Research Article

Revolutionizing DNA Extraction: A Cost-effective Approach for Genomic DNA Retrieval from Dried Blood Spots

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Keywords

Dried blood spots, DNA extraction, CTRC, GAPDH, Polymerase chain reaction

Abstract

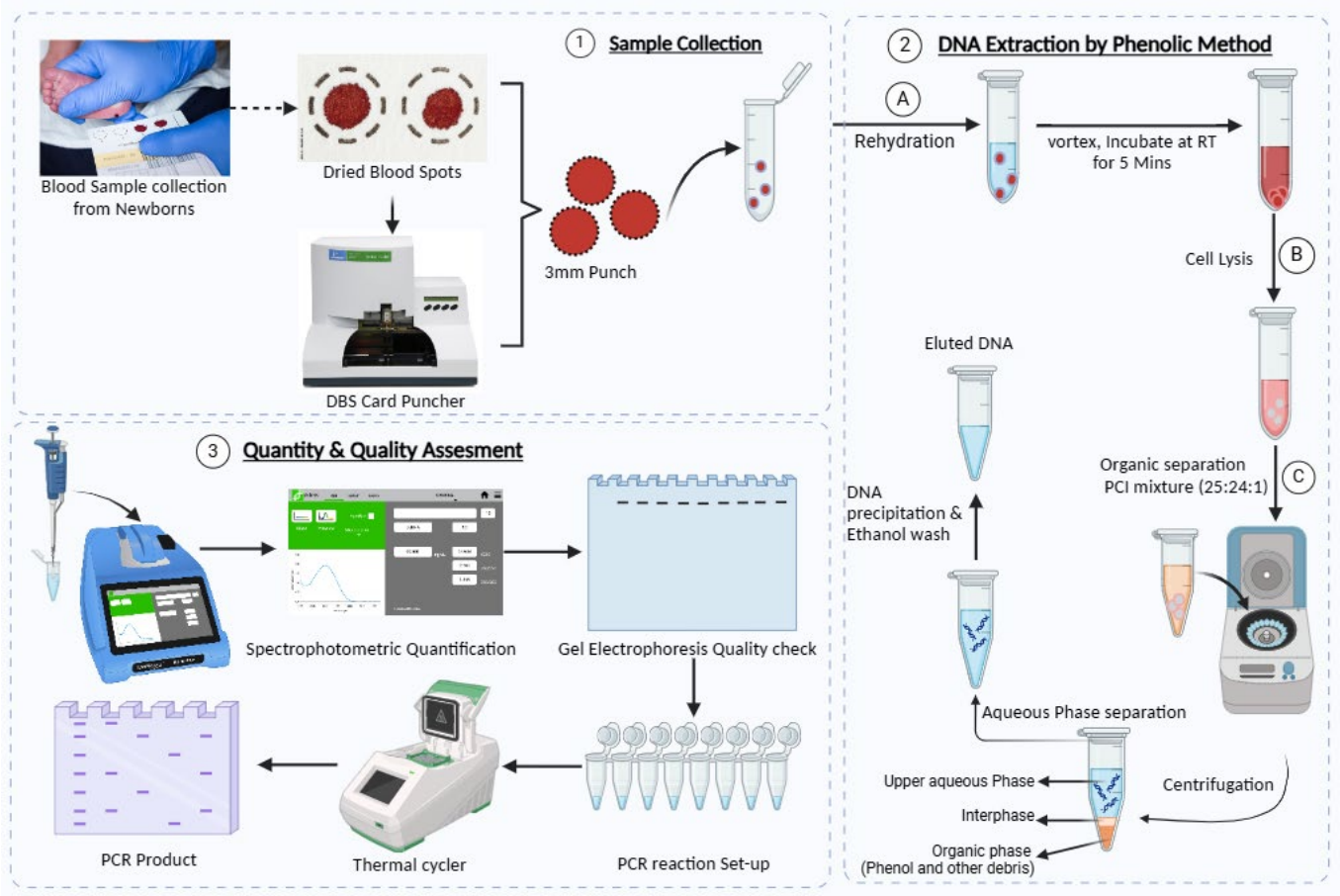
Background: This study introduces an efficient, cost-effective laboratory- derived method for extracting genomic DNA from dried blood spots (DBS) by optimizing the organic separation phenol method.

