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SIGNIFICANCE OF 260/230 RATIO AND STRATEGIES FOR OPTIMIZED AND IMPROVED PCR OUTCOME

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Abstract

Background: High-quality human DNA samples and associated information are essential for biomedical research, particularly for specific downstream applications. Ensuring the usefulness and long-term functionality of DNA products is crucial. Quality indicators, such as purity and integrity, are vital for assessing DNA quality. This study examines the quality indicators in DNA samples from 12 blood samples and discusses optimization techniques to address low-quality DNA samples for downstream applications. Additionally, the study underscores the importance of DNA quantification and qualification procedures to ensure proper sample management, minimize unreliable results, reduce costs, and prevent sample wastage. **Aim:** To examine the importance of 260/230 ratios in DNA quality assessment and propose optimization techniques for improved PCR outcomes. **Material and Methods**: Blood samples were obtained via venepuncture, stored at 4-8°C and DNA was extracted using the QIAamp DNA Blood Mini Kit. DNA purity was determined using UV spectrophotometer, categorizing samples based on the 260/230 ratio. Three PCR reactions were conducted for the MTNR gene, with one including BSA as an additive. Optimization techniques involved adjusting annealing temperature and primer concentrations. **Results**: Reactions lacking additives showed weak bands in samples with 260/230 <1.5, while BSA-enhanced reaction exhibited significantly stronger bands, indicating improved amplification efficiency. **Conclusion:** It is essential to consider 260/230 ratio as DNA purity indicator for improved amplification. Addition of PCR additives BSA is better strategy for amplification in the samples with poor 260/230 ratio.

Keywords: DNA, Spectrophotometry, 260/230 Ratio, Bovine Serum Albumin, Optimization Techniques.

INTRODUCTION

Deoxyribonucleic acid (DNA) serves as valuable resource for researchers, providing access to stable genomic DNA for genetic analysis and the exploration of pathophysiological and prognostic factors in various diseases [1]. Before employing DNA samples in analytical techniques, it is imperative to assess their quality and usability using DNA quality indicators, including DNA purity and integrity. The absorbance ratio at 260/280 nm is commonly utilized to gauge DNA purity, with a ratio around 1.8 indicating purity; however, deviations (≤1.6) may suggest the presence of contaminants like proteins or phenol [2,3]. Conversely, RNA presence can elevate this ratio, cautioning against DNA over-quantification. Additionally, the 260/230 ratio serves as a secondary purity measure, typically ranging between 2.0 and 2.2 for pure DNA, with lower values possibly indicating contaminants such as proteins, guanidine HCl, EDTA, carbohydrates, lipids, salts, or phenol, with the less concentrated sample

showing a lower 260/230 ratio due to salt absorbance at 230 nm [4,5] Moreover, the choice of solvent used can influence DNA absorption, with acidic solutions leading to underestimation and basic solutions to overestimation of the 260/280 ratio. Additionally, conventional spectrophotometric methods for DNA quantification may be prone to inaccuracies due to variations in light scattering, exposure to leaching chemicals, and other factors. To address these challenges and optimize PCR results, researchers have explored various optimization techniques [6]. One such technique involves the use of additives such as bovine serum albumin (BSA) in combination with organic solvents like dimethyl sulfoxide (DMSO) or form amide to enhance PCR amplification yield, particularly for GC-rich DNA targets [7]. Studies have shown that this combination significantly improves PCR efficiency without compromising specificity, making it a cost-effective and reliable approach for PCR optimization [8,9]. We aim to investigate the significance of the 260/230 ratios and explore optimization techniques to improve PCR results, particularly in cases of low-quality DNA samples. Building upon existing literature, we will delve into additional optimization techniques and methodologies to enhance the accuracy and reproducibility of DNA quantification and PCR amplification.

MATERIALS AND METHODS

Ethical Considerations:

Ethical approval was obtained from the institutional review board for human subject research. Informed consent was obtained from all participants prior to sample collection.

DNA Sample Collection:

Blood samples were obtained from 12 individuals via standard venipuncture techniques using anticoagulant vials containing EDTA and processed within 2-3 days of collection.

DNA Extraction

Genomic DNA was isolated from the blood samples using the QIAamp DNA Blood Mini Kit (QIAGEN; Germany) following the manufacturer's protocol. This kit utilizes a silica-gel membrane to selectively bind DNA while allowing contaminants to pass through. PCR inhibitors, including divalent cations and proteins, were efficiently removed in two wash steps, ensuring the purity of the extracted nucleic acid. The eluted DNA was obtained in either water or a buffer provided with the kit, ready for use in downstream PCR procedures.

