REVIEW

High-resolution DNA melting analysis for simple and efficient molecular diagnostics

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High-resolution melting of DNA is a simple solution for genotyping, mutation scanning and sequence matching. The melting profile of a PCR product depends on its GC content, length, sequence and heterozygosity and is best monitored with saturating dyes that fluoresce in the presence of double-stranded DNA. Genotyping of most variants is possible by the melting temperature of the PCR products, while all variants can be genotyped with unlabeled probes. Mutation scanning and sequence matching depend on sequence differences that result in heteroduplexes that change the shape of the melting curve. High-resolution DNA melting has several advantages over other genotyping and scanning methods, including an inexpensive closed tube format that is homogenous, accurate and rapid. Owing to its simplicity and speed, the method is a good fit for personalized medicine as a rapid, inexpensive method to predict therapeutic response.

Keywords: DNA melting, genotyping, heteroduplexes, HLA matching, LCGreen® dye, melting temperature, mutation scanning

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High-resolution melting is a new method for DNA analysis introduced in 2002 by a collaboration between academics (University of Utah, UT, USA) and industry (Idaho Technology, UT, USA). As the simplest method for genotyping, mutation scanning and sequence matching, its popularity is growing. No separations or processing of the samples is required. After PCR amplification, melting curves are generated by monitoring the fluorescence of a saturating dye that does not inhibit PCR.

When combined with rapid-cycle PCR [1], high-resolution melting is an ideal solution for personalized DNA diagnostics. For example, warfarin is a commonly used anticoagulant with a narrow therapeutic range. If the dose is not right, either serious bleeding or clotting may occur. The required dose of warfarin is modified by sequence variants in genes that affect its metabolism. The genotyping of three loci explains much of the variance in the required dose [2]. Rapid genotyping to determine appropriate dosing can be critical in emergency surgery. Rapid-cycle PCR (<15 min) followed by high-resolution melting (<2 min) provides a rapid solution. To give another example, a patient with typhoid fever requires rapid treatment. However, there are genetic variants of *Salmonella* that result in resistance to the commonly used antibiotics. These variants can be detected by high-resolution melting in order to direct alternative antibiotic therapy. Not only are these methods fast, but they are inexpensive because real-time thermal cyclers and covalently-labeled probes are not required [3].

Thermal melting of DNA was historically monitored by UV absorbance. For high-quality melting curves, µg amounts of DNA and rates of 0.1–1.0°C/min were required. In contrast to absorbance, fluorescence analysis of DNA melting is more sensitive, and only nanogram amounts are needed, conveniently provided by PCR amplification. Methods that monitor DNA melting by fluorescence have become popular with the advent of real-time PCR [4] and were introduced 10 years ago with the LightCycler® [5–7]. Capillary sample formats and smaller sample volumes allowed better temperature control, enabling much faster melting rates of 0.1–1.0°C/s. SYBR® Green I was introduced as a sensitive, convenient dye for PCR product melting analysis.

High-resolution DNA melting with saturation dyes

Modern high-resolution DNA melting is enabled by novel saturation dyes and high-resolution instruments. With SYBR Green I, it is difficult to guarantee saturation of the PCR product with dye as only limited concentrations can be used before it inhibits PCR. Although single-base genotyping with SYBR Green I has been reported [8–10], the results have been questioned [11] and, in our hands, is not robust [12,13]. Much better results are possible with a new generation of saturation dyes, specifically developed for high-resolution melting. These dyes, under the tradename LCGreen®, are compatible with PCR over a wide range of concentrations. Single-base variants and small

insertions or deletions are easily detected and genotyped with LCGreen dyes. Alternative saturating dyes other than the LCGreen family (LCGreen I and LCGreen Plus) are beginning to appear [14,15], although no comparative studies are available.

High-resolution DNA melting instruments

In addition to saturation dyes, new instrumentation was necessary to fully empower high-resolution melting techniques. The first high-resolution melting instrument (HR-1, Idaho Technology) was developed with the goal of making DNA melting as precise and accurate as possible in order to investigate the potential of the technique. Single samples are analyzed in LightCycler capillaries surrounded by a metal ingot heated by a resistance coil. Amplification is performed in a LightCycler (Roche) or the low-cost RapidCycler II (Idaho Technology). Analysis is rapid (1–2 min) for a throughput of approximately 45 samples/h. At 0.3°C/s, approximately 65 points are acquired and plotted per °C without any smoothing of the data. Genotyping [12,13], mutation scanning [16–18] and sequence matching [19] were all first demonstrated on this instrument. Demand for a 96- or 384-well plate format led to the introduction of the LightScanner® (Idaho Technology) [20–23]. Paired with standard plate thermal cyclers, the throughput of such a system is very high with many thermal cyclers funneling into one LightScanner. With a melting turnaround of 5 min, over 4000 samples can be analyzed per hour on a 384-well LightScanner if enough thermal cyclers are available.

