Biochemical Society Transactions www.biochemsoctrans.org

Mutation scanning using high-resolution melting

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Abstract
Mutation scanning techniques are used to detect sequence variants without the need for prior knowledge of the identity or precise location of the variant, in contrast with genotyping techniques, which determine the status of a specific variant. High-resolution melting is a recently developed method that shows great potential as a mutation scanning technique. Sensitivity and specificity for mutation detection are extremely high and the technique also has advantages of cost and throughput. Practical considerations for successful mutation scanning by high-resolution melting are also discussed in this review.

Principle of HRM (high-resolution melting)
HRM is a simple, PCR-based method for detecting DNA sequence variation by measuring changes in the melting of a DNA duplex. Melting of double-stranded DNA molecules is influenced by several factors. Some of these, such as the length, GC content and sequence, are properties of the individual molecule [1]. Others are not: these include the ionic strength of the buffer solution, the DNA concentration and the presence of substances such as DMSO or betaine [2,3]. For HRM analysis, duplexes may be formed from the two strands of a PCR amplicon [4–6] or from an oligonucleotide probe and an amplicon strand [7–9]. Amplicon melting has been used for both genotyping and mutation scanning [5,6]. Probe melting is restricted to detecting variation within the probe-binding site. It has generally been used for genotyping applications [10], although there is scope for limited scanning because the presence of variants under the probe other than the target variant also perturbs probe melting [7].

A homozygous sequence variant usually changes the T_m of the duplex. Where there is an exchange between G:C and T:A base pairs, the change in T_m is relatively large: approx. 0.8–1.4°C [6]. If, however, the bases swap strands but the base-pair does not change, the change in T_m is smaller, becoming undetectable if there is also nearest-neighbour symmetry [6]. A heterozygous sample contains four duplex species (Figure 1A) and its observed melting curve is a composite of the four individual melting curves. The contribution from the relatively unstable heteroduplexes changes the shape of the heterozygous melting curve (Figure 1B) [5,6].

Duplex melting is generally monitored using intercalating dyes, although fluorescently labelled primers have also been used [4]. These dyes, which bind to double-stranded but not single-stranded DNA, fluoresce when bound but not after release on duplex melting. Dyes such as LCGreen, able to saturate available binding sites at a concentration compatible with PCR, are necessary for successful HRM. As a consequence of dye redistribution during melting, non-saturating dyes, such as SYBR Green, are biased against low-temperature melting species and do not detect heteroduplexes [5,11].

HRM is a simple method: after PCR, carried out in the presence of a suitable dye, the product is heated while the level of fluorescence is measured. As the temperature rises and the duplex passes through its melting transition, dye is released and fluorescence intensity is reduced. Although several instruments capable of performing the fluorescence acquisition exist, they vary in performance, with those designed for HRM giving a more satisfactory outcome [6,11–14].

Low-resolution melting data and HRM data in a genotyping context are often viewed as a derivative plot ($-dF/dT$ against T), whereas for mutation scanning, a different approach to data analysis is taken [4]. A group of raw melting curves show variation in both the fluorescence and temperature axes (Figure 2A). Normalization of fluorescence before and after the melting transition (Figure 2B) corrects fluorescence variation caused by factors such as variable amplicon levels. Temperature variation poses more of a problem: variations in the observed T_m are caused not only by sequence-dependent T_m changes but also, for example, by buffer differences between samples and by non-uniformity across microtitre-format melting instruments [15]. These extrinsic variations are of similar magnitude to sequence-dependent changes in T_m [6,15]. Normalization of temperature, known as temperature shifting (Figure 2C), is a necessary correction for these variations, but at the expense of homozygote detection [5]. Finally, the melting curves are often converted into a subtractive difference plot (Figure 2D) in which the sample melting curves are shown relative to a wild-type control curve. Difference plots are more informative than derivative plots [4,5], and can make even subtle changes in the shape of the melting curve readily apparent [10,14].

