

High Throughput Molecular Beacon-Based Assays for Genotyping Common *HFE* Variants

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Short Title: HFE: p.C282Y and p.H63D genotyping

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Abstract

Background: Hereditary Hemochromatosis (HH) is an autosomal recessive disorder highlighted by iron-overload. Two popular mutations in *HFE*, p.C282Y and p.H63D, have been discovered and found to associate with HH in different ethnic backgrounds. p.C282Y and p.H63D diagnosis is usually made by restriction enzyme analysis. However, the use of this technique is largely limited to research laboratories because they are relatively expensive, time-consuming, and difficult to transform into a high throughput format. Single nucleotide variations in target DNA sequences can be readily identified using molecular beacons. Molecular beacons are quenched probes with a loop and hairpin structure, and they become fluorescent only by specific recognition of their target.

Results: We developed high throughput homogeneous real-time PCR assays using molecular beacon technology, to genotype p.C282Y and p.H63D variants.

Representative samples for all p.C282Y and p.H63D genotypes were assayed by restriction enzyme analysis and direct sequencing for comparison with the newly developed molecular beacon-based real-time PCR assay. Complete concordance was achieved by all three assay formats. Homozygotes (mutant and wildtype) and heterozygotes were readily identified by the allele specific beacons as reported by the associated fluorophore in the real-time assay developed in this study.

Conclusion: These assays may be reliably applied as a diagnostic test or large scale method for population screening.

Background

Hereditary Hemochromatosis (HH) is an autosomal recessive disorder highlighted by iron-overload [1]. HH was recognized more than a century ago as a condition with three symptoms: diabetes, skin bronzing, and cirrhosis [2]. In Caucasians, HH affects 3–8 in every 1000 individuals, with an approximately 10% prevalence of heterozygous carriers [3]. The molecular basis for HH was completely unknown until the identification in 1996 of a gene on chromosome 6p, designated *HLA-H*, subsequently renamed *HFE* [4]. A cysteine-to-tyrosine substitution at position 282 in the HFE protein (p.C282Y), has been observed in the majority of HH patients, with frequencies ranging from 64% to 100% in subjects (from different geographic areas) who have hemochromatosis [5-7]. A substitution of histidine by aspartic acid at position 63 (p.H63D) in the HFE protein, was also linked to an additional 1-10% of HH cases [5]. Large population-based studies are required to definitively establish the prevalence of these mutations on an ethnic and or geographic basis. A recently described third mutation, which substitutes cysteine for serine at position 65 (p.S65C) of the HFE protein, is enriched in HH individuals who have a mild form of the disease and lack the p.C282Y or p.H63D mutations [8]. The overall impact of this third mutation is currently unclear.

The diagnosis of hemochromatosis was previously based on a combined clinical and laboratory assessment including physical examination, elevated transferrin saturation, and serum ferritin. Pedigree studies have boosted the acceptance of HH genetic screening in several countries [9]. The most established screening test for HH is transferrin saturation. The sensitivity of transferrin saturation for the diagnosis of HH has been reported to be

94% based on a population screening study from Busselton, Australia [10]. Today, the diagnosis of HH has shifted from clinical symptoms and biochemical assessment towards genetic testing. Genetic testing for *HFE* mutations now plays a central role in confirming the clinical diagnosis of HH. The molecular methods used to screen for the p.C282Y mutation include allele-specific oligonucleotide hybridization [11], restriction enzyme analysis [11], and oligonucleotide ligation assays [4]. However, the use of all of these techniques is largely limited to research laboratories because they are relatively expensive, time-consuming, and difficult to transform into a high throughput format.

Molecular beacons are single-stranded oligonucleotide probes with a stem-and-loop hairpin structure [12]. The loop domain of the molecular beacon is complementary to the target sequence and contains the mutation site usually embedded centrally. The molecular beacon is labeled with a fluorophore at one end and a quencher, 4'-(4-dimethylaminophenylazo) benzoic acid (DABCYL), at the other end. When the specific target is encountered, the molecular beacon forms a hybrid with the target leading to conformational changes that position the fluorophore away from the quencher. As a result, fluorescence of the molecular beacon is restored signaling presence of the associated allele. Under optimal conditions, instability of mismatched hybrids increases the specificity of molecular beacons, making them the perfect probes for single nucleotide polymorphism (SNP) genotyping or for detection of point/small mutations. Molecular beacons can each be conjugated to different fluorophores enabling simultaneous detection of any genotype in a closed tube homogeneous assay [12-14].