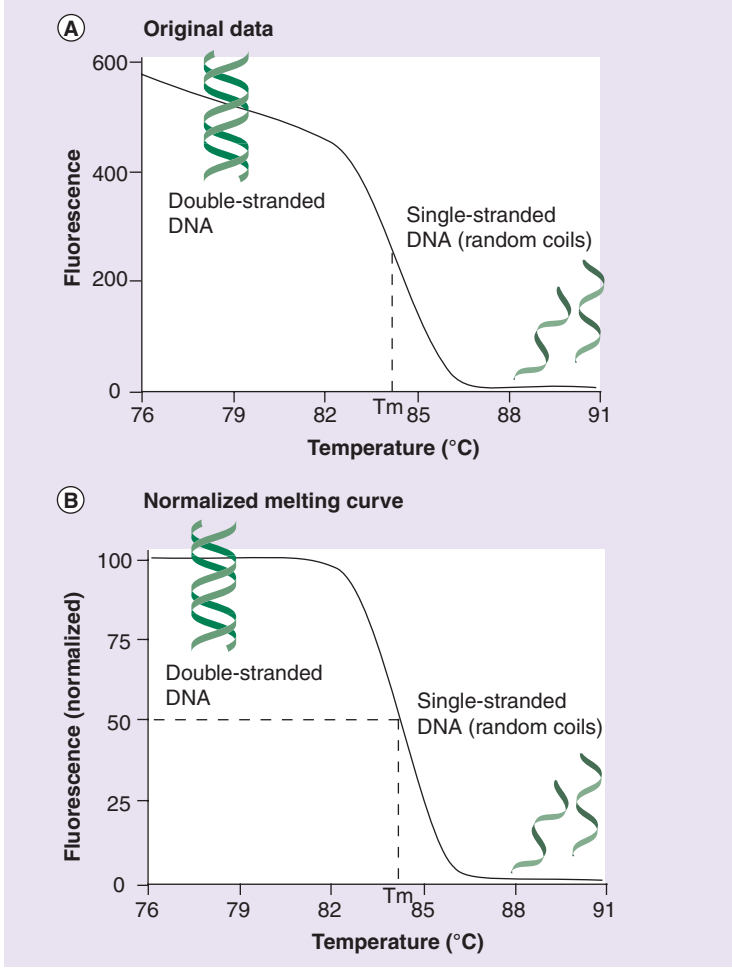
Recently, some real-time thermal cyclers have been modified to incorporate high-resolution melting, including the LC480 (Roche) and the Rotor-Gene 6000 (Corbett). These instruments approach high-resolution data quality by melting at slower rates. For example, the HR-1 melts at 0.3°C/s, taking just over a minute to pass through a 20°C range. In contrast, the Rotor-Gene temperature ramps are defined as '°C'. Under recommended conditions (0.1°C), a 2 s hold is performed at each 0.1°C step in temperature. The resulting actual measured rate is 0.017°C/s, producing 10 points/°C and requiring 20 min for the same temperature range. Although this is 18-times slower than the HR-1, the extra time is necessary to improve the data quality. Not surprisingly, comparative studies indicate that, in general, instruments dedicated to high-resolution melting perform better than real-time instruments adapted to high-resolution melting [24–26]. However, there is convenience in having both functions (amplification and melting analysis) combined in one instrument, and some prefer to interpret melting data in the context of real-time data. Nevertheless, integrated real-time data comes at a cost. The HR-1 remains the gold standard in melting quality and the LightScanner provides the highest throughput. Melting resolution is directly correlated to performance, that is, scanning sensitivity and specificity and genotyping accuracy. Most conventional real-time thermal cyclers do not perform well compared with high-resolution instruments. The detailed technical performance of 16 different melting instruments was recently compared in a series of reports [24–26].

High-resolution melting methods have been compared with other techniques in recent reviews [27–30]. In what follows, the fundamentals of DNA melting analysis will first be covered, followed by applications, including testing for known sequence variants (genotyping), identifying similarities or dissimilarities in DNA (sequence matching) and screening for mutations (scanning).

Fundamentals of fluorescent DNA melting analysis

Certain dyes fluoresce strongly in the presence of double-stranded DNA. The most familiar of these is ethidium bromide, giving the red bands often observed in electrophoresis gels. Asymmetric cyanine dyes, such as SYBR Green I and LCGreen, are even brighter and are the dyes of choice in fluorescence melting analysis and real-time PCR [4]. In order to generate a melting curve, the sample is heated through a range of temperatures, while fluorescence is continuously collected (Figure 1A). Any double-stranded DNA present will fluoresce strongly at low temperatures. As the temperature is increased, the fluorescence will decrease, at first slowly, and then, at a characteristic temperature the fluorescence rapidly drops, reflecting the melting of DNA into single strands. The melting temperature (Tm) of a DNA duplex is characteristic of its GC content, length and sequence and is the temperature at which the normalized fluorescence is 50% (Figure 1B). Accurate calculation of Tm first requires background removal before normalization. The major component of background is linear and arises from a physical

Figure 1. Fluorescent DNA melting analysis.



(A) Original fluorescence data showing a linear decrease of fluorescence at low temperature, followed by a rapid decrease centered around the melting temperature (Tm). Fluorescence is low when the DNA is single stranded. **(B)** The original data is normalized between 0 and 100% after background subtraction so that the curve is horizontal outside of the transition.

attribute of fluorescence: as the temperature is raised, fluorescence decreases. At lower temperatures, an exponential component of background becomes apparent that arises from dye binding to high concentrations of primers. Methods to remove linear [4] and exponential [31] background have been described and are incorporated into commercial high-resolution melting software.

