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Original Article

Improved DNA Extraction Protocol from Frozen Blood of Patients Who Underwent Systemic Chemotherapy

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Abstract

Introduction: The quality of the extracted genomic DNA is largely reduced in patients who are exposed to chemotherapeutic treatment, which is usually encountered in the commonly used DNA extraction methods that are not designed to isolate DNA from those patients. In this study, a special non-organic protocol was developed to overcome the negative effects of chemotherapeutic drugs on the quality of extracted DNA.

Materials and Methods: Numerous modulations were applied in the washing, suspension, and lysis approaches to compensate for the harmful impacts of the chemotherapeutic drugs on the quality of genomic DNA. Obvious purity and adequate yields of the extracted DNA were demonstrated in the suggested protocol. The validity of this protocol for digestion with restriction endonucleases, conventional PCR, and real-time PCR was confirmed.

Results: This protocol proved satisfactory values of absorbance ratio (1.8 ± 0.02 and 2.1 ± 1.2, for $A_{260/280}$ and $A_{260/230}$, respectively) and adequate yields of DNA (10 ± 2.24) µg/100 µl. The validation experiments proved the efficacy of extracted DNA for downstream applications of molecular biology.

Conclusions: The utilization of this method entails a useful approach for extracting molecular biology-grade DNA without having inhibitors against common enzymes used in molecular biology even after exposing patients to several sessions of chemotherapy.

Keywords: DNA Extraction, Normal, Patients, Protocol, Frozen Blood

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Introduction

The treatment of cancer has largely been based on the use of chemotherapeutic drugs. Some of these drugs are regarded as potent DNA-damaging agents, which have a long history of employment in cancer chemotherapy.[1-3] It is well documented that patients receiving chemotherapy have higher levels of DNA damage in blood cells than those patients who have not been exposed to chemotherapy.[4-8] These DNA-damaging drugs are not only associated with the overall instability of gDNA, but they go beyond this limit by having a direct impact on essential biological reactions.[9] It is well-documented that each chemotherapeutic drug has its own mechanism of action.[10,11] However, all chemotherapeutic drugs are recognized as DNA-damaging agents due to their straightforward effect on DNA replication and repair to prevent tumor metastasis.[12] Thus, chemotherapy drugs exert a negative interference with DNA replisome and directly inhibit DNA replication.[13] Some of these negative effects have been attributed to the presence of purine or pyrimidine nucleoside analogues that directly impair the activity of DNA polymerases.[14,15] The chemotherapy drugs induce their negative impact on the DNA by incorporating themselves as base analogues instead of thymine or uracil.[16] These drugs contain a fluoride atom at the 5-carbon position on the ring preventing the addition of the next nucleobase on the strand and terminating chain elongation, which may induce apoptosis.[17] Therefore, such drugs may largely hamper interacting enzymes with the extracted DNA and may lead to a possible failure for polymerases involved in the polymerase chain reaction (PCR) and sequencing, site-specific endonucleases involved in restriction fragment length polymorphism (RFLP),[18] or even other downstream clinical applications that require enzymatic intervention. Due to their high efficacy in inhibiting replication *in vivo*, the ability of chemotherapeutic drugs to prohibit DNA replication *in vitro* cannot be eliminated. With the progressively prescribed chemical treatments of tumors, the negative impact of these drugs on the extracted DNA is increasingly being confronted. However, in addition to the commonly known methods of gDNA extraction,[19-21] other numerous protocols have increasingly been reported from blood.[22-24] Despite the

immense progress made in DNA extraction, little attention has been paid to reduce the difficulty of undergoing downstream experiments for DNA samples extracted from patients who are exposed to regular chemotherapeutic sessions. Due to the difficulties associated with sampling of DNA from tumor patients' entire blood,[25-27] no reliable practical solution for this technical issue has been precisely outlined. For these reasons, the present study was aimed to design a standard protocol to extract genomic DNA of those patients, which can be utilized in the diagnostic and genotyping experiments without being compromised by chemotherapeutic treatment.

