Multiplex Genotyping by Small Amplicon Melting and Unlabeled Probes: Application to HFE genotyping

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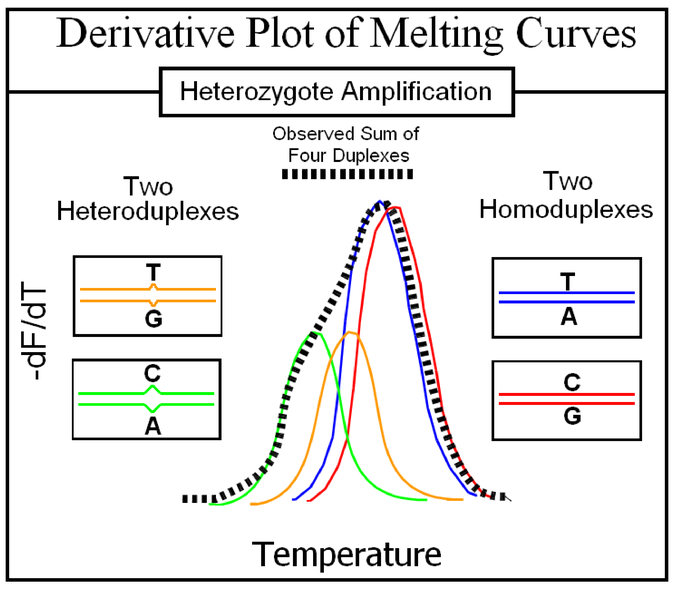
Abstract

Simple methods of genotyping by melting analysis without labeled probes have recently appeared. Small amplicon genotyping requires only two PCR primers and a generic DNA dye. Unlabeled probe genotyping incorporates an additional 3'-blocked unlabeled probe. Small amplicon and unlabeled probe genotypes can be extracted from the same melting curve after multiplex PCR. This combined method was applied to genotyping all common alleles of the HFE gene (HLA-H). Mutations in the HFE gene are responsible for hereditary hemochromatosis, an autosomal recessive disorder of iron metabolism.

PCR was performed on a LightCycler (Roche). High resolution melting analysis was performed on an HR-1 and a LightScanner (Idaho Technology). The dsDNA fluorescence dye LCGreen Plus (Idaho Technology) was used to monitor PCR amplification and subsequent melting analysis. The PCR reaction used two primer sets. One amplicon incorporates H63D and S65C and the other C282Y. The unlabeled probe covers the H63D mutation and T189C polymorphism but does not span the S65C site. An unlabeled probe is needed to genotype the H63D site because a C is exchanged for a G which, due to the nearest neighbor parameters of that particular sequence, makes the Tm of the homozygous mutant the same as wild type.

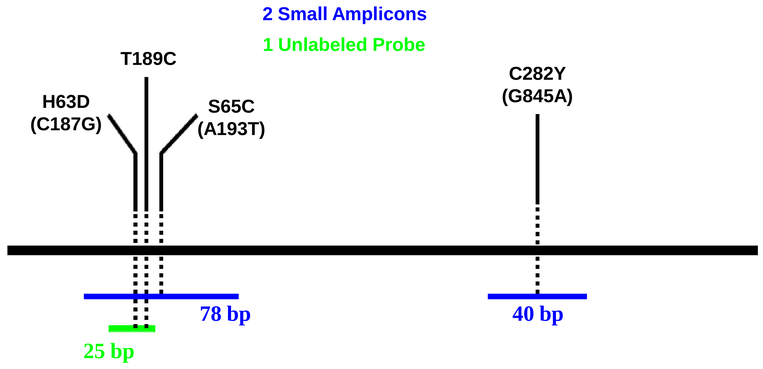
There are three main mutations associated with hemochromatosis: C282Y (G845A), H63D (C187G) and S65C (A193T). There is also a polymorphism, T189C which is a wobble at the third base of a histidine codon. The nine most common genotypes of HFE were all differentiated, including wild type, homozygotes for C282Y and H63D, heterozygotes for C282Y, H63D, S65C and T189C and the compound heterozygotes C282Y/H63D and H63D/S65C. Multiplex genotyping by melting of small amplicons and unlabeled probes is simple, inexpensive, and rapid. Regions of considerable sequence variation can be genotyped in a single color, closed-tube system by rapid PCR followed by high-resolution melting analysis. Real-time monitoring of PCR is not necessary.

High Resolution Melting



Schematic representation of the DNA melting analysis of a heterozygous SNP. The observed melting curve is the sum of 4 DNA duplexes : 2 homozygotes and 2 heterozygotes. These 4 duplexes are formed after PCR by denaturing the amplicons and then rapidly cooling to below the annealing temperature. This forces some of the amplicons to form heteroduplexes.

Multiplex Schematic



Nearest Neighbor Symmetry of H63D The Need for a Probe

wild type	mutant
5′atCat	5′atGat
3′taGta	3′taCta

PCR Protocol

C282Y Primers	0.025 uM each
H63D Primers	F 0.1/R 0.5 uM
H63D Probe	0.4 uM

Note: H63D Primers are Asymmetric

H63D

```
Forward   5'-tgggctacgtggatga
Reverse   5'-aaacccatggagttcgg
Probe     5'-gctgttcgtgttctatgatCatgag-P
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C282Y

```
Forward   5'-tggggaagagcagagatatac
Reverse   5'-tgggtgctccacctg
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3 mM $MgCl_2$, 200 uM each dNTP, 0.4 U/10 ul KlenTaq, 500 ug/ml BSA, 5 ng Template DNA

Thermocycling - Total Time

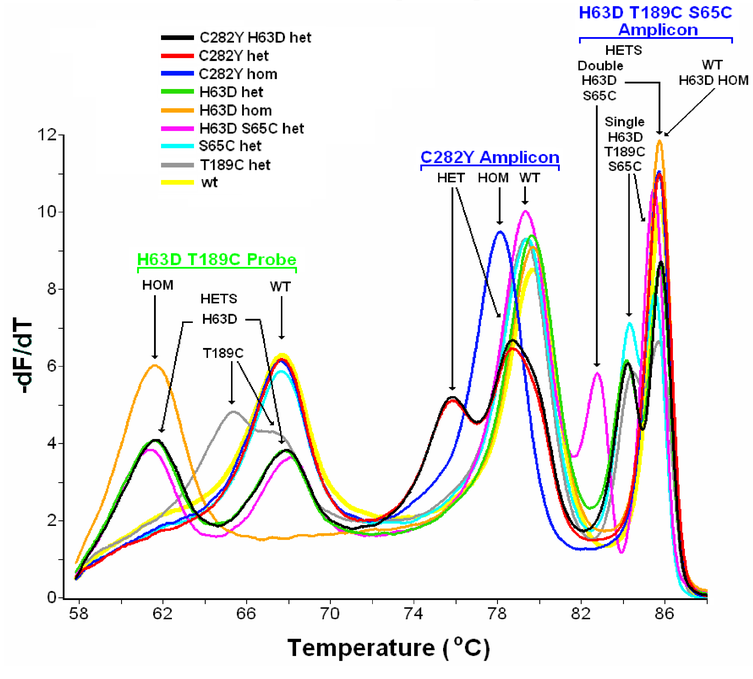
LightCycler 17 min

Block Cycler 48 min

1 cycle	50 cycles	1 cycle
94 ºC / 15s	94 ºC /1s 60 ºC /1s 75 ºC /2s ramp@ 2ºC/s	94 ºC/1s 45 ºC/15s

HR-1

3 min 30 s per Sample



LightScanner

8 min per Plate