

Evaluation of a modified salt-out method for DNA extraction from whole blood lymphocytes: A simple and economical method for gene polymorphism

Mohammad Shokrzadeh, Abbas Mohammadpour*

Pharmaceutical Research Center, Department of Toxicology and Pharmacology, Faculty of Pharmacy, Mazandaran University of Medical Sciences, Sari, Iran

ARTICLE INFO

*Corresponding author:

Abbasbiology@yahoo.com

Article history:

Received: Mar 18, 2018

Accepted: May 27, 2018

Keywords:

DNA extraction,
Salt-out method,
Whole blood lymphocytes,
Economical method

ABSTRACT

Extraction of high-quality and-quantity DNA is a fundamental requirement for genetic research. It is very important to address the use of DNA extraction methods that are simple and cost-effective in gene polymorphism with large number of samples. This study was designed to investigate the optimal DNA extraction from lymphocytic cells by salt-out method. In this study, 200 blood samples of the two groups of patients and control were collected and transferred to Ethylenediaminetetraacetic acid-containing tubes. Afterwards, DNA was extracted from 1 ml of blood cells by modified salt-out method. Furthermore, three parameters in this research were evaluated, including quality (optimal density at 260 nm), quantity (DNA concentration) by electrophoresis, and efficiency of extracted DNA or polymerase chain reaction (PCR) status. The findings revealed that extracted DNA had excellent concentration and purity. The obtained results of electrophoresis confirmed the absence of any fragments in the extracted DNA. The PCR of the extracted DNA were successful, indicating lack of inhibitors in the reaction. According to the results of this study, this modified method can be used as a simple, efficient, and economical method for DNA extraction.

Citation: Pharm Biomed Res 2018;4(2): 28-32.

Introduction

The isolation of DNA, RNA, and protein is very important in genetic analysis and molecular diagnosis (1). Moreover, a DNA extraction with high concentration and purity is required to succeed in molecular biology techniques (2). In the past, the process of extraction nucleic acids was complex, time-consuming, and labor-intensive. However, there are now many specialized methods for extracting genomic DNA that differ widely in terms of the initial volume of blood, extraction time, safety, cost-effectiveness, yield, and purity of DNA (3). Manual methods for nucleic acid extraction have changed over time through commercial offers (e.g., DNA extraction kits that contain most of the compounds needed to isolate the nucleic acid). However, most of these methods require significant dilution of the sample, repeated centrifugation steps, and removal of supernatant levels, which makes it difficult to isolate DNA from a small amount of biological samples (4).

In the selection of suitable DNA isolation method, especially when there is a large number of samples, the speed and accuracy of the assay should be maximal, while the risk of contamination and cost of the assay should be minimal (5). Different compounds are usually used for successful DNA isolation. Detergents (to lyse the cell and denature the nucleoprotein complex), Proteinase K (to digest proteins), chelating agents (to bind to bivalent cation and inactive DNase), RNase A (to remove RNA contaminants), organic solvents, including phenol,

chloroform, and isoamyl alcohol (to purify DNA in most common procedures) are the examples (6, 7). Most of the current methods use toxic substances (phenol or guanidine isothiocyanate) and are not cost-effective because of the use of Proteinase K or RNase A. In the last few years, researchers used the salting method instead of toxic material and they used detergents and NaCl solution instead of incubation with enzyme for protein precipitation, which led to the correction of the existing methods (8).

The salting-out method is a simple and non-toxic DNA extraction technique, introduced by Miller et al., that isolates a high-quality DNA from the whole blood (9). In the standard salting-out method, proteins K and RNase are added to them after the lysis of cells. Saturated NaCl was needed for the proteins to precipitate out of the solution. The samples are centrifuged and the DNA is separated from the supernatant via washing it with ethanol detergent (10). In this approach, we modified the standard salting-out method to extract DNA from whole blood. Therefore, DNA isolation was performed using available materials, as well as a non-enzymatic and rapid method. It should be noted that this technique is fast and cost-effective for the use in laboratory settings.

