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Real-Time PCR for the Rapid detection of common β-Thalassemia mutation in Gujarat

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Abstract- The inherited Haemoglobinopathis are autosomal recessive disorder including beta Thalassemia. Out of 200 different mutations causing β Thalassemia, IVS 1-1, IVS 1-5, Codon 41-42, Codon 8-9,and Codon 619 Bp deletion are most common in Gujarat Thalassemic patients. Rapid technique of prenatal diagnosis of only IVS 1-1, IVS 1-5 and Codon 8-9 have been standardized in our lab and Prenatal Diagnosis of these mutations was implemented. The Roche Light Cycler 480 (LC 480) system was evaluated for qualitative diagnosis of common mutations in β Thalassemia in Hb beta gene. A total number of 60 whole blood samples from carrier couples , 11 CVS and 9 Amniotic fluid samples were obtained from antenatal mothers .For testing; LC480 system is more suitable and versatile .Real time PCR platform in a routine laboratory settings for the diagnosis of common mutations in β Thalassemia as noted above is the basis of the present study.DNA Samples used for amplification where obtained from CVS or Amniotic fluid and whole Blood samples .The Standardized mutations in our lab were detected using 2 sets of probes and one common set of universal primer ,usually distinguishing mutants from the normal alleles. In Prenatal diagnosis of βThalassemia mutations accuracy and the speed are of paramount importance taking only one and half hours by the entire procedures in our present study.

Index Terms- Beta Thalassemia, Real time PCR, MPLC, Prenatal Diagnosis

I. INTRODUCTION

Around 7% of the world populations are carriers of globin gene mutations in the α or β globin gene clusters (1).In view of above, Indian Red Cross Society ,Gujarat State Branch carried out population screening for beta Thalassemia ,during the period from 2004 -2011. It was observed from the carrier screening that 1000 infants are born each year with homozygous β Thalassemia disorder in Gujarat. (2) Hence there is need to develop cost effective diagnosis technique for prenatal diagnosis.

The Aim of the present Study was to develop new Real Time PCR assay protocol for the diagnosis of above 3 common mutations in β Thalassemia in Gujarat on the Light Cycler 480 system.

II. MATERIALS AND METHODS

2.1 Patients and Samples

60 Blood samples in EDTA vacuttes were collected in our lab from the antenatal couples and CVS /Amniotic fluid samples were collected by gynecologist from the antenatal mothers (3) .In a Study of the molecular basis of β Thalassemia in Gujarat 5 different mutations have been identified (4). Amongst them only 3 mutations (IVS 1-1, IVS 1-5, and Codon 8-9) were standardized in our lab. For Control 10 Blood samples were also collected from healthy individuals. The test was performed after obtaining written consents from parents.

III. DNA EXTRACTION

For prenatal diagnostic assay 20 microlitre of amniotic fluid that is free of maternal blood or a pinhead sized clean chorionic villus sample (CVS) free of maternal tissue were obtained. A 20 microlitre blood sample was collected from each parent to confirm the diagnosis, before the amniocentesis or the CVS was performed.

For the isolation of DNA, proprietary magnetic glass particles and specialized MagNA Pure LC kits were used. DNA binds to Magnetic Glass Particles (MGPs) in the presence of a chaotropic salt at a pH>7.0. MGPs have a glass (silica) surface and a magnetic core. Nucleic acids are bound to the silica-surface of the MGPs in the presence of isopropanol and high concentration of chaotropic salts, which remove water from hydrated molecules in solution. Polysaccharides and proteins do not bind to the beads and are removed by sequential washing steps. Pure nucleic acids are then eluted from the beads by applying low-salt conditions and heat.

Once bound to the surface of the MGPs, the nucleic acids gets separated from the solution with a magnet. The advantage of this isolation technology is that it requires no centrifugation or any other manual steps (5-8).

IV. DESIGN OF OLIGONUCLEOTIDE PRIMERS AND PROBES:

PCR and mutation detection by melting curve analysis were performed on the LightCycler, which can simultaneously measure emitted signals from two different fluorophores. Forward primer ,

New A 5'-gCTgTCATCACTTAgACCTCA-3' and Reverse primer New B 5'-CACAgTgCAgCTCACTCAg-3' annealing in the beta-globin at positions 42-63 and 629-611 respectively are used to amplify the 457 bp region spanning the IVSI-1, IVSI-5 and codon 8-9 mutations.

