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## Diagnostic method validation: High resolution melting (HRM) of small amplicons genotyping for the most common variants in the *MTHFR* gene

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### Abstract

**Objectives:** According to OECD guidelines methods implemented in a diagnostic laboratory should be properly validated prior their implementation. For this purpose we selected genotyping by High Resolution Melting (HRM) of small amplicons using common variants in *MTHFR* as a model.

**Design and methods:** We selected previously typed samples on which selected analytical validation-related parameters relevant to DNA diagnostics — specificity, sensitivity, precision, robustness and ability to perform reliable calls were evaluated.

**Results:** Correct genotype was assigned in 375/381 (98.4%) for c.677 C>T (rs1801133: C>T; p.A222 V) and in 102/104 (98.1%) for c.1298 A>C (rs1801131: A>C; p.E429A) of all cases. Low analytical failure rate and very high specificity/sensitivity were achieved. Similarly, precision and robustness were consistent.

**Conclusions:** We have successfully validated HRM of small amplicons using common *MTHFR* variants as a model. We proved that this technique is highly reliable for routine diagnostics and our diagnostic validation strategy can serve as a model for other applications.

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**Keywords:** DNA diagnostics; Genotyping; High resolution melting (HRM); Diagnostic validation; Methylentetrahydrofolate reductase gene (*MTHFR*); Organization for Economic Cooperation and Development (OECD) guidelines; Small amplicons

### Introduction

Although direct DNA sequencing is considered as a “gold standard” for genotyping of known or unknown mutations, it still remains relatively expensive, laborious and time consuming. Different methods have been developed to simplify the detection of novel mutations, with the most common techniques being based on restriction enzyme analysis [1], allele-specific amplification [2], ligation based assays [3], single-base extension [4], fluorogenic ASO hybridization probes [5,6] and pyrosequencing [7].

High Resolution Melting (HRM) is a simple, rapid and low-cost mutation scanning method [8–11]. Its advantage is the fact that PCR amplification and melting curve analysis are performed within the same tube or plate, without any post-PCR

processing. This feature is particularly important for a routine diagnostic setting. HRM is based on computer analysis of DNA melting transitions, whereby it is possible to record more than 25 readings per 1 °C [12], via monitoring of the change in fluorescence that results from gradual temperature-dependent release of a saturating ds-DNA binding dye [8,9].

Since HRM is based on thermodynamic differences between DNA fragments, it has been used in particular for scanning of heterozygous sequences. However, in its original form, discrimination between homozygous genotypes is more difficult, because the difference between homozygous sequence melting profiles is usually merely represented by a slight shift in the melting temperature ( $T_m$ ), but not by a change of the melting curve profile [13]. Therefore, HRM has been adapted for analysis of polymorphic SNPs via PCR amplification of small amplicons. Such a reduction of the amplicon length results in a broader divergence between melting profiles and increases the sensitivity of the technique, which then could be used not only

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for scanning, but also for accurate genotyping. Moreover, differences between homozygous wild type and homozygous mutant DNA fragments are thus more apparent [14].

Due to the advantageous features of the HRM of small amplicons this technique is currently being rapidly introduced into diagnostic laboratories for genotyping of disease-associated genes. According to the “OECD guidelines for Quality Assurance in Molecular Genetic Testing” [15], there is an obligation for diagnostic laboratories to provide high quality results. Therefore, all methods implemented within a routine setting must be duly validated prior to their diagnostic use.

In this study, we utilized suggested methodology and evaluated selected analytical validation-related parameters as stipulated by ISO15189 [16], QSOP 23 [17], the American College of Medical Genetics [18], the International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH) [19], recommendations of the Czech Clinical Biochemistry and Medical Genetics societies [20] or as published elsewhere [21]. Parameters mostly relevant for the diagnostic setting comprise sensitivity, specificity, precision (reproducibility and repeatability) and robustness [18–21].

