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ORIGINAL ARTICLE

Optimization of conditions to extract high quality DNA for PCR analysis from whole blood using SDS-proteinase K method

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Abstract In case of studies associated with human genetics, genomics, and pharmacogenetics the genomic DNA is extracted from the buccal cells, whole blood etc. Several methods are exploited by the researchers to extract DNA from the whole blood. One of these methods, which utilizes cell lysis and proteolytic properties of sodium dodecyl sulfate (SDS) and proteinase K respectively, might also be called SDS-PK method. It does not include any hazardous chemicals such as phenol or chloroform and is inexpensive. However, several researchers report the same method with different formulas and conditions. During our experiments with whole blood DNA extraction we experienced problems such as protein contamination, DNA purity and yield when followed some SDS-PK protocols reported elsewhere. A260/A280 and A260/A230 ratios along with PCR amplification give a clear idea about the procedure that was followed to extract the DNA. In an effort to increase the DNA purity from human whole blood, we pointed out some steps of the protocol that play a crucial role in determining the extraction of high quality DNA.

1. Introduction

Genomic DNA isolation is a key step in various clinical related studies including genetics, genomics, gene polymorphism, DNA fingerprinting and gene sequencing. These studies utilize a number of techniques that include, but are not limited to, agarose gel electrophoresis, restriction fragment length polymorphism (RFLP), real time/polymerase chain reaction (RT/PCR), Sanger-sequencing and microarrays. Whole blood is

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the primary source of genomic DNA in most of the clinical investigations associated with genetics, genomics, pharmacogenomics, genetic diseases and epidemiology (Gao et al., 2015; Lu et al., 2016; Rzehak et al., 2016; Visvikis et al., 1998). Buccal swab or saliva is also used to extract DNA for a number of PCR based studies (Küchler et al., 2012; Ng et al., 2004).

Whole blood yields ample amount of DNA, which is extracted by several methods (Chacon-Cortes et al., 2012; Ghaheri et al., 2016). A very common method includes phenol–chloroform extraction (Di Pietro et al., 2011). The same method may be applied to extract the DNA from tissues as well. However, due to the hazardous nature of phenol and chloroform it remains a major concern when it is used for DNA extraction. A different method utilizes sodium dodecyl sulfate (SDS) and proteinase K. It is often utilized for extraction of genomic DNA from different types of biological samples including whole blood (Goldenberger et al., 1995; Hassani and Khan, 2015; Murray et al., 2016).

Extraction procedure of DNA from whole blood may be divided into three main steps (i) Red blood cell (RBC) lysis and removal, (ii) White blood cell (WBC) lysis and protein removal, (iii) DNA extraction and washing. Based on previously reported formulas and protocol of SDS-PK method we extracted DNA from human whole blood. During the course of the whole procedure of DNA extraction it was noticed that the experimental procedures in all the above three steps, along with reagents and their concentration, play a crucial role in determining the yield's quantity, quality, and integrity. Several modifications were done to fine tune the extraction of a high quality DNA from human whole blood. The present manuscript highlights several crucial points which were identified in the SDS-PK method of DNA extraction from human whole blood. Identification of these points ensured the optimization of experimental protocol and conditions which directly affect the quality and quantity of DNA yield.

2. Materials and methods

2.1. Chemicals and reagents

Sodium dodecyl sulfate, ethylenediaminetetraacetic acid, disodium (disodium EDTA), tris–HCl and proteinase-K were purchased from Bio Basic Inc. Canada. SYBR green PCR master mix was purchased from Applied Biosystems, UK. Ammonium chloride and absolute ethanol were purchased from Avonchem, UK; ammonium acetate was purchased from Winlab UK; sodium bicarbonate from Fluka, Switzerland; sodium chloride from BDH, UK and RNAse A was purchased from Sigma–Aldrich, USA. All other chemicals were of highest purity grade.

