Diagnostics

High-resolution melting curve analysis on the LightCycler® 480 PCR system

Roche Applied Science´s LightCycler® family of real-time PCR systems offer fast, accurate and versatile platforms for genetic variation research. The new plate-based LightCycler® 480 System provides the temperature homogeneity and optical characteristics required for high-performance melting-curve analysis (MCA). On the level of data acquisition and available detection channels, this new instrument opens the way to more advanced applications in the emerging field of gene scanning where amplicons can be screened for unknown sequence variations with low efforts in time and cost.

Principles of MCA for genetic variation analysis

Post-PCR MCA has become a robust and well-established method to characterize amplicons, for applications that include microbiological identification or the detection of mutations and single nucleotide polymorphisms (SNPs). In the case of SNP studies, the goal could be to detect the presence of different allelic variants of a given SNP in a sample set or to identify polymorphic positions at which previously unknown SNPs occur without prior knowledge of the exact allele. This latter approach is especially useful as a screening technique to reduce the number of sequencing reactions required to detect new variants[1].

The LightCycler 480 System (instrument for 96- and 384-well plates; **Fig. 1**) implements a modular approach toward hardware, software and reagents that allows users to answer both types of questions by using a broad range of detection chemistries and data analysis algorithms according to the user's specific research needs.

Known SNPs present in PCR amplicons can be investigated with sequence-specific, labeled probes that bind with different strength to the different alleles or allele combinations in the SNP-containing region, depending on whether they fully match the target sequence or contain mismatches. Another detection principle makes use of special DNA-binding dyes (for example, LC Green Plus) whose binding characteristics allow them to be used at high concentrations without inhibiting PCR. Because of their saturating, homogeneous staining of PCR products, these dyes give sharp, unique melting profiles that allow differentiation between homo- and heterozygous samples, and often even between different homozygous genotypes[2].

For either method, melting-curve raw data are generally represented by plotting fluorescence over temperature, with the relevant temperature range and acquisition rate (data resolution) being higher with generic high-resolution dyes than with labeled probes (**Figs. 2**, **3**). However, they can be analyzed more conveniently by looking at the negative first derivatives (-d*F*/d*t*), revealing melting temperatures as peaks (**Figs. 2b**, **3b**). A comparison of data derived from homozygous wild types, homozygous mutant and heterozygous samples can reveal differences in peak number, peak position or both. The use of sequence–specific labeled probes provides reliable results and clear separation (**Fig. 2b**), with heterozygotes showing two peaks, and homozygotes only one peak at different positions for wild type and mutant. If high-resolution dyes are used instead of probes, the differences obtained between melting peaks are often detectable, but sometimes not big enough to allow a clear differentiation of homozygotes (**Fig. 3b**).

Figure 1 | The LightCycler 480 system.

The LightCycler 480 Genotyping Software analyzes the shape of melting curves obtained with probes. It can also perform an automated calling of the corresponding sample genotypes, by grouping samples with similar melting-curve shape together, either automatically or (optional) based on standards included in the experiment (data not shown).

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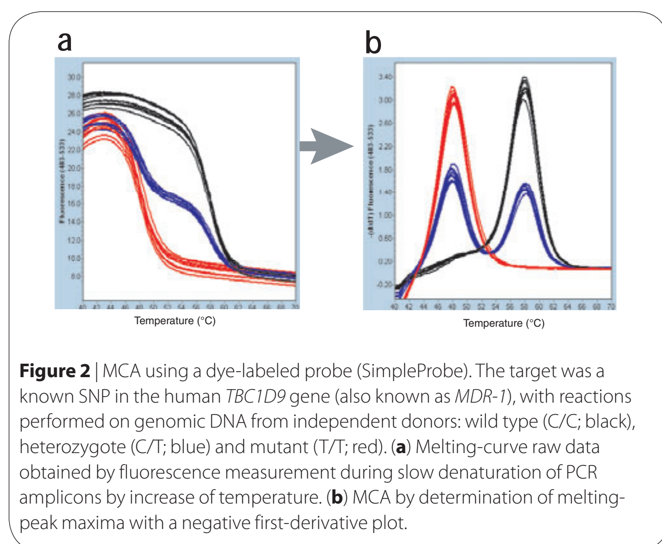

Figure 2 | MCA using a dye-labeled probe (SimpleProbe). The target was a known SNP in the human *TBC1D9* gene (also known as *MDR-1*), with reactions performed on genomic DNA from independent donors: wild type (C/C; black), heterozygote (C/T; blue) and mutant (T/T; red). (**a**) Melting-curve raw data obtained by fluorescence measurement during slow denaturation of PCR amplicons by increase of temperature. (**b**) MCA by determination of melting-peak maxima with a negative first-derivative plot.

High-resolution melting detects new sequence variants

By definition, the scanning of amplicons for unknown variations cannot be done with sequence-specific probes because the location of a potential sequence variation is not known. Therefore, generic DNA-binding dyes with saturating binding characteristics must be used, and melting signals must be analyzed at high resolution.

