

High-Resolution DNA Melting Analysis: Advancements and Limitations

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For the Focus Section on HRMA Technology

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ABSTRACT: Recent advances in fluorescent dyes, methods, instruments and software for DNA melting analysis have created versatile new tools for variant scanning and genotyping. High resolution melting analysis (HRM or HRMA) is faster, simpler, and less expensive than alternative approaches requiring separations or labeled probes. With the addition of a saturating dye before PCR followed by rapid melting analysis of the PCR products, the sensitivity of heterozygote scanning approaches 100%. Specificity can be increased by identifying common polymorphisms with small amplicon melting, unlabeled probes or snapback primers to decrease the sequencing burden. However, some homozygotes require mixing for identification. Furthermore, different heterozygotes may produce melting curves so similar to each other that, although they clearly vary from homozygous variants, they are not differentiated from each other. Nevertheless, the experimental return for minimal effort is great. This focus issue of *Human Mutation* includes a concise, timely review on high resolution melting, a comparison to denaturing gradient gel electrophoresis, integration with qPCR for copy number assessment, combined amplicon scanning and unlabeled probe genotyping from a single melting curve, and applications to the mitochondrial genome and to *BRCA1*.

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PCR product melting analysis in combination with real-time PCR was first introduced with the LightCycler[®] (Wittwer et al., 1997). Unlike most DNA analysis techniques, no processing or separations were necessary. The stability of DNA duplexes was monitored by the fluorescence of SYBR[®] Green I as the temperature of the solution was increased. Although gross differences between PCR products were distinguished by melting temperature (T_m), subtle sequence variation was considered beyond the reach of fluorescent melting analysis. Several years later, high resolution DNA melting (HRM or HRMA) appeared as a rapid method for genotyping known variants or scanning for unknown variants (Wittwer et al., 2003). The scanning and genotyping applications of high resolution melting have been recently reviewed (Erali et al., 2008; Farrar et al., 2009; McKinney et al., 2009.; Reed et al., 2007; Wittwer et al., 2009).

This issue of *Human Mutation* highlights the technology, including 7 articles focused on methods and applications. A good

place to start is the concise, cogent review by Vossen et al. (2009), including both a general introduction and ongoing development efforts of considerable promise. Genotyping examples include *APOE* typing by amplicon melting and distinguishing 10 different alleles under one unlabeled probe. The potential for clone characterization is discussed, as well as replacing ethidium bromide-stained gels for PCR product characterization. As previously noted (Reed et al., 2007), some, but not all, simple sequence repeat genotypes can be distinguished. Because homozygotes can be difficult to detect directly, a scheme for combining rows on a 96-well microtiter plate after PCR is proposed, followed by repeat melting to detect any heterozygotes formed by mixing. A method to determine gene dosage is introduced by comparing melting curves of unknowns to mixtures of known alleles. The Vossen et al. (2009) review is filled with new applications, supporting the assertion that HRMA is a versatile genetic tool destined for general use. Nevertheless, its main application remains gene scanning.

Similar to all PCR-based techniques, large insertions or deletions are not typically detected during gene scanning by melting. Therefore, an additional technique such as multiplex ligation-dependent probe amplification (MLPA) or quantitative PCR (qPCR) is needed to assess gene dosage. In this issue, Rouleau et al. (2009) combine qPCR for detection of large rearrangements with high resolution melting to detect small variants. In 90 min on a single instrument, they determine both exon-by-exon gene dosage and scan the mismatch repair gene *MLH1* for variants. The 19 exons and additional control targets were amplified in triplicate on a 384-well plate, allowing the analysis of 5 patients at once. Samples included 14 wild type controls and 62 patients, including 45 single base changes, 9 small deletions (1–17 bp), 3 small insertions (1–2 bp), 3 duplications (1–3 exons), and 2 large deletions (1–3 exons). All rearrangements (3 duplications, 2 deletions) and small variants previously detected by MLPA and denaturing high pressure liquid chromatography were detected by the combined technique. The method provides a powerful first step in targeted gene assessment, especially when a significant fraction of deleterious variants arise from large rearrangements. As the precision of qPCR and melting further improves with time, the number of replicates required may decrease, making the approach even more accessible.

Some of the limitations of high resolution melting are defined by comparison to denaturing gradient gel electrophoresis (DGGE) in this issue (Tindall et al., 2009). Samples that displayed distinct DGGE patterns across complex targets (up to 4 variant loci and 12 genotypes) were selected. DGGE was optimized by denaturing gradient (starting range 40–60%, ending range 65–80%), electrophoresis running time (6.0, 7.5 hours, or overnight), constant (9%) or gradient (9–12%) polyacrylamide concentrations, and voltage (110–150 V), using a 60°C running temperature and 1 or 2 GC-tails on the primers. High resolution melting was

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optimized by including a proprietary additive for GC-rich targets when the GC% was between 65 and 72.5%.

