



Robert Pryor<sup>1</sup>, Michael Liew<sup>2</sup> Robert Palais<sup>3</sup>, and Carl Wittwer<sup>1,2</sup>  
<sup>1</sup> Dept. of Pathology, Univ. of Utah School of Medicine, SLC, UT 84132.  
<sup>2</sup> Institute for Clinical and Experimental Pathology, ARUP, SLC UT 84108  
<sup>3</sup> Dept. of Mathematics, Univ. of Utah, SLC, UT 84112

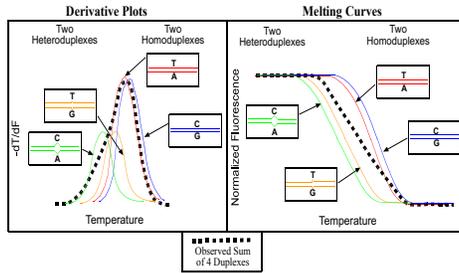


**Abstract**

Homogeneous PCR methods for genotyping single nucleotide polymorphisms (SNPs) usually require fluorescently labeled oligonucleotide probes or allele specific amplification. High-resolution melting of amplicons with the DNA dye LCGreen I (Idaho Technology) is a homogeneous, closed-tube method of heteroduplex detection that does not require probes or real-time PCR (Wittwer et al, Clin Chem 2003;49:853-60). We adapted this system to genotype SNPs after rapid cycle PCR (12 min) of small amplicons (<50 bp). All possible SNPs were systematically studied with engineered plasmids. In addition, the clinical SNP targets, factor V (Leiden) G1691A, HFE C187G, beta globin (HbS) A17T, MTHFR A1298C, and prothrombin G20210A were studied.

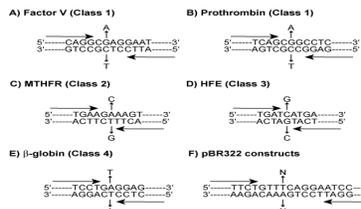
In all cases, heterozygotes were easily identified because the heteroduplexes produced changed the shape of the melting curve. In most cases, homozygous polymorphisms were also distinguishable from each other by melting temperature (T<sub>m</sub>) shifts. When the amplicon size is small, these differences are large enough that they can usually be seen on regular (low-resolution) real-time instruments. However, about 15-20% of SNPs are A/T or G/C exchanges with very small T<sub>m</sub> differences between homozygotes. These differences require high-resolution instrumentation (HR-1, Idaho Technology) for complete genotyping. Even with high-resolution analysis, one-quarter of A/T and G/C SNPs show nearest neighbor symmetry, and, as predicted by this model, the homozygotes cannot be resolved. In these rare cases, adding 15-20% of a known homozygous genotype to unknown samples produces different amounts of heteroduplexes and clustering of the melting curves according to genotype. The method is simple, rapid, and inexpensive, requiring only PCR, a DNA dye, and melting instrumentation.

**Introduction to Melting Analysis**



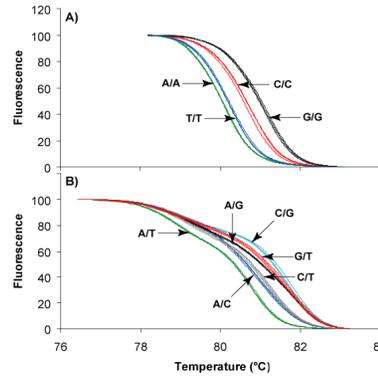
**Figure 1.** Schematic representation of the DNA melting analysis of a heterozygous SNP. The observed melting curve is the sum of 4 DNA duplexes : 2 heterozygotes and 2 homozygotes. These 4 duplexes are formed after PCR by denaturing the amplicons and then rapidly cooling to below the annealing temperature. This forces some of the amplicons to form heteroduplexes.

**Primer Design Flanking SNP Site**



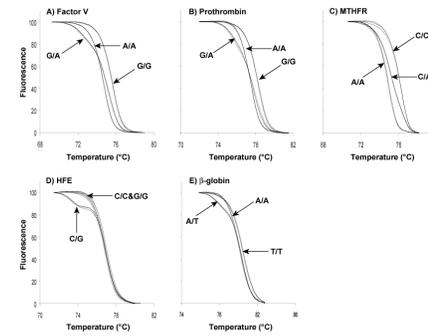
**Figure 2.** Primer sets for amplification of engineered and clinical SNP targets. Designed using SNPWizard (dnawizards.path.utah.edu).