The Tm of a PCR product is a convenient metric, but it is only one point on the melting curve. More information is contained in the complete melting curve than in the Tm. The shape of the melting curve is used extensively in sequence matching and mutation scanning as an indicator of heteroduplexes formed from heterozygous DNA.

Cost

Cost advantages of high-resolution melting are derived from the simplicity of the technique. The only reagent required is a saturating DNA dye that costs less than the PCR reagents/container. Available hardware ranges from real-time instruments at more than US$50,000, down to $15,000 [3]. All instrument options cost significantly less than a denaturing high-pressure liquid chromatography (DHPLC) setup for mutation scanning. Additional cost advantages include the time saved and errors avoided because the method is closed-tube, and the ability to perform both genotyping and scanning on one platform with one generic reagent.

Workflow

The saturating dye is added into the PCR before amplification, so no sample processing or additions are necessary after PCR has begun. DNA extraction and quantification are usually performed before PCR. For best results, all test and control DNA should be prepared in the same way and added into the PCR at the same concentration. However, good results can also be obtained from crude DNA preparations, such as those prepared from dried blood spots without quantification [32]. If care is taken to prevent undesired side reactions through PCR optimization and the reaction is run into the plateau phase for all samples, the initial DNA concentration can vary between samples by at least 100-fold.

PCR optimization

Robust, specific PCR is critical when results depend on the PCR product melting profile. Use of a gradient thermal cycler and gel electrophoresis is still one of the best methods for optimization of conditions, and varying the Mg^{2+} concentration usually allows multiple targets to be amplified under identical conditions.

Genotyping

Although there are many methods of genotyping, closed-tube methods have strong advantages for the clinical laboratory, point-of-care diagnostics and personalized medicine. Since no processing is required between amplification and analysis, the need for automation and risk of contamination are eliminated. These methods conventionally use allele-specific labeled probes, often a fluorescent dye and a quencher that separate during amplification by hydrolysis and/or loss of secondary structure [28]. In order to genotype correctly, two probes, one matching the wild-type sequence and

another matching the mutation sequence, are usually required. Typically, the fluorescence is measured in a real-time PCR machine once each cycle during annealing or extension.

Genotyping by melting

Genotyping by closed-tube melting analysis was introduced in 1997 [33]. The method is inherently more powerful than allele-specific methods as many different alleles are distinguished and hybridization is monitored over a range of temperatures, rather than only at a single temperature. Before the advent of high-resolution melting analysis, labeled probes were usually necessary for single-base genotyping by melting. Either fluorescence resonance energy transfer [33,34] or guanosine quenching [35] produced the probe melting curves necessary for genotyping. Depending on the sequence under the probe, different alleles resulted in different probe melting temperatures. Heterozygous PCR products were easily distinguished from homozygous samples by a double peak on derivative melting curve plots. Both fluorescence color and Tm were exploited for multiplexing [36]; for example, genotyping of HbC, HbS and HbE of human β-globin [37].

Genotyping of PCR products by high-resolution amplicon melting

High-resolution melting analysis enables genotyping without probes, even when the sequence change is only a single base. Consider an A>C variation with possible genotypes A/A, A/C and C/C (Figure 2A). If a small amplicon is generated with PCR primers that bracket the variable locus, all three genotypes are easily distinguished. The A/A and C/C curves are similar in shape with the Tm of the C/C homozygote approximately 1°C higher than that of the A/A homozygote. The melting curve of the A/C heterozygote differs in shape from that of the homozygotes with a more gradual transition over a larger temperature range. The greater range results from melting four different duplexes: two homoduplexes (A/A and C/C) and two heteroduplexes (A/C and C/A).

High-resolution genotyping without probes (direct PCR product genotyping) was first reported using fluorescently-labeled primers [12]. A 113 bp fragment of β-globin was amplified bracketing the HbS, HbC, and HbE single-base loci. All homozygotes (AA, SS, CC and EE) and heterozygotes (AS, AC, AE and SC) tested were distinguished. Genotyping became more difficult as the amplicon size and the distance from the labeled primer increased. For detection, the labeled primer had to be in the same melting domain as the sequence variant. This problem was solved in 2003 with the introduction of saturation dyes [13].