Quantification and Assessment of DNA Purity Using Spectrophotometry

The purity and concentration of extracted DNA were assessed using the Eppendorf Bio Spectrometer (Eppendorf; Germany), measuring absorbance at 260/280 nm and 260/230 nm.

Categories:

Based on the 260/230 ratio, which is considered optimal between values of 2.0 and 2.2, DNA samples were classified into four groups:

1. Best quality: ratios between 2 and 2.2.

2. Borderline quality: ratios between 1.5 and 1.9
3. Low quality: ratios ranging between 1 to 1.4
4. Very low quality: ratios less than 1

PCR Reactions:

Three PCR reactions were conducted as follows:

1. PCR reaction with 50ng DNA concentration without BSA in a total volume of 20µl.
2. PCR reaction with 50ng DNA concentration without BSA in a total volume of 50µl (Dilution).
3. PCR reaction with 50ng DNA concentration with BSA additive in a total volume of 20µl.

PCR Amplification

For PCR analysis in this study, 50 ng of DNA from each sample was amplified for the MTNR gene. The amplification reactions consisted of 1 U Taq DNA polymerase (Qiagen; Germany), 0.15µM dNTPs (Thermo Scientific; USA), and 0.3µM each of forward and reverse primers for MTNR gene amplification. No additives were used in the first two reactions, while BSA was included as an additive in the third reaction. The PCR product, expected to be 434bp in size, was visualized by agarose gel electrophoresis using gelDoc. Additionally, DNA Ladder 50bp (Thermo Scientific; USA) was utilized as size references.

PRIMER SEQUENCE (5'-3')	Tm (^{0}C)	Ta (^{0}C)	AMPLICON SIZE
TTTTTGTGCTGCAAATGGGTTAAAGAGG (Forward Primer)	57	61.1	439
GAGCCTTTGTTCAGAACCATGCTGCTTA (Reverse Primer)	60		

PCR Optimization Techniques:

Various optimization techniques were employed to improve PCR results, including:

Annealing temperature

Annealing temperature optimization involves finding the temperature at which primer binding to the template DNA is most efficient, leading to optimal amplification. Initially, a range of annealing temperatures was tested to determine the temperature that yielded the highest specificity and efficiency for the target gene. This involved setting up PCR reactions with a gradient of annealing temperatures, typically ranging from 55°C to 65°C. After amplification, the PCR products were analyzed by agarose gel electrophoresis to assess the intensity and specificity of the band. The annealing temperature 61.3°C resulted in the most specific amplification of the MTNR gene, indicated by a clear and intense band on the gel with minimal non-specific amplification, was selected for further experiments.

Adjusting primer concentrations

This involves preparing PCR reactions with varying concentrations of forward and reverse primers, such as 0.1µM to 1.0µM, in increments of 0.1µM. After amplification, the PCR products are analyzed by agarose gel electrophoresis to evaluate the intensity and specificity of the bands. The primer concentration of 0.375µM was selected that produced the most intense and specific band for the target gene.

PCR cycling conditions

Table 1: Optimization of PCR cycling conditions

Temperature	Time	Cycles
95°C	4 minutes	1
95°C	25 seconds	40
61.3°C	30 seconds	
72°C	30 seconds	
72°C	5 minutes	1

Additives

PCR additives are crucial for overcoming challenges in amplifying GC-rich sequences. Compatible solutes aid in preventing secondary structure formation and non-specific hybridization, especially beneficial for GC-rich and low DNA quantity samples. Sugars like trehalose, glucose, and glycerol stabilize enzymes such as Taq polymerase against thermal inactivation, enhancing amplification efficiency and specificity. Trehalose acts as a stress-responsive molecule, preventing protein denaturation and serving as a lyoprotectant in PCR reagents. Sucrose enhances yield and specificity, particularly for short amplicons, while glycerol strengthens hydrophobic interactions and aids in nucleic acid strand separation. Proline alters DNA stability at high concentrations, enhancing PCR without inhibiting enzyme activity. Organic solvents like dimethylsulfoxide (DMSO) and formamide enhance PCR by preventing secondary structure formation, regulating DNA and primer melting temperature, and reducing non-specific amplification. DMSO facilitates DNA sequencing and isothermal amplification but can inhibit polymerase activity at high concentrations. Proteins such as bovine serum albumin (BSA) protect DNA polymerase from inhibitors, stabilize enzyme activity, and enhance PCR efficiency, particularly useful for challenging sample types. Tetraalkyl-ammonium (TAA) salts and other additives offer potential for improving PCR under diverse conditions, collectively enabling robust analysis of GC-rich sequences and challenging samples.Tungsten disulphide (WS2), a layered transition metal sulphide nanomaterial, enhances PCR specificity and efficiency by adsorbing single-stranded DNA, reducing primer mis-annealing and dimer formation, and altering primer-DNA binding kinetics and DNA polymerase activity without compromising fidelity [10,11].