Although reviews and reports on the use of HRM for mutation scanning and genotyping were published previously [8,10,11,22–25], there is no report on diagnostic validation of this technique as required by OECD and/or ISO guidelines [15,16]. Furthermore, we feel that there is a particular need to provide examples of proper validation strategies for genotyping of SNPs by HRM of small amplicons due to its increasing use in diagnostics [11].

Therefore, the aim of this work was diagnostic validation of small amplicon genotyping by HRM using as model the examination of common variants of the *methylenetetrahydrofolate reductase (MTHFR)* gene: c.677 C>T (rs1801133: C>T; p.A222V) and c.1298 A>C (rs1801131: A>C; p.E429A). These variants are associated with a measurable reduction of enzymatic activity of *MTHFR*, in particular in the case of the c.677 C>T variant, and have been proposed to be in association with several disorders related to impaired folate metabolism such as neural tube defects, infertility, thrombosis or some types of cancer [26–30].

We hope that our study would serve as an example for diagnostic validations of other molecular genetic techniques applied in routine practice.

## Materials and methods

### DNA template preparation

Since the main objective of our study was to validate HRM genotyping we have randomly selected DNA aliquots from patients examined at our Department for inherited thrombophilia prior to assisted reproduction [31]. All patients or their legal representatives signed an informed consent approving general/anonymous research use of respective specimens and this study was approved by the Internal Ethics Committee of University Hospital Motol. Genomic DNA was extracted from blood using

Puregene™ “Genomic DNA Purification Kit” (Gentra Systems, MN, U.S.A.; currently distributed by Qiagen, Germany) and diluted to 10 ng/μL using the “DNA Hydration Solution” provided by the manufacturer.

All samples used in this study were previously genotyped for respective *MTHFR* variants by an alternative technique and were selected retrospectively with the aim to have enough samples for each genotype. The rs1801133: C>T variant was genotyped utilizing the RHA Kit Thrombo™ (Labo Biomedical Products, The Netherlands) and/or by RFLP-based (Restriction Fragment Length Polymorphism) typing [32], while the rs1801131: A>C variant, only RFLP-based analysis was used [33].

### Primer design and annealing temperature

Primers were designed to amplify a small fragment surrounding the polymorphisms and avoid the presence of other sequence variations in the primer region (Table 1). Primer  $T_m$  and general suitability were calculated using FastPCR software, version 4.0.27 [34]. In order to select the optimal annealing temperature ( $T_a$ ) for our assay, we performed a gradient PCR within the range of 10 °C using the median temperature from the  $T_a$  range proposed by the FastPCR software as the starting point. Gradient PCR was performed using the PTC-220 thermocycler (MJ Research, MA, U.S.A.) under the PCR cycling and HRM conditions described in the next section.

After the gradient PCR reaction and for the purpose of the optimization of  $T_a$ , we initially performed HRM on the LightCycler® 480 Real-Time PCR System (Roche Diagnostics, Germany) followed by 4% agarose gel electrophoresis in order to detect spurious bands. The  $T_a$  with an optimal melting profile and associated with no unspecific amplification products, and which could be used for amplification of both SNPs, was selected.

### PCR conditions and HRM acquisition

PCR amplification for rs1801133: C>T and rs1801131: A>C variants were performed under the same conditions in a 96-well plate in the LightCycler® 480 Real-Time PCR System. Reaction volume was 10 μL; 2 μL of genomic DNA (10 ng/μL) was added to 8 μL of reaction master mix consisting of 1× LightCycler® 480 High Resolution Melting Master (containing the proprietary ds-DNA saturating binding dye), with 2.5 mM

Table 1  
Primer sequences and amplicon sizes used for rs1801133: C>T and rs1801131: A>C variants HRM small amplicon genotyping.