Materials and Methods

Preparation of Reagents

Lysis buffer 1 consisted of 102.69 g (0.3 M) of sucrose Merck (Germany), 1.6 gr (10 mM) of Tris-HCl Merck

(Germany), and 1.02 gr (5 mM) of MgCl₂ Merck (Germany) diluted with distilled water in a total volume of 900 ml 10 ml of Triton X 100 Merck (Germany), added to micro tube and shaken for 30 min until a homogeneous solution was obtained. Final pH was adjusted to 8 and stored at -20 °C.

Lysis buffer 2 contains 0.88 gr of NaCl Merck (Germany), 1.75 g of ethylenediaminetetraacetic acid Sigma Aldrich (USA), 4.5 g of Tris-HCl Merck (Germany), and 10 ml SDS Merck (Germany) diluted with distilled water in a total volume of 100 ml. Final pH was adjusted to 8 and stored at 4 °C.

Lysis buffer 3 consisted of 5 M sodium perchlorate Sigma Aldrich (USA) and 35 g of sodium perchlorate (NaClO₄ H₂O) dissolved in 50 ml of distilled water and kept at room temperature.

DNA extraction by modified salting out method

A total of 200 blood samples were collected and stored at 4 °C. In this study, DNA was extracted using modified salting-out method. In this method, 1 ml of total blood was added to 1.5 ml micro tube and filled with lysis buffer 1. The mixture was incubated on ice for 15min. This led to the fragility of the red blood cells membrane. Afterwards, it was centrifuged at 6000 rpm for 4 min and two thirds of the supernatant containing the red blood cells was discarded because the red blood cells did not have a nucleus or a genetic material. The remainder of the solution containing white blood cells was suspended with a small amount of lysis buffer 1. Later, lysis buffer 1 was added and incubated for 10 min at room temperature. It was then centrifuged at 6000 rpm for 4 min. This step was repeated 5 times, until finally a white-pink pellet was observed.

Lysis buffer 1 destroyed red blood cells. The 500 µl of lysis buffer 2 was added to the pellet obtained from the previous step and gently taped into the micro tube until the pellet was completely dissolved (if necessary, this can be piped). The micro tube was then placed in a water bath at 50 °C for 50 min. Lysis buffer 2 was for lysing the white blood cell membrane and nucleus membrane. In the next step, 300 ml of sodium perchlorate was added to the micro tube and inverted until the proteins were denatured, separated, and accumulated in a brown mass. The net charge on the solubility of protein can be positive or negative. By adding sodium perchlorate, this salt covers all charges of proteins, and eventually the charge of proteins is zero. Therefore, the protein becomes insoluble and cloudy.

In the next step, 35 µl of cold chloroform was added to the micro tube under a fume hood and was inverted 10 times, until a milky-colored pellet was observed. It was then centrifuged at 12000 rpm for 10 min. At the end of this step, three phases were observed. The organic phase, which was at the bottom and contained chloroform, the middle phase contained insoluble proteins, and the water phase contained DNA. Then the

supernatant was discarded and transferred to the new micro tube. The 70 µl of cold ethanol (96%) was added to the micro tube and shaken a few times. At this stage, a DNA cluster was observed. Micro tube was centrifuged at 12000 rpm for 10 min to pellet down DNA. Finally, at the bottom of the micro tube, a small amount of pellet in the form of a white cluster was visible and the alcohol was placed on top of the tube. After centrifuge process, the supernatant was removed and 200 µl of ethanol (70%) was added to the remaining pellet. In this case if salt is present on the DNA cluster, dissolve in the water phase. This was then centrifuged at 12000 rpm for 10 min and again the supernatant was discarded. The micro tube containing DNA was placed at room temperature to dry completely. After drying, 80 µl sterile distilled water was added to dissolve the DNA. The extracted DNA was then placed at -20 °C.

Determination of the quality and quantity of isolated DNA

The concentration and purity of the extracted genomic DNA were evaluated by spectrophotometer and measured by the ratio of DNA optical density (A₂₆₀) and protein optical density (A₂₈₀). To determine the probability of DNA degradation, electrophoresis was performed on 1% agarose gel with a 15000 bp ladder.

