International Journal of
Life Sciences
International Peer Reviewed Open Access Refereed Journal

Special Issue A 11: January 2018
UGC Approved Journal No 48951

Original Article | Open Access

Application of FTA card and novel primers for amplification and sequencing of human mitochondrial cytochrome *b* (CYTB) gene

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Manuscript details:

Available online on
http://www.ijlsci.in

ISSN: 2320-964X (Online)
ISSN: 2320-7817 (Print)

Editor: Dr. Arvind Chavhan

Cite this article as:
Chaudhary Ruchira, Sairkar Manisha and Sairkar Pramod (2018) Application of FTA card and novel primers for amplification and sequencing of human mitochondrial cytochrome *b* (CYTB) gene, *Int. J. of. Life Sciences*, Special Issue, A11: 31-40.

ABSTRACT

MTCYB gene produces a protein which is a component of respiratory chain complex III. This gene is also important in phylogenetic relationships between and among species. Presently many hereditary carcinomas are also associated with the alterations of this gene. The aim of the current study is to develop primers for amplification and sequencing of complete human mitochondrial cytochrome b (CYTB) gene and use of the FTA card for isolation and preservation of mitochondrial DNA. DNA from 6 blood samples were isolated by the phenol chloroform method as well as by the FTA card method which was further used for the PCR amplification and sequencing process. Novel human mitochondrial cytochrome b (CYTB) gene specific primers (two sets) were designed and examined for amplification and sequencing. DNA trapped on FTA cards and DNA isolated by the phenol chloroform method produced a brighter clear band of approximately 1320 bp with both designed primers. During sequencing reverse and forward primers of both primer sets were produced good quality DNA sequence and prepared consensus sequences covers complete cytochrome b (CYTB) gene. Whatman FTA Classic card was successfully used for storage and amplification of Human mitochondrial DNA as well as, two sets of Human mitochondrial cytochrome b (cyt b) gene specific novel primers namely MVM-MT-Cyb-01 and MVM-MT-Cyb-02.

Keywords: FTA classic card, cytochrome b gene, PCR amplification, sequencing, human mitochondria.

INTRODUCTION

Mitochondria are found in all eukaryotes, their number and location vary from single to several thousand according to cell types. Mitochondrial

(mt) genomes consist of a circular DNA molecule (16,569 base pairs) consisting of 37 genes, including 13 protein-coding genes, 2 ribosomal (r)RNA encoding genes, 22 transfer (t)RNA encodinggenes, and one major noncoding control region (Boore 1999, Chan 2006). Each mitochondrion contains 2 to 10 copies of the mtDNA. Ten times higher mutation rate was observed in *mt*DNA compared to nuclear DNA (Howell *et al,* 2003). In forensic applications, using *mt*DNA enables identification of victims, (Pereira *et al,* 2010, Pakendorf and Stoneking 2005) during mass disasters like war or terrorism (Budowlea *et al,* 2005).

*mt*DNA alterations, either deletions-insertions or base substitutions causes different cancers in human (Brandon *et al,* 2006), including breast cancer (Plak *et al,* 2009), ovarian cancer (Aikhionbare *et al,* 2007), colorectal cancer (Aikhionbare *et al,* 2004), gastric cancer (Bi *et al,* 2011, Sui *et al,* 2006), hepatic cancer (Yin *et al,* 2010), lungs cancer (Hosgood *et al,* 2010) prostate cancer (Ray *et al,* 2009), thyroid cancer (Gasparre *et al,* 2010) and head and neck cancer (Allegra *et al,* 2006).

MTCYB is encoded by the guanine-rich heavy (H) strand of the *mt*DNA and located between nucleotide pairs (nps) 14747 and 15887 (Anderson *et al,* 1981; Wallace 1994). The *MTCYB* gene encompasses 1140 nps of *mt*DNA and encodes a single polypeptide without introns. In the mitochondrion of eukaryotes, cytochrome *b* produces a protein which is a component of respiratory chain complex III (EC 1.10.2.2) also known as Ubiquinol-cytochrome c reductase. These are involved in electron transport, pumping of protons to create a Proton Motive Force (PMF). The proton gradient is finally used for the generation of ATP. Cytochrome *b* is an integral membrane protein of approximately 400 amino acid residues that has 8 transmembrane segments. Four conserved histidine residues are postulated to be the ligands of the iron atoms of these two heme groups (Howell 1989; Esposti *et al,* 1993).

