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Functional assessment of DNA extraction methods from frozen human blood samples for Sanger sequencing analysis

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ABSTRACT

The quality of input DNA is crucial for obtaining significant inferences from molecular techniques like Sanger sequencing and Next Generation Sequencing experiments. Many of the extraction methods are suitable for retrieving quality DNA from fresh blood and tissue samples, regardless of the isolation principle. However, while isolating DNA from frozen blood samples, processed tissue samples or low-quality samples, careful selection of suitable extraction methods is extremely important. Moreover, there is no standard protocol recommended for genomic DNA extraction from stored blood samples, particularly those stored in a Biobank, for applications like Sanger sequencing. Consequently, we have systematically compared different commercial DNA isolation kits with a modified manual extraction method for blood samples frozen for up to three years and assessed their quality, yield and suitability for PCR, Real-Time PCR and Sanger sequencing. The manual DNA extraction method was improved by incorporating a few modifications: a lower NaCl concentration was used for precipitating DNA and excluded the use of phenol. The modified method provided the maximum DNA yield from stored blood. Although all the methods tested were suitable for recovering DNA from stored blood, the modified method described here may be preferred for large-scale applications as it provides cost-effective ways to obtain large quantities of quality DNA. Most importantly, the DNA isolated by the modified method appears to be more stable in long-term storage at -80°C.

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Introduction

Recent advancements in high-throughput techniques such as Next Generation Sequencing (NGS), Sanger sequencing and single-cell sequencing have revolutionized molecular genetics by addressing the biological questions at the genome-wide scale. The potential of these techniques to evaluate multiple genes simultaneously makes them excellent molecular tools in a wide range of research and clinical medicine applications including cell biology and signaling, the molecular basis of human disorders, cancer genetics, genetic and metabolic disorders, linkage analysis and mutation detection (1,2). Although the demand for contemporary molecular techniques like NGS has intensely expanded, the cost of the technique continues to be a significant constraint to executing high-throughput sequencing of the genome routinely. Due to the high running cost, it has become obligatory to guarantee the quality of the input materials and reagents in order to minimize undesirable errors in the final outcome. This is significantly challenging especially when the biological samples are to be stored for a long time, such as in Biobanks.

The selection of appropriate methods for DNA extraction from blood stored in a Biobank is very important for sequencing reactions. The efficiency of any DNA extraction procedure greatly depends on its robustness and capability to provide good-quality DNA with a high yield (3). Besides these, the protocols should also be consistent, time-saving, cost-effective and applicable for large-scale extraction. The choice of extraction method also depends on the type of sample used, the sample volume available, the nature of the downstream assay and the timeframe available for extraction (4). Additionally, the method should also be capable of extracting DNA with the maximum feasible elimination of inhibiting chemicals and other impurities that may interfere with diverse future applications (3,5). Nowadays several methods are being developed for the extraction of DNA from blood samples. The majority of the commercially available kits, as well as most manual methods, are optimized for the extraction of DNA from fresh blood. Neither of them is intended specifically for

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the extraction of DNA from samples that have been stored improperly or have been stored for a long duration (4).

Nucleic acid extraction has been regarded as a limiting factor since the quality of input DNA influences the outcome of every sequencing test (6). As biological specimens also vary greatly in both quality and quantity coupled with the labor-intensive nature of the procedure, DNA extraction is more prone to technical anomalies. The salting-out method, first introduced by Miller et al. (7) is one among the various traditional extraction procedures that are widely used in research and clinical laboratories. Since the laborious manual technique demands a larger initial sample volume, incorporation of PCR-interfering reagents and more time consumption, these extraction methods have been replaced by commercial kits (8). The kit methods based on chromatography, columns, silica membrane, or magnetic beads matrix have been shown to be very efficient in terms of time and yield than salting out and Phenol-Chloroform methods (9).

Although myriads of extraction methods are available for isolating DNA, there is no defined standard protocol recommended for genomic DNA extraction for any sequencing experiments (3). Despite several studies have compared the effectiveness of various commercial kits for fresh blood samples, there are limited or no comprehensive studies that compared commercial kits with salting out the method for extracting DNA from stored blood (6). Additionally, the suitability of DNA extracted from frozen blood using commercial kits and salting out for conducting genotyping, Real-time PCR and PCR-RFLP were reported previously; however, there have been no such studies so far that compared these methodologies for Sanger sequencing (6). Also, the DNA samples stored in Biobanks are being increasingly explored for the evaluation of the molecular basis of diseases; knowing disease stratification; assess drug response together with dosage and adverse events (10). Therefore, DNA quality for biobanks should be top-notch, in order to maintain stability during long-term storage. Hence, this study aimed to test the most suitable method for DNA extraction from frozen blood samples. For this, DNA was isolated from whole blood stored at -20ºC for up to 3 years using six commercially available kits and compared with a modified method. After comparing the quality, quantity and purity of isolated DNA, their compatibility with downstream applications like Real-time PCR was compared. Further, we have compared the efficacy of this DNA for gene sequencing using Sanger's method. Finally, the stability of isolated DNA in storage at -80°C for three years was evaluated.