In this report we describe the design and validation of 2 molecular beacon-based real-time PCR assays for the detection of the common HH mutations, p.C282Y and p.H63D.

Results

C282Y and H63D Genotyping of *HFE* by PCR/Restriction Enzyme Analysis

Both of p.C282Y and p.H63D mutations alter restriction-enzyme sites, providing a method for genotyping. c.845G>A creates a recognition site for *RsaI*; c.187C>G abolishes the DNA sequence recognition site for *MboI*. *RsaI* was used for restriction analysis of an amplicon (357 bp undigested; Figure 1A, lane 2) containing the p.C282Y mutation site of *HFE*. Representative restriction analysis using the control samples is shown in Figure 1. Following *RsaI* digestion a homozygous wild type sample (GG) produces two fragments (Figure 1A, lane 3: 250 and 107 bp), whereas a homozygous mutant sample (AA) results in three fragments (Figure 1A, lane 4: 250, 78, and 29 bp). For heterozygotes, four fragments (Figure 1A, lane 5: 250, 107, 78, and 29 bp) were present following *RsaI* digestions. Similarly *MboI* was used for restriction analysis of an amplicon (294 bp undigested; Figure 1B, lane 2) containing the p.H63D mutation site of *HFE*. Following *MboI* digestion a homozygous wildtype sample (CC) produces three fragments (Figure 1B, lane 3: 138, 99 and 57 bp), whereas a homozygous mutant sample (GG) generates two fragments (Figure 1B, lane 4: 237 and 57 bp). For heterozygote sample (CG) four fragments (Figure 1B, lane 5: 237, 138, 99, and 57 bp) were present following digestion.

Direct Sequencing of *HFE* Amplicons Containing the p.C282Y and p.H63D sites

Direct sequencing of amplicons containing the p.C282Y and p.H63D sites of *HFE* were performed on both strands for all 23 patients and three control samples. Sequencing results of all 23 samples and the controls were 100% concordant with that achieved using PCR and restriction analysis.

p.C282Y and p.H63D Genotyping by Molecular-Beacon Based Real-Time PCR

Assays

p.C282Y and p.H63D genotyping was carried out by using two independent molecular beacon-based homogeneous assays. Representative results of assays for C282Y and p.H63D using sequence validated controls are shown in Figures 2 and 3 respectively. These results were generated using the Corbett 3000 system; identical profiles are also observed when the assay is run on the ABI Prism 7900 HT sequence detection system. Ct values were detected in these assays usually between cycles 25 and 30, and indicate the presence of specific allele(s) depending on the positive signal(s) that are detected. For both of these assays, wild type and variant allele-detecting molecular beacons were labeled with FAM and TET respectively. FAM channel only positive signal indicated a homozygous wild type genotype. Conversely TET channel only positive signal indicated a homozygous mutant genotype. FAM positive/TET positive channels indicate a heterozygote genotype. p.C282Y and p.H63D real-time assay cycling parameters were optimized to be identical thus permitting simultaneous analysis of both genotypes, albeit in independent reactions. Genotyping of p.C282Y and p.H63D variants was accomplished by real-time PCR amplification of C282Y- and H63D-containing amplicons

in the presence of the allele-specific molecular beacons for these variants. Relative fluorescence was monitored using the Rotor Gene 3000 real-time thermal cycler, and representative genotyping results are shown in Figures 2 (p.C282Y) and 3 (p.H63D). Using this real-time PCR method, genotyping of all 23 test samples was in complete concordance with the results achieved using restriction enzyme analysis and direct sequencing.

High Throughput Population Genotyping for p.C282Y and p.H63D

Here we analyzed a total of 540 DNA samples obtained from anonymized newborn dry bloodspots. DNA was amplified from these spots using whole genome amplification (WGA) technology. WGA provides a comprehensive whole genome coverage and minimal loci bias [15]. The WGA method provides unlimited quantities of DNA directly from a variety of samples types including dried blood spots. p.C282Y and p.H63D genotyping was facilitated by the use of the two molecular beacon assays which we developed and that were readily applicable for genotyping of a large number of samples. Using this approach on a cost per sample basis in respect of both reagents and time the molecular beacon based assay was more economical than either restriction enzyme analysis or direct sequencing. The overall p.C282Y genotyping resulted in no mutant alleles being detected as all subjects were homozygous (GG) for the wildtype allele. This result indicates the mutant or variant A allele to be very rare (frequency < 0.001) in the Saudi population. On the other hand, p.H63D genotyping analysis showed genotypes as follows: 380 (70.4%) subjects were wildtype homozygotes (CC), 129 (23.9%) subjects were heterozygotes (CG) and 31 (5.7%) were homozygote mutants (GG). Based on these

genotypes, the population allele frequencies are 0.823 and 0.177 for the normal (C) and risk (G) alleles respectively at this locus.

