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Quantitative heteroduplex analysis for single nucleotide polymorphism genotyping

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Abstract

High-resolution melting of polymerase chain reaction (PCR) products can detect heterozygous mutations and most homozygous mutations without electrophoretic or chromatographic separations. However, some homozygous single nucleotide polymorphism (SNPs) have melting curves identical to that of the wild-type, as predicted by nearest neighbor thermodynamic models. In these cases, if DNA of a known reference genotype is added to each unknown before PCR, quantitative heteroduplex analysis can differentiate heterozygous, homozygous, and wild-type genotypes if the fraction of reference DNA is chosen carefully. Theoretical calculations suggest that melting curve separation is proportional to heteroduplex content difference and that the addition of reference homozygous DNA at one seventh of total DNA results in the best discrimination between the three genotypes of biallelic SNPs. This theory was verified experimentally by quantitative analysis of both high-resolution melting and temperature-gradient capillary electrophoresis data. Reference genotype proportions other than one seventh of total DNA were suboptimal and failed to distinguish some genotypes. Optimal mixing before PCR followed by high-resolution melting analysis permits genotyping of all SNPs with a single closed-tube analysis.

Keywords: High-resolution melting; SNP; Mixing; Spiking; Genotyping; Heteroduplex analysis; Quantitative TGCE analysis; Nearest neighbor symmetry

Heteroduplex analysis is a popular technique to screen for sequence variants in diploid DNA. After PCR, heteroduplexes are analyzed by separation techniques such as conventional gel electrophoresis [1–3], denaturing high-pressure liquid chromatography (dHPLC)[1][4], and temperature-gradient capillary electrophoresis (TGCE) [5]. Recently, heteroduplexes have been detected directly in solution after PCR by high-resolution melting analysis. Either labeled primers [6] or a saturating DNA dye [7] were used to detect a change in shape of the fluorescent melting curve resulting from heteroduplexes. High-resolution melting of PCR products from diploid DNA has been used for mutation scanning [8–11], HLA matching [12], and genotyping [7,13].

Heteroduplex analysis techniques using separation are seldom used for genotyping because different homozygotes are usually not resolved. Both dHPLC and TGCE usually fail to detect homozygous single nucleotide polymorphisms (SNPs) and small homozygous insertions and deletions. If suspected, these homozygous changes can be detected by mixing the PCR product of the unknown sample with the PCR product from a known homozygous reference sample. The mixture is denatured and then cooled to form heteroduplexes, and the separation is repeated. If heteroduplexes are detected in the mixture, the two samples are of different genotype. Two sequential analyses and manual processing are required, however, exposing the concentrated PCR product to the laboratory and increasing the chance of PCR product contamination of subsequent reactions.

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[1] *Abbreviations used:* dHPLC, denaturing high-pressure liquid chromatography; TGCE, temperature-gradient capillary electrophoresis; SNPs, single nucleotide polymorphisms.

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High-resolution melting can usually distinguish different homozygotes by a difference in melting temperature. Complete genotyping of human SNPs by high-resolution melting is possible in over 90% of cases using short PCR products [13]. However, with some SNPs, the melting curves may not distinguish the homozygous mutant from wild-type. Typically, this is due to a nearest neighbor thermodynamic symmetry [14,15] where the bases adjacent to the SNP are identical on both DNA strands and the SNP consists of an interchange between complementary bases. An example is the SNP causative for hemochromatosis (HFE) H63D, 187C > G. The two homozygous genotypes 5′-TCA-3′ and 5′-TGA-3′ have identical nearest neighbor pairs (TC/AG and CA/GT). For complete genotyping of these "symmetric" SNPs, post-PCR mixing and separation studies could be performed, but the advantage of closed-tube analysis is then lost. It is known that when DNA mixtures are amplified by PCR for heteroduplex detection, the strand proportions of different genotypes before and after amplification do not change [2,3]. This suggests that complete genotyping of these SNPs should also be possible by mixing unknown and reference samples before PCR instead of after.

Depending on both the fraction of reference DNA and the genotype of the unknown sample, different amounts of heteroduplexes that should allow discrimination of all genotypes will be produced. Previously, we empirically determined that such discrimination was possible with the addition of 15% homozygous reference DNA to 85% of unknown DNA prior to PCR [13]. However, the optimal percentage of reference DNA was not known.

We now present a predictive mathematical model for the heteroduplex content of mixtures with regards to the fraction of homozygous reference DNA added and the DNA of unknown genotype. The resultant heteroduplex content determines the extent to which the melting curve deviates from wild-type samples and the relative intensity of the heteroduplex TGCE peaks. The reference DNA fraction (wild-type) that optimally distinguishes genotypes is $\frac{1}{7}$ of the total DNA, resulting in predicted heteroduplex contents of 0 (wild-type), $\frac{12}{49}$ (heterozygote), and $\frac{24}{49}$ (homozygous mutant) This prediction was tested by amplifying various fractions of reference wild-type DNA mixed into each genotype, followed by quantitative high-resolution melting curve analysis and TGCE.