Methodology: DBS samples, collected via heel prick from 50 neonates as a part of routine newborn screening, were processed using an optimized phenol method that employs lysis buffers with minimal concentrations of proteinase K and phenol:chloroform:isoamyl alcohol (PCI) reagent.

Results: The extracted genomic DNA exhibited a concentration range of 50 to 200ng/µl, with purity levels (A260/280) falling within the range of 1.4 to 1.6, as measured by nanodrop. Gel electrophoresis, post polymerase chain reaction (PCR) amplification, consistently revealed distinct, non-degraded bands for both a 345-bp fragment (Chymotrypsin C, CTRC gene) and a 250-bp fragment (Glyceraldehyde-3-phosphate dehydrogenase, GAPDH gene) across all samples.

Conclusion: The method utilizes routine consumables readily available in basic molecular biology laboratories, circumventing the need for expensive kits. It holds significant promise for genetic diagnostics and research applications, particularly in situations where DBS serves as a means of collecting and preserving samples from individuals in remote areas.

Graphical abstract



Highlights

- Refined phenol-based method that offers cost-effective means of extracting genomic DNA from dried blood spots on filter paper.
- Key attributes of this approach include its simplicity and use of PCI (25:24:1) reagent for superior DNA yield.
- A reliable choice that is economically advantageous for further molecular investigations involving DBS specimens.

Introduction

Molecular diagnostic tests, such as polymerase chain reaction (PCR), restriction enzymatic digestion, and recombinant DNA selection are essential tools in contemporary research and clinical practice [1]. The reliability and accuracy of these tests hinge upon the quality of the starting material: the genomic DNA. Traditionally, whole blood has been the preferred source for DNA extraction. However, this method is not without its challenges, including the need for stringent storage conditions, transportation precautions, and the risk of spillage. In contrast, dried blood spots (DBS) offer a convenient alternative. They are easy to collect, simple to store without the need for specialized facilities, cost-effective to prepare, and do not require the separation of serum and plasma. Moreover, DBS can be treated as non-infectious samples, as most viruses and microorganisms are rendered inactive during the drying process. Their transportability, even from remote areas, makes DBS an optimal choice for sample collection [2]. Furthermore, DBS resolves the issue of obtaining venous blood samples, which can be challenging, especially in neonates.

The adoption of DBS for clinical and research purposes has a notable history, dating back to Dr. Robert Guthrie's pioneering use of DBS samples in 1963 to analyze phenylalanine levels in infants, aiding in the identification of phenylketonuria. Since then, DBS has found diverse applications in disease screening [3,4], drug monitoring [5], genetic analyses [6], and epidemiological studies, particularly in resource-constrained settings with suboptimal storage and transportation conditions. Notably, DBS has been instrumental in DNA methylome analysis [7] and the identification of congenital cytomegalovirus (CMV) infections [8,9], which can lead to hearing impairments.

While alternative methods, such as salt extraction, exist for genomic DNA retrieval, they may not provide the high yields and purity necessary for low-DNA content samples such as DBS. . Methods relying on substances such as Chelex [10-12] and InstaGene Matrix [13-16], though effective, may not be the most cost-efficient solutions. Commercial kits, including QIAamp DNA Mini Kit [16-18], provide convenience but can be expensive and yield low recovery rates. Additionally, when collecting blood samples on filter paper, limitations arise from the marginal amount of DNA obtained from a single blood drop, further compounded by the presence of cellulose fibers from the filter paper itself.

In light of these considerations, we have developed a simple yet highly efficient method for genomic DNA extraction from DBS. Built upon the foundational phenolic method, our approach has been meticulously optimized to ensure both quality and quantity. The phenol extraction method, though more complex, was chosen due to its higher efficacy in removing protein and lipids, yielding higher-quality DNA suitable for downstream applications like PCR. We have assessed the integrity of the extracted DNA through PCR amplification, confirming its suitability for downstream molecular analyses. In this study, we present our methodology and results, demonstrating the robustness of our optimized phenol-based DBS DNA extraction method. To validate our approach, we utilized DBS samples collected from newborns as a part of routine screening. We believe that our streamlined method addresses several of the limitations associated with DNA extraction from DBS and provides a valuable tool for researchers and clinicians alike.

