Clinical Chemistry 56:9
1500–1501 (2010)

Citation Classic

Making DNA Melting Useful

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Featured Article: Wittwer CT, Reed GH, Gundry CN, Vandersteen JG, Pryor RJ. High-resolution genotyping by amplicon melting analysis using LCGreen. Clin Chem 2003;49:853–60.[3]

DNA melting (the denaturation of DNA by heat) is a fundamental property of DNA that can be used as an analytical tool. Classic studies of DNA melting monitored the absorbance near 260 nm as the temperature was increased and required large (microgram) amounts of DNA and analysis times of hours. Although DNA melting was an accurate research tool, these limitations relegated DNA melting to the specialized laboratory, away from most research laboratories and clinical testing.

Fluorescence analysis of DNA melting as a companion to real-time PCR was introduced in 1996 with the LightCycler®, the product of university academics and the small company Idaho Technology *(1)*. In 1998, Roche Applied Science adopted the LightCycler worldwide. The PCR conveniently produces nanogram amounts of DNA that can easily be monitored with fluorescence. DNA fluorescence typically increases during real-time PCR and decreases during melting analysis. For the first time, no handling, processing, or separation of the sample after the PCR was required for analysis.

Methods for both probe and PCR product (amplicon) melting were developed for the LightCycler and were later incorporated into other real-time instruments. DNA melting monitors stability across temperature as a "dynamic dot blot," an approach that is inherently more robust than the prior single-temperature "static" analysis. Single-base variants, such as the *F5* [coagulation factor V (proaccelerin, labile factor)] Leiden variant, were genotyped by melting hybridization probes *(2)*, and in 2003 these tests became the first genetic tests cleared by the US Food and Drug Administration. Real-time PCR with SYBR® Green I became more popular than with fluorescently labeled probes because of the cost and the added benefit of assessing PCR product purity by melting after amplification. The characteristic melting curves of amplicons after the PCR usually allowed the differentiation of unique products *(3)*. Usually but not always. Small differences, such as a single-base change in 1 copy of diploid DNA, were difficult to detect.

Real-time PCR instruments, including the carousel LightCycler, did not focus on melting quality. In 2000, we initiated a project with Idaho Technology to build a high-resolution melting instrument. Our goal was to see what additional information might be obtained if melting-curve quality were improved. The resulting instrument, the HR-1, used a LightCycler capillary surrounded by an aluminum ingot with a wound resistive coil. Melting rates of 0.1 °C/s to 0.3 °C/s produced 40–120 points per degree Celsius, all of which could be plotted and analyzed without approximation or smoothing. We learned that "high resolution" did not necessarily mean "slow," because melting curves of excellent quality were acquired over 30 °C in <2 min. As with the PCR, melting analysis does not have to be slow to be good. We also learned that new analysis methods, including normalization and difference plots, were required to display the small differences revealed by high-resolution melting. Heterozygotes were best detected by comparing melting-curve shapes after "curve overlay" or "temperature shifting," rather than by their melting temperatures, or T_ms.

High-resolution amplicon melting was first demonstrated with PCR products amplified with a fluorescently labeled primer *(4)*. Fluorescein was attached to the 5′ end of one primer, and the fluorescence intensity was sensitive to whether the DNA was single- or double-stranded. After the PCR, any variation within the melting domain that included the labeled primer was detected, demonstrating that even single-base changes could be genotyped. A fluorescently labeled primer was required, however, and variants in other domains were not seen. If fluorescence could be generated with dyes rather than with labeled probes, both concerns might disappear. SYBR Green I, however, produced variable results.

