Comparison of two methods of DNA extraction for amplification by real-time PCR using blood samples

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Abstract:
Extraction of DNA is one of the most basic and critical steps affecting molecular-based techniques. There are many popular DNA isolation methods from human whole blood for efficient genomic DNA extraction. This study was aimed to compare the genomic DNA quality and quantity between modified chemical Non-Phenolic Non-Enzymatic Rapid-Method and commercially available silica column-based blood DNA isolation kit (QIAamp DNA Blood Mini Kit). Genomic DNA was extracted from nineteen female Libyan students using both protocols was evaluated using gel electrophoresis, Nanodrop spectrophotometric analysis, and real time PCR assay. Results revealed significant differences among the two methods (40.55 ± 37.65 ng/µL for modified chemical; 22.84 ± 6.1 ng/µL for Qiagen QIAamp Kit; P= 0.04). The DNA purity (OD260/OD280) of DNA obtained from both methods produced the highest purity DNA, with differences being not statistically significant. Our results showed that modified chemical method was the best choice to DNA extraction from whole blood samples in large scale because this method was found cheap enough with good yield over commercial kits especially in the poor laboratories. On the other hand, this method is providing a sufficient quantity and quality of DNA for real- time PCR analysis.
Keywords: DNA extraction, Whole blood, modified chemical method, QIAamp DNA Blood Mini Kits.

Introduction

There is no doubt that the use of deoxyribonucleic acid (DNA), ribonucleic acid (RNA), and protein samples is of the great importance in development of molecular biology and life sciences. Successful use of available downstream applications will benefit from the use of high-quantity and high-quality DNA. Therefore, genomic DNA extraction is a key step in laboratory procedures required to perform PCR, genetics, genomics, gene polymorphism, DNA fingerprinting and gene sequencing. Genomic DNA for genetic studies can be produced from any tissue. However, for the past 40 years, DNA has extracted from whole blood where DNA extraction is a fundamental process in many diagnostic, identification and research applications and it has been widely used in many facilities around the world. [4,10 ,12]

Numerous studies have documented that PCR-based analyses of genomic DNA composition may be affected by the DNA extraction protocols applied, being able to prepare human genomic DNA from whole blood with high quantities of pure, intact, double stranded, highly concentrated, not contaminated genomic DNA from blood is important for successful and reliable large scale genotyping analysis [2,7,14]

Thus, the efficacy of DNA isolation among modified standard chemicals method and silica column-based Blood DNA isolation kit may vary in different characteristics that are of importance to test such as time for processing, yield, purity and integrity of DNA, cost and simplicity of process. Consequently, it can be useful to measure such parameters in comparison of different available methods for DNA isolation. DNA analysts and educational institutions would thus have more criteria for the selection of methods for particular needs [5,6,15].

We have compared a modified chemical Non-Phenolic Non-Enzymatic Rapid-Method and commercially available silica column-based blood DNA isolation kit (QIAamp DNA Blood Mini Kit), then studied and evaluated the genomic DNA quality and quantity from whole blood samples obtained from nineteen female Libyan students by using gel electrophoresis, Nanodrop spectrophotometric analysis, and real time PCR assay. Thus, the objectives of this study are to determine method that provide the cheapest, astest and easiest way to purify f total DNA for future research and development of a high throughput.

Aims:

To compare two DNA extraction protocols for final DNA concentration, purity, quality, cost and use of template for real- time PCR assay.

Material and methods:

Resource Population and Sample Collection

Blood samples were collected from nineteen healthy Libyan students in 5ml tubes by venipuncture in EDTA tube and stored at –20 °C. Collected samples were processed for DNA extraction .The collection of the whole blood samples was conducted in the national center of disease control\ Tripoli.