For constitutional variants, Tindall et al. (2009) found that 100% (26/26) of the samples with one or more heterozygous loci were distinguished from wild type by high resolution melting. This compares well to a recent compilation of 19 studies for constitutional variants with an overall sensitivity of 99.3% ($n = 839$) and a specificity of 98.8% ($n = 2,659$) (Farrar et al., 2009). For somatic variants in samples with admixed wild type DNA, Tindall et al. (2009) found that 5/8 (63%) single heterozygotes, and 2/2 (100%) double heterozygotes were distinguished from wild type. Eighteen prior studies with somatic variants report somewhat better results with an overall sensitivity of 96.9% and specificity of 97.1% for heterozygous variants (Farrar et al., 2009).

Although HRMA heterozygote detection is excellent, Tindall et al. (2009) focus on a different question; whether different heterozygotes are distinguishable from each other. To the extent that each heterozygote follows a unique melting path, specific genotyping is possible. They clearly present data that reveals the risks of direct genotyping when the primary purpose is scanning. Although different heterozygotes often have unique melting curves, many melting curve clusters are present that include different genotypes. From the data presented, the success rate of correctly differentiating between heterozygotes can be calculated. Restricting the analysis to constitutional variants, 83% of different heterozygotes were distinguishable from single heterozygotes ($n = 41$ pairwise comparisons). This is somewhat lower than a prior estimate of 93% ($n = 40$) obtained from *CFTR* analysis (Montgomery et al., 2007). Together, these 2 studies suggest that the misclassification risk is about 10%. That is, about 90% of the time different heterozygotes will follow different melting paths that are distinguished using current technology. One method to verify the identity of 2 heterozygotes is to mix them together either before or after PCR and compare the melting curve of the mixture to the individual samples (Zhou et al., 2004). Tindall et al. (2009) also demonstrate for the first time that some, but not all, *cis/trans* haplotypes are distinguishable by high resolution melting.

Tindall et al. (2009) also detected of 5 out of 7 (71%) constitutional homozygous variants and 2/5 (40%) homozygous or hemizygous somatic variants. This compares to an average homozygote detection rate of 75% ($n = 60$) from 5 prior studies using constitutional variants (Farrar et al., 2009). Successful discrimination between homozygous variants that melt in a single domain depends mostly on their T_m . The ability to use absolute temperature differences for genotyping depends on the temperature precision of the instrument (Herrmann et al., 2006) and the consistency (ionic strength) of compared samples (Seipp et al., 2007). Most single base variants (84%) result in T_m differences of about 1°C, although 12% cluster around 0.25°C and some (4%) are predicted to be identical by nearest neighbor thermodynamics (Liew et al., 2004). Many homozygous insertions and deletions (e.g., p.F508del in *CFTR*) are also difficult to detect. The standard solution is to mix unknowns with wild type DNA, converting homozygous variants to heterozygotes, although this doubles the amount of work required. Outside of mixing, one method to improve homozygote detection is to include internal temperature standards (Gundry et al., 2008). This allows calibration to correct for temperature variation as well as buffer differences between samples.

Interestingly, high rates of homozygous variant detection are reported for both *BRCA1* (93%, $n = 14$) (van der Stoep et al., 2009) and mitochondrial DNA (96.5%, $n = 428$) (Dobrowolski et al., 2009) in this issue. In both cases, the PCR product lengths

are longer than typical, with a 625 bp fragment included in the *BRCA1* study and an average length of 519 bps in the mitochondrial scan. Contrary to heterozygote detection where shorter PCR products are better, these results suggest that longer products may be preferred for homozygote detection. Longer products commonly have multiple melting domains, and since sequence variants typically only affect one domain, the shape of the multiple-domain melting curve will change. In contrast, short PCR products have only one domain and homozygous variants result in little if any shape change. In general, shape differences are easier to detect than T_m differences. Curve shapes are easily compared by “temperature shifting” or “curve overlay” in software, used by both van der Stoep et al. (2009) and Dobrowolski et al. (2009). Curve overlay increases the sensitivity of heterozygote detection by comparing curve shapes. It also appears to increase the sensitivity of homozygote detection in long (multiple domain) products, but may decrease the sensitivity of homozygote detection in small (single domain) products. Hence, analysis both with and without curve overlay is important to maximize detection of heterozygous and homozygous variants.

