Mutation Scanning Using High Resolution Melting or dHPLC: a Performance Comparison Study

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Introduction

High Resolution Melting (HRM) is a novel and promising screening technique used for mutation analysis and detection. Its application prevents the need to sequence all the exons of a gene when looking for novel mutations. The method is therefore time- and cost-effective for the analysis of large genes comprising many exons.

In the research project described here, we compared HRM on the LightCycler® 480 System with denaturing high-performance liquid chromatography (dHPLC), a more conventional method for mutation scanning. A WAVE 4500 HT System has been used for many years in our accredited human genetic laboratory for screening human samples by dHPLC, to identify unknown mutations that can then be characterized in detail by sequence analysis.

We asked whether it was possible to transfer dHPLC assays to the HRM method, with as few changes as possible in primer sequence or assay optimization. The establishment of assays and testing involved 12 exons of the human BRCA1 gene, linked to breast and ovarian cancer, in previously characterized research samples, and – if available – plasmids serving as controls. These samples were analyzed in parallel, using both the WAVE and the LightCycler® 480 System. Results obtained with these methods were compared with each other in terms of sensitivity and specificity.

Materials and Methods

Template preparation

Genomic DNA was prepared from 200 µl whole blood per sample using the MagNA Pure LC System.

PCR setup

Based on previously established PCR conditions, the first step was to determine the optimum annealing temperatures and Mg^{2+} concentrations for PCR using the LightCycler® 480 High Resolution Melting Master Kit on the LightCycler® 480 Instrument.

First, the optimum Mg^{2+} concentrations for all PCR fragments of the BRCA1 gene were established with a touchdown PCR program (annealing temperatures from +65 to +53°C). Mg^{2+} concentrations between 1.5 and 3.5 mM were evaluated, and 5–10% DMSO was added to some of the preparations. We used a 3-step protocol, including initial denaturation for 2 minutes at +94°C; 35 cycles of +94°C, 30 sec / T°ann, 30 sec / +72°C, 1 minute; final elongation at +72°C for 7 minutes, and final cooling to room temperature.

Results and Discussion

For mutation analysis of the BRCA1 gene, we had previously designed primers for amplification of 34 fragments, covering 23 exons. The resulting amplicons have been used in many routine procedures, such as PCR and dHPLC analysis, and certain fragments had also been sequenced. Twelve of these 34 PCR fragments were selected for High Resolution Melting (HRM) analysis with the LightCycler® 480 System and dHPLC in comparison. In this study, the same primers were used as previously for dHPLC and sequencing. For most, but not all, selected fragments, both wild-type and heterozygous or homozygous mutation controls were available. Below, for exons 9, 11 and 20, the comparative analysis obtained by dHPLC and HRM is discussed in detail for six of the twelve analyzed fragments. For the analysis using the LightCycler® 480 System´s Gene Scanning Software module, the wild-type version of the plasmid was always selected as the baseline for comparison.

Exon 9

The PCR product of exon 9 has a size of 292 bp, is rich in repeats, and contains two known mutated sites. Three different mutation controls were available, *i.e.*, the genomic heterozygous mutation control (het-G; mutation +64delT IVS9) inside intron 9 and one heterozygous and one homozygous mutation control as plasmid (het-P and hom-P; mutation c.676C>A). Both mutations are located near the center of the PCR fragment. Three unknown samples A, S and K were analyzed (Figure 1). The difference between the heterozygous controls (het-P and het-G) and the wild

type (Wt-P) was clearly visible both by HRM (left) and by dHPLC (right). In addition, both methods provided an unambiguous result for the samples S and K (both het-G). Sample A looked similar to hom-P in dHPLC but different from all other samples in HRM. This finding was further confirmed by sequencing, showing that the genotype of sample A was hom-G. In addition, the HRM method allowed the demonstration of a clear difference between het-P and hom-P research samples, while this small difference was not easily detected by the dHPLC method.

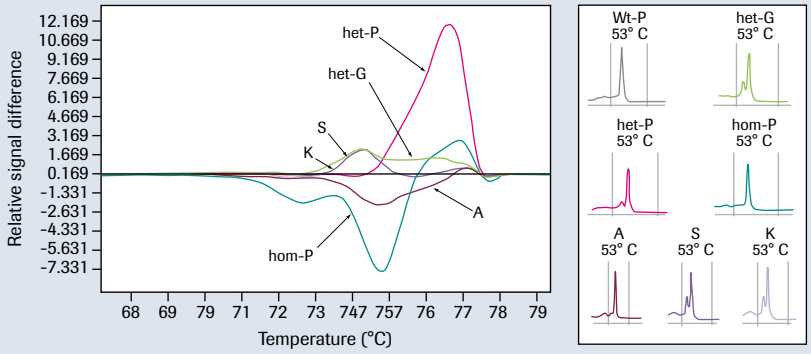

Figure 1: Exon 9. Comparison of mutation scanning results for Exon 9, using high resolution melting (left) and dHPLC (right).

