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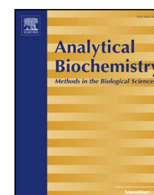

Quantifying variant differences in DNA melting curves: Effects of length, melting rate, and curve overlay

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ABSTRACT

High resolution DNA melting of PCR products is a simple technique for sequence variant detection and analysis. However, sensitivity and specificity vary and depend on many factors that continue to be defined. We introduce the area between normalized melting curves as a metric to quantify genotype discrimination. The effects of amplicon size (51–547 bp), melting rate (0.01–0.64 °C/s) and analysis method (curve shape by overlay vs absolute temperature differences) were qualitatively and quantitatively analyzed. To limit experimental variance, we studied a single nucleotide variant with identical predicted wild type and homozygous variant stabilities by nearest neighbor thermodynamic theory. Heterozygotes were easier to detect in smaller amplicons, at faster melting rates, and after curve overlay (superimposition), with some p-values $< 10^{-20}$. As heterozygote melting rates increase, the relative magnitude of heteroduplex contributions to melting curves increases, apparently the result of non-equilibrium processes. In contrast to heterozygotes, the interplay between curve overlay, PCR product size, and analysis method is complicated for homozygote genotype discrimination and is difficult to predict. Similar to temperature cycling in PCR, if the temperature control and temperature homogeneity of the solution are adequate, faster rates improve melting analysis, just like faster rates improve PCR.

Introduction

Fluorescent melting analysis was first identified as an effective method to characterize PCR products in 1997 using real time PCR instruments [1]. Melting analysis has evolved into "high resolution" DNA melting as a simple and inexpensive technique for genotyping, variant scanning, methylation analysis, sequence matching, and relative copy number assessment [2–4].

A basic but limited way to characterize melting curves is by their T_m, the temperature at which half of the duplex DNA has been denatured to single stranded DNA. With fluorescence melting analysis, T_m is best estimated by removing the fluorescence background and evaluating the temperature at 50% fluorescence [5]. Easier, but less correct, is using the peak fluorescence on negative derivative curves, often done incorrectly without background removal.

Entire melting curves are much more informative than T_ms. Changes in T_m and melting curve shape are the two primary features used in high resolution melting analysis. Qualitative visual assessment of high resolution melting curves is usually sufficient to allow clustering and genotyping with high sensitivity and specificity [6]. A few studies have looked at more quantitative approaches. These include genotyping based on thermodynamic considerations [7], and statistical software for genotype analysis [8]. We elected to use a simple quantitative metric intuitively used in visual genotype discrimination, the area between curves.

The formation of heteroduplexes during the later stages of PCR produce shape changes that are characteristic of heterozygous samples, while homozygotes differ mainly in the temperature of similarly shaped melting curves. Shape changes are highlighted by curve overlay that superimposes melting curves to minimize temperature differences, originally introduced as "temperature shifting" [9]. Background removal and fluorescence normalization on the Y-axis are first performed to stretch all melting curves between 0 and 100% fluorescence. Then, curves are superimposed by translation along the X-axis. The process of fluorescence normalization defines an area between melting curves that can be used visually and quantitatively as a difference metric. Further overlay by translation along the Y-axis defines an area for visual and quantitative assessment of shape differences. We used these metrics to evaluate the effects of PCR product size, melting rate, and curve overlay on genotype discrimination, factors whose effects are not completely

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resolved in the literature.

The influence of amplicon size on genotype discrimination is different for heterozygotes vs homozygotes. Although there is ample evidence that heterozygote discrimination becomes more difficult as the PCR product length increases [10], homozygote discrimination is more complicated. With homozygotes, genotyping accuracy does not always degrade consistently as the PCR product length increases, even when the surrounding sequence is identical [11]. Furthermore, it may be easier to identify homozygotes in PCR products with multiple domains [6], and a high homozygote detection sensitivity of 96.5% has been reported for PCR product sizes of 301–669 bps [12].

The influence of melting rate on genotype discrimination is uncertain. In deference to thermodynamics, most instruments use slow rates that approach equilibrium to presumably increase discrimination, for example, 0.01 °C/s [11,13]. However, there is early evidence that faster melting increases the prominence of heteroduplex peaks [14]. Furthermore, one study across nine different instruments found a trend ($r = 0.3$, $p = 0.057$) that correlates faster melting instruments to better genotyping accuracy [11]. Recently, a microfluidic study found that when nL volumes are used to achieve fine temperature control and homogeneity, small amplicon genotyping is better at faster melting rates [15]. Contrary to popular belief, such evidence suggests that faster melting may improve genotype discrimination.

The utility of overlay is often debated in high resolution melting analysis. Overlay does make heterozygote identification visually clearer, but at the loss of absolute temperature information that is particularly helpful in homozygote differentiation. One solution is to use overlay to identify heterozygotes, and then separately identify homozygotes without overlay. However, some homozygotes have very close or identical T_ms [16], and small differences in shape may become important. For example, on some instruments, homozygous genotypes are not separated unless overlay is applied [11].

To systematically investigate how PCR product size, melting rates and overlay affect genotyping, we used area calculations to exhaustively analyze one single nucleotide variant with three genotypes (AA, AT, and TT). Four bracketing PCR products of different lengths were used with seven melting rates between 0.01 and 0.64 °C/s that were measured on the most precise melting instrument available with a replicate SD of 0.018 °C [13,17]. Melting curves were evaluated both qualitatively and quantitatively using the area between curves as both visual and quantitative discrimination metrics.