DNA Extraction Methods

DNA was extracted from the samples with the following two different protocols. All methods described next:

Method 1: (Modified chemical method)

This protocol describes the standard method for nucleic acid purification by extraction with chemicals. After that DNA extracted by this protocol: 500 µl of blood and 1000 µl of red cell lysis buffer (0.01 M Tris-HCl pH 7.6, 320 mM sucrose, 5 mM MgC12, 1% Triton X 100 and distilled water) were added to a 1.5 ml eppendorf tube and mix. Then centrifuged at 7000 rpm for 2 minutes and the supernatant was discarded and repeated step two or three more times in order to clean the white blood cells from residual of hemoglobin. The tubes were placed on tissue paper for few seconds downward. Then 400 µl of nucleic lysis buffer (0.01 M Tris-HC1, 11.4 mM sodium citrate, 1 mM EDTA, 1 % sodium dodecyl sulphate) was added to the eppendorf tube. Then 100 µl of saturated NaCl (5M) and 600 µl of chloroform were added to the tubes and mixed the tube on a rotating blood mixer at room temperature. After that tubes centrifuged at 7000 rpm for 2 minutes, and the aqueous layer was then carefully removed from each sample and placed into a new tube. Then 800 µl of cold (-20°C) absolute ethanol, after that shaking the tubes and vortex it. DNA appears as a mucus-like strand in the solution phase. Then centrifuged at 12000 rpm for 1 minute after centrifugation, the supernatant was discarded, and the pellet was

dried at room temperature. The pellet was resuspended in 50 µL of TE or deionized water and stored at 4°C or -20°C for later uses (Bailes et al 2007).

Method 2: QIAamp DNA Mini Kit (commercial kit)

(QIAGEN, USA) were tested. In this protocol DNA was extracted as described by the manufacturer's instructions. In this method proteinase K (20µL) and Buffer AL (200µL) were added to blood samples (200µL) and incubated at 56°C for 10min. Absolute ethanol (200µL) was added, and the mixtures were transferred to the QIAamp spin columns. These were centrifuged at 6,000g for 1 min, and then the waste tube was discarded. The columns were washed with 500µL of buffer AW1 and then again with 500µL of AW2. The purified DNA was eluted from the spin columns with 200µL of buffer AE into clean sterile microfuge tubes and stored at -20°C until further analysis.

Evaluation of DNA Extraction

- **Concentration and Purity Determination**

Quantity and Purity of DNA extracted with each method was assessed using the Thermo Scientific™ NanoDrop Lite Spectrophotometer. Absorbance was measured at wavelengths of 260 and 280 (A260 and A280, respectively) nm, the concentration of the sample was determined from the 260 nm absorbance reading and the purity from the 260/280 nm ratio. An absorbance quotient value of $1.8 \leq$ ratio (R) ≤ 2.0 was considered to be good, purified DNA. A ratio of $\leq$ 1.8 is indicative of protein contamination, where as a ratio of ≥ 2.0 indicates RNA contamination.

- **DNA Integrity**

The quality and integrity of all genomic DNA samples were assessed by agarose gel by electrophoresis apparatus (Micro – Bio -Tec, Horisontal agarose). Each DNA sample was analyzed on 1% agarose gel in 1 × Tris Acetate-EDTA buffer containing 2µl SYBR® Gold Nucleic Acid Gel Stain, under conditions: 70V and 120 mA for one hour. The bands were visualized using MultiDoc-It™ Imaging System by UVP (Cambridge, UK). Each DNA sample was graded, according to the electrophoretic migration of sample DNA compared with a known molecular weight marker (Gelpilot 100 bp Ladder, QIAGEN), and the images were captured.

- **Real-Time PCR amplification**

DNA quality differences among the two sampling methods were also evaluated by Real-Time PCR using (Roter- Gene Q, QIAGEN) thermocycler. In a real time PCR assay a positive reaction is detected by accumulation of a fluorescent signal. The threshold cycle (Ct) is defined as the number of cycles required for the fluorescent signal to cross the threshold line. Ct level is inversely proportional to the amount of target nucleic acid in the sample. human haemochromatosis (HFE) gene was used as a reference gene in order to determine the DNA quality and quantity, a real time PCR reaction mixture containing 2µLof DNA sample, 0.75µL of forward and reverse primers and Eva Green master mix kit (Metabion, Germany) in a final reaction volume of 25 µL was made using nuclease-free water. The primer sequences used for HFE being forward: ACA TGG TTA AGG CCT GTT and reverse: ATG TGA TCC CAC CCT TTC produced amplicons of 245 base pairs (bp). The thermal profile for H63D polymorphism was contained one cycle of an initial denaturation step (Taq polymerase activation) at 95°C for 4 min followed by 40 cycles of denaturation step at 95°C for 30 sec, annealing step at 57°C for 30 sec, and extension step at 72°C for

20 sec. Determination of amplification was computed as a Ct value using the Rotor-Gene 2.1.0.9 software supplied by the manufacturer.