Materials and Methods

Ethics

The Institutional Ethical Committee approval was obtained for the present study (Ref. No: JSS/MC/PG/1098/2021- 22 dated 22/04/2021)

Collection and storage of samples

The DBS were collected from 50 newborns within 48 to 72 hours of birth using the heel prick method on Whatman 903 filter paper as a part of routine newborn screening. Subsequently, these DBS samples were stored in a clean box at 4°C following the biochemical screening and were later utilized for DNA extraction. Additionally, whole blood was collected from normal healthy individuals using an EDTA vacutainer, which served as a positive control.

DBS DNA extraction

1. DBS Puncher *(DBS Puncher®, Revvity)* was used to punch out three 3mm diameter circles from the DBS samples into a 2 ml microcentrifuge tube *(QSP 2.0 ml MCT tube, Thermo Fisher Scientific)*.
2. The DBS spots were rehydrated by adding 300µl of solution A [155mM NH4Cl (SRL), 0.1mM EDTA (SRL), 1mM KHCO3 (SRL)] with 2 minutes of vigorous vortex (DLAB). This mixture was then incubated at room temperature for 5 minutes and the subsequent removal of supernatant using a pipette after a short spin in a mini centrifuge *(D1008E Mini centrifuge, DLAB)* for 30 seconds.
3. To the remaining mixture, 210µl of solution B [200µl of lysis buffer (20mM Tris *(SRL)*, 40mM EDTA *(SRL)*, 300mM NaCl *(SRL)*, 2.5%SDS *(SRL)*), 10µl of Proteinase K (20mg/ml) *(HiMedia)*] was added followed by invert mix 4-5 times and incubated at 56°C for 30 minutes.
4. The Genomic DNA was then extracted from the mixture by adding 300µl of phenol:chloroform:isoamyl alcohol mixture (25:24:1) *(SRL)* and gently invert mix the tube 5-6 times. The mixture was centrifuged *(Refrigerated Centrifuge, Hettich)* at 10000rpm for 5 minutes at room temperature and the obtained aqueous phase was transferred to a fresh 1.5 ml microcentrifuge tube *(QSP 1.5 ml MCT tube, Thermo Fisher Scientific)*.
5. The nucleic acid was recovered by standard precipitation, 40µl of 1.5M sodium acetate (pH 5.2) *(HiMedia)*, an equal volume of ice-cold isopropyl alcohol *(SRL)*, mixed well and centrifuged at 12000 rpm for 15 minutes at 4°C.
6. The clear supernatant was discarded, and the pellet was washed with 500µl of 70% ice-cold ethanol *(DNA Diluent, HiMedia)* two times. The obtained pellet was dried at room temperature until there was no trace of ethanol, and the genomic DNA was resuspended in 25µl of 1X-TE *(SRL)* buffer by gentle finger flicking and stored at -20°C *(Dixell, Antech Instruments)*.

The use of chloroform and isoamyl alcohol in conjunction with phenol ensured proper separation of the aqueous (DNA-containing) phase from the organic phase. Chloroform aids the extraction of proteins and lipids, pulling them into the organic phase, while isoamyl alcohol minimizes foaming, enhancing the efficiency of phase separation. This solvent combination helps reduce phenolic contamination in the aqueous phase, which contains the DNA. Residual phenol and other contaminants were removed by washing the extracted DNA with 70% ethanol.

DBS DNA extraction by the Kit method

DNA extraction from the DBS samples was also carried out using a magnetic bead-based kit, Mag genome dried blood spot DNA extraction kit (Xpress DNA Dried blood spots, MagGenome Technologies Pvt Ltd. India) following the manufacturer's protocol.

Table 1 clearly compares and contrasts the method optimised in this study with the previously established methods [19,20].

Table 1: Comparison of Phenol baseline protocol and optimized phenol protocol for DBS DNA extraction.

