Original article

A Comprehensive Comparison of DNA Extraction Using Fresh and Stored Bloods in Molecular Hematology Diagnostic Study

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Abstract:

Objective: Blood is the main source of DNA in molecular biology. It provides a high DNA quality and quantity. In this study, we compare the quality and quantity of DNA isolated from stored blood that has been kept at -40°C for one-year to that of fresh blood.

Materials and Methods: Twelve fresh and stored blood samples were randomly selected for this study. Nucleo Spin® Blood L kit was used to isolate the DNA from the samples. The integrity and intensity of DNA were examined through 1.6% agarose gel precast with SYBR® safe DNA stain. The DNA samples were further examined through PCR amplification and Sanger sequencing.

Results: There was no significant difference in quality and quantity of isolated DNA from fresh blood and stored blood samples. The high intensity of an intact DNA band as well as the success in PCR amplification and sequencing are indicators of high DNA quality. ***Conclusion:*** Proper storage of patients'left-over whole blood sample at -40°C offers an acceptable alternative for DNA resources in molecular study.

Keywords: DNA extraction; fresh blood; stored blood; molecular hematology

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Introduction:

Blood is the standard sample used in the diagnosis of various diseases.It has valuable biological information for laboratory investigation. It was the main source of DNA and the most easily accessible source from diagnostic laboratories. DNA isolated from blood was widely used inforensics[1]; diseases diagnosis[2,3]; mutation and polymorphism identifications[4,5]; neonatal methylation study[6]; and pathogens detection in human and livestock[3,7,8]. In hematology, DNA isolated from blood was used in diagnosis of leukemia[9], thalassemia[10], polycythemia[11] and G6PD deficiency[12]. Blood provide high yield and high quality of DNA as compared to other sources such as saliva, hair, Guthrie cards, and buccalcells[13,14]. Blood sample from routine laboratory service is stored at 4°C for up to 4 weeks. However, storage at 4°C for long-term period has a negative impact on the yield and quality of DNA[15,16]. On the other hand, the yieldand quality of DNAisolated from blood stored at multiple temperaturesincluding 45°C, 37°C, 25°C, 4°C, -20°C or -70°C for 24 hours was not statistically significant[16]. A working quantity of DNA can be isolated from blood stored at multiple temperatures above 4°C[17,18]. The DNA isolated from blood stored at room temperaturefor a period of one week was still good in terms of quality[17]. Inconsistently, another study showed that lower DNAyieldwere obtained from blood incubated at 37°C as compared to blood stored at lower temperatures[15].

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Immediate processing of blood will minimize the DNA degradation and the drop in DNA yield. Blood samples are usually frozen when immediate processing cannot be done. Storage of blood at -20°Ceven for a short period has been reported toreducethe DNA yield and increasethe risk of protein contamination[15]. Likewise, another study showed that even though blood storage at -80°C for 1 week cause the DNA quantitydropped,the sample still produced DNA with acceptable quality[19]. Conversely, DNA isolated from whole blood stored at -20°C for 30 years is reliable and is a potential resource for genotyping studies[20].

Hitherto, there are no studies published on the impact of one-year storage of left-over blood samples at -40°C on yieldand quality of DNA. Therefore, this study will compare the qualityand quantity of DNA from fresh blood and stored blood samples. The study is aimed to prove that left-over whole bloodstored at -40°C could be used as an option for DNA resources in molecular studies.

Materials and Methods:

Sample collection

An approximately two ml of fresh blood wascollected from 12 randomly volunteered subjects through venipunctureand were placed in ethylene diamine tetra aceticacid (EDTA) tube (BD Vacutainer®, USA).Fresh blood samples were immediately processed for the DNA extraction.Meanwhile, forcomparison purpose, 12 stored bloodsin EDTA tube were randomly selected from the storage freezer. The storedblood was the left-over whole bloodfrom routine hematology tests and has been stored for one year at -40°C. Due to the limited volume of stored blood samples, only one ml per samplewas collected for the DNA extraction.The study is complianced with ethical standards and was approved by Human Research Ethics Commitee of Universiti Sains Malaysia.