SNP	Primer sequences	Amplicon size (bp)
rs1801133:	5'-GAAGGAGAAGGTGTCTGCG	45
C>T	5'-AGCTGCGTGATGATGAAATC	
rs1801131:	5'-GGGAGGAGCTGACCACTGAA	50
A>C	5'-GTAAAGAACGAAGACTTCAAAGACT	

Table 2  
Settings for the analysis of *MTHFR* variant genotyping.

SNP	Pre-melting normalization (°C)	Post-melting normalization (°C)	Temperature shift	Sensitivity
rs1801133: C>T	73.0–75.0	82.0–84.0	5	Auto-group: 0.7 In-run standards: 1.0
rs1801131: A>C	70.0–72.0	79.7–81.7	5	Auto-group: 0.7 In-run standards: 1.0

Legend: “Auto-group” calculation was used for the comparison of the control sample replicates for the three genotypes when reproducibility, repeatability and robustness parameters were evaluated. “In-run standards” calculation was used to analyze blinded samples using control samples for the three genotypes analyzed as melting standards.

MgCl<sub>2</sub> (Roche Diagnostics, Germany) and 0.5 μM of forward and reverse primers. For this study only one batch of the commercial master mix was used. The PCR program started with an initial denaturation of 10 min at 95 °C, continued with 40 cycles of 10 s at 95 °C, 15 s at 60 °C and 10 s at 72 °C. This program also allows one step for heteroduplex formation by heating to 95 °C for 30 s and cooling down to 40 °C for 1 min. For HRM, the plate was heated from 65 °C to 95 °C performing 25 acquisitions per 1 °C.

### HRM analysis

Melting curve analysis was performed using the Light-Cycler® 480 Gene Scanning software version 1.2 (Roche Diagnostics, Germany). All the samples with a late amplification, as monitored by real-time PCR or associated with fluorescence of less than the 60% of the maximum, were excluded from the analysis. According to manufacturer’s recommendations these could generate unreliable melting profiles. The