Materials and methods

Oligonucleotides were obtained from Integrated DNA Technologies and quantified by absorbance at 260 nm (A_{260}). Four oligonucleotides of sequence CCAGCTGTT CGTGTTCTATGATXATGAGAGTCGCCGTGTG and its complement CACACGGCGACTCTCATYATCATA GAACACGAACAGCTGG where X and Y were either C or G were purified by HPLC. Homoduplexes (X = C, Y = G or X = G, Y = C) or heteroduplexes (X = C, Y = C or X = G, Y = G) of the HFE 187C > G SNP were formed by binary combinations.

Nine whole-blood samples (from three people of each of the three HFE genotypes, wild-type, homozygous 187C > G, and heterozygous 187C > G) were obtained from ARUP laboratories after identifications were removed. Human genomic DNA was extracted from these samples (QIAamp DNA Blood Kit, Qiagen), concentrated by ethanol precipitation, and quantified by A_{260}. One of the wild-type samples was selected as the reference and mixed with the other samples prior to PCR. The final fraction of reference DNA ranged from 0 to 1. For each DNA sample, 21 different reference fractions and the original unmixed sample were amplified by PCR and analyzed by high-resolution melting and TGCE.

PCR

For high-resolution melting analysis, 40-bp products were amplified in a LightCycler (Roche) [13]. Ten-microliter reaction mixtures consisted of 25 ng of genomic DNA, 3 mM $MgCl_2$, 1× LightCycler FastStart DNA Master Hybridization Probes master mix, 1x LCGreen Plus (Idaho Technology), 0.5 μM forward (CCAGCTGTTCGTGTTC TATGAT) and reverse (CACACGGCGACTCTCAT) primers, and 0.01 U/μl *Escherichia coli* UNG (Roche). The PCR was initiated with a 10-min hold at 50 °C followed by a 10-min hold at 95 °C. Rapid thermal cycling was performed between 85 and 63 °C at a programmed transition rate of 20 °C/s for 40 cycles. After denaturation at 94 °C and rapid cooling to 40 °C, a melting curve was generated on the LightCycler at 0.05 °C/s to confirm the presence of amplicon. All DNA samples with a common reference fraction were amplified together, along with two heterozygous control samples with no added reference.

For TGCE analysis, a 242-bp product was amplified on a ABI 9700. Reactions components were as given above, except that 0.4 μM forward (CACATGGTTAAGGCCT GTTG) and reverse (GATCCCACCCTTTCAGACTC) primers were used. The PCR was initiated with a 10-min hold at 25 °C and a 6-min hold at 95 °C. Thermal cycling consisted of a 30-s hold at 94 °C, a 30-s hold at 62 °C, and a 1-min hold at 72 °C for 40 cycles, followed by a 7-min hold at 72 °C. The samples were then heated to 95 °C for 5 min followed by a cooling over 60 min to 25 °C for heteroduplex formation.

Analysis by high-resolution melting

Synthesized oligonucleotide duplexes (0.5 μM) were melted in PCR solution (see above). Prior to high-resolution melting analysis, all samples (synthesized and PCR generated) were heated to 94 °C and cooled to 40 °C at a programmed rate of 20 °C/s. High-resolution melting curves were obtained with an HR-1 instrument (Idaho Technology) by melting at a rate of 0.3 °C/s from 65 to 85 °C. Data were analyzed by custom software written in LabView (National Instruments) as previously described [7]. After normalization and removal of background fluorescence, data were usu-

ally temperature-shifted by superimposing their high-temperature regions where only the most stable homoduplexes remain. The difference between melting curves was quantified as the maximum vertical distance between curves. The heteroduplex proportion of a curve was estimated by this distance (from wild-type), normalized so that unmixed heterozygous samples equaled 0.5. Difference plots of the temperature-shifted data were created by subtracting the average of wild-type curves from sample curves to highlight the variation between genotypes.

Analysis by TGCE

PCR amplicons were transferred to 24-well TGCE trays, diluted 1:1 with 1× FastStart *Taq* polymerase PCR buffer, and overlaid with mineral oil. TGCE was performed using the Reveal mutation discovery system, reagents, and Revelation software (Spectrumedix) as previously described [16]. DNA samples were injected electro-kinetically at 2 kV for 45 s, resulting in peak heights ranging from 5000 to 40,000 intensity units with ethidium bromide staining. Optimal results were obtained when the temperature was ramped from 60 to 65 °C over 21 min and data were acquired over 35 min. Sequential camera images were converted to plots of image frame number (time) versus intensity units (DNA concentration).