We investigated dyes other than SYBR Green I by looking for their ability to detect the heteroduplexes produced by the PCR after amplification of a heterozygote. In collaboration with Idaho Technology, we synthesized and characterized >30 asymmetric cyanine dyes. The dyes most sensitive to heteroduplexes be-

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[3] In 2009 alone, this report was cited more than 75 times.
Received; accepted April 13, 2010.
Previously published online at DOI: 10.1373/clinchem.2010.146175

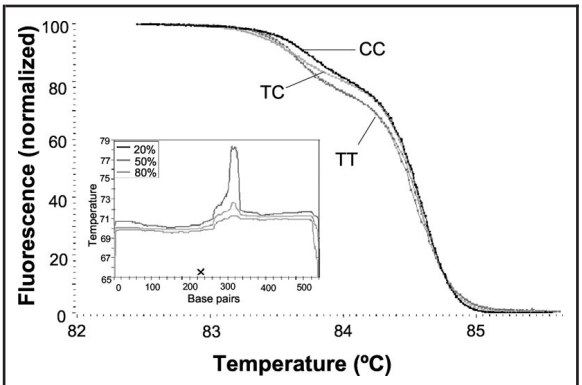

Fig. 1. High-resolution melting curve genotyping of a single-nucleotide polymorphism within a 544-bp fragment of *HTR2A*.

Duplicate samples of each genotype (CC, TC, and TT) are shown. The data were normalized and temperature shifted to superimpose the curves between 10% and 20% fluorescence. The inset shows a predicted melting map of the homoduplex and the position of the polymorphism in the lower melting domain (marked as X). Experimental melting curves also showed 2 apparent melting domains. All genotypes were similar in the higher melting temperature domain but differed in the lower melting domain.

came the LCGreen family of dyes. These dyes do not inhibit the PCR, even at saturating concentrations, and have become known as heteroduplex-detecting or "saturating" DNA dyes.

With a new instrument, new software, and a saturating dye, high-resolution amplicon genotyping was finally enabled *(5)*. Single-base heterozygotes and homozygotes could now be distinguished, as, for example, in the genotyping of all combinations of hemoglobin A, S, and C alleles. Even in amplicons greater than 500 bps with multiple melting domains, single-base variants can be directly genotyped by melting alone (Fig. 1). Because the entire amplicon between the PCR primers affects melting, the method can screen or scan for genetic variants, thereby replacing other technologies dependent on physical separations (denaturing HPLC, single-strand conformation polymorphism analysis, denaturing gradient gel electrophoresis). Investigators now had available a simple method for assessing genetic variation that required minimal effort.

Since 2003, the response to the high-resolution melting technology has been strong and sustained. Publications continue to increase exponentially with no sign of decay. Most major vendors include high-resolution melting options for their real-time instruments. Abbreviations abound, and many specifics remain hotly debated, with manufacturers vying for commercial advantage. In addition to genotyping and variant scanning, new applications continue to appear, including sequence matching and methylation analysis *(6)*. Future advances may include better predictive models and melting curve reference libraries.

It has been a very good decade for DNA melting.

Author Contributions: *All authors confirmed they have contributed to the intellectual content of this paper and have met the following 3 requirements: (a) significant contributions to the conception and design, acquisition of data, or analysis and interpretation of data; (b) drafting or revising the article for intellectual content; and (c) final approval of the published article.*

Authors' Disclosures of Potential Conflicts of Interest: *Upon manuscript submission, all authors completed the Disclosures of Potential Conflict of Interest form. Potential conflicts of interest:*

Employment or Leadership: C.T. Wittwer, Idaho Technology.
Consultant or Advisory Role: None declared.
Stock Ownership: C.T. Wittwer, Idaho Technology.
Honoraria: None declared.
Research Funding: C.T. Wittwer, Idaho Technology. DNA-melting methods were developed under several government and industrial grants to C.T. Wittwer, including from the NIH (GM072419, GM073396, GM082116), the state of Utah (Center of Excellence for Homogeneous DNA Analysis), the ARUP Research Institute for Experimental and Clinical Pathology, and Idaho Technology.
Expert Testimony: None declared.

Role of Sponsor: The funding organizations played no role in the design of study, choice of enrolled patients, review and interpretation of data, or preparation or approval of manuscript.

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