V. Probes

Four sequence-specific fluorescent-labeled,
1) FITC IVI.5.6 5'-
TgCCCAgTTTCTATTggTCTCCTTAAACCTgTC--FL ,
2) Red705- IV1 5'-LC705-
TgTAACCTTgATACCAACCTgCCCA—PH
3) Sensor 5'-LC 640-gACTCCTgAggAgAAgTCTgC—PH
4) Anchor+ 5'CCTCAAACAgACACCATggTgCACC—FL

Probes were designed in collaboration with Tib Molbiol (Berlin, Germany) to span the IVSI-1, IVSI-5, Codond 8-9 mutations. The probe sequences were based on the wild type or the mutant sequence depending on the mutation. This is to maximize the difference in the probe melting temperature between the wild type and the mutant genotype (9). For example, Probe FITC IVI.5.6 was based on the wild type sequence and the difference in the probe melting temperature between the wild type (64.6 deg centigrade) and the Probe FITC IVI.5.6 is labeled with fluorescein at the 3' end and probe Red705- IVI is labeled with fluorescein at the 5' end. Upon hybridization, the two probes come in close proximity resulting in fluorescence resonance energy transfer (FRET). During FRET, the FITC IVI.5.6 fluorescein is excited by the LC light source donating part of the excitation energy to the acceptor fluorophore of probe Red705 which then emits energy that is measured by the optical unit of the Light Cycler instrument.

VI. LightCycler- PCR (LC-PCR) Protocol

LC-PCR is performed using 5 microlitre DNA, 4 microlitre genotyping master, 0.2 microlitre of specific fluorescein labeled probes and 0.5 microlitre of primers with the LightCycler-Faststart DNA Master Hybridization Probes Kit (Roche Biodiagnostics, Mannheim, Germany) according to the manufacturer's recommendations in a final reaction volume of 20 microlitre using disposable glass capillaries. Cycling was started after a 5-min incubation period at room temperature to enable UNG activity. Forty amplification cycles are performed as follows: Denaturation and AmpliTaqGold activation (95 deg C for 10 Sec), annealing (58 deg C for 20 Sec), and extension (72 deg C for 25 Sec).

The ramp rates are programmed at 4.4 deg C/Sec from denaturation to annealing, 2.2 deg C/Sec from annealing to extension and 4.4 deg C/Sec from extension to denaturation. At the end of the amplification, a programme for melting curve was set , analysis was added as follows: Heating to (95 deg C for 1 min), cooling to (40 deg C for 1 min), heating slowly at 85 deg C without holding time and a final recooling to 40 deg C for 30 Sec. Genotyping is decided after melting curve analysis is done by comparing the melting peaks of the suspected patient with that of individuals with known genotypes.(10-13)

This Technique is based on the employment of Real time PCR Quantification and melting curve formation for the mutation detection. This Method relies on the use of fluorescent –Labeled oligonucleotides probes hybridizing to complementary sequences harboring the mutation, hence producing curves with melting peaks that clearly distinguish between the wild type and the mutant genotypes

The temperature at which a DNA strand separates or melts when heated can vary over a wide range, depending on the sequence, the length of the strand , and the GC content of the strand. This effect is more pronounced for short DNA hybrids and is thus, the basis for probe based genotyping analysis.

In the case of hybridization probes, the separation of targets-probe hybrids results in the spatial separation of the fluorescence resonance eregy transfer (FRET) partners and in a drop of fluorescence from the reporter dye at a certain temperature. The melting temperature or Tm, is defined as the point at which half the probes have melted off their target DNA sequence.

VII. Results

The Light Cycler method was standardized by analyzing DNA from 80 beta Thalassemia heterozygotes and homozygotes as per the availability. All the cases were known, and previously diagnosed from RDB and ARMS PCR technique.18 samples for IVS1-1, 15 samples for IVS 1-5, and 15 samples for codon 8-9 were analysed. A separate experiment was set up with each mutation with the appropriate pair of acceptor and donor probes. After melting curve analysis, the presence or absence of a mutation could be recognized by a noticeable shift in the melting peak as compared to wild type or known genotype control.

IVS1-1 mutation

A melting peak from Red 705 acceptor probe FITC donor probe using primer NEW A New B was observed at 58 ° centigrade indicating the IVS 1-1 mutation (Fig. 1)which accounts for 13.74 % in Gujarat Population. It was distinct from the melting peak of the wild type, observed at 67° centigrade. An IVS 1-1 homozygous individual shows a single peak at 58° centigrade (Fig 2). As compared to single peak at 67° centigrade for a homozygous normal individual. A heterozygous individual displays two peaks, one at 58° and one at 67° centigrade.

