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Use of FTA in nucleic acid research: An optimization study for G6PD gene with FTA

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Abstract : In the present study the standardization and then modification has been done in the protocol of Whatman FTA cards for G6PD gene which plays an important role in RBCs metabolism. The present modification showed better result in PCR program for G6PD gene. The results suggest that the optimization was best observed with 2 discs instead of 1 disc as per standard protocol given in the literature. It is noticed that best results were observed with 10 pmol of forward and reverse primers and 12 µl of master mix instead of 15 µl for total 20 µl reaction mixtures. The annealing temperature should be 61°C which is more sensitive instead of 60°C as reported in the protocol. It is also suggested that the present alteration in the protocol is best suited for Indian conditions as the study showing clean and sharp peaks in the electropherogram of 5 G6PD samples.

Key Words : G6PD, PCR, Whatman FTA Classic cards

Introduction

G-6-PD (Glucose-6-phosphate dehydrogenase) deficiency is an inherited condition in which the body does not have enough G-6-PD enzymes to help RBCs function normally because it has been identified as the only NADPH (Nicotinamide Adenine Dinucleotide Hydrogen Phosphate) producing enzyme in RBCs that is activated during oxidative stresses (Filosa *et al.,* 2006). G-6-PD deficiency is relatively common in the population exposed to malaria and the incidence of G-6-PD deficiency seems to be relatively high in places where *falciparum* malaria has been a life-threatening factor for centuries (Motulsky and Allison, 1960). Researchers have found evidences that the parasite that causes this disease does not survive well in G-6-PD deficient cells. This is a selective advantage against malaria among the population where malaria was once endemic. An incidence of 25-28 % G-6-PD deficiency among tribes of Bastar district (High risk malarial region) has been reported (Tomar *et al*, 1983).

This clinical characterization of the enzyme makes it significant for population genetic studies. Genetically, it is an X-linked disorder located on the telomeric region of the long arm of the X-chromosome (band Xq28). The G-6-PD gene is 18.5 kb in size and consists of 13 exons, which are the regions of the DNA that code for the enzyme and 12 introns, which are intervening sequences (Scriver *et al.,* 1995). A mutant G-6-PD enzyme may differ person to person as it can be in the form of point mutations or can range from one to several base pair deletions or substitution in the DNA.

The present paper is thus a part of large study plan of finding such type of mutation and variants in the tribes of M.P. And therefore it deals with the very basic and important process of standardization of the working protocol of FTA (Fast Technology of Analysis) that best suited to Indian laboratory conditions. It includes the preparation of DNA template and optimization of its PCR (Polymerase Chain Reaction) program to screen G6PD gene mutation, by sequencing of the amplicons. For the purpose, the use of Whatman FTA classic card is remarkably an advanced and convenient way to collect and isolate the nucleic acid samples for analysis. In the Present paper we have optimized the FTA protocol for G-6-PD gene and its PCR program for DNA amplification and we have confirmed the optimized conditions via sequencing.

FTA — Fast Technology of Analysis

Leigh Burgoyne was an inventor of FTA technology. He wanted to collect nucleic acid samples (especially from blood), safely transport and store these prior to analysis by PCR or RFLP (Restriction Fragment Length Polymorphism). It was essentially required a convenient medium that would preserve the nucleic acid material and could facilitate fast analysis of multiple samples for a population based study. The idea for the trade mark 'FTA' was chosen to mean Fast Technology of Analysis of nucleic acids. USA patented Whatman FTA cards are impregnated with a chemical reaction that lyses cell membranes and denatures proteins upon contact. (Fig. I)