2.2. Blood samples

All the procedures were done according to ethical guidelines. Fresh blood samples were obtained from King Khalid University Hospital. Scientific and ethical approval was granted for these experiments by the Institutional Review Board, College of Medicine and King Khalid University Hospital, with approval number 15/004/IRB (Research Project No. E-14-1193). Blood samples were collected from healthy individuals in heparinized vacutainers and stored at 2–8 °C. Collected samples were processed for DNA extraction within 72 h of collection.

2.3. DNA extraction method

Genomic DNA from the whole blood was extracted in the following three steps.

2.3.1. RBC lysis and removal

In a 15 ml centrifuge tube 2 ml blood was taken and 8 ml RBC lysis solution was added which contained NH_4Cl (ammonium chloride) (150 mM), $NaHCO_3$ (sodium bicarbonate) (10 mM) and disodium EDTA (0.1 mM). Tubes were placed in tube rotator for 5 min. All tubes were centrifuged for 10 min at 300× gravitational force. Supernatant was discarded and the white cell pellet was resuspended in 500 µl phosphate buffered saline (PBS) containing NaCl (sodium chloride) (137 mM), KCl (potassium chloride) (2.7 mM), Na_2HPO_4 (sodium phosphate dibasic) (10 mM) and KH_2PO_4 (potassium phosphate monobasic) (1.8 mM), and pH adjusted to 7.4. This RBC lysis and removal step was repeated for three times and at the end, a RBC-free clean white pellet was obtained and resuspended in 500 µl phosphate buffered saline (PBS).

2.3.1.1. Technical point. Initially RBC lysis was done in two steps instead of three, as mentioned above, and it was noticed that extracted DNA analysis in NanoDrop 8000 (Thermo Scientific, Wilmington, USA), A260/A280 ratio was found to be less than what it is expected (1.8).

2.3.2. WBC lysis

In WBC suspension 1.5 ml lysis buffer (pH 8.0) containing tris–HCl (20 mM), disodium EDTA (0.1 mM) and NaCl (25 mM) was added along with 500 µl sodium dodecyl sulfate (SDS, 10%) and 50 µl freshly made proteinase K (10 mg/ml PBS). This mixture was incubated at 50 °C in a water bath for two hours. This incubation is a minimum period to obtain a clear lysed cell solution, if the cell pellet is visible after two hours of incubation, the time may be extended until a clear solution is obtained.

2.3.2.1. Technical point. 10 µl of freshly made proteinase K (10 mg/ml PBS) along with incubation at 55 °C in water bath for two hours. and RBC lysis with two steps of washing could not yield highly pure DNA as observed by NanoDrop. However, increasing the concentration of proteinase K, incubation at 50 °C, and three washing steps in RBC lysis were found to have a positive effect on purity of the DNA.

2.3.3. DNA extraction and collection

After incubation the samples were removed from the water bath and cooled down at room temperature. 500 µl of 7.5 M ammonium acetate was added in each sample and gently vortexed until the solution was homogenous. In this mixture, which is approximately 3 ml, 7 ml of chilled absolute ethanol was added and sample tubes were inverted until a condensed DNA pellet was visible. This DNA pellet was collected with the help of a wide bore pipette tip attached to a100 µl micropipette.

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DNA pellet was transferred to a 1.5 ml Eppendorf tube and washed with 500 µl, 70% ethanol. Ethanol was carefully removed using a micropipette and DNA pellet was left in the tube and air dried at room temperature. After the pellet was dried, 100–200 µl tris–EDTA (TE) buffer (pH 8.0) was added and the tubes were incubated at 37 °C to allow the DNA pellet to form a clear, homogenous solution. All the samples were stored at −20 °C.

2.3.3.1. Technical point. Washing of the DNA pellet with 70% ethanol appeared to be crucial in ensuring purity of DNA in terms of A260/A230 ratio of absorbance. Bypassing this step exhibited severe effect on this ratio indicating non-removal of ethanol and/or ammonium acetate. This may be mainly because of ammonium acetate as it shows significant absorbance near 230 nm.

2.4. Determination of DNA concentration and purity

Concentration and purity of the DNA were measured in a NanoDrop 8000 (Thermo Scientific, USA). A260/A280 and A260/A230 ratios were monitored to assess any possible contamination by protein, organic solvents, salts, carbohydrate etc. The machine was set in single sample mode and TE buffer was used as sample blank.