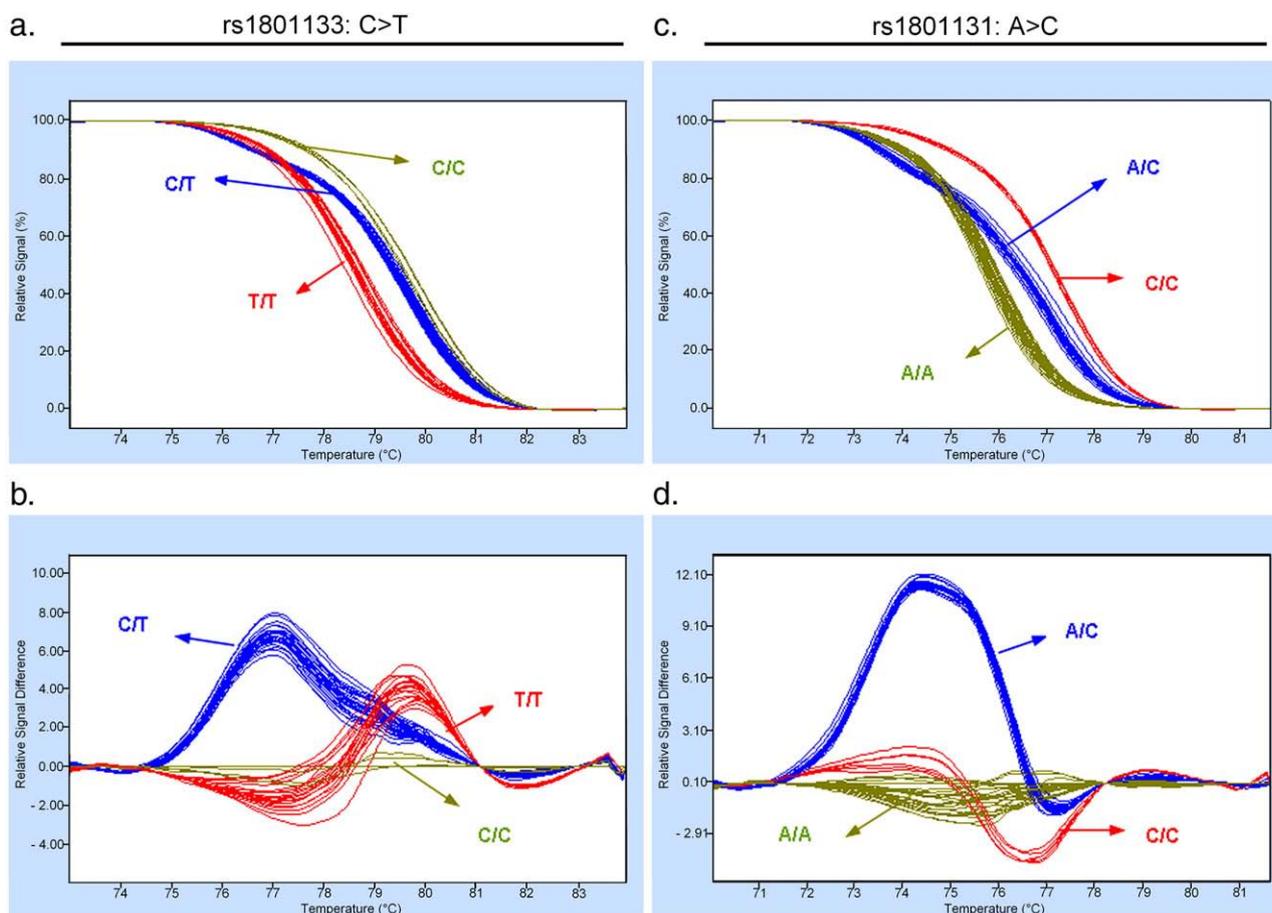


Fig. 1. Normalized plots, and normalized and temperature shifted difference plots for the genotyping of rs1801133: C>T and rs1801131: A>C variants. In order to calculate sensitivity and specificity, samples were blinded. From the normalized melting curves for rs1801133: C>T and rs1801131: A>C (panels “a” and “c”, respectively) it is possible to distinguish both homozygous groups by their  $T_m$  variation (C/C wild type homozygous and T/T homozygous for the variation for rs1801133: C>T and A/A wild type homozygous and C/C homozygous for the variation for rs1801131: A>C), heterozygous samples (C/T and A/C, respectively) have a different melting curve shape.

normalization settings were exactly the same for each experiment performed (Table 2).

#### Validation parameters

**Sensitivity and specificity:** sensitivity is the probability of a positive test result in the presence of a risk allele (heterozygous and homozygous for risk allele samples) expressed as the ratio between true positivity (TP) and the sum of true positivity and false negativity (FN):  $TP/(TP+FN)$ , while specificity is the probability of a negative test result of the test in the absence of risk alleles (homozygous wild type samples), expressed as a ratio between true negativity (TN) and the sum of true negativity and false positivity (FP):  $TN/(TN+FP)$  [18–21]. To evaluate sensitivity and specificity we ran two replicates (i.e. same sample ran two times within the same run) of each genotype as melting control standards and one replicate of tested samples. Altogether we tested 381 samples for rs1801133: C>T and 104 samples for rs1801131: A>C, proportionate to their availability in our laboratory.

For the validation of HRM of small amplicons, these samples were organized into blinded groups. For specificity evaluation we examined 178 negative samples for rs1801133: C>T variant, while for rs1801131: A>C, 46 negative samples were

analyzed. To determine sensitivity, we analyzed 203 positive samples for rs1801133: C>T variant and 58 positive samples for rs1801131: A>C.

**Intra-run precision–repeatability:** is the comparison of results within a single series in parallel within a single day performed by one analyst [20]. To test repeatability a single analyst ran 10 replicates of each genotype control sample within the same run.