Discussion

Three genotyping methods were compared in this study independently using DNA samples representing all possible genotypes of the p.C282Y and p.H63D mutations of HFE. We have developed and validated two new molecular beacon-based real-time PCR genotyping assays, and performed a head to head comparison against the restriction enzyme digestion and direct sequencing reference methods.

Both the new real-time PCR assays provided unambiguous genotyping in total concordance with reference methods. These real-time assays were simple, inexpensive, fast, and less labor intensive in relation to restriction digestion-based mutation analyses and direct sequencing. Therefore, the closed-tube allelic discrimination assays developed by this study have several advantages over other mutation detection techniques: a) the hairpin-shaped molecular beacons are extremely specific in distinguishing single base-pair variants; b) there is significant reduction of post-PCR cross-contamination risk, and c) the technology can readily be adapted to a high-throughput format (e.g., 96 or 384-well assays). For this purpose, we used the molecular beacon based assay for analysis of 540 random anonymized DNA samples derived from Saudi newborn subjects, using the 384-well genotyping format and the ABI Prism 7900 HT sequence detection system. Genotyping could be performed in less than two hours per run allowing the analysis of thousands of DNA samples per day. Such a true high

throughput setting is essential to the performance of large population-based studies for the establishment of allele frequencies. The establishment of population based allele frequencies for common disease related mutations or variants are a necessary prelude to the clinical use of genotyping of these loci. Based on these results, the two assays which we have developed possess adequate target specificity and are readily performed in a high throughput format to facilitate population based studies.

Population *HFE* studies for most of the world regions are missing, and previous genotyping studies indicated that siblings of non-expressing (asymptomatic) homozygotes have been found to have iron overload [9, 16]. In the Saudi population incidence of the *HFE* A or risk allele of P.C282Y, which is the primary mutation underlying HH in other populations, was extremely low (<0.001). Alternately, incidence (0.177) of the C or risk allele underlying the *HFE* p.H63D mutation was relatively common based upon reports in other populations [4, 17, 18]. Collectively the data suggests that the Saudi population is either at low risk for HH or that novel mutations of *HFE* may play a role in this population. The high incidence of p.H63D would elevate risk but does not have as strong an association with HH as p.C282Y [10, 19-21]. Sequencing of *HFE* in clinically well characterized patients of Saudi origin with HH is indicated to clearly establish the role of p.H63D or novel *HFE* mutations in this population.

Methods

DNA Samples

We utilized diagnostic samples received by our laboratory for *HFE* genotyping. Routinely, these samples are assayed using a restriction digest based methodology. A total of 23 samples of known genotypes for p.C282Y and p.H63D were selected and anonymized prior to being used in assay development and validation. For population studies, we have utilized a total of 540 anonymized newborn dry bloodspots as a source of template DNA. Samples collection and enrollment in this study was approved by our institutional review board (IRB) according to the Helsinki Declaration and guidelines.

Whole Genome Amplification (WGA)

DNA was amplified from dried blood spots using a modified WGA method [22]. A Repli_g 100 kit (Qiagen, Valencia, CA) was used for amplification. In brief, 1.2 mm punch biopsies were derived from the dried blood spots using a Harris Micro Punch (Whatman Inc, USA), and dispensed into 96-well plates. The punch biopsies were then soaked with 20 μ l of PBS, followed by addition of 20 μ l of lysis solution A according to the manufacturers recommendations. The plates were allowed to sit on ice for 10 minutes to allow release of nuclei from the discs. Each sample was then neutralized by adding 20 μ l of solution B. From each lysis mix, 2 μ l was transferred to 8 μ l of the WGA reaction mix present in each well of a 96-well micro plate, bringing the final reaction volume to 10 μ l per sample. Reaction mixtures were incubated for 16 hr at 30°C on an MJ Research thermocycler (MJ Research, Watertown, MA, USA) and terminated by

heating to 65°C for 5 min. Dilutions from the crude amplification mixture were directly used for genotyping.