Polymerase Chain Reaction

In order to control the extracted DNA and the absence of inhibitors in the polymerase chain reaction (PCR), the extracted DNA was used as a template for amplification of the CYP2D6 gene using a pair of primer (Table 1). Amplification reactions were performed with 50-100 ng extracted DNA in a volume of 25 µl in a reaction mixture, including 12.5 µl of Master Mix 2X, 1 µl of each primer (concentration: 10 pM), and 1U Taq polymerase DNA enzyme. The PCR amplification program started with one initial denaturing step at 94 °C for 5 min and continued with 35 cycles at 94 °C for 30s, 62 °C for 30s, and 72 °C for 20 s. The program was completed with a final step at 72 °C for 10 min. The PCR products were then analyzed by electrophoresis on 1.5% agarose gel (11).

Table 1 primers of CYP2D6 gene used in this study

primer	sequences	PCR product
Forward	- ACAGTCAACACAGCA GGTTAC-3'5	250 bp
Reverse	- GCAGTATGGTGTGT TCTGGAAG-3'5	

Results

The purity and concentration of the isolated genomic DNA were calculated by Nano Drop system to evaluate the quality and quantity of parameters. Table 2 shows the spectrophotometric data and the mean concentration for the genomic DNA isolated from 10 blood samples by modified salt-out method. The yield of DNA was within the range of 20-48 $\mu\text{g}/\text{ml}$ and the mean OD 260/280 ratio was 1.80, which are indicative of good deproteinization in this DNA isolation method.

The integrity of all genomic DNA extracted from blood samples was investigated by electrophoresis on 1% agarose gel. The presence of bands with high intensity and molecular weight indicated high integrity and non-degradation during the extraction process in the three extracted samples (Figure 1). As can be seen in Figure 2, the PCR amplification of CYP2D6 gene with all genomic DNA samples was performed successfully, which indicates lack of inhibitor for Taq polymerase enzyme in the final solution.

Table 2 Purity and quantity of DNA extracted from human blood samples by this research procedure

Sample code	($\mu\text{g}/\text{ml}$) DNA quantity	(OD _{260/280}) Purity
P1	39	1.89
P2	34	1.65
P3	42	1.91
P4	27	1.62
P5	33	1.66
P6	35	1.82
P7	45	1.93
P8	37	1.88
P9	30	1.79
P10	31	1.81

Discussion

The extracted genomic DNA from whole blood samples is used in molecular genetic studies, which is effective in diagnostic and personalized medicine (12). Rivero et al. used formalin-fixed and/or paraffin-embedded tissues to extract DNA using formalin (to precipitate protein) and isopropanol (to precipitate DNA). However, in the current study, the whole blood samples were used for the DNA extraction since the blood samples were usually the specimen of choice to extract the genomic DNA in

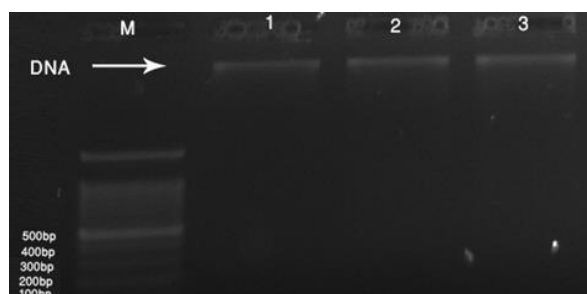


Figure 1 Agarose gel electrophoresis of genomic DNA isolated by this research procedure

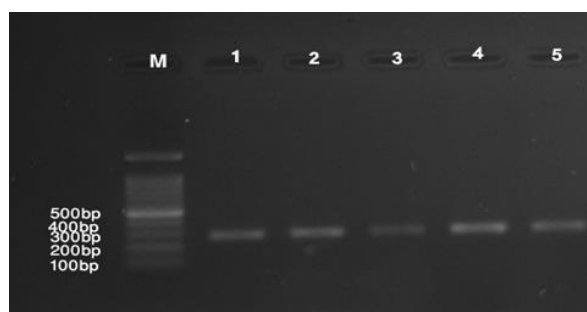


Figure 2 Agarose gel displaying the result of PCR reaction performed on DNA extracted by this research

molecular biology studies (13). The modified phenol-chloroform method, boiling method, and commercial DNA extraction kit have been common methods of DNA extraction in most recent studies (14). Furthermore, there are currently various methods to extract DNA from peripheral blood lymphocytes by phenol acceptable chloroform.