**Inter-run precision–reproducibility:** is the comparison of results between the series — on different days (day to day reproducibility) [20]. For reproducibility (inter-run precision) parameter test, a single analyst prepared one sample from each genotype in triplicate, with that same analyst repeating the procedure on three different days.

**Robustness:** is the ability of a method to remain unaffected by minor modifications [18–21]. To evaluate robustness we ran one sample from each genotype in triplicate. The tested parameters were DNA template amount, annealing temperature, cycle number, analyst variation and pipetted volume variation. We added 10 ng, 20 ng, 50 ng and 100 ng of DNA into the reaction, respectively, for the evaluation of DNA template amount variation. Annealing temperature was modified within the range of  $\pm 1$  °C. Cycle number variation was assessed adding or decreasing by two cycles in the PCR program. Three

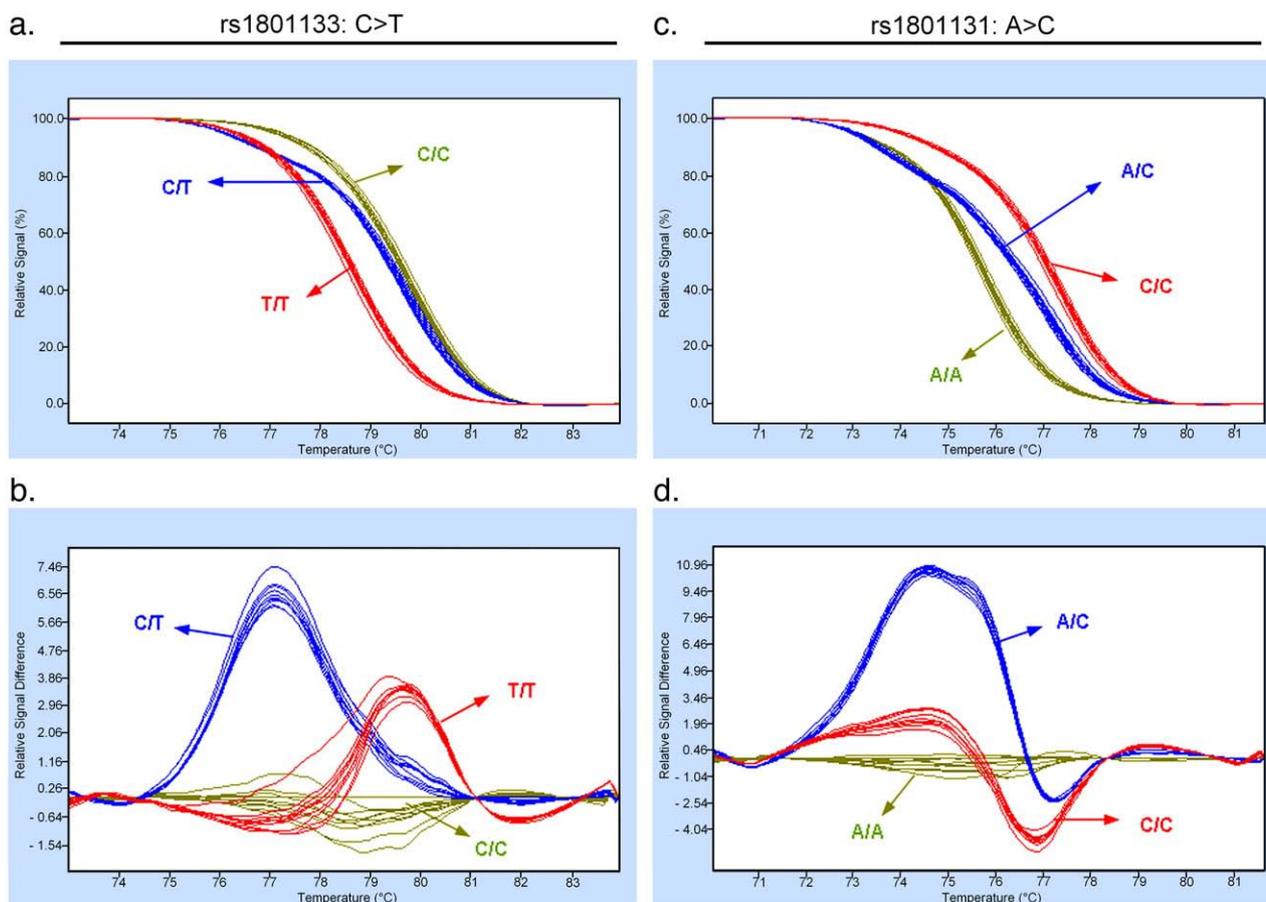


Fig. 2. Repeatability. Normalized plots, and normalized and temperature shifted difference plots for the genotyping of rs1801133: C>T and rs1801131: A>C variants. The same control sample for a genotype was analyzed in 10 replicates. Panels “a” and “c” represent normalized melting curves, while panels “b” and “d” depict difference plots for the genotyping of rs1801133: C>T and rs1801131: A>C, respectively.

different analysts repeated the same PCR and HRM procedure in order to assay analyst variation. For examining the influence of reaction master mix and DNA volume variation during pipetting, we modified respective volume in both instances by  $\pm 0.5 \mu\text{L}$  and  $1.0 \mu\text{L}$ .

## Results

### HRM genotyping

The analytical failure rate was 6 out of 381 for rs1801133: C>T and in 102 out of 104 calls for rs1801131: A>C, while proper software-based genotype assignments (“calls”) were obtained in the remaining cases. In aggregate, from the normalized melting curves and difference plots we were able

to clearly distinguish three genotype melting profiles (Fig. 1). Individually, all calls corresponded to previous genotype assignments.

### Sensitivity and specificity

Since we did not have any errors (no false calls) in genotyping assignments made by the software, we reached 100% sensitivity and specificity for both tested SNPs.

### Intra-run precision–repeatability

All genotype replicates grouped together with regard to their melting pattern in both tested SNPs. Additionally, melting profiles were the same for each sample in all 10 replicates (Fig. 2).