2.5. Determination of DNA integrity by real-time polymerase chain reaction (RT-PCR)

Extracted DNA's integrity was determined by RT-PCR amplification of *CYP2C9* using forward (5′-GCCTGAACCCA TAGTGGTG-3′) and reverse (5′-GGGGCTGCTCAAAATC TTGATG-3′) primers. All the procedures were completed in Applied Biosystems (California, United States) Step One Plus Real Time PCR. Briefly, in a 25 µl reaction mixture, 12.5 µl SYBR Green Master Mix (2x), 1 µl template DNA (containing 100–200 ng DNA), 1 µl forward primer (5 pmol/µl), 1 µl reverse primer (5 pmol/µl), and 9.5 µl H_2O. Steps in thermal cycle included 60 °C 30 s, 1 cycle; 95 °C 5 min, 1 cycle; 95 °C 15 s – 60 °C 30 s – 72 °C 30 s – 40 cycles; 60 °C 30 s, 1 cycle post PCR read. ΔCt values were obtained for the determination of valid amplification.

2.6. Statistical analysis

Statistical analysis of the data was done using GraphPad Instat software (GraphPad Software Inc., La Jolla, CA). Means were obtained from the DNA concentration, A260/A280 and A260/A230 ratios. Means were presented as mean ± S.D. Significant differences were analyzed between data from different methods by ANOVA and Tukey–Kramer Multiple Comparison Test. $P < 0.05$ was considered significant.

3. Results

*3.1. **DNA quality**, with optimized method*

DNA concentration measured by NanoDrop, revealed a high yield of total genomic DNA from whole blood (Table 1). Total yield ranged 19–75 µg of DNA/ml blood. The average yield was 39 µg of DNA/ml blood from 23 different samples. A260/A280 ratio is an indicator for level of protein contamination and for pure DNA it is 1.8. The average A260/A280 ratio was 1.81 ± 0.05 (Table 1). A260/A230 ratio, an indicator of organic contamination was found to be 2.07 ± 0.07 (Table 1), for uncontaminated DNA it is reported to be 2–2.2. Thus results indicate the high purity of DNA extracted from the method reported in the present manuscript.

Table 2 includes the observations of NanoDrop data after DNA extraction without optimization of the method (10 µl of proteinase K (10 mg/ml PBS), incubation at 55 °C in a water bath for two hours and RBC lysis with two steps of washing only). A260/A280 ratio obtained was 1.68 ± 0.1 which was lower than the expected value of 1.8. Similarly

Table 1 Various samples of DNA extracted from whole blood. Their concentration, 260/280 and 260/230 ratios were obtained by NanoDrop 8000.

Sample number	DNA concentration ng/µl	260/280 ratio	260/230 ratio
1	401.2	1.86	2.16
2	386.0	1.86	2.14
3	683.8	1.81	2.12
4	514.3	1.86	2.18
5	479.2	1.85	2.12
6	346.6	1.71	2.13
7	531.3	1.67	2.17
8	830.7	1.87	1.91
9	323.3	1.76	2.13
10	413.9	1.81	2.07
11	612.6	1.71	2.09
12	699.4	1.78	2.10
13	459.4	1.85	2.02
14	655.9	1.77	1.95
15	290.6	1.85	2.05
16	516.6	1.85	2.06
17	253.4	1.86	1.91
18	374.3	1.87	2.07
19	1005.0	1.85	2.04
20	510.1	1.79	1.96
21	623.4	1.81	2.13
22	832.7	1.84	2.10
23	332.6	1.87	2.10
Mean ± S.D.	525 ± 193	1.81 ± 0.05	2.07 ± 0.07

Table 2 DNA extraction with 55 °C incubation for two hr. and low proteinase K concentration (10 µl of 10 mg/ml). Following table highlights the protein and organic contamination in terms of 260/280 and 260/230 ratios respectively.