TGCE data were fit to exponential decay distributions, one for each peak. Each exponential distribution was one-sided (i.e., zero to the left of the initial peak) and an iterative solution for the amplitudes and decay rates of successive peaks was obtained. The heteroduplex proportion was obtained by dividing the sum of the amplitudes of the two heteroduplex peaks by the sum of all peaks. This ratio was then adjusted by a factor (close to 1) so that heteroduplex controls were equal to the expected value of 0.5.

Results

Experimental melting curves of the thermodynamically symmetric SNP HFE H63D are shown in Figs. 1 and 2. Duplexes melted in Fig. 1 were formed by annealing equal concentrations of synthetic 40-mer oligonucleotides. After normalization of the melting curves between 0 and 100% fluorescence, both homoduplexes (wild-type TCA and mutant TGA) show identical melting transitions as predicted by nearest neighbor thermodynamics. Melting curves of the two pure heteroduplexes are distinguishable from each other and destabilized by about 3 °C from the homoduplexes. A mixture of all four strands in equal concentrations simulates melting of a heterozygous DNA sample after PCR amplification, resulting in a composite melting curve between the homoduplex and the heteroduplex melting curves.

Fig. 2A shows the melting curves produced from amplifying the three possible genotypes of H63D from human genomic DNA. Similar to the study with synthetic oligonucleotides, the homozygous mutant and wild-type samples are superimposed. The melting curve that results from amplification of heterozygous DNA includes both homoduplex and heteroduplex components, resulting in a curve

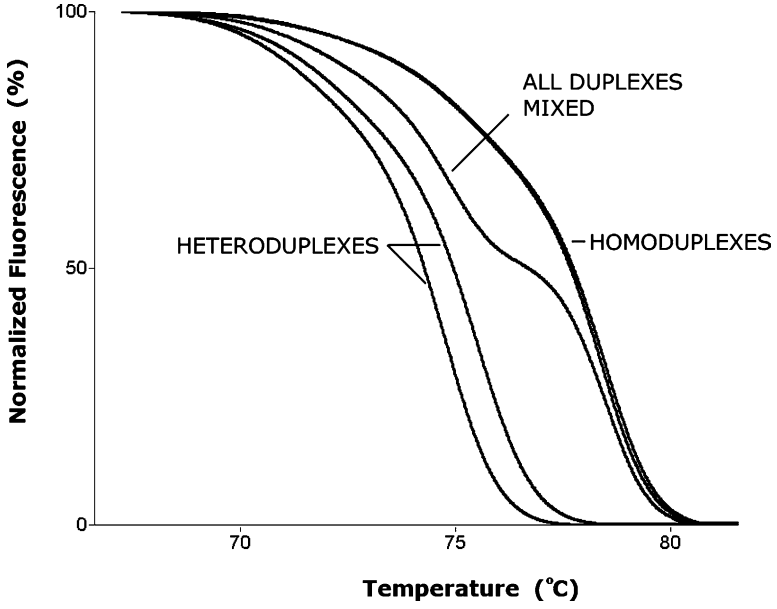

Fig. 1. Melting curves of synthesized oligonucleotide homoduplexes, heteroduplexes, and their mixture for an SNP with nearest neighbor symmetry. Normalized melting curves of synthetic 40-bp homoduplexes and heteroduplexes are shown. The sequences of the duplexes are the same as the HFE H63D (187C > G) PCR products used in the rest of the study. Binary mixtures of strands with complementary bases (C:G or G:C) at the SNP form homoduplexes that have identical melting curves because of nearest neighbor symmetry. Strands with mismatched base pairs at the SNP form heteroduplexes with destabilized melting curves (C:C less stable than G:G). Equal parts of all strands were mixed and the resulting melting curve in the center resembles that of the genomic heterozygous sample after PCR amplification (see Fig. 2).

