METHODS

Human Mutation

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Diagnostic Guidelines for High-Resolution Melting Curve (HRM) Analysis: An Interlaboratory Validation of *BRCA1* Mutation Scanning Using the 96-Well LightScanner™

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For the Focus Section on HRMA Technology

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ABSTRACT: Genetic analysis of *BRCA1* by sequencing is often preceded by a scanning method like denaturing gradient gel electrophoresis (DGGE), protein truncation test (PTT) or DHPLC. High-resolution melting curve (HRM) analysis is a promising and economical method for high-throughput mutation scanning. The EuroGentest network (www.eurogentest.org) aims to assist with the introduction of novel technologies in the diagnostic setting. Therefore, we have performed a thorough and high-standard interlaboratory evaluation and validation of HRM, in collaboration with Idaho Technology, the manufacturer of the LightScanner™ (LS). Through this detailed study of 170 variants, we have generated guidelines for easy setup and implementation of HRM as a scanning technique for new genes, which are adaptable to the quality system of an individual diagnostic laboratory. This validation study includes the description of a *BRCA1*-specific mutation screening test using the 96-well LS. This assay comprises 40 amplicons and was evaluated using a statistically significant elaborate panel of variants and control DNA samples. All heterozygous variants were detected. Moreover, genotype analysis for nine common polymorphisms created a fast screening and detection method for these frequently occurring nonpathogenic variants. A blind study using a total of 28 patient-derived DNA samples resulted also in 100% detection and showed an average specificity of 98%, indicating a low incidence of false positives (FPs).
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KEY WORDS: high-resolution melting curve analysis; HRM; *BRCA1*; diagnostic validation

Introduction

The *BRCA1* gene (MIM# 113705) is involved in susceptibility to breast and ovarian cancer with a very high penetrance rate [Castilla et al., 1994; Claes et al., 2003; Couch and Weber, 1996; Deffenbaugh et al., 2002; Easton et al., 1995; Ford et al., 1998]. Approximately 3 to 5% of breast cancers are caused by germline mutations in the breast cancer genes *BRCA1* and *BRCA2* [Beck et al., 1993; Guldberg and Guttler, 1993; Narod and Foulkes, 2004]. After the *BRCA1* and *BRCA2* (MIM# 600185) genes were identified, genetic testing became available and is now routinely offered to women from high-risk families. Over 1,590 different heterozygous mutations have been reported in the *BRCA1* gene; these mutations have been named the "Breast Cancer Information Core" (BIC) of the Breast Cancer Mutation Database, hosted by the National Human Genome Research Institute (NHGRI) (http://research.nhgri.nih.gov/projects/bic) [Ozcelik et al., 1996; Goldgar et al., 2004]. Mutations are scattered over all exons; therefore, genetic tests for this gene require a mutation scanning analysis of the entire coding region of the *BRCA1* gene. In most diagnostic laboratories, this procedure is currently performed using direct sequence analysis, often preceded by denaturing gradient gel electrophoresis (DGGE) [Beck et al., 1993; Guldberg and Guttler, 1993], protein truncation test (PTT) [den Dunnen and van Ommen, 1999; Ozcelik et al., 1996], or denaturing high-performance liquid chromatography (DHPLC) [Gross et al., 1999; Liu et al., 1998]. However, all these techniques are time-consuming and/or expensive. Moreover, considering the increasing number of requests for *BRCA1* scanning tests, the demand for a fast and reliable scanning technique is high.

High-resolution melting curve (HRM) analysis is a potentially useful new method for fast genotyping and high-throughput mutation scanning of disease-related genes in genome diagnostics [Herrmann et al., 2006; Montgomery et al., 2007; Wittwer et al., 2003]. The procedure is simple and consists of PCR, followed by a short melting step and subsequent analysis. This post-PCR analysis method scans entire amplicons and detects sequence variations using a saturating double-stranded DNA (dsDNA) binding dye, such as LCGreen Plus (Idaho Technology, Salt Lake City, UT) [Wittwer et al., 2003]. The melting profile of the PCR product depends on its GC content, length, sequence, and heterozygosity, and mutations in the sequence will give rise to heteroduplexes that change the shape of the melting curve when compared to the wild-type (wt) melt profile [Herrmann et al., 2006; Montgomery et al., 2007; Wittwer et al., 2003]. Although several tests using this new technique have been described; we present the first interlaboratory assessment study for diagnostic use that includes a thorough evaluation and validation of HRM analysis on the LightScanner™ (LS) (Idaho Technology), which employs a statistically significant large panel of more than 150 variants for *BRCA1* only. This ensures that the number of variant samples tested is large enough that, under the assumption that the sensitivity point estimate will be 100%, the lower bound of the 95% confidence interval will be at or above 98%.

Moreover, we composed a list of diagnostic guidelines that can also be applied for setting up and interpreting HRM scans for

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

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other genes, and we describe a validated HRM test for the *BRCA1* gene using the 96-well LS that includes genotype analysis of nine common polymorphisms.

Materials and Methods

Interlaboratory Validation by EuroGentest

EuroGentest is a European Network of Excellence aiming at harmonizing genetic testing services throughout Europe. One key objective of EuroGentest is to set up evaluation and validation programs for new techniques and tests in diagnostics. In this respect, we performed this validation study for HRM for *BRCA1* on the 96-well LS in close collaboration with the Center for Human Genetics in Leuven (Belgium), the Institute of Biology and Medical Genetics in Prague (Czech Republic), the LS manufacturer Idaho Technology, and the LS distributor BIOKÉ (Leiden, the Netherlands). Validation of the HRM technology was performed according to international guidelines ISO15189, Medical laboratories.

DNA Samples

DNA samples used for the initial validation study were all patient-derived and isolated from whole blood using the PUREGENE™ nucleic acid purification method on the Autopure LS robotic workstation (Gentra Systems, Minneapolis, MN) according to validated diagnostic isolation procedures; the samples were diluted to 10 ng/μl. DNA concentrations were measured using the Nanodrop® ND-1000 Spectrophotometer (Isogen Life Science, Ijsselstein, The Netherlands).

Robustness of the HRM tests was evaluated further by using DNA samples isolated by four different DNA isolation procedures, including the Autopure LS (Gentra), the Chemagen procedure (Chemagen AG, Baesweiler, Germany), manual phenol extraction, and the QIAamp® DNA Kit (Qiagen, Venlo, The Netherlands).

The panel of DNA samples tested for *BRCA1* included 170 variants and 197 wt controls, which were all verified by direct sequencing using the Big Dye Terminator method (Applied Biosystems, Nieuwerkerk a/d Ijssel, The Netherlands). Supp. Table S1 lists all variants tested and indicates the classification, type, and distribution of the mutations. Mutations are indicated according to HGVS mutation nomenclature guidelines and DNA mutation numbering system used is based on cDNA sequence. For the blind tests, 28 patient-derived DNA samples were selected, together with two wt control samples. Note that 2 of the 28 patient-derived DNA samples were also included in the evaluation of the cohort of variants.

Note: We have consent from the patients to use the DNA that remains after diagnostic testing in the framework of quality of care (re)assessment; we comply with the Federation of Biomedical Scientific Societies (FMWV) Code: "Proper for Proper Secondary Use of Human Tissue" (2002).