- **Statistical analysis**

Means of amounts of DNA and ratios were compared by Paired t-Test: Two Sample for Means. P-values of less than 0.05 were considered evidence of statistical significance.

Results

Method Comparison

The quality and quantity of genomic DNA extracted from blood samples is a key feature which should be considering when choosing a protocol. The results from DNA extractions compared by Nanodrop spectrophotometer, gel electrophoresis methods and real time PCR demonstrated as the following:

- **Quantification of DNA extracted from modified chemical and QIA amp DNA blood Mini Kit methods**

DNA concentration measured by Nano Drop is usually calculated from DNA absorbance reading at 260 nm. Purity of nucleic acid samples is assessed in a 260/280 absorbance ratio, and values in the range of 1.8–2.0 are generally considered acceptable. Blood samples from nineteen individuals were used to determine the concentration of the DNA and quality of the extracted DNA from two different DNA extraction protocols; revealed a high yield of total genomic DNA from whole blood as shown in Table 1. The largest amount of DNA extracted from modified chemical method with a mean ± SD of 40.55 ± 37.65 ng/µl, and lower amounts were obtained from Silica column (QIA amp DNA Blood Mini Kit) method with a mean ± SD of 22.8± 6.1 ng/µl as shown in fig.1. Results for blood samples from modified chemical method were significantly different to those obtained from Silica column (QIA amp DNA Blood Mini Kit) method (P = 0.04).

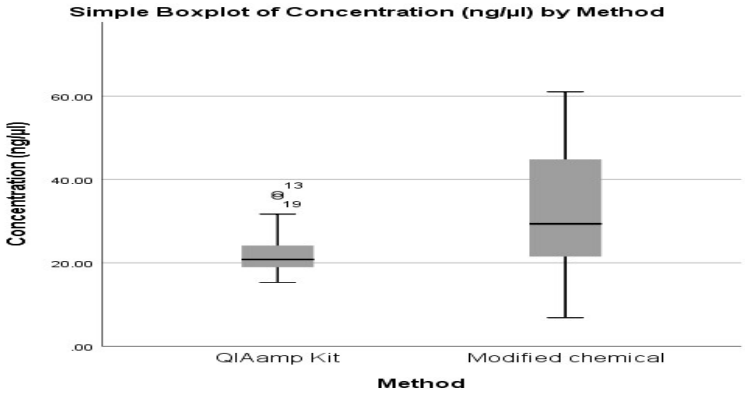

Fig.1. Histogram showing the mean concentrations of DNA obtained from samples extracted by modified chemical and QIA amp DNA blood Mini Kit methods. Values are means. of results from 19 volunteers.

The purity of DNA obtained was assessed by the ratio of absorbance at 260 nm and 280 nm. The mean values of the optical density 260/280 ratios were (1.8 ± 0.06 and 1.8 ± 0.04; n = 19 and n = 19) for genomic DNA purified from modified chemical and QIA amp DNA blood Mini Kit methods respectively with not statistically significant difference for samples (P = 0.42) as shown in fig.2.

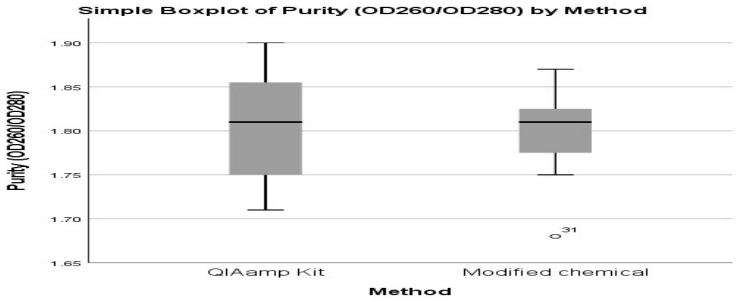

Fig.2. Histogram showing the mean purity of DNA obtained from samples extracted by modified chemical and QIA amp DNA blood Mini Kit methods, as indicated. Values are means of results from19 volunteers.