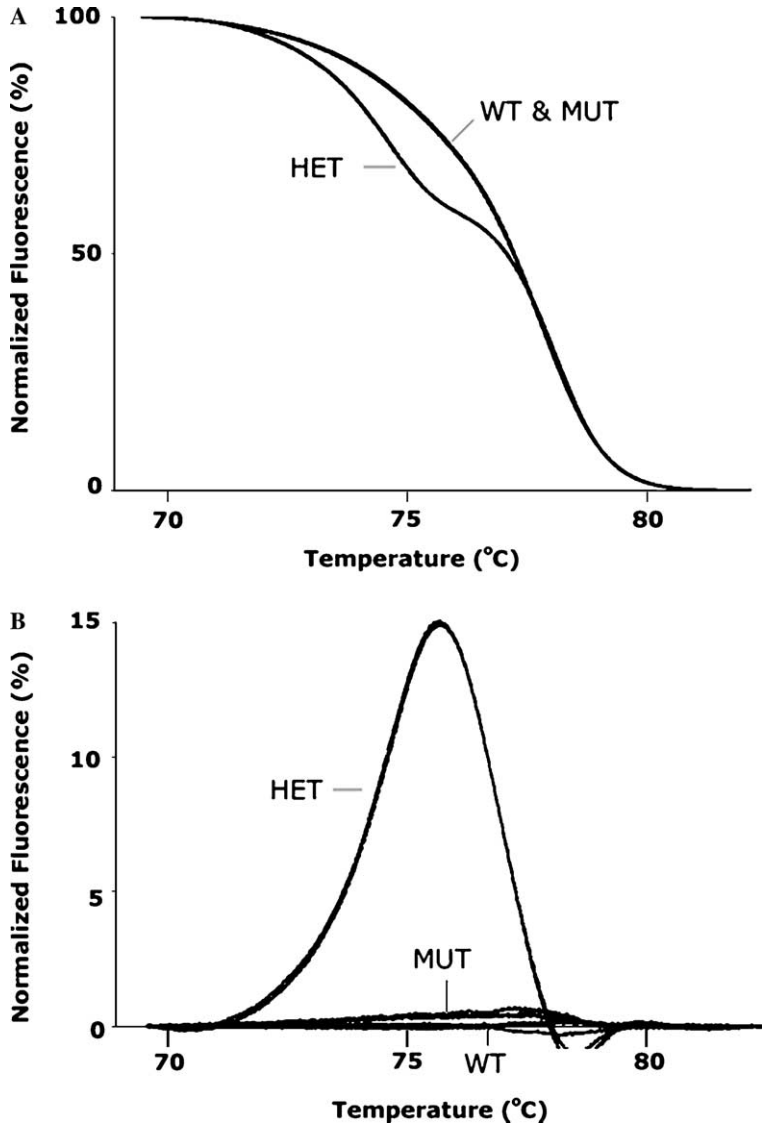

Fig. 2. Melting curves of an SNP with nearest neighbor symmetry after PCR amplification. The same sequence as that in Fig. 1 was amplified from genomic DNA by PCR and melting curves were obtained for wild-type (WT), homozygous mutant (MUT), and heterozygous (HET) genotypes. For each genotype, three different DNA samples are displayed. (A) Normalized, temperature-shifted melting curves. All six homozygous samples appear to trace the same path. Melting curves of heterozygous samples show a low temperature transition from the contribution of heteroduplexes, as predicted from Fig. 1. (B) Difference of each curve from the mean wild-type curve.

very similar to that observed when all synthetic duplexes are mixed (Fig. 1). Fig. 2B shows the same data presented as a difference plot where each sample is subtracted from the average wild-type curve. Although heterozygotes are readily identified on such plots, the two different homozygotes cannot be distinguished.

When a homozygous reference DNA is added to each sample before PCR, all genotypes can usually be distinguished, depending on the fraction of reference DNA present. Fig. 3 shows both the theoretical prediction and the experimental data correlating the fraction of reference wild-type DNA (x) to the resulting heteroduplex proportion. A detailed derivation of our heteroduplex model is presented as Supplementary Material. If the sample genotype is wild-type, the mixture has a heteroduplex content of zero for all values of x. If the sample is homozygous mutant, the mixture has heteroduplex content $m(x) = 2x(1-x)$, shown as a symmetric curve with a peak at $x = 0.5$ in Fig. 3. This formula agrees with intuition that when x is 0 or 1, the heteroduplex content is 0, and when x is 0.5, equal amounts of wild-type DNA and homozygous mutant DNA result in a heteroduplex content of 0.5. If the sample is heterozygous, the mixture has heteroduplex content $h(x) = \frac{1}{2}(1 - x^2)$. This formula predicts a steadily decreasing curve with a maximum of 0.5 for the heteroduplex content of a heterozygote without added reference (Fig. 3). The reference DNA fraction predicted to maximize the difference between any two genotypes is $x = \frac{1}{7}$, shown as the thin vertical line in Fig. 3. At $x = \frac{1}{7}$, the heteroduplex content of the homozygous mutant sample,