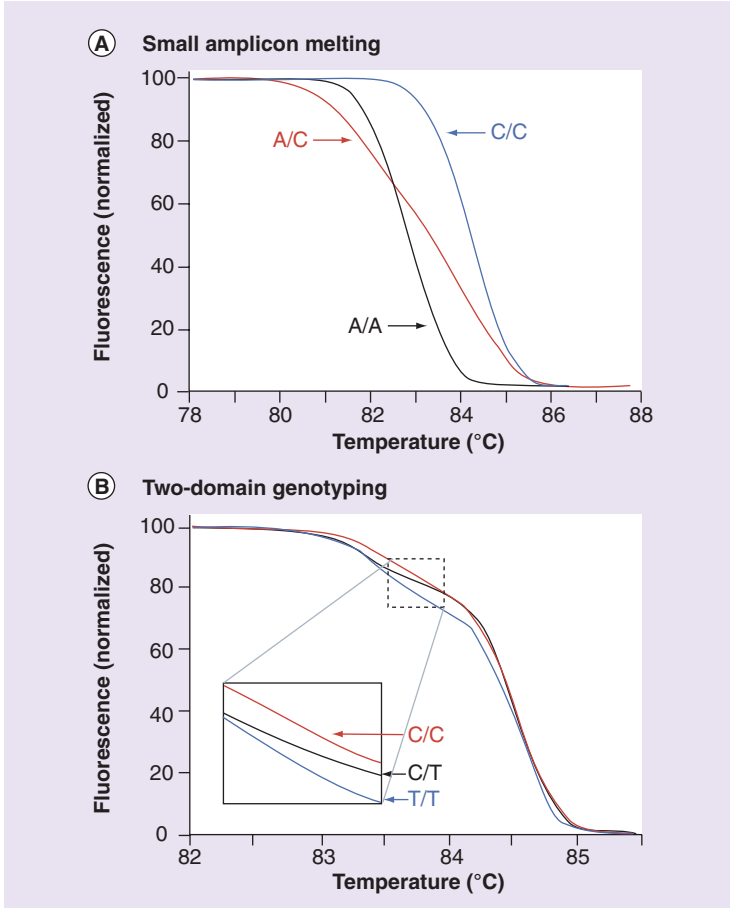
With saturation dyes, the PCR product is labeled along its entire length, so that all melting domains are detected. This is demonstrated in Figure 2B, where all genotypes of a C/T single base variant in a two-domain melting curve are shown. The difference between genotypes is revealed in the lower temperature domain, while the upper melting domain is constant between genotypes. The differences between genotypes are greater for smaller amplicons (Figure 2A) than for large amplicons (Figure 2B).

For single-base genotyping, heterozygotes are easy to identify because of the change in curve shape. However, not all homozygotes can be distinguished by Tm [38]. Approximately 84% of all human single-base changes result in an A:T to G:C interchange with a Tm difference of approximately 1°C in small amplicons. In the remaining 16%, the base pair is inverted or neutral (A:T to T:A or G:C to C:G) and the Tm difference is smaller. In approximately 4% of human single base changes, nearest-neighbor symmetry predicts no difference in Tm. In such a case, mixing is necessary for complete genotyping. If mixing is performed after PCR is complete, a known homozygote is mixed into each unknown homozygote and the mixture melted again. Alternatively, a known genotype can be added into all samples before PCR and quantitative heteroduplex analysis is performed [39].

Different heterozygotes can often be distinguished from each other by differences in curve shape. In one study, all 21 random pairs of unique heterozygotes were distinguishable by high-resolution melting of small amplicons [40]. In another study of 24 exons in two genes, all common variants were distinguishable from disease-causing variants and each other [41]. However, not all heterozygotes can be distinguished. For example, identical nearest-neighbor changes may occur at different locations within the same amplicon, such as the same mutation at different cysteine residues in the *RET* proto-oncogene [42].

High-resolution amplicon melting has been applied to both human (diploid) and microbial (monoploid) genotyping. Human targets include disease-associated variants in β-globin [12,13,38], cystic fibrosis [12,13,23,43], factor V [23,38],

Figure 2. Genotyping by amplicon melting.



(A) Single-base genotyping by small amplicon melting. **(B)**. Single-base genotyping within a large DNA fragment (544 bp) that melts in two domains. Panel B is reprinted with permission of AACC [13].

prothrombin [38], 5,10-methylenetetrahydrofolate reductase [38,44] and hemochromatosis proteins [38,39], platelet antigens [21,45], lactase [21], cytochrome P450 *2C9* [46] and methylation of the *MGMT* promoter region [15]. Microbial targets include mycobacterial typing using *hsp65* [47], bacterial speciation using the 16s rRNA gene [48], identifying *gyrA* mutations that cause quinolone resistance in *Salmonella* [3] and *Aspergillus* speciation [49].