Materials and methods

Generation of PCR products for melting

Four fragment lengths (51, 100, 272 and 547 bp) covering the same single nucleotide variant in human genomic DNA [rs3213784, c. 3405-29A > T] in *CPS1* (carbamoyl-phosphate synthase 1, mitochondrial)] were amplified in 96-well plates on a CFX96™ (Bio-Rad) PCR instrument. PCR was performed in fifty μL volumes per well containing 100 ng genomic DNA, 200 μM each deoxynucleotide triphosphate, 2 U KlenTaq™ (AB Peptides), 440 ng of TaqStart antibody (Clontech), 2 mM $MgCl_2$, 50 mM Tris (pH 8.3), 500 μg/mL bovine serum albumin, 0.5 μM primers and 1 × LCGreen® PLUS (BioFire Defense). The 3 genotypes of genomic DNA (AA, TT and AT) were confirmed by sequencing. The primers for the 51, 100, 272, 547 bp PCR products were: AGTCAAGTCT AGTATTAGCATAAACCT and AAGGAAGGGGAAAAAAAGCAG; ATAG GTTGTCTGGAACTGTTCTG and TCATAGCAGACCCACTGGAA; TTGGT TGATTGTCCTGGTGA and CAGTCACTACAAAGAAATTGGACA; CAGAA AGGGCAAACTTTGGA and GGAGACTAGAGGGTAGAAGAGGAAA, respectively. PCR was performed with an initial denaturation at 95 °C for 2 min, followed by 35–42 cycles (35 cycles for 51 and 100 bp; 40 cycles for 272 bp; 42 cycles for 547 bp) of denaturation at 95 °C (10 s for 51 and 100 bp; 15 s for 272 and 547 bp), annealing at 65–67 °C (10 s at 65 °C for 51 bp; 15 s at 65 °C for 100 and 272 bp; 15 s at 67 °C for 547 bp), and extension at 72 °C (10 s for 51 bp; 15 s for 100 and 272 bp; 20 s for 547 bp), then 95 °C with a 30 s hold, and cooling to 15 °C. PCR products were melted on a LightScanner® 96 (BioFire Defense) to confirm the purity of each well and finally pooled by genotype for each fragment length.

Melting curve acquisition

Ten μL PCR products in triplicate for all combinations of three genotypes (AA, AT, TT) in four fragment lengths (51, 100, 272, and 547 bp) for melting at 7 different rates (0.01, 0.02, 0.04, 0.08, 0.16, 0.32, 0.64 °C/s) were loaded into 252 LightCycler capillaries (Roche Diagnostics) and kept at 0–4 °C before melting. Each sample was equilibrated at 54 °C in an HR-1™ high resolution melting instrument (BioFire Defense) and melted between 65 and 88 °C for the 51 and 100 bp PCR products, and 65–90 °C for the 272 and 547 bp PCR products. The LED power was adjusted automatically by the instrument at the beginning of each run to achieve 90% of the detector maximum at 65 °C. Melting times ranged from 42 min at the 0.01 °C/s rate to 39 s at the 0.64 °C/s rate. The temperature recorded by the HR-1 instrument is from a resistive thermal device (RTD) positioned inside a metal cylinder that surrounds the capillary. The RTD temperature was compared to the sample temperature at different melting rates with a J-type thermocouple placed inside a 10 μL sample within a capillary.

Melting curve analysis

All data were analyzed on custom software [5,9]. A LabView version is available from RAP and analysis can also be performed with a web version (https://www.dna.utah.edu/uv/uanalyze.html), both freely available for non-commercial use. Briefly, exponential fluorescence background was first removed after setting temperature regions for background fitting below and above the melting transition. The curves were then normalized between 0 and 100% so that the vertical axis was proportional to the percent helicity of the DNA for direct comparison to predicted melting curves (https://www.dna.utah.edu/umelt/umelt.html). At this stage, optional data overlay may be performed to focus on melting curve shape rather than absolute temperatures. For overlay, the mean temperature of each curve was calculated by integration of a least squares quadratic fit of temperature as a function of normalized fluorescence in the 5–15% range. Finally, the data was presented as either background subtracted and normalized curves (normalized fluorescence), or further transformed to difference plots or negative derivative plots, all with or without temperature curve overlay.

Quantification of melting curve differences

The area between two melting curves on a background subtracted, normalized plot was used as a metric to quantify the difference between curves. The area was calculated as the integral of the absolute difference between two curves across temperature on normalized, background-subtracted melting curves. The within or intraclass difference was the average area of all pairs of curves of the same genotype, that is, AA vs AA, TT vs TT, and AT vs AT ($n = 9$ for triplicate samples of each genotype). The AA vs TT homozygote interclass difference was the average area of all pairs that included one AA and one TT genotype ($n = 9$). Since the AA and TT genotypes had very similar melting curves (predicted ΔT_m of 0.00 °C), the heterozygote vs homozygote interclass difference used was the average of all pairs that included one AT genotype and one AA or TT genotype ($n = 18$). T_ms were defined as either: 1) the temperature at 50% fluorescence on normalized plots, or 2) the peak fluorescence on negative derivative plots. Peak height ratios and interpeak ΔT_ms of heterozygotes were derived from negative derivative plots.

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