Table 2: PCR enhancers or additives concentrations used in PCR

Mechanisms/PCR enhancers	Concentration(s) used in PCR
Betaine	5 M [10]
Trehalose	0.2 mol/L [11]
Sucrose	2–10% [12]
Glycerol	5–10% [13]
Proline	3–5.5 M [14]
Homoectoine	0.1–0.5 M [15]
DMSO	2–10%, with 5% more commonly used [16]
Formamide	5% [17]
BSA	200–400 ng/µL [18]
TAA	10–100 Mm 10–20 mM [19]
WS_2	1–15 µg/mL [19]

Utilization of PCR Additives, BSA to Improve Amplification:

The beneficial effects of BSA were observed in the absence of any other additive. Since most of the PCR inhibitors in the samples analyzed in these experiments were also substances that BSA can bind to, the beneficial effects of BSA were proposed to prevent these inhibitors from interacting with DNA (Taq) polymerase.

BSA concentration gradient

BSA was initially prepared at a concentration of 5 µg/µl. The 20 µl PCR reactions were set up using BSA volumes ranging from 0.3 µl to 0.9 µl, with increments of 0.1 µl, to create a concentration gradient.

RESULT

The assessment of DNA quality was conducted based on 260/280 and 260/230 ratios, categorizing the samples into four quality groups. Samples with ratios between 2 and 2.2 were classified as having the best quality DNA, exhibiting a mean 260/280 ratio of 1.83 and a mean 260/230 ratio of 2.15, with an average DNA concentration of 32.57 ng/µl. Those with ratios between 1.5 and 1.9 fell into the borderline quality category, showing a mean 260/280 ratio of 1.86 and a mean 260/230 ratio of 1.67, with an average DNA concentration of 46.70 ng/µl. The low-quality category comprised samples with ratios ranging from less than 1 to 1.5, displaying a mean 260/280 ratio of 1.82 and a mean 260/230 ratio of 1.26, with an average DNA concentration of 24.73 ng/µl. Finally, samples with ratios less than 1 were categorized as very low quality, showing a mean 260/280 ratio of 1.68 and a mean 260/230 ratio of 0.51, with an average DNA concentration of 28.13 ng/µl. These findings underscore the association between DNA quality categories and the corresponding mean values of 260/280 and 260/230 ratios, along with the mean DNA concentration within each category

Table 2: DNA Quality Assessment Based on 260/280 and 260/230 Ratios by Category

Category	Sample No.	DNA Concentration in ng/µl	260/280	260/230
1	1	40.2	1.85	2.43
	2	21.3	1.81	2.01
	3	36.2	1.84	2.02
2	4	91.3	1.86	1.83
	5	19.5	1.77	1.53
	6	29.3	1.95	1.66
3	7	16.7	1.72	1.21
	8	25.6	1.86	1.15
	9	31.9	1.88	1.42
4	10	17.5	1.63	0.58
	11	12.7	1.72	0.4
	12	54.2	1.7	0.55

BSA concentration gradient

Initially, BSA was prepared at a concentration of 5µg/µl. PCR reactions were then set up using varying volumes of BSA (ranging from 0.3µl to 0.9µl in 0.1µl increments) to establish a concentration gradient.

Table 3: BSA Gradient: their concentration and volume

BSA Gradient (20µl reaction)		
S. no.	**concentration in µg/µl**	**Volume in µl**
1.	0.05	0.2
2.	0.075	0.3
3.	0.1	0.4
4.	0.125	0.5
5.	0.15	0.6
6.	0.175	0.7
7.	0.2	0.8
8.	0.225	0.9

Following amplification, the PCR products underwent agarose gel electrophoresis to evaluate band intensity and specificity. Notably, the BSA volume of 0.8 µl was identified as the optimal condition, demonstrating the most robust and specific band corresponding to the target gene. This outcome suggests that a BSA volume of 0.8 µl provides optimal support for PCR amplification, enhancing both the intensity and specificity of the amplified product as in figure 1.