Materials and Methods

Blood sample collection

Fifteen healthy volunteers were included in the study and the use of human specimens was approved by the Institutional Ethics Committee of SCTIMST. After obtaining informed consent from each individual, 10 ml of blood was collected in BD Vacutainer K_2EDTA tubes (Becton Dickinson, Franklin Lakes, NJ). In order to test the appropriate method for extraction of DNA from stored samples, the blood samples were frozen at standard conditions in a password protected -20°C freezer (X-Cold, Angelatoni Life Science, Italy) located in an access-controlled laboratory with an automatic temperature logger. The storage conditions were automatically monitored and the data on any deviation can be retrieved through the inbuilt USB port. The blood samples were categorized into three study groups according to the duration of storage (one, two and three years of storage). After a specified storage duration, the samples were subjected to DNA extraction.

DNA extraction

In this report, we have compared six kit-based methods with the modified manual method. The kits used were as follows.

Method 1: Xpress DNA blood mini kit (MagGenome, USA, Cat. No.: MG17BI-01).

Method 2: Purelink genomic DNA mini kit (Invitrogen, USA, Cat. No.: K1820-01).

Method 3: DNeasy blood and tissue kit (Qiagen, Germany, Cat. No.: 69504).

Method 4: GeneJet genomic DNA purification kit (ThermoFisher Scientific, USA, Cat. No.: K0782).

Method 5: DNA isolation kit for mammalian blood (Roche Germany, Cat. No.: 11667327001).

Method 6: QIAamp DNA blood midi kit (Qiagen, Germany, Cat. No.: 51185).

For the manual method, the salting out protocol

Table 1. Specifications of seven DNA extraction methods used.

	Method 1	Method 2	Method 3	Method 4	Method 5	Method 6	*Method 7
Starting blood volume	200 µl	200 µl	200 µl	200 µl	5000 µl	1500 µl	1500 µl
Incubation temperature (°C)	56	55	56	56	37	70	55-60
Enzyme used	Proteinase K	Proteinase K	Proteinase K	Proteinase K	Protein precipitation solution	Proteinase K	Proteinase K
Separation technique	Magnetic beads	Column based	Column based	Column based	Precipitation	Column based	Modified method as described below
Elution volume	100 µl	100 µl	100 µl	100 µl	100 µl	100 µl	100 µl
Elution buffer	Blood elution buffer	Genomic elution buffer	Buffer AE	Elution buffer	TE buffer	Buffer AE	TE buffer

*modified method.

(Method 7) developed by Miller et al. (7) was modified and used. The modified DNA extraction method combines salting out with solvent extraction with the following modifications. Compared to the solvent extraction method, this method has excluded the use of phenol. Likewise, this method uses a lower NaCl concentration (4M) for precipitating DNA, instead of the 6M NaCl used in the salting out method. Five samples each stored for three different years were used for extraction by all the chosen methods except for method 5, where only one sample was used due to the unavailability of a large initial blood volume (5 ml) requirement. The frozen blood samples were thawed at room temperature for approximately 30 minutes to 1 hour and DNA was extracted according to the manufacturer's protocol. The specifications of each method are listed in Table 1. The extracted DNA was stored at -20°C in aliquots. In addition, aliquots of DNA samples isolated by Method 1, Method 5 and Method 7 were stored at -80°C for checking the stability of DNA, on storage. DNA was also extracted from fresh blood by Method 4, Method 5, Method 6 and Method 7.

DNA quantity and quality check

The yield and quality of each extracted DNA were assessed by measuring the concentration (ng/µl) and $A_{260/280}$ and $A_{260/230}$ ratios in a Nanodrop One Spectrophotometer (ThermoFisher Scientific, USA). The possible degradation of DNA during isolation was analyzed by electrophoresis on 0.7% agarose gels in 1X TAE buffer (Biorad, USA). DNA (1 µl) was loaded onto each well and the gel was run at 80 V for 1 hour. A DNA ladder with a size range of 250 bp to 10 kb (NEX-GEN DNA ladder, Genetix, India) was also loaded on the gel for determining the molecular weight. The DNA bands obtained were visualized with the aid of a gel documentation system (G: Box, Syngene, United Kingdom).

Amplification and detection of APOP gene

The concentration of DNA was quantified using a micro-volume UV-VIS spectrophotometer and diluted to a concentration of 50 ng/µl for further analysis. A fragment of the APOP gene located on chromosome 6 (363 bp) was amplified in a 25 µl reaction volume consisting of 10.5 µl nuclease-free water (Takara, Japan), 12.5 µl Emerald Amp® MAX PCR Master Mix (Takara, Japan), 0.2 µM Forward (APOP F):5' GGCCATAGACTTCCTACC 3' and Reverse (APOP R):5'GCAGTTGTTTAGAAGTTGAGGACC3' primer set (Sigma-Aldrich, USA). The reaction conditions were as follows: initial denaturation at 94ºC for 2 minutes, denaturation at 94ºC for 15 seconds, annealing at 58ºC for 30 seconds and extension at 72ºC for 40 seconds for 35 cycles and final extension at 72ºC for 7 minutes. Following the PCR reaction, the single amplicon obtained was analysed by 1% agarose gel electrophoresis and the images were captured by the gel documentation system (after staining with ethidium bromide (10 mg/ml). The PCR products were purified using PureLink Quick Gel Extraction and PCR Purification Combo Kit (Invitrogen, USA) and eluted in 30 µl Elution Buffer (Invitrogen, USA) for sequencing.