Materials and Methods

Reagents/Tools Used

100 bp ladder marker (BioLabs Inc., cat. no. N3231S), 1 kb ladder marker (BioLabs Inc., cat. no. N3232L), 1.5 ml capacity microfuge tubes, 5 ml capacity Vacutainer tubes, *Bam*HI Restriction enzyme (BioLabs Inc., cat. no. R0136S), Disodium ethylene diamine tetraacetic acid (EDTA; Calbiochem, cat no. 324503), Ethanol absolute (Merck-Millipore, cat. no. 107017), Glacial acetic acid (Merck-Millipore, cat. no. 137130), *Hin*fI Restriction enzyme (BioLabs Inc., cat. no. R0155S), Lyophilized primers (Bioneer), Methanol (Merck-Millipore, cat. no. 107018), Nuclease-free tri-distilled water (BioLabs, cat. no. B1500S), *Rsa*I Restriction enzyme (BioLabs Inc., cat. no. R0167S), Sodium acetate (NaAc; Merck-Millipore, cat. no. 106268), Sodium dodecyl sulfate (SDS; Merck-Millipore, cat. no. 817034), and Tris-hydrochloride (Tris-HCl; Merck-Millipore, cat. no. 108219).

Solutions Used

Cell suspension buffer: 2 mM EDTA (pH 8.0), Cell lysis buffer: 0.2% SDS, 20 mM Tris-Cl (pH 8), DNA washing buffer: 60% ethanol in 5 mM Tris-Cl (pH 8.0), DNA elution buffer: 10 mm Tris-Cl (pH 8), 1 mM EDTA, Luna® Universal qPCR Master Mix (New England Biolabs, cat. no. #M3003), PCR premix (Bioneer, cat. no. K-2012), Protein precipitation buffer: 3 M sodium acetate, adjusted to pH 4.5 by acetic acid, Tris-acetate-EDTA (TAE) buffer: 40 mM Tris-acetate; 2 mM EDTA (pH 8.3), Tris-borate-EDTA (TBE) buffer: 2 mM of EDTA, 89 mM of Tris-Borate (pH 8.3), Washing buffer: 10% methanol and 18mm Tris-Cl (pH 7.5).

Equipment Used

Agarose gel electrophoresis unit (CleaverScinetific, cat. no. MSCHOICE7), Centrifuge equipped with a fixed angle rotor of FA-24x2 (Eppendorf, cat. no. 5405000514), Gel documentation imaging system (JUNYI Electrophoresis, cat. no. JY04S-3C), Gradient PCR thermocycler (Eppendorf, cat. no. 6331000017), Micropipettes (Eppendorf, P-200, and P-1000), nanodrop spectrophotometer (Biodrop, part no. 80-3006-51), Rotating mixer of 48x1.5/2.0ml capacity (Benchmark Scientific, cat. no. R5010), Rotor-Gene Q Real-Time PCR System (Qiagen, cat. no. 9001620), Water bath (Thermo-Scientific, cat. no. TSSWB15).

Blood Sampling

To verify the detailed accuracy of our suggested protocol in extracting DNA from patients who underwent systemic chemotherapy, a large-scale sample size was included in this study. The total number of blood samples collected from patients was 207. Those patients that were included in this study had suffered from several grades of various types of tumors (54, 48, 31, 20, 19, 16, 11, and 8 samples of the bladder, colorectal, breast, ovarian, non-small cell lung, pancreatic, cervical, and hepatocellular carcinomas, respectively). All those patients had previously been exposed to systemic chemotherapeutic sessions which included short (32-94 min) and long (245-638 min) infusions with 5-FU (uridine-thymidine analogue). From each involved patient, only 500 µl was specified for this study, which was routinely provided by personnel of Marjan Teaching Hospital in Babylon. Written Informed consent was obtained from each included patient who was involved in this study. All blood samples were frozen under -20 °C until being processed for DNA extraction.