Unlabeled probe genotyping

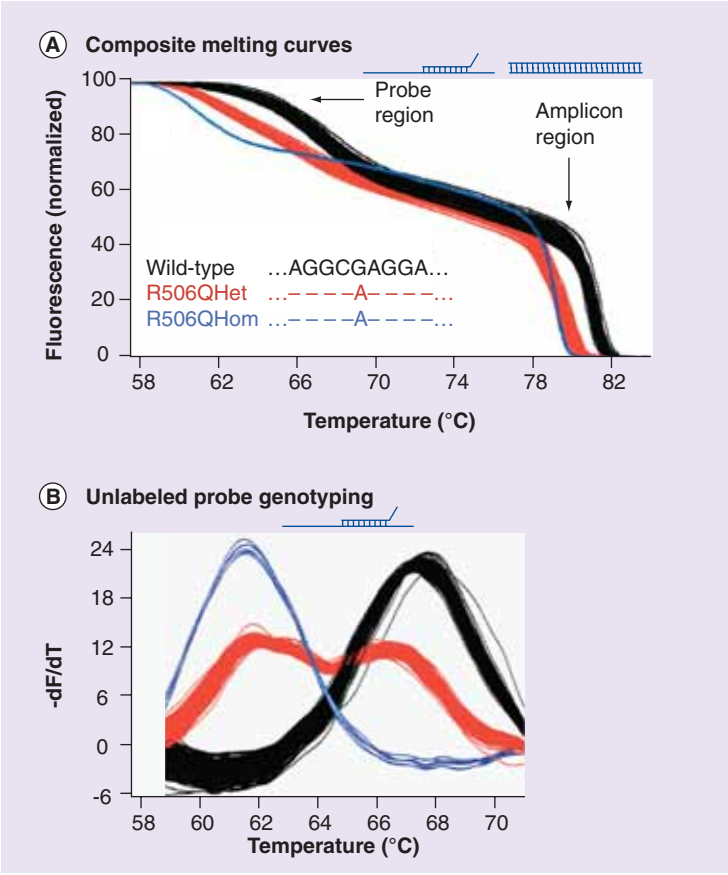
An interesting variation on genotyping with saturation dyes is to include an unlabeled probe. In addition to the full-length PCR product, the probe produces additional melting data focused on the region under the probe. Unequal primer concentrations are used to generate one strand of DNA in excess. Some of the excess strand hybridizes to the complementary unlabeled probe. Both probe and amplicon duplexes will be saturated with dye, giving melting regions for both the probe and the amplicon. Such a composite melting curve for factor V Leiden genotyping is shown in Figure 3A. Each region alone provides unambiguous genotyping. Considered together, cross-validation provides an additional level of confidence. When only the unlabeled probe region is considered, the melting curves are usually plotted as derivative plots (Figure 3B).

Genotyping with unlabeled probes was first published in 2004 [50]. To prevent polymerase extension, the probes are usually blocked at the 3´-end, often with phosphate, although other blockers are more stable [51]. High-resolution melting improves the quality of the melting curves and allows more variants to be distinguished from each other. However, unlabeled probe genotyping can be performed on lower resolution instruments, including the LightTyper® and the LightCycler [50], as long as appropriate data analysis software is available [31]. The probes are usually present during PCR, although they can be added after amplification is complete without breaking the closed-tube environment [52].

Unlabeled probe analysis allows fine discrimination of variants under the probe. Probes can be designed to mask certain variants or segments by incorporating deletions, mismatches or universal bases [53]. Multiple unlabeled probes can interrogate different amplified regions. For example, two unlabeled probes strategically positioned within exon 10 of the cystic fibrosis gene were used to genotype six different variants [23]. Unlabeled probes are helpful when amplicon melting alone does not provide adequate detail in highly polymorphic regions. Unlabeled probe genotyping can also be combined with scanning for unknown variants [23]. Any sequence variation between the primers will affect amplicon melting, while only a variant under the probe will affect probe melting.

Unlabeled probe and amplicon genotyping were recently compared [21]. Unlabeled probe genotyping was successful in all cases. By contrast, successful amplicon genotyping depends on the Tm difference between homozygotes [38,39] and the instrument resolution [24–26]. Genotyping accuracy is better with smaller amplicons as the Tm differences are magnified and the chance of unexpected variants between the primers is less. Most variants can be directly genotyped by amplicon melting, but a small minority requires mixing

Figure 3. Simultaneous genotyping and scanning.



(A) Composite normalized melting curve showing transitions in both the probe and amplicon regions (factor V Leiden locus). **(B)** Derivative plot of the probe region from part A after background removal by exponential subtraction. Portions of this figure are reprinted with permission of AACC [23].

with a known genotype [39]. Internal temperature controls can be used to improve amplicon genotyping accuracy, especially on lower resolution instruments [21,44].

Targets genotyped with unlabeled probes include factor V [23,54], cystic fibrosis [23,50], human platelet antigens [21], the *RET* proto-oncogene [42], lactase [21] and hereditary hemorrhagic telangiectasia [41].

Repeat typing

Tandem repeats are scattered throughout both eukaryotic and prokaryotic genomes and are highly polymorphic. A number of techniques have been used for repeat typing, such as gel and capillary electrophoresis [55], capillary arrays [56], microchip capillary electrophoresis [57], mass spectrometry [58] and hybridization arrays [59]. High-resolution melting of PCR-amplified repetitive regions is an interesting option for repeat typing that would be rapid and amenable to high-throughput analysis, while minimizing the danger of contamination.

High-resolution melting has been used to detect 6–102 bp internal tandem duplications in the juxtamembrane domain of the FMS-like tyrosine kinase 3 gene that are associated with acute myelogenous leukemia [60]. Internal duplications were identified based on variation in the melting curve shape compared with wild-type amplicons.