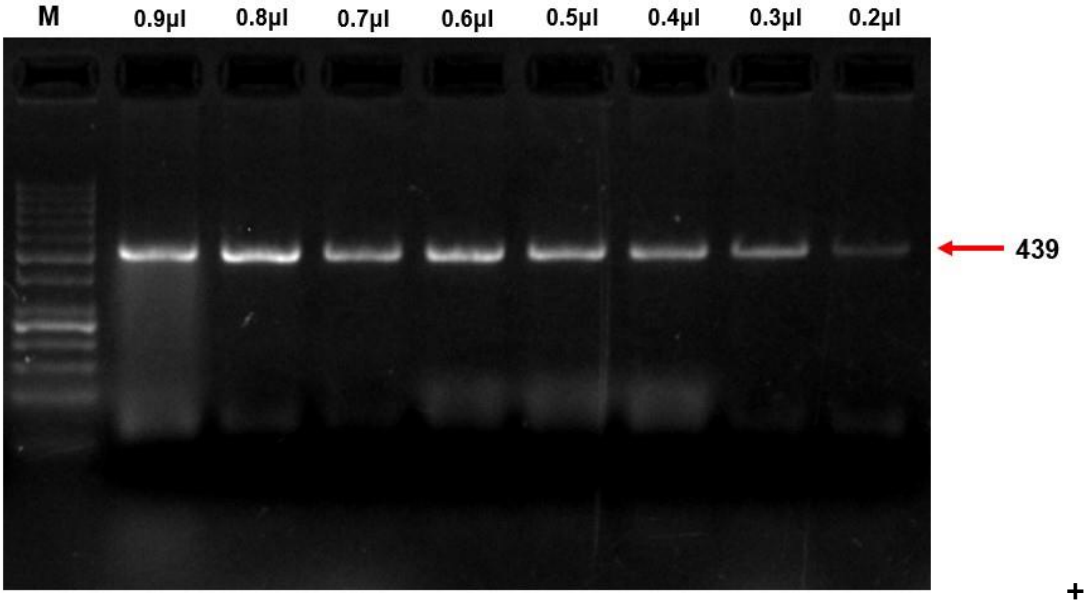

Figure 1: BSA gradient PCR products -agarose gel electrophoresis to evaluate band intensity and specificity

DNA samples were subjected to PCR amplification targeting the MTNR gene. Three PCR reactions were conducted: reaction 1 without any additives, reaction 2 also without any additives, and reaction 3 containing BSA as an additive.

Reaction 1

In reaction 1, where no additives were included, PCR amplification of the MTNR gene was observed, but the bands visualized in the electrophoresis appeared faint or light. Notably, only samples 1 and 2 exhibited intense bands, indicating more robust amplification in good quality DNA samples compared to other samples under these conditions.

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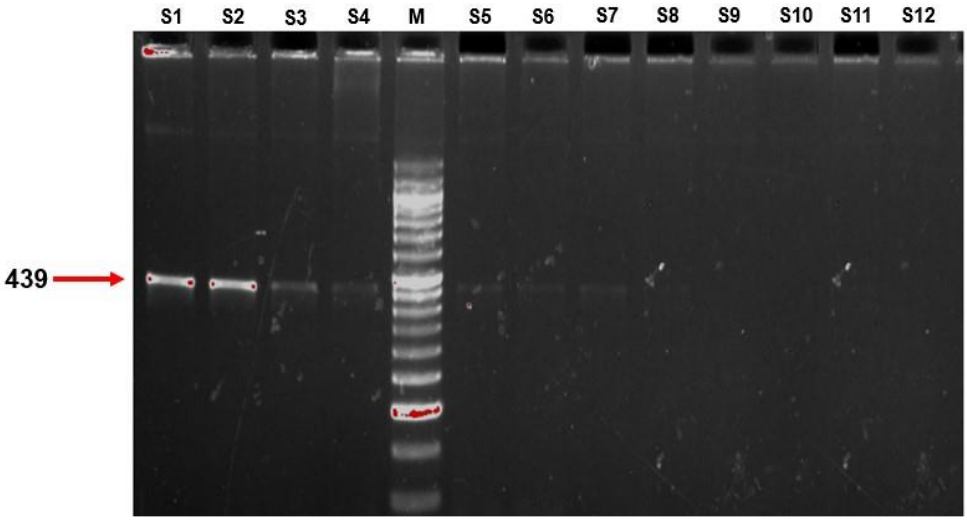