All gradient PCR assays for optimization of the PCR annealing temperature (Ta) were performed using a pool of four individual Autopure-derived genomic DNA samples at a concentration of 10 ng/μl. This way all PCR optimization results were obtained from a mixed DNA sample that represents an average DNA purity as obtained upon isolation by the Autopure LS.

Validation Criteria for Determining Sensitivity and Specificity of the Technique

Validation of a mutation scanning method is limited to the assessment of a selected panel of variants. It is unfeasible and technically impossible to validate all possible occurring variants that can arise; therefore, a statistically reliable number of variants needs to be tested for the validation to establish the sensitivity of the method at a chosen confidence level. According to the "rule of three" we can say with a confidence interval of 95% that the probability of detecting a false negative, given a study of n samples with no false negatives, is 3/n [Hanley and Lippman-Hand, 1983]. Consequently, if we take the conservative point of view that the lower bound of the 95% confidence interval of the test sensitivity should be at or above 98%, then we would have to achieve perfect detection of at least 150 variants.

Because all possibly occurring mutations cannot be tested, it is important to select and examine a representative assortment of variants that comprises all types of substitutions and/or small deletions and insertions at various locations in the amplicon. We included variants at various locations ranging from 2 bp away from the PCR primer (e.g., c.4837A>G in MEX16B) to the middle of the amplicon, representing all 12 possible types of substitution (75%) and different nucleotide insertions, duplications, deletions, or indels of 1 to 62 bp (25%). Moreover, although only heterozygous variants are considered to be potential pathogenic mutations for the *BRCA1* gene, we have also included a set of 14 individual homozygous variants to examine their detection efficiency.

Selection Primer Sets

A large panel of 66 primer pairs was evaluated for HRM of *BRCA1*. The primers were newly designed or derived from current primer sets in use for DGGE and sequence analysis of *BRCA1*. All primer sets contain an M13 forward and reverse sequence tail, respectively, in order to allow direct sequencing analysis, and were synthesized by Biolegio (Nijmegen, The Netherlands). The design of most new primers was performed using the LightScanner Primer Design software package, version 1.0 (PD-v1.0; Idaho Technology). Primers were completely homologous to the reported *BRCA1* gene sequence, accession L78833, NM_007294.2, NT_025965.11. The nucleotide sequence of the primers was judged according to the criteria described in Supp. Table S2A. The final selection of validated primer set is shown in Supp. Table S3.

Optimization of HRM PCR

Optimal PCR Ta for all primer sets was evaluated using a gradient PCR setup of 55°C to 67°C on the MJ-PTC 200 PCR machine (Bio-Rad Laboratories B.V., Veenendaal, The Netherlands). The PCR was performed in a 10-μl volume using 20 ng of genomic DNA, 4 μl LS Mastermix (LCGreen® Plus dye), 3 pmol forward primer, 3 pmol reverse primer, and water (molecular grade) (Supp. Table S4). Mineral oil (15 μl per reaction), necessary for the LS melt step was already added before starting the PCR. The PCR was initiated with a 10-min hold at 95°C. Thermal cycling consisted of a 20-second hold at 95°C, a 30-second hold at the indicated Ta (in Supp. Table S3), and a 40-second hold at 72°C for 40 cycles. Finally, reactions were elongated for 5 min at 72°C and heteroduplexes were generated by adding a step at 95°C for 1 min and cooling the reactions to 25°C. Ramp speed of the PCR machine was set at 2.5°C/second.

Only primer sets that fulfilled the criteria as described in Supp. Table S2B were qualified for further HRM evaluation and the most optimal Ta was selected.

Genotype Analysis of Common Polymorphisms

Nine unlabeled oligonucleotide probes were designed and examined for the detection of heterozygous and homozygous

frequently occurring polymorphisms of *BRCA1*. Probes and characteristics are listed in Supp. Table S5. Probe criteria were set as described previously and indicated in Supp. Table S2C [Montgomery et al., 2007]. All probes carried a block at the 3′ end omitting participation in the PCR reaction and 3′ exonuclease activity. We tested both the 3′ phosphate- and the 3′-C3 carbon (phosphoramidite)-spacer block. In addition we examined HPLC purified probes and compared results. All unlabeled probe-containing PCR reactions were performed using an asymmetric dilution of the forward and reverse primers of 1:5 at the same final primer concentration of 6 pmol, as used for the standard PCR and 5-pmol probe. Different numbers of PCR cycles were tested and all PCR reactions were performed as depicted in Supp. Table S4 using the optimal Ta (Supp. Table S3).

Optimization and Defining Instrument Setting Per Amplicon on the LS

PCR reactions were carried out on the Biometra thermocylcer (Westburg, Leusden, the Netherlands) as described above using 4titude Framestar plates (BIOKÉ) as recommended for HRM on the LS. Subsequently, samples were melted in the LS type HR96 (Idaho Technology and BIOKÉ) according to operating instructions using two different melt ranges of either 55°C to 98°C or 70°C to 98°C at a hold temperature of 50°C and 65°C, respectively.

The basic data analysis of the melt curves was performed using the supplied Call IT 1.5 Software according to the LS manual supplied by Idaho Technologies.

Both gene scanning and genotyping by HRM are performed by simple analysis of a single melting profile. Sequence variants are identified as groups that exhibit similar melting profiles and, if applicable, these groups can be genotyped for frequently-occurring polymorphisms. The scanning settings were assessed per amplicon by analyzing both the selected wt and all variant DNA samples (as listed in Supp. Table S1) and according to the guidelines described in this study.

Interlaboratory Testing of 10 Selected Amplicons

Interlaboratory performance of the test was evaluated in two laboratories using 10 different amplicons. Selected samples and matching amplicons are listed in Table 1. LS Mastermix, 10 primer sets and 37 DNA samples, including 22 wt samples and 27 variant samples, were sent to both laboratories indicated. Note that several variant DNA samples were used as wt control for other fragments. The Center of Human Genetics in Leuven performed additional validation experiments by testing a set of 19 DNA samples purified by Chemagen as indicated in Table 2. All PCR reactions were carried out on either the PTC200 (Bio-Rad) or the Biometra Thermocycler (Westburg) using the same conditions as defined by the diagnostic laboratory in Leiden. All plates were melted in the LS and the raw data files were evaluated by both laboratories themselves and sent to Leiden for reevaluation using the specifically defined Call IT 1.5 amplicon scanning settings of Leiden.

BRCA1 Blind Tests for 28 Patient-Derived DNA Samples

The blind tests included two complete *BRCA1* mutation scanning rounds using a selection of the 40 best performing primer sets that encompass the entire *BRCA1* gene. Two series of each 14 patient-derived DNA samples, one negative wt control and one blank control were tested. The 40 amplicon reactions were distributed over seven 96-well plates according to their Ta, in groups of six amplicons, as depicted in Supp. Figure S1.