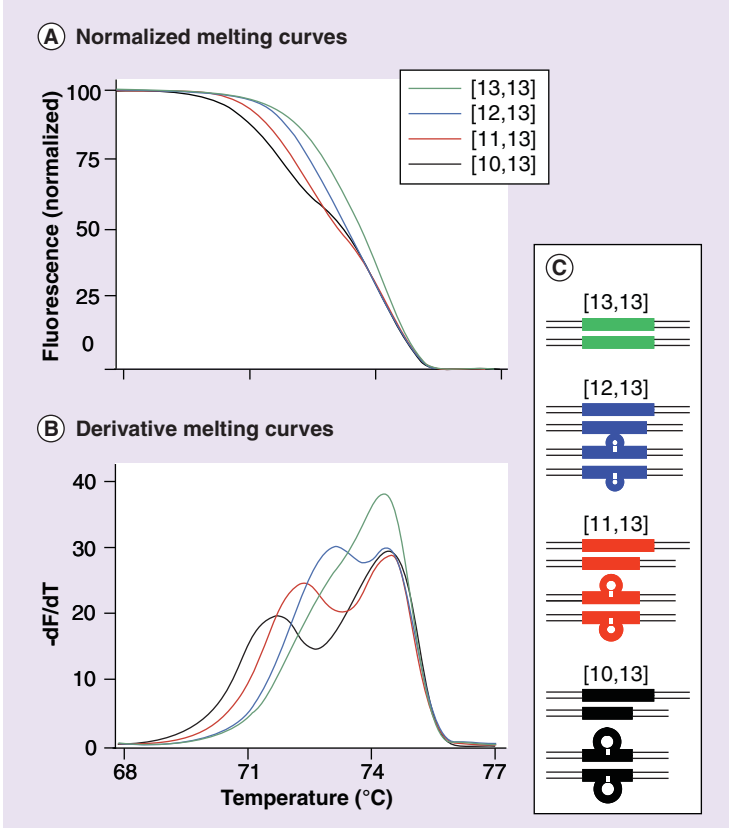
More difficult than the detection of the presence of duplications is complete genotyping of short tandem repeats. For example, can high-resolution melting be used to eliminate the need for electrophoresis in repeat genotyping? Although this remains a difficult problem, Figure 4 shows progress toward a solution [Unpublished Data]. A polymorphic tetranucleotide repeat region was amplified with primers just outside the repeats. Resulting normalized and derivative melting curves are shown in Figure 4. The difference in stability between hetero- and homoduplexes appears proportional to the size difference between the two alleles. These results show that at least some genotypes can be differentiated from each other, although complete genotyping of highly polymorphic repeats remains a future goal.

Sequence matching

In some cases, complete genotyping of the target DNA is less important than determining whether DNA sequences match. This scenario occurs in tissue transplantation, genotype–phenotype correlation and forensics. That is, sequence knowledge of the genotype is not needed, but sequence identity is. For example, in living-related organ transplantation, siblings are usually genotyped for HLA to obtain the best major histocompatibility match. This involves serotyping or genotyping at several loci, usually HLA A, B, C and DR by often laborious means. However, what is really important is to find a compatible sibling, that is, one with HLA sequence identity. For each available sibling, there is a 25% chance of a complete match.

HLA sequence identity (matching) by high-resolution melting was demonstrated using the highly polymorphic HLA-A locus in all seven cases of shared alleles among two individuals [19]. HLA genotype identity was suggested when two individuals had the same melting curves. Identity was confirmed by comparing the melting curve of a 1:1 mixture with the individual melting curves. If the samples are not identical, different heteroduplexes are formed that change the

Figure 4. Genotyping a tetranucleotide repeat.



(A) Normalized, temperature-shifted melting curves of locus D5S818 with the number of repeats indicated. **(B)** Derivative plots of the same samples. **(C)** Duplexes formed by the four genotypes tested.

shape of the melting curve. The potential to reduce a very complex genotyping problem to a simple, closed-tube, rapid process is attractive.

Scanning for sequence variants by high-resolution melting analysis

Many methods for mutation scanning (as opposed to genotyping) have been developed to screen for differences between the two copies of DNA within an individual (Figure 5). These techniques include single-strand conformational polymorphism analysis (SSCP) [61], denaturing gradient gel electrophoresis (DGGE) [62], DHPLC [63], temperature gradient capillary electrophoresis (TGCE) [64] and even mass spectroscopy [65]. Sequencing provides both genotyping and scanning at the same time, but requires extensive automation, instrumentation and analysis. All of these methods require separation of the sample on a gel or other matrix, some after additional processing, enzymatic or chemical reactions. Any processing increases the risk of contamination in future reactions because PCR products are exposed to the environment. Some of the methods are manual and labor intensive, while others are complex and require specialized instrumentation. Many are based on detection of heteroduplexes (mismatched duplexes) formed after amplification of heterozygous DNA. The need for processing the sample after PCR is a severe disadvantage.

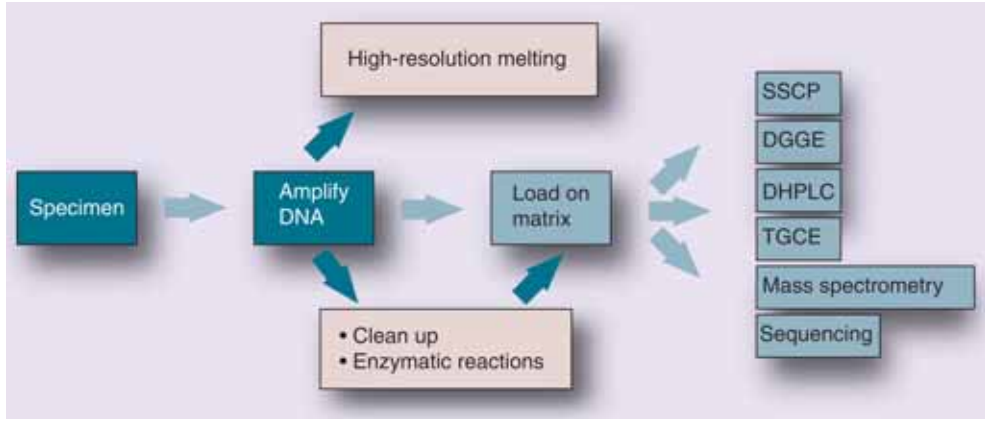
High-resolution melting analysis is a scanning method that does not require any processing, reagent additions or separations after PCR. Ideal melting rates are 0.1–0.3°C/s, so that the analysis is usually complete in 1–5 min. The sensitivity and specificity are better than DHPLC [43]. Single-base changes, insertions and deletions can all be detected, as long as the PCR primers bracket the variation. This limitation is similar to sequencing: deletions of entire genes and exons will usually go undetected.