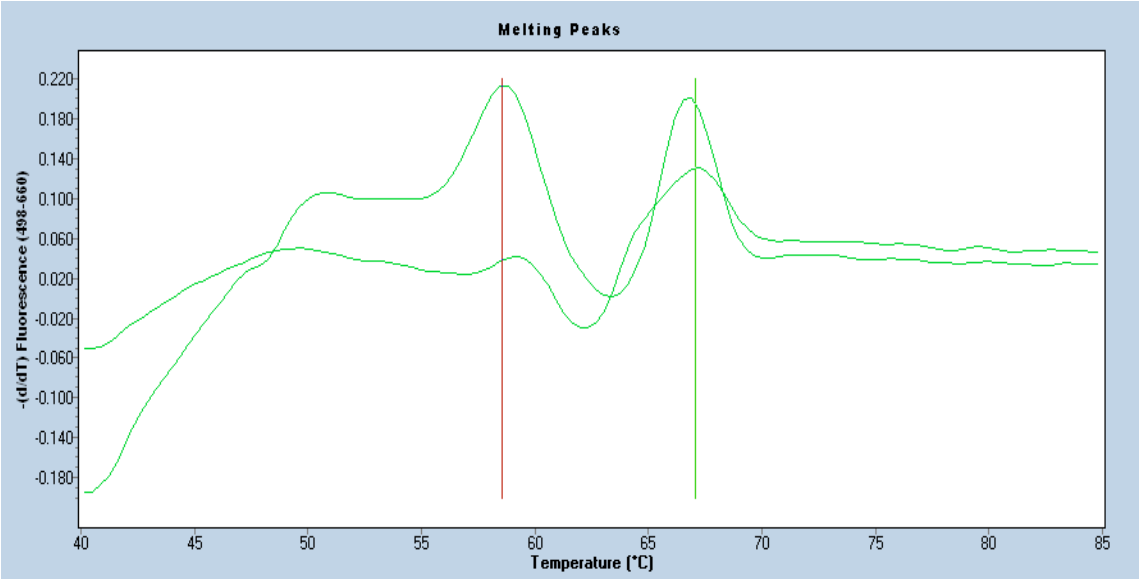

Figure 1- Melting curves for the heterozygous IVS 1-1 mutations in β Thalassemia

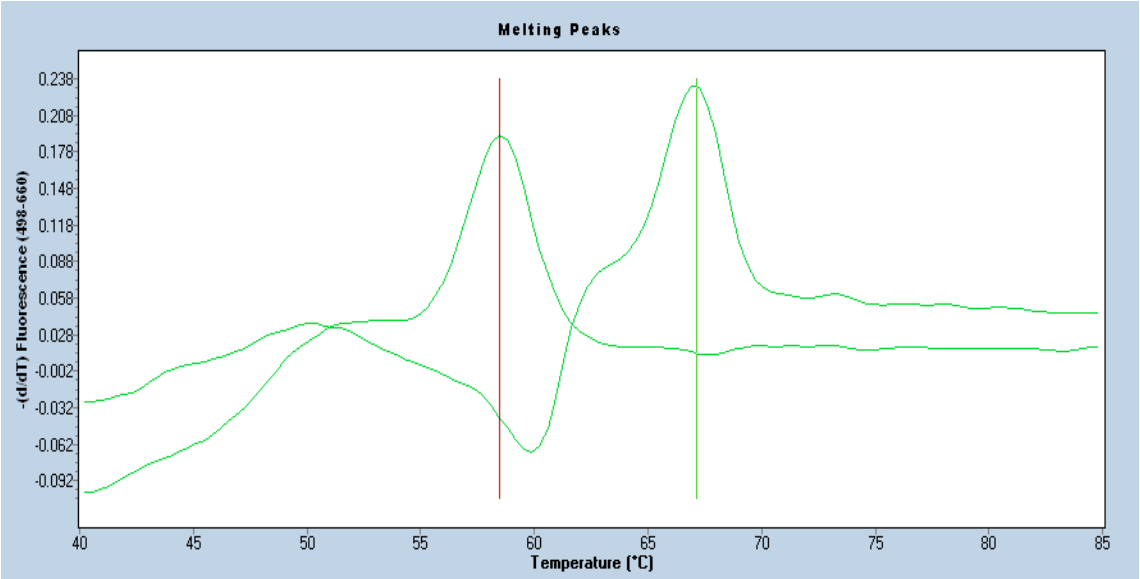

Figure 2- Melting curves for the Homozygous IVS 1-1 mutations in β Thalassemia

IV1- 5 mutation

A melting peak from Red 705 acceptor probe FITC donar probe using primer

New A New B was observed at 59° centigrade indicating the IVS 1-5 mutation which accounts for 41.34 % in Gujarat Population. It was distinct from the melting peak of the wild type, observed at 67° centigrade. An IVS 1-5 homozygous individual showed a single peak at 59° centigrade (Fig 4), as compared to single peak at 67° degree centigrade for a homozygous normal individual. A heterozygous individual displays two peaks, one at 59° and one at 67° centigrade (Fig 3). However the shift of temperature in between IVS 1-1 and IVS I-5 is close to each other. In such cases, the IVSI-1 mutation was identified by use of ARMS PCR *(14)*, And the IVSI-5 mutation was confirmed by digestion with *Eco*RV *(15)*.

