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High-Resolution Melting Analysis (HRMA)—More Than Just Sequence Variant Screening

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For the Focus Section on HRMA Technology

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ABSTRACT: Transition of the double-stranded DNA molecule to its two single strands, DNA denaturation or melting, has been used for many years to study DNA structure and composition. Recent technological advances have improved the potential of this technology, especially to detect variants in the DNA sequence. Sensitivity and specificity were increased significantly by the development of so-called saturating DNA dyes and by improvements in the instrumentation to measure the melting behavior (improved temperature precision combined with increased measurements per time unit and drop in temperature). Melt analysis using these new instruments has been designated high-resolution melting curve analysis (HRM or HRMA). Based on its ease of use, simplicity, flexibility, low cost, nondestructive nature, superb sensitivity, and specificity, HRMA is quickly becoming the tool of choice to screen patients for pathogenic variants. Here we will briefly discuss the latest developments in HRMA and review in particular other applications that have thus far received less attention, including presequence screening, single nucleotide polymorphism (SNP) typing, methylation analysis, quantification (copy number variants and mosaicism), an alternative to gel-electrophoresis and clone characterization. Together, these diverse applications make HRMA a multipurpose technology and a standard tool that should be present in any laboratory studying nucleic acids. Hum Mutat 30:1–7, 2009. © 2009 Wiley-Liss, Inc.

KEY WORDS: mutation detection; methodology; melt curve analysis; HRM; HRMA

The Hardware

Several suppliers produce systems for high-resolution melting curve analysis (HRM or HRMA). Overall, these systems perform as can be expected but resolution may differ significantly [Herrmann et al., 2006, 2007]. The most sensitive system currently available is the HR-1 (Idaho Technology Inc., Salt Lake City, UT) generating fluorescence data from 55–95°C at a temperature transition rate of 0.1°C/sec and 200 data points/°C. Some assays, for example, multiplex SNP-typing [Seipp et al., 2008], critically depend on this resolution. The HR-1 uses capillaries and can analyze only one sample at a time; other capillary systems analyze up to 32 samples. Microtiter plate systems are more popular, facilitating the analysis of 96 or 384 samples simultaneously. Another distinction is whether the system is used for HRMA only or whether it is a combined (real-time) polymerase chain reaction (PCR) and HRMA instrument. As nicely demonstrated by Rouleau et al. [2009], combined qPCR and HRMA instruments allow detection of both quantitative (deletion/duplication) and qualitative (nucleotide) changes in one assay.

In most systems small well-to-well temperature differences exist that negatively influence sensitivity. To bypass this problem, Seipp et al. [2007] added control temperature calibration probes: one melting at low and one at high temperature. The analysis software can use the melt positions of these probes to compensate for temperature differences between wells, decreasing T_m SD by 38% [Seipp et al., 2007] notably increasing sensitivity. Note that the software available on HRMA instruments is an important element determining the ultimate sensitivity achieved [Herrmann et al., 2007], and not all packages yet facilitate the use of temperature calibration probes.

LC-Green was the first saturating dye available [Wittwer et al., 2003]; now there are many more, including LCGreen® + (Idaho Technology Inc.), Syto9® (Invitrogen, Carlsbad, CA), EvaGreen® (Biotum) and LightCycler® 480 ResoLight Dye (Roche, Indianapolis, IN). Pricing of the dyes differs significantly; all have slightly different characteristics, and they often demand slightly different PCR buffers and conditions.

Implementation of HRMA is quite straightforward. In the most simple setting, requiring no modifications of existing conditions, dye is added post-PCR directly prior to melting. This simple approach allowed us, for example, to immediately discriminate all possible apoE alleles (Fig. 1A). ApoE typing with other techniques is often problematic, mostly requiring typing of each allele separately. Ultimately, performing HRMA in a closed-tube assay by adding the dye pre-PCR is more attractive. For existing assays this demands modification of PCR conditions, usually increasing Mg^{2+} concentration 2–3 μM and the annealing temperature by 1–5°C will be sufficient. When Mg^{2+} addition raises the T_m above the maximum instrument temperature, additives like DMSO (10%) or betaine (0.5 M) can be added to lower the T_m. For difficult cases, a gradient PCR-cycler can be used to quickly determine the most optimal annealing temperature.