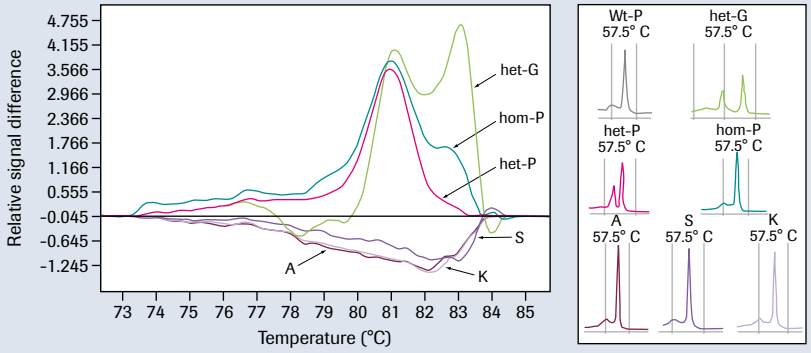

Figure 2: Exon 11A. Comparison of mutation scanning results for Exon 11A, using high resolution melting (left) and dHPLC (right).

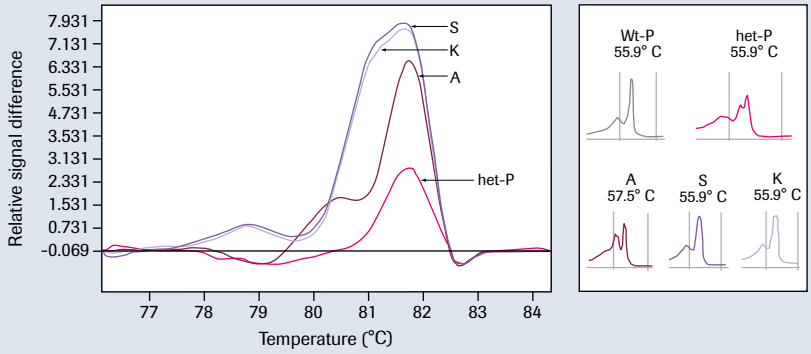

Figure 3: Figure 11E. Comparison of mutation scanning results for Exon 11E, using high resolution melting (left) and dHPLC (right).

Exon 11A

Exon 11A comprises a PCR fragment of 394 bp. It is a relatively long fragment carrying two known mutations, with many thymidine repeats in its front part. Three mutation controls were used for this fragment, *i.e.*, the genomic heterozygous mutation control (het-G; mutation c.969_970insTCATTAC) plus one heterozygous and one homozygous control for a specific SNP (het-P and hom-P; mutation c.999A>T), both available as plasmids. Both mutations are >50 bp away from the forward and reverse primers. Three unknown research samples A, S, and K were analyzed. The two mutations het-G and het-P were clearly differentiated by dHPLC and HRM analysis (Figure 2). With either method, all three unknown samples A, S and K were found to correspond to the wild type. Compared with dHPLC, the HRM analysis gave a better resolution in some cases, allowing a clear differentiation between *e.g.*, hom-P and the wild type, Wt-P.

Exon 11E

The PCR product of exon 11E has a size of 445 bp, which is relatively long for both dHPLC and HRM analysis. The control used for this examination was mutation c.2187A>T in heterozygous constellation (het-P). This mutation is located closer to the 3´-region of the PCR fragment and was clearly differentiated by both dHPLC and HRM. Again, the three research samples A, S, and K were examined for mutations in this exon. Both methods allowed detection of the difference between the samples S and K on the one hand and sample A on the other hand. Subsequent sequencing demonstrated that S and K carry mutation c.2196G>A in heterozygous constellations and A carries another mutation c.2201C>T in a heterozygous constellation. Both systems, HRM and dHPLC, identified this difference clearly (Figure 3).