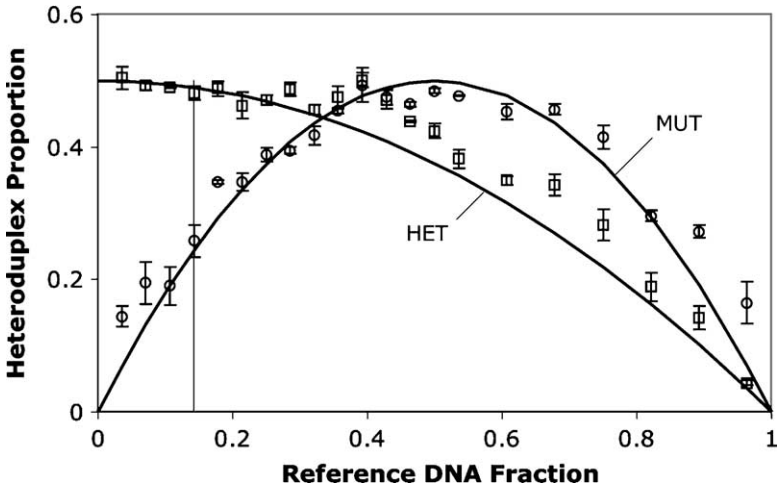

Fig. 3. Dependence of heteroduplex proportion on genotype and the fraction of added wild-type DNA. When wild-type reference DNA is mixed with homozygous mutant or heterozygous DNA, the resultant heteroduplex proportion depends on the fraction of reference DNA (x) in the mixture. For homozygous mutant (MUT) DNA, the heteroduplex proportion is $2x(1-x)$. For heterozygous (HET) DNA, the heteroduplex proportion is $1/2(1-x^2)$. The optimal separation between genotypes occurs when the reference DNA fraction is $\frac{1}{7}$ (left vertical line), giving heteroduplex proportions of $\frac{0}{49}$ (WT), $\frac{12}{49}$ (MUT), and $\frac{24}{49}$ (HET). When the reference DNA fraction is $\frac{1}{3}$, both MUT and HET samples have the same heterodupex proportion ($\frac{4}{9}$) and cannot be differentiated. Detailed derivations are provided as Supplementary material. For each reference DNA fraction, high-resolution melting experiments were performed in triplicate. The heteroduplex proportion was estimated from the maximum fluorescence difference between the melting curves of wild-type samples and those of each mixture, scaled to make the value for unmixed heterozygous samples equal to 0.5. The mean estimated heteroduplex proportion from these experiments is shown for homozygous mutant (circles) and heterozygous (squares) samples, and the standard deviation is indicated with error bars.

$m(\frac{1}{7}) = \frac{12}{49}$, is exactly half of the heteroduplex content of the heterozygous sample, $h(\frac{1}{7}) = \frac{24}{49}$.

Experimental melting curve data confirming the model are also displayed in Fig. 3. The peak heights on difference plots (e.g., Fig. 2B) were used to establish the heteroduplex proportion resulting from the addition of various fractions of wild-type, reference DNA. These values were scaled so that the heteroduplex proportion of unmixed heterozygotes was 0.5, allowing a direct comparison of the experimental values to the model. The correlation between the experiment and the model satisfies $R^2 > 0.99$ for the heterozygous samples and $R^2 > 0.98$ for the homozygous samples.

Melting curves of the different H63D genotypes at various values of reference DNA fraction are shown in Figs. 4 and 5. Fig. 4 shows the melting curves corresponding to the optimal reference DNA fraction, $\frac{4}{28} = \frac{1}{7}$. Melting curves of the three genotypes are well separated and easily classified by the observer's eye or by automatic hierarchal clustering. Difference plots (Fig. 4B) show that the peak height of the heterozygous genotype is approximately half that of the homozygous genotype, as predicted by theory and Fig. 3.

Fig. 5 shows melting curves corresponding to suboptimal reference DNA fractions. Figs. 5A and B show melting curves with reference DNA fractions of $\frac{9}{28}$ and $\frac{14}{28}$, respectively. As predicted by Fig. 3, it is difficult to differentiate homozygous mutant and heterozygous samples. Fig. 5C shows the melting curves at a reference DNA fraction of $\frac{19}{28}$, near the predicted value with the best separation at reference DNA fractions greater than $\frac{1}{3}$. Although the homozygous and heterozygous mutant genotypes are distinguishable, much better separation is obtained at the optimal reference DNA fraction of $\frac{1}{7}$.

The dependence of heteroduplex proportion on the reference DNA fraction was also studied by TGCE. Results similar to those of the melting analysis were obtained with a correlation between model and experiment of $R^2 > 0.97$ for both heterozygous and homozygous samples (data not shown). TGCE traces at the theoretically optimal reference DNA fraction of $\frac{1}{7}$ are shown in Fig. 6. The heteroduplex peaks of the mixtures with homozygous mutant DNA appear approximately halfway between those of the heterozygous and wild-type genotypes. Replicates of a common genotype cluster well, though not as perfectly as their melting curves. Mixtures with suboptimal reference DNA fractions $\frac{9}{28}$ and $\frac{14}{28}$ failed to distinguish homozygous and heterozygous mutant samples, similar to results obtained by melting analysis (data not shown).