HRMA demands no big changes in the laboratory and does not require specific skills; it is a simple PCR performed under slightly modified conditions and in the presence of a specific dye. The most expensive element is acquisition of the instrument. However, with €10, 000; 50, 000, depending on the system, even this compares

Additional Supporting Information may be found in the online version of this article.

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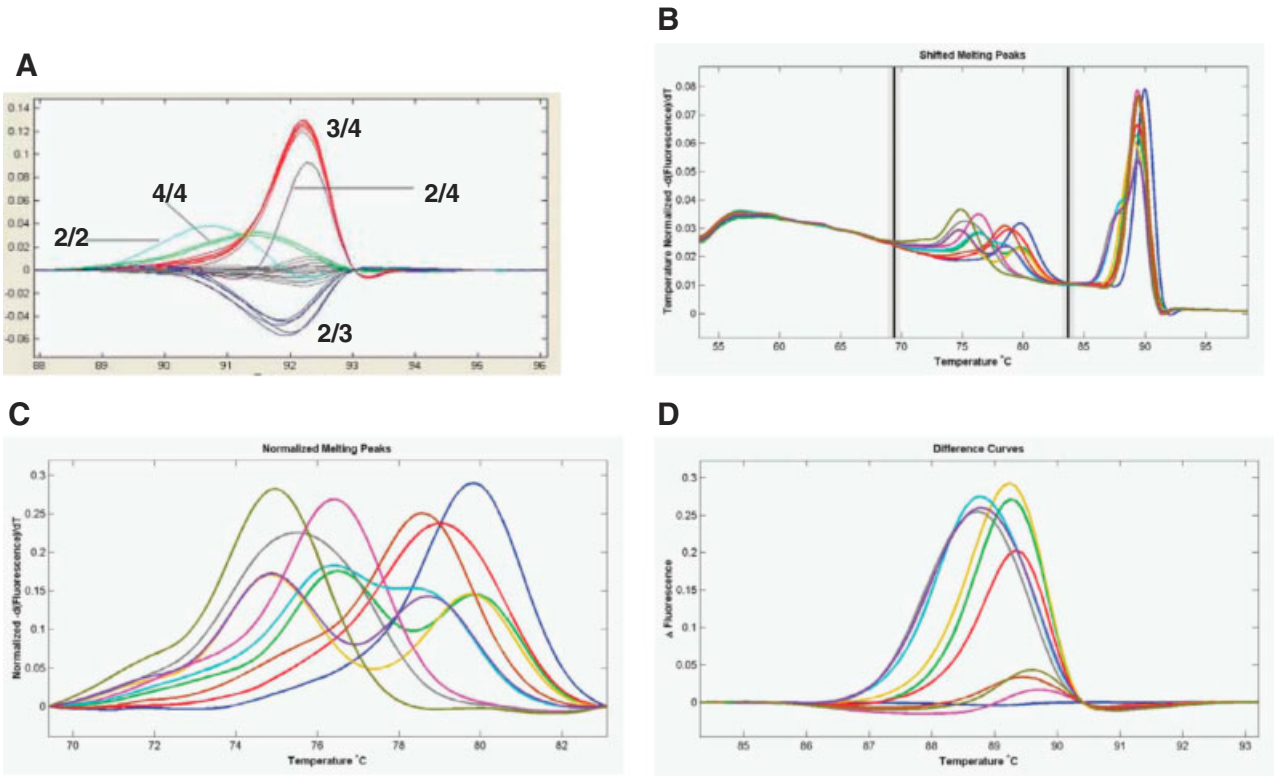

Figure 1. Sequence variant detection using HRMA. **A**: detection of all six possible ApoE-alleles combinations; LC-Green+was added post-PCR, before HRMA (the PCR contained 10% DMSO). The ApoE 3/3 allele was set as standard (horizontal gray line). **B–D**: SNP typing using an unlabeled amino-blocked melt probe covering three independent variants in the first exon of the MBL2 gene (Roos et al., in preparation). **B**: overall derivative plot; **C**: enlargement of the low (melt probe) and **D**: high (PCR fragment) T_m peaks. Using the combined melt profiles all 10 possible alleles can be clearly discriminated.

favorably to some other technologies. Because HRMA, unlike many other techniques (e.g., SSCA, dHPLC, DGGE, or capillary electrophoresis), does not require post-PCR separation, significant cost savings are achieved. Furthermore, HRMA is a nondestructive method, and subsequent analysis by, for example, gel-electrophoresis or sequencing, can still be performed after melt analysis.

