Spiking to Enhance Genotyping by High-Resolution Melting Without Probes

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Abstract

High-resolution melting of small amplicons is a homogeneous DNA analysis technique that can genotype most SNPs. A small proportion of homozygous SNPs are near the limit of the resolution of this method. We estimate the frequency of such cases based upon an in silico analysis of the melting temperature differences corresponding to the 96 possible nearest-neighbor configurations of over 5 million SNPS in the current human SNP database. We describe a homogeneous method for distinguishing melting curves of the unresolved homozygous SNPs from those of the wild-type and heterozygous DNA, and compare a mathematical model of the technique with experimental data.

Introduction to Melting Analysis

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.) Heterozygotes are 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 (Tm) shifts. However, about 15-20% of SNPs are A/T or G/C exchanges with very small Tm 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.

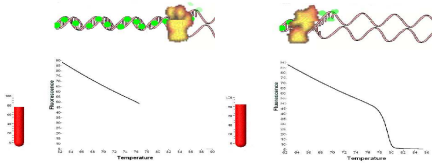


Figure 1. Double-stranded DNA captures intercalating dye molecules which fluoresce in this configuration. As temperature rises, DNA denatures. The dye molecules return to solution and their fluorescence decreases. This transition occurs very rapidly near a critical "melting temperature",Tm. Both Tm and the Fluorescence-Temperature graph, the melting curve, are extremely sensitive to DNA sequence in general and especially to base-mismatches which occur upon melting and re-annealing heterozygous DNA.

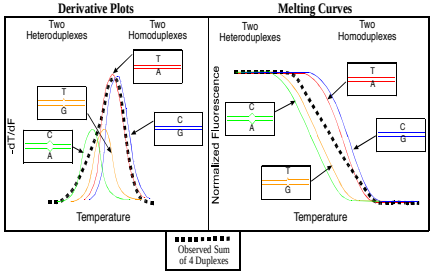


Figure 2. Schematic representation of the DNA melting analysis of a heterozygous SNP. The observed melting curve is the sum of signals produced by 4 types of DNA duplexes: 2 homoduplexes and 2 heteroduplexes. These are formed by denaturing the complementary amplicons produced by PCR and then rapidly cooling to below the annealing temperature. This forces some of the strands to pair into heteroduplexes.

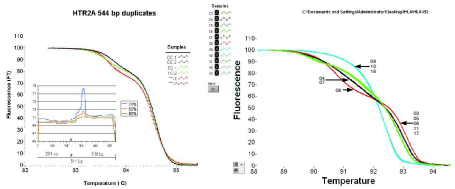


Figure 3a. Resolution. On the left are melting curves corresponding to single base changes in a 544bp amplicon. Two samples (black), two homozygous mutant (red), and two heterozygous mutant (green) curves are shown, and the genotypes are easily classified.

Figure 3b. Applications. On the right seventeen members of a family were analyzed by melting curve analysis. A highly variable region important for transplantation (HLA) was used. The melting curves group into clusters. Family members who are compatible for transplantation have melting curves in the same cluster. The same principle can be applied to forensics or microbe identification.

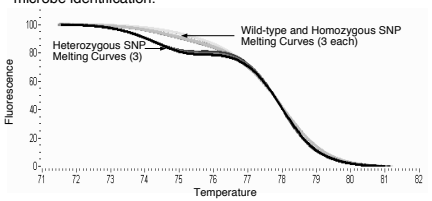


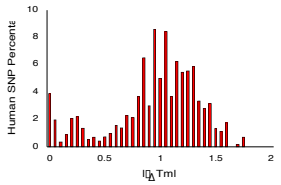
Figure 4. Exceptional Cases. About 15-20% of SNPs are A/T or G/C exchanges with very small Tm differences between homozygotes. These differences require high-resolution instrumentation (HR-1, Idaho Technology) for complete genotyping. Even with high-resolution analysis, one-quarter of A/T and G/C SNPs show nearest neighbor symmetry, and, as predicted by this model, the homozygotes cannot be resolved. In this figure, three replicates each of wild-type and mutant homozygous genotype melting curves overlap (upper curves). The lower curves are three replicates of the heterozygous genotype.

Genome-wide analysis of SNPs and their nearest-neighbor characteristics.

Figure 5. We analyzed the NIH database of Human SNPs, (URL: http://www.ncbi.nlm.nih.gov/SNP/index.html, Build 120, March, 16, 2004), of over 5 million standard SNPs, for the relative frequency of occurence of 16 types of nearest-neighbor configurations for the six types of SNPs.