Real-Time PCR

The amplification of APOP gene was performed using real time PCR (Biorad CFX96 Real-time system, USA). Each 10 µl reaction consists of 2.5 µl sterile water, 0.25 µl Buffer, 5 µl Power Track SYBR Green Master Mix (Applied Biosystems, USA), 0.2 µM of each primer sets and 1 µl of DNA template (50 ng/ µl). The PCR cycling regime was as follows: initial denaturation at 94ºC for 2 minutes followed by 35 cycles of denaturation at 94ºC for 15 seconds, annealing at 58ºC for 30 seconds, extension at 72ºC for 40 seconds and final extension at 72ºC for 7 minutes. Cycle threshold values were used to express the data of the APOP gene, preferably in smaller Ct values. The samples extracted by different methods were used and each sample was run in duplicates.

Sequencing PCR and capillary electrophoresis of APOP gene

Sequencing PCR was carried out using the purified PCR products as a template. Each 10 µl PCR reaction consisted of 6.1 µl of sterile water, 1 µl of PCR purified product (50 ng/ µl), 2 µl of sequencing buffer, 0.5 µl of sequencing mix (Big Dye 3.1 cycle sequencing kit, Applied Biosystems, USA) and 0.4 µl of any one of the primer pair. Amplification was performed in a thermal cycler (Applied Biosystems, USA). The PCR cycling profile is as follows: initial denaturation at 96ºC for 1 min followed by 25 cycles of denaturation at 96ºC for 10 seconds, annealing 50ºC for 0.5 seconds, extension at 60ºC for 4 minutes and finally hold at 4ºC. Post-sequencing clean-up was performed by EDTA (Sigma Aldrich, USA) / Ethanol (Himedia, India) precipitation method. The precipitates were dissolved in 10 µl HiDi formamide (Applied Biosystems, USA), heat-denatured and snap-chilled on ice. The samples were loaded onto an automated genetic analyzer for capillary electrophoresis (ABI 3500 series, Applied Biosystems, USA). All sequences were analyzed by manually inspecting each trace file using Four Peaks (Nucleobytes).

Results

Comparative analysis of extraction methods: quality, quantity and yield of DNA

A total of seven methods were employed in the study for extraction of DNA, in which four methods (1-4) relied on low blood volume (200 µl) while the rest (methods 5-7) used higher volumes (1.5 ml to 5 ml). Out of all the extraction methods, the highest yield was obtained for DNA extracted using the modified method, Method 7 (Table 2). The DNA yield obtained by different extraction methods were represented in Figure 1.

The yield and quality of each extracted DNA were then assessed by measuring the concentration (ng/µl) and $A_{260/280}$ and $A_{260/230}$ ratios in a Nanodrop Spectrophotometer. The spectrophotometric evaluation revealed variations in DNA concentrations and quality obtained by each method for the tested samples (Table 3).

The possible degradation of DNA during isolation was further analyzed by electrophoresis on 0.7% agarose gels. The results of electrophoresis showed single, distinct high molecular weight DNA bands with some smearing pattern and without any RNA contamination (Figure 2 A-G). Among all the methods, method 7 had the least or no smearing pattern observed. To check whether the mild degradation observed (smearing seen in Figure 2) is due to the storage of blood, available freshly isolated DNA was checked for its integrity by agarose gel electrophoresis (Figure

Table 2. The average DNA yield was calculated from five independent blood samples (n=5) stored for one (2021), two (2020) and three (2019) years. Due to the high initial blood volume (5 ml) requirement, only one sample was extracted for each year using Method 5.

*Method	DNA Yield (µg)		
	2019	2020	2021
Method 1	4.48	4.912	6.166
Method 2	2.268	2.772	2.416
Method 3	2.302	1.834	2.368
Method 4	4.114	3.8	4.664
Method 5	17.036	14.378	26.118
Method 6	8.986	6.224	9.52
Method 7	43.418	41.764	51.778

***Method 1:** Xpress DNA blood mini kit; **Method 2:** Purelink genomic DNA mini kit; **Method 3:** DNeasy blood and tissue kit; **Method 4:** GenJet genomic DNA purification kit; **Method 5:** Roche DNA isolation kit for mammalian blood; **Method 6:** QIAmp DNA blood midi kit; **Method 7:** Salting out.