Applications

Mutation Detection

HRMA has been developed for the detection of DNA sequence variants and it was applied first for genotyping [Wittwer et al., 2003]. Simplicity, low cost, ease of use, and a high sensitivity/specificity have been the most prominent features, making HRMA an attractive new tool for genotyping and application in diagnostic labs. In Supp. Table S1 we give a comprehensive list of all (human) gene-based assays we could find. HRMA systems come with powerful software tools facilitating automated scoring that is very robust, although a quick manual check remains advisable. Using HRMA for mutation detection has been reviewed recently [Erali et al., 2008]; therefore, we will make general comments only. Furthermore, this issue of *Human Mutation* contains several papers describing application of HRMA for sequence variant detection, elegantly demonstrating its current state of the art (see also Supp. Table S1).

Tindall et al. [2009] compared two instruments and fluorescent dyes in particular regarding the detection of combinations of DNA variants present in GC-rich fragments. They demonstrated the current limitations of HRMA and called for caution when using it as the sole method to make a clinical diagnosis. Nguyen-Dumont et al. [2009] showed the power of including a melt probe to improve identification of rare variants in combination with a known SNP as well as to dramatically reduce sequencing effort. Van Der Stoep et al. [2009] followed a similar approach designing a sequence variant screen covering the BRCA1 gene, including melt probes against known frequent SNPs. In the setting of the EuroGenTest consortium, the authors went through the effort to perform an elaborate interlaboratory evaluation and validation of HRMA and generated guidelines for setting up and implementing it as a scanning technique for new genes. In a blind study on 28 patient samples the protocol resulted in a 100% detection sensitivity at a specificity of 98%, indicating a low incidence of false positives. Rouleau et al. [2009] used the possibility provided by some instruments to perform quantitative PCR and HRMA in one instrument to scan for both quantitative (deletions/duplications) and qualitative nucleotide changes in one assay. Finally, Dobrowolski et al. [2009] described the use of HRMA to scan the entire 16.6 kb human mitochondrial genome (mtDNA) for sequence variants in less then 2 hr. Identification of mtDNA variants is complicated, as many are heteroplasmic, with the variant allele present at highly variable percentages. The fact that the authors successfully identified variants present at levels ranging from 1–100% heteroplasmy nicely shows the sensitivity of the assay as well as the power of HRMA to detect quantitative changes (see Quantification).

Although HRMA of fragments up to 600 bp and more has been reported, our experience is that the technology is more sensitive for smaller fragments. For fragment screening, fragments of

150–250 bp are used, that is, in general, one fragment per exon. When assays are designed to type specific variants (SNP typing) we target fragment sizies of 80–100 bp. We find it critical for high sensitivity that the melt profile contains not more then one to two melt domains. When fragments contain more melt domains chances increase that not all variants are detected. Today the design of new assays is simplified by the availability of powerful design programs, often delivered together with the instrument.

In early HRMA experiments we have seen that a second and sometimes even a third melt of the PCR products may improve results. Similarly, especially when the concentration of the DNA fragments to analyze differ considerably, results can be improved by adding 1 µl high salt buffer (1.0 M KCL, 0.5 M Tris-HCl [pH = 8.0]) followed by a new melt (Fig. 2). Salt addition may increase resolution and improve clustering, but success of the method is unpredictable and depending on the samples, fragments, and variants analyzed.

When a few simple rules are taken into account (i.e., avoid long fragments and multiple melt domains), designing a HRMA screen for a gene is rather straightforward. We have successfully designed assays to screen a range of genes of which the DMD gene with ~90 fragments covering 79 exons was the largest (Al-Momani et al., in press). As Nguyen-Dumont et al. [2009] and Van Der Stoep et al. [2009] show, it is time and cost saving to include unlabeled melt probes to positively identify known nonpathogenic variants. This prevents unnecessary sequencing of such fragments, while it at the same time safeguards against overlooking other variants in the same fragment (see Presequence Screening).