HRM in practice: evaluation and application
Although amplicon HRM has been used as a genotyping technique [4,5,16], it is as a scanning technique that it finds wider application [10,17–42]. Together, these studies have examined

Key words: amplicon, genotyping, high-resolution melting (HRM), mutation detection, mutation scanning, sequence variant.
Abbreviations used: HRM, high-resolution melting.
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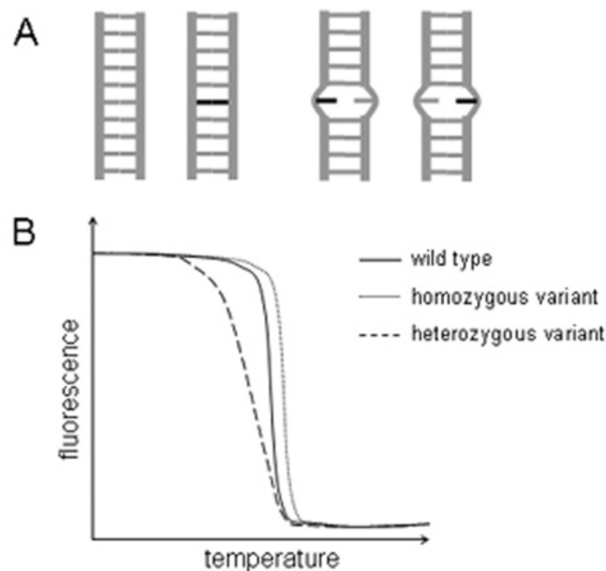
Figure 1 | Homoduplexes, heteroduplexes and their melting curves
(**A**) The four duplexes formed after PCR from a heterozygous sample: two fully matched homoduplexes, one wild-type and one mutant, and two mismatched heteroduplexes, formed from the pairing of a mutant strand with a wild-type strand. (**B**) Schematic melting curves from wild-type, homozygous and heterozygous samples. The homozygous curve has a different T_m from the wild-type curve, whereas the heterozygote curve has a different shape from the wild-type.



hundreds of mutations in many different genes. Human genetic disease has been the largest area of application, with investigations into autosomal dominant, recessive and X-linked disorders. The other main application has been the identification of somatic mutations acquired by human tumours.

The sensitivity of heterozygote detection has been remarkable, with many of the studies able to detect all heterozygous mutations examined [16–19,21,23–25,27,30,31,33,34,41,42]. Even where false negative results have been reported, they represent a small proportion of the total, suggesting that the true sensitivity indeed approaches 100%. All categories of substitution are detectable, as are insertions and deletions small enough to be amplified by PCR. Mutations are detectable at any location in the amplicon, including those within a few base pairs of the primers [19,27].

In principle, temperature shifting during data analysis renders homo- or hemi-zygous mutations undetectable, but in practice many homozygous mutations are detected [23,25,27,36,41,42], suggesting that the shape of the melting curve is in fact sometimes changed in homozygotes [5]. Hemi- or homo-zygous mutations are most likely to be encountered when screening genes mutated in X-linked conditions and in recessive conditions where common or founder mutations are frequent. Pre-PCR mixing of such samples with wild-type template [19,20,27,35,36] can avoid this problem, as can extending screening to obligate heterozygote carriers [41]. Internal temperature calibration using 3′-blocked double-stranded oligonucleotides increases the discrimination of homozygous mutations from wild-type in short amplicons [15,43] and may also prove to be applicable in a scanning context. Screening for sequence variation in haploid organisms on the basis of changes in T_m rather than melting curve shape has been described in [44,45].

Greater amplicon length and lower GC content may have an association with false negative results, but the relationship does not seem to be straightforward [42,46]. Location of the variant within the fragment can clearly affect the magnitude of the change to the melting curve for an individual variant [46], but again, there appears to be no simple relationship between location and sensitivity. Adjustment of PCR primers [22] or analysis parameters [23,26] allowed detection of some variants initially scored as false negative. Others were caused by clerical errors [10] or were associated with sample source and preparation [26,28] and a few remained unaccounted for [20].

In tumour specimens, or in constitutive DNA from individuals mosaic for a germline mutation, the proportion of mutant DNA may deviate considerably from the 50% usually present in a heterozygote. Several studies have quantified the minimum level of mutant DNA detectable by HRM [22,26,29,30,42]. The lower limits defined are rather variable: some of this variation appears to be mutation specific [29,30] and there may also be an association with amplicon length [42]. A minority of mutations may have a lower detection limit in the range of 10–25%; many are detectable at 5% or lower. Clearly, HRM is more sensitive to low-level mutations than DNA sequencing [26,32,42]. Nevertheless, several false negative results were found when screening tumour samples for somatic mutations [26,29,32,38]. It seems that, in many cases, the proportion of mutant DNA in the sample fell below the detection threshold: either the same variant was clearly detected in other samples containing a larger proportion of tumour cells [29,32] or became detectable on re-preparation of the sample to contain a greater proportion of tumour [38].