***HFE* Genotyping by Restriction Enzyme Digestion**

Restriction digestion was carried out as described [4]. Briefly, two amplicons encompassing the p.C282Y and p.H63D mutation sites were generated by PCR prior to restriction digestion analysis. Amplification was performed in total volume of 25 µl. PCR reactions were carried out using an MJ Research thermocycler (MJ Research, Watertown, MA, USA). Each PCR reaction contained 100 ng gDNA, 1X PCR buffer, 1.5 mM MgCl₂, 0.25 mM dNTPs, 0.5 µM forward/reverse primers, and 1U *Taq* polymerase (Qiagen, Valencia, CA). The primers TGGCAAGGGTAAACAGATCC and CTCAGCTCCTGGCTCTCAT were used to generate a 357 bp genomic fragment that contained the c.845G>A mutation site in the *HFE* gene. Primers ACATGGTTAAGGCCTGTTGC and CTTGCTGTGGTTGTGATTTTCC were used to generate a 294 bp genomic fragment that contains the c.187C>G mutation site in the *HFE* gene. In each instance, amplification was performed after an initial activation step for *Taq* polymerase of 15 minutes at 95°C followed by 35 cycles each of 1 min at 95°C, 1 min at 56°C, and 1 min at 72°C. A final extension step of 10 min at 72°C was also used. Following PCR, restriction enzyme digestion reactions were set up as follows: 10 µl PCR product, 2 µl restriction enzyme (RE), and 1X RE buffer. *RsaI* (10 u/µl), and *MboI* (10 u/µl) (Stratagene, La Jolla, CA, USA), were used to digest the c.845G>A and c.187C>G mutation-containing fragments respectively. Digestion products were run on a 2% agarose gel for 30 min at 120V.

PCR and Direct Sequencing

Amplicons encompassing the p.C282Y and p.H63D mutation sites of HFE were generated by PCR amplification using gDNA samples. Primer pairs used generated 388 bp and 486 bp fragments for p.C282Y and p.H63D respectively. Sequencing reactions were performed using an Amersham ET Dye Terminator sequencing kit (Amersham Biosciences, Piscataway, NJ, USA) following the manufacturers instructions. Sequencing reactions were desalted and unincorporated nucleotides removed using ethanol precipitation, and then re-suspended in a formamide EDTA solution for injection on a MegaBACE 1000 capillary electrophoresis system (Molecular Dynamics, Sunnyvale, CA, USA). Sequence analysis was performed using the SeqMan module of the Lasergene software package (DNA Star Inc, Madison, WI, USA) by comparing alignments of multiple samples with each other and a Genbank reference sequence (U60319).

Genotyping by Molecular Beacon Assays

Real-time small scale PCR assays were performed in 0.2 ml clear flat top tubes (Phenix, Hayward, CA, USA) for use with the Rotor Gene 3000 (Corbett Research, Sydney, Australia). For large scale assays, 384-well clear optical reaction plates were used with the ABI Prism 7900 HT sequence detection system (Applied Biosystems, Foster City, CA). PCR mixtures consisted of 1X qPCR mastermix plus (Eurogentec, Seraing, Belgium), 0.5 μ M forward/reverse primers, 0.25 μ M of each molecular beacon probe,

and 20 ng of gDNA template. Reaction tubes were subjected to an initial 2 min incubation at 50°C (Uracil-N-glycosylase will eliminate carry over amplicon contaminants in this step), then a 10 min incubation at 95°C for *Taq* polymerase activation, followed by 40 PCR cycles. Cycling conditions were 95°C denaturation for 10 sec, 58°C annealing for 30 sec, and 72°C extension for 30 sec. A negative control (deionised water) was included in each assay. The following allele-specific molecular beacons and primers were designed and used in the assays (all were sourced from Proligo, Paris, France):

C282 assay:

Wild type beacon: FAM-ccggc AGATATACGT**G**CCAGGTGG gccgg-DABCYL

Mutant beacon: TET-ccgcc GAGATATACGT**A**CCAGGTGGA ggcgg-DABCYL

Forward primer: GGCTGGATAACCTTGGCTGT

Reverse primer: GATCACAATGAGGGGCTGAT

p.H63D assay:

Wild type beacon: FAM-ccgctg CTATGATCATGAGAGTCGCC cagcgg-DABCYL

Mutant beacon: TET-ccgctg CTATGATGATGAGAGTCGCC cagcgg-DABCYL

Forward primers: CTTTGGGCTACGTGGATGAC

Reverse primer: TGGCTTGAAATTCTACTGGAAA

In the beacon sequences, underlined lower case bases refer to the complementary arms. Bolded and capitalized bases represent the allelic variants at each locus. Genotype calling of DNA samples was determined by measuring the threshold cycle (Ct) value for each sample. Fluorescence data was collected during each annealing step throughout cycling

at which point the molecular beacon is bound to its complementary target amplicon. Results were displayed as an amplification plot for fluorescence versus cycle number. The threshold cycle is the cycle at which the fluorescent signal is first reported above the set baseline fluorescence. The baseline fluorescence was set at 10% above the average fluorescence value of PCR cycles 1 through 18. Genotyping of DNA samples was based on positive threshold cycles for FAM, TET, or both. Positive Ct values were usually detected between cycles 25-30, and indicated the presence of specific allele(s).

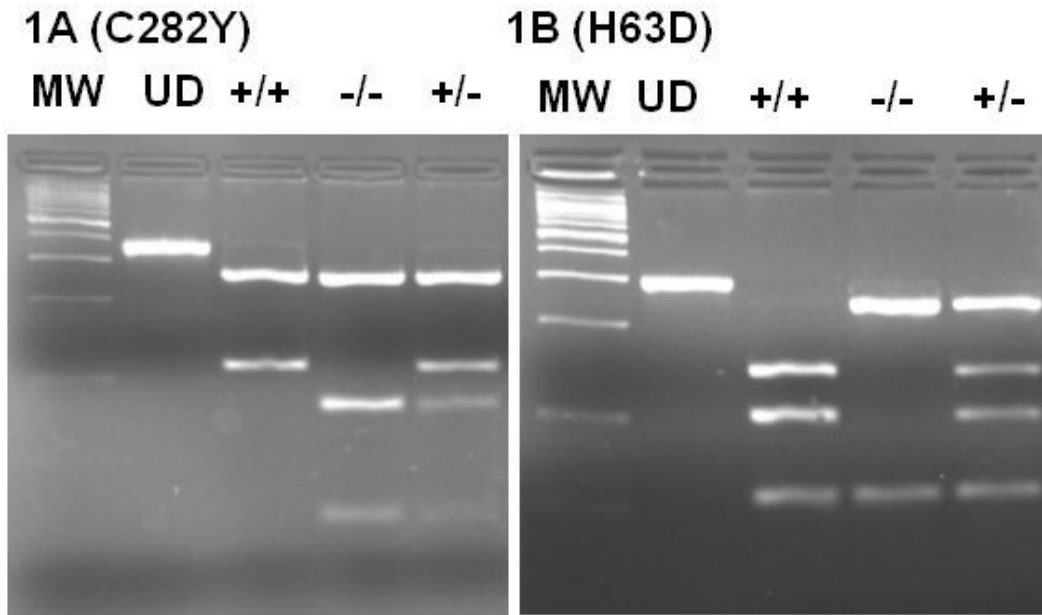
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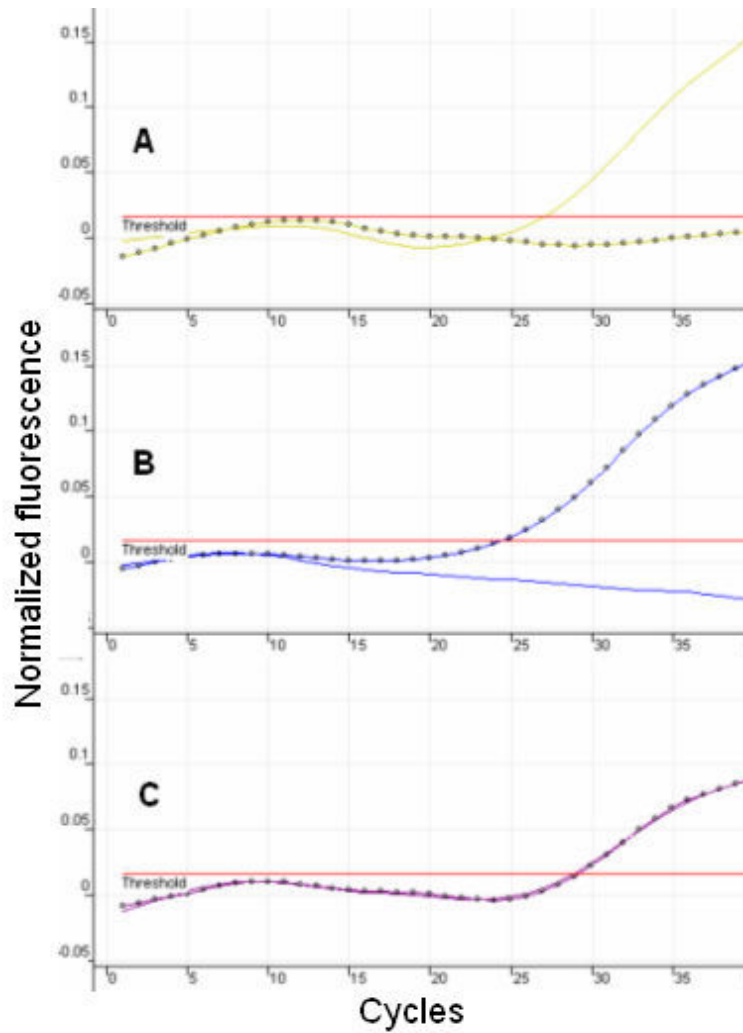
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Figure 1.