DNA Extraction Protocol

Five main steps were conducted in this extraction protocol; cell washing, cell lysis, protein precipitations, DNA precipitation, and DNA recovery. In the cell washing step (Figure 1a), the frozen blood sample that was placed in a vacutainer tube was thawed at room temperature and mixed gently. Then, only 500 µl of the blood was transferred to an Eppendorf tube of 1.5 ml capacity. Up to 1 ml of washing buffer was added to the blood sample, mixed, and incubated at ambient temperature for 10 min, or 5 min in a rotating mixer. The mixture was centrifuged at 10000 g for 2 min. After discarding the supernatant, 1 ml of washing buffer was added again, and the tube was inverted several times to wash the pellet and then centrifuged at the same speed for 10 sec. The supernatant was discarded and the pellet was gently suspended with 1 ml of washing buffer by back-and-forth pipetting with a wide orifice tip and centrifuged for 1 min. The previous step was repeated twice, or once a yellowish-white precipitate of leukocytes appears). In the cell lysis step (Figure 1b), the pellet was suspended with 200 µl of cell suspension buffer and mixed several times to remove cellular aggregations. Then, 200 µl of cell lysis buffer was added to the generated homogenate, mixed, and then left at room temperature for 5 min. In the protein precipitation step (Figure 1c), proteins were denatured by mixing cellular lysate with 100 µl of protein precipitation buffer for vortexed for 20 sec. Afterward, the suspension was centrifuged at