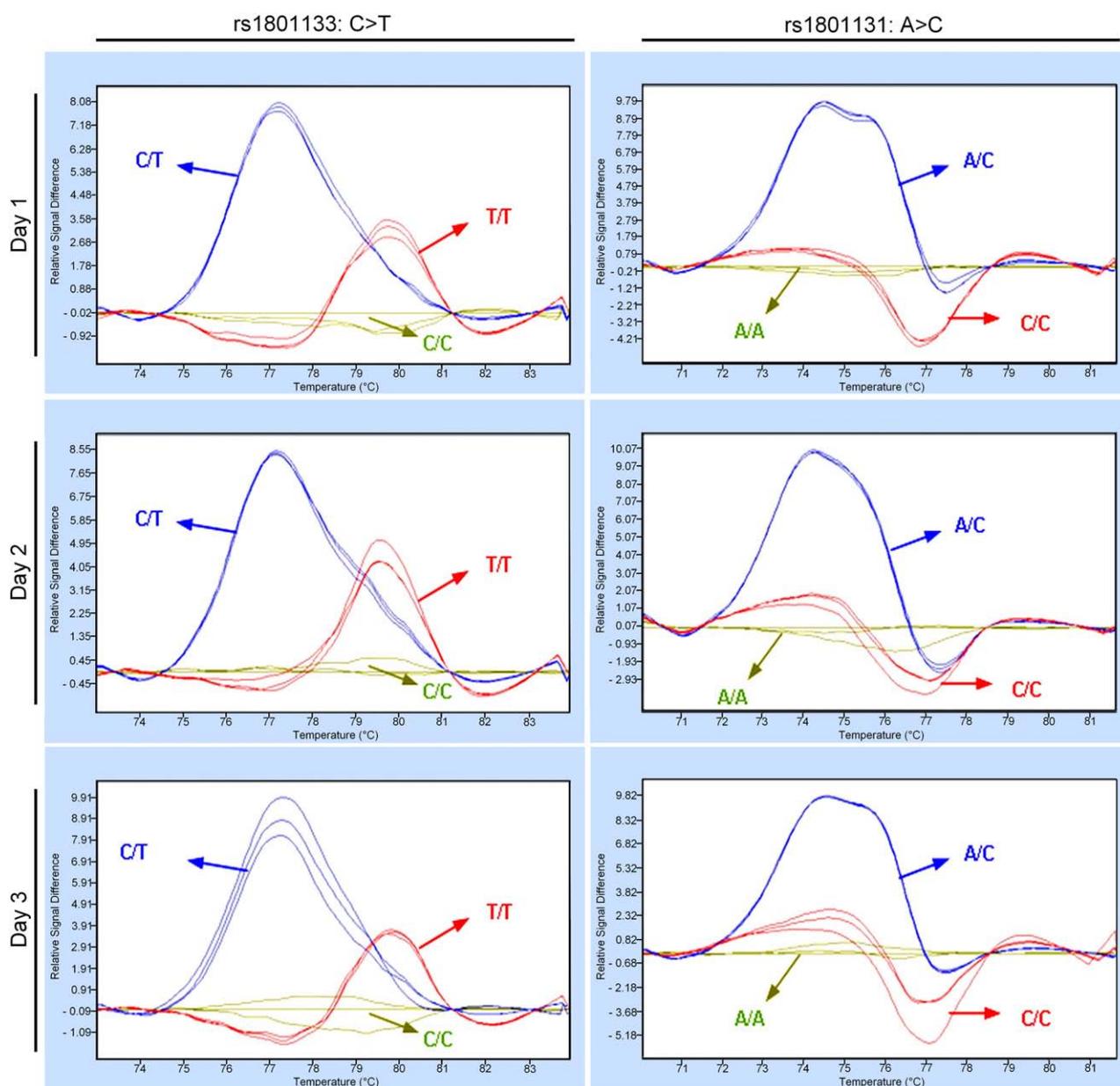


Fig. 3. Day-to-day reproducibility. Normalized and temperature shifted difference plots for the genotyping of rs1801133: C>T and rs1801131: A>C performed on three different days by the same analyst are shown. Control samples for each genotype were run in triplicates. Each genotype group is indicated by arrows.