Sample number	DNA concentration ng/µl	260/280 ratio	260/230 ratio
1	276.7	1.54	0.71
2	519.0	1.78	1.34
3	239.8	1.68	1.02
4	889.0	1.75	1.19
Mean ± S.D.	481 ± 298	1.68 ± 0.1	1.065 ± 0.27

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A260/A230 ratio was 1.065 ± 0.27 which is much lower than the expected value of 2–2.2. These data were found to be significantly lower (A260/A280, $P < 0.01$; A260/A230, $P < 0.001$), when compared with Table 1 data.

Table 3 highlights the significance of washing of DNA pellet with 70% ethanol to remove contamination of ammonium acetate. DNA quality measurement in NanoDrop, without washing in 70% ethanol, exhibits a significant reduction in A260/A230 ratio, which was found to be 1.37 ± 0.23. However, A260/A280 ratio remains as it was expected, (1.81 ± 0.06) (Table 3). A260/A230 ratio was found to be significantly lower ($P < 0.001$) when compared with optimized A260/A230 ratio (Table 1). Difference between A260/A280 ratios was not found to be significant.

Table 3 Various samples of DNA extracted from whole blood without pellet washing (70% ethanol). Their 260/230 ratios indicate contamination, probably by ammonium acetate and ethanol.

Sample number	DNA concentration ng/μl	260/280 ratio	260/230 ratio
1	347.3	1.71	0.84
2	691.1	1.8	1.53
3	546.6	1.84	1.52
4	679.4	1.83	1.32
5	876.6	1.81	1.37
6	372.8	1.92	1.51
7	628.8	1.81	1.64
8	587.9	1.75	1.26
9	361.5	1.85	1.42
Mean ± S.D.	565 ± 179	1.81 ± 0.06	1.37 ± 0.23

3.2. DNA integrity

DNA integrity was checked in terms of PCR amplification where products were monitored in real time. Drug metabolizing gene *CYP2C9* was amplified in 10 randomly selected DNA samples (A260/A280 ratio- 1.81 ± 0.05; A260/A230 ratio- 2.07 ± 0.07). All the samples showed amplification in real time (Fig. 1) and the ΔCt was recorded at 23 for all the samples except one for which it was 22.

4. Discussion

The two main advantages of the present method is that it utilizes non–hazardous chemicals and it is cost effective. It takes a total of 3.5 h. in complete procedure, which is comparable with costly DNA extraction kits available in the market. This method is optimized to extract genomic DNA from fresh