Discussion

The objective of this study was to find the optimal fraction of homozygous reference DNA in an unknown sample added before PCR to distinguish all genotypes by quantitative heteroduplex analysis. Theoretical considerations suggest that this optimal fraction is $\frac{1}{7}$ and a detailed derivation is provided as Supplemental material. High-resolution melting analysis [13] and TGCE [5] were used to provide experimental verification of the theory.

Our quantitative heteroduplex model is based on three assumptions. First, when two genotypes of DNA are mixed, we assume that PCR generates all four strands with equal efficiency. Therefore, at the end of PCR, the relative proportion of these strands, whether in homoduplex or heteroduplex form, is the same as that of the corresponding

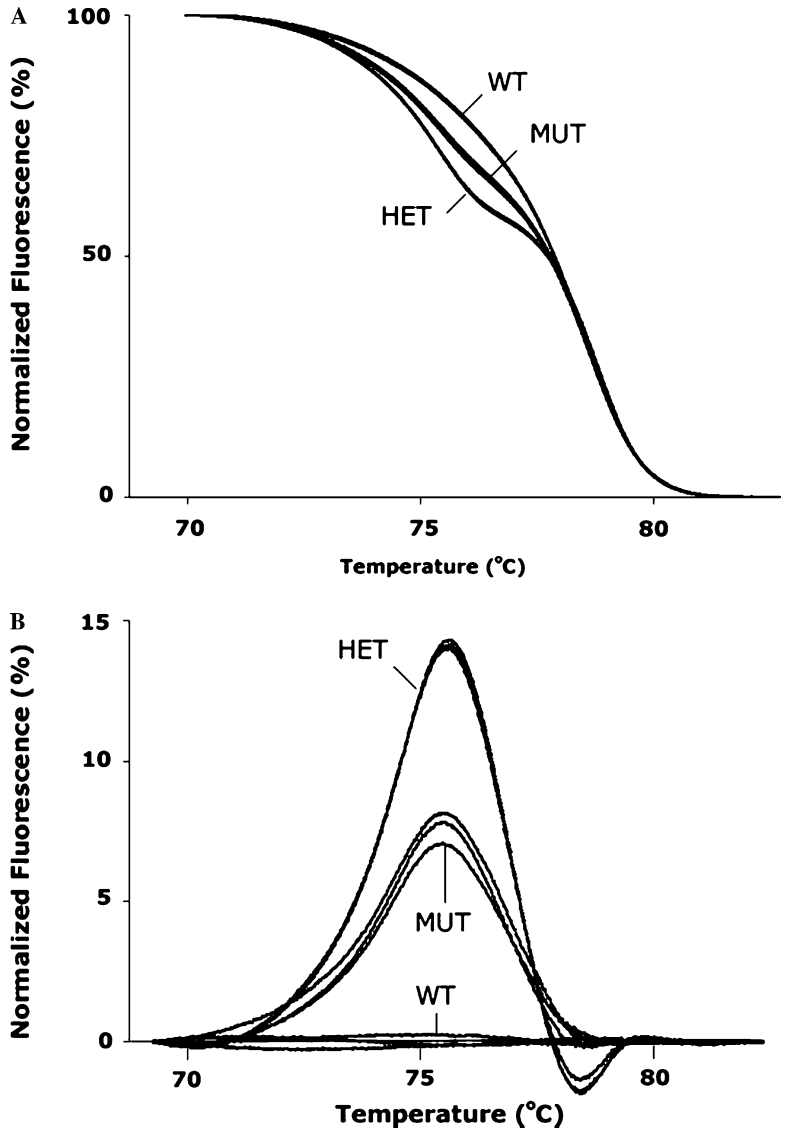

Fig. 4. Melting curve analysis of an SNP with nearest neighbor symmetry after addition of an optimal amount of wild-type DNA and PCR amplification. Triplicate samples of each genotype (WT, wild-type; HET, heterozygous; MUT, homozygous mutant) were mixed with wild-type DNA to obtain a reference DNA fraction of $\frac{1}{7}$. (A) After amplification, melting, and normalization, homozygous mutant curves were equidistant from the wild-type curves and the barely altered heterozygous curves. All samples of the same genotype appear as single curves because of the reproducibility of high-resolution analysis. (B) Individual curves can be seen when the difference of each curve with the mean wild-type curve is displayed. The heterozygous and homozygous curves have the same shape and peak, while their magnitude varies by a factor of two, as predicted (Fig. 3). The heteroduplex content of the homozygous mutant mixture is 0.5 times that of the heterozygous mixture.

strands in the initial template. Second, when PCR products are denatured and annealed after amplification, the proportion of homoduplex and heteroduplex species formed is given by the product of the relative concentrations of each strand; i.e., there is no preference for association with exactly vs nearly complementary strands. Third, the model assumes that the normalized melting curve resulting from such a mixture of duplexes is given by the weighted average of the melting curves corresponding to each duplex, with coefficients given by their relative proportion in the mixture.