It should be noted that the shape of an HRMA curve in itself is usually not sufficient to type a specific variant [Tindall et al., 2009]; to achieve this either a melt probe should be added or the fragment should be sequenced. The power of adding an unlabeled melt probe to discriminate specific variants or multiple alleles is astonishing. An example is shown in Figure 1B–D (Roos et al., in preparation). Using a melt probe containing the wild-type sequence we were able to discriminate all 10 possible alleles deriving from a series of three closely spaced variants in the MBL2 gene.

The cheapest block available to prevent extension during PCR of the melt probe is a 3′ phosphate. Unfortunately, this block is unstable, and after time undesired additional melt peaks may emerge. Other blocks are more stable but also more expensive. Zhou et al. [2008] elegantly solved this issue by using a so-called snapback primer, that is, a 5′ tailed primer including a loop region and a sequence complementary to its extension product, covering the variant to scan.

A potentially weak point of HRMA is the detection of homozygous variants. Although recent developments have further improved resolution [Gundry et al., 2008], the difference for some variants (e.g., A–T to T–A changes) are so subtle that they can easily be missed. Especially when samples from different sources have to be analyzed, sample-to-sample variation and thus experimental noise increases and subtle changes might go undetected. Therefore, in sequence variant scanning applications (clinical diagnostics) we consistently use sample mixing to generate hetero-duplexes. First, samples are melted to obtain a standard melt profile. Next, using the scheme shown (Fig. 3, designed for DMD, an X-linked disease), samples are mixed and then a second melt curve is generated. The simple mixing scheme results in two heteroduplexes for each sample (note that sample mixing can be easily automated). Homozygotes will result in two wells with heteroduplexes, greatly improving their detection. Heterozygotes will be detected from the standard premixing melt curves, although they will usually be obvious from the mixed samples as well (1:3 ratio, see Quantification). The scheme shown will only fail when samples with identical variants are mixed but this can be simply prevented by adding more controls or by mixing samples from unrelated individuals only. It should be noted that the scheme presented (Fig. 3) has the potential to discriminate hemizygous from true homozygous alleles as well.

It should be noted that the sensitivity of HRMA to detect heterozygotes is much better than that of DNA sequencing (see Quantification). Consequently, when HRMA indicates the presence of a variant that cannot be confirmed using sequencing it might well be that this variant is present in a relatively low fraction of the sample (somatic mosaicism). Other techniques, for example, cloning+sequencing or single molecule dilution+PCR and HRMA, might be required to confirm these variants.

Pressequence Screening

Using HRMA for presequence screening, in particular for larger genes, may yield significant cost savings; Provaznikova et al. [2008] reported avoiding unnecessary sequencing of more than 85% for the MYH9 gene. In the example of the DMD gene, where 79 exons need to be analyzed (Al-Momani et al., in press), assuming bidirectional sequencing costs €10 per exon, screening a patient amounts to ~€800. PCR per exon (€1) and HRMA (€0.10 dye) would cost ~€90 per patient. Assuming 5 exons show a melt shift requiring verification by sequence analysis (including one pathogenic variant, three nonpathogenic variants and a false positive)