Exon 11H

The PCR product of exon 11H has a size of 329 bp and a low GC content of 40%. Due to its relatively high adenine content, the identification of this product with the dHPLC system is relatively difficult. The mutation control available for this exon was mutation c.2765T>A in homozygous and heterozygous constellations (hom-P and het-P). Both dHPLC and HRM allowed the detection of this mutation in heterozygous constellation compared with the wild type, Wt-P. However, neither HRM nor dHPLC was able to clearly differentiate hom-P from wild-type controls (Figure 4). The three research samples A, S, and

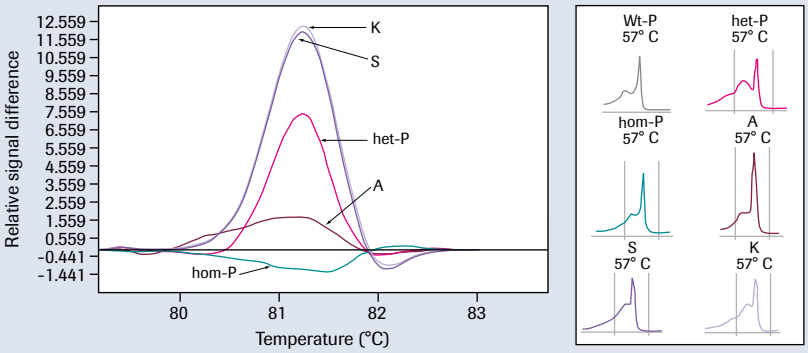

Figure 4: Exon 11H. Comparison of mutation scanning results for Exon 11H, using high resolution melting (left) and dHPLC (right).

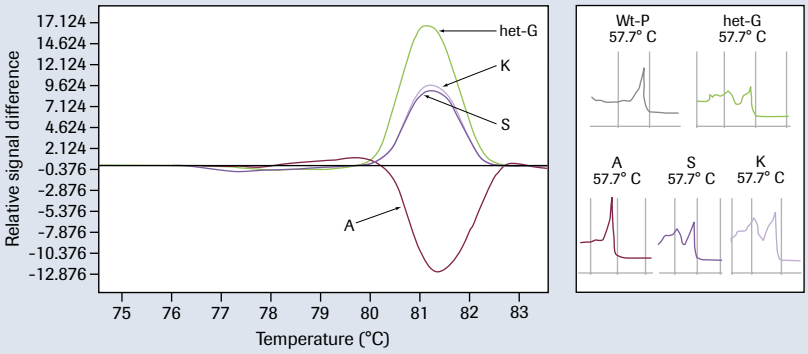

Figure 5: Exon 11K. Comparison of mutation scanning results for Exon 11K, using high resolution melting (left) and dHPLC (right).

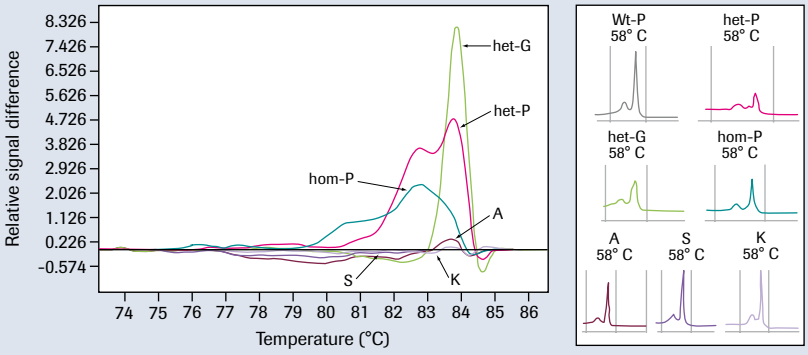

Figure 6: Exon 20. Comparison of mutation scanning results for Exon 20, using high resolution melting (left) and dHPLC (right).

K were examined. The two samples S and K carry mutation c.2731C>T in heterozygous constellation. This difference was demonstrated by both the dHPLC and the LightCycler® 480 System and was verified by sequencing. Sample A was found in the same group as hom-P and het-P: It was concluded that it is different from the wild type and samples S, K, but only sequencing would be able to characterize it completely.

Exon 11K

The PCR product of exon 11K has a size of 313 bp. Mutation c.3667A>G in heterozygous constellation was analyzed (het-G) and research samples A, S, and K were examined. Samples S and K, both carrying mutation c.3667A>G in heterozygous constellation, were clearly identified both with dHPLC and on the LightCycler® 480 System (Figure 5). In addition, the LightCycler® 480 System allowed detection of the difference between sample A, carrying mutation c.3667A>G in homozygous constellation, and the wild type. This difference was much more difficult to see using dHPLC.