Compared to the previously mentioned methods, the salt-out extraction method is relatively rapid and simple to use. Additionally, there are a large number of studies which lent support to the efficiency of this method (13). In a study conducted by Radheshyam Maury et al., the DNA concentration obtained by salt-out method was $40.8 \pm 4.8 \mu\text{g}/\text{ml}$ and the DNA purity was 1.9 ± 0.07 , meaning that the concentration of DNA extracted by this method is enough to conduct further PCR reactions (3). In many salting extraction methods expensive enzymes, such as Proteinase K and RNase A, are used. Although these enzymes create a suitable DNA extraction method, it is not economical to use them (6).

In the current study, DNA extraction from whole blood cells was performed using the non-enzymatic (salting-out) method, and the obtained quantity of DNA was $40.8 \pm 4.8 \mu\text{g}/\text{ml}$. This amount was sufficient to carry out further PCR reactions for genetic researches, such as microsatellite and single nucleotide polymorphism analysis. Likewise, Suguna et al. extracted acceptable quality and yield of genomic DNA using non-enzymatic salting method. The obtained results showed that despite lack of proteinase and RNase, digestion of protein and

RNA were performed well (9). Therefore, efficient DNA isolation was possible even in small laboratories using this useful technique. Similar to the study conducted by Gaaib et al., the removal step of the prolonged digestion of samples with Proteinase K (overnight) minimized the extraction time, which was one of the important criteria for choosing the appropriate DNA extraction method (2). Electrophoresis of extracted DNA on agarose gel is an important criterion for calculating DNA concentration (15). As shown in Figure 1, the results of the gel electrophoresis obtained from this study showed that quality and yield of the extracted DNA was acceptable and there was no smear on the sample lane (no degradation of nucleic acids). In fact, the presence of Ethylenediaminetetraacetic acid in lysis buffer 2 (responsible for releasing the genomic DNA of the white blood cells by lysing the cell membrane and nucleus membrane) helps to maintain the integrity of DNA. The reason for this is that most enzymes involved in the degradation of nucleic acids need divalent ion cofactors (usually magnesium) to promote activity (16). A quick assessment of the purity of nucleic acid samples is very important in molecular biology. It is expected that the ratio of spectrophotometric absorbance of the pure extracted DNA from 260 nm to that of 280 nm ranged 1.8-2. Protein or phenolic contamination reduces A260/280 ratio and RNA contamination increases this ratio (17). According to the results of spectrophotometry, the purity of extracted DNA in this method was satisfactory (Table 2). The results of several studies to find the suitable method for DNA extraction from a variety of samples, such as tissues, peripheral blood, and bone marrow showed that the quantity and concentration of DNA could not be an accurate criterion for the comparison of extracted DNA by different methods.

This study focused on the modified DNA extraction method using simple and inexpensive laboratory equipment and it does not have any comparative concept with recent studies. The DNA efficiency or PCR status is the main criterion for comparing the extracted DNA. The results of the PCR amplification indicated the extracted DNA has been degraded or not degraded during the extraction process (18). The PCR multiplication of CYP2D6 gene with the isolated DNA was performed successfully through our proposed method, indicating there was no prominent inhibitory material for Taq polymerase in the final solution (lipid, protein, and high calcium concentrations are potential inhibitors of PCR) and extracted DNA was of good quality.

Conclusion

The proposed DNA extraction method described uses simple and available laboratory materials and equipment, and needs no expensive materials (K and RNase proteins), which results in a significant reduction of DNA extraction cost. Moreover, it decreases the time of

operation by eliminating the step of prolonged enzyme digestion. In short, our method is a simple, fast, and inexpensive method, which can be used for genetic analysis in medical laboratories and research centers with financial and time limitations. In addition, our results indicate that the DNA produced by this modified method with high quality could be used for PCR-based experiments, especially for gene polymorphism studies in a human population.

Acknowledgments

In this study the data were extracted from a thesis submitted by Mr. Abbas Mohammadpour in partial fulfillment of the requirement for the degree of PhD. This study was supported by grant from the Mazandaran University of Medical Sciences, Sari, Mazandaran, Iran (No. 2393).

Declaration of Interest

The authors report no declarations of interest.

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