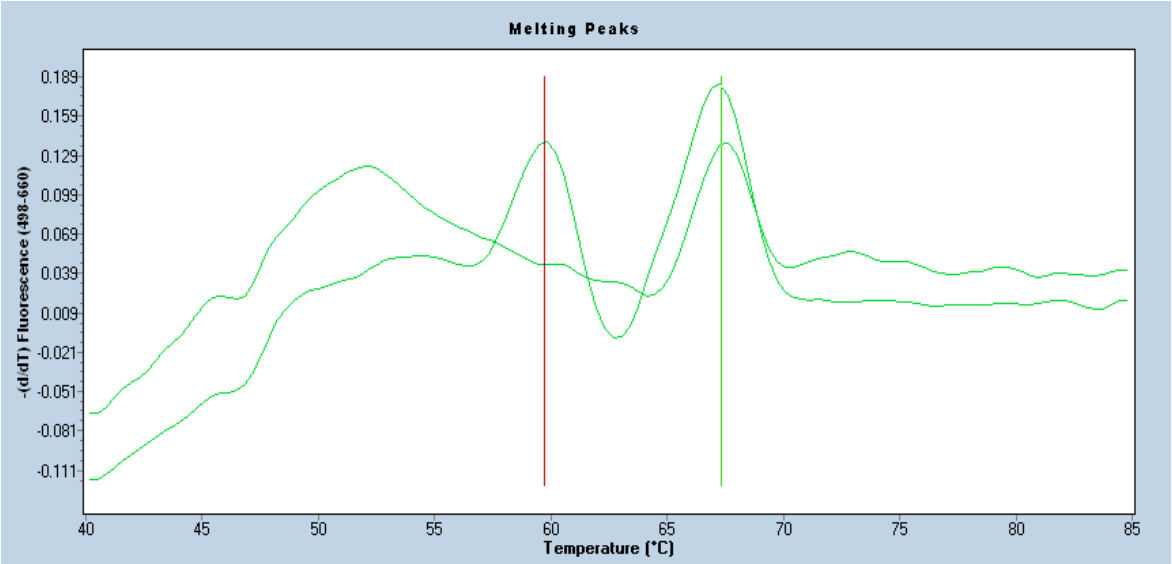

Figure 3- Melting curves for the heterozygous IVS 1-5 mutations in β Thalassemia

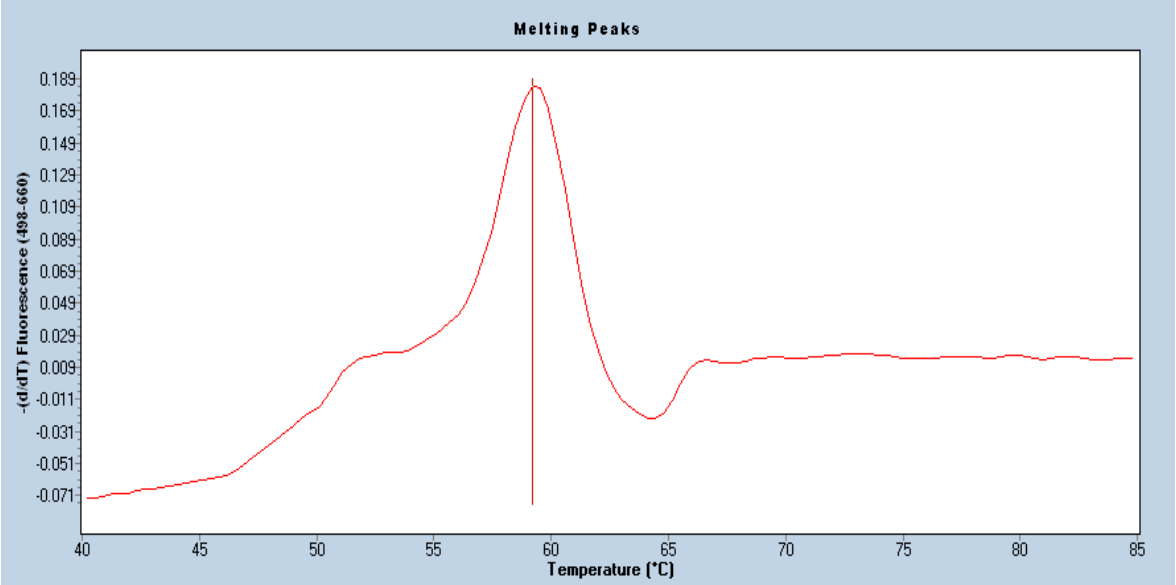

Figure 4- Melting curves for the homozygous IVS 1-5 mutations in β Thalassemia

CD 8-9

A melting peak from Sensor acceptor probe and Anchor +donor probe using primer NEW A New B was observed at 58 ° centigrade indicating the codon 8-9 mutation which accounts for 7.70 % in Gujarat Population. It