Specificity, the proportion of true negatives correctly identified as such, is an important parameter, especially with high-throughput applications, because every false positive result generates unnecessary downstream sequencing. Accurate determination of specificity requires analysis of large numbers of known wild-type samples. Several studies have estimated the specificity of HRM, with most finding that it is in the range of 90–100% [16,22–24,26,28,33–35,38,46]. Specificity may be higher in shorter amplicons [46] and seems to have been adversely affected by sample quality in some [24,42] but not all [28] cases.

HRM: considerations for experimental design

Effective amplicon design is an important consideration for successful HRM. Robust and well-optimized PCR is needed for maximum sensitivity and specificity [23,35], and melting behaviour of the amplicon has an impact on the results [22,41]. The number of melting domains in an amplicon is determined

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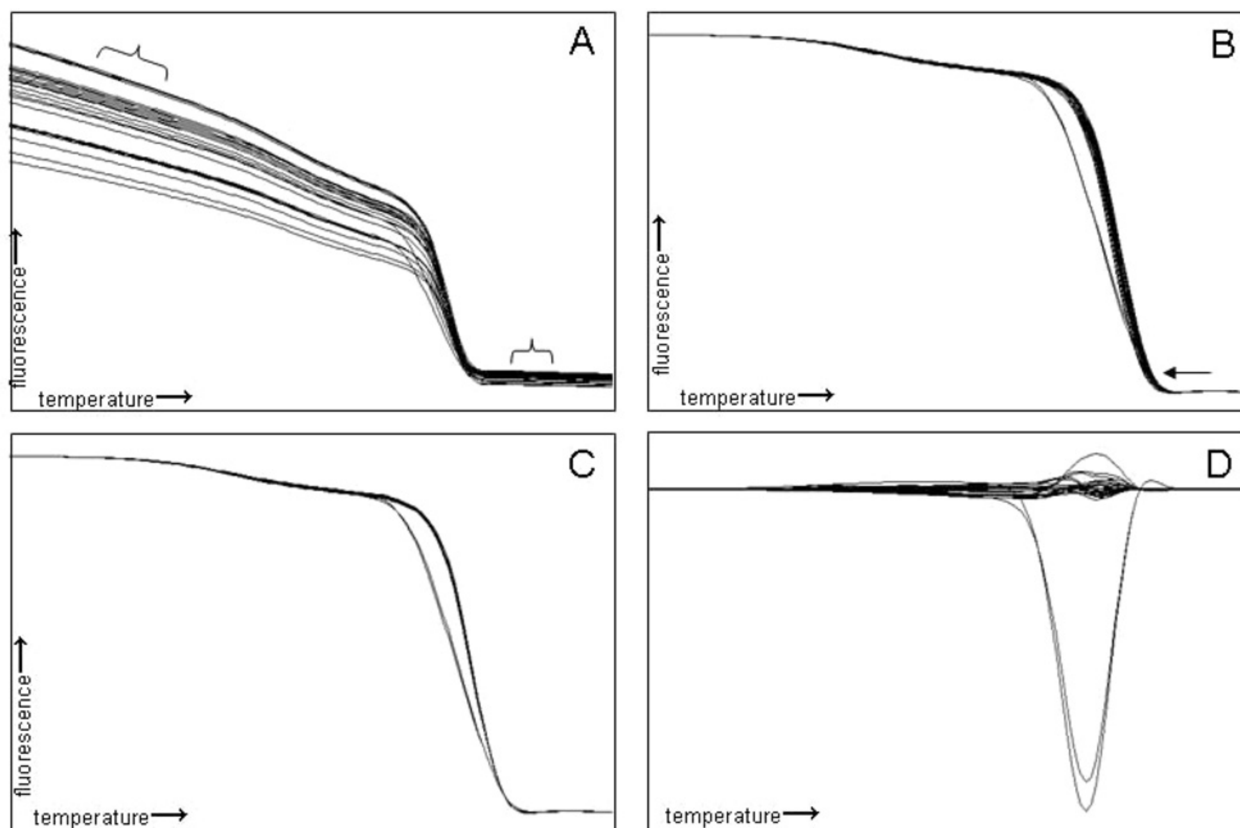
Figure 2 | **Processing of HRM mutation screening data**

(**A**) Raw melting data. The pre- and post-melting regions of the curve selected for fluorescence normalization are indicated by curly brackets. (**B**) Fluorescence-normalized melting data. The region of the curve selected for temperature normalization (temperature shifting) is indicated with an arrow. (**C**) Temperature-shifted, fluorescence-normalized melting data. (**D**) Subtractive difference plot.