Table 1. Interlaboratory Validation for BRCA1: EUGT Test-Set

Exon	Amplicon	GC%	Wts tested[a]	Mutation[b]	Variant classification	Length (bp)
3	MEX3	35	2	c.81–6T>A	MUT	347
8	MEX 8	39	2	c.442–34T>C	POL	320
				c.536A>G;		
				c.442–34T>C	UV+POL2	
				c.442–34T>C	POL2	
11	MEX 11A-L	39	2	c.1067A>G	POL2	625
				c.1067A>G	POL	
				c.825C>T	UV	
				c.1016delA	MUT	
7	MEX 7	34	2	c.302–3C>G	MUT	279
				c.441G>C	UV (1X)	
				c.302–41T>C	UV	
9	MEX 9	31	2	c.591C>T	POL	187
				c.548–17G>T	UV	
11	MEX 11J	36	2	c.3113A>G	POL	378
				c.3113A>G;		
				c.3119G>A	POL	
				c.3113A>G	POL2	
				c.2989_2990dupAA	MUT	
11	MEX 11-6	37	2	c.1648A>C	UV	320
				c.1621C>T	MUT	
				c.1525A>G	UV	
11	MEX 11-7	37	2	c.1865C>T	UV	272
11	MEX 11-8	40	3	c.1961delA	MUT	254
				c.2019delA	MUT	
				c.2014A>T	MUT	
11	MEX 11-5	38	3	c.1292dupT	MUT	316
				c.1456T>C	UV	
				c.1289dupA	MUT	

MUT, heterozygous pathogenic variant; UV, unclassified variant; POL heterozygous polymorphism; POL2, homozygous polymorphism.
[a]Number of wild type samples tested.
[b]Mutations are indicated according to HGVS mutation nomenclature guidelines and DNA mutation numbering system used is based on cDNA sequence NM_007294.2. Nucleotide numbering reflects cDNA numbering with +1 corresponding to the A of the ATG translation initiation codon in the reference sequence, according to journal guidelines (www.hgvs.org/mutnomen). The initiation codon is codon 1.

The analysis was carried out applying the selected and fixed scanning settings obtained from the primary evaluations study. In addition all these plate specific settings were saved per plate as specific "Scanning Analysis" and "Genotype Analysis," including the subset annotation, localization of lower and upper normalization settings, and "Auto Group" sensitivity level for the analysis of the amplicon and, if applicable, the same was done for the genotype analysis using the unlabeled probe.

Results

Selection of *BRCA1* Primer Sets and Primary Optimization

In total we evaluated a panel of 66 primer pairs for HRM analysis of the *BRCA1* gene that often included two or more overlapping sets per region. Based on criteria mentioned in Supp. Table S2A and B, the 58 sets gave rise to good PCR and HRM results. Products that gave either low yield, additional side products, poor melting curves, or more than two melting domains were disqualified. All primer sets that were designed using the IT PD-v1.0 performed very well. Primers that performed poorly in HRM analysis were designed before applying less stringent criteria or had an amplicon length of 420 bp and longer. In addition, these primers often scored poorly when judged by the PD-v1.0 or gave rise to many hits with other genomic regions upon a NCBI BLAST search.

Table 2. Interlaboratory Validation for BRCA1: DNA Samples Leuven

Exon	Amplicon	Wts tested[a]	Mutation[b]	Variant classification	Length (bp)
3	MEX3	2	c.133A>C	UV	347
8	MEX 8	2	c.442–34T>C	POL	320
			c.442–34T>C	POL2	
			c.470_471delCT	MUT	
11	MEX 11A-L	2	c.693G>A	UV	625
			c.744C>G	UV	
			c.844_850dupTCATTAC	MUT	
			c.1016delA	MUT	
7	MEX 7	2	c.441G>C	UV	279
9	MEX 9	2	c.591C>T	POL	187
11	MEX 11J	2	c.2920T>C	UV	378
			c.3119G>A	POL	
11	MEX 11-5	2	c.1292delT	UV	317
			c.1418A>G	UV	
			c.1525A>G	UV	
11	MEX 11-6	2	c.1487G>A	POL	320
11	MEX 11-7	2	c.1865C>T	UV	272
11	MEX 11-8	2	c.1878A>G	POL	254
			c.2005A>T	UV	

MUT, heterozygous pathogenic variant; UV, unclassified variant; POL heterozygous polymorphism; POL2, homozygous polymorphism.
[a]Number of wild-type samples tested.
[b]Mutations are indicated according to HGVS mutation nomenclature guidelines and DNA mutation numbering system used is based on cDNA sequence NM_007294.2. Nucleotide numbering reflects cDNA numbering with +1 corresponding to the A of the ATG translation initiation codon in the reference sequence, according to journal guidelines (www.hgvs.org/mutnomen). The initiation codon is codon 1.

Evaluation and Defining Guidelines for Optimal HRM Analysis Per Amplicon on the LS

In order to perform a thorough and elaborate analytical evaluation of various amplicons, we initially extensively examined all 58 well-performing primer sets for the detection of variants through HRM using the elaborate panel of 170 variants and 197 negative control (wt) patient-derived DNA samples as listed in Supp. Table S1. Some variants and many wt samples could be used for multiple amplicons resulting in a total of 248 and 352 different variant (including homozygous variants) and wt control HRM reactions, respectively. All melting curves were analyzed using the software program Call IT version 1.5. First, subsets were selected per amplicon, the "Curve Shift" setting was left at the default setting of 0.050 and the grouping of the curves was performed using the "Auto Group" option at high sensitivity. During our examination of all 58 primer sets, we established additional critical criteria for adjusting instrument and software settings to create optimal HRM analysis per amplicon on the LS. An overview is given in Supp. Table S6. This includes the positioning of normalization bars and defining relative fluorescence levels and sensitivity levels, but also criteria related to the melting curve profiles that need to be fulfilled. The following guidelines were established. First of all we tested the criteria of the lower and upper normalization bars and concluded that the optimal width per set of bars should be in a range of 1°C to 2.5°C. The location of the bars should be close around the melt domain and can be adjusted to optimal position by establishing a straight horizontal line at the start of the normalized melting curve (Fig. 1). Figure 1A illustrates the optimization of MEX11-13E, the left column shows the result of melting curves upon incorrect setting of the normalization regions. The obtained aberrant normalized melting curve profiles are indicated with a red circle in plot 2, and plot 3 shows the subsequent incorrect variant calling. The right column depicts the correct normalization setting. Variant curves and congruent mutations are called correctly and are indicated in lower right of plot 6. Note sensitivity levels were kept fixed at 3.0 ("Auto Group" high). Figure 1B shows a similar comparison of correct and incorrect normalization settings for amplicon MEX11-15; again sensitivity levels were kept fixed at 3.0.

The optimal sensitivity level was determined by analyzing all 170 different variants and 197 different wt samples for the 58 amplicons. The level of sensitivity at the "Auto Group" menu option was adjusted for each individual amplicon and all amplicons were set at sensitivity "High." The maximum sensitivity level that can be applied was established by testing a series of 5 to 10 individual wt curves and allowing a limited detection of 0 to 5% (average) false positives (FPs). We recommend designing new primers for sets that yield a maximum sensitivity level below 2.7. Subsequently, the minimum sensitivity of the amplicon was established by analyzing the available variants. Again, new primers were designed for sets that yielded a sensitivity level below 2.7. Optimal sensitivity was reached when all variants could be detected and no more than an average of 5% FP reactions were observed in the wt series (excluding reactions that fail in the PCR).