Mutation scanning by high-resolution melting depends on the melting of heteroduplexes that distort the shape of the melting curve (Figure 6). This distortion can be seen by comparing the normalized melting curves of a homozygous standard to a heterozygous sample [12,13]. In order to focus on comparing curve shape, the bottoms of the curves are superimposed by shifting the curves along the temperature axis until they are overlaid (Figure 6A). As the difference between curves is small, it is often magnified by plotting the difference between samples (Figure 6B). Each curve is usually subtracted point-by-point from the homozygous reference (or an average of all wild-type curves analyzed). Although difference curves look similar to derivative melting curves (Figure 6C), they should not be confused. Derivative curves are commonly used in melting curve genotyping [33,34]. However, because they require data smoothing, derivative curves in high-resolution display should be used cautiously, despite their familiarity.

The sensitivity and specificity of scanning for heterozygous single-base changes were systematically studied using a set of engineered plasmids [17]. All possible base changes were considered in PCR products from 50 bp to 1 kb in a background of 40, 50 or 60% GC content. For PCR products less than 400 bp, sensitivity and specificity were 100%. In PCR products 400–1000 bps in length, sensitivity was 96.1% with a specificity of 99.4%. The position of the variant within the PCR product did not affect scanning accuracy.

Although designed to detect heterozygotes, high-resolution scanning often detects homozygyous changes as well. As discussed previously,

Figure 5. Workflow of different mutation scanning methods.



DGGE: Denaturing gradient gel electrophoresis; DHPLC: Denaturing high-pressure liquid chromatography; SSCP: Single-strand conformational polymorphism analysis; TGCE: Temperature gradient capillary electrophoresis.

96% of human single-base changes have homozygotes that differ in Tm and should be detectable. What is more surprising is that most homozygotes are detectable by curve shape changes alone, that is, after temperature shifting has been performed. Even though many homozygotes can be detected, it is still wise to mix an unknown sample with a known wild-type sample for detection of hemizygous variants (X-linked or Y chromosome) or if homozygous variants are likely.

The need for controls is controversial. The cautious will include wild-type controls, but they are not necessary when variants are rare and many samples are analyzed. The suspicious will include negative controls without template, although such controls must be checked at the original data stage and not normalized (you cannot normalize a melting transition when it is not there). The compulsive will include positive controls, even though the variants identified will most likely be different from the positive controls included. One place where positive controls are useful is in the identification of common polymorphisms, that is, variants that are not of interest. As discussed above under sequence matching, identical amplicon melting curves are strong evidence of sequence identity. Identity can be confirmed by mixing with a standard and re-melting, small amplicon genotyping, unlabeled probe genotyping, or sequencing. In one example of scanning 24 exons [41], benign polymorphisms were present in 96% of normal samples, greatly reducing the positive predictive value of mutation detection. When common polymorphisms were identified by amplicon melting, the positive predictive value for mutation detection increased to 100%. Melting curves of the same genotype can be mathematically clustered together, eliminating the guesswork of genotype assignment. In the large majority of cases, common polymorphisms can be eliminated by amplicon melting alone. Secondary genotyping or sequencing is seldom necessary.

