Optimization of Isolation Protocol for cell free DNA

Sajid Ali[1], Asif Mir[2], Syed Hussain[3], Muhammad shehr Yar Ali Khan Niazi[4], Hasan Shuja[5], Abubakar[6]

Abstract— The purpose of the study is to optimize cell free DNA extraction method and compare them with the existing protocols, some of the methods are costly and have limitations. This paper is going to overcome the limitations by simplifying the extraction methods. To optimize the protocol, we extracted cell free DNA with different methods and compare them with the existing protocols. The results of the study demonstrate that the quantity of cell free DNA by our method were high compared to existing protocols, the success of our method over existing protocol are, that the whole process can be performed in room temperature and also the isolation of cell free DNA from the healthy individual.

The study suggests that the optimization of the protocol overcome many crucial areas like, keeping cell free DNA in low temperature, high cost, time consuming methods.

Index Terms— cell free DNA, cell free fetal DNA, half-life of cfDNA, non-invasive prenatal testing, prenatal screening, tumor circulatory DNA

1 INTRODUCTION

Cell free DNA (cfDNA) is double stranded DNA circulating in the blood stream of human. [1]. Its fragments are present in mono-nucleosomes that are cell-unbounded DNA, these are attached to either binding protein or phospholipids on the outer cell surface. These small fragments of DNA represent the whole genome. [2]. Some cfDNA are not able to pack in the nucleosome and are digested by a suit of enzymes present in the cell. Only approximately 150 nucleotides (nt) DNA are packed in the nucleosome while remaining are digested. [3]. In healthy individuals, cfDNA is mainly due to the production of cellular components of blood, while in cancer condition it is due the production of cellular components of blood, as well as tumor DNA circulating in blood stream (ctDNA). [4]. The fragment length of cfDNA varies in healthy person, tumor patients and in gestational women, by using prenatal SNP genotyping cell free DNA in gestational women are slightly greater in size (166 bp) than in healthy person (143 bp). [5].

The isolation of cell free DNA is main barrier in non-invasive diagnosis, we investigated in our study different simple and cost-effective method to evaluate and validate cfDNA isolation. Our research aim is to optimize a simple, less cost effective and accurate method for isolation of cfDNA. We compared our methods and materials with commercial kits. To study cfDNA is the most difficult due to DNA contamination, low quantity, and small DNA fragmentation. Thus, it is essential to develop a precise protocol in order to evaluate the quantity of cfDNA. In our study of research, we have focused on collection, centrifugation, and storage and DNA isolation from biological source such as plasma.

2 Half-life of cfDNA

The level of cfDNA is determined by the amount of DNA is released into the blood stream and the DNA clearance process. In healthy individual the levels of cfDNA are low due to less amount cell death as compared to in cancer and in gestational case, and also cfDNA are rapidly cleared. The estimate half-life of healthy individual's cfDNA varies from minutes to few hours, the half-life varies from 4 minutes to 2 hours.

In gestational women average half-life of cffDNA 06 minutes to an hour. he half-life of ctDNA also varies according to the types and stages of the cancers. The half-life of neck and head cancer is about 23-53 minutes. While studies show that the half-life of colorectal cancer is approximately 114 minutes. Eradication of the cfDNA from the body occur in the liver, spleen and kidney, liver is the main source of nucleosome elimination, as the cfDNA are packed in the nucleosome and the liver remove nucleosome from the blood stream within 10 minutes. [6].

- *Sajid Ali BS degree program in biotechnology in International Islamic University, Pakistan, PH-+92 331 9139573* E-mail*: sajidbsbt1043@iiu.edu.pk*
- *Asif Mir Head of Department, Biological Sciences, International Islamic University, Pakistan, E-mail: asif.mir@iiu.edu.pk*

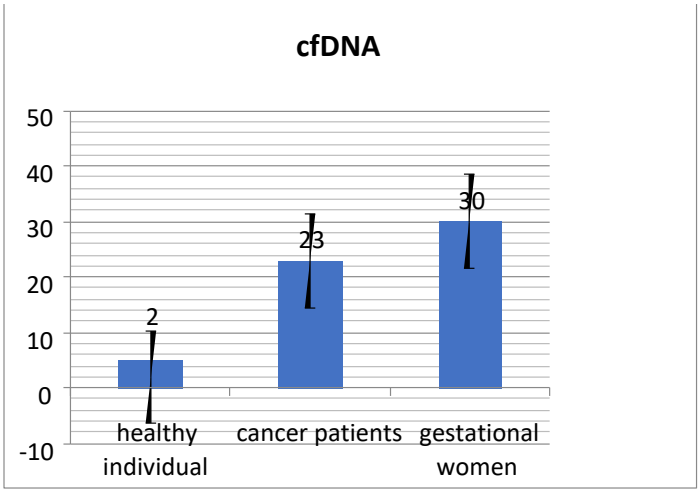

Fig.1 half-life of cfDNA, healthy individual, cancer patients and gestational.