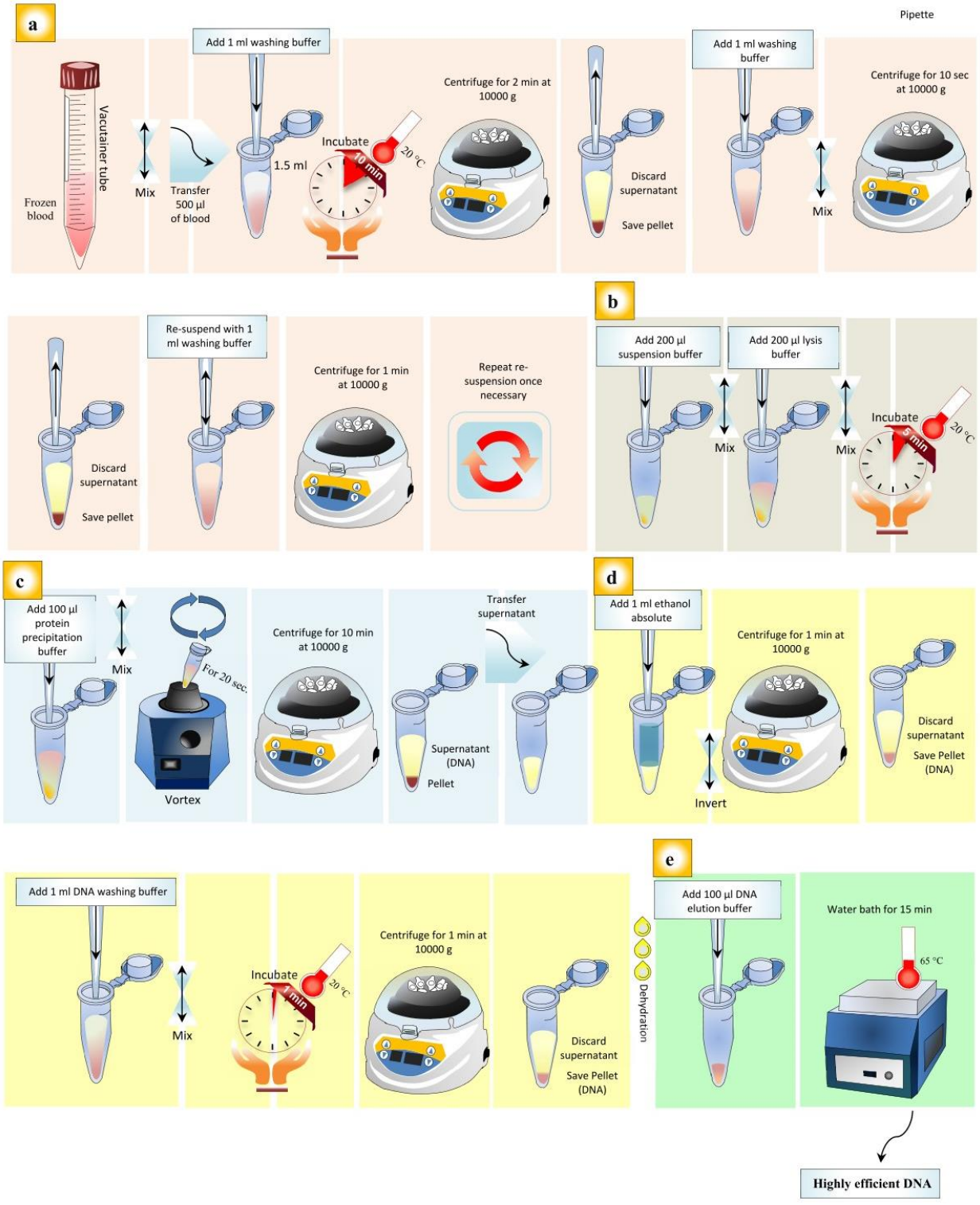

Figure 1. A Schematic Diagram for the Described Modified DNA Extraction Steps of this Study. This protocol is made of five main steps; a) cell washing, b) cell lysis, c) protein precipitations, d) DNA precipitation, and e) DNA recovery.

10000 g for 10 min. The supernatant containing DNA was transferred to a new 1.5 ml centrifuge tube. In the DNA precipitation and washing step (Figure 1d), up to 1 ml of absolute ethanol was added to the supernatant until the appearance of ideal DNA threads was achieved. Subsequently, centrifugation at 10000 g for 1 min was performed. The supernatant was discarded and 1 ml of DNA washing buffer was added and mixed well with the pelleted DNA and left for 1 min to re-suspend DNA. The suspension was centrifuged at 10000 g for 1 min and the supernatant was discarded. In the DNA recovery step (Figure 1e), the pelleted DNA was exposed to direct dehydration at room

temperature, then it was mixed with 100 µl of DNA elution buffer. The mixture was incubated for 15 min at 65 °C in a water bath to speed up recovery.

Qualitative and Quantitative Evaluation of the Isolated DNA

The concentration and purity of the isolated DNA were analyzed by a Nanodrop spectrophotometer. DNA concentration measurements were recorded as µg/100 µl, while DNA purity was taken from the ratio obtained from the A260/280 absorbance formula.

Digestion with Restriction Endonucleases (RE)

The extracted DNA was exposed to digestion with several REs to confirm the absence of any possible inhibitor for the biological activity of these enzymes.[28] Randomly selected samples of gDNA were digested with three different REs, *Bam*HI (5′…GGATCC…3′), *Hin*fI (5′…GANTC...3′), and *Rsa*I. (5′…GTAC…3′), following the instruction manuals recommended by manufacturers' suppliers (BioLabs Inc., 240 County Road, USA). Briefly, 1 µg of gDNA was diluted with 18 µl of distilled water. Then, 2 µl of 10X RE-mix and 10U of each selected RE were added. The mixture was incubated overnight at 37 °C, and analyzed by standard 1.5% (w/v) agarose gel electrophoresis in TBE buffer.

Electrophoresis

The integrity of the gDNA was assessed by a direct running of a series of gradually increasing concentrations of gDNA in a standard 0.8% (w/v) agarose gel electrophoresis unit in a TAE buffer and photographed by a gel documentation imaging system. The quality was also considered in the gel as the integral gDNA migrates as a relatively well-defined band while degraded gDNA takes a smeared migration pattern.[28]

Conventional PCR

DNA amplification was performed to ensure the validity of the extracted DNA to act as a competent template for PCR reaction and to confirm the absence of any possible inhibitor for *Taq* DNA polymerase activity. Ten pairs of specific primers were designed by the primer BLAST server[29] (Table 1). A total reaction volume of 20 µl containing a lyophilized PCR premix, 0.7 µl of 10µM of both forward and reverse primers, 1 µl of the extracted DNA template, and 17.6 µl nuclease-free tri-distilled water, was used for PCR amplification. The optimum annealing temperatures for the designed primers were empirically determined using a gradient thermal cycler. The PCR program was set by initial denaturation at 94 °C for 4 min, followed by 30 cycles of denaturation at 94 °C for 30 sec, annealing temperature ranged from 59 to 61.3 °C for 30 sec, and elongation at 72 °C for 30 sec, and was concluded with a final extension at 72 °C for 5 min. Subsequently, PCR products were assessed by 1.5% agarose gel electrophoresis. PCR products were exposed to sequencing reactions from both termini according to instructions of the sequencing laboratories (Macrogen, Geumchen, Seoul, Korea).