Both the "Normal" and "High" sensitivity levels were evaluated for all variants. Although the vast majority of variants could easily be detected using a "Normal" sensitivity setting at the "Grouping" standard, "Auto Group" at level "Normal" 2.7, some variants were only visible at a sensitivity level "High." Several of these less easily detectable variants were homozygous variants. However, also some heterozygous variants, such as c.135-15_135-12delCTTT and c.2898delT in the respective amplicons MEX05 and MEX11IB could only be detected using the "Auto Group" "High" sensitivity setting. Figure 2 depicts the analysis of these variants and compares the results obtained with "Normal" and "High" sensitivity. Clearly, grouping of all curves needs to be performed at high sensitivity level to be sure these variants are detected. Due to this observation we chose to select a "High" sensitivity level for all amplicons to accomplish a detection level as optimal as possible and to reduce the risk of missing important pathogenic mutations such as c.2898delT.

In general, "Auto Group" sensitivity level "High" was often set higher than 2.7 to reach the most optimal and stringent detection level. In case few variants were available, we focused mainly on the quality of the detection by using a large panel of negative control samples and selected only primer sets that yielded wt melt curves that were located closely together and gave few FPs, never more than 5%. Figure 3 shows the importance of testing a large series of wt samples. Both plots depict HRM of 15 wts; however, only amplicon B gave rise to wt melt curves that are in close range of each other and that were all correctly called by the software. In contrast, amplicon A results in wt curves that are much more broadly dispersed from one another and give rise to four FP reactions. The latter result would give rise to a low sensitivity level and subsequent potential loss in variant detection.

Evaluation Results Using All 58 Amplicons

Evaluation of HRM for all 58 amplicons applying the general guidelines described in the previous section and using the panel of variant and wt DNA samples resulted in the detection of all tested heterozygous variants representing 220 analyses (+28 homozygous variant analyses). This data was in full coherence with the results obtained by sequence analysis. According to the "rule of three" this results in a sensitivity of at least 98.6% with a confidence interval of 95%. Of the homozygous variants, only one could not be detected by two different primer sets (MEX11J and MEX11-13C), namely

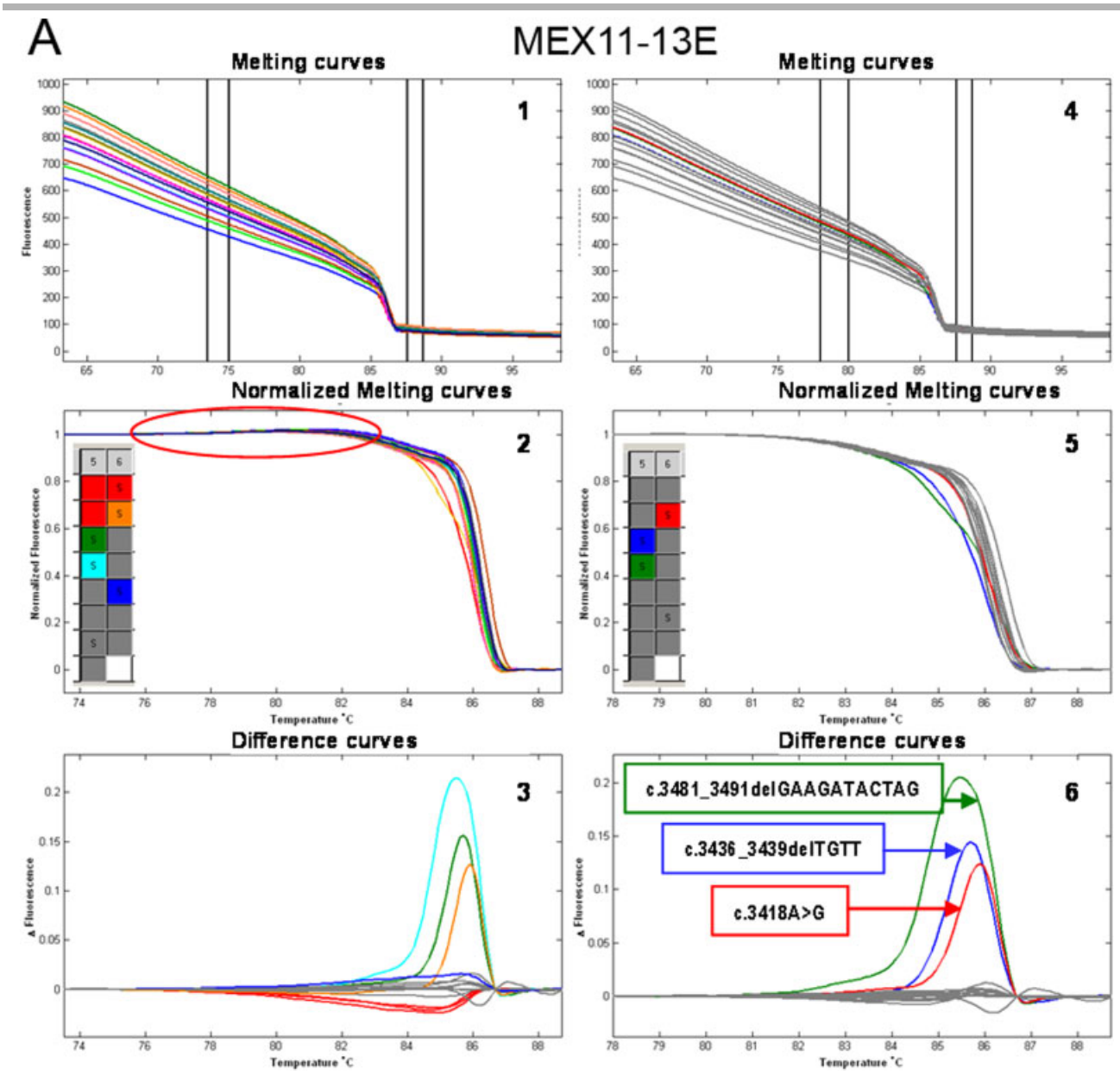

Figure 1. **A**,**B**: Adjusting "Normalize Setting" for optimal melt curve analysis. Evaluation of optimal adjustment of normalization bar settings is illustrated for two amplicons. A: MEX11-13E: HRM analysis of 12 wt DNA samples and three variant samples of amplicon MEX11-13E. First left column depicts results of melting curves upon incorrect setting of normalization bars, resulting in aberrant normalized melting curve profiles as indicated with a red circle (2). Lowest plot 3 shows subsequent incorrect variant calling with several FP calls. Right column depicts correct normalization setting. Variant curves and corresponding mutations are called correctly and are indicated in lower right plot (6). No FPs are detected. Note that sensitivity levels are kept fixed at 3.0. **B:** MEX11-15: HRM analysis of 14 wt DNA samples and one variant sample of amplicon MEX11-15. First left column depicts results of melting curves upon incorrect setting of normalization bars, resulting in aberrant normalized melting curve profiles as indicated with a red circle (2). Lowest plot 3 shows subsequent incorrect variant calling. Right column depicts correct normalization setting. Variant curves and congruent mutations are called correctly and are indicated in lower right plot (6). No FPs are detected. Note that sensitivity levels are kept fixed at 3.0.

c.3113A>G. In total, 26 out of 28 homozygous variants reactions were detected, representing 14 unique homozygous variants. This results in a detection ratio of 93%. In Supp. Figure S2 the variant detection by several amplicons is illustrated, including MEX07 (A), MEX08 (B), MEX11-10 (C), MEX16B (D), and MEX24 (E). For amplicon MEX11-10, seven different variants were tested, including the homozygous polymorphism c.2082C>T and several different deletions ranging from 1 to 5 nucleotides. Another homozygous polymorphism (POL2), c.4837A>G, is shown for amplicon MEX16B, of which the melt curve is relatively close to the wt curves but clearly visible and reproducibly detected. This polymorphism is located only two nucleotides from the start site of the forward primer located at c.4835. For the other amplicons the variants are shown in triplicate or duplicate to illustrate repeatability of the tests. All identical variants grouped nicely together at the indicated sensitivity levels.