by the distribution of G:C base-pairs. While the requirement to cover the entire region of interest, usually coding exons and splice sites, is paramount, various ways of manipulating primer design and location to improve the melting characteristics of the amplicon have been devised. Algorithms that predict melting behaviour from sequence have been used to optimize primer location [17,22,24,27,31,41]. Amplicon melting has been modified by using GC-clamped primers [41] or mismatched primers of reduced GC content [22]. HRM has been successfully performed in amplicons of 38–1000 bp [6,46], but most studies have used fragment sizes in the range of approx. 100–300 bp. T_m changes are greater in smaller amplicons [4] and evidence also suggests that the magnitude of the change to a heterozygote melting curve, and hence the heterozygote detection-sensitivity, are also greater in smaller amplicons [4,42,43,47]. In one study, a control mutation, missed when located in an amplicon with a complex melt profile, could be seen easily when the primers were redesigned to amplify two shorter overlapping fragments [22]. Nested PCR has been successfully applied, for example, where genomic complexity necessitates long-range PCR to mediate specific amplification of the sequence of interest without co-amplification of homologous sequences [47].

Samples from many sources are suitable for HRM. Many of the applications have been in human genetic disease, and DNA extracted from peripheral blood lymphocytes is a widely used source. It has been noted that when samples prepared in different laboratories or by different extraction methods are run alongside one another they can produce visibly different melting curves [15,23]. Whole genome amplification methods have been used in conjunction with HRM [18,26,33], although one study found a reduction in sensitivity in whole-genome-amplified material [26]. Other sample sources have included dried blood spots [36], buccal cells [33] and frozen tumour specimens [22,26,48]. Archived samples in the form of methanol-fixed tissue [28], formalin-fixed paraffin-embedded tissue [24,26,28,34,38,40] and cytology slides [28,29,39] have also been analysed successfully, although, again, sensitivity may be reduced [26,28]. Although the amount of template has been quantified before PCR in most studies, there may be reasonable tolerance of variation in quantity provided that samples amplify well [24,33].

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Where multiple samples contain the same variant, the mutant melting profiles group together tightly [10,17,27, 31,37], whereas, in many cases, different variants in the same amplicon [14,17,20,21,26,37] or compound heterozygotes [14,36] produce distinct profiles. However, as it has been shown that different variants can give rise to indistinguishable profiles [10,18,23,33], it would be unwise to assume that the shape of the melting curve is diagnostic of the underlying variant, and specific confirmation of the causative variant is prudent. PCR products are not consumed by HRM, so positive samples can be transferred directly to sequencing for mutation identification and confirmation [27,35].

The presence of a high-frequency benign polymorphism within an amplicon can be challenging for HRM, as for other scanning techniques, because polymorphisms are detected as effectively as disease-causing mutations. Consequently, in the presence of a common polymorphism, the positive predictive value of a variant profile can be low [10] and the need to confirm the identity of variants means that excess sequencing has to be carried out [24,27,34,35,38,39]. Simple avoidance is not always possible, particularly for polymorphisms close to or within exons. PCR using primers containing a mismatch to both alleles of the polymorphism has been described [24]. Combined probe and amplicon melting of asymmetric PCR products is a further option [9,10,49]. Other solutions make use of the observation that biological replicates have reproducible melting profiles. A 1:1 mix of a test sample believed to be heterozygous for a specific variant with a confirmed heterozygous control will produce a melting profile identical with the two unmixed samples if both contain the same variant but not if they contain different variants [47]. More recently, a redundant screening strategy has been devised [35,36]. In addition to HRM screening of exons, short-amplicon genotyping HRM assays are performed. The results of exon scanning are compared with the genotyping result. If concordant, the genotype is assigned without the need for sequencing. If the results are inconsistent or if the exon melting profile does not group with the polymorphism control, then the sample is sequenced.

Concluding comments

HRM has been shown to have high sensitivity and specificity, with a performance as good as, if not better than, other commonly used mutation scanning techniques [50]. There are other attractions: only a single analysis condition is used and it is not necessary to tailor this to the individual amplicon, in contrast with many other widely used methods [31,50]. HRM is a closed tube system, which not only reduces the potential for contamination, but also increases sample throughput because there is no requirement for physical separation of DNA molecules [50]. HRM offers significant savings in cost and turnaround time [16,19,37,40,47] when compared with other scanning methods. Ease of use, high sensitivity and specificity, low cost and rapid turnaround make HRM an attractive choice for mutation scanning, especially in high-throughput environments.

Acknowledgements

I am grateful to Mike Churchman and Phil Chambers for helpful comments on the manuscript.

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Received 27 October 2008
doi:10.1042/BST0370433

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