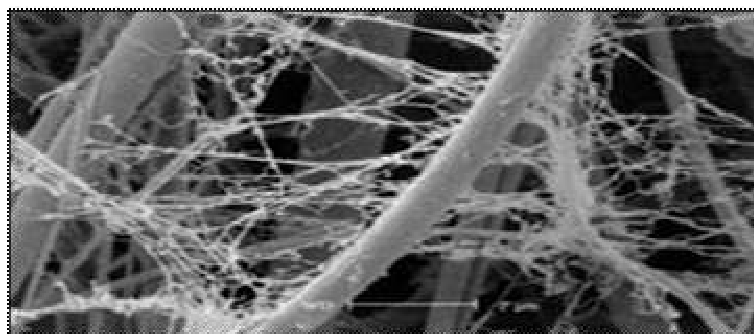

Fig. 1. Electron micrograph of entrapped DNA into FTA matrix

Materials and Methods

Only after consent of the subjects, random sampling has been done in malarial prone areas of M.P. About 1 ml blood is temporarily collected in EDTA (Ethylene Di-amine Tetra Acetic Acid) vaccutainers, kept in thermocol icebox and carried to laboratory to store it at 4°C till further processing on to FTA Classic cards.

A) DNA preparation using FTA cards

The preparation of DNA was performed using the Whatman FTA classic cards. The methodology is based on step wise optimization with the efficient and cost effective alteration in the protocol that best suited to Indian laboratory conditions. The use of Whatman FTA classic cards is as follows:

1. Label the Whatman FTA classic cards properly and pour the blood sample about 200-250µl on the circle.
2. Allow the sample to dry for 1 hr at room temperature and store it at the same.
3. Remove (punch out) a small disc (1.2 mm) from a dried sample spot and place it into a PCR tube.
4. 200µl of washing reagent was added to PCR tube and incubated for 15-20 minutes at room temperature, discard all spent FTA reagent by pipetting.
5. The process was repeated for two washes with FTA purification reagent.
6. The same process was repeated twice with 100 µl TE (Tris EDTA) buffer and the entire spent TE buffer was removed and discarded by pipetting.
7. The disc was allowed to dry at room temp for overnight and then it is ready for further PCR amplification.

B) Genetic analysis

i) PCR amplification (2720 AB Thermal Cycler)

The optimization of PCR condition includes the preparation of DNA template (number of FTA discs), primer concentration, volume of PCR master mix and cycling conditions to get better results in domestic conditions. PCR program commonly used for G6PD is 94°C for 2 min, 94°C for 30 sec, 60°C for 30 sec, 72°C for 60 sec, for 45 cycles72°C for 4 min and finally 4°C for infinity (Beutler and Yoshida, 1993) and 94°C for 2 min, 94°C for 30 sec, 60°C for 60 sec, 72°C for 30 sec, for 34 cycles 72°C for 4 min and finally 4°C for infinity (Guindo *et al*, 2007) but the condition for PCR were modified with the use of FTA cards and optimized as: 94°C for 2 min, 94°C for 30 sec, 61°C for 30 sec, 72°C for 1 min, for 40 cycles 72°C for 4 min and finally 4°C for infinity, after working on different annealing temperatures (54.9°C. 55.1°C, 55.8°C, 56.8°C, 58.1°C, 59.5°C, 61.0°C, 63.7°C, 64.8°C, 65.6°C, 66°C).

The PCR condition was standardized with different number of FTA discs (represents the amount of DNA) starting from 2 to 4. The details are shown in Table I. The primer sequences chosen were of exon 3 and taken from NCBI (National Centre for Biological Information) (G6PD forward: ATACTTCTGTGGAGTGGCAGTGTT and Reverse: CTTGTCCCACTCCCCA AGTC). Optimization of primer concentration was done with 15pmol and 10pmol. The master mix was used in the volume of 10µl and 15 µl to make up the total reaction volume of 15 µl and 20 µl respectively. By observing the better results with 10 pmol of primer concentration, the condition were further modified in order to minimise the use of PCR master mix to make it more cost effective for population genetic studies. And it was done by taking 12 µl of master mix and 6 µl of water (Table II). Following the PCR, the products of 260bp were electrophoresed at 100V in 1% agarose gel. The PCR products were then visualized under UV light in transilluminator. On successful obtaining of a single band devoid of any primer-dimer, the PCR products were then sequenced.