DNA isolation

Nucleo Spin® Blood L kit (Macherey-Nagel, Germany) was used to isolated the DNA fromfresh and stored blood samples.The protocols were according to the manufacturer's instructions[21]; Two ml of fresh bloods were added with 150 µl Proteinase K and 2.0 ml of buffer BQ1; The samples were vigorously vortexed for 10 seconds before incubated at 56°C for 20 minutes and vortexed once during the incubation;The samples were cooled down to roomtemperature for 10 minutes before the addition of 2.0 ml of absolute ethanol and laterinverted 10 times;Threeml of samples (lysate) were loaded into thecolumn located in a collection tube and centrifuged for three minutes at 4,500 xg; All the remaining lysate samples were loaded into the respective columnand centrifuged for another five minutes at 4,500 xg; The columns were then inserted into anew 15 ml collection tubeand centrifugedfor two minutes at 4,500 xgafter the addition of 2.0 ml of buffer BQ2;For a second wash, another 2.0 ml of buffer BQ2 was added to thecolumns and centrifuged for 10 minutes at 4,500 xg; The columns were again inserted into a new 15ml collection tube and 200 µl of preheated buffer BE at 70°C was directly applied to the centerof the columns;The columns were incubated for five minutes at roomtemperature, followed by centrifugation for two minutes at 4,500 xg.For stored bloods, 1.0 ml of phosphate buffered saline (PBS) (Qiagen, USA) was added into one ml of samplesbefore the addition of 150 µl of Proteinase K and 2.0 ml of buffer BQ1 (Macherey-Nagel, Germany).There are two differences in isolating the DNA from stored bloods; 1)Incubation time at 56°C with Proteinase K and buffer BQ1was 30 minutes instead of 20 minutes for fresh bloods; 2) During the incubation, the storedbloods were vortexed twice instead of once for fresh bloods. The rest of the steps were the same as in extraction of fresh bloods.The final volume of DNA samples in buffer BE was 200 µl with dissimilar concentrations.

DNA concentration and purity

The concentration and purity of extracted DNA was measured using NanoDropND-1000 spectrophotometer (Nano-Drop Technologies, USA). Absorbance of 230, 260 and 280 nm was measured on each sample respectively.The ratios of 260/280 nm and 260/230nm provide an estimation of DNA purity. For 260/280nm ratio, the value of 1.8-2.0 was considered as purified DNA. A ratio value of less than1.8 indicates protein contamination and a ratio value of more than 2.0 indicates RNA contamination. Expected 260/230 nm ratiovalue was commonly greater than accepted 260/280 nm ratio value. Lower values of 260/230 nm ratio indicatesthe presence of contaminants such as carbohydrates, salts or organic solvents which absorb at 230 nm. The concentration and purity of DNA were measured threetimes in each sample and the average was calculatedto be taken as the mean value.

DNA integrity

TheDNA integrity was examined through agarose gel electrophoresis. A 9 µl of total DNA samples were loaded onto 1.6 % agarose gel precast with SYB® safe DNA stain (Life Technologies, USA) in 1X lithium borate(LB) buffer (Fast Better Media,

LLC)for 40 minutes at100 Volts.

Polymerase chain reaction (PCR)

The DNA samples were subjected to PCR amplification to check the intactness and the presence of any inhibitory material that can interfere with the amplification. For this purpose, human growth hormone (HGH) and GP.Vw genes were amplified in a 20 µl volume, consisting of10 µl of 1X High-Fidelity PCR Master Mix with HF Buffer (Thermo Fisher Scientific, USA), 2 µl of g DNA (10 ng), 1 µl of each primer sets(0.5 µM) and 6 µl of nuclease free water (Qiagen, USA). Thermal cycling was conducted in a C100 Thermal Cycler (Bio-Rad, USA) with the following PCR program: initial denaturation at 94°C for 15 minutes (1 cycle); denaturation at 94°C for 20 seconds, annealing at 65°C for 20 seconds, extension at 72°C for 20 seconds (35 cycles);concluding with 10 minutes at 72°C (1 cycle) and ∞ minute at 4°C. The details of primers used were shown in (Table 1). The PCR products were examined on 2% agarose gel precast with SYB®safe DNA stain, electrophoresed in 1X LB buffer for 40 minutes at 100 volts to assess the size and integrity prior to sequencing.After electrophoresis, the digital images were analyzed with a 2.0 Blue Light Transilluminator (Life Technologies, USA). The presence of a band for each primer set indicates the PCR success.