Table 1. The Designed Primers of the *PTEN, LEPR, Kras,* and *ATM* genes by NCBI primer BLAST tool

No.	Primers' code	Sequence (5′-3′)	Length (bp)	Annealing temp. (°C)	NCBI accession number
1	*PTEN*-F	TGCTGCAGTCCATTGAGCATA	658	60.1	NC_000010.11
	PTEN-R	GCTGTGGTGGGTTATGGTCTT			
2	*LEPR*-F	GCAGCTCTGAAAGGGGTTGTA	103	61.1	NC_000001.11
	LEPR-R	CGGTAATCAGCTGTGGGACT			
3	*Kras*-F	ACTCCTCTTGACCTGCTGTG	107	59.8	NC_000012.12
	Kras-R	AATCCAGACTGTGTTTCTCCCTT			
4	*ATM*-F	GCCATTCTATGGTAGCCCCC	345	61.9	NC_000011.10
	ATM-R	TGCAAAAACTCACCTCAAGCA			

Real-time PCR

More confirmation of the validity of the extracted DNA was conducted using real-time PCR to provide a further indication for the presence of amplifiable DNA in extracts of blood samples. One set of primer pairs was designed to partially amplify 167 bp of *GTA* gene; forward primer 5′-TATTTCTGCATGGGCCAGGG-3′, and reverse primer 5′-ACTCGGAGAAACAAAGTGCCT-3′. Real-time PCR amplifications were conducted using a Rotor-Gene Q Real-Time PCR System. A universal Luna® qPCR Master Mix was employed for the amplification of different extracted DNA concentrations (1000-1 ng). All samples were run in duplicates, and the Ct values, amplification efficiency, y-intercept, slope, dynamic range, and other parameters were calculated following the manufacturer's instructions (Rotor-Gene Q software version 2.1).

Comparison with Other Methods

Ten representative samples of our method were compared with three manual methods for DNA extractions, namely Adeli and Ogbonna, 1990;[30] Nassiri et al., 2005;[31] Lahiri and Nurnberger, 1991.[32] The parameters used for comparisons included the amount of starting blood, time of extraction, proteinase K utilization, RNase utilization, organic solvents-based hazard, quality of genomic DNA (A260/280), and quantity of genomic DNA (µg/100 µl).

Results

This protocol of DNA extraction was validated after conducting a series of conventional experiments in molecular

Table 2. Yield and Purity of the Genomic DNA Extracted from Frozen Blood Obtained from Normal Donors and Patients who are Exposed to Chemotherapy. Values are Expressed as means±SD

Frozen blood sample	DNA yield (µg/100 µl)	$A_{260/280}$	$A_{260/230}$
Normal	13 ± 4.22	1.79 ± 0.04	2.1 ± 0.8
Patient	10 ± 2.24	1.80 ± 0.02	2.1 ± 1.2