**Engineered SNP pBR322 Plasmids**



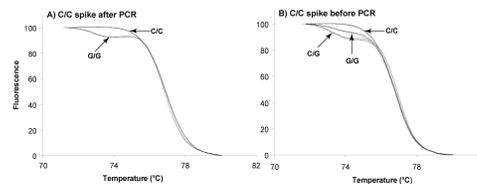
**Figure 3.** Normalized, high-resolution melting curves of all possible SNP genotypes at one position using engineered plasmids. Three samples of each genotype were analyzed and included four homozygotes (A) and six heterozygotes (B).

**Clinical Samples**



**Figure 4.** Normalized, high-resolution melting curves from: A) factor V Leiden G1691A (Class 1), B) prothrombin G20210A (Class 1), C) MTHFR A1298C (Class 2), D) HFE C187G (Class 3), and E) β-globin A17T (Class 4) SNPs. Three individuals of each genotype were analyzed and are displayed for each SNP.

**Spiking Experiments**



**Figure 5.** Genotyping at the HFE C187G locus by adding wild type DNA to each sample. In A) wild type amplicons were mixed with amplicons from three individuals of each homozygous genotype after PCR. In B) 15% wild type genomic DNA was added to the DNA of three individuals of each genotype before PCR.

**Validation of Assay**

MARKER	GENOTYPES	HybProbe	Amplicon melting
Factor V G1891A	Wild type	35	35
	Heterozygous	35	35
	Homozygous mutant	34	34
Prothrombin G20210A	Wild type	8	8
	Heterozygous	3	3
	Homozygous mutant	11	11
MTHFR A1298C	Wild type	6	6
	Heterozygous	7	7
	Homozygous mutant	7	7
HFE C187G	Wild type	4	4 <sup>a</sup>
	Heterozygous	4	4
	Homozygous mutant	4	4 <sup>a</sup>
β-globin A17T	Wild type	3	3
	Heterozygous	3	3
	Homozygous mutant	3	3

**Figure 6.** Genotype concordance using adjacent hybridization probes (HybProbe™) and small amplicon, high resolution melting analysis (Amplicon melting). All samples were originally genotyped by ARUP (Factor V, prothrombin, MTHFR and HFE) or Pediatrx Screening (β-globin) as clinical samples with adjacent hybridization probes and melting curve analysis.

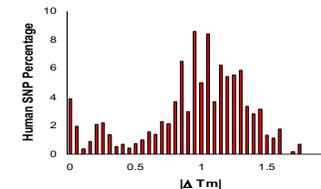
<sup>a</sup>Genotyping required spiking with homozygous DNA

**Frequency of Theoretical ΔTm of SNPs**

Class	SNP (frequency) <sup>a</sup>	Homoduplex Matches (# of Tms)	Heteroduplex Mismatches (# of Tms)	Example (Figure Number)
1	C/T or G/A (0.675)	C::G and A::T (2)	C::A and T::G (2 or 1) <sup>b</sup>	3B, 4A, 4B
2	C/A or G/T (0.169)	C::G and A::T (2)	C::T and A::G (2 or 1) <sup>b</sup>	3B, 4C
3	C/G (0.086)	C::G (2 or 1) <sup>b</sup>	C::C and G::G (2)	3B, 4D, 5
4	T/A (0.070)	A::T (2 or 1) <sup>b</sup>	T::T and A::A (2)	3B, 4E

**Figure 7.** SNP classification according to the homoduplexes and heteroduplexes produced. SNPs are specified with the alternative bases separated by a slash, for example C/T indicates that one DNA duplex has a C and the other a T at the same position on the equivalent strand. Base pairing is indicated by a double colon and is not directional.

<sup>a</sup> Human SNP frequencies from: Venter et al, Science 2001;291:1304-51.  
<sup>b</sup> The number of predicted thermodynamic duplexes depends on the nearest neighbor symmetry around the base change. One quarter of time, nearest neighbor symmetry is expected, that is, the position of the base change will be flanked on each side by complementary bases. For example, if a C/G SNP is flanked by an A and a T on the same strand (Fig. 2D), nearest neighbor symmetry occurs and nearly identical homoduplex Tms are expected (as observed in Fig. 4D).



**Figure 8.** In silico estimation of the T<sub>m</sub> difference between homozygous genotypes of small amplicon SNPs. The frequency distribution is adjusted for the relative occurrence of each SNP class in the human genome (see Figure 7). The larger the ΔT<sub>m</sub>, the easier it is to differentiate the homozygous genotypes. Approximately 4% of human SNPs have a predicted ΔT<sub>m</sub> of 0.00°C and are expected to require spiking with known homozygous DNA for genotyping of the homozygotes.