Table (1) PCR primers used in this study

Primers	Sequences (5'- 3')	Product size (bp)	Reference
F1[a]	CAGCATTTCTCTAAAGGCTAAATAAGAAGATGTA	296	[22]
RIN[a]	CATATGTGTCCCGTTTGTGCA		
HGH5580F[b]	TGCCTTCCCAACCATTCCCTTA	434	[23]
HGH5967R[b]	CCACTCACGGATTTCTGTTGTGTTTC		

[a]F1 and RIN amplified a fragment of 296 bp specific for glycophorinVerweyst (GP.Vw). GP.Vw is one of *GYP (A-B-A)* hybrids in the Miltenberger blood group.

[b]HGH5580F and HGH5967R amplified a 434 bp fragment of the human growth hormone (HGH) gene as an internal control.

Sanger sequencing

The PCR productswere sent to 1stBASE laboratories SdnBhd (Malaysia) for DNA sequencing using HGH5580F, HGH5967R, F1 and RIN primers. The resulting sequence traces were qualitatively evaluated for baseline noise and peak shape. The identity of products was analyzed using BioEdit software and blasted against Gen Bank(http://www.ncbi.nlm.nih.gov/genbank/).

Statistical analysis

The data were presented as mean ± standard error of the mean (SEM). The normally-distributed data were analyzed using parametric independent t-test.The statistical analysis was performed using statistical product and service solutions(IBM SPSS, 23.0 Version) software.The significant value was set as $p< 0.05$.

Results:

Concentration, yield and purity of DNA at differentsamplingages

Spectrophotometric data of DNA extracted from fresh and stored bloodswere shown in Table 2.The concentration, yield and purity of extracted DNA from fresh and storedbloods were not statistically significant with $p> 0.05$ (Table 2).Therefore, one-year storage at -40°C did not affect the yield and purity of the extracted DNA.

Integrity of DNA

Regardless sampling time, both extracted DNA showed intact single band with high intensity (Figure 1).

Validation by PCR

The extracted DNA from fresh and stored bloodswassuccessfully amplified by F1/RIN and HGH5580F/HGH5967R primer sets (Figure 2). The target size of PCR products of GP.Vw and HGH were 296 and 434base pair, respectively. The PCR products showed good intensity with no apparent non-specific amplification products.

Sequence success

High quality DNA sequences were obtained from fresh and stored blood samples. The sequencing chromatograms results proved that good template was amplified by the primers (Figure 3). In general, the PCR products of the extracted DNA from fresh and stored bloodsyielded sequencing data with little background noise, well-formed and evenly spaced peaks.

Table (2) Comparison of quality and quantity of extracted DNA from fresh and stored bloods.

Whole bloods	DNA concentration[c] (ng/ul)	DNA Yield[c] (µg)	OD 260/280[d]	OD 260/230[e]
Fresh (*n*=12)				
1	247.65	49.53	1.89	2.31
2	249.73	49.95	1.87	2.32
3	251.26	50.25	1.88	2.37
4	267.86	53.57	1.89	2.44
5	257.83	51.57	1.87	2.39
6	273.50	54.70	1.88	2.39
7	237.77	47.55	1.87	2.40
8	243.36	48.67	1.86	2.38
9	249.14	49.83	1.87	2.36
10	249.57	49.91	1.86	2.41
11	264.93	52.99	1.87	2.33
12	233.38	46.68	1.88	2.42
Mean ± SEM	252.17 ± 3.46	50.43 ± 0.69	1.87 ± 0.002	2.38 ± 0.01
Stored (*n*=12)				
1	250.41	50.08	1.85	2.36
2	273.47	54.69	1.84	2.40
3	226.41	45.28	1.84	2.50
4	240.32	48.06	1.85	2.34
5	257.59	51.52	1.85	2.23
6	240.32	48.06	1.86	2.38
7	242.46	48.49	1.85	2.37
8	237.62	47.52	1.86	2.24
9	253.38	50.68	1.85	2.28
10	221.53	44.31	1.89	2.42
11	231.94	46.39	1.94	2.37
12	272.84	54.57	1.87	2.38
Mean ± SEM	245.70 ± 4.77	49.14 ± 0.95	1.86 ± 0.008	2.36 ± 0.02
*p*value	0.285	0.285	0.188	0.412