The results indicate that, the purity of genomic DNA extracted from the blood samples by modified chemical method is not significantly different than that from the QIA amp DNA blood Mini Kit methods. However, the DNA concentration from QIA amp DNA blood Mini Kit methods is lower when compared to the blood samples by modified chemical method as reported in table1:

Table1:- The DNA concentration and purity:-

DNA Sample	Concentration ng/µl Silica column (QIA amp DNA Blood Mini Kit) method	OD260/OD280	Concentration ng/µl modified chemical method	OD260/OD280
1	18.8	1.73	44.83	1.81
2	28	1.90	40.13	1.75
3	21.5	1.71	51.6	1.82
4	23.6	1.72	50.05	1.76
5	20.8	1.72	28.9	1.79
6	19.8	1.83	42.2	1.83
7	22.2	1.79	61.02	1.78
8	22	1.75	12.98	1.80
9	20	1.79	23	1.77
10	15.3	1.75	182.3	1.87
11	24.7	1.81	21.5	1.86
12	31.7	1.85	29.8	1.68
13	36.5	1.78	28.5	1.79
14	18.9	1.86	24.4	1.83
15	17.4	1.87	44.6	1.81
16	20.6	1.81	16.1	1.82
17	17.3	1.86	50.2	1.87
18	19	1.85	11.5	1.75
19	35.9	1.89	6.83	1.81
mean ±SD	(22.8± 6.1ng·µL^{-1})	(1.8 ±0.06)	(40.5 ± 37.6 ng·µL^{-1})	(1.8 ±0.04)

- **Gel Electrophoresis analysis of isolated DNA**

Integrity of the extracted DNA was assessed by agarose gel electrophoresis. Gel electrophoresis revealed that high-molecular-weight non-degraded genomic DNA was obtained with two methods and the results are shown in Fig. 3.

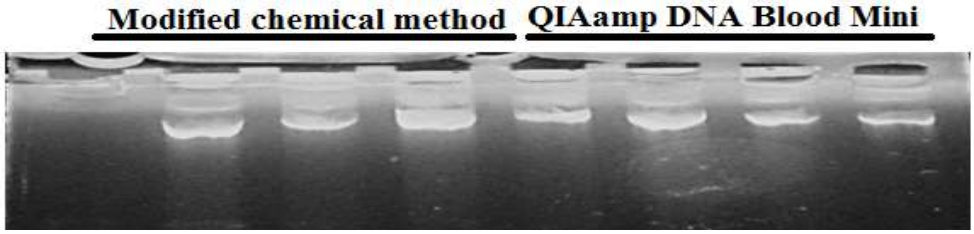

Fig.3. Photographs of agarose gels 1% showing the results of genomic DNA extraction obtained from samples from whole blood samples:. lanes 2, 3, 4: samples from modified chemical method; lanes 5, 6, 7, 8 : samples from QIA amp DNA Blood Mini Kit method

- **Amplification of the HFE gene by real-time polymerase chain reaction (PCR)**

All samples were successfully amplified by Real-Time PCR, using DNA samples extracted using both methods. Four samples representing PCR products are illustrated in Figure 4. All of them used HFE gene as a reference for Ct values. The resulting Ct values were averaged and used for statistical analysis as shown in (Table 2).

Table2:- The Ct values for genomic DNA purified from modified chemical and QIA amp DNA blood Mini Kit methods:-

DNA Sample	QIA amp DNA blood Mini Kit	Ct	modified chemical method	Ct
1		22.62		21.99
2		20.78		22.84
mean ± SD		(21.7 ± 1.3)		(22.42 ± 0.6)

The Ct values ranged from 20.78 to 22.84 and the Ct mean values are 22.42 ± 0.6 and 21.7 ± 1. 3for genomic DNA purified from modified chemical and QIA amp DNA blood Mini Kit methods respectively. There is no difference between mean Ct values between samples extracted using both techniques (p=0.34).