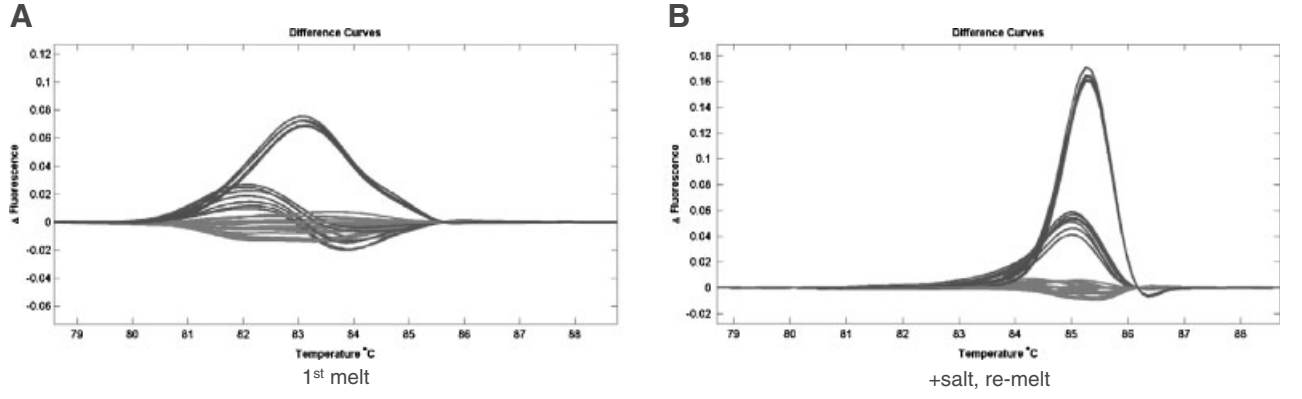

Figure 2. Effect of high-salt. **A**: HRMA analysis of a series of samples. **B**: The same samples as in **A** analyzed after addition of 1 µl 1.0 M KCl/0.5 M Tris-HCl (pH = 8.0) and remelting. Note the improved separation and sharpening of the three groups.

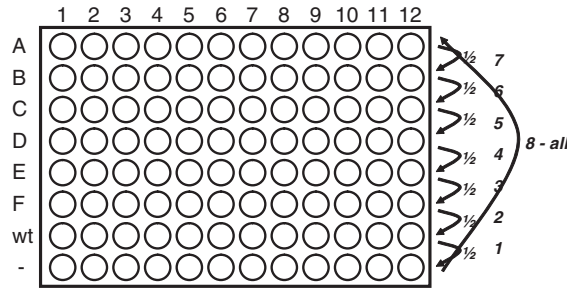

Figure 3. Generating heteroduplexes. To ensure the detection of all homozygous variants the scheme shown can be used. Wells 1–12 contain PCR products from different fragments (exons) amplified for each sample (patients A–F and wt = wild-type control). Row 8 is empty. After a first HRMA mixing starts with taking half of the sample from row 7 (wt, 5–10 µl of the PCR product) and transferring this to row 8. Subsequently, half the volume of from row 6 is transferred to row 7, half of row 5, to row 6, etc. Finally, the content of row 8 is transferred to row 1 and the fragments are melted again.

would add €50, making a total of €140. A cost saving of more then €650 per patient that can be further reduced when melt probes are included to confirm the presence of the most frequent nonpathogenic variants. In fact, based on these figures, HRMA prescreening of a five-exon gene is already cost-effective. In addition, to detect somatic changes or heteroplasmy [Dobrowolski et al., 2009], HRMA seems more sensitive then sequencing (see Quantification).

SNP typing

HRMA for SNP-typing can be very attractive. Assay design is cheap, simple, and fast. When in a specific region one or two PCRs covering a SNP are designed, at least one and usually both will give a good assay. To increase sensitivity, the fragment should preferably be small (80–100 bp) and when a choice is available (e.g., in a haplotype block) one should select a G to A variant (predicted to give the largest melt shift) [Reed and Wittwer, 2004]. Addition of an unlabeled melt probe [Nguyen-Dumont et al., 2009; Zhou et al., 2008] is recommended, giving a double check in the assay and improved scoring for homozygote variants. When ordered primers arrive, it should not take more then one or two PCRs to test amplification conditions and the assay is ready. Assay costs are then just PCR, and cost for assay design (an often largely underestimated factor) are negligible. Unless thousands of samples need to be typed, dye cost should compare favorably to assays including specific labeled probes (e.g., TaqMan). HRMA sample throughput can be increased using robotic plate loading, available as an extension on some systems or by installing an additional loader.

Based on the positive results described above one wonders whether variable number tandum repeats (VNTRs), especially CA repeats, could be typed using HRMA (Fig. 4). Although in the example shown all curves can be clearly discriminated, we found that all possible allele combinations are so many that heterozygous CA repeat typing cannot be performed with certainty. It is possible though in small families to detect differences between family members and to use HRMA to study loss of heterozygosity (LOH) in heterozygous samples. As indicated by the experiments of Intemann et al. [2009], it can be speculated that addition of a melt probe spanning the smallest and/or largest allele might increase resolution further.