Comparison and identification of species in the same genus or the same family could be well studied by an aide of sequence variability of cytochrome *b* (Tsai *et al,* 2007). Phylogenetic studies of cytochrome *b* gene propose new classification schemes that better reflect the phylogenetic relationships among the species (Schall and Denis 2010, Castresana 2001). Alteration in the *MTCYB* gene causes many diseases like hypertrophic cardiomyopathy (Valnot *et al,* 1999; Andreu 2000; Hagen *et al,* 2013) mitochondrial myopathies (Andreu *et al,* 1999) various tumors (Fliss *et al,* 2000; Liu *et al,* 2001), bladder cancer (Dasgupta *et al,* 2008) heteroplasmic (Legros *et al,* 2001) chronic lactic acidosis (Mancuso *et al,* 2003) etc.

FTA technology is a novel method designed to simplify the collection, shipment, archiving and purification of nucleic acid from a wide variety of biological sources (Yoshida *et al,* 1995). The FTA™ purification method is very mild and similar to detergent based protocols (Montgomery and Sise, 1990) compared to traditional phenol based purification methods (Maniatis et al, 1989, Mullen *et al,* 2009).

In this study, genomic DNA isolated through FTA card and phenol chloroform method was used for the amplification of human mitochondrial cytochrome *b* gene. Two primer sets were designed for amplification and sequencing of complete human mitochondrial cytochrome *b* (cyt *b*) gene.

MATERIALS AND METHODS

Collection and storage of samples

A total of 6 samples were collected from Cancer Hospital and Research Centre Gwalior, Madhya Pradesh, India in the form of fresh blood. Fresh blood was collected in EDTA tubes and stored at 4°C. FTA classic card (Whatman Inc., 2009) was used for the storage of samples at room temperature. About 100 µl of blood sample was applied to labelled FTA card and allowed to dry at room temperature. These cards were directly used for PCR amplification of genomic DNA.

Isolation of genomic DNA from Whole Blood using the phenol chloroform method

About 0.5 mL of blood sample was taken and 1.5 mL of erythrocyte lysis buffer I (10 mM Tris HCl (pH 8.0), 320 mM Sucrose, 5 mM $MgCl_2$, 1% Triton X) was added. This mixture was centrifuged at 3600 rpm for 10 minutes and the supernatant was discarded. Precipitate was dissolved in 0.8 mL of lysis buffer II (400 mM Tris HCl, 60 mM EDTA, 150 mM NaCl, 1%SDS added after autoclaving) and 0.2 mL of 5M sodium perchlorate by vigorous shaking. When precipitate dissolved completely, an equal volume of Phenol:Chloroform: Isomylalcohol (25:24:1) was added, mixed gently and centrifuged at 3000 rpm for 10 minutes. The aqueous

layer so formed was collected in a fresh centrifuge tube, mixed thoroughly with equal volume of chloroform and centrifuged at 3000rpm at 10 minutes. The aqueous layer was collected again in a fresh centrifuge tube and ice cold ethanol was added to precipitate DNA. The precipitated DNA was washed twice with 70% ethanol, air dried and dissolved in 0.1 mL TE buffer (10 mM Tris HCl (pH 7.5), 1m M EDTA, pH 8.0). Quantity and quality of isolated DNA were measured using spectrophotometer (Sambrook and Russell, 2001).

Isolation of genomic DNA from Whole Blood using FTA Card

Three discs were removed from the center of the FTA card using the 2.0 mm Harris Micro Punch tool and collected in 200 µl PCR amplification tubes. About 200 µl FTA purification reagent was added to each tube, capped and inverted twice and incubated for 5 minutes at room temperature. FTA reagent was pipetted up and down twice and discarded as much reagent as possible and this step was repeated once again with 200 µl of FTA reagent. Then 200 µl of TE Buffer was added and inverted twice and incubated at room temperature for 5 minutes. Finally discs were allowed to completely air dry for 1 hour at room temperature (Whatman Inc., 2009).