Exon 20

The PCR product of exon 20 has a size of 380 bp. For this exon, the genomic mutation control c.5382_5383insC in heterozygous constellation and the plasmid mutation controls c.5350G>C were examined in heterozygous and homozygous constellations. The samples A, S, and K were examined. Both dHPLC and HRM allowed differentiation of the heterozygous genomic mutation control (het-G; c.5382_5383insC) and the plasmid mutation control (het-P; c.5350G>C) in the heterozygous constellation. The plasmid mutation control c.5350G>C in homozygous constellation (hom-P) was clearly differentiated from the wild type only via HRM analysis (Figure 6). The three samples A, S, and K demonstrated no mutation and were therefore identified as wild type with both methods. In total, the result obtained for this exon was very clear.

Discussion and Conclusions

Using the BRCA1 gene as a study model, it was demonstrated by comparative analysis of two gene scanning methods, the more traditional dHPLCS and the more recently developed High Resolution Melting, that both systems are highly comparable with respect to sensitivity and specificity. However, the comparative analysis performed in our laboratory also demonstrated that the LightCycler® 480 System is much more convenient to use than the dHPLC method, since the HRM method is homogeneous and both the PCR and subsequent analysis take place in the same instrument with no need to open the reaction tubes. A further benefit of the LightCycler® 480 System is the option to use both plasmids and genomic DNA as target material, thus permitting the addition of wild-type material immediately prior to the PCR. Subsequent sequencing of the PCR product, following enzymatic or column purification, can also be performed without prob-

lems, due to the special properties of the DNA-binding dye in the LightCycler® 480 High Resolution Melting Master, which is quite different from SYBR Green I in this regard. Although the dHPLC method allows the use of both plasmids and genomic DNA as target material, wild-type material is normally added to the reaction mixture after the PCR (requiring the tubes to be opened), because this approach significantly improves the dHPLC results. However, this means that an additional labor-intensive work step must be carried out, with the inherent risk of contamination. Another benefit when performing HRM using the LightCycler® 480 System compared with dHPLC is the much reduced need for servicing and maintenance. High-performance liquid chromatography instruments in general require many more service calls than a plate PCR thermocycler. In addition, the daily handling and regular maintenance procedures of the dHPLC system are much more complex and time-consuming. We observed that HRM analysis can be more difficult in some cases when the mutations are in proximity (≤16 bp away) to one of the PCR primers. This phenomenon did not occur to the same extent for analyses by dHPLC. When transferring assays from the dHPLC to the HRM method, redesign of some of the primers may therefore be required in certain cases. Bearing in mind that in this study we did not carry out any primer redesigns, the number of assays that were successfully transferred without any optimization was remarkably high. Even in cases where primer dimers were present (as detected by classical melting curve analysis; data not shown), the data were analyzed satisfactorily. Although plasmids were not available as controls for all exons and mutations, samples with new, unexpected variants (*e.g.*, exon 9 in sample A) were amplified and easily characterized further by sequencing.

Neither HRM nor dHPLC analysis permitted reliable conclusions with regard to the exact genotype of a fragment based on the melting or migration pattern of a mutated fragment alone. To fully characterize research samples differing from the wild-type controls, we therefore always included a sequencing step. This applied to results for both the HRM and the dHPLC method. With respect to the analysis of the heterozygous constellation of mutations, both systems demonstrated unambiguous results. However, analysis with the LightCycler® 480 System also allowed us to detect a difference between the heterozygous and the homozygous constellation of mutations (*e.g.*, BRCA1 exon 11K, exon 20). Although this differentiation can be achieved with the LightCycler® 480 System, it is still strongly recommended that the analysis be completed by comparing the homozygous mutation control with the wild type, because the recognition of the mutation in the homozygous constellation strongly depends on fragment-specific factors, such as amplicon size and GC content.

In summary, the transfer of gene scanning protocols from well-established PCR/dHPLC-based protocols to HRM on the LightCycler® 480 System proved to be straightforward, allowing us to achieve very clear results for PCR fragments having a medium size of ≤500 bp with a moderate GC content. This means that very little effort is required to replace dHPLC Systems with the LightCycler® 480 System for mutation scanning and detection in biomedical research and testing. ■

References

1. de Juan I et al. (2009) Breast Cancer Res Treat 115:405–414
2. Takano et al. (2008) BMC Cancer 8:59

Order INFO

Product	Pack Size	Cat. No.
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LightCycler® 480 High Resolution Melting Master	5 x 1 ml	04 909 631 001