A weak point of HRMA is that it cannot easily be applied in multiplex mode, that is, to type variants in several different

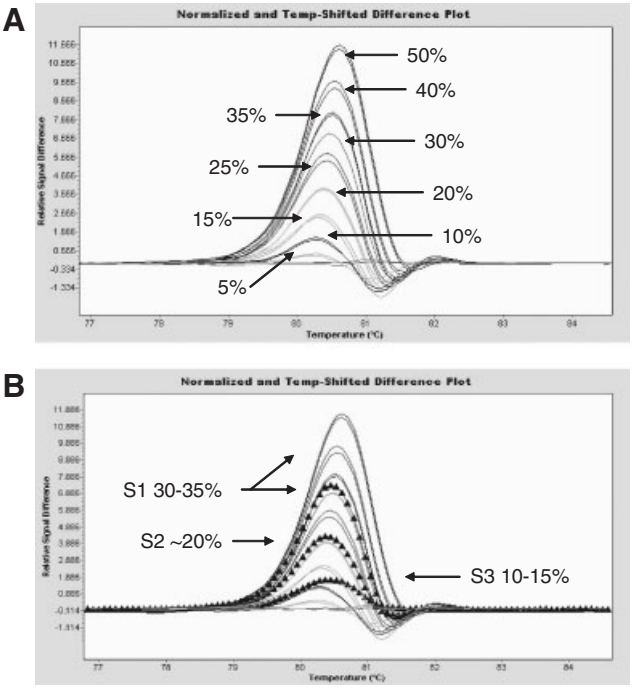

Figure 5. Quantification using HRMA. **A**: Dilution series for a pathogenic A > G variant in APC exon 8. **B**: Superposition of the HRMA profiles from three mosaic samples (S1–S3) and the estimation of the level of somatic mosaicism. Pyro-sequencing of the samples gave estimates of 30, 19, and 7%, respectively.

fragments at the same time. Theoretically, multiplexing can be achieved by exploiting color differences, temperature differences, or both. Seipp et al. [2008] used the simplest approach, that is, T_m-differences to successfully design a quadruplex genotyping assay. However, such design is time consuming and its success critically depends on high sample DNA quality and the sensitivity of the HRMA system used.

Methylation

Lately, genomic studies frequently involve the analysis of epigenetic marks, especially methylation at CpG dinucleotides in relation to gene expression, imprinting, and cancer (Ehrich et al., 2006). A critical step in these procedures is bisufite treatment of the genomic DNA, changing unmethylated C nucleotides (but not methylated Cs) to Us. HRMA can be used in such studies in two stages. First, success of the bisulfite treatment can be checked by PCR of a control genomic segment devoid of methylated CpGs and comparison of its melt profile with that of a 100% converted fragment (cloned) and an untreated sample. The closer the melt profile resembles that of the 100% converted fragment, the better the bisulfite treatment worked [Worm et al., 2001]. Second, HRMA can be used to determine the percentage of C to U conversion, either directly, in combination with a melt probe [Maat et al., 2007], or after cloning (see Clone Characterization).

Quantification—Mosaicism and Copy Number Variant (CNV) Confirmation

Depending on the melt shift obtained, HRMA can also be used for quantitative analysis; the larger the shift, the smaller the

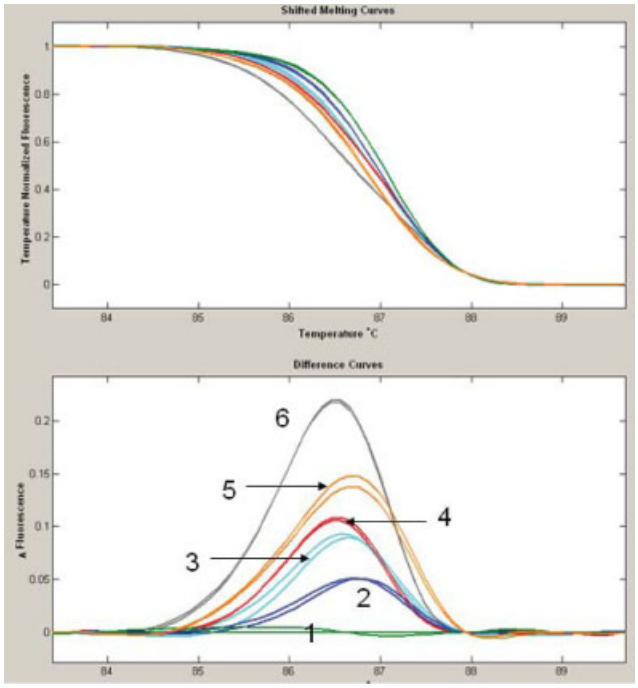

Figure 4. CA repeat analysis using HRMA. Six different DNA samples were analyzed in duplicate, all heterozygotes. Top panel: normalized temperature shifted melting curves. Bottom panel: derivative plot. Allele lengths were 1 = 18/21, 2 = 17/21, 3 = 14/17, 4 = 14/18, 5 = 15/21, 6 = 14/21.