3 Existing protocols

we compared our method with two commercially available kits and a proposed method, the quantity if cfDNA extracted by different methods are compared with the optimized method. [3].

Protocols	DNA concentration (ng/µl)	OD 260/280 (nm/nm)	OD 260/230 (nm/nm)
Suggested protocol	248.79 ± 14.07	1.62 ± 0.04	1.11 ± 0.14
AccuPrepTM Genomic DNA Extraction Kit	66.15 ± 15.42	1.69 ± 0.09	1.15 ± 0.13
13 QIAamp DNA Blood Mini Kit	46.26 ± 15.81	1.00 ± 0.07	0.6 ± 0.07
Optimized method	246.79 ± 14.01	1.67 ± 0.05	1.01 ± 0.17

Tab.1 comparison between suggested protocol, AccuPrepTM Genomic DNA Extraction kit, QIAamp DNA Blood mini kit and Optimized method.

4. Materials and methods

Ethical approval:

This study was conducted as the project of Bachelor Degree. The ethical clearance has been taken from the institutional bioethics committee of International Islamic University. The participants were briefed about the objective of study and written informed consent was taken from all participants prior to enrollment.

Sample population

Data of 40 donors, healthy persons, gestational women, and tumor patients were retrieved from local laboratories in Islamabad capital territory, including information related to age, predisposition to any other diseases and statistical analysis was done. The sample taken are categorized as following 40 samples were taken from healthy individuals. 5 samples were taken from tumor patients. 5 samples were taken from gestational women of different trimester.

5 DNA Extraction

The extraction of cfDNA was carried out by following the proposed protocol and some important changes was made which did not affect the quantity or quality of cfDNA but we focused to improve both the quality and quantity, and cost.

0.9 ml of blood was taken in 1.5 ml Eppendorf tube, centrifuged for 5 minutes at 10,000 rpm to extract 0.3 ml plasma, added 0.6 ml of the lysis solution and mixed for 5 to 10 minutes. Incubated in water bath for 20 minutes at 65 degrees. Centrifuged for 10 minutes at 10,000 rpm. Discarded the supernatant leaving behind the pallet. Added 0.6 ml digesting buffer and slightly mixed. Incubated again in water bath for 20 minutes at 65 degrees. Centrifuged for 10 minutes at 10,000 rpm. Discarded the supernatant, washed with 70 % ethanol 0.05ml and kept for 20 minutes for drying, Added 0.05ml T.E buffer.

6 BLOOD COLLECTION AND PROCESSING

The blood of different individuals was collected in various places of Islamabad and carried to human genome lab in different times ranging from 10 minutes to 8 hours and were processed accordingly. Out of 30 processed samples in total, 3 samples were tested positive and remaining 27 were negative. The failure and successfulness were happened due changing the number of different solutions and time required for centrifugation and incubation. The half-life of cfDNA were also checked and verified. Blood processing were also done in different pH.

7 Result

We collected 40 samples, 30 were of clinically healthy individuals, 5 samples were of gestational women, and 5 samples of cancer patients. Samples were delivered to the Human genome lab at different time in room temperature. Sample processing were also done in various time to confirm the half-life of cfDNA of different cases.

In case of healthy individual's sample processing, we added various amount of lysis buffer and digestion solution at different time. We concluded that the mean half-life of cfDNA in healthy individual were 7 minutes in which cfDNA bands in gel electrophoresis were obvious.

Blood amount	Blood processing time	Digesting solution	Lysis solution	Results
1ml	2 minutes	3ml	1ml	negative
900µl	2 minutes	1ml	1ml	Positive
900µl	6 minutes	600µl	600µl	Positive
900µl	10 minutes	600µl	600µl	negative

Tab.2 Blood processing in different quantities of Digesting Buffer and lysis solution.

