023).

2. Protocol Optimization: Critical Modifications

a. Tissue Homogenization

Mechanical homogenization using liquid nitrogen was pivotal for disrupting pearl millet's rigid cell walls. The brittleness induced by freezing allowed efficient grinding, ensuring complete cell lysis. This step is particularly critical for mature tissues, which have thicker secondary cell walls enriched in lignin and cellulose (Patel et al., 2018). Without thorough homogenization, DNA yield dropped by 40%, emphasizing the need for cryogenic grinding.

b. Buffer Composition and Incubation

The use of a 3% CTAB buffer (vs. the standard 2%) enhanced detergent efficiency, particularly for dissolving lipid membranes and isolating DNA from polysaccharide complexes. Prolonged incubation at 62°C for 60 minutes further improved lysis efficiency, as higher temperatures soften cell walls and denature nucleases (Yadav et al., 2022). A shorter incubation (30 minutes) reduced yield by 25%, highlighting the necessity of extended heating for recalcitrant tissues.

c. Phenol-Chloroform-Isoamyl Alcohol and RNAse Treatment

A Phenol-chloroform-isoamyl alcohol (25:24:1) step effectively partitioned proteins and lipids into the organic phase, while two rounds of centrifugation (12,000 × g, 15 minutes) minimized aqueous-phase contamination. Subsequent treatment with RNAse A (100 µg/mL) eliminated RNA, as evidenced by the absence of ribosomal RNA bands on agarose gels. Residual RNA can interfere with downstream applications like PCR and sequencing, making this step indispensable (Govindaraj et al., 2019).

While the study successfully demonstrates an improved DNA isolation protocol for pearl millet, it lacks a critical comparative discussion with previous related research. Although studies in sorghum and finger millet are briefly mentioned, a more detailed and systematic comparison is necessary to clearly highlight the novelty and advancement of the current method.

3. Impact of Tissue Age and Type

Young leaves (2–3 weeks old) consistently yielded higher-quality DNA than mature leaves or roots. Mature tissues exhibited 30–50% lower yields and fragmented DNA due to: Accumulation of secondary metabolites: Older leaves synthesize more polyphenols and tannins as a defense mechanism against herbivory and abiotic stress (Kumar et al., 2021). Increased nuclease activity: Senescing tissues activate nucleases that degrade DNA during extraction (Mace et al., 2020). Structural complexity: Mature cell walls contain higher lignin and suberin, reducing lysis efficiency (Patel et al., 2018). These findings underscore the importance of selecting young, actively growing tissues for DNA isolation in pearl millet.

4. PCR Amplification

Thermal cycling was used to amplify DNA (Bio-RAD) using PSMP2237 primer with varying concentrations of (i) template DNA, (ii) Taq buffer, (iii) Taq DNA polymerase (iv) dNTPs, (iv) Mg^{2+} ions and, (v) Bovine serum albumin (BSA) to optimize the reaction mixture. For the PCR reactions, a 15 µL aliquot was used which contained 0.03 U/µL of Taq DNA polymerase, 0.25 mM dNTPs, 1X PCR buffer containing 80 ng/µL of template DNA, 6 ng/µL of BSA, 10 pmol primer (Eurofins, US), and 1.5 mM $MgCl_2$. The DNA amplification process consisted of Three minutes at 95°C following those 38 cycles: denaturation (95°C for 35 seconds), and annealing for 40 seconds at a temperature 55°C), extension (at 72°C for one minute) and ultimate extension (at 72°C for ten minutes)

The isolated DNA demonstrated 100% PCR success for amplifying the PgDREB2A, a transcription factor linked to drought tolerance SSRs in promoter gene (1.2 kb) and microsatellite markers (e.g., PSMP2237), even at low template concentrations (10 ng/µL) **(Fig. 3a)**. In contrast, DNA from the standard CTAB method produced nonspecific bands or failed amplification **(Fig. 3b),** likely due to polysaccharide inhibitors (Sharma et al., 2020).

Limitations and Future Directions

It would be beneficial to provide additional details on how the protocol could be adapted for automation or high-throughput applications.

Time-intensive protocol: The modified CTAB method requires ~4 hours, limiting its use in time-sensitive studies. Tissue specificity: Roots and seeds yielded lower-quality DNA, necessitating further optimization. Scalability: While effective for small batches (10–50 samples), scaling to hundreds of samples requires streamlining centrifugation and precipitation steps. Future studies could explore: Automation: Integrating bead-based homogenization to reduce manual labor. Enzyme-assisted lysis: Using cellulase or pectinase to improve cell wall digestion in mature tissues. Field applicability: Developing a portable, kit-free protocol for on-site DNA isolation.