was distinct from the melting peak of the wild type, observed at 64° centigrade. Codon 8-9 homozygous individual shows a single peak at 58° centigrade (Fig 5), As compared to single peak at 64° centigrade for a homozygous normal individual. A heterozygous individual displays two peaks, one at 58° and one at 64° centigrade (Fig 6).

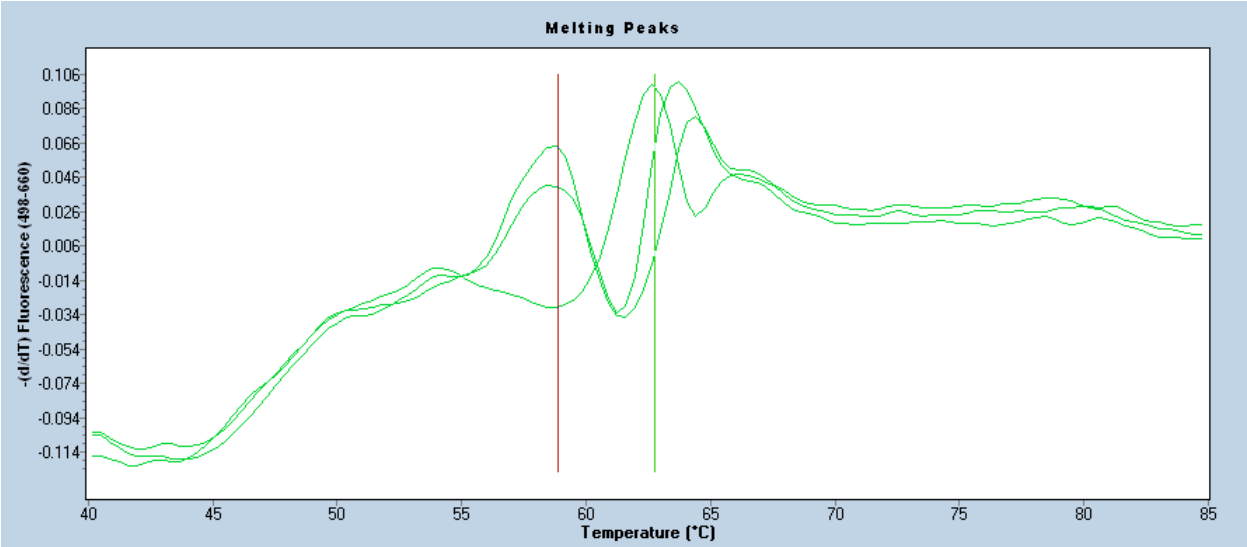

Figure 5- Melting curves for the heterozygous Codon 8-9 mutation in β Thalassemia