Unknown sequence variations in diploid organisms such as humans become apparent in heterozygous samples during post-PCR MCA owing to the presence of heteroduplex DNA. When amplified and melted, these samples show melting curves with different profiles than those derived from homozygous wild-type or mutant samples (**Fig. 3a**). The LightCycler 480 Gene Scanning Software analyzes these differences by first normalizing the data and then temperature-shifting the curves such that differences in the melting temperature (T_m) between homozygotes with very similar curve shape disappear, and heterozygotes stand out more clearly owing to their differently shaped curves (**Fig. 3c**). In cases where T_m differences between homozygotes are big enough, these can be displayed by omitting the temperature-shift step. By finally plotting the difference in fluorescence for each sample compared with the wild type, heterozygote samples can be easily identified (**Fig. 3d**).

A choice of SNP discovery and analysis methods

The LightCycler 480 Instrument is also compatible with other mutation-detection methods, but MCA has the advantage of being based on a robust, post-PCR biophysical measurement. MCA reveals more information than other real-time PCR-based mutation analysis methods, such as deriving genotype information from the PCR process itself. Less sequence information is needed to design a genotyping assay, and allele-specific oligonucleotides are not required because the same dye or probe can be used for all known or unknown alleles present and investigated. For presequencing, a gene-scanning approach such as MCA, performed with a high-resolution dye, offers greater convenience and throughput than traditional methods (for example, denaturing high-performance liquid chromatography).

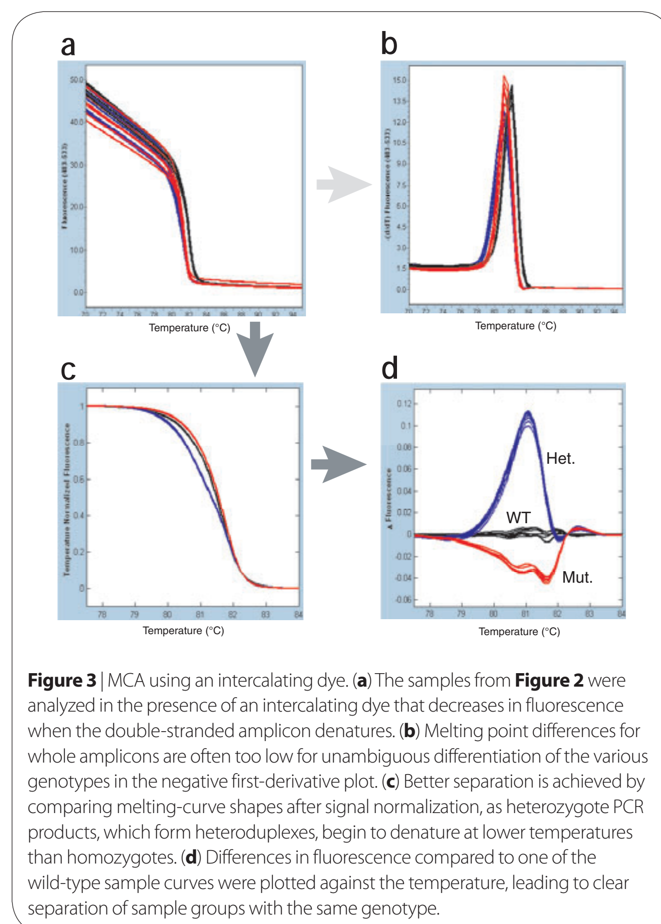

Figure 3 | MCA using an intercalating dye. (**a**) The samples from **Figure 2** were analyzed in the presence of an intercalating dye that decreases in fluorescence when the double-stranded amplicon denatures. (**b**) Melting point differences for whole amplicons are often too low for unambiguous differentiation of the various genotypes in the negative first-derivative plot. (**c**) Better separation is achieved by comparing melting-curve shapes after signal normalization, as heterozygote PCR products, which form heteroduplexes, begin to denature at lower temperatures than homozygotes. (**d**) Differences in fluorescence compared to one of the wild-type sample curves were plotted against the temperature, leading to clear separation of sample groups with the same genotype.

Conclusion

The LightCycler 480 System is a versatile platform for the discovery and analysis of genetic variation, and is presently the only available plate-based real-time PCR system allowing mutation scanning by high-resolution melting[3]. The optional LightCycler 480 Genotyping and Mutation Scanning Software can be combined with optimized PCR master mixes for both genotyping and gene scanning. The system thus provides highly accurate results based on the analysis of melting-curve profiles.

Additional information about the LightCycler 480 System and the mutation detection approaches described here is available from Roche Applied Science (http://www.lightcycler480.com). LIGHTCYCLER is a trademark of Roche. Patent and license disclaimer information is available online (http://www.lightcycler.com).

1. von Ahsen, N. Two for typing: homogeneous combined single-nucleotide polymorphism scanning and genotyping. *Clin. Chem.* **51**, 1761–1762 (2005).
2. Herrmann, M.G. *et al.* Amplicon DNA melting analysis for mutation scanning and genotyping: cross-platform comparison of instruments and dyes. *Clin. Chem.* **52**, 494–503 (2006).
3. Dujols, V. *et al.* High-resolution melting analysis for scanning and genotyping. in: *Real-Time PCR.* (ed, Tevfik, D.) 155–169 (Taylor and Francis, Abingdon, UK 2006).

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