Table I: Details of number of FTA discs, primers, master mix conditions

No. of discs	**2**	**2**	**3**	**3**	**4**	**4**
Primers (10 pmol)	2	2	2	2	2	2
Master mix	10	15	10	15	10	15
Water	3	3	3	3	3	3
Total	**15**	**20**	**15**	**20**	**15**	**20**

Table II : Modification in PCR master mix

No. of discs	**2**	**2**
Primers (10 pmol)	2	2
Master mix	12	15
Water	6	3
Total	**20**	**20**

ii) Sequencing of the PCR product

The sequencing of the PCR products was outsourced from Sequencher Tech Pvt. Ltd., Ahmedabad (Gujrat), India.

C) Data analysis

Following the practical work the obtained sequences were analyzed using appropriate software tools such as ChromasPro, Codon Code Aligner, clustal-X and online available BLAST programs.

Results and Discussion

The results of the standardization of the annealing temperature are shown in (Fig. II). The results shows that optimization of working condition for G6PD gene was best observed with 2 discs of Whatman FTA classic cards and best results were obtained with 10 pmol of both forward and reverse primers (Fig. III). 15 pmol primer concentrations also show bands but with primer dimer in all the discs (Fig. IV). In case of PCR mater mix, the best results were obtained in 20 µl reaction mixture volume with 2 discs (Fig. III). Although DNA bands were also present with 15 µl volume with 3 and 4 discs, but the band intensity was very light that cannot be suggested for further sequencing.

Sequencing results of 5 samples with forward primer were obtained which was essential, in order to confirm the optimized conditions. As shown in (Fig. V), the peaks are clean and sharp without any noise indicate, proportionate primer concentration and it confirms the accurate use of 10 pmol of primer (Fig. VI). The sequence of 240bp was obtained, when the expected gene size was 252bp. This supports the fact that the primer concentration and PCR cyclic condition is accurately optimized and it can varies only as per gene

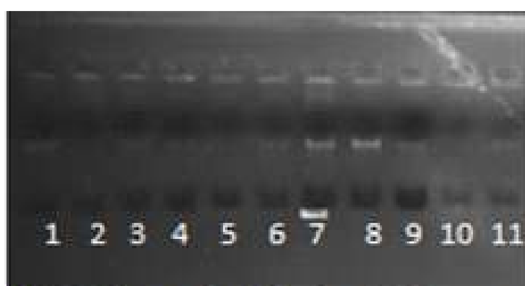

Fig. II. Gel picture of optimization of different annealing temperatures (54.9°C, 55.1°C, 55.8°C, 56.8°C, 58.1°C, 59.5°C, 61.0°C, 63.7°C, 64.8°C, 65.6°C, 66°C). Lane 7 with annealing temperature 61.0°C shows sharp DNA band.

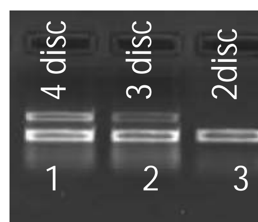

Fig. III. Gel picture showing DNA bands with 10 pmol of primer in different number of FTA disc.

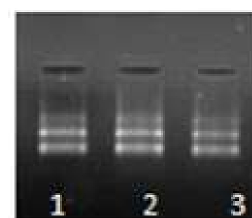

Fig. IV. Gel picture showing DNA bands with 10 pmol of primer in different number of FTA disc.