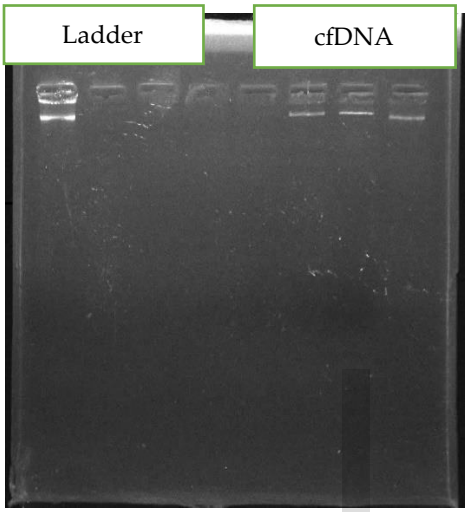

Fig.1 Gel electrophoresis bands of cfDNA.

Cell free fetal DNA was extracted from gestational women by amniocentesis, extracted cffDNA was confirmed by amplification of β-globin gene (GenBank accession: NG_000007.3), and was run on SDS PAGE.

Gene	Primer	Amplicon size(bps)
βglobin gene	Forward primer 5′-ACCTCACCCTGTGGAGCCAC Reverse primer 5′-CCCCTTCCTATGACATGAACT	376bp

tab. 2 primer of β-globin gene.

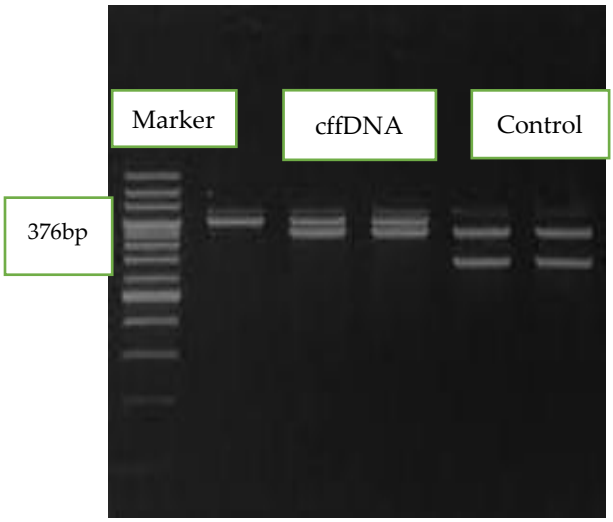

Fig.3 PAGE shows result of thalassemia major.

CONCLUSION

In our research study our aim was to optimize cost effective and precise method for isolation of cfDNA and compared our method with different commercially available methods. The solutions we used in our research were less in quantity as compared to the existing protocols. We isolated large quantity of cfDNA from healthy individuals. Some of most important sides we have proved that really does resist the isolation of cell free DNA were pH of the digesting solution, little quantity of cfDNA and half-life. The half-life of cfDNA of healthy individual were very short, we extracted cfDNA after 5 minutes from the collection of whole blood. We performed 2nd isolation, when cfDNA processing was done after 7 minutes of the collection of cfDNA and result was negative, so wan can now conclude that the half-life of cfDNA is not more than 6 minutes. The same case was with the pH of digesting buffer, in 7.5 and 8.5 the result was negative, so the best pH for digesting buffer was 8.

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Bibliography

[1] g. z. l. q. j. L. B. Y. a. T. W. y. xue, ""Sequencing Shorter cfDNA Fragments Decreases the False Negative Rate of

Non-invasive Prenatal Testing," *Forntiers in Genetics,* p. 07, 2020.

[2] .. L. W. H. a. D. W. B. E. S. Taglauer, ""cell-free fetal DNA in the maternal circulation as an indication of placental health and disease," *Placenta,* vol. 01, no. 2014, p. 06, 2014.

[3] C. L. C. a. L. L. E. Chen, "Cell-free DNA concentration and fragment size as a biomarker for prostate cancer," *Sci Rep,* vol. 01, no. 2021, p. 05, 2021.

[4] J. Y. a. Y. Q. Y. Hou, ""Factors affecting cell-free DNA fetal fraction: statistical analysis of 13,661 maternal plasmas for non-invasive prenatal screening," *Hum Genomics,* vol. 01, no. 2021, p. 04, 2021.

[5] E. Obeng, "Apoptosis (programmed cell death) and its signals - A review," *British Journal ofGeneralPractice,* vol. 1, no. 2017, p. 03, 2017.

[6] I. Rafi, "use of cell-free fetal DNA in Down syndrome screening," *British Journal ofGeneralPractic,* vol. 1, no. 2017, p. 04, 2017.