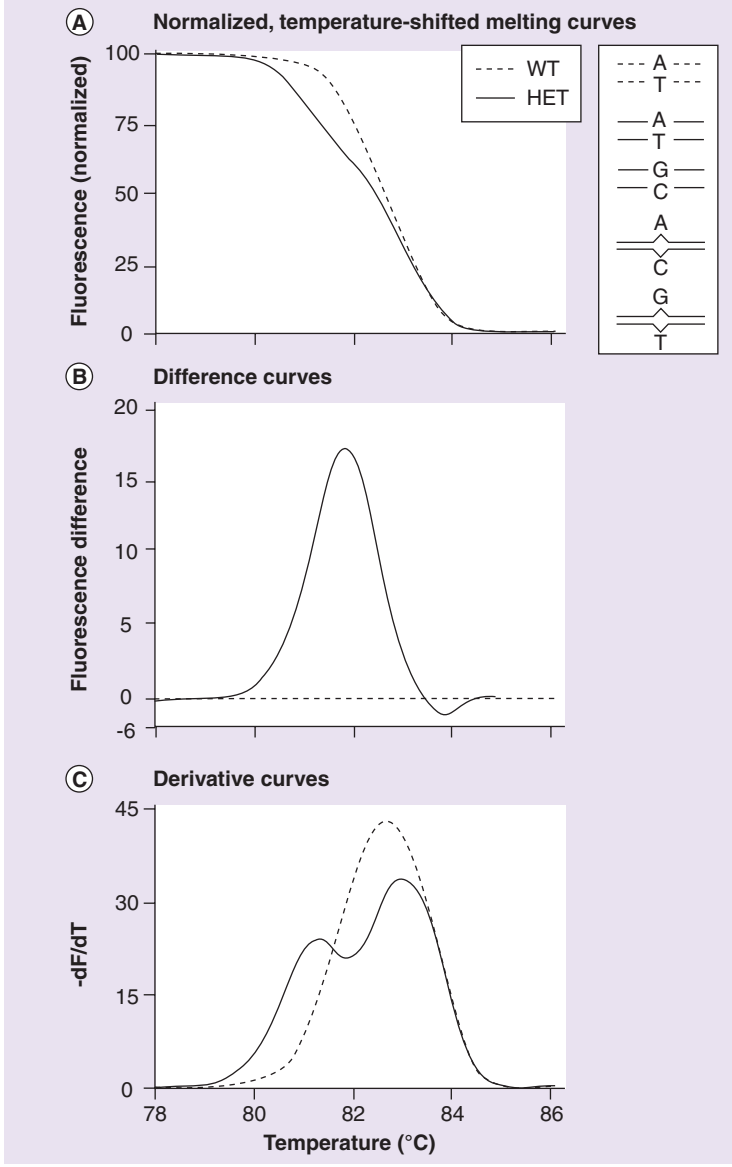
Mutation scanning by high-resolution melting has been reported for *c-kit* [18,66–68], medium-chain acyl-CoA dehydrogenase [16], primary carnitine deficiency [69], *RET* [42,70], epidermal growth factor receptor [71–74], exostoses 1 and 2 [22], gap junction protein β1 [20], *K-ras* [14,74], phenylalanine hydroxylase [32], v-raf murine sarcoma viral oncogene homolog B1 [66,74], *p53* [74], *HER2* [71,72], hereditary hemorrhagic telangiectasia [41], and some exons of the cystic fibrosis gene [23,43].

Future perspective

High-resolution DNA melting provides very simple solutions for genotyping, sequence matching and mutation scanning. The technique is new, but expanding rapidly as high-resolution instruments and dyes become available. As fluorescently-labeled probes and real-time PCR are not required, high-resolution methods have cost and simplicity advantages over other closed-tube genotyping approaches. Those few variants that cannot be identified by amplicon melting can be genotyped with unlabeled probes. The complexity and cost of labeled probes for genotyping is destined to make them obsolete.

Sequence matching by high-resolution melting can be used when exact genotyping is not necessary. For example, siblings considering living-related organ transplantation can be rapidly and

Figure 6. Melting curve plots useful in mutation scanning.



HET: Heterozygous DNA (A>G single-base change); WT: Wild-type homozygote.

inexpensively matched for HLA compatibility, replacing laborious, expensive genotyping methods. Specific genotypes correlate to melting curve shape and position, so that genetic variation can be visualized on a two-dimensional difference plot for genotype–phenotype correlation. Finally, identity may be established through melting analysis of variable regions such as HLA or single-base changes, although requirements of DNA purity and quality may limit practical applications.

High-resolution melting is currently the best method for mutation scanning because no processing or separations are required and the cost is minimal. When implemented correctly, 95–99% of the need for sequencing disappears. Since all sequencing first requires PCR amplification, high-resolution melting can be inserted into the sequencing process. Mutation scanning by melting is nondestructive, so that any positive samples can be further processed for sequencing if simpler methods for identification (matching and genotyping) fail.

So, what does the future hold? High-resolution melting can be extended to interrogate RNA sequence variability by standard reverse-transcriptase PCR. In combination with real-time PCR, detection, quantification and genotyping are all feasible on the same sample in one assay. Such analysis could be applied, for example, to hepatitis C where detection, quantification and genotyping are all clinically relevant. Application to *in situ* PCR has not been explored but may be feasible. The possibility of direct detection without PCR is also unexplored, but may be possible for high copy number plasmids or double-stranded viruses.

Disclosure

Aspects of high-resolution melting are licensed from the University of Utah to Idaho Technology. CTW has equity interest in Idaho Technology. GHR and JOK have nothing to disclose.

Executive summary

- High-resolution DNA melting provides the simplest methods for genotyping and mutation scanning.
- High-resolution DNA melting provides rapid analysis (1–5 min after PCR) without reagent additions or separations.
- The resolution of melting instrumentation is critical for accuracy, sensitivity and specificity – most real-time thermal cyclers do not perform well.
- The DNA dye used is critical – heteroduplex detection is enabled with saturating double-stranded DNA dyes.
- Fluorescently-labeled probes and real-time PCR are not required.
- Genotyping methods include amplicon melting and unlabeled probes.
- High-resolution melting analysis is a closed-tube approach for fast, accurate and high-throughput mutation scanning.
- High-resolution melting analysis offers low-cost analysis compared with many alternatives.

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