

# Mutation Scanning of the Cytidine Deaminase Gene by High-Resolution Melting Curve Analysis Using the LightCycler® 480 System

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## Introduction

The purpose of the study was to screen sequence variations of the cytidine deaminase (CDA) gene in tumor samples. CDA plays a key role in the tumoral and hepatic catabolism and inactivation of gemcitabine, a nucleoside analog extensively used in chemotherapeutic treatment of solid tumors. Recent studies have demonstrated that the CDA gene is highly polymorphic, displaying a variety of single nucleotide polymorphisms (SNPs) and one insertion-deletion in African- and Caucasian-American individuals. Genetic variation in CDA might explain the observed interindividual differences in both the therapeutic and toxic responses to gemcitabine-based treatment of cancer. We investigated sequence variations in CDA using a novel gene scanning method based on post-PCR analysis of high-resolution melting curves. The method reveals sequence variants due to distinct patterns in melting curve shape, allowing limitation of sequencing to those samples that actually contain anomalous sequence information. The LightCycler® 480 System is a multiwell-plate-based system integrating PCR and high-resolution melting capabilities, including an optimized master mix and analysis software. We tested the LightCycler® 480 System and also compared it with the LightScanner Instrument (Idaho Techn.)

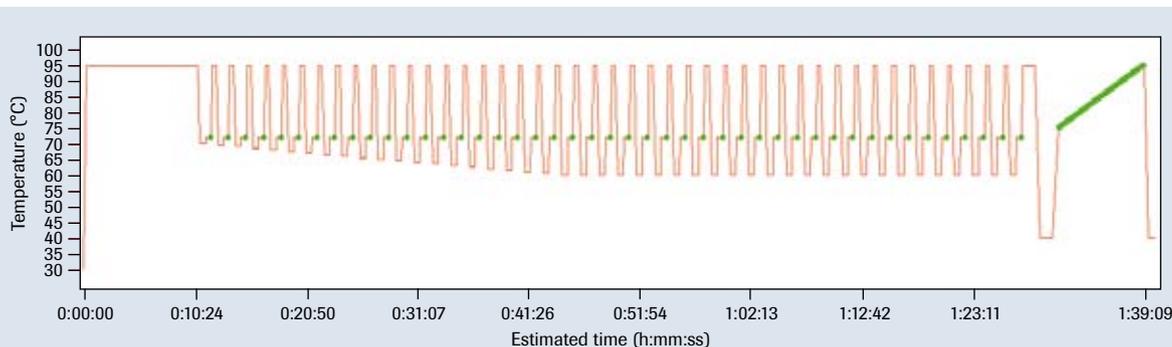
## Materials and Methods

The LightCycler® 480 High Resolution Melting Master Mix containing a proprietary saturating HRM dye was used as

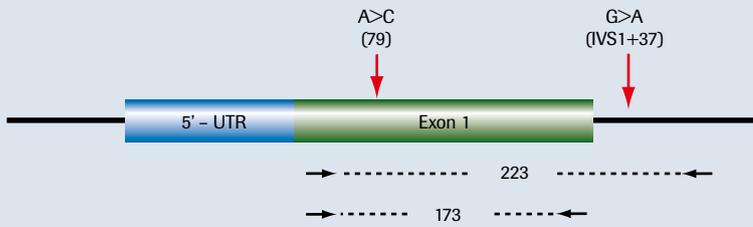
described in the pack insert. 1 µl of a 20 ng/µl solution of each DNA sample (derived from 46 individuals) was added to 9 µl of PCR mix in 96-well plates, as well as 3 mM MgCl<sub>2</sub> and 0.5 µM of each primer. PCR was carried out using a touchdown protocol, with annealing temperatures ranging from 70°C to 60°C. High-resolution melting curve data were obtained at a rate of 25 acquisitions per °C (Figure 1). Fluorescence data were visualized using normalization, temperature-shifting, and difference plotting [1], and then analyzed using the automated grouping functionalities provided by the LightCycler® 480 Gene Scanning Software. Amplicons displaying sequence variations were subsequently subjected to DNA sequencing.

## Results

An overview of the analyzed CDA gene fragment is given in Figure 2. High-resolution melting analysis showed that 22 of 46 DNA samples were heterozygous for a sequence change in a 173-bp-fragment derived from exon 1 (Figure 3). Sequencing showed that the underlying variation corresponded to a known SNP resulting in a Lys27Gln change. All other samples had wild-type sequences, and data obtained by high-resolution melting were 100% concordant with sequencing data. When a longer amplicon was investigated, high-resolution melting revealed the presence of another variant in two cases (shown in green in Figure 4). Sequencing confirmed that these two samples

