Transfering PCRs to HRM-assays on the LightCycler® 480 System – Examples for BRCA1

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Introduction

High-resolution melting curve analysis (hrMCA) is an attractive technique to scan for unknown mutations in genes. To evaluate how easy or difficult it is to design hrMCA assays using the LightCycler® 480 Instrument, we selected 3 different fragments in exon 11 of the BRCA1 gene, designed an MCA assay, and tested its sensitivity to detect known variants.

Materials and Methods

Starting from our normal PCR conditions, we first determined the optimal conditions to perform the reaction in the LightCycler® 480 High Resolution Melting master mix containing a saturating DNA dye. After raising the Mg^{2+}-concentration to 2–3 mM, PCR conditions were tested by running a temperature gradient of 55–65ºC using a block cycler. PCR and hrMCA were then performed on a LightCycler® 480 Instrument to confirm that the PCR products gave good melting curves. Agarose gels were run to confirm that clean products of the expected length had been obtained.

Results and Discussion

For all 3 fragments good amplfication was obtained at a broad temperature range (Figure 1). Our experiments showed that we were able to obtain good amplification products with only minimal adjustment of the original PCR conditions (touchdown PCR is another option to consider and has been used successfully, for example, in a study by Evrard et al. [1]).

To determine whether known DNA variants could be detected, we analyzed a set of 24 samples, including one no template control (NTC). The samples included the DNA variants listed in Table 1. All samples were amplified in duplicates in a 96- and a 384-well microtiter plate. In total, 276 reactions were performed, and none failed. Amplification and MCA results obtained in 96- and 384-well format were comparable but a slightly better melting resolution was obtained with the 384-well format.

BRCA1-fragment 1 is 246 bp long and covers variants A, B, C, D, and E. All variants except the homozygous variant C were readily detected (Figure 2). Next to the wild types, three main groups of curves can be seen. Variants A and B could not be discriminated from each other. BRCA1-fragment 2 is 268 bp long and covers variants F, G, H, I, J, and K. All variants except F and J were easily discriminated from wild type and from each other (Figure 3). Variants F and J could not be detected because in both cases the mutation was located underneath one of the primers, hence only the wild type allele was amplified. BRCA1-

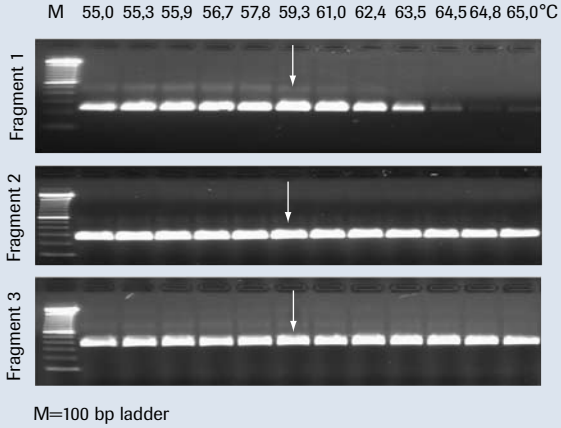

Figure 1: Determination of optimal annealing temperatures for the three BRCA1-fragments. The temOperatures chosen for further experiments are indicated by the arrows.

Table 1: DNA variants.

DNA variant	Mutation	Description
A	c.[2989_2990dupAA]+[3113A>G]	heterozygous pathogenic variant and heterozygous polymorphism
B	c.3113A>G	heterozygous
C	c.3113A>G	homozygous
D	c.3119G>A	heterozygous polymorphism
E	c.[3113A>G]+[3119G>A]	double heterozygous
F	c.3436_3439delTGTT	heterozygous pathogenic variant
G	c.[3485delA]+[3548A>G]	heterozygous pathogenic variant and heterozygous polymorphism
H	c.3548A>G	heterozygous
I	c.3548A>G	homozygous
J	c.[3548A>G]+[3640G>A]	heterozygous polymorphism and heterozygous unclassified variant
K	c.[3548A>G]+[3627A>G]	homozygous polymorphism and heterozygous unclassified variant

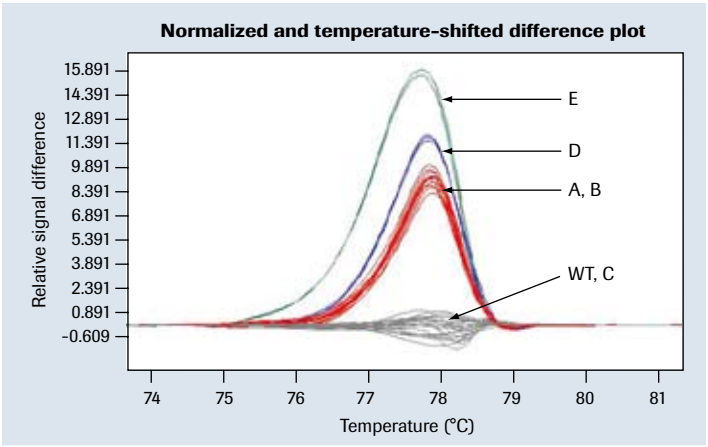

Figure 2: Analysis of BRCA1-fragment 1.