Figure 2: Reaction 1: PCR Amplification of MTNR Gene without Additives

Reaction 2

In reaction 2, where the reaction volume was increased to 50 microliters to investigate the effect of dilution on the quality of the post-amplification product, the bands observed in samples 1 to 5 were intense, while the bands in the remaining samples appeared faint. This difference in band intensity suggests that higher reaction volumes might have impacted the efficiency or concentration of PCR products, resulting in varied amplification outcomes across different samples.

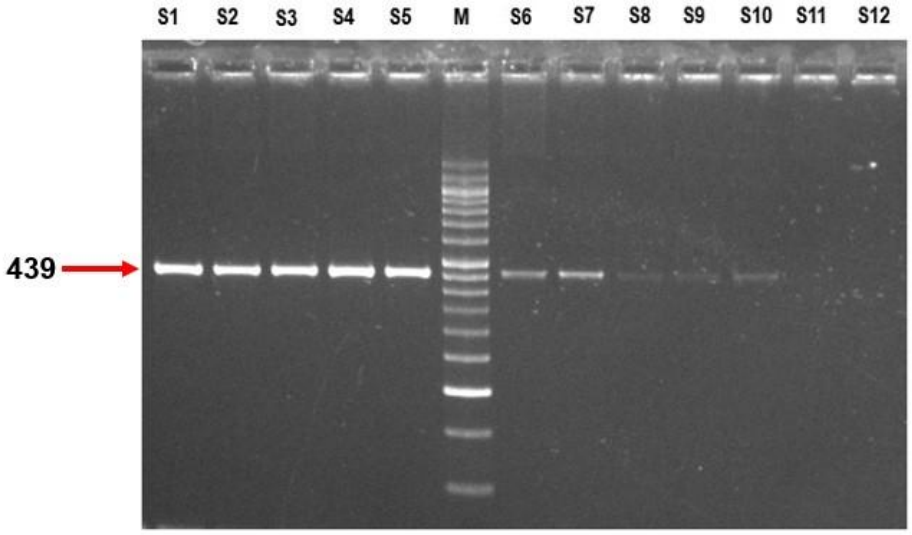

Figure 3: Reaction 2: PCR Amplification of MTNR Gene without Additives

Reaction 3

In contrast, in reaction 3 where BSA was added as an additive, a significant enhancement in the amplification yield of the MTNR gene was observed, with visibly stronger bands in all the samples observed in electrophoresis. These findings indicate that the addition of BSA as an additive in PCR reactions improves the amplification efficiency of the MTNR gene, resulting in more robust amplification products.

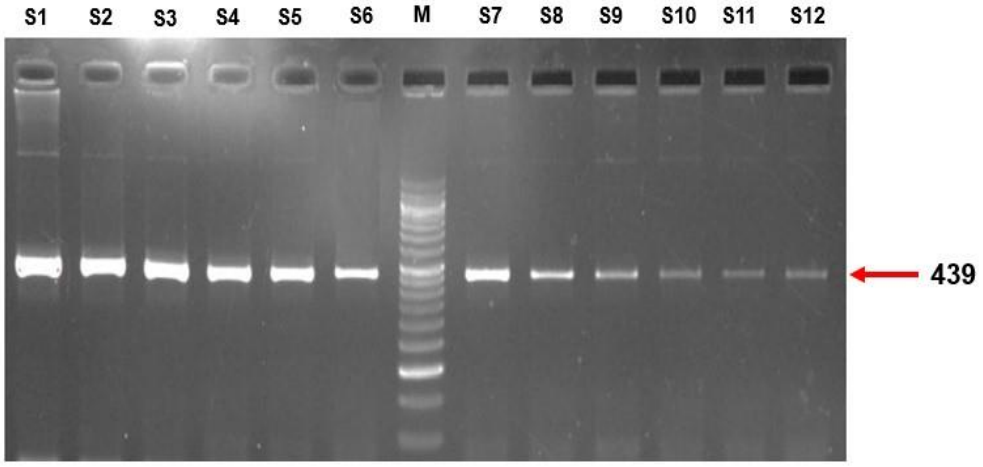

Figure 4: Reaction 3: PCR Amplification of MTNR Gene without Additives

DISCUSSION

The significance of DNA quality and purity in molecular biology experiments, particularly in PCR amplification, cannot be overstated. In this study, we explored the importance of DNA purity indicators, focusing on the 260/230 ratio, and investigated strategies for optimizing PCR results, especially in scenarios involving suboptimal DNA quality.