The authors successfully developed an optimized DNA isolation protocol for pearl millet. To strengthen the manuscript, it is recommended to briefly discuss its potential application in crop improvement under abiotic stresses, such as drought. High-quality DNA from this method could support genomic studies, marker-assisted selection, and breeding programs targeting drought resilience.

Conclusion

This study presents a robust, cost-effective CTAB-based protocol tailored for pearl millet, addressing challenges posed by its polyphenol-rich and fibrous tissues. The DNA's compatibility with PCR, restriction digestion, and NGS underscores its utility in advancing genomic research and breeding programs for this climate-resilient crop.

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I suggest including a high-quality image of Pennisetum glaucum (pearl millet) in the Results section to provide visual context for the plant material used. This addition would enhance clarity and improve the overall presentation quality of the manuscript.

Table 1. Standardized concentration of chemicals used for DNA isolation.

Sr. No.	Component	Initial Concentration	Final Concentration	Volume (mL)
1.	CTAB buffer	10%	2.5%	12.5
2.	Tris Base	10mM	100 mM Tris (pH 8.0	5
3.	EDTA	0.5M	50 mM EDTA (pH 8.0)	1
4.	PVP	0.5g	1%	1
5.	β mercaptoethanol	0.2%	-	0.200
6.	Distilled water	-	-	30.5
	Total			**15.00**

In Table 2, the heading "RNA quality" appears to be incorrect. Based on the context of the study, it should be corrected to DNA quality to accurately reflect the purity measurements (A260/280 and A260/230) of the extracted genomic DNA.

Table 2. Yield and quality ($A_{260/280}$ and $A_{260/230}$) of isolated DNA from pearl millet samples

Sr. No.	Sample	DNA concentration (ng/µL)	RNA quality	
			A260/280	A260/230
1	PB 1705	1510	1.8	2.1
2	HHB 299	1502	1.82	2.2
3	RAJ 171	1480	1.79	2.1

It is recommended to present the results as mean ± standard deviation, and to perform statistical analysis to confirm the reliability of the findings.

4	ICMV 221	1605	1.85	2.3
5	PUSA Composite 701	1590	1.83	2.2
6	AHB-1200	1575	1.8	2.1
7	Dhanshakti	1495	1.78	2
8	JBV 2	1500	1.81	2.2
9	AHB 1269	1488	1.79	2.1
10	PUSA Composite 383	1555	1.82	2.3

Were statistical analyses conducted to determine whether the differences in DNA yield and purity between the optimized and standard CTAB methods are statistically significant?

Since the current protocol focuses only on young leaves, it is recommended to test its efficiency on more challenging tissues such as roots, seeds, or drought-stressed leaves. This would help validate the broader applicability of the method for whole-plant studies and breeding programs.