When the identity of likely homozygous variants is known, genotyping with unlabeled probes (van der Stoep et al., 2009; Vandersteen et al., 2007), snapback primers (Zhou et al., 2008), or small amplicon genotyping (Dobrowolski et al., 2007; Dobrowolski et al., 2009) can be performed with the same instruments and dyes that are used for scanning. In this issue, (Nguyen-Dumont et al., 2009) elegantly demonstrate simultaneous scanning and genotyping using unlabeled probes. An unlabeled probe was included in asymmetric PCR such that both probe/product and full length product duplexes were produced. From a single melting curve, both genotyping data (at low temperature) and scanning data (at high temperature) were extracted. Two exons of *ATM*, each including a common variant that interferes with standard scanning were analyzed by high resolution melting on 384-well plates. Simultaneous scanning and genotyping of 1356 subjects was performed. For analysis, the curves were grouped by probe/target melting (by the genotype of the common variant) and amplicon scanning was performed on each group. Up to 9 different genotype combinations were distinguished and the curve clusters were completely concordant to sequencing.

Simultaneous scanning and genotyping allows better differentiation of multiple variants, the major concern of Tindall et al. (2009). Furthermore, the sequencing burden from common variants can be dramatically reduced. Nguyen-Dumont et al. (2009) suggest that simultaneous scanning and genotyping be considered for PCR products with variant frequencies >1%, which in the case of *ATM* is about 15% of the amplified fragments. The authors suggest particular value in large-scale screening projects. By including 9 unlabeled probes to genotype common polymorphisms, the sequencing burden of *BRCA1* analysis was decreased from 17.8% to 4.8% (van der Stoep et al., 2009).

Two reports in this issue detail large HRMA scanning studies. In the first, the human mitochondrial genome was scanned in 36 overlapping fragments, each 301-658 bps in size (Dobrowolski et al., 2009). Variants were detected in each of 33 patient samples with characterized mitochondrial mutations down to 1% heteroplasmy. Scanning 125 healthy donors identified 431 unique variants, of which 22% were novel and less than 3% were heteroplasmic. Of the homoplasmic variants, 96.5% were identified without mixing. Comprehensive scanning was performed in less than 1 day on a single 384- or two 96-well plates.

Small amplicon melting was used to identify specific mutations associated with aminoglycoside hearing loss.

The second clinical application reported here is scanning the breast cancer susceptibility gene, *BRCA1* (van der Stoep et al., 2009). As part of the EuroGentest consortium, the study included 170 *BRCA1* variants and 197 control samples all verified by sequencing. Three quarters of the variants were single base substitutions, and one quarter were insertions, duplications, deletions or indels of 1 to 62 bp. Using 40 PCR products to cover the entire coding region, all heterozygotes were detected for a sensitivity of 100% and a specificity of 97.6%. Based on the number of variants analyzed and a 95% confidence interval, van der Stoep et al., calculate a sensitivity of at least 98.3%. Unlabeled probes were used to decrease the sequencing burden from polymorphisms. Interlaboratory testing confirmed the accuracy and robustness of the method.

Van der Stoep et al. (2009) also provide diagnostic guidelines for developing and implementing HRMA scanning assays. Annealing temperature optimization on a gradient cycler using primers with M13 tails simplify the workflow. Four different DNA isolation methods were compared and did not influence scanning accuracy. Although different reconstitution buffers can affect absolute Tms (Seipp et al., 2007), temperature shifting to compare curve shapes appears to eliminate any differences. DNA concentrations were varied at least 4-fold without affecting melting results.

High resolution melting is a relatively new technique and the seven articles in this focus issue further expand and define its limits. It is simple, rapid, and inexpensive, but depends strongly on good PCR, instruments and dyes. Heterozygote detection does not depend on the type of single base substitution or the variant position within the PCR product (Reed and Wittwer, 2004; van der Stoep et al., 2009). Small insertions and deletions may be somewhat more difficult to detect than substitutions (van der Stoep, et al., 2009). Accuracy is critically dependent on the resolution of the instrument (Herrmann et al., 2006). Saturating DNA dyes are not required for some HRMA applications like methylation analysis where heteroduplexes are at best a distraction. However, scanning and genotyping are entirely dependent on heteroduplex identification and different dyes are variably effective. For example, LCGreen[®] Plus detects heterozygotes better than SYTO[®] 9, which is better than EvaGreen[®], which is better than SYBR Green I (Farrar et al., 2009). Any effect of amplicon GC content is difficult to dissect from its effect on PCR and must await further data. The dependence of scanning accuracy on PCR product length has been studied on the HR[™]-1 (Reed and Wittwer, 2004) and LightScanner[®] instruments (McKinney et al., 2009.), revealing more errors as the length increases above 400 bps. However, the optimal number of melting domains for HRMA remains controversial. For example, Tindall et al. (2009) argue strongly for small products and against multiple domain melting, while half of the targets used by Rouleau et al. (2009) have more than one domain and most of the PCR products used by Dobrowolski et al. (2009) have two or more domains, all in this issue. Controversy and competition may be good signs for this fledgling utilitarian method.

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