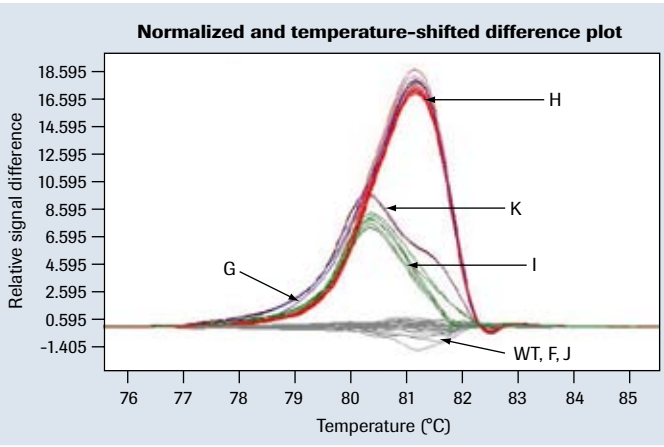

Figure 3: Analysis of BRCA1-fragment 2.

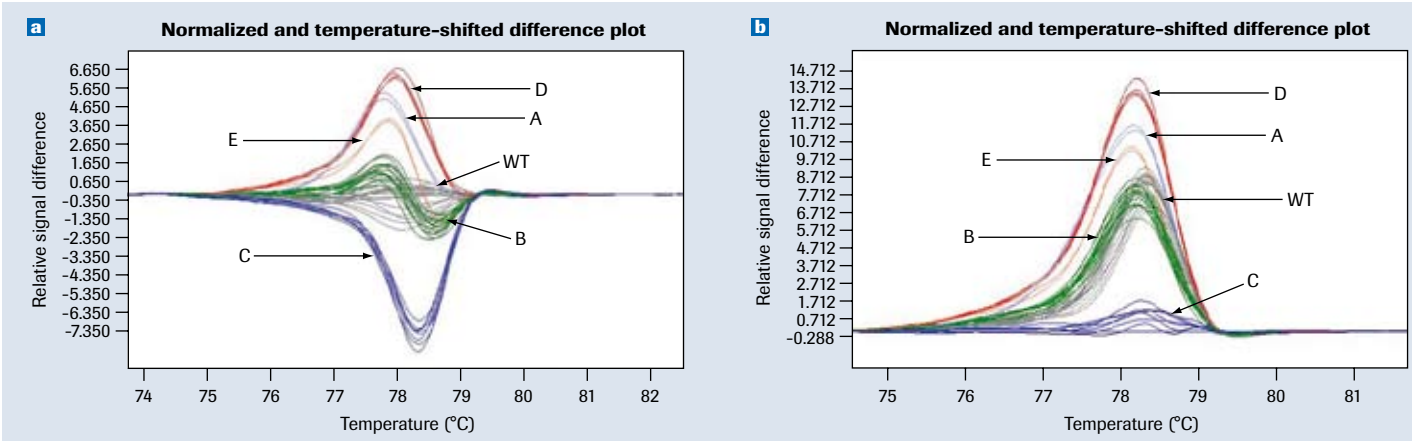

Figure 4: Analysis of BRCA1-fragment 3.

fragment 3 is 378 bp long, overlaps with fragment 1, and covers the same variants; A, B, C, D, and E. Although the picture becomes rather complex, all variants were easily discriminated (Figure 4). It should be noted that in such cases, for clear visualization, it might help to select instead of the wild type one of the variants as the reference (compare Figure 4a and 4b). The functionality to do this is provided in the LightCycler® 480 Gene Scanning Software module. Interestingly, mutation C which could not be resolved from the wild type in fragment 1 (Figure 2), is easily detected in this larger fragment.

Conclusions

Designing and optimizing assays for amplification and melting curve analysis on the LightCycler® 480 Instrument using High Resolution Melting Master Mix with the help of a PCR system facilitating running a temperature gradient turned out to be simple and straightforward. When a block cycler is not available, touchdown PCR can be considered. All heterozygous variants analyzed were readily discriminated from wild type, indicating that a good sensitivity can be expected when the assay is used to scan for unknown heterozygous variants. Detection of homozygous variants using hrMCA has been described and was observed in our study. It should be noted, however, that for efficient detection of homozygous variants it is better to spike in wild-type DNA. Analysis using a 96- and 384-well format gave similar results, sometimes with a slightly better resolution using the 384-well format. As a result of this positive outcome, in collaboration with FP6-project EuroGentest (Nienke van der Stoep), we are now setting up an hrMCA-screen for the entire BRCA1 and 2 genes to be applied in DNA diagnostics. ■

References

1. Evrard A *et al.* (2007) Biochemica 3:13–14

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Order INFO

Product	Pack Size	Cat. No.
LightCycler® 480 Instrument	1 instrument (384 well)	04 545 885 001
LightCycler® 480 Gene Scanning Software	1 software package	04 980 336 001
LightCycler® 480 High Resolution Melting Master	500 reactions	04 909 631 001