The specificity of the HRM for all amplicons was addressed by evaluating a total of 197 different wt DNA samples. This resulted in 352 wt reactions for the complete evaluation of all 58 amplicons. In total, 12 FP reactions were observed (3.4%), resulting in a specificity

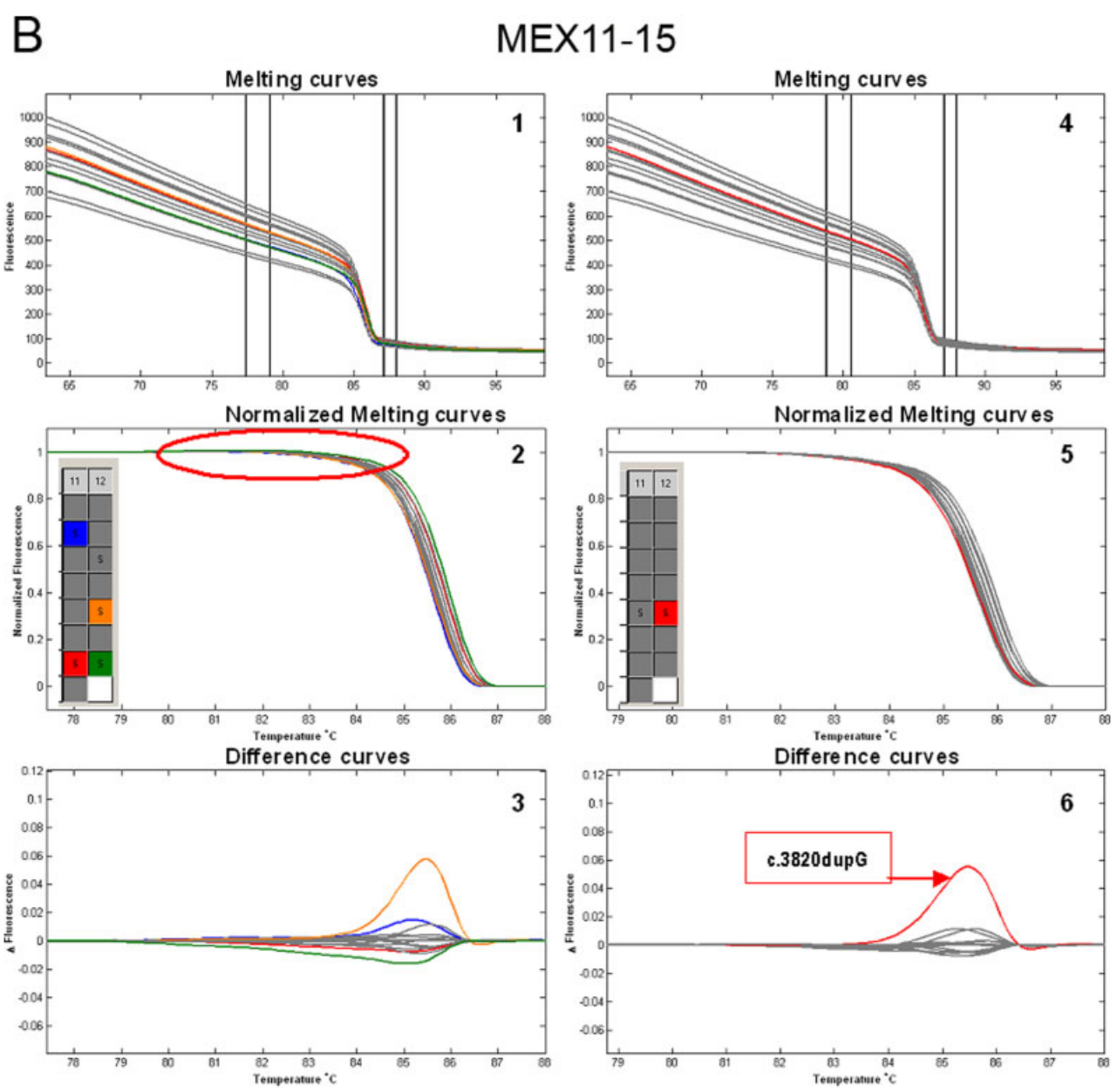

Figure 1. *Continued.*

of 96.6%. The detection of FPs varied per amplicon and overall a good reproducibility of the melt curves per amplicon was observed using this broad panel of Gentra-derived DNA isolations.

Eventually, based on our results and the described criteria for primers and HRM analysis, we subsequently selected the 40 best-performing primer sets that encompassed the entire coding region of *BRCA1*. This primer selection is depicted in Supp. Table S3 and results in an increased specificity of 97.6% and a sensitivity of at least 98.3% with a confidence interval of 95% (180/180 heterozygous variant reactions). Supp. Table S7 gives an overview of the LS software settings applied.

Interlaboratory Testing of 10 Selected Amplicons

Next, we tested the analytical performance and reproducibility of a selection of primer sets in two other laboratories (EuroGentest member). For this interlaboratory performance test we selected 10 different amplicons ranging in size, GC content, and HRM performance, including variants that gave melt profiles close to the wt control melt curve (Table 1). Apart from amplicons that harbored variants that were easily detected, we also included the largest amplicon MEX11A-L tested (625-bp-long) and the amplicon MEX11J that harbors the homozygous variant c.3113A>G, which could not be detected in the evaluation studies performed in Leiden. In both laboratories all tests were performed with identical consumables, samples, and PCR conditions as described in Materials and Methods. All HRM reactions were examined as described in Materials and Methods with "Auto Group High." Both laboratories detected all heterozygous variants, indicating good reproducibility of the HRM tests. Again the homozygous variant c.3113A>G could not be detected by amplicon MEX11J. For the large amplicon MEX11A-L (624-bp-long) all variants were correctly detected, including the one nucleotide deletion of variant c.1016delA that displays a melt