Sample results for A/C SNPs are reported below, to the left. From this information, we can perform in silico prediction of melting curves and melting temperatures. The histogram below to the right gives the resulting distribution of Tm differences. The small leftmost peak represents the 4% of exceptional cases which require additional care for accurate genotyping. Updated overall SNP class frequencies determined from this search are: C/T or G/A (0.662), C/A or G/T (0.176), C/G (0.088), T/A (0.074).

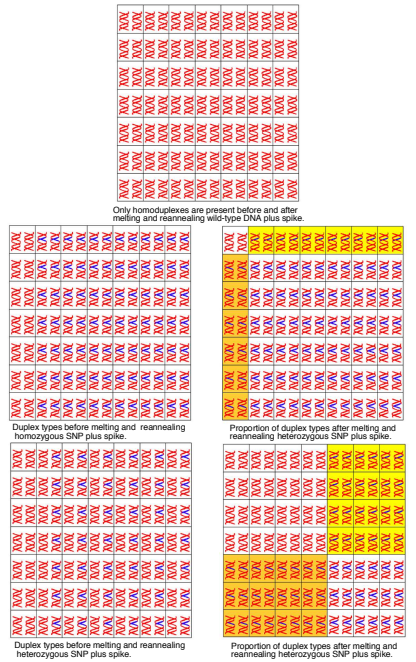
5'	SNP	3'	
A	A/C	A	88848
A	A/C	C	53983
A	A/C	G	26747
A	A/C	T	41793
C	A/C	A	49425
C	A/C	C	49869
C	A/C	G	26896
C	A/C	T	44862
G	A/C	A	39888
G	A/C	C	30967
G	A/C	G	22879
G	A/C	T	29987
T	A/C	A	38825
T	A/C	C	32228
T	A/C	G	14747
T	A/C	T	55633



How spiking with additional known wild-type DNA before PCR distinguishes unresolved genotypes.

Figure 6a. Without spiking. Above is wild-type DNA and a homozygous SNP which have similar melting temperatures and form no heteroduplexes upon melting and reannealing. Below is the corresponding heterozygous SNP. If we assume strands re-anneal independently after melting, 50% of the strands form heteroduplexes (yellow boxes), providing a significant change in melting curves and temperatures.

Figure 6b. With spiking. If additional known wild-type (a "spike") added to the an unknown sample before PCR, then after melting and reannealing, wild-type sample DNA is unaffected and still forms no heteroduplexes. A spiked homozygous SNP will form heteroduplexes and a spiked heterozygous SNP will form fewer than 50% heteroduplexes.



Experimental Validation of the Model

Figure 7. SNP melting curve separation according to the homoduplexes and heteroduplexes produced. If we assume that the separation of melting curves with homoduplexes which behave similarly is proportional to the concentration of heteroduplexes formed by melting and reannealing, the separation of each genotype from the wild-type can be modeled as a quadratic function of the proportion of spike in the mixture. Below, these curves are scaled and superimposed over the experimental maximum differences between standardly normalized melting curves of three melting curve replicates of each genotype and the mean wild-type melting curve, for a variety of spike proportions. The experimental results and the simple mathematical model reflect each other quite nicely. (Green: wild-type, Red: homozygous SNP, White: heterozygous SNP.)

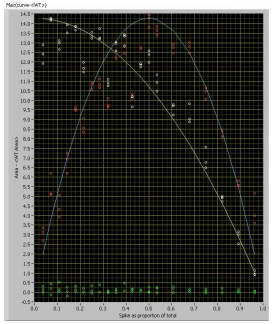
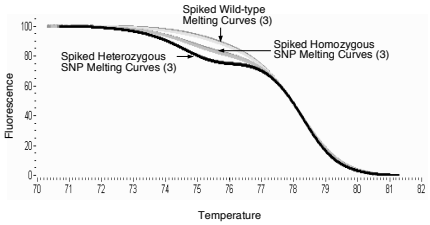


Figure 8. Spiking with an appropriate proportion resolves all exceptional cases. In this figure, the same replicate unknowns from figure 4 have been spiked with additional wild-type DNA. The melting curves now cluster into easily distinguished genotype classes.



Additional References

1. M. Liew, R. Pryor, R. Palais, C. Meadows, M. Erali, E Lyon, and C.T. Wittwer, Genotyping of Single-Nucleotide Polymorphisms by High-Resolution Melting of Small Amplicons, Clinical Chemistry, July 1, 2004, 50(7)

2. L. Zhou, J. Vandersteen, L. Wang, T. Fuller, M. Taylor, B. Palais, C.T. Wittwer, High-resolution DNA melting curve analysis to establish HLA genotypic identity. Tissue Antigens. July, 2004.