quantitative differences that can be detected. To quantify the fraction of variant molecules frequently used technologies are dideoxy sequencing (resolution limit down to 20–30%) and pyro-sequencing (down to 1–10%). Although powerful, pyro-sequencing is labor-intensive, costly, and demands specific equipment. We have successfully applied HRMA for the quantification of different alleles [Aten et al., 2009; Bruder et al., 2008], and shown that detection in steps of 12.5% (1 in 8) is usually possible [Aten et al., 2009]. Application for the detection of the level of somatic mosaicism in colon cancer is shown in Figure 5. Using a dilution series as a ruler we could readily determine the level of somatic mosaicism in three patients suspected to carry a specific pathogenic variant down to a level of 5–10%. The data obtained matched perfectly with those obtained using pyro-sequencing [Hes et al., 2008], yet the assay was less expensive and simpler to perform. It should be noted that when melt probes are included resolution can be improved to below 5%. Other qHRMA applications are the determination of differences in allelic expression, based on the presence of a variant in the mRNA and the detection of heteroplasmy in mtDNA [Dobrowolski et al., 2009].

Recently, we used the same approach to estimate the fraction of cells carrying a somatic deletion identified in one individual from an identical twin pair [Bruder et al., 2008]. The approach can also be used to confirm CNVs (both deletions and duplications) detected using whole genome SNP arrays while screening patients of diverse diseases for genomic rearrangements. Depending on the setting, techniques like FISH, MAPH [Armour et al., 2000], MLPA [Schouten et al., 2002], MAQ [Suls et al., 2006], and qPCR can be applied to confirm the array findings. However these techniques are either costly or demand considerable time to develop. For confirmation with HRMA one can use any SNP from the suspected region. Samples homozygous for the two opposite alleles (AA and BB) of these SNPs are used to generate a reference ruler as well as a 1:1 mix with the sample potentially carrying the rearrangement. Assume the test sample carries the A allele and is either A0 or AA. When mixed 1:1 with a homozygous BB sample the deletion is confirmed when the melt profile comigrates with the 1:2 AA:BB sample mix (A0BB). The deletion is absent when the melt profile comigrates with the 1:1 AA:BB sample mix (AABB). A duplication will be confirmed when the 1:1 mix with a homozygous BB sample comigrates with the 3:2 AA:BB sample mix (AAABB).