Phenol baseline protocol	Optimized phenol protocol
Rehydration	
300µl of rehydration solution, vortex for 1-2 minutes & incubation at R.T for 5 mins.	300µl of rehydration solution, vortex for 2 minutes & incubation at R.T. for 5 mins.
Protein digestion	
160µl of double distilled H2O,	***None***
2X Proteinase K lysis buffer containing 20mg/ml, vortex for 20 seconds.	200µl of lysis buffer, 10 µl of Proteinase-K (20mg/ml), ***invert mix 4-5 times.***
Incubate at 56^{o}C for 1hr (with intermittent vortex for 10 seconds).	incubate at ***65^{o}C for 30 mins.***
Genomic DNA extraction	
200µl of buffered phenol and 200 µl of chloroform and isoamyl alcohol mixture (24:1), with subsequent vortex for 30 seconds. The mixture is centrifuged at 10,000 rpm for 4 minutes at room temperature.	***250µl of phenol: chloroform: isoamylalcohol (25:24:1) reagent and gently invert mix the tube 5-6 times.*** The mixture was centrifuged at 10,000 rpm for 5 minutes at room temperature.
DNA precipitation	
To the aqueous phase, 40µl of 3.0 M sodium acetate (pH-5.2) and 400µl isopropyl alcohol is added, mixed, and centrifuged at 10,000 rpm for 4mins at R.T. The supernatant is removed, and the pellet is washed with 70% ethanol, air dried & dissolved in 50µl of 1X TE buffer.	To the aqueous phase, add 40µl of 3.0 M sodium acetate (pH-5.2) and an ***equal volume of isopropanol.*** The solution was centrifuged at ***12,000 rpm for 15mins at 4^{o}C.*** The supernatant was removed, and the pellet was washed with 70% ethanol, air-dried & dissolved in ***25µl of 1X TE buffer.***

Estimation of DNA concentration and quality

The concentration of the extracted DNA samples was determined using a Nanodrop spectrophotometer (DeNovix 11). The absolute concentration was calculated by measuring the absorbance at 260nm (A260), with an assumption that an A260 of 1.0 corresponds to 50ng/µl of DNA. Additionally, the DNA quality was assessed by determining the A260/280 ratio and performing gel electrophoresis to evaluate its integrity.

PCR amplification of *CTRC and GAPDH* genes

A PCR amplification was conducted to assess the quality of the extracted DNA, determined by the successful generation of PCR products visible as bands on an agarose gel. For this purpose, a pair of endogenous control primers were synthesized (Bioserve Biotechnologies, India Pvt. Ltd) (Table 2). These primers targeted the *CTRC* and *GAPDH* genes and were employed to assess the suitability of extracted DNA from dried blood spots. The *CTRC* primer set yielded a 345-bp PCR product, while the *GAPDH* primer set produced a 250-bp PCR product, both of which were visualized on an agarose gel.

Table 2: The primers sequence of an endogenous control gene.

Sl.No.	Gene	Primer sequence
1	*CTRC* Forward primer	5'AAGGACAATGGGAACACTCTCT3'
2	*CTRC* Reverse primer	5'TCAGGTATGGGGTGCGACAG3'
3	*GAPDH* Forward primer	5'CCACTCCTCCACCTTTGACG3'
4	*GAPDH* Reverse primer	5'CCACCACCCTGTTGCTGTAG3'

The PCR reactions were conducted using the Takara Bio Inc. Thermocycler machine. Each reaction had a total volume of 20µl, comprising 1µl of genomic DNA (50ng), 10pmol of each primer, 10mM of dNTPs (TaKaRa), 0.75unit of Taq DNA Polymerase (TaKaRa), 1X buffer with 1.5mM MgCl2 (TaKaRa). For positive control, 50ng of the extracted genomic DNA from whole blood collected in an EDTA vacutainer was used, without the need for an enhancer and additional MgCl2. The PCR cycling conditions consisted of an initial denaturation at 94°C for 5 minutes, followed by 30 cycles of denaturation at 94°C for 30 seconds, annealing at 59°C for 30 seconds, extension at 72°C for 30 seconds, and a final extension at 72°C for 5 minutes. Approximately 5µl of the obtained PCR product was subjected to electrophoresis on a 2% agarose (HiMedia) gel stained with ethidium bromide (HiMedia). Electrophoresis was conducted in Tris-acetate-EDTA buffer (TAE) (HiMedia) and the resulting bands were compared to a 100bp DNA ladder (Gene Direx).