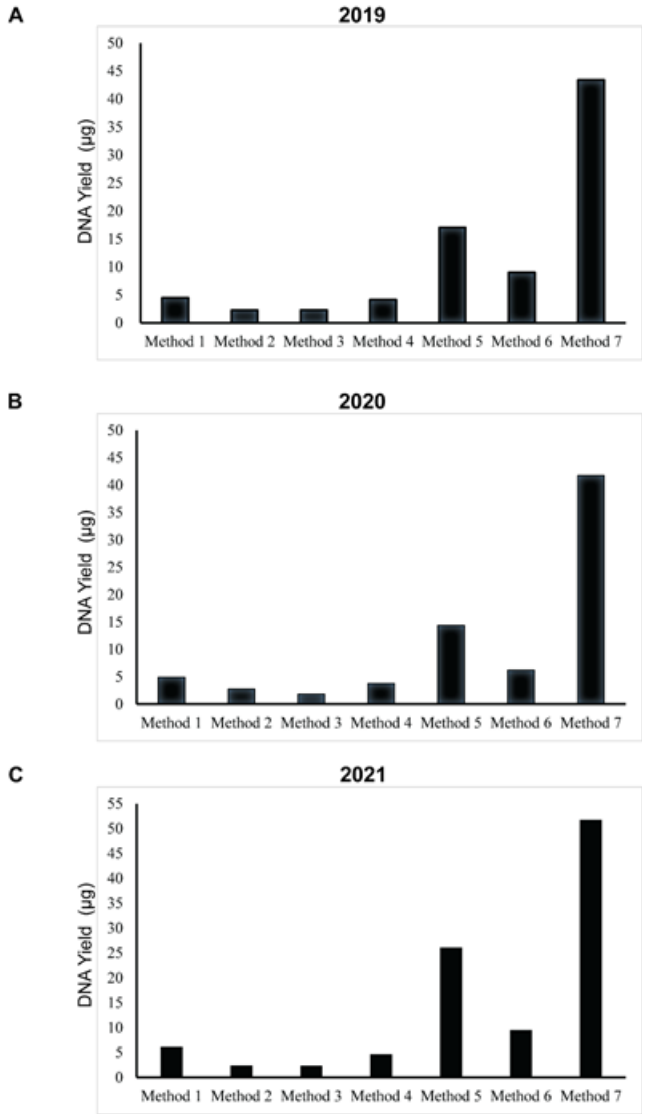


Figure 1. The yield of DNA. The DNA yield for each extraction method was calculated and the average value derived from the 5 independent samples was plotted, except for Method 5. Due to the high initial blood volume (5 ml) requirement, only one sample was extracted for each year using Method 5. The DNA yield was obtained for the five independent samples for **(A)** 2019 **(B)** 2020 and **(C)** 2021.

2H). From the gel image, it can be seen that the pattern was similar to that observed for DNA isolated from stored samples with minimal degradation observed in method 7. The spectrophotometric data of these freshly isolated DNA indicate that both the $A_{260/280}$ and $A_{260/230}$ ratios were at the desired range for the four isolation methods tested (Table 4). Although there was variability in quality and quantity among the method used, the modified method (method 7) seems more suitable for the extraction of DNA from stored samples.

Assessment of DNA quality for RT-PCR applications

The RT-PCR assay targeting the APOP gene yielded a single sigmoidal curve (Figure 3) for all the tested samples. The presence of a single peak in the melt curve analysis further add on to the purity of the DNA extracted using different methods. All the methods yielded comparable Ct values for the APOP gene amplification. There were no statistically significant differences in amplification for all the protocols used for extraction ($P>0.05$), although, Method 7 (salting-out) and Method 4 (GeneJet genomic DNA purification kit) gave the lowest Ct values.

Assessment of DNA quality for sequencing

PCR amplification of APOP gene using DNA extracted by the different methods showed a single band corresponding to 363 bp (Figure 4).

The purified PCR products were subject to Sanger sequencing. The electropherogram showed that the results were identical for the DNA isolated by all seven methods (Figure 5 A-C). The results obtained showed that in every sequence, the base-specific quality score is similar and all the reactions provided the expected sequence as verified by the BLAST tool (Figure 5D). There were no overlapping peaks or background noise (secondary lower peaks) observed in any of the chromatogram data. From the assembly of reverse and forward primer sequencing data, it can be concluded that all the methods tested were very efficient in providing suitable sequencing quality DNA from frozen samples.

Effect of storage on the stability of isolated DNA

The suitability of long term stored DNA for sequencing were analysed by agarose gel electrophoresis using three samples each isolated by Method 1 (Xpress DNA), Method 5 (Roche) and Method 7 (salting out) that were stored at -80°C (Figure 6A). The gel image showed that there was

Table 3. Spectrophotometric evaluation of DNA isolated from stored blood samples. Average Concentration and absorbance ratios ($A_{260/280}$ and $A_{260/230}$) of DNA extracted from five independent blood samples (n=5) stored for one (2021), two (2020) and three years (2019).

*Method	DNA concentration (ng/µl)			$A_{260/280}$			$A_{260/230}$		
	2019	2020	2021	2019	2020	2021	2019	2020	2021
Method 1	44.8	49.12	61.66	1.77	1.78	1.76	1.75	1.73	1.75
Method 2	22.68	27.72	24.16	1.66	1.65	1.68	1.84	1.26	1.49
Method 3	23.02	18.34	23.68	1.85	1.97	1.88	2.84	1.67	1.21
Method 4	41.14	38	46.64	1.75	1.75	1.77	1.37	1.50	1.67
Method 5	851.8	718.9	440.6	1.65	1.79	1.86	0.91	1.20	1.94
Method 6	89.86	62.44	95.2	1.82	1.82	1.83	3.16	2.66	2.18
Method 7	434.04	417.76	517.78	1.81	1.82	1.86	1.63	1.63	1.95

***Method 1:** Xpress DNA blood mini kit; **Method 2:** Purelink genomic DNA mini kit; **Method 3:** DNeasy blood and tissue kit; **Method 4:** GenJet genomic DNA purification kit; **Method 5:** Roche DNA isolation kit for mammalian blood; **Method 6:** QIAmp DNA blood midi kit; **Method 7:** Modified method.