p value < 0.05 is considered to be statistically significant

[c]Higher values are desirable

[d]Values in range of 1.8 to 2.0 are desirable

[e]Values above 2.0 are desirable

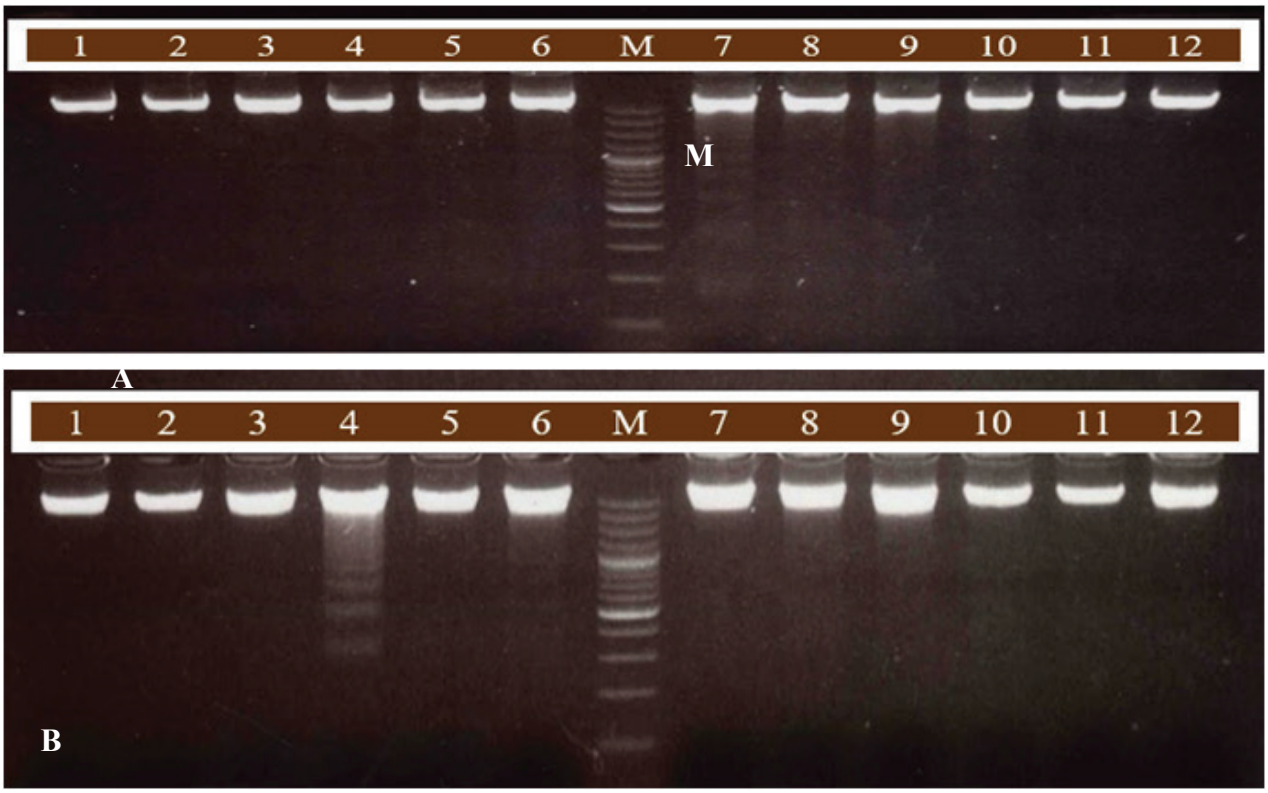

Figure 1. Electrophoretic analysis of total DNA from fresh and stored bloods on 1.6% agarose gel. (A) Total DNA from fresh bloods; (B) Total DNA from stored bloods; Lanes 1-12: Blood samples 1-12; M: 100 bp DNA ladder plus (Thermo Fisher Scientific, USA).