The 260/230 ratio, a critical metric for assessing DNA purity, is indicative of potential contaminants that can affect downstream applications like PCR. We observed variations in this ratio among different DNA samples, reflecting the presence of substances such as proteins, salts, or phenol. Notably, samples with lower 260/230 ratios exhibited reduced DNA quality, potentially impacting the efficiency and accuracy of subsequent molecular analyses. The 260/280 values are highly critical for proper amplification. This ratio should also be considered along with 260/280 to achieve better amplification efficiency.

To address these challenges and enhance PCR performance, we implemented various optimization techniques. One key strategy involved the use of additives, specifically bovine serum albumin (BSA), to improve amplification efficiency. BSA, known for its ability to bind to PCR inhibitors, proved effective in enhancing PCR outcomes, particularly in low-quality DNA samples. Our findings align with previous studies demonstrating the beneficial effects of BSA in PCR optimization, underscoring its utility as a cost-effective and reliable additive for amplifying challenging DNA templates [7,8].

Our study investigated the optimization of PCR conditions, which included fine-tuning annealing temperature, adjusting primer concentrations, and optimizing cycling parameters. These adjustments were essential to maximize the efficiency of DNA amplification, ensuring specific and robust amplification of target sequences, particularly in cases of suboptimal DNA quality.

Moreover, our PCR results demonstrated the impact of BSA on amplification yield. In the absence of additives, PCR efficiency varied among different DNA samples, with some showing weaker amplification signals. However, the addition of BSA notably enhanced amplification outcomes, as indicated by the increased intensity of bands observed in agarose gel electrophoresis. Interestingly, dilution of samples also

improved band intensity, particularly in high-quality DNA samples, even without the use of BSA.

This improvement underscores the importance of optimizing PCR conditions and utilizing additives to overcome challenges associated with low-quality DNA templates.

Moreover, the establishment of a BSA concentration gradient allowed us to identify the optimal BSA volume (0.8 µl) for maximizing PCR efficiency. This finding provides practical insights into the dosage of BSA needed to achieve optimal amplification results in PCR assays.

CONCLUSION

The study underscores the importance of DNA quality assessment and optimization strategies in PCR-based molecular analyses. By leveraging additives like BSA and fine-tuning PCR conditions, researchers can enhance the reliability and reproducibility of PCR experiments, even when working with low-quality DNA samples. Moving forward, further research into novel additives and optimization techniques will continue to advance the field of molecular biology, facilitating more robust and accurate genetic analyses across diverse applications.

Abbreviations

DNA: deoxyribonucleic acid PCR: Polymerase chain reaction; BSA: Bovine serum albumin; DMSO: Dimethyl sulfoxide; TAA: Tetraalkyl-ammonium; WS2: Tungsten disulphide

Competing interests

The authors declare that they have no competing interest.

References

1) Ghatak, S., Muthukumaran, R. B. and Nachimuthu, S. K. (2013) “A simple method of genomic DNA extraction from human samples for PCR-RFLP analysis,” *Journal of biomolecular techniques: JBT*, 24(4), p. jbt.13-2404-001. doi: 10.7171/jbt.13-2404-001.

2) Bunu, S. *et al.* (2020) “Determination of serum DNA purity among patients undergoing antiretroviral therapy using NanoDrop-1000 spectrophotometer and polymerase chain reaction,” *Biomedical and Biotechnology Research Journal (BBRJ)*, 4(3), p. 214. doi: 10.4103/bbrj.bbrj_68_20.

3) Lawaju, B. R. and Yan, G. (2024) “Assessment of common factors associated with droplet digital PCR (ddPCR) quantification of Paratrichodorus allius in soil,” *International journal of molecular sciences*, 25(6), p. 3104. doi: 10.3390/ijms25063104.