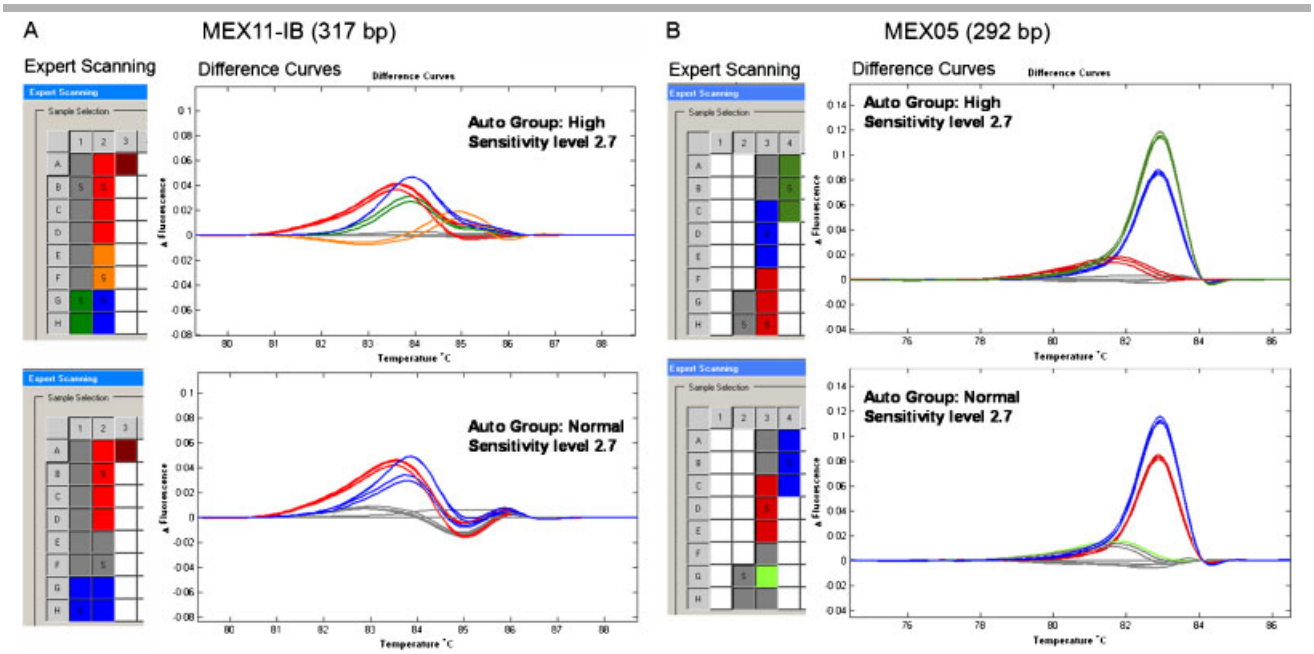

Figure 2. **A,B:** High sensitivity level is needed for detection of certain deletion variants. A comparison of mutation detection analysis is shown for two amplicons using either High sensitivity level vs. Normal sensitivity level. In (A) variant detection for of several variants of amplicon MEX11I-B at normal and high sensitivity levels is illustrated. The pathogenic mutation c.2898delT (wells E2-F2) is clearly detected at high sensitivity level (upper plot orange curve) but detection is lost at normal sensitivity level. Similarly, the mutation c.135-15_135-12delCTTT of amplicon MEX05 depicted in (B) is not consistently detected at normal sensitivity level but shows a well reproducible curve (red) at high sensitivity level. Samples and the location in the 96-well plate are indicated below the plots. WT curves are in depicted in gray.

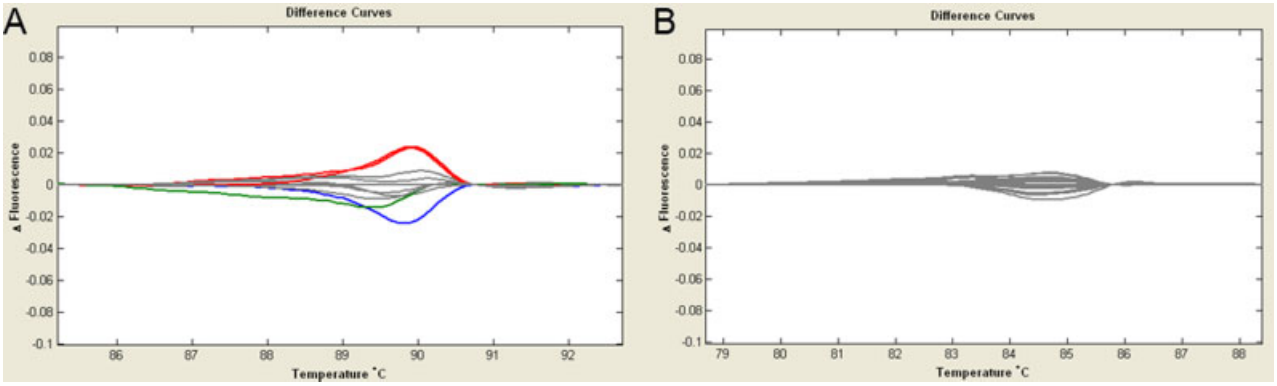

Figure 3. Curve profile of optimal wt melt curves. **A,B:** Comparison of two primer sets A and B and the examination of their HRM performance; each difference plot depicts the HRM analysis of 10 different wt samples. A: A typical bad profile of widely distributed wt curves, which indicates poor HRM quality and gives rise to FP reactions as indicated in two red, one green, and one blue curves. B: Shows the example of an optimal performing primer set for HRM; here all 10 different wt samples arrange close together and show little curve variation. Grouping is at standard "Auto Group" and sensitivity is set at High level 3.

curve which is close to the wt curves and that is occasionally not detected at normal sensitivity. Examination of all raw data from the LS melt files using the instrument settings defined by Leiden gave rise to identical results, indicating good reproducibility of LS performance and test results in general from all the different laboratories.

In addition to repeating the DNA samples from Leiden, Leuven performed additional validation experiments by testing a set of 19 additional DNA samples purified by Chemagen in combination with the wt control DNA samples from Leiden purified with the Gentra method (Table 2). Again all variants were detected. This implies that this DNA extraction method does not interfere with the HRM results and samples isolated by both these methods can be combined in one test.

In conclusion, these results indicate good interlaboratory reproducibility, even for more critical amplicons such as the large 625-bp fragment for exon 11, and show little influence of the DNA purification method.

Genotyping of Common *BRCA1* Polymorphisms

BRCA1 is known for harboring several common polymorphisms, resulting in the recurrent detection of variants for several amplicons. Because HRM has shown that different variants can group into the same melting curve profile, one can never conclude that variants that are detected as one group (color) all represent the same known variant and/or polymorphism. Consequently confirmation by

sequence analysis would be essential for all variants detected. However, for *BRCA1* this would result in the sequence analysis of all detected polymorphisms, which occur in around 10 to 20% of the PCRs. Therefore, we designed and tested nine unlabeled probes to identify the frequently-occurring polymorphisms of *BRCA1*, to avoid unnecessary sequence analysis upon detection of these nonpathogenic variants. In Supp. Table S5, we listed the probes and the corresponding amplicon used for this study. All probes were complementary to the reverse strand except for amplicon MEX11-4, and were designed using the criteria described by Wittwer et al. [2003] and in Supp. Table S2C. Due to the overlap of some amplicons, the presence of several polymorphisms was confirmed both directly and indirectly through probe analysis of the overlapping amplicon. For example, POL c.4837A>G occurs both in MEX16A and MEX16B, but is only confirmed by probe analysis for MEX16A.

Previous studies performed their genotype PCRs using 50 to 55 cycles; however, by using a slightly higher end concentration of primers, we obtained correct and good genotype results at 40 cycles. This made combination of amplicons with and without probes on the same plate feasible even though the unlabeled probe genotyping requires an asymmetric PCR. Finally, all probes correctly detected their specific polymorphism as illustrated for polymorphisms c.4308 T>C and c.4837A>G. of exon 13 and 16, respectively, in Supp. Figure S3A and B. Both the 3′ phosphate- and a 3′-C3 carbon-spacer block gave good and similar results. However, due to better stability (especially at 4°C) the Idaho Technology research team recommends employing the C3-block. The use of HPLC purified probes did slightly improve the grouping and subsequent detection of the polymorphisms.