Results

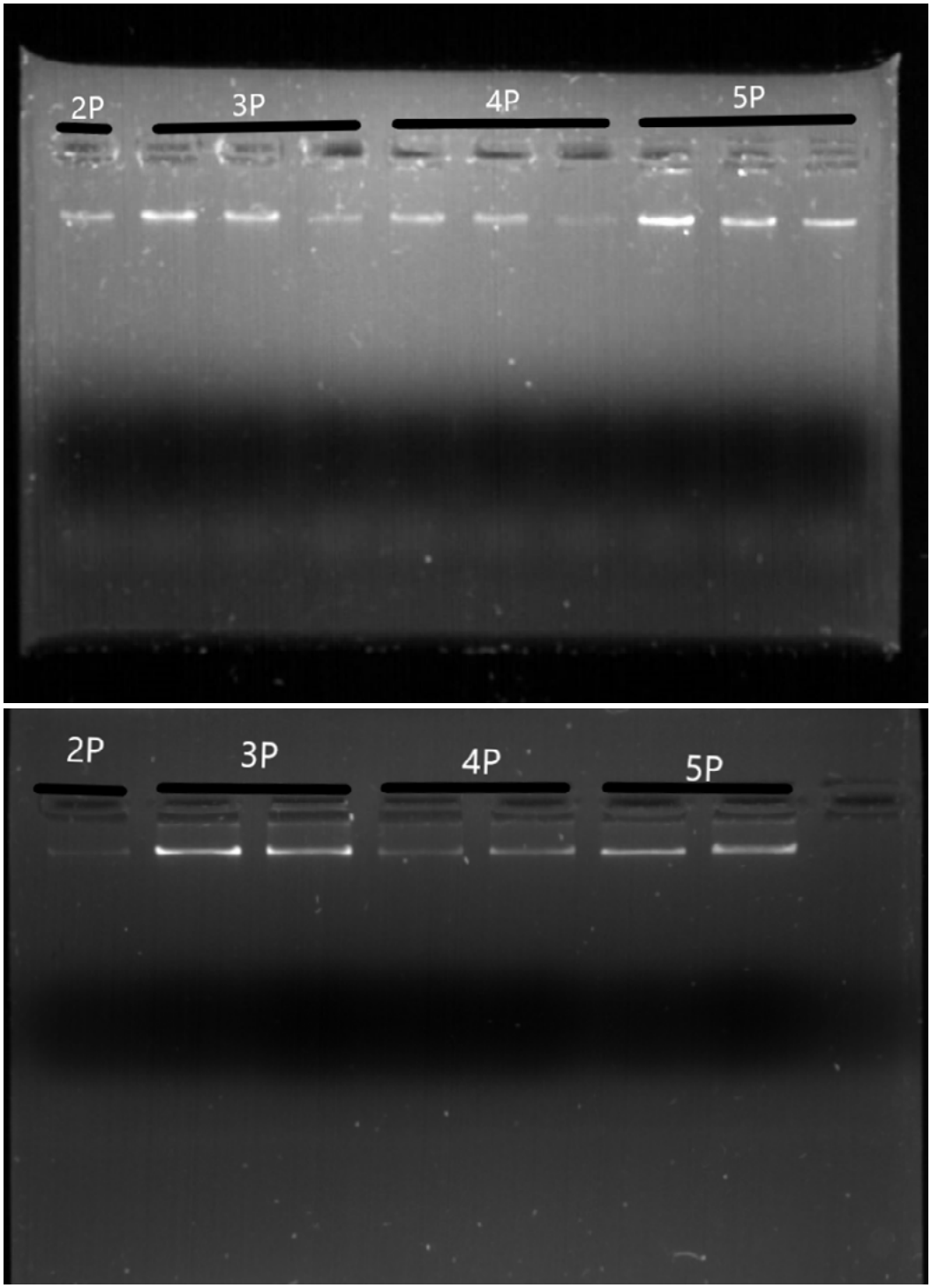
Comparison of DBS DNA isolation methods (Quantity and Quality)