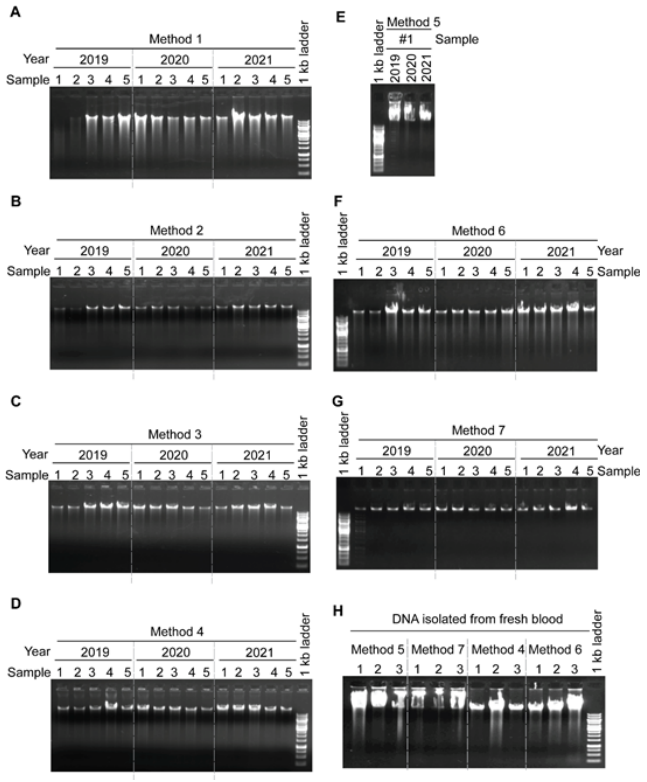
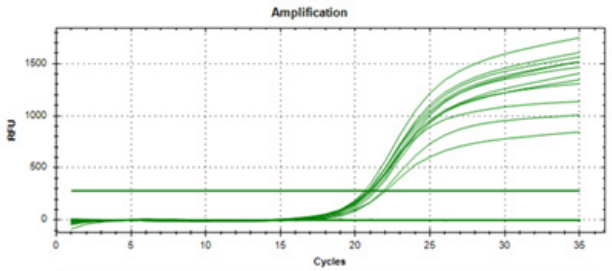


Figure 2. DNA quality check by agarose gel electrophoresis. 1 µl of DNA isolated by different methods was subjected to agarose gel electrophoresis on a 0.7 % gel and stained with ethidium bromide. The sample number represents the five independent samples stored in the year 2019, 2020 and 2021. From five independent samples, Genomic DNA was extracted by Method 1 (**A**), Method 2 (**B**), Method 3 (**C**), Method 4 (**D**), Method 5 **(E),** Method 6 (**F**) and Method 7 (**G**). (**H**) Agarose gel image showing genomic DNA extracted from fresh blood using Method 5 (lanes 1–3), Method 7 (lanes 4–6), Method 4 (lanes 7–9) and Method 6 (lanes 10–12). The 1kb DNA ladder with a size range of 250 bp to 10 kb was also loaded in each gel. The images were captured using the gel documentation system.



Method	Ct Values
Method 1	21.20
Method 2	21.14
Method 3	21.45
Method 4	20.78
Method 6	21.41
Method 7	20.81

Figure 3. Real-time PCR amplification plot of the APOP gene. The amplification plot for the APOP gene with Relative Fluorescence Units in y-axis and the number of cycles represented in x-axis. The DNA extracted using all the 7 methods as mentioned in Materials and Methods was used for RT-PCR. All the reactions were carried out in triplicates for every five independent samples isolated using different methods. Ct values were obtained in RT-PCR with the DNA templates recovered using 6 different extraction methods (n=3).

considerable degradation in all three samples isolated by method 5 while the DNA appears to be intact for method 1 and the modified method (Method 7) (Figure 6B). However, the spectrophotometric analysis showed that the concentration and the quality were not affected by storage (Table 5). Finally, the suitability of this stored DNA for Sanger sequencing was tested (Figure 6C). Although the DNA quality was poor on storage for method 5, we were able to obtain the desired PCR product and the comparable sequence read in Sanger sequencing.

Discussion

Obtaining pure and intact DNA is the primary requirement for the success of all molecular biology techniques. Hence selection of appropriate methods for the extraction of DNA is crucial. The efficiency of any DNA extraction procedure greatly depends on its robustness and capability to provide good quality DNA with a high yield. Besides these, the protocols should also be consistent, less time-consuming, cost-effective and applicable for large-scale extraction. Researchers from across the world have published a number of DNA extraction methods that have been shown to be reliable and effective at producing a large enough quantity of high-quality DNA from fresh human blood. But none of them are specifically designed

Table 4. Spectrophotometric evaluation of DNA isolated from fresh blood samples. Average concentration and absorbance ratios ($A_{260/280}$ and $A_{260/230}$) of DNA extracted from three independent fresh blood samples (n=3).

*Method	DNA concentration (ng/µl)	$A_{260/280}$	$A_{260/230}$
Method 5	1050	1.86	2.09
Method 7	1047.87	1.9	2.39
Method 4	85.4	1.82	2.01
Method 6	179.5	1.84	2.54

***Method 5:** Roche DNA isolation kit for mammalian blood; **Method 7:** Salting out; **Method 4:** GenJet genomic DNA purification kit; **Method 6:** QIAmp DNA blood midi kit.