4) García-Alegría, A. M. *et al.* (2020) “Quantification of DNA through the NanoDrop spectrophotometer: Methodological validation using standard reference material and Sprague Dawley rat and human DNA,” *International journal of analytical chemistry*, 2020, pp. 1–9. doi: 10.1155/2020/8896738.

5) McKee, A. M., Spear, S. F. and Pierson, T. W. (2015) “The effect of dilution and the use of a post-extraction nucleic acid purification column on the accuracy, precision, and inhibition of environmental DNA samples,” *Biological conservation*, 183, pp. 70–76. doi: 10.1016/j.biocon.2014.11.031.

6) Lucena-Aguilar, G. *et al.* (2016) “DNA source selection for downstream applications based on DNA quality indicators analysis,” *Biopreservation and biobanking*, 14(4), pp. 264–270. doi: 10.1089/bio.2015.0064.

7) Farell, E. M. and Alexandre, G. (2012) “Bovine serum albumin further enhances the effects of organic solvents on increased yield of polymerase chain reaction of GC-rich templates,” *BMC research notes*, 5(1). doi: 10.1186/1756-0500-5-257.

8) Qamar, W., Khan, M. R. and Arafah, A. (2017) “Optimization of conditions to extract high quality DNA for PCR analysis from whole blood using SDS-proteinase K method,” *Saudi journal of biological sciences*, 24(7), pp. 1465–1469. doi: 10.1016/j.sjbs.2016.09.016.

9) *QIAamp DNA Blood Kits* (no date) *Qiagen.com*. Available at: https://www.qiagen.com/zh-us/products/discovery-and-translational-research/dna-rna-purification/dna-purification/genomic-dna/qiaamp-dna-blood-kits (Accessed: April 24, 2024).

10) Rees, W. A. *et al.* (1993) “Betaine can eliminate the base pair composition dependence of DNA melting,” *Biochemistry*, 32(1), pp. 137–144. doi: 10.1021/bi00052a019.

11) Spiess, A.-N., Mueller, N. and Ivell, R. (2004) “Trehalose is a potent PCR enhancer: Lowering of DNA melting temperature and thermal stabilization of taq polymerase by the disaccharide trehalose,” *Clinical chemistry*, 50(7), pp. 1256–1259. doi: 10.1373/clinchem.2004.031336.

12) Sakhabutdinova, A. R., Chemeris, A. V. and Garafutdinov, R. R. (2020) “Enhancement of PCR efficiency using mono- and disaccharides,” *Analytical biochemistry*, 606(113858), p. 113858. doi: 10.1016/j.ab.2020.113858.

13) Sairkar, P. *et al.* (2013) “Optimization of DNA isolation process and enhancement of RAPD PCR for low quality genomic DNA of Terminalia arjuna,” *Journal, genetic engineering & biotechnology*, 11(1), pp. 17–24. doi: 10.1016/j.jgeb.2013.02.001

14) Iakobashvili, R. (1999) “Low temperature cycled PCR protocol for Klenow fragment of DNA polymerase I in the presence of proline,” *Nucleic acids research*, 27(6), pp. 1566–1568. doi: 10.1093/nar/27.6.1566

15) Schnoor, M. *et al.* (2004) “Characterization of the synthetic compatible solute homoectoine as a potent PCR enhancer,” *Biochemical and biophysical research communications*, 322(3), pp. 867–872. doi: 10.1016/j.bbrc.2004.07.200.

16) Obradovic, J. *et al.* (2013) “Optimization of PCR conditions for amplification of GC-rich*EGFR*promoter sequence: Optimization of PCR conditions,” *Journal of clinical laboratory analysis*, 27(6), pp. 487–493. doi: 10.1002/jcla.21632.

17) Sarkar, G., Kapelner, S. and Sommer, S. S. (1990) “Formamide can dramatically improve the specificity of PCR,” *Nucleic acids research*, 18(24), pp. 7465–7465. doi: 10.1093/nar/18.24.7465.

18) Kreader, C. A. (1996) “Relief of amplification inhibition in PCR with bovine serum albumin or T4 gene 32 protein,” *Applied and environmental microbiology*, 62(3), pp. 1102–1106. doi: 10.1128/aem.62.3.1102-1106.1996.

19) Zhang, D. *et al.* (2019) “Enhancement of the polymerase chain reaction by tungsten disulfide,” *RSC advances*, 9(17), pp. 9373–9378. doi: 10.1039/c8ra09689a.