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Integrity of Genomic DNA Isolated From Heparin Treated Human Leukocytes By A Modified Protocol

Karuvaje Thriveni[1], Anisha Raju[1]
1.DepartmentOf Biochemistry, Kidwai Memorial Institute Of Oncology, Dr.M.H.Marigowda Road, Bangalore, India. 560029.email: thrivenibhat@yahoo.com

Abstract: *This study is focused on a simple method of genomic DNA isolation from human blood collected on heparin and EDTA anticoagulant. It was known heparin may interfere with DNA testing in polymerase chain reaction amplification process. To overcome this, a modified protocol was followed. Here, TRizol and SDS were used to denature the membrane proteins and nucleoprotein complexes. Unlike standard/conventional procedure, excess proportion of isoamyl alcohol might had helped to reduce foaming and to obtain clear aqueous and organic layers. Sodium acetate wash steps helped to neutralize the DNA charge and precipitated it easily with added ethanol. The yield and integrity of genomic DNA was higher in heparin treated blood sample and tested by single nucleotide polymorphism assay and could be stored for a longer period at -20°C. The protocol was simple to perform, sensitive, economic and enabled us to obtain good yield, DNA integrity, purity and better PCR performance.*
Keywords*: Heparin, leukocytes, genomic DNA, SDS, TRizol*

I. Introduction

The important aspect which could potentially affect DNA isolation and performance of molecular based assays are sample collection, storage and handling of human whole blood specimens. In the diagnostic and research laboratory, PCR based methods are routinely used and require a large number of good quality genomic DNA with high purity. As described by scientists [1], the common procedure involved in DNA extraction are disruption of cells, denaturation of nucleoprotein complexes, inactivation of nuclease enzymes, removal of organic contaminants and finally precipitation of DNA. Several methods for isolation of genomic DNA from leukocytes are available including commercially available kit method. It is possible to store kit isolated genomic DNA for longer period because of lack of organic impurities and DNase. The standard procedure for genomic DNA isolation requires EDTA blood samples incubated with proteinase K for overnight[2,3]. Many studies implicated that blood sample collected on heparin anticoagulant is not ideal for PCR analysis[4], as it inhibits restriction enzymes and Taq polymerase due to binding with higher affinity of these enzymes to heparin than to DNA[5,6].

The crucial steps in diagnostic and molecular biology laboratory during sample collection were choice of preservatives, fractionation of components and storage[7]. Most of the assays require whole blood in an anticoagulated tube consisting of plasma, buffy coat and RBCs. While biochemical assays need heparin preserved or coagulated blood consisting of serum[8,9]. The anticoagulants should be carefully chosen, since various anticoagulants differ in their mechanism of action and affect certain laboratory applications. Heparin accelerates the inactivation of clotting factors like thrombin by binding to antithrombin III. Anticoagulant EDTA has metal chelating property with calcium and magnesium which play an important role for some blood-based assays but adversely affect others. In general, EDTA is an ideal anticoagulant for DNA based assays but is not suitable for cytogenetic analyses[4].

Carpi et al in 2011, reviewed DNA extraction methods used in a wide range of biological sources, including many methods used on whole blood samples[10]. The three evaluated features for nucleic acid isolation methods such as use of toxic compounds, cost per sample, and time required were summarized. Chacon-Cortes et al[11] evaluated cost-effectiveness and time efficiency of three available DNA extraction techniques from whole blood samples: a traditional salting out method, a modified salting out method, and a commercially available kit based on a solid-phase DNA extraction method QIAamp® DNA blood maxi kits and found modified salting out method is more simpler and cost effective.

Some studies even show that blood collected on heparin anticoagulant has a similar integrity and purity as any other anticoagulant, if genomic DNA is isolated by commercial kit method. It has been proposed that heparin occupies DNA binding sites for polymerases. This prevents the polymerase from binding to promoter DNA which is required in a wide range of molecular biological assays. Most of the diagnostic procedures require patient's DNA for PCR amplification, which is easily extracted with high yield from heparin treated white blood cells. According to Yokota M et al, heparin may be extracted along with the DNA, and interference level of 0.002U for 50 µL PCR reaction mixture will affect the analysis[12].

Prolonged incubation with proteinase K is a time consuming and expensive method, so an alternative method of deproteinization of DNA is developed from blood samples[13,14]. The modified protocol described here is fast, simple to perform, sensitive, economic and several gDNA are isolated simultaneously from heparin treated blood samples. The quality is tested by PCR amplification of lymphotoxin alpha (LTα) single nucleotide polymorphism assay of +252A>G (rs 909253), and quantity of gDNA is sufficient for several PCR amplifications and stable for longer periods.