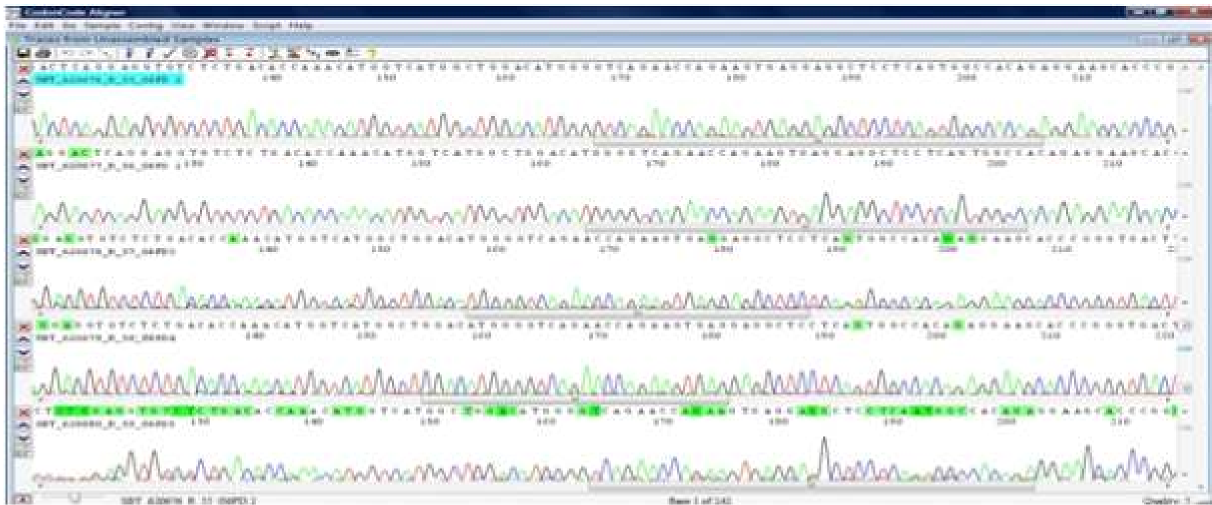

Fig. V. Electropherogram of 5 G6PD samples showing clean and sharp peaks

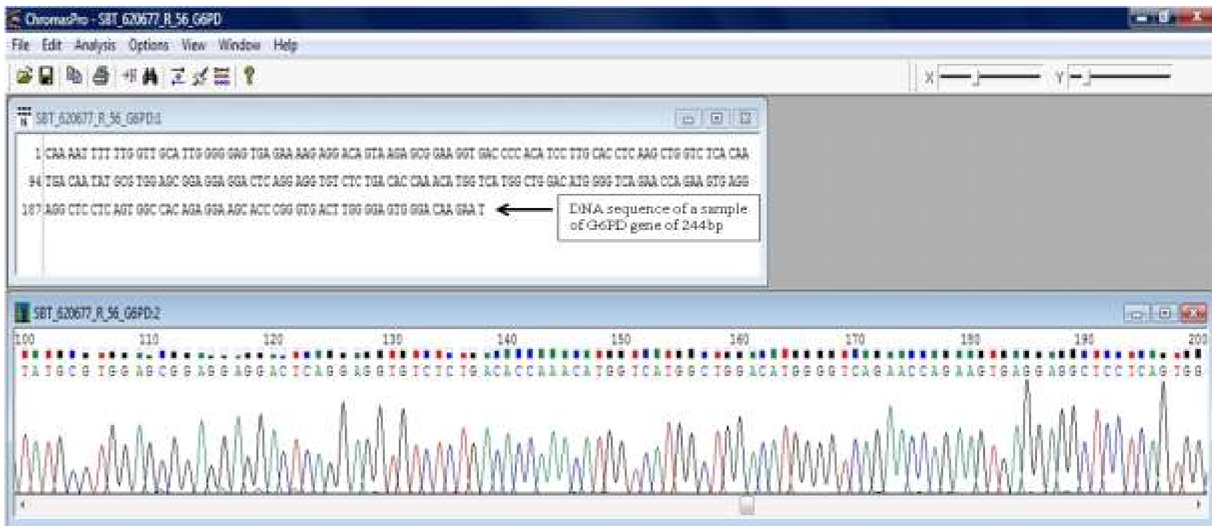

Fig. VI. Electropherogram of 5 G6PD samples showing DNA sequence of more than 240bp

and the primers that researchers are using.

Conclusion

This work concludes that in the field of molecular biology, among different methods of DNA isolation, the use of Whatman FTA classic cards is a very efficient and convenient technique of DNA collection, isolation and even preservation. The present paper has thus optimized the FTA protocol for DNA isolation and amplification for G-6-PD gene in order to make the population genetic studies more efficient and practically more convenient.

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