As a consequence of this model, the expected difference between two melting curves is the difference in their heteroduplex proportions multiplied by a universal curve. The heteroduplex proportion depends on the genotype and reference DNA fraction, while the universal curve does not. The universal curve is the difference between the mean homoduplex and the mean heteroduplex melting curves. The temperature at the maximum difference between curves is constant. However, the magnitude of that difference and the area between curves are directly proportional to the difference in the heteroduplex proportion between samples. That is, for any particular PCR product and SNP, the position and shape of the difference curve remains constant at different allele fractions, while its magnitude and area are proportional to the difference in heteroduplex proportions.

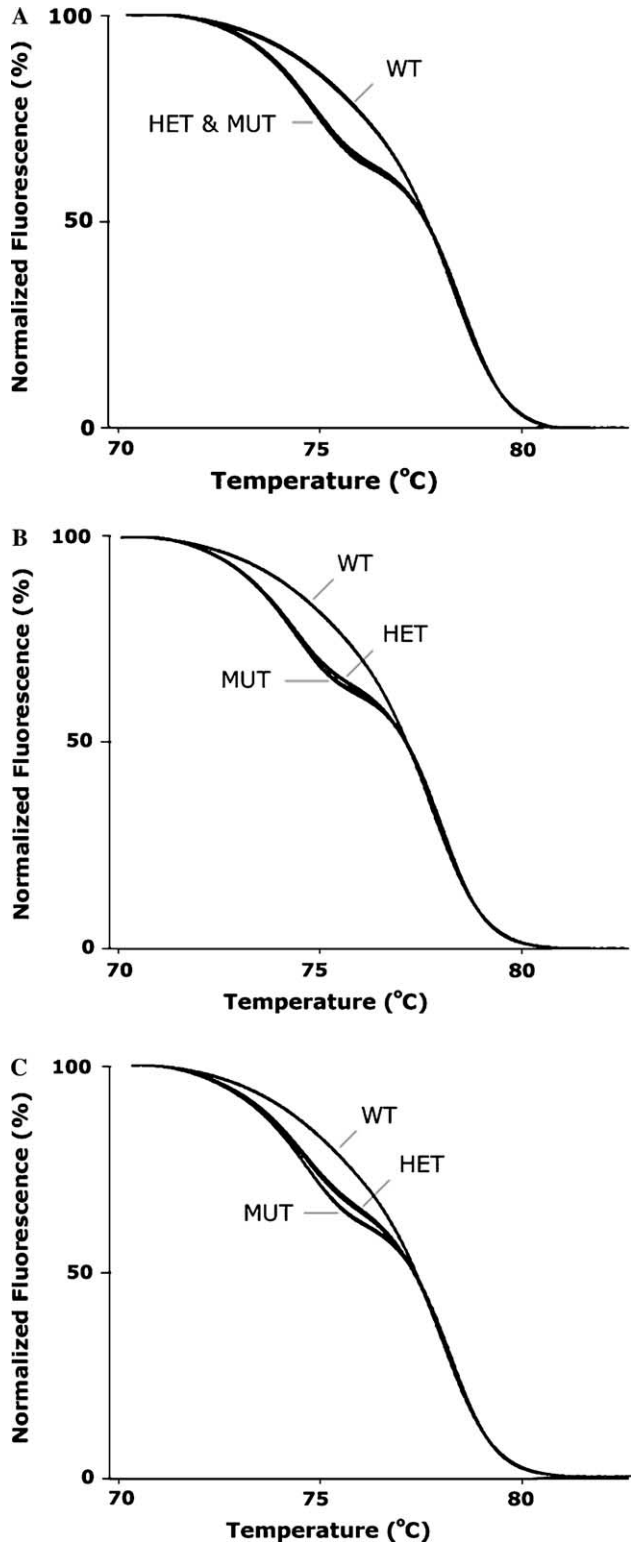

Fig. 5. Melting curve analysis of an SNP with nearest neighbor symmetry after addition of suboptimal amounts of wild-type DNA and PCR amplification. Three samples of each genotype (WT, wild-type; HET, heterozygous; MUT, homozygous mutant) were mixed with different amounts of wild-type DNA to obtain different suboptimal reference DNA fractions. Wild-type DNA fractions of $\frac{9}{28}$, $\frac{14}{28}$, and $\frac{19}{28}$ are shown in A–C, respectively, and can be compared to the predicted heteroduplex proportions of Fig. 3. Samples of the same genotype usually appear as single curves because of the reproducibility of high-resolution analysis.