II. Materials &Methods

This study was a part of breast cancer research work on cytokines. Ethical approval for the study was granted by the Institute and informed consent was obtained from each individuals. In general, heparin anticoagulant blood was collected for the routine biochemical analysis in the department of Biochemistry. But for the study purpose, 3mL blood samples were collected on each of heparin and EDTA tubes separately by trained professionals. Samples were centrifuged at 3000rpm for 10 minutes; cells and plasma were separated and stored at -20ºC.

The following chemicals were required for the study and obtained from Sigma Aldrich (USA) and Merck (USA).

Sodium EDTA (MW 372.24), NH_4Cl , $KHCO_3$, NaCl, TRizol (Sigma), Sodium dodecyl sulphate(SDS 10%), Tris Equilibrated saturated phenol 10 mM, TrisHCl pH 8.0, Chloroform, Isoamyl alcohol, 100% Ethanol, and Sodium Acetate.

1. Lysis Buffer: 200 µL of 0.5 M Sodium EDTA 8.29 g of NH_4Cl and 1.0 g of $KHCO_3$ was dissolved in 1000 mL of distilled water.
2. SE Buffer: 4.39 g of NaCl and 50 µL of sodium EDTA were dissolved in 1000 mL of distilled water.
3. Sodium Acetate 3M pH 7: 246g of sodium acetate was dissolved in distilled water. Final volume was made up to 1000 mL with distilled water.
4. TEbuffer pH (8.0)commercially available

The RBCs were lysed with 3mL lysis buffer. The tubes were centrifuged, forming a leukocyte pellet in the bottom. The supernatant, containing haemoglobin, and other soluble components from the lysed red cells were poured off leaving a relatively clean leukocyte pellet. The pellet was resuspended in the same lysis solution, and the tube was again centrifuged and decanted. The pellets were further washed with 3mL SE buffer. The leukocytes of heparin and EDTA extracted pellet was treated separately with 1 mL 1M NaCl, 150 µL TRizol and 20 µL of SDS and the mixture was incubated at 55 ºC for 1 to 2 hrs followed by vigorous pipetting. The mixture was added to a fresh 2 mL microcentrifuge tube (MCT) and equal volume of phenol: chloroform: isoamyl alcohol (1:1:1) was added and incubated at room temperature (RT) for 10 minutes. The tubes were then centrifuged at 13000 rpm for 10 minutes. The upper aqueous layer was carefully transferred to a fresh 2 mL MCT and if required step was repeated. In the next step, 400µL of chloroform: isoamyl alcohol (24:1) was added to aqueous layer and the vials were mixed by inverting and incubated at RT for 10 minutes and centrifuged at 13000 rpm for 10 minutes. The upper aqueous layer was carefully transferred to a fresh MCT for further use. The steps were repeated in order to remove phenol. To the aqueous layer, 200 µL of 3M sodium acetate and 1 mL of isopropanol was added[13]. Further DNA was precipitated by the addition of alcohol. The supernatant was discarded and pellet was suspended in 800 µL of 100% ethanol. Solution was kept in -20º C for 1 to 2 hrs[15]followed by centrifugation at 13000 rpm for 10 minutes. Supernatant was discarded and pellet was dissolved in 70% cold ethanol and centrifuged at 13000 rpm for 10 minutes. Again, supernatant was discarded and the pellet was dried under vacuum. Pellet was resuspended in 0.5X TE buffer.

III. Results and Discussion

The genomic DNA isolated from both heparin and EDTA anticoagulant tubes were comparable in their quality and quantity. The concentration was measured using Eppendorf biospectrometer at 260 nm and was in the range of 800 to 3,000 ng/µL with an absorbance value of 1.7 to 1.8. The integrity of genomic DNA was tested by PCR amplification of lymphotoxin alpha (LTα) gene polymorphism of +252A>G (rs 909253) on heparin and EDTA treated blood samples. The amplified products of 368 base pair were obtained with the use of forward (5'CTCCTGCACCTGCTGCCTGGATC) and reverse (5'GAAGAGACGTTCAGGTGGTGTCA) primers [16]. The PCR master mix consisted of 200 ng/µL of DNA, 1 µM primers (Sigma), 200 µM of each dNTP, Taq DNA polymerase 1X buffer with 1.5 mM $MgCl_2$ and 2.5 units Taq Polymerase. After heating at 95°C for 10 minutes, PCR reactions were performed for 31 cycles consisting of heat denaturation (96°C for 60 seconds), annealing (65°C for 60 seconds), and extension (72°C for 120 seconds). The PCR amplified products were analyzed on 2% agarose gel stained with ethidium bromide (Fig 1) and it showed better amplified products in heparin treated blood samples.