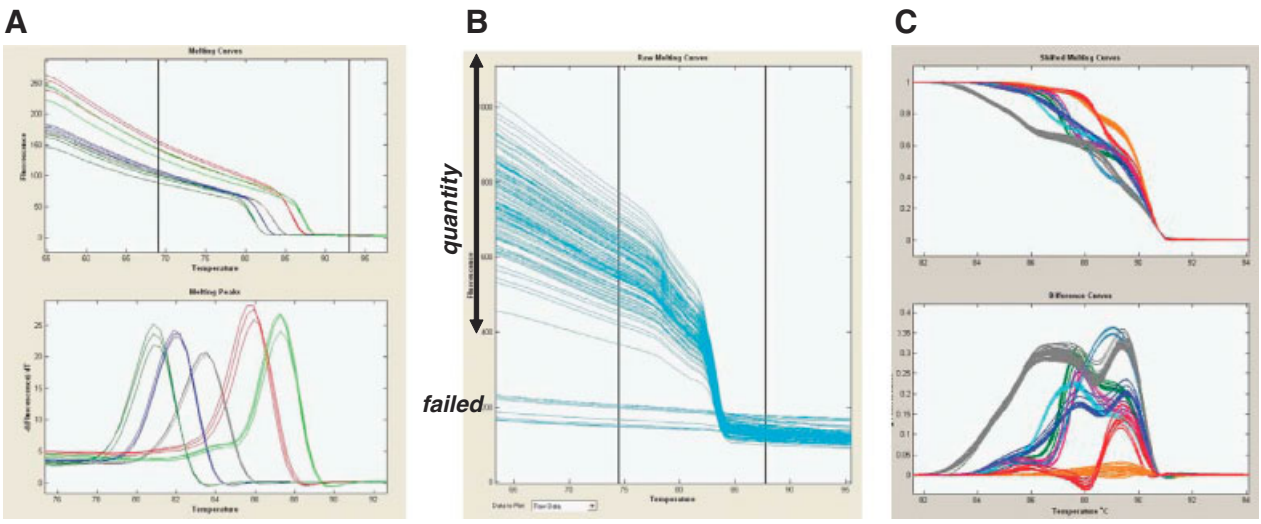

Figure 6. HRMA as alternative for gel electrophoresis. **A**: Analysis of a series of five different PCR fragment; because the fragments have clearly different melt profiles all five fragments can be analyzed in one analysis. When the melt profiles partly overlap, analysis can be done per fragment. **B**: Analysis of a PCR performed on 96 different samples. Some PCRs failed (only background) fluorescence, yield of the others can be estimated from the level of fluorescence. Purity, including absence of primer dimers, can be checked by analysis of the HRMA difference plots (not shown). **C**: HRMA after insert PCR of 384 phage display clones after second round selection. Several clear groups of melt profiles are identified, an indication that the clones contain identical inserts. Note that to identify all groups present, clones recognized after a first analysis need to be removed and software grouping must be repeated. This procedure has to be repeated until no further groups are recognized.

Alternative for Gel Electrophoresis

The current standard to check the result of a PCR or digestion is analysis of the product using agarose gel electrophoresis and ethidium–bromide staining. Identity of the fragment is characterized by its length, purity by the absence of other fragments, and yield by the strength of the fluorescence of the band. HRMA is an attractive alternative; identity characterized by the melting profile, purity by the absence of distortions from the control melt curve (and absence of additional melt peaks), and yield by the amount of fluorescence signal (Fig. 6). The advantages of using HRMA are clear; one does not have to pour gels and use hazardous chemicals (ethidium–bromide), melting is faster then electrophoresis, and data analysis can be performed automatically. Furthermore, because it is a nondesctructive method, when HRMA would not give clear results fragments can still be analyzed on gel.

In our laboratory HRMA is quickly replacing gel electrophoresis for the characterization of PCR products. Dye is added post-PCR (1 µl LC-Green + [10 × stock] per 10 µl sample), sample is 5 min incubated at 95°C, cooled down to room temperature, and melted. Figure 5A shows an example where five different fragments were analyzed, all clearly discernable by their individual melt behavior. Primer–dimer formation would be recognized by the presence of a melt peak at low T_m. Out et al. (manuscript submitted) used HRMA instead of gel electrophoresis to check amplification as well as to determine long-range PCR yield guiding equimolar pooling before sequencing of the MUTYH gene. The authors could successfully show detection of nearly all variants in the expected frequencies down to 0.5% (1/200 chromosomes).

Clone Characterization

Several studies generate large series of clones that need to be sequenced to determine their identity. These include in vitro mutagenesis experiments, methylation studies, cDNA cloning to determine levels of differential splicing and/or allelic expression, and phage display selections. HRMA provides an attractive tool to prescreen the clones to detect those that share the same insert and those that differ, generating considerable savings for subsequent sequencing. An example is shown in Figure 6C, showing the result of a second round phage display selection. The experiment resulted in several groups of clones with identical melt curves, indicating that the experiment was successful in positively selecting several different phage display clones. Subsequent sequencing of representative clones per group confirmed the HRMA results; sequence differences between groups and sequence identity within groups (van Roon-Mom et al., submitted). Previously, clone inserts were fingerprinted using restriction digestion and gel electrophoresis, a less sensitive and much more laborious method [Verheesen et al., 2006].

Conclusion

The advantageous characteristics of HRMA make it a technology that quickly attracts a range of new users. Its ease of use, simplicity, flexibility, low cost, nondestructive nature, superb sensitivity, and specificity, make HRMA the method of choice to screen patients for pathogenic variants. As reviewed above, HRMA has several attractive additional applications, making it a versatile multipurpose analytical tool to analyze nucleic acids in general. Because HRMA is still a rather young technology, one can only expect exciting further developments. The company Fluidigm markets a nanoliter qPCR system [Spurgeon et al., 2008], today facilitating the analysis of 96 samples × 96 PCR assays (i.e., 9,216 assays simultaneously); imagine the power of such a system when it would facilitate HRMA.

Acknowledgments

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