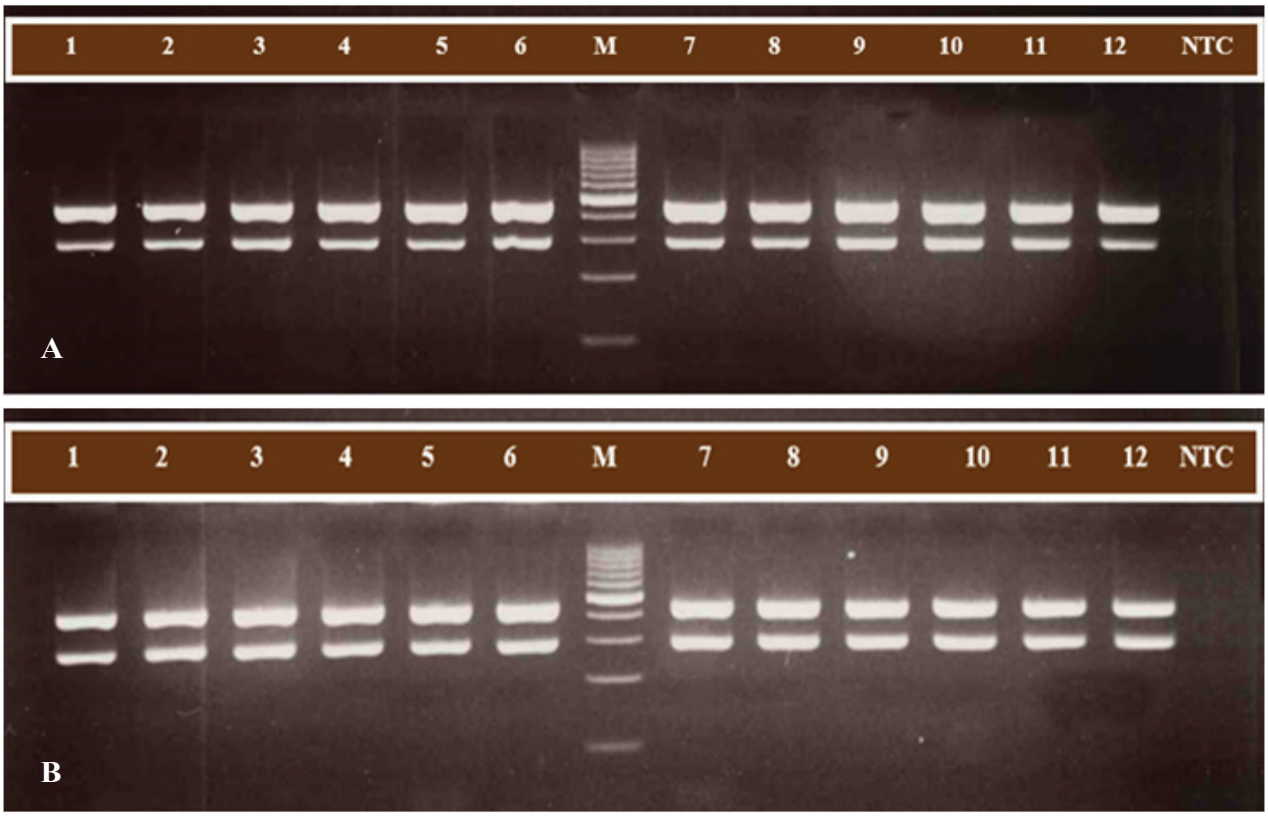

Figure (2)PCR amplification of HG Hand GP.Vw genes using fresh and stored bloods on 2% agarose gel. (A) Fresh bloods, (B) Stored bloods; Lanes 1-12: sample 1-12; M: 100 bp DNA ladder (Thermo Fisher Scientific, USA); NTC: Non-template control. HGH= Human Growth Hormone, GP.Vw= Glycophorin Verweyst