Design of human mitochondrial cytochrome *b* (CYTB) gene specific primers

The reference sequence of human mitochondrial DNA (ref: NC_012920.1, GI: 251831106) was downloaded from NCBI database and a fragment of this reference sequence (location of mitochondrial DNA: 14500 to 16200) was used for designing of cytochrome *b* gene specific primers. The parameters like, 1200-1400 bp product length, 19 to 26 bp (optimum 20 bp) primer size, up to 60% GC contents, melting temperature of the primer (Tm) between 57 to 61^0C were followed during designing of primers by PRIMER 3 software (Rozen and Skaletsky 2000).

Amplification and sequencing of *MTCYB* gene

The designed *MTCYB* gene specific primers were examined by amplification of cytochrome *b* gene through polymerase chain reaction. 50 µl of the PCR reaction consisting of 25 µl 2x red dye PCR mix, 1 µl (10 pico mole) each reverse and forward primers and 22 µl sterile DNAse, RNAse free water with 1 µl (25ng/ 1µl) of the isolated genomic DNA. In the case of FTA Card method, instead of genomic DNA, 3 purified FTA discs were added.

Amplification was performed on the automatic thermal cycler (ABI) and the PCR conditions consisting of an initial denaturation (one cycle) at 94 °C for 6 minutes, 30 cycles of 1 minute at 94°C, 1 minute at 59°C and 2 minutes at 72°C and a final extension at 72°C for 10 minutes (Matsuda *et al,* 2005 Anderson *et al,* 1981). About 5 µl of PCR products were resolved on 1 % agarose gel with 100bp DNA ladder and remaining PCR product were purified using Medox-Easy Spin Column PCR Cleanup Minipreps kit. These purified PCR products were sequenced at Samved BioTech Pvt. Ltd. Ahmedabad.

Data analysis

Obtained DNA sequence was visualized and analyzed by software SeqScanner 2 including quality of the sequence, Contagious read length (CRL) Q+16 values. Consensus sequences were prepared by MEGA6 (Tamura et al, 2013) software. Similar sequences were searched and annotated using online software BLAST (Basic Local Alignment Search Tool) provided by NCBI (National Centre of Biotechnology Information). Examined primers were submitted in probe database and DNA sequences were submitted in Gene bank database of NCBI.

RESULTS

DNA Isolation from fresh blood by traditional method

Genomic DNA was isolated in triplicate from each of the six samples by the traditional phenol chloroform method. The quantity of obtaining genomic DNA was in the range of 128.27±4.0197 to 185.58±10.9378, while ratio of A_{260}/A_{280} was observed between 1.73±0.0153 to 1.89 ±0.0208 (Table 1). These DNA produces prominent and clear band on 1.5% agarose gel.

Designing of Novel Primers

Reference sequence of *Homo sapiens* mitochondrion genomic DNA (ref: NC_012920.1, GI: 251831106) was downloaded from the NCBI gene bank in the FASTA format. This sequence was trimmed from both 5' and 3' ends and finally a 1400 bp sequence (ref location: 14501 to 15900) covering complete Cytochrome *b* gene was obtained. Using this sequence, specific primer was designed for amplification of complete *MTCYB* gene by Primer3 software. Two suitable sets of reverse and forward primers were selected from the list of primers and named as MVM-MT-Cyb-01 and MVM-MT-Cyb-02.

These primers were 19 to 21 bases long and their melting temperature was in the range of 64.9 to 65.5 ˚C. The product sizes of MVM-MT-Cyb-01 and MVM-MT-Cyb-02 primers were 1312 and 1317 bp respectively (Table 2).