The genomic DNA isolated from DBS samples using the optimized phenol method exhibited superior quality compared to the kit-based method (Table 3). The concentration of DNA ranged between 50-200ng/µl and the purity (A260/280) ranged from 1.4 to1.6 (Table 3, Fig.1). The integrity of DNA was assessed by resolving it in a 1% agarose gel electrophoresis, stained with ethidium bromide. In all DBS samples isolated using the phenol method, a distinct, bright band without any degradation was consistently observed (Figure 1).

Table 3: The genomic DNA concentration of Dried blood spot samples measured by DeNovix DS-11 Spectrophotometer.

DBS DNA Samples – Optimized Phenolic Method					DBS DNA Samples – Kit Method				
Sl.No.	Concentration (ng/µl)	Absorbance A260	A260/280 ratio	A230/260 ratio	**Sl.No.**	Concentration (ng/µl)	Absorbance A260	A260/280 ratio	A230/260 ratio
1	92.91	1.858	1.47	1.07	**1**	48.48	0.977	1.30	0.82
2	127.73	1.554	1.63	1.39	**2**	50.03	1.000	1.40	0.78
3	167.00	1.340	1.45	1.05	**3**	51.75	1.035	1.42	0.77
4	193.82	1.028	1.45	1.47	**4**	54.66	1.093	1.39	0.72

Figure 1: Representative of 1% Agarose Gel Electrophoresis of isolated DBS DNA samples by manual Phenolic method and the Xpress DBS DNA MagGenome Kit method.