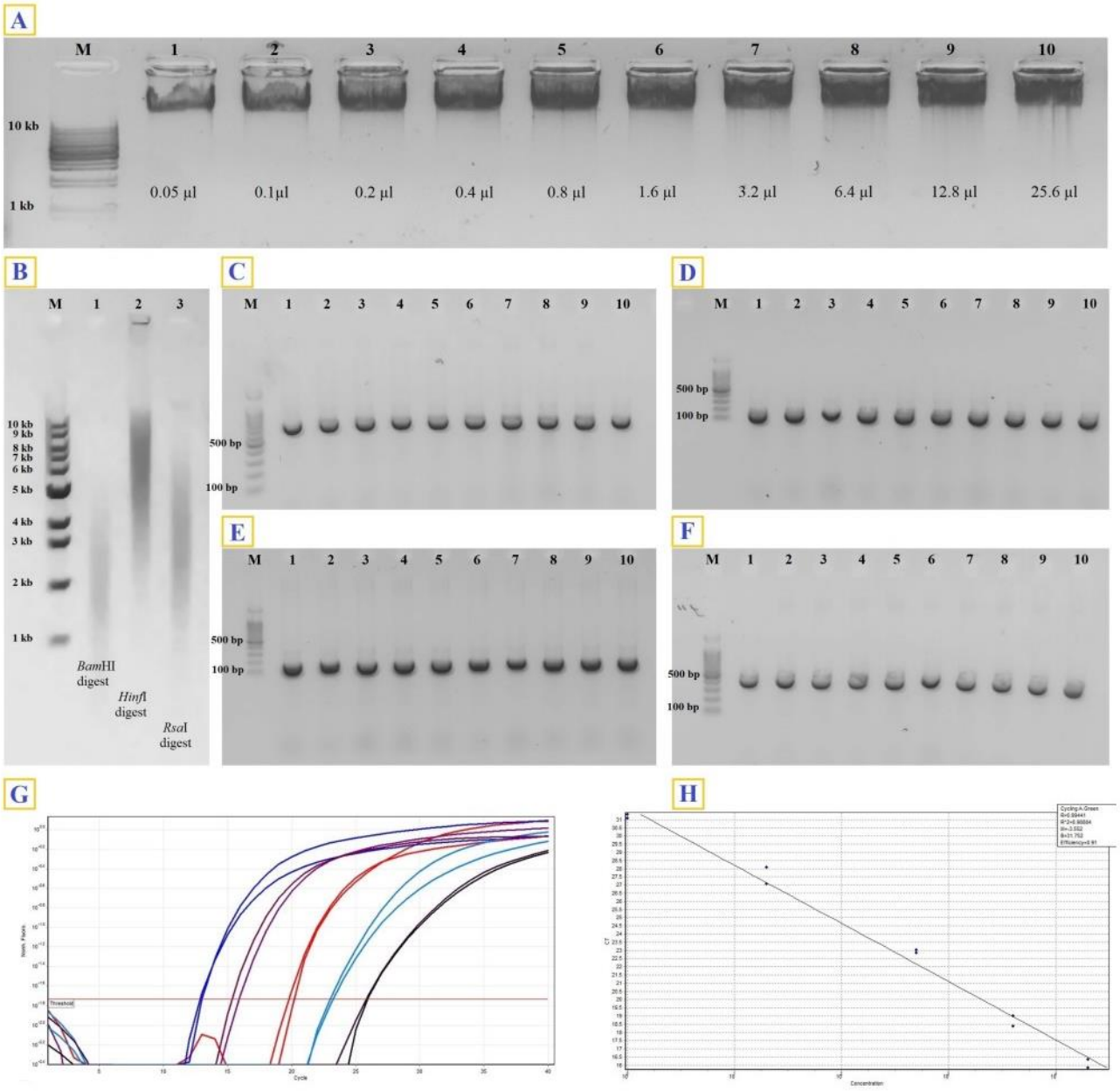

Figure 2. Validation of the DNA Extraction Protocol Using Several Techniques. A) 0.8% agarose gel electrophoresis of various gradual concentrations of genomic DNA; B) DNA digestion with restriction endonucleases; C-F) agarose gel electrophoresis of 658 bp, 103 bp, 107 bp, and 345 bp PCR products, respectively; G-H) real-time PCR for 167 bp PCR products. The symbol M refers to the ladder marker.

biology. The conducted spectrophotometric results showed a high yield and purity of the DNA extracted by this protocol. The average yield of isolated DNA was estimated to be 10 ± 2.24 µg per 100 µl (Table 2). Furthermore, the absorbance ratio scored highly acceptable levels as they were valued 1.78 into 1.81, and 2.0-2.21 for $A_{260/280}$ and $A_{260/230}$ respectively, which signified low levels of contamination. However, relatively higher amounts of DNA were obtained from normal samples, which was simply attributed to the absence of any exposure to DNA impairment agents of 5-FU found in the received chemotherapeutic sessions.