Robustness: The Effect of DNA Purification, Concentration, and Sample Variation

To determine the effect of the DNA isolation method, we compared the results of several HRM tests using DNA samples purified by different methods including phenol extraction, Qiagen columns purification, and automated Chemagen and Gentra (Autopure) DNA isolations. PCR and HRM analysis criteria were identical for all tests as described above. Results showed that the DNA isolation method applied did not specifically influence the HRM results and differently-isolated DNA samples could easily be examined and compared in one test. The minor signal variation among the samples was similar to the signal variation observed in series of identically isolated DNA samples.

In addition to the variation in DNA isolation methods, we also examined the influence of different DNA concentrations for the detection of wt samples and variant samples. Three amplicons, MEX07, MEX11-13C, and MEX11-13F, were evaluated in duplex using a DNA concentration range of 50, 20, 10, and 5 ng per reaction. For each amplicon, two wt control DNA samples were tested together with one variant. We observed no deviating results when using 20, 10, or 5 ng per reaction and all curves correctly grouped together; only the reactions that contained an input of 50 ng DNA per reaction occasionally (3/18), gave rise to a slightly deviating curve (data not shown).

In conclusion, HRM is a robust assay and the detection of variant or wt samples is not hampered when HRM reactions contain four times less DNA than usual (down to 5 ng). An input of a higher amount of DNA (2.5×) can occasionally result in a deviating curve and therefore could give rise to an FP reaction. Note that if samples give rise to poor amplification results this will always result in an aberrant melt curve, indicating that further analysis for this sample is necessary.

Guidelines for Interpretation of HRM Data

The analysis of both the amplicon melt curves and, if applicable, the additional probe melt curves, results in a large set of data that requires correct interpretation. The combination of probe data and amplicon data especially needs extra attention. Based on all our observations during this elaborate evaluation study, we propose a list of guidelines that can be applied for the interpretation of the HRM data, which is described in detail in Supp. Table S8. In principle, all curves that deviate from the wt curve and appear in a different color in the difference plots using validated fixed settings potentially contain a variant in the sequence and need to be sequenced. This can be done directly on the HRM PCR sample. In case aberrant melt curves are visible for some reactions, repeating the melt step of the same plate again can improve the results (for details see Supp. Table S8). Standard rule is that once a sample is tested as a wt curve, the sample will not contain a heterozygous variant even if it shows some deviation in the other melt step.

Note that the unlabeled probe genotype analysis will only confirm the presence (heterozygous or homozygous) or absence (wt) of the examined common polymorphisms. But it does not exclude the presence of an additional variant in the total amplicon and the results should always be combined with the melt results of the complete amplicon. More general aspects are indicated in Supp. Table S8.

Final Validation Step: Blind Tests Using 28 DNA Samples and the 40 Best-Performing Primer Sets

Finally, we performed two complete *BRCA1* specific HRM mutation scans using the 40 best-performing primer sets and two series of 15 DNA samples each, including one wt control. Again, we compared the performance of the HRM method with the current "gold standard" for mutation detection; i.e., sequence analysis. PCR reactions were performed as described previously and analyzed using the same fixed scanning analysis settings that were defined during the assessment of the large cohort of variants.

All 18 heterozygous variants, also identified with sequence analysis, were detected in the blind studies and include pathogenic mutations, unclassified variants, and infrequent polymorphisms resulting in 100% detection (see Supp. Table S9A and B). In addition, genotype analysis for nine common polymorphisms detected all 154 polymorphisms correctly and omitted sequence analysis of an additional 147 (13%) polymorphism-containing reactions. Note that upon probe detection of only one homozygous polymorphism in one series of 14 samples it was essential to sequence this PCR since we can never exclude the presence of an additional mutation in such samples. We detected 1.4% (8/560) and 2.5% (14/560) of FPs and three and four negative reactions (no product) per series, respectively, resulting in an average specificity of 98%. Table 3 shows a summary of all data and demonstrates that in total (two series) only 54 out of 1,120 PCR reactions (4.8%) would require subsequent sequence analysis. Indicating the large reduction in sequence analysis realized after prescanning the samples with HRM and applying genotype analysis for nine common polymorphisms. Due to the presence of M13 tags, sequence analysis could be performed directly on the HRM PCR reactions.

In summary, we again observed good reproducibility and high specificity of the selected primer sets using the previously fixed instrument settings.

Discussion

In this study we have not only performed an extensive evaluation and validation of HRM on the IT LS, but we also

Table 3. Summary of Two Blind Studies (28 Samples)*

	Series 1 [% (n)][a]	Series 2 [% (n)][a]
New VAR	1.6 (9)	1.6 (9)
FP	1.4 (8)	2.5 (14)
N.R	0.5 (3)	0.7 (4)
FN	0 (0)	0 (0)
POL/POL2	11.8 (66)	15.7 (88)
Single POL2[b]	1.3 (7)	–
Total sequence reactions[c]	4.8 (27)	4.8 (27)

New VAR, total of variants detected excluding the common polymorphisms; FP, false positive; N.R., no reaction and/or no clear melting curve observed; FN, false negative; POL, heterozygous polymorphism; POL2, homozygous polymorphism.
*PCR reactions per series = 560.
[a]Percentage of total samples analyzed per series (number of reactions in parentheses).
[b]Probe-mediated detection of a single POL2 per set of 14 reactions.
[c]Total number of reactions that would need subsequent sequence analysis.

include an interlaboratory assessment of HRM, a genotype analysis of nine common polymorphisms, the validation of a primer set for *BRCA1* mutation scanning, and most of all a list of diagnostic guidelines that can be applied for setting up HRM for other genes.

Previous studies have compared different HRM platforms and examined many individual HRM tests for specific mutations or small sets of samples [De et al., 2008; Takano et al., 2008; Reed and Wittwer, 2004], but these studies did not include interlaboratory testing nor such an elaborate panel of selected and validated variant and wt samples for one gene that were all verified by sequence analysis. A recent literature study reviewing the quality of various scanning techniques for *BRCA1* and *2*, indicated the need for more statistically significant studies that thoroughly verify the diagnostic accuracy of new mutation scanning techniques that include confidence intervals of the results [Gerhardus et al., 2007]. Therefore we tested a panel of variants exceeding the minimal number of 150 samples in order to reach a satisfactory lower bound of the 95% confidence interval of the assay sensitivity.

In this study, we compared HRM analysis with sequence analysis, which is the current gold standard for mutation detection in most diagnostic laboratories. Our study, using 170 individual variant and 197 individual wt DNA samples, indicate that HRM analysis is a highly sensitive method that detected all heterozygous mutations (157 samples) with a sensitivity of 100%. This results in a final statistical sensitivity point estimate of 100% with a 95% confidence interval of 98.3 to 100.0% (for 180 heterozygous variant reactions) when using the 40 selected primer sets. In total 58 amplicons, were thoroughly evaluated and this resulted in 248 different variant analyses and 352 wt analyses. Using the large panel of amplicons we already observed a specificity of 96.6%. This was increased to an average of 98.1% in the final blind test, which includes large sets of wt sequence-encoding amplicons and employs the selected 40 best-performing primer sets. Common *BRCA1* polymorphism could be easily detected using nine specific unlabeled probes that harbored the SNP, omitting unnecessary sequence analysis of 10 to 20% reactions per series. Evaluation in two independent diagnostic laboratories of 10 different amplicons that vary in size, melt profiles, and GC content yielded identical results. Moreover, the raw data files from these laboratories were analyzed using settings selected by Leiden, and again equal results were obtained, indicating good reproducibility of the melt file profiles derived from the individual LS instruments.