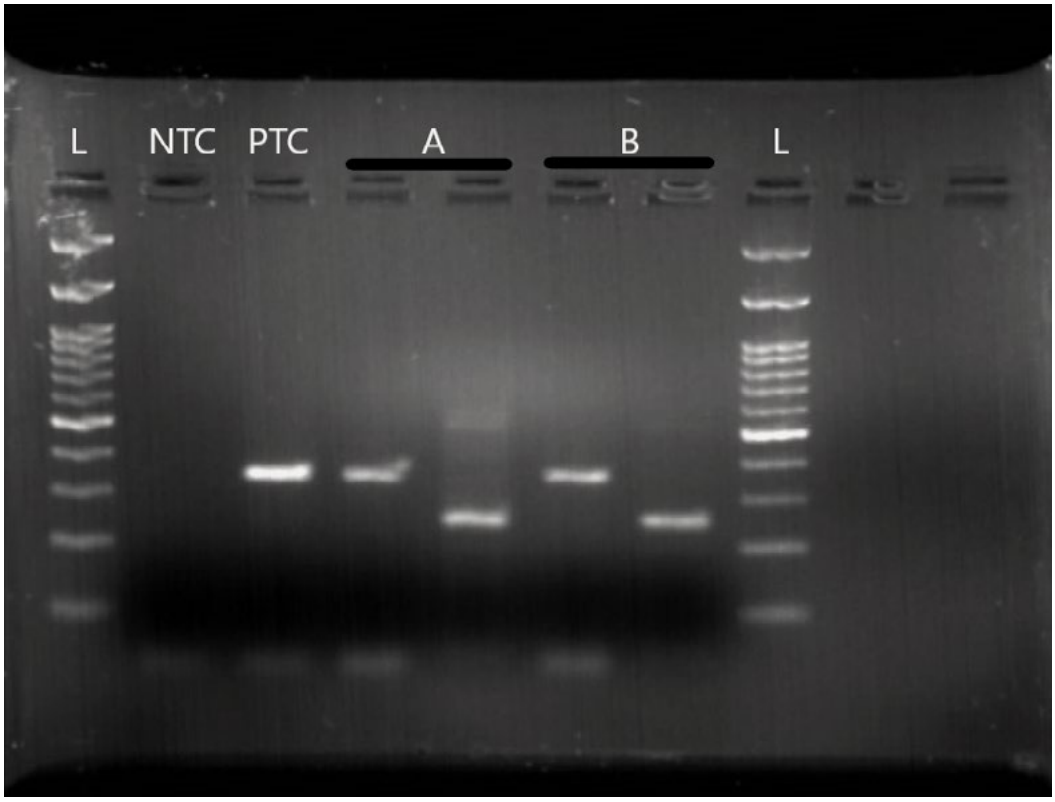


A (optimized Phenolic method)- Lane 1: 2punches, Lane 2-4: 3punches, Lane 5-7: 4punches, Lane 8-10: 5punches of DBS (3mm).; B (Kit method) Lane 1: 2punches, Lane 2-3: 3punches, Lane 4-5: 4punches, Lane 5-6: 5punches of DBS (3mm). Distinct bright bands without any degradation were observed in all the samples.

PCR Product Gel-Electrophoresis

To assess the presence of PCR inhibitors in the genomic DNA obtained from DBS samples, we subjected the isolated DNA to PCR amplification targeting the *CTRC & GAPDH* endogenous genes. As a positive control, we used genomic DNA extracted from the whole blood of a normal healthy human. The results demonstrated the presence of PCR products of the expected sizes: a 345-bp fragment of the *CTRC* gene and a 250-bp fragment of the *GAPDH* gene in all the DBS-extracted DNA samples by both kit and manual methods. Notably, no bands were observed in the negative control and positive control showed *CTRC & GAPDH* gene amplification (Figure 2).

Figure 2: Representative of 2% Agarose Gel Electrophoresis of PCR products of two different targeted genes.



Lane 1-DNA Ladder (100bp); Lane 2-Negative control; Lane 3-Positive control; A: Kit method Lane 4-CTRC gene (345bp), Lane 5-GAPDH gene (250bp); B: Manual method Lane 6-CTRC gene, Lane 7-GAPDH gene.

Discussion

This study aimed to develop a cost-effective in-house protocol for extracting genomic DNA from the dried blood samples collected on Whatman 903 filter paper. DBS sampling is most frequently used to assess small-molecule analytes in newborn screening for metabolic disorders, epidemiological studies, and biobanking due to its ease of collection, transportation, and long-term storage stability in clinical laboratories [21,22]. Given the historical reliance on DBS for over a century and its use in metabolic disease screening for the past five decades, it is crucial to optimize DNA extraction methods that are both efficient and economically viable [23].

Previous studies have explored various DNA extraction methods from DBS, emphasizing the need for methods that ensure high purity and yield while minimizing cost and complexity. For instance, Kumar et al. evaluated the efficacy of column-based and magnetic bead-based methods. While these methods provide sufficient DNA for genetic studies, they were associated with significant drawbacks, including a 5-10% loss of magnetic beads and consequently, genomic DNA [24]. Such losses are particularly problematic when working with limited samples or in resource-limited settings. Storm and colleagues compared commercially available kits, including the Chelex-100, QIAamp DNA Mini Kit, and InstaGene Matrix, with a TE buffer-based method. Although Chelex-100 showed fewer PCR inhibitors and was effective in chelating polyvalent metal ions, the cost and availability of these kits, particularly in remote areas, present significant challenges [25]. Moreover, methods such as the QIAamp DNA Mini Kit, despite being widely used, still involve multiple steps that could introduce variability and reduce DNA recovery, particularly when combined with the inherent variability of DBS samples [25]. Ghantous et al. further highlighted the variability in DNA yield across different methods, noting that even optimized protocols, such as the combination of GenSolve and Qiagen kits, resulted in DNA yields ranging from 3-40 ng/µl [7]. Such variability is concerning, especially for applications requiring high DNA concentrations or when working with samples stored over extended periods, where the integrity of the DNA may be compromised.

Similarly, Barsa Baisalini Panda and colleagues explored several extraction methods, including Methonol-based and PBS-based methods, in addition to Chelex-100 and TE buffer-based protocols. Methanol-based extraction, while cost-effective due to the low price of methanol, involves time-consuming steps with repeated drying and overnight incubation. The PBS-based method, which is similar to the conventional phenol-chloroform extraction technique, also requires prolonged overnight treatment with lysis buffer and proteinase K. While the Chelex-100 method is rapid, its high cost and limited availability in remote areas make it less feasible in certain settings. Importantly, the PCR

results using the optimized phenolic method were comparable to those obtained with commercial kits, with the phenolic method being particularly advantageous for large sample sizes due to its cost-effectiveness, approximately one-third of the cost of kit-based methods [26].