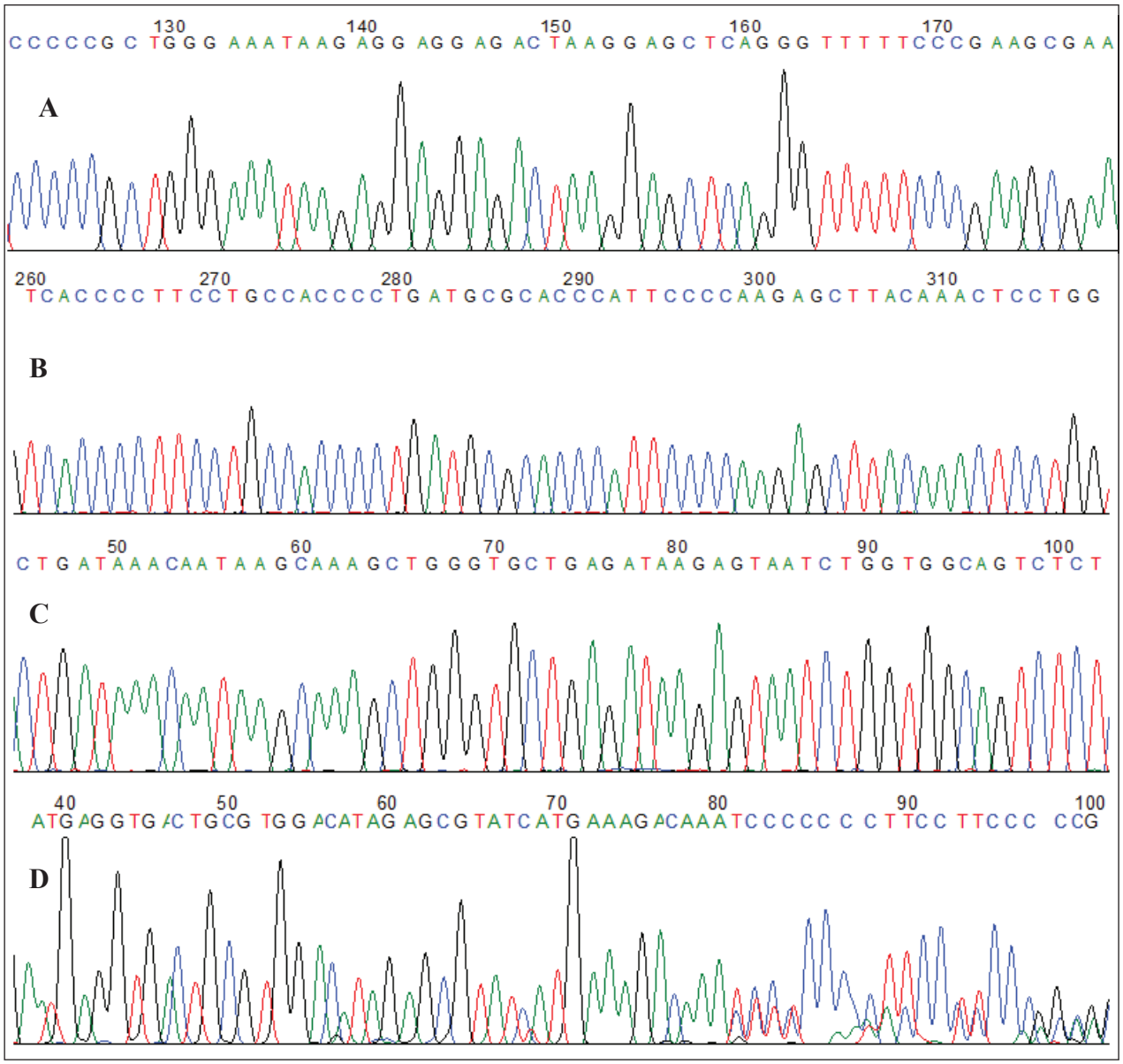

Figure 3: DNA Sanger sequence traces. Amplification by (A) HGH5580F (B) HGH5967R (C) F1 and (D) RIN, respectively. (A) and (D) were stored blood. (B) and (C) were fresh blood. Sequencing chromatograms showed that evenly-spaced peak with only one color and little noise. However, figure 3(D) showed little overlap peaks with different color which can be considered as good template as most distinct peaks and no ambiguity nucleotides reading.