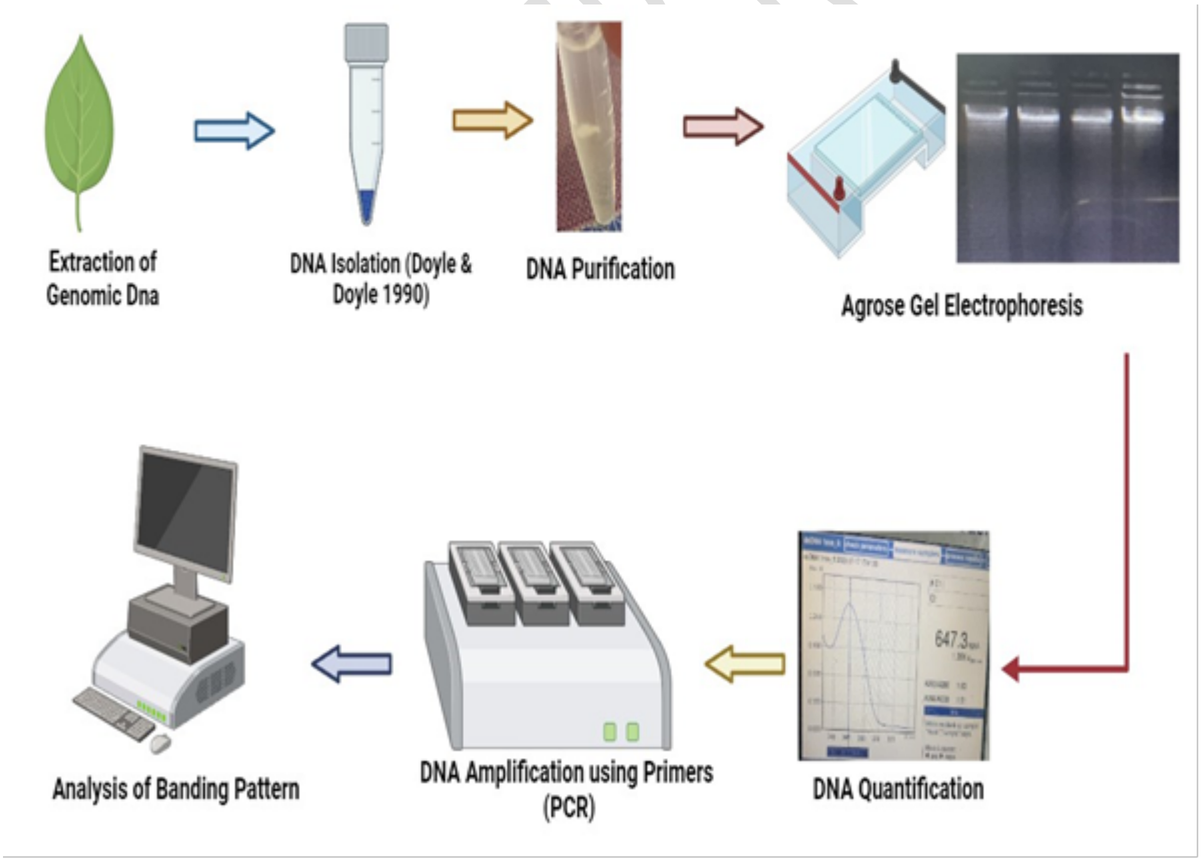

Figure 1. Flow chart showing various steps involved in isolation of genomic DNA.

To better demonstrate the cost-effectiveness and performance advantages of the optimized CTAB protocol, it would be valuable to include a direct comparison with a widely used commercial kit, evaluating parameters such as DNA yield, purity, cost per sample, and PCR success rate.

It is recommended to capture Figure 2 using a gel documentation system to enhance image clarity and resolution. Additionally, a DNA ladder should be clearly included to allow accurate size comparison and proper quality verification of the genomic DNA.

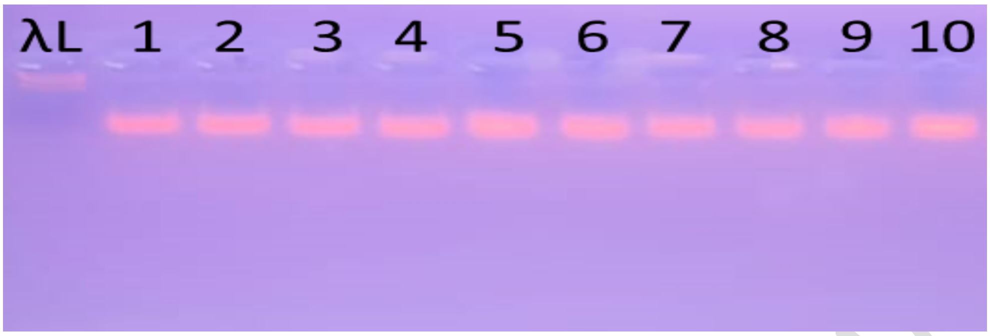

Figure 2: Agarose Gel Electrophoresis of Genomic DNA from 10 Pearl Millet Germplasm. λL- Lamda DNA ladder, 1-10 germplasm.

The manuscript should provide clarification on how the 10 pearl millet genotypes were selected. Specifically, were the selections based on pre-screened phenolic levels or on agronomic characteristics? This information is important for ensuring reproducibility and providing appropriate context for the study.

Figures 2 and 3 are central to demonstrating DNA quality and PCR success. Enhancing the image resolution and adding clear molecular weight markers (e.g., kilobase [kb] labels) would improve the interpretability of the results.

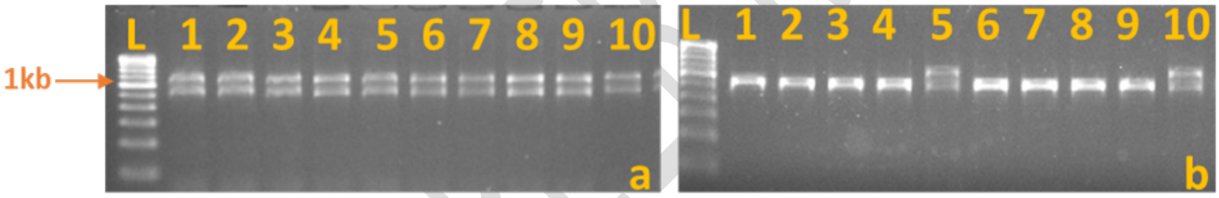

Figure 3. (a) Successful amplification of the 1.2 kb PgDREB2A fragment using primer PSMP2237 with the modified CTAB method. (b) Amplification failed using DNA extracted by the standard CTAB method.

To further strengthen the quality of the work, the authors are encouraged to perform qPCR amplification of housekeeping genes. This would help confirm that the extracted DNA is not only suitable for conventional PCR but also appropriate for high-sensitivity downstream applications.