These predictions are supported by data showing that the shape and position of the difference curves remains nearly constant, while the peak height and area depend on the heteroduplex proportion difference. For experimental demonstration, an SNP with nearest neighbor thermodynamic identity between the wild-type and the homozygous mutant DNA was chosen. The added reference DNA was wild-type, though homozygous mutant DNA could have been used without affecting the results. Adding reference DNA to samples before PCR allows complete genotyping if the amounts of heteroduplexes formed after PCR are sufficiently different between all three genotypes. When wild-type reference DNA is mixed with wild-type samples, no heteroduplexes result. However, when wild-type DNA is added to heterozygous or homozygous mutant DNA, the amount of heteroduplexes formed after PCR depends on the genotype and the fraction of wild-type DNA present. The melting curve of a heteroduplex-enhanced homozygous mutant sample moves away from the stationary wild-type melting curve, while the melting curve of a heteroduplex-reduced heterozygous sample moves toward the other two and eventually crosses between them. Both melting analysis and TGCE results follow the quadratic behavior of the model qualitatively and quantitatively over the full range of reference DNA fractions. The optimal fraction of reference DNA for genotype discrimination by theory and experiment was $\frac{1}{7}$. This fraction should be independent of amplicon size and type of sequence variant.

TGCE is not usually used for heteroduplex quantification. The two heteroduplexes are delayed several frames compared to homoduplexes and may be separated from each other also. TGCE peaks exhibit simple mathematical behavior that makes it possible to separate and quantify the relative contributions of the heteroduplexes. Agreement between the results of TGCE analysis, melting curve experiments, and theory not only confirms the theory but also indicates that both of these methods can be used for estimation of heteroduplex content of samples mixed with reference DNA or of pooled samples.

Most heteroduplex-based techniques do not detect homozygous sequence variants. An exception is high-resolution melting analysis where most, but not all homozygous SNPs can be detected [13]. The technique is attractive as a closed-tube method that allows both scanning for any sequence variant and specific genotyping without probes [7,8,13]. Scanning sensitivity and specificity approach 100% for PCR products less than 400 bp [8]. The utility of high-resolution amplicon melting for genotyping was recently demonstrated in one study where 21 of 21 pairs of different heterozygotes within the same amplicon were distinguishable [17]. However, some homozygous variants (4–16% of human SNPs) have very similar, if not identical, melting transitions compared to wild-type [13]. Detection of these SNPs usually requires

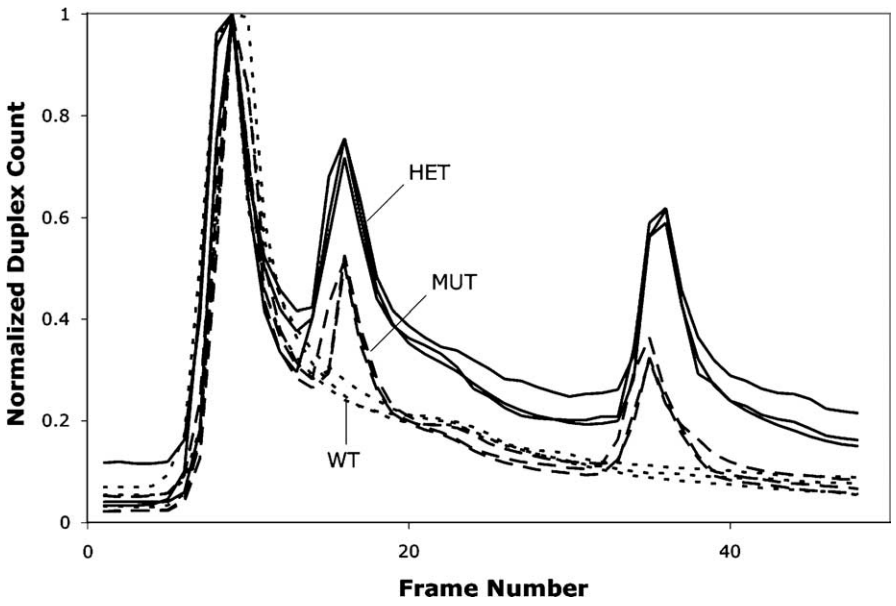

Fig. 6. TGCE data of an SNP with nearest neighbor symmetry after addition of an optimal amount of wild-type DNA and PCR amplification. Triplicate samples of each genotype were mixed with wild-type DNA to obtain a reference DNA fraction of $\frac{1}{7}$. After amplification and separation by TGCE, data were normalized by shifting and scaling the largest peaks to a common location and magnitude. Homozygous mutant curves were approximately equidistant from the wild-type curves and the barely altered heterozygous curves.

the addition of a reference amplicon after PCR is completed, followed by a second heteroduplex analysis. This study demonstrates that complete genotyping in a single step is possible if a reference homozygous DNA is added to the unknown samples before PCR and quantitative heteroduplex analysis is performed. It provides an experimentally validated model for performing this analysis that also determines the optimal amount of reference DNA to be added.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ab.2005.08.010.

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