The electrophoretic experiments performed showed clear integrity of the extracted DNA samples, which indicated no smear or other contaminating particles even when very high

concentrations of DNA were being analyzed (Figure 2a). Most importantly, the readability of this DNA for digestion by restriction enzymes was tested (Figure 2b). Clear activity for the utilized REs was detected and DNA samples were easily degraded by these enzymes. Another layer of confirmation came from conventional PCR, in which specific bands were observed in all amplified loci (Figure 2c-f). More verifications were obtained from qPCR experiments, in which the extracted DNA by our protocol showed high purity and inhibitor-free amplification reactions. Results showed successful amplification of low and high concentrations of DNA (1 ng - 1 µg), respectively. An accepted dynamic range for the extracted DNA was achieved with calculated efficiency and R-values of 0.91 and 0.99 respectively (Figure 2g-h). These successive experiments demonstrated the absence of any significant inhibitor for the enzymes used in DNA digestion, conventional PCR, and quantitative PCR reactions and indicated that the isolated DNA was of excellent quality. To provide further validation of this protocol, PCR products were exposed to numerous Sanger dideoxy-sequencing reactions. These sequencing reactions showed clear electropherograms for PCR amplicons with obvious, distinct, and non-interfering nucleic acids peaks.

Table 3. Comparison between this Method and Three Manual Methods for Genomic DNA Extraction from Blood. Ten representatives of samples are used in the conducted comparison.

Parameter used	This method	Method#1	Method#2	Method#3
Amount of starting blood	0.5 ml	1 ml	5 ml	5 ml
Time of extraction	1 h	1.5 h	2.5 h	1 h
Proteinase K utilization	No	No	No	No
RNase utilization	No	No	No	Yes
Organic solvents-based hazard	No	Yes	No	No
Quality of genomic DNA (A260/280)	1.80 ± 0.1	1.83 ± 0.6	1.72 ± 0.16	1.81 ± 0.80
Quantity of genomic DNA (µg/100 µl)	13 ± 0.22	8.2 ± 0.18	6.9 ± 0.13	16.2 ± 1.06

Method#1, #2, and #3 refer to Adel and Ogbonna, 1990; Nassiri et al., 2005; Lahiri and Nurnberger, 1991, respectively.

This method was compared with three known methods for genomic DNA extraction from blood.[30-32] Our suggested method was found to be superior in terms of the reduced amount of the starting blood, reduced time of processing, absence of handling with the hazardous organic solvents, and optimum quality of the obtained DNA. Though the method of Lahiri and Nurnberger showed higher amounts (16.2 ± 1.06 µg/100 µl) and optimum quality (1.81 ± 0.80) of the extracted DNA, larger amounts of blood samples sizes are required for this method, which exceeded the amounts required in our methods by ten folds. Whereas the other two methods did not compete with our methods and with the method of Lahiri and Nurnberger, 1991, respectively.

Discussion

Several types of chemotherapeutic drugs have fluorescent compounds that inhibit the activity of many enzymes used in molecular diagnostics. One of these commonly used drugs is 5-FU, which is an effective pyrimidine analogue that is being used to treat several types of malignancies.[33-36] The uridine-thymidine analogue, a synthetic form of 5-FU, differs from deoxycytidine by the addition of two fluorine atoms at the 2′ -position of the sugar. The triphosphate form of 5-FU functions as a substrate for DNA polymerases involved in DNA synthesis.[37,38] After 5-FU is incorporated as a pyrimidine analogue, DNA synthesis is terminated. Therefore, 5-FU has an inevitable inhibitive role against interactive enzymes in molecular genetics.[39] This role has been ascribed to the presence of this chemotherapeutic drug, which has eliminated any competency of the extracted DNA for downstream standard genotyping experiments.

Despite the crucial DNA damaging effects caused by therapeutic drugs for cancer patients, no parallel light is directed to evaluate the association of these damaging effects with the efficacy of common protocols used in DNA extraction from those patients. It was hypothesized that chemotherapeutic drugs may cause DNA-damaging potential leading to increased mutational load.[40] Some other protocols recognized numerous mechanisms through which the chemotherapeutic drugs can damage DNA and explained their interfering role against DNA architecture.[41] Moreover, Chemotherapy-induced DNA damage in peripheral blood was quantified by a flow cytometry method.[42] However, the direct effect of chemotherapy on the quality of DNA in peripheral blood has not been investigated and the impact of chemotherapy on the total platform of peripheral blood may be underestimated.