Examination of designed primers

MVM-MT-Cyb-01 and MVM-MT-Cyb-02 were synthesized and supplied by Sigma Aldrich, India. DNA samples isolated by the phenol chloroform method and trapped on FTA cards were amplified for the authentication of developing novel primers. Amplified PCR products were resolved on 1.5% agarose gel and the result revealed that the both the primers produced prominent band of approximately 1320 bp in all the screened samples.

Sequencing of *MTCYB* gene using developed primers

The amplified product of six samples was sequenced using reverse and forward primers of both set MVM-MT-Cyb-01 and MVM-MT-Cyb-02. Contiguous read length (CRL) of sequences obtained by reverse primer of MVM-MT-Cyb-01 was observed in the range of 898 to 1048 bp with a mean of 957.667± 61.976 bp and in the range 987 to 1043 bp with a mean of 1022.833± 22.825 by forwarded primer of MVM-MT-Cyb-01. While CRL of sequences obtained by reverse primer of MVM-MT-Cyb-

Table 1: Quantitative and qualitative estimation of isolated DNA

SL	Sample ID	Ratio of A_{260}/A_{280}	Concentration (ng/µl)
1.	B-01	1.76±0.0116	128.27 ±4.0197
2.	B-02	1.81±0.0306	185.58 ±10.9378
3.	B-03	1.89±0.0208	163.76 ±2.8725
4.	B-07	1.73±0.0153	175.15 ±2.1854
5.	B-08	1.75±0.0321	169.15 ±2.4856
6.	B-09	1.79±0.0265	182.15 ±3.1075

Table 2: Sequence of designed primers

SL	Name	Code	Sequence (5'--------3')	Total Length	Temp.	Product size
1.	MVM-MT-Cyb-01	P1-f	CACGGACTACAACCACGACC	20 bases	65.5 ˚C	1312 bp
		P1-r	TTGGGTGCTAATGGTGGAGTT	21 bases	65.4˚C	
2.	MVM-MT-Cyb-02	P2-f	TCGCACGGACTACAACCAC	19 bases	64.9˚C	1317 bp
		P2-r	CTTTGGGTGCTAATGGTGGAG	21 bases	65.4˚C	

Table 3: Consensus sequences of primer MVM-MT-Cyb-01 and MVM-MT-Cyb-02

SL	Sample ID	Obtained Sequences (bp) by Primer MVM-MT-Cyb-01			Obtained Sequences (bp) by Primer MVM-MT-Cyb-02		
		Reverse (CRL)	Forward (CRL)	Consensus sequences	Reverse (CRL)	Forward (CRL)	Consensus sequences
1.	B-01	898	1027	1140	941	1123	1259
2.	B-02	1010	1041	1180	1072	1056	1248
3.	B-03	945	1036	970	1043	1035	1011
4.	B-07	1048	1003	1082	1073	1130	1251
5.	B-08	890	987	1011	991	1236	1246
6.	B-09	955	1043	1170	949	1134	1262
Mean±SD		957.667± 61.976	1022.833± 22.825	1092.167± 86.806	1011.5± 59.551	1119± 70.818	1203± 99.075

02 were recorded in the range of 941 to 1073 bp with a mean of 1011.5± 59.551 bp and forwarded primer produced 1035 to 1236 bp with a mean of 1119± 70.818 bp.

The contiguous read length (CRL) is the longest uninterrupted stretch of bases with quality, higher than a specified limit (QV16+). In the evaluation of the quality of each base, not only the best quality value of that base was used, but also those of adjacent bases within a specified window size. According to the contiguous read length (CRL) both the developed primers were produced good quality of readable sequences. 1092.167± 86.806 bp mean consensus sequence with minimum 970 and maximum 1180 bp and 1203± 99.075 bp mean consensus sequence with minimum 1011 and maximum 1262 bp were prepared using reverse and forward primers of primer MVM-MT-Cyb-01 and primer MVM-MT-Cyb-02 respectively (Table 3).