The genomic DNA bands obtained from heparin sample were intact and were less smeared even after 10-12 months of storage as compared to those obtained by the previous conventional standard method (Fig 2a&2b). Organic impurities and DNase might damage DNA during prolonged storage in the convention method. Generally, successful nucleic acid purification required some important steps: effective disruption of cells; denaturation of nucleoprotein complexes; inactivation of nucleases [17]. Although phenol, a flammable, corrosive could denature proteins rapidly, it did not completely inhibit DNase activity. This problem could be solved by using a mixture of phenol: chloroform: isoamyl alcohol [18].Guanidinium thiocyanates present in TRizol were a chaotropic agent used in protein degradation and prevented theactivity of RNase and DNase along with SDS. The basic pH was maintained at which RNA was not stable [19]. Also smaller amount of TRizol was not enough to maintain the integrity of the RNA. Denaturation of proteins by SDS and TRizol helped to retain the protein layer for further molecular analysis. Because of the use of isolated leukocytes heparin might not be interfered in PCR amplification. But several wash steps were also included to minimize heparin interference during genomic DNA isolation.

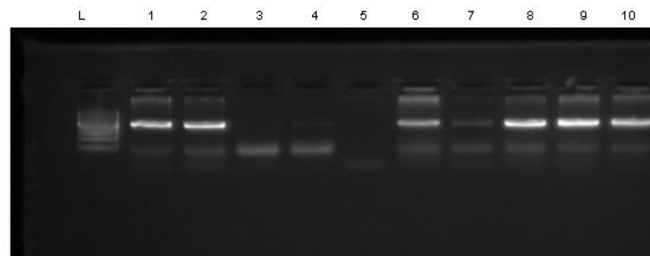

Fig 1: LTα gene PCR amplification. The 2% agarose gel picture shows PCR amplificatied product of 368 base pair LTα gene from genomic DNA samples isolated from EDTA and heparin anticoagulant blood samples by modified protocol. Wells 1-5 are for EDTA and wells 6-10 are for heparin samples.

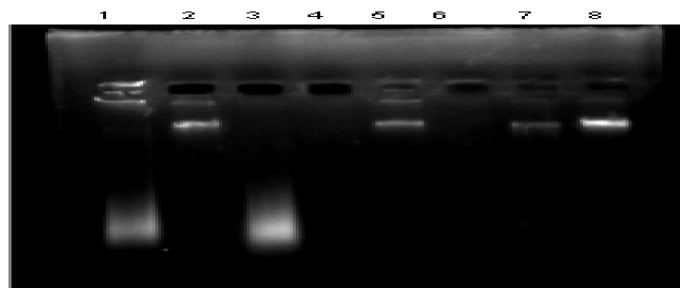

Figure 2a shows 1% agarose gel picture of genomic DNA isolated from heparin preserved leukocytes by conventional standard protocol stored for 10-12 months. The samples in well 1, 3, 4 and 6 have completely degraded. While those in well 2, 5, 7 and 8 are full of smears.

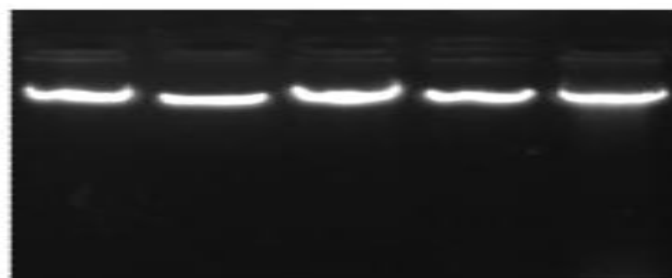

Figure 2b shows 1 % agarose gel picture of genomic DNA isolated from heparin treated leukocytes by the modified protocol after 10-12 months of storage.

IV. Conclusion

The yield of genomic DNA was higher in heparin preserved blood samples. The purity and integrity of gDNA was good and PCR amplicon were used for sequencing studies. The conventional phenol: chloroform: isoamyl alcohol was slightly modified to 1:1:1 instead of 25:24:1 proportion. This probably helped to reduce the phenol contamination caused during conventional method and storage of intact genomic DNA was possible for a longer period.

This modified protocol involved both salting out process and conventional phenol-chloroform procedures, where unlike other salting out procedure proteinase K was not used. Thus, this is simpler

inexpensive method, and heparinized blood samples can also be used for pure genomic DNA isolation and isolated DNA sample might be stored for a longer period at -20°C. A good source of biomarkers could be analyzed using blood samples collected on heparin tubes.

Conflicts of interest: The authors declare that there is no conflict of interest regarding the publication of this paper.
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