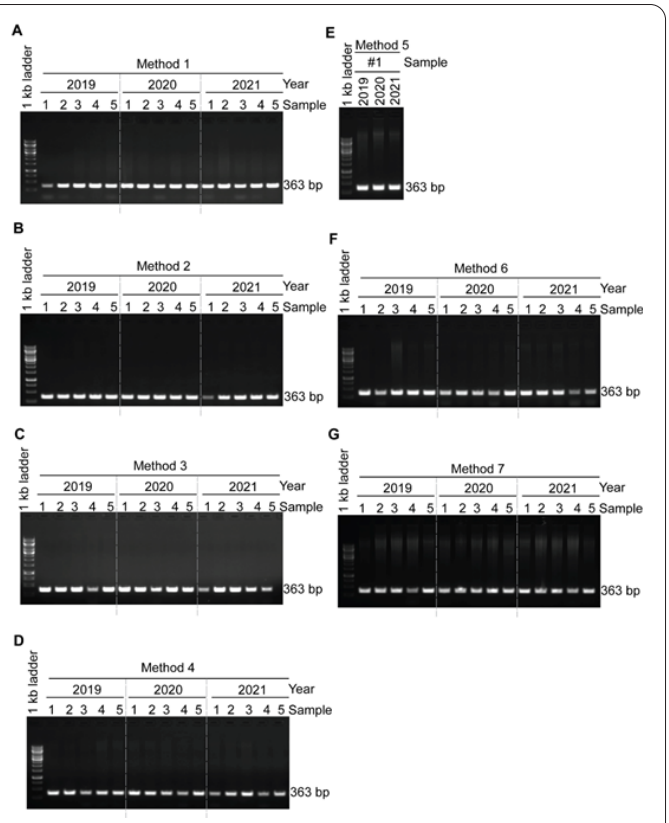


Figure 4. Agarose gel electrophoresis of PCR amplification product of APOP gene. The amplified APOP fragment (363 bp) obtained using five independent genomic DNA isolated by seven different methods and stored from 2019, 2020 and 2021, were electrophoresed on 1% agarose gel and stained with ethidium bromide. The PCR product was obtained by Method 1 (**A**), Method 2 (**B**), Method 3 (**C**), Method 4 (**D**), Method 5 (**E**), Method 6 (**F**) and Method 7 (**G**). The 1kb DNA ladder with a size range of 250 bp to 10 kb was also loaded in each gel. The images were captured using the gel documentation system.

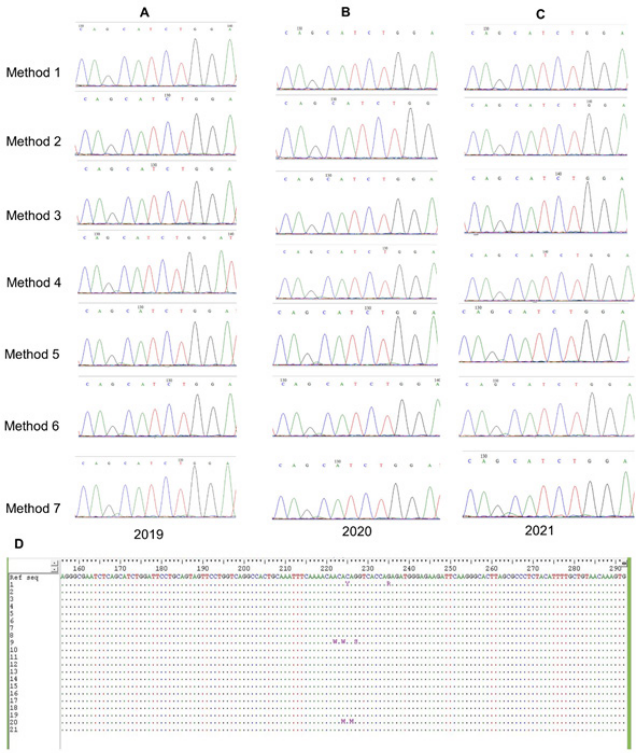


Figure 5. The APOP gene sequencing by automated Sanger's method. Representative images of APOP gene fragments were detected from the DNA obtained by seven different methods. The sequence obtained for the samples stored in the year 2019 (**A**), 2020 (**B**) and 2021 (**C**). (**D**) Multiple Sequence Alignment of a set of 21 nucleotide sequences of APOP gene from genomic DNA extracted using Method 1 (1, 2, 3), Method 3(4, 5, 6), Method 2 (7, 8, 9), Method 4 (10, 11, 12), Method 7 (13, 14, 15) Method 6 (16, 17, 18) and Method 5 (19, 20, 21) using BioEdit sequence alignment editor software. The nucleotide alignment was viewed using sequence colour in option and by view conservation by plotting identities to a standard as a dot.

for the extraction of DNA from blood samples that have been stored improperly or been stored for a long duration. In view of this, the present study focused on evaluating the efficiency of different methods in extracting DNA from stored frozen blood as well as their suitability in downstream applications. In addition, an effort was also made to determine which extraction method produced DNA suitable for long-term storage.

Six kit-based methods and a modified manual method were employed in the study for the extraction of DNA from blood samples, that were frozen at standard conditions in a password protected -20°C freezer, after a specified storage duration. All the tested methods were successful in extracting DNA from the samples. Spectrophotometer and electrophoretic analysis was further performed to assess the concentration, purity and integrity of the extracted DNA. The spectrophotometric ratio $A_{260/280}$ is usually used to evaluate the purity of DNA. Additionally, the $A_{260/230}$ ratio is also considered a second measurement of evaluation. An $A_{260/280}$ ratio of 1.8-1.9 and an $A_{260/230}$ ratio above 2.0 is considered pure for DNA (11). In the present work, most of the methods tested gave DNA with desirable 260/280 nm absorbance ratios of approximately 1.8, regardless of the storage duration; except for methods 2, 4 and 5. The other quality parameter, 260/230 ratios were near the expected value of 2 for methods 6 and 7 while for other methods, the values were below 2, indicating the possibility of salt contamination or residual carryovers (like EDTA, guani-

Table 5. Spectrophotometric evaluation of stored DNA samples. Average concentration and absorbance ratios ($A_{260/280}$ and $A_{260/230}$) of three independent DNAs (n=3) were extracted using three different methods and stored at -80°C for three years.