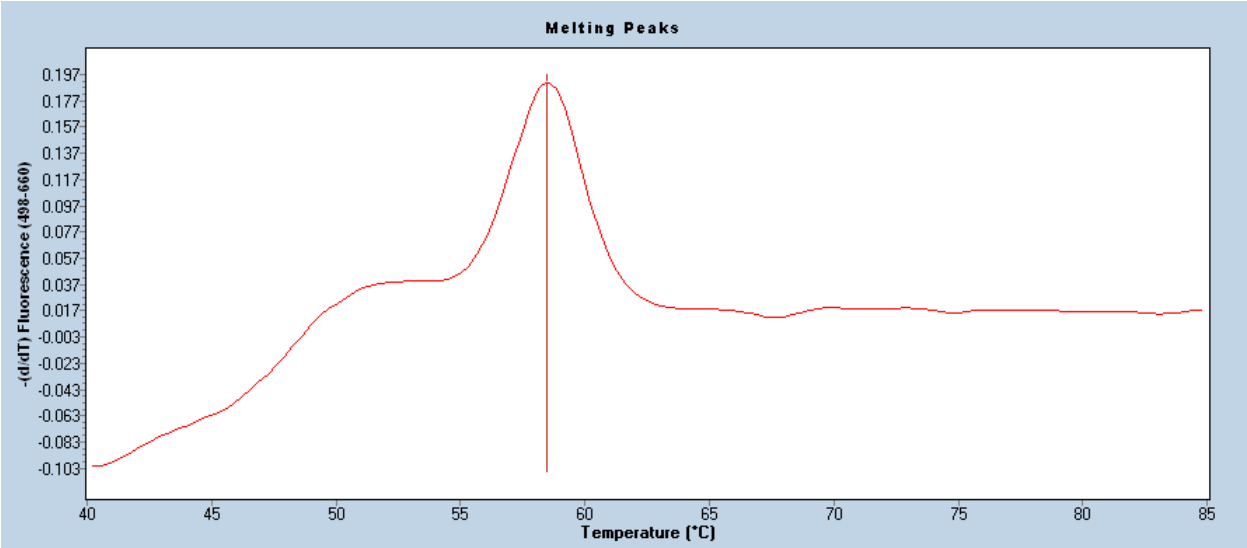

Figure 6- Melting curves for the homozygous Codon 8-9 mutations in β Thalassemia

VIII. DISCUSSION

The β–globin gene is a relatively small gene(,<2000 bp)located on the short arm of chromosome 11(16).Although more than 180 mutations have been reported for β Thalassemia syndrome, the spectrum of mutations and their frequencies in most population usually consist of a limited number of common mutations and a slightly larger number of rare mutations(17).

Mutation characterization in carriers is a pre requisite when offering prenatal diagnosis to couples at risk for having an affected child and frequently needs to be achieved as quickly as possible. With the aim of rapid DNA analysis, Real time PCR has became an important tool in both research and routine clinical diagnosis. According to the published data the cumulative gene frequency of haemoglobinopathis in India is 42%.werears the frequency in western India is 34% of the total population. Every year 1200 homozygous beta Thalassemics are born, in western India and 1000 in Gujarat as per the annual activity report of Indian Red Cross society Gujarat State Branch India.

To prevent this birth a cost effective and less time consuming technique was developed i.e. Real time PCR assay has been developed which allows an easy assay of β Thalassemia mutations.

In western India more than 15 mutations have been found out but among them 5 accounts for the common mutations and rest are classified under rare mutations. In this study we designed a protocol for at least three common mutations which are common in western India.

An analogous light cycler method has been described for genotyping samples. This sample carry mutations which are very close to each other, hence Tm calling method was used to distinguish the melting curves. The peaks were different for different mutations in both the cases of homozygotes as well as in heterozygotes. Due to the close proximity of Snp's a single primer and two different sets of probe worked for 3 common mutations in Gujarat population i.e. for IVS 1-1, IVS 1-5 and codon 8-9; but out of the three mutation as above one single different sets of probes were designed for codon 8-9 i.e. sensor and anchor+ . Even then, this method was cost effective, rapid and allows easy detection among known samples with less than 2 hours. It was also observed that in cases where common probes have been used, the TM was different for wild type and hence no ambient results were produced.

Overall the present method was cost effective reliable, accurate and rapid.

IX. Conclusion

The LC 480 Systems, coupled with Magna pure Compact instrument(Roche Diagnostics) for automated DNA extraction, constitutes a suitable and versatile Real time PCR platform in a routine laboratory setting for the diagnosis and monitoring of common mutations in beta Thalassemia.

Conflict of Interest

Authors have no conflict of interest.

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