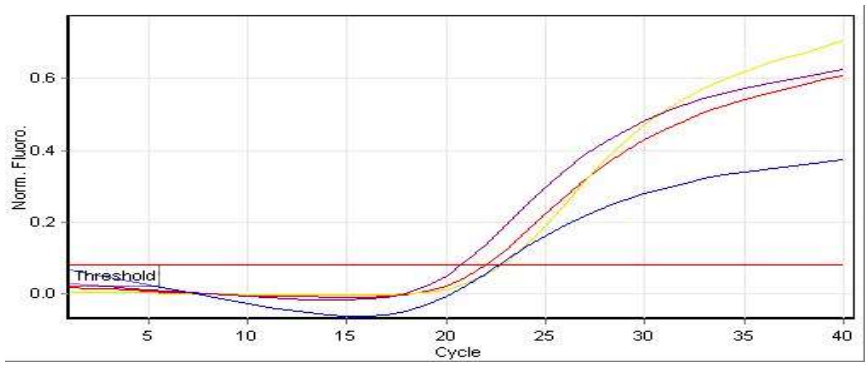

Fig.4. Amplification of HFE gene using real time PCR Amplification plot of the same DNA extracted by modified chemical (red, yellow lines) and QIA amp DNA blood Mini Kit (blue ,violet lines) methods.

Discussion

The ideal extraction method should fit the following criteria: it should be sensitive, quick, and easy to use, and depending on the country in which it is used it may be important to minimize specialized equipment or biochemical knowledge . It should also pose minimum risk to users, as well as avoid possible cross contamination of samples. Finally, and most importantly, the DNA extraction technique chosen should be able to deliver pure DNA samples ready to be used in downstream molecular applications [3, 9. 13]

In the present study, the DNA quality and quantity by the different protocols are in agreement with the Nanodrop Spectrophotometer measurements, gel electrophoresis and real time PCR . This study performed a comparison of the performance of two methods for DNA isolation from human blood. Table 1 summarizes the comparison of DNA concentration and DNA purity. In general, DNA obtained from modified chemical method was more than that obtained from Qiagen QIAamp Kit, with differences being statistically significant (40.55 ± 37.65 ng/µL for modified chemical; 22.84 ± 6.1 ng/µL for Qiagen QIAamp Kit; P= 0.04). The DNA purity (OD260/OD280) of DNA obtained from both methods produced the highest purity DNA, with differences being not statistically significant. Although DNA from all the samples was suitable for PCR, to evaluate the DNA isolation procedures for high throughput Real time PCR analysis, we isolated DNA from 19 blood samples using both methods . After DNA isolation, quantification of 38 samples showed a variable range of DNA concentrations (Table 1). Given these concentrations, we used 2µL of the DNA solution in 25µL real time PCR amplifications for analysis at H63D for HFE gene; we obtained good data for real time PCR amplification of four different extracted DNA samples as indicated toward the integrity of the samples and supported the observations made in the present investigation Fig. 4 . In economic feature, the price for one DNA extraction assay by modified chemical method (0.96 $ USD) is three times cheaper than using QIAamp® DNA Mini Kit (2.74$ USD). The modified chemical method may also spend lower cost as the proteinase K is not used. In summary, the results of this study demonstrate that the modified chemical method performed better than other commercial method evaluated for the extraction of DNA from human blood with simple method, low cost, high concentration and exceptional purity, the modified chemical method is a good DNA extraction method

can be used in any laboratory. It only required about 40 to 55 minutes completing isolation where speed of sample processing time is a paramount need . QIAamp columns performed good concentration and purity but also involved high cost for DNA isolation. On the other hand, the main purpose of collecting DNA is for future studies and our Real-Time PCR was successfully performed for the two methods.

Conclusion

In conclusion, our results showed that modified chemical method was the best choice to DNA extraction from whole blood samples in large scale because this method was found cheap enough with good yield over commercial kits especially in the poor laboratories. On the other hand, this method is providing a sufficient quantity and quality of DNA for real- time PCR analysis.

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