Phenol-based methods are often criticized for the potential risk of contamination, our protocol minimizes this issue through the use of phenol: chloroform: isoamyl alcohol mixture. Chloroform facilitates the separation of proteins and other organic contaminants into the organic phase, while isoamyl alcohol prevents foaming and aids in more efficient phase separation. Together, these components ensure a clean separation of the DNA-containing aqueous phase, thereby reducing the risk of phenolic contamination. Furthermore, multiple ethanol wash steps were performed to remove any remaining phenol residues.

While non-toxic methods such as salt extraction offer simplicity, our optimized phenol: chloroform: isoamyl alcohol protocol demonstrates several advantages, particularly when working with DBS. Organic solvent extraction consistently yielded higher DNA concentrations (50-200 ng/µl) and improved purity compared to salt extraction, which may not perform as well with limited sample volumes such as those found in DBS. Additionally the phenol method effectively removes proteins and cellular debris, which can interfere with downstream applications, making it a more robust solution for these challenging sample types. Salt extraction, while non-toxic, is better suited for larger sample volumes where DNA yield is not as much of a constraint. The higher yield and purity obtained through our optimized method justify its use over salt extraction in this context, particularly for laboratories working with limited or low-volume samples like DBS. The optimized phenolic method demonstrated several advantages over commercial kits, including a significantly lower cost and fewer steps, which reduces the likelihood of human error. While phenol is a toxic reagent, its benefits in terms of yield and purity in genomic DNA extraction from challenging samples like DBS justify its use. Importantly, the method proved effective in amplifying targeted genes using conventional PCR technique. This suggests that the method is suitable for mutation studies, which require precise and reliable DNA amplification. Further refinement of non-toxic alternatives, such as salt extraction, may provide safer but equally effective methods in the future. However, at present, the phenol-based approach offers a significant advantage in terms of both quality and quantity of DNA retrieval.

Limitations

Despite its advantages, the phenolic method does present some limitations. The purity of the extracted DNA was lower compared to commercial kits, potentially due to the presence of cellulose fibres and phenol residues, which could interfere with downstream applications. Additionally, the uneven distribution of blood on the filter paper may result in variable DNA yields, a factor that warrants further investigation. The methods effectiveness across different storage conditions and durations also remains to be validated, which is critical for ensuring its applicability in diverse research and clinical settings.

Future Directions

Further studies should focus on optimizing the phenolic method further to enhance DNA purity, perhaps by incorporating additional purification steps to remove contaminants such as cellulose fibers. It is also essential to validate the method's performance in highly demanding diagnostic techniques, such as microarray analysis and next-generation sequencing (NGS), to confirm its broader applicability. Additionally, exploring the effects of storage conditions, such as temperature and duration, on DNA integrity and yield from DBS will provide critical insights into the methods robustness and reliability in biobanking and long-term epidemiological studies.

Conclusion

In conclusion, our DNA extraction method holds significant promise for genetic diagnostics and research applications, particularly in situations where DBS serves as a successful means of collecting and preserving samples from individuals in remote areas. While further optimization is required to address its limitations, the method provides a valuable solution in regions where access to freezers is limited. Moreover, DBS emerges as a potential sample source for biobanking in epidemiological studies.

Ethics statements

The study was approved by the Institutional Ethical Committee of JSS Medical College, Mysuru (India) via approval letter number JSS/MC/PG/1098/2021-22 dated 22/04/2021. Informed consent was obtained from the human subjects.

Author contributions

Akila Prashant: Conceptualization, Methodology, Writing-Reviewing and Editing. Haripriya S Kundapura: Writing-Original draft preparation, Investigation. Anju Srinivas: Method Validity tests, Data curation. Manju Hosuru Chikkalingaih & Anshu Kumar Yadav: Visualization, investigation. Prashant Vishwanath and Suma M. N: Supervision.

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