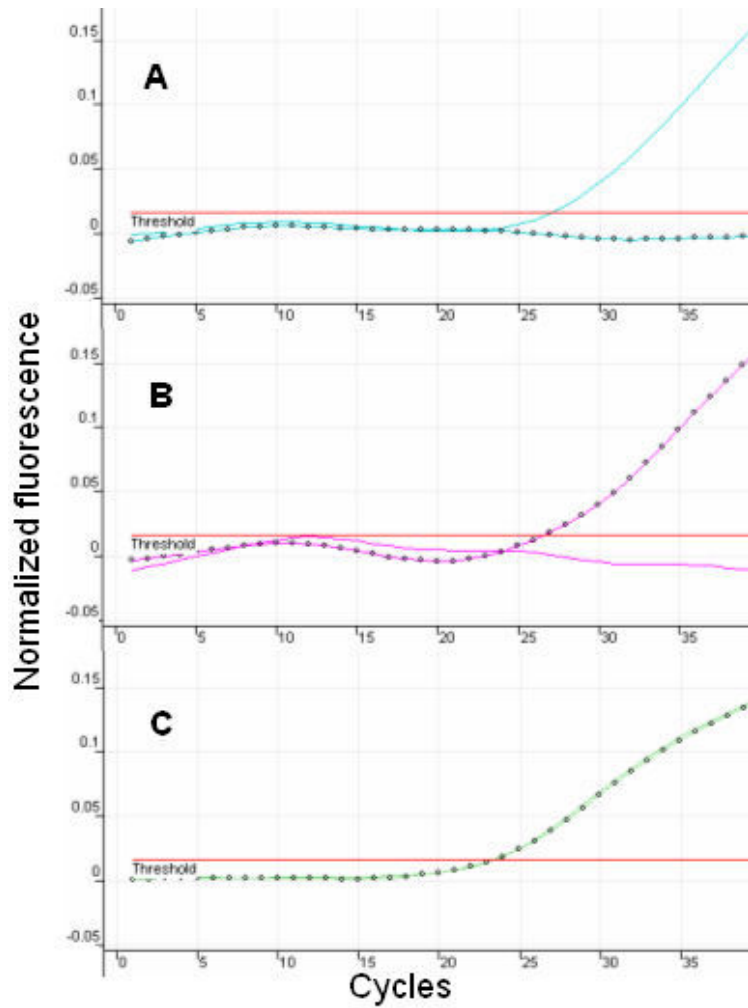
2% agarose gel showing the restriction digestion fragments for detection of p.C282Y (1A) and p.H63D (1B) mutations. Undigested (UD), normal (+/+), mutant (-/-), and heterozygote (+/-).

Figure 2:



FAM signal; (—); TET signal (—○—). Representative real-time PCR genotyping results for p.C282Y variants are shown. (A) Normal (FAM positive, TET negative), (B) mutant genotype (FAM negative TET positive), and (C) heterozygote (FAM positive TET positive).

Figure 3:



FAM signal; (—); TET signal (—○—). Representative real-time PCR genotyping results for p.H63D variants are shown. (A) Normal (FAM positive, TET negative), (B) mutant genotype (FAM negative TET positive), and (C) heterozygote (FAM positive TET positive).