The newly suggested method for genomic DNA extraction was compared with three known methods. The aim of this comparison is to determine which method is superior based on several criteria. The newly suggested method for genomic DNA extraction from blood shows to be a favorable option due to its ability to work with reduced starting blood, shorter processing time, absence of hazardous solvents, and overall high-quality DNA yields. Though the method of Lahiri and Nurnberger, 1991 showed higher DNA yields and quality, it requires significantly larger blood sample sizes. Whereas the other two known methods of Adeli and Ogbonna, 1990 and Nassiri et al., 2005 were not competitive in comparison to the suggested method.

Though considerable variations of DNA concentrations were reported in cancer patients,[43,44] our protocol has proven to be superior after conducting several experiments of validation. According to our results, the persisting inhibiting effects of the chemotherapeutic agents were eliminated in our protocol. This evidence may be attributed to the substantial modifications applied in cell washing, in which the majority of these counterfeiting agents were eradicated. After a series of efforts, we have managed to use 10% methanol in cell washing instead of other available solutions to solve this issue. The utilization of methanol induces a reduction in polarity and surface tension in such a way cellular chemotherapeutic agents would be eliminated alongside other cellular sediments.[45] Furthermore, several trials were performed to make this protocol more familiar. The chemical composition described in this method was designed to include commonly available chemicals to provide further simplicity for routine investigations without being compromised by greater labour input by having more steps and chemicals in removing 5-FU. This piece of evidence – in turn – is very beneficial in terms of reducing costs and efforts without scarifying DNA in these samples. Add to that, the non-organic/non-enzymatic steps have also proven more feasibility of our protocol as it does not require the hazardous inclusion of toxic organic solvents,[23] or the extended incubation with proteinase K.[46] However, the utilization of this protocol is not restrained to these chemotherapeutic cases. Instead, it can be used to extract DNA with considerable efficiency for molecular biology experiments. The DNA quality obtained by our protocol is highly acceptable as it is found to be around 1.8, while the DNA yield is sufficient for performing at least 50 PCR experiments. After validating this protocol through a series of experiments, a confirmation to obtain the highest purity grade needed in RFLP, PCR, and qPCR experiments was provided. In addition to the application of this protocol for DNA extraction in essential experiments, it is confirmed to be competent for sequencing protocols. This evidence was based on many sequencing reactions conducted on PCR products that showed clear chromatograms with optimal baselines, which implies no possible contamination for sequencing reactions. The observed distinct results for many validating experiments showed a non-controversial efficacy of this method. In light to numerous validations that have been carried out for this method, we suggest employing this protocol in DNA extraction from patients who have undergone systemic chemotherapeutic sessions. Therefore, it can be stated that this is the first DNA extraction protocol specially designed to remove all fluorescent inhibitors found in the DNA extracted from patients undergoing chemotherapy with high efficiency.

Conclusion

All experimental validations performed have confirmed the competency of the presently suggested protocol to eliminate the fluorescence agents found in chemotherapeutic drugs. The chemical compositions of the DNA extraction protocol have eradicated the unfavourable interaction of 5-FU against endonucleases and polymerases used in molecular biology experiments. In addition to the advent feasibility of the present protocol for normal samples, we strongly recommend it in DNA extraction from patients who have been intensively exposed to chemotherapy.

Authors' Contributions

HOH Designed the work, collected the blood samples, and conducted all the experiments. MBSA interpreted results, analyzed data, and wrote the manuscript. The authors read and approved the final version of the manuscript.

Ethical Approval

The present sampling procedures have been approved by the research ethics committee of Babylon Health Directorate, Ministry of Health, Iraq (numbered 6086, dated 13/10/2018). Verbal and written consents were obtained from all patients and controls before being involved in the study as their personal data were asked to be anonymous. The Research Ethics Committees approved this step.

Data Availability

This manuscript was previously published as a preprint and is freely available online (https://www.preprints.org/manuscript/202009.0683/v1). Other data underlying this article will be shared on reasonable request to the corresponding author.

Conflict of Interest Disclosures

The authors declare that they have no conflicts of interest.

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