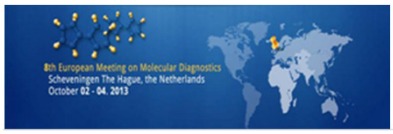

OPTIMISATION OF AN IN-HOUSE MULTIPLEX REAL-TIME PCR FOR THE SIMULTANEOUS DETECTION OF 3 MAJOR MUTATIONS IN THE HEMOCHROMATOSIS (HFE) GENE ON THE ROTOR GENE Q

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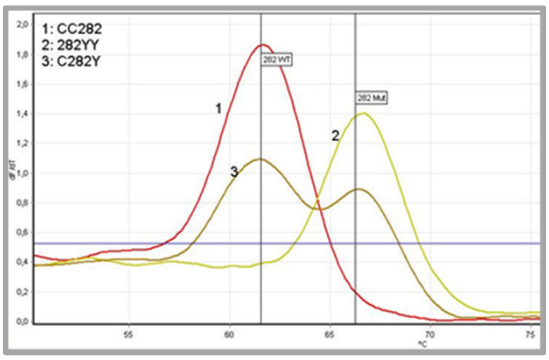
Background

Hereditary hemochromatosis (HH) is the most frequent genetic disease in Europe characterized by excessive iron overload that leads to multi-organ failure. The most prevalent genotype in HH is homozygosity for C282Y mutation of the HFE gene. 2 additional mutations, H63D and S65C, appear to be associated with a milder form of HH

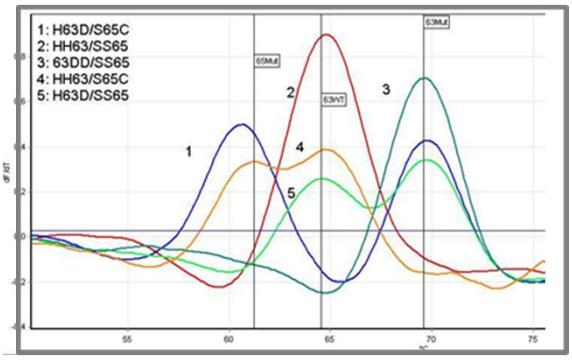
Methods (1)

The mutations are detected by a multiplex real-time PCR followed by melting curve analysis with adjacent fluorescent probes using the quenched-FRET principle.
Primers and probes were chosen from the literature (C.B Moysés et al, Brazilian J of Med and Biological Research 2008).

The C282Y FRET probe is designed to match the mutated allele sequence and is labelled with FAM. Therefore melting occur earlier in samples containing the wild type (WT) sequence (61.55 C) than in those containing the mutated sequence (66.24 C).
Heterozygous samples show two melting peaks,



The H63D/S65C FRET probe is designed to be complementary to the mutated/wild type allele, respectively, and is labelled with a different fluorophore (JOE). The difference in melting temperature among H63D wild type, H63D mutant and S65C alleles is greater than 3 C, allowing accurate determination of all 6 possible allele combinations. 3 different bins are defined at temperatures 61.23 C (65Mutation), 64.54 C (63WT) and 69.60 C (63Mutation).



Methods (2)

DNA from 35 patients and 23 external quality controls was extracted from EDTA blood using the MagNA Pure platform (Roche, DNA I High Performance protocol). 3 µl DNA was added to 18 µl mastermix. The mastermix used was LC480 Probes Master (Roche). The final concentration of the reverse primers and the probes was 0.2 µM. The forward primers had a final concentration of 0.06 µM.

PCR profil on the Rotor Gene Q: 95 C for 5 min as the initial denaturation step, followed by 50 cycles of 95 C for 10 s, 55 C for 20 s, and 72 C for 20 s.
After the PCR 3 hold steps were added: 20 s 95 C, 1 min 40 C, 1 min 50 C,
Thereafter, melting curve analysis was performed, starting from 50 C and proceeding until 88 C, at a linear rate of 0,5 C every 4 s.

The results were compared with the CE/IVD labelled “Hereditary Hemochromatosis” kit of AutoImmun Diagnostika (AID), which is a line probe assay.

Results

All possible genotype combinations for C282Y and H63D were included in this study. As the AID kit can not discriminate between the homozygous 63 DD allele mutation and the compound heterozygosity H63D and S65C, one sample was discordant. This sample was sent to another lab for confirmation.
The results for the S65C mutation were for all 23 quality controls concordant with the expected results.

Conclusion

The genotyping method is accurate, rapid, cost-effective and less time consuming than the line probe assay and is therefore implementated in our daily routine

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