Annotation and submission

Similar sequences of obtained sequences were searched in the gene bank database (NCBI) using BLAST software one by one. Obtained sequences were annotated as per provided information by BLAST search. According to that 3 tRNA region, namely TRNE (tRNA- Glutamine; GeneID:4556; HGNC:7479; MIM:590025), TRNT (tRNA-Threonine; GeneID:4576; HGNC:7499; MIM:590090) and TRNP (tRNA-Proline; GeneID:4571; HGNC:7494; MIM:590075) and one coding region (gene- MTCYB; GeneID:4519; HGNC:7427; MIM:516020) were observed in obtained sequences. Out of 6 obtained sequences all sequences having TRNE gene, which was partially at 3' end. According to BLAST search gene- *MTCYB* was observed in obtained sequences which produces protein Cytochrome *b* (protein_id="YP_003024038.1"). Gene *MTCYB* was observed in the all obtained sequences, out of them complete gene or coding region was observed in 5 sequences. A TRNT gene was observed in 5 obtained sequences. TRNP gene was observed in 5 obtained sequences. After successful annotation of the sequences, these sequences were submitted in NCBI gene bank database and gene bank ID was allotted for all 6 sequences. Furthermore the information of the sample used in this study was also submitted in the NCBI BioSample database.

Wet laboratory authenticated primers were submitted in the NCBI probe database after successful validation of wet laboratory amplification and sequencing. After submission, accession no Pr032250587 with Probe ID 32250587 was assigned to primer set MVM-MT-Cyb-01 and accession no Pr032250588 with Probe ID 32250588 was assigned to primer set MVM-MT-Cyb-02.

DISCUSSION

Blood is the main source of DNA for genotype-related studies in humans. A rapid, efficient, and cost-effective method for the isolation of genomic DNA from whole blood is needed for screening a large number of samples (Subbarayan *et al,* 2002). Phenol chloroform method for genomic DNA isolation showed good quality and quantity. These methods are reliable and have a long history of isolation of genomic DNA from human blood samples (Parzer and Mannhalter 1991; Albarino and Romanowski 1994; Wang 1994; Robbins *et al,* 1995; Rudbeck and Dissing 1998; Sambrook and Russell 2001). Time to time, many researchers made various alterations in this method for recovering DNA from different part of the human body, like white blood cells (Ikuta *et al,* 1992), buccal cells (Cozier *et al,* 2004) buccal swabs (Walker *et al,* 1999), formalin–fixed paraffin–embedded tissue sections (Rivero *et al,* 2006).

In this study FTA classic card was used for preservation of genomic DNA at room temperature. These FTA cards were successfully used for amplification of mitochondrial DNA. According to the best of our knowledge, this is the first report in which mitochondrial DNA was amplified using the FTA card. FTA is a simple technology that reduces the steps of DNA collection, transportation, purification and storage and subsequently reduces the cost and time required to process DNA to the final step of purified DNA ready for the downstream application (Rajendram *et al,* 2006; Whatman Inc., 2009). The FTA cards are firstly developed for Phenylketonuria screening in newborns by Guthrie and Susi in 1963, later these cards are used in medical and forensic science for detecting DNA using the polymerase chain reaction (Vanek *et al,* 2001). It has been also used for isolation of genomic DNA from animal samples (Crabbe 2003; Harvey 2005; Borisenko *et al,* 2008), bacterial DNA or viral RNA from different biological samples (Lampel and Orlandi 2002; Lampel *et al,* 2004; Warren *et al,* 2005). Presently FTA cards are used in both DNA and RNA based studies of many plants (Roy and Nassuth 2005; Tsukaya *et al,* 2005; Adugna *et al,* 2011; Sairkar *et al,* 2013).