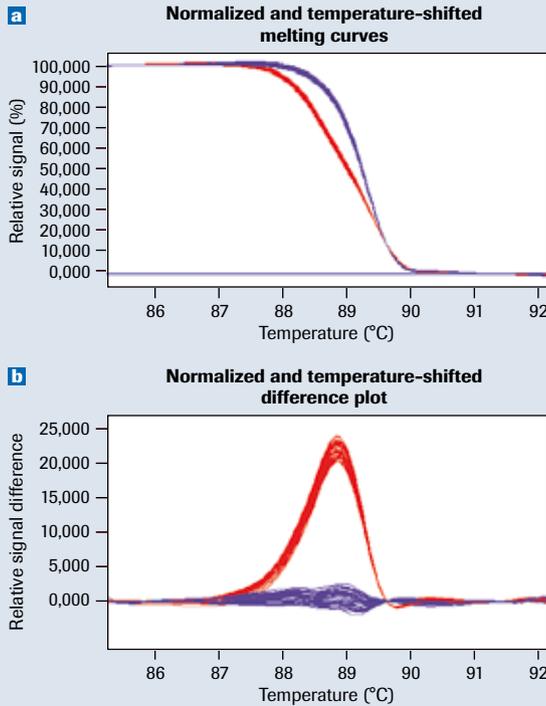


**Figure 1: Overview of the PCR and high-resolution melting analysis temperature profiles used.** This standard touchdown PCR Protocol was suitable for all amplifications performed in the study.

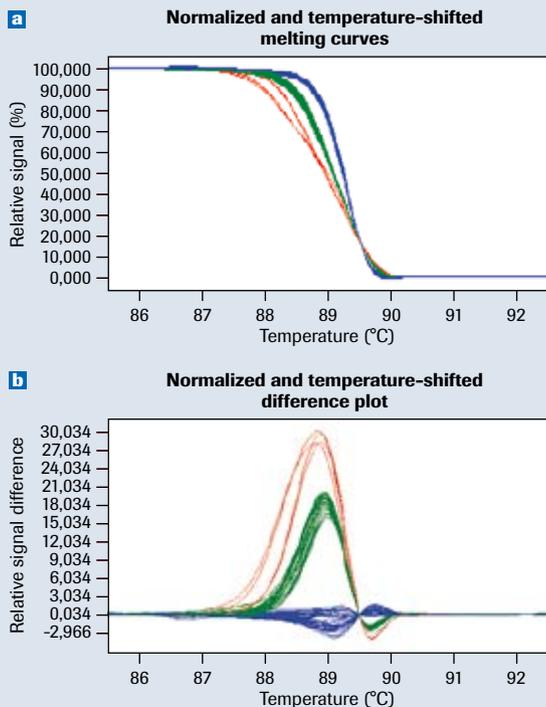


**Figure 2: Location of PCR primers and variable positions in the investigated CDA gene.**

**Figure 3: High-resolution melting analysis of a 173-bp-fragment derived from the human CDA gene.** Wild-type samples are shown in blue, heterozygote mutants (SNP A to C at position +79) in red.



**Figure 4: High-resolution melting analysis of a 223-bp-fragment reveals additional variation.** The presence of an additional intronic variation in two samples led to an additional group (shown in red, two replicates per sample) identified by the LightCycler® 480 Gene Scanning Module.



were heterozygotes for the 2 SNPs A79C and IVS1+37 G>A. They were easily distinguishable in the difference plot chart from samples that harbored A79C alone.

## Conclusions

Sensitivities and specificity of HRM-based gene scanning on the LightCycler® 480 System were comparable to that obtained with previously used, more conventional scanning methods (e.g., dHPLC), with the advantage that results were obtained much faster and with much less hands-on time. Performance also was comparable with the one obtained on the LightScanner platform; however, as an integrated PCR/HRM platform, the LightCycler® 480 System allowed concomitant online control of amplification efficiency and reaching of the plateau phase. In contrast to the LightScanner, it did not require oil overlay on plates. In our hands, the LightCycler® 480 Gene Scanning Software was easy to use, detecting 100% of heterozygotes. When the default sensitivity settings were applied, we were able to identify heterozygous single base changes in PCR products (up to 622 bp in our experiments) with an optimal sensitivity and specificity (not shown). Differentiation of homozygote wildtypes and mutations turned out to be highly dependent on various target-specific factors, including amplicon length and GC content. Spikes of wild-type DNA added to all samples and comparison to unspiked reactions has been shown by others to provide a valuable approach to addressing this point. We found that gene scanning results were best when the amount of starting DNA was standardized as much as possible; which was not always easy to do, since blood samples were derived from various origins so that DNA quality varied. The melting protocol proposed by Roche proved to be suitable for most of our experiments. The LightCycler® 480 High Resolution Melting Master was easy to use because all components – including a novel HRM dye – are provided in one tube. The fact that MgCl<sub>2</sub> was provided separately allowed us to adapt the concentrations to the one known from PCR protocols optimized previously.

In summary, our results show that the LightCycler® 480 System provides a novel, integrated solution to scanning gene fragments for sequence variations based on post-PCR melting curve analysis at high resolution. ■

## References

1. BIOCHEMICA 2007 1:17–19
2. Mercie C et al. (2007) Pharmacogenet Genomics, in press

Product	Pack Size	Cat. No.
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LightCycler® 480 High Resolution Melting Master	500 reactions	04 909 631 001

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