*Method	DNA concentration (ng/µl)	$A_{260/280}$	$A_{260/230}$
Method 1	75.3	1.80	2.55
Method 7	1704.73	1.88	2.39
Method 5	2943	1.86	2.24

***Method 5:** Roche DNA isolation kit for mammalian blood; **Method 7:** Salting out; **Method 4:** GenJet genomic DNA purification kit; **Method 6:** QIAmp DNA blood midi kit.

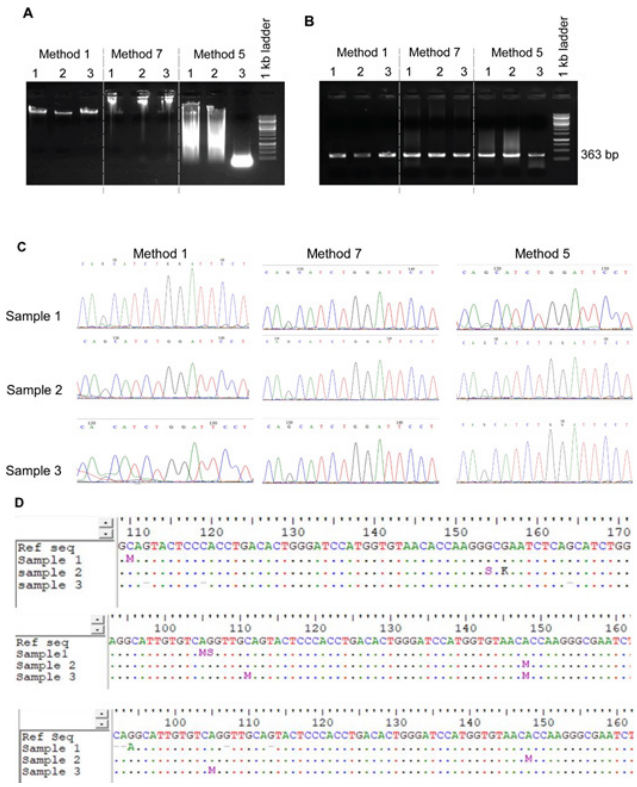


Figure 6. Agarose gel electrophoresis, PCR and Sanger sequencing of stored DNA samples. (**A**) Agarose gel images of three independent samples of genomic DNA isolated by Method 1, Method 7 and Method 5, stored for three years at -80°C. Lanes 1,2,3 represents three different samples (**B**) The PCR amplified APOP fragment (363 bp) from the stored DNA samples. The 1kb DNA ladder with a size range of 250 bp to 10 kb was also loaded in each gel. The images were captured using the gel documentation system. (**C**) Representative images of APOP gene fragments were detected from the three independent DNA stored for three years. (**D**) Multiple Sequence Alignment of APOP gene from genomic DNA extracted using Method 1, Method 7, and Method 5 using BioEdit sequence alignment editor software.

dine hydrochloride, etc.) in the extraction mixture (12). These results were on par with the studies conducted on DNA extraction from frozen blood by Bulla et al. and Chen et al. stating that the blood DNA yield is impacted after long-term storage (13,14). The studies conducted by Schroder and Steimer on the stability of methylation patterns in stored EDTA blood samples using pyrosequencing showed that DNA methylation levels increased with storage time (15) and the increased methylation was responsible for the reduction in yield of genomic DNA from long-term frozen samples. It was suggested that this could be due to the difference in stabilities of methylated and non-methylated DNA or changes in cell type proportions. Analyzing DNA integrity using agarose gel electrophoresis is crucial prior to any downstream applications as it leads to errors in both qualitative and quantitative data. The DNA extracted by all the methods showed the presence of single, distinct DNA bands with some smearing pattern and without any RNA contamination. In order to further clarify whether the mild degradation observed is due to the storage of blood, DNA was isolated from few fresh blood samples by 4 methods (Methods 4-7). The agarose gel pattern and the absorbance ratios were consistent with the data obtained for the DNA isolated from stored blood. This suggests that despite long-term storage and exposure to numerous chemical washes during extraction, there was only minimal DNA degradation.

Even though all the seven methods tested were successful in extracting DNA, variations in yield were observed across the methods. The yield of DNA was highest for the modified manual extraction method when compared to other extraction methods tested. The modified method also gave the highest yield of DNA throughout sample storage. Hence the modified method (method 7) seems more suitable for the extraction of DNA from stored samples. However, analysis with a longer duration of storage may be required to further test whether the modified method is capable of extracting DNA with high quality. The suitability of the extracted DNA for downstream applications was additionally evaluated using Real time PCR and Sanger sequencing techniques.