Based on this evaluation study, we composed a list of diagnostic guidelines for setting up, analyzing, and interpreting HRM for new genes, as summarized in Supp. Tables S2, S6, S7, and S8. This way, our extensive evaluation can not only greatly facilitate the set up for *BRCA1* but also of new mutation scanning tests in diagnostic laboratories using HRM and will avoid the evaluation of very large panels of variants.

However, the following critical issues will also need to be initially addressed when setting up a new test. First, HRMC testing for a new gene is only valuable when a large part of the samples and amplicons generate wt sequences and/or harbor the repetitive detection of a common polymorphism. In case a specific exon or DNA fragment of the gene can contain many different variants it is recommended to perform direct sequence analysis for this particular gene region. Second, the current evaluation concerns a gene with amplicons that have an average GC content ranging from 31% to 54%. Previous studies have shown that high GC content ($>60\%$) can be a critical factor in obtaining optimal PCR and HRM results (Technology Assessment on HRM as reported by Helen White, National Genetics Reference Laboratory [NGRL], Wessex, United Kingdom; http://www.ngrl.org.uk/Wessex/downloads_reports.htm). Similarly, low GC content can also limit the detection of variants and most of all reduce sensitivity. Consequently, we recommend performing a more elaborate variant analysis for such high-GC– or low-GC–containing amplicons to evaluate the sensitivity and reproducibility of the tests while using fixed scanning settings. Reducing the size of the amplicon to 200 bp or even less can be one option to increase the mutation detection sensitivity. Again, more related technical details can also be found in the study by Helen White (NGRL).

Third, although we and others have shown that many homozygous variants can be detected, it is essential to realize that not all are found. Hence the detection of these variants will always require spiking with other (wt) PCR reactions. Finally, it is important to note that changing the reagents indicated in this study can lead to poor results. The dye is an especially crucial factor and should always be saturating. Today many more dyes are available and are also being evaluated for HRM. However, although it has been shown that several dyes give rise to good reproducibility for detection of specific known variants, so far their performance for mutation scanning analysis has not been shown in an statistically valuable study. Clearly this latter type of analysis demands excellent performance quality; therefore, we recommend thorough evaluation tests when using a new fluorescent dye.

An important guideline that resulted from our study is the evaluation of large series of wt samples per amplicon. The wt melt curves should always cluster close to each other for each amplicon in the difference plot. This way high sensitivity levels can also be selected when no variants are available for evaluation, and variant curves located close to the wt curve can be detected; moreover, it circumvents the detection of frequent FP scores. We recommend reevaluating the results again after performing the first series of diagnostic scanning tests. Note that when wt samples give large variation in the individual curves and adjustment of Ta or Mg concentration give no improvement, one should always consider developing new primer sets. Do note that this applies for amplicons which fall in the indicated GC content range tested in this study.

Based on our evaluation, we selected the "Auto Grouping" at "High" sensitivity level from the software menu. Although the fast majority of variants could easily be detected using the "Normal" sensitivity setting, some variants were only visible at this high setting. Several of these undetectable variants were homozygous variants; however, two heterozygous variants, namely c.135-15_135-12delCTTT and c.2898delT could also only be detected using the "High" sensitivity setting. Due to this

observation, we choose to select this level for all amplicons to accomplish a detection level as high as possible and to reduce the risk of missing important mutations such as c.2898delT. Because we selected for amplicons that have their wt melt curves in close range, we could easily increase the sensitivity level and only observed a very moderate increase of FPs compared to "Normal" sensitivity. Correspondingly, we observed a high specificity of 98% in the final blind tests using the "High" sensitivity levels for all amplicons, which is similar to the specificity found in the study by De Leeneer et al. [2008], who applied a "Normal" sensitivity level. Unfortunately, the two critical "del" mutations that needed a high sensitivity level in our investigation were not tested in this particular study. It may be that lower sensitivities could be applied to some amplicons and that the adjustment might be amplicon-dependent. However, since both examples concern small deletions, which occur less frequently than the substitutions during mutation scans for *BRCA1*, they were present less often in our cohort and could not be tested for all amplicons. Future tests using additional high numbers of such variants will need to confirm whether this is indeed applicable and not intrinsically related to the detection of some small deletions. Do note that 26 deletion variants in our variant panel are detected at normal sensitivity level, although c.1961delA is also closer to the wt curves at normal sensitivity. Because all 130 substitutions (including all possible nucleotides at various locations) were detected as close as up to two nucleotides away from the primer, it appears that, in general, deletions are more critical to detect and it is advisable to evaluate this type of variants more extensively.

In our study we noticed that some melt profiles could be significantly improved upon repeating the melt step in the LS. Such second melts can reduce the detection of FP samples in the test, as explained extensively in point 2 of Supp. Table S8.

Note also that the software can not always discriminate different variants in the same amplicon. Therefore, common polymorphisms should never be judged only by their similarity in melt profiles, but should always be confirmed by probe or sequence analysis to exclude the presence of a mutation with an identical melt profile. Also in our study, the overlap in melt profiles was not always simply explained by similarity of the substitution and short distance in location of the two variants. For example, c.2014A>T could not be discriminated from c.2019delA in amplicon MEX11-8, and c.1067A>G resulted in a similar melt profile as c.1209dupT in amplicon MEX11-4. However, in contrast to the observation in a previous study [De et al., 2008], we were able to distinguish the SNPs c.3113A>G from c.3119G>A and the pathogenic mutations c.2934T>G and c.2989_2990dupAA (data not shown). The latter two could also be easily distinguished at normal sensitivity level. This indicates first of all the obvious influence of elevating the sensitivity level from "normal" to "high" sensitivity for better distinction of variants, but also the influence of using different primer sets for the same gene region. The latter suggests that a small shift in nucleotide composition of the amplicon tested can significantly improve the detection. Notably, the mutation c.2989_2990dupAA is located only 20 bp away from the reverse primer in MEX11I-B, whereas the other one is more or less in the middle of the amplicon, which could perhaps explain the clear difference in melt profiles.

As indicated, we used the "Primer Design" (PD-v1.0) software as supplied by Idaho Technology for the design of 10 new primer sets to replace either moderate performing primers or omit the presence of a rare SNP under the primer. All sets were indicated in green by the PD primer list (indicating a theoretical good quality) and gave very good PCR products and HRM results. Therefore, we can recommend using the PD software for the design of new primer sets. Of course primers still need to be examined for SNPs and number of hits in a BLAST or BLAT search.

In summary, we conclude that HRM is a rapid and sensitive post-PCR mutation scanning method that can easily be applied in diagnostics to scan genes for various mutations. All results were in coherence with the earlier sequence data, indicating at least a similar sensitivity level when compared to this technique. The use of M13 tags greatly facilitates the direct sequence analysis of samples that show a variant melt curve and create a fast workflow. We do recommend taking note of the critical features mentioned in this study, which should be specifically addressed when applying HRM for mutation scanning analysis.

We have summarized all recommendations and guidelines that can be considered when setting up and performing HRM for other genes in the online supporting information. Finally, we supply a validated set of PCR primers for mutation scanning analysis of the *BRCA1* gene on the LS using identical test conditions.

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