Discussion:

Highquality and quantity of DNA yield is the first priority for laboratories that performmolecular analysis from clinical sample.Thus, we comparedthe impact of different sample age on DNA quality and quantity from whole blood samples. Based on our results, DNA quality and quantity were not affected by different sample ages(Table 2). DNA recovery from stored blood was similar to that of fresh blood and was sufficient for initiating downstream PCR and Sanger sequencing reactions. This might be due to the presence of EDTA as an anticoagulant resulting in high quality of DNA[24]. DNA degradation is inhibited by the presence of EDTA[16].

There were no significant differences in the mean concentration, yield and purity of the extracted DNA from fresh and storedbloods. The quantity of DNA was generally consistent for both blood samples. The extracted DNA showed good quality for molecular analysis with regards to PCR and sequence success. This is similar with other study showing that DNA extraction using Nucleo Spin® Blood L kit (Macherey-Nagel, Germany) resulted in high quality of DNA with successfulPCR amplification and sequencing [25]. The purity of the extracted DNA was confirmed by spectrophotometer and was calculated as the

260/280 OD ratio and 260/230 OD ratio. A value of 260/280 OD ratio above 1.8 was generally accepted as pure DNA[26]. Ratios lowerthan 1.8 indicates the presence of protein contamination that was absorbed strongly at or near 280 nm[27,28]. Ratioshigher than 2.0 indicate the presence of RNA contamination[29]. In our study, the 260/280 OD ratio wasabove of 1.8 and less than 2.0; indicating the extracted DNA to be free from protein and RNA contaminations (Table 2). A secondary measure of nucleic acid purity was based on the 260/230 OD ratio[26,30]. The values of 260/230 OD ratio for pure nucleic acid were often higher than the respective 260/280 OD ratio values[31]. We found that the ratio at 260/230 nm wasabove 2.0, which confirmedthe extracted DNA to be of good quality[30]. Lower ratios may indicate the presence of phenols, aromatic compounds, peptides and carbohydrates contaminants which absorb near 230 nm[29,32].

The DNA was intact and showed high intensity, indicating high DNA quality from both samples (Figure1). Amplification of DNA fragments of less than 1000 bp could be readily identified from small amounts (10 ng) of DNA from both samples.The high quality of isolated DNA used in PCR reaction was demonstrated by clear and recognizable bands (Figure 2). Additional analysis of these fragments via Sanger sequencing (Figure 3) confirmed that one-year storage of left-over whole bloods at -40°C did not affect the DNA quality as well as DNA yield. DNA degradationduring the long-term storage could be prevented by storing the blood below -20°C[33]. PCR and sequencing results suggested that the quality of DNA isolated from fresh and stored blood was equal in terms of functional integrity.

Therefore, in conclusion, one-year storage of patients' left-over whole blood at -40°Cmay provide an alternative option for laboratories to perform molecular studies. However, the final choice of storage method is based on individual laboratory services.

Conclusions:

We demonstrated that storage at -40°C did not affect the quality and quantity of extracted DNA. Therefore, we believe that this finding will enable clinicians, researchers, and epidemiologists to use frozen stored blood from left-over sample as a source of DNA sample. This will reduce repeated venepuncture procedure to patients in cases where molecular study is indicated.It is well known that the fresh sample is the first choice and the best way to isolate high molecular weight DNA. However, sometimes fresh samples cannot be obtained or will be obtained in limitedquantity and one has to rely on previously stored samples.

Conflict of interest: There is no conflict of interest exists.

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