Since many large-scale genotyping techniques now involve PCR-based amplification steps, real-time PCR has been used as a method for determining the quantity and quality of DNA for downstream applications. In this regard, an RT-PCR assay targeting the APOP gene was carried out. Even though analogous research has been carried out previously, it would be hard to compare our current findings with those of others as none has performed a comprehensive evaluation using similar extraction methods. Furthermore, there were considerable variabilities in the samples selected for DNA isolation and quality assessment in each study. Koshy et al. (16) for instance, evaluated DNA recovery from human whole blood using the phenol-chloroform (PCI) method to the Rapid-method (RM) and a commercial kit (Origin blood DNA Kit), whereas Psifidi et al. investigated eleven different techniques, including PCI method, an in-house magnetic beads-based procedure, and modified silica-based commercial kits in DNA from ovine blood (6). Psifidi et al. reported that the modified silica-based kit and in-house magnetic bead-based procedure have achieved the lowest Ct values (6). The conclusion proposed by Psifidi et al. appears to be relatively consistent with our current real-time data since the

current investigation clearly shows the lowest Ct value for DNA extracted using method 4 (commercial kit). Additionally, the interaction of any PCR inhibitory material that is carried after the DNA extraction can be the cause of the highest Cq value and the delayed amplification displayed by the DNA extracted using the remaining techniques (17). Since salting out employs a larger salt concentration for precipitation and because residual salts may be present, higher Ct values were anticipated. However, interestingly, in our study, the salting out method yielded the lowest Ct value. Overall, the range of Ct values obtained (Ct APOP ≈ 20-21) suggests that all the methods used could extract DNA from stored blood and that are suitable for further sequencing experiments. Since the salting-out method has not previously been compared with kit methods for the isolation of DNA from frozen blood samples, this result demonstrates that our modified method is also very efficient in extracting excellent-quality DNA for RT-PCR analysis.

Sanger sequencing is considered the gold standard for detecting the sequence of nucleotide bases in DNA and is a commonly used method in laboratories for genetic analysis. As for a molecular technique, sequencing also requires high-quality DNA so as to strengthen the accuracy of the result. Initially, a PCR reaction using APOP gene-specific primers was performed to investigate the intactness of the genomic DNA and to ascertain whether any inhibitory materials were impeding the reaction. The amplified products were homogenous single bands in all the methods used and for the DNA isolated from frozen blood samples for 1, 2 and 3 years (2019, 2020, 2021). This result suggests that both kit methods and modified methods could give sufficient quality and quantity of DNA from frozen blood for further sequencing reactions. The amplified PCR products were purified and subject to Sanger sequencing. Because of its relative sensitivity, the electropherogram detection of the sequence shows that the results were identical for the DNA isolated by all seven methods. From the assembly of reverse and forward primer sequencing data, it can be concluded that all the methods tested were very efficient in providing suitable sequencing quality DNA from frozen samples.

The future research and clinical applications of DNA stored in a DNA bank is vast. Therefore, it is important to check the quality of DNA and its suitability for sequencing tests after long-term storage in a -80°C freezer. We have checked the quality of three independent DNA samples stored for three years, by agarose gel electrophoresis. Three samples each isolated by Method 1 (Xpress DNA), Method 5 (Roche) and Method 7 (salting out) that were stored at -80°C for three years were analysed. We were unable to compare the quality of DNA isolated by other kits on storage, due to the unavailability of those stored samples. The results implied that there was considerable degradation in all three independent samples isolated by method 5 while the DNA appears to be intact for method 1 and the modified method (Method 7). However, the spectrophotometric analysis showed that the concentration and the quality were not affected by storage. This indicates that the choice of isolation method should also be based on the stability of isolated DNA, in long-term storage. Finally, the suitability of this stored DNA for Sanger sequencing was tested. Although the DNA quality was poor on storage for method 5, we were able to obtain the desired PCR product and the comparable sequence read in Sanger sequencing. In the future, it would be interesting to test whether further storage of DNA would be suitable for long read PCR reactions and other sequencing tests.

Conclusion

Although there was variability in concentration, yield and quality, the outcome of this study suggests that the six commercial extraction kits and the modified manual salting out methodology can provide quality DNA that is sufficient for Sanger sequencing and real-time PCR assays. Since higher DNA concentrations are preferred for various simultaneous downstream applications and storage at DNA banks for future applications, the modified salting-out method appears to be superior when compared with the kit-based protocols. Furthermore, when the required equipment and skilled laboratory personnel are available, the use of the modified salting out method is a cost-effective option for handling incorrectly stored blood as well as for higher sample volumes. At the same time, commercial DNA extraction kits can be recommended for isolating DNA from rare and small quantities of clinical or forensic samples in order to obtain high-quality DNA for immediate testing. Another important parameter to be considered is the stability of this isolated DNA on long-term storage at -80°C. We recommend that DNA isolated by the modified manual method may be worthy for DNA storage in a biobank in order to maintain the quality for the long term.

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Interest conflict

The authors declare that there is no conflict of interest.

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Availability of data and material

The raw data generated in this study are available from corresponding authors on request.

Author Contributions

Conceptualization of the study by Pravi Vidyadharan, Srinivas Gopala, Cibin T Raghavan and Madhusoodanan Urulangodi with contributions from all other authors. Methodology and data collection were performed by Pravi Vidyadharan, Santhi CKV and Anjali SM. Data analysis was performed by Srinivas Gopala, Cibin T Raghavan and Madhusoodanan Urulangodi. The first draft of the manuscript was written by Pravi Vidyadharan, Santhi CKV, Anjali SM and Madhusoodanan Urulangodi and all authors edited and provided inputs on previous versions of the manuscript. All authors read and approved the final manuscript.

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Ethics approval and consent to participate

The studies performed with human subjects were in accordance with the ethical standards of the Institutional Ethics

Committee of Sree Chitra Tirunal Institute for Medical Sciences and Technology, Thiruvananthapuram, and with the 1964 Helsinki Declaration and its later amendments or comparable standards. Accordingly, this study has been reviewed and approved with Ethical Approval No. SCT/IEC/1383/June-2019. Informed consent was obtained from all the participants in the study.

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