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Single tube melting temperature assay for rapid and sensitive detection of the most frequent hemocromatosis mutations, C282Y and H63D

We report the development of a single-tube assay for rapid genotyping of the two mutations C282Y and H63D of the HFE gene, commonly detected by PCR-RFLP and responsible for 80% of cases of hereditary hemochromatosis. The method, named T melting curve analysis, discriminates between amplification products by their melting temperatures.

Sir,

The eight hemochromatosis (HH) patients in this study included: two homozygotes for C282Y, two homozygotes for H63D, two compound heterozygotes and two heterozygotes for C282Y. Four healthy individuals, two heterozygotes for H63D and two without HFE mutations, were also studied.

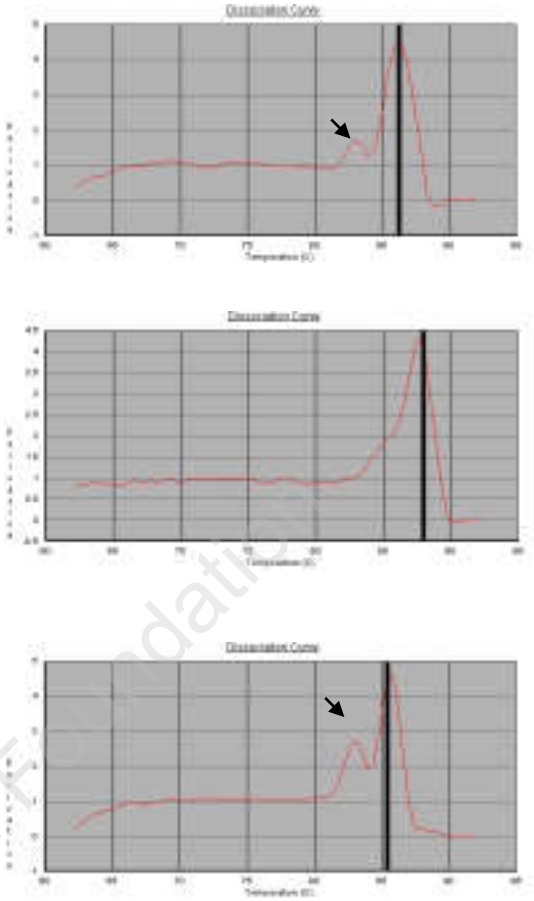

Figure 1. Melting profile of the wild type (A), mutated (B) and heterozygous (C) genotypes at position 63 of HFE gene. On the X axis there are the temperatures at which fluorescent data was collected (see materials and methods), the Y-axis shows the first derivative of the above data. The vertical bold bar highlights the melting point at which the amplicons reassociate. PCR has been optimized in order to better discriminate the different genotypes. A good Tm profile is represented in panel B because of low presence of primer dimers, still present in panels A and C, as the lower peak which is indicated by arrows. Nevertheless, even the heterozygous (C) and the wild type (A) are easily distinguishable from the primer dimer product.

DNA was isolated from peripheral whole blood using standard protocols. The HFE genotyping was performed by PCR-RFLP using the procedure described by Bacon *et al*.[1]

The melting temperature assay is based on the ability to distinguish between PCR amplification products by their melting temperature (Tm).[2] The single tube fluorescent PCR assay uses a specific primer set, with a high annealing temperature, and the SYBR green I fluorescent dye, able to detect double stranded DNA products. The temperature at which double-strand templates dissociate depends on the product length, GC content and sequence structure. The

PCR product has a distinct Tm, depending on base substitutions, single point mutations and insertions-deletions.[3] The PCR was performed on the 5700 Sequence Detection System (PE Biosystems). The analyzed samples were run in quadruplicate and in 3 different PCR runs. No template controls (NTCs) were run in order to verify non-specific PCR product formation.

For genotyping the mutations C282Y and H63D, genomic DNA was run in a primer optimization matrix (primers' concentrations ranging from 50 to 300mM). Forward/reverse primer combinations 50/300, 50/50 and 300/50 gave no aspecific products. Using these primer combinations in the presence of target DNA, the PCR product was detectable after 27, 34 and 30 cycles, respectively. Because lower cycle thresholds are related to a higher sensitivity of the test, the 50/300 mM combination was chosen. Three different PCR reactions were done for each sample, and each amplification was repeated four times. The resulting Tm profiles of three representative genotypes at codon 63 are shown in Figure 1.

The mutations found in 12 individuals are summarized in Table 1. For each subject, the HFE genotype and the Tm mean values (± standard deviation) are shown. We successfully characterized the mutant alleles of the patients, confirming the data obtained by PCR-RLFP (data not shown). The homozygous C282Y genotype shows a Tm mean value of 84.7°C, distinct from those of heterozygous (83.8°C) and wild type homozygous (83.0 °C) individuals. Also for position 63, different Tm mean values characterize mutant homozygous (86.2°C), heterozygous (85.4°C) and wild type homozygous (88.0°C) subjects.

We have developed an easy, inexpensive, automated method for determining the genotypes of the two mutations typical of hemochromatosis. The method is not dependent on fluorescent probes and eliminates post-PCR processing. The reproducibility of the method allowed us to test several subjects; all the samples were successfully and clearly genotyped; the Tm genotyping study was confirmed by PCR- RLFP.

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Table 1. Tm profiles in 12 individuals with different HFE genotype at position 282 and 63.

Cases	Genotype of codon 282	Genotype of codon 63	Tm at position 282 mean value (°C)	Tm at position 282 SD	Tm at position 63 mean value (°C)	Tm at position 63 SD
1	+/+	-/-	84.70	0.03	88.00	0.07
2	+/+	-/-	84.70	0.03	88.00	0.01
3	-/-	+/+	83.00	0.00	86.20	0.02
4	-/-	+/+	83.00	0.10	86.20	0.01
5	+/-	+/-	83.80	0.18	85.40	0.10
6	+/-	+/-	83.80	0.02	85.40	0.02
7	+/-	-/-	83.80	0.15	88.00	0.10
8	+/-	-/-	83.80	0.20	88.00	0.20
9	-/-	+/-	83.00	0.01	85.40	0.12
10	-/-	+/-	83.00	0.02	85.40	0.10
11	-/-	-/-	83.00	0.56	88.00	0.02
12	-/-	-/-	83.00	0.11	88.00	0.20

Tm: Melting tempetature; SD: Standard deviation; +: mutant allele; -: wild type allele.

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Screening for (--^{SEA}) α-globin gene deletion in β-thalassemia carriers and prevention of hydrops fetalis

In Southeast Asia, where thalassemia is prevalent, heterozygous carriers of both β-thalassemia mutation and the (--^{SEA}) α-globin gene deletion are encountered. These individuals are potentially at risk of being parents to off-springs affected by β-thalassemia major, hemoglobin (Hb) H disease, and Hb Bart's hydrops fetalis. We propose screening for the (--^{SEA}) α-thalassemia mutation in all β-thalassemia carriers to prevent hydrops fetalis.

Sir,

Hb Bart's hydrops fetalis, caused by deletion of all four α-globin genes, is associated with a dismal prognosis for the affected fetus, who will usually die in utero during the third trimester or shortly after birth.[1] Moreover, there is an increased incidence of serious maternal complications in these pregnancies. Preg-