In this study, the mitochondrial cytochrome *b* gene was amplified for detection of SNPs in breast and ovarian

carcinoma. Cytochrome *b* of eukaryotic mitochondria (*MTCYB*) is an important enzyme of respiratory chain complex III, also known as the bc1 complex or Ubiquinol-cytochrome c reductase. *MTCYB* is an imperative membrane protein of 380 aminoacid that possibly has 8 transmembrane segments (Howell 1989; Esposti *et al,* 1993). The DNA sequence of *MTCYB* is commonly used to determine phylogenetic relationships between organisms due to its sequence variability. It is considered to be most useful in determining relationships within families and genera (Castresana 2001). *MTCYB* has been used for a diversity assessment at the species level (Meyer and Wilson, 1990; Irwin *et al,* 1991; Normark *et al,* 1991; Cantatore *et al,* 1994; Lydeard and Roe 1997; Kumazawa and Nishida 2000) and at the population levels (Sturmbauer and Meyer 1992; Rocha-Olivares *et al,* 1999; Kirchman *et al,* 2000; Lovejoy and de Arau´jo 2000). The phylogenetic effectiveness of the *MTCYB* gene has been studied at several taxonomic levels among animals taxa (Irwin *et al,* 1991; Moritz *et al,* 1992; da Silva and Paton 1993; Graybeal 1993; Lamb and Lydeard 1994; Moore and DeFilippis 1997; Nunn and Stanley 1998) and particularly in fish taxa (Ortí and Meyer 1996, 1997; Lydeard and Roe 1997; Martin and Bermingham 1998; Zardoya and Doadrio 1999; Lovejoy and de Arau´jo 2000).

In this study two primers were developed for amplification of complete *MTCYB* gene, which produced 1312 and 1317 bp fragments and these primers were also successfully used for capillary based Sanger sequencing. In this study, 3 tRNA regions, namely TRNE (tRNA- Glutamine), TRNT (tRNA- Threonine) and TRNP (tRNA-Proline) and one coding region CDS (gene- *MTCYB*) were observed in obtained sequences. These results revealed that the developed primers may be used for amplification and sequencing of complete CDS regions. In previous studies, many primers were developed and used for amplification of a *MTCYB* like, Kocher *et al,* (1989) developed a set of primers which amplified a 307-base-pair segment of the cytochrome *b* gene not only from humans but also from most other vertebrates. Kent and Norris (2005) developed a specific multiplexed primer set based on mitochondrial cytochrome *b*. A "universal" primer pair has been designed and validated to amplify a 464 bp segment of the cytochrome *b* gene (Unseld *et al,* 1995) while Edwards *et al,* (1991), Krajewski *et al,* (1992) and Krajewski and Fetzner (1994) developed two primer sets each for amplification of mitochondrial cytochrome *b* gene.

Universal PCR primers generated several different anomalous sequences during amplifying a portion of the cytochrome *b* gene and this force the researcher to believe it to be nuclear pseudogenes (Mirol *et al,* 2000; Smith *et al,* 1992; Arctander, 1995; Collura *et al,* 1996; Zhang & Hewitt, 1996a, b). This problem may be overcome by complete amplification of gene and in this study, we amplified 1317 bp fragment which contained tRNA region, namely TRNE (tRNA- Glutamine; GeneID:4556; HGNC:7479; MIM:590025), TRNT (tRNA-Threonine; GeneID:4576; HGNC:7499; MIM:590090), TRNP (tRNA-Proline; GeneID:4571; HGNC:7494; MIM:590075) and one coding region (gene- *MTCYB*; GeneID:4519; HGNC:7427; MIM:516020). Amplification of *MTCYB* gene using our developed primers may reduce the chances of confusion between nuclear and mitochondrial copies.

CONCLUSION

In the present study, two novel primers were developed for amplification and sequencing of *MTCYB* gene. We conclude that, the FTA card is suitable for amplification of mitochondrial DNA as well as suitable for the preservation of DNA at room temperature. These primers and FTA card may also be used for study of large samples with complete mitochondrial DNA.

Acknowledgment

The authors are grateful to Dr. (Mrs) Abha Gargav, Principal, Govt. Motilal Vigyan Mahavidyalaya, for providing the opportunity to work in this prestigious academic institution. The authors also like to thank to Dr. BR Shrivastav, Director, Cancer Hospital & Research Institute (CHRI), Gwalior and Dr. Archana Shrivastav, Principal, College of Life Sciences, Gwalior for providing blood samples.

Conflicts of interest: The authors stated that no conflicts of interest.

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