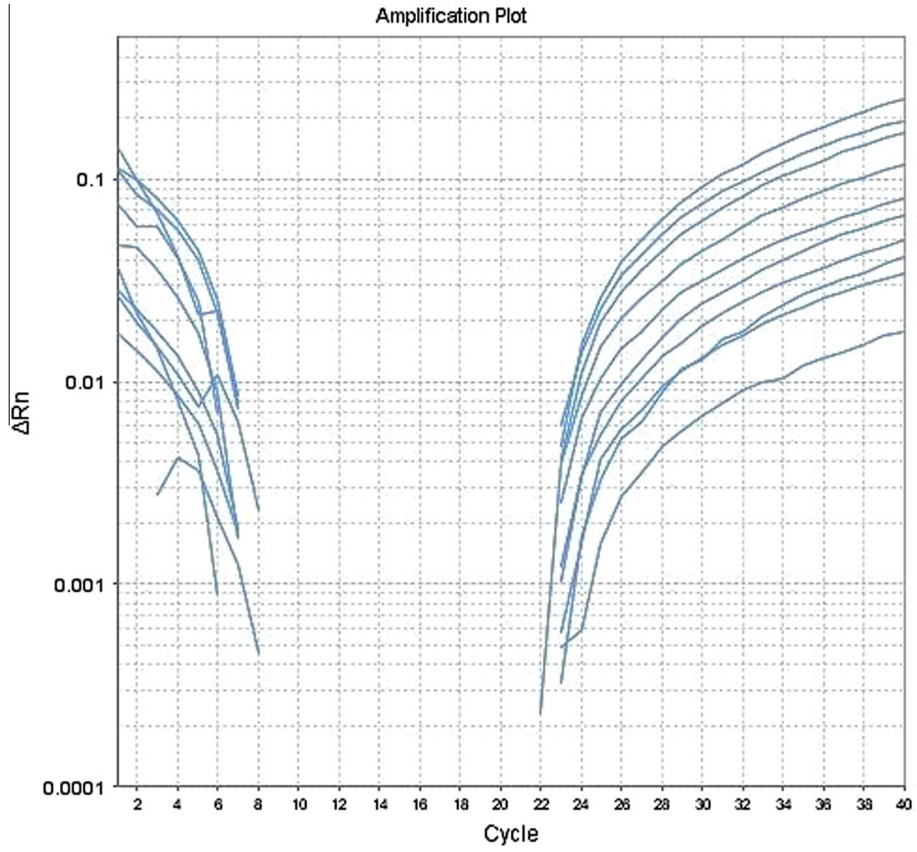

Figure 1 Amplification plot for the gene *CYP2C9* exhibits the integrity of DNA samples extracted from whole blood. 10 randomly selected DNA samples were amplified, which show ΔCt around 23.

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whole blood. While extracting DNA by this method, the following points should be considered.

- Proteinase K, a broad spectrum protease, plays a central role in the present method and is responsible for digestion of all the proteins in the cell lysate. This is why an appropriate concentration of this enzyme is necessary in the lysate. During the present experimentation different volumes of the enzyme stock (10 mg/ml PBS) were investigated for its effect on protein contamination in DNA samples. 10 mg/ml stock of proteinase K is commonly used in various protocols; however, different volumes of this stock were used. 10 µl of the freshly made enzyme stock removed the bulk of proteins, however it was found to be insufficient in achieving the desired A260/A280 ratio of the DNA sample. Few experiments were done using 20 µl of Proteinase K stock and mixed results were observed (data not given). However, 50 µl of the same stock was capable of removing all the proteins efficiently and desired A260/A280 ratio of around 1.8 could be achieved (Table 1). In the case of high throughput DNA sequencing based analysis similar method may be applied with an addition of 10 µl of RNAse A (5 mg/ml PBS) to remove any traces of RNA from the sample.
- Incubation temperature also appeared to play a significant role in the present investigation. 55 °C incubation temperature appears to negatively affect the proteinase K activities, which was apparent by protein contamination in DNA samples. At 50 °C proteinase K from the same stock efficiently digested all the proteins. Time of storage and repeated freeze thaw cycles of the proteinase K stock also affected the efficiency of the enzyme. These observations boil down to the following three factors that play a significant role in this method in association with proteinase K activity. (i) Proteinase K concentration, (ii) temperature and (iii) storage condition of the enzyme.

PCR amplification of the extracted DNA samples indicated toward the integrity of the samples and supported the observations made in the present investigation (Fig. 1). As it is described that the whole blood DNA extraction is done in three major steps, the optimization cannot be completed without inclusion of all of these steps.

- Careful removal of RBCs in the first step by three times washing with lysis solution is crucial in ensuring high purity of DNA.
- In a similar manner, the last step of washing DNA pellet with 70% ethanol removed all the contaminating salt from the DNA pellet. In the present method it is effective in removal of ammonium acetate contamination.
- Air drying of the pellet at room temperature is also important to remove the remaining ethanol from the sample.
- The pellets must not be over dried; it may cause some difficulties when dissolving the pellets in TE buffer.

5. Conclusion

Experimental conditions were optimized to extract high quality genomic DNA from fresh human whole blood, which can be used for different PCR based analysis. With the present method the goal of A260/A280 ratio of 1.8 could be achieved by the optimization along with significantly improved A260/A230 ratio. The present method is cost effective when compared with commercial DNA extraction kits, however, time consumption by this method, as compared with the kits, restricts the number of samples processed and it may be recommended for the labs working with less number of samples. The proposed method can also be used to extract DNA from fresh whole blood from rodents, rabbits, birds etc.

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