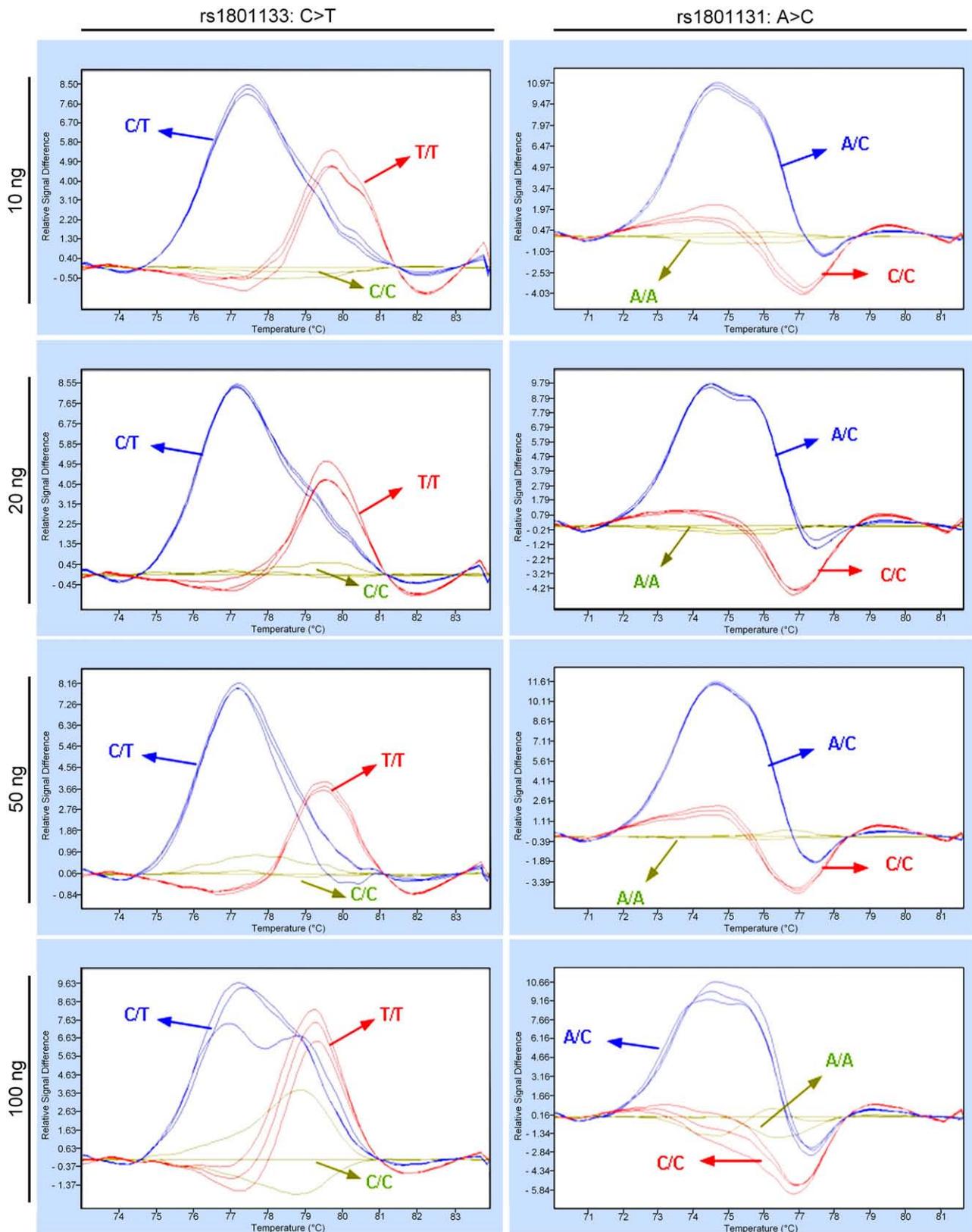


Fig. 4. DNA template amount variation. Normalized and temperature shifted difference plots for the genotyping of rs1801133: C>T and rs1801131: A>C are shown. 10 ng, 20 ng, 50 ng and 100 ng of gDNA template per 10  $\mu$ L of reaction were tested. Each genotype group is indicated by arrows.

### Inter-run precision–reproducibility

There was no difference in the calls and difference plots when the same procedure was repeated on different days (Fig. 3).

### Robustness

Template DNA amounts ranging from 10 ng to 50 ng provided correct grouping within the three different genotype groups (Fig. 4). Only when 100 ng of DNA was used in the reaction, melting profiles became unreliable. An annealing temperature variation of  $\pm 1$  °C did not change the ability of the method to render correct grouping (data not shown). An increase in the cycle number, for both tested SNPs, did not

modify the correct grouping nor did influence the melting profile. There was no detectable difference when we decreased the PCR program by two cycles for rs1801131: A>C, whereas a decrease in two cycles for rs1801133: C>T modified the melting profiles (Fig. 5).

Three different analysts repeated the same procedure in order to group same control samples (in three replicates) within the different genotype groups. With regard to the grouping ability, neither the software calls, nor the melting profiles varied (data not shown).

A difference in the melting profile was observed only when the same sample was analyzed by adding 7.0  $\mu$ L of reaction master mix instead of 8.0  $\mu$ L, and when template DNA volume added was 3.0  $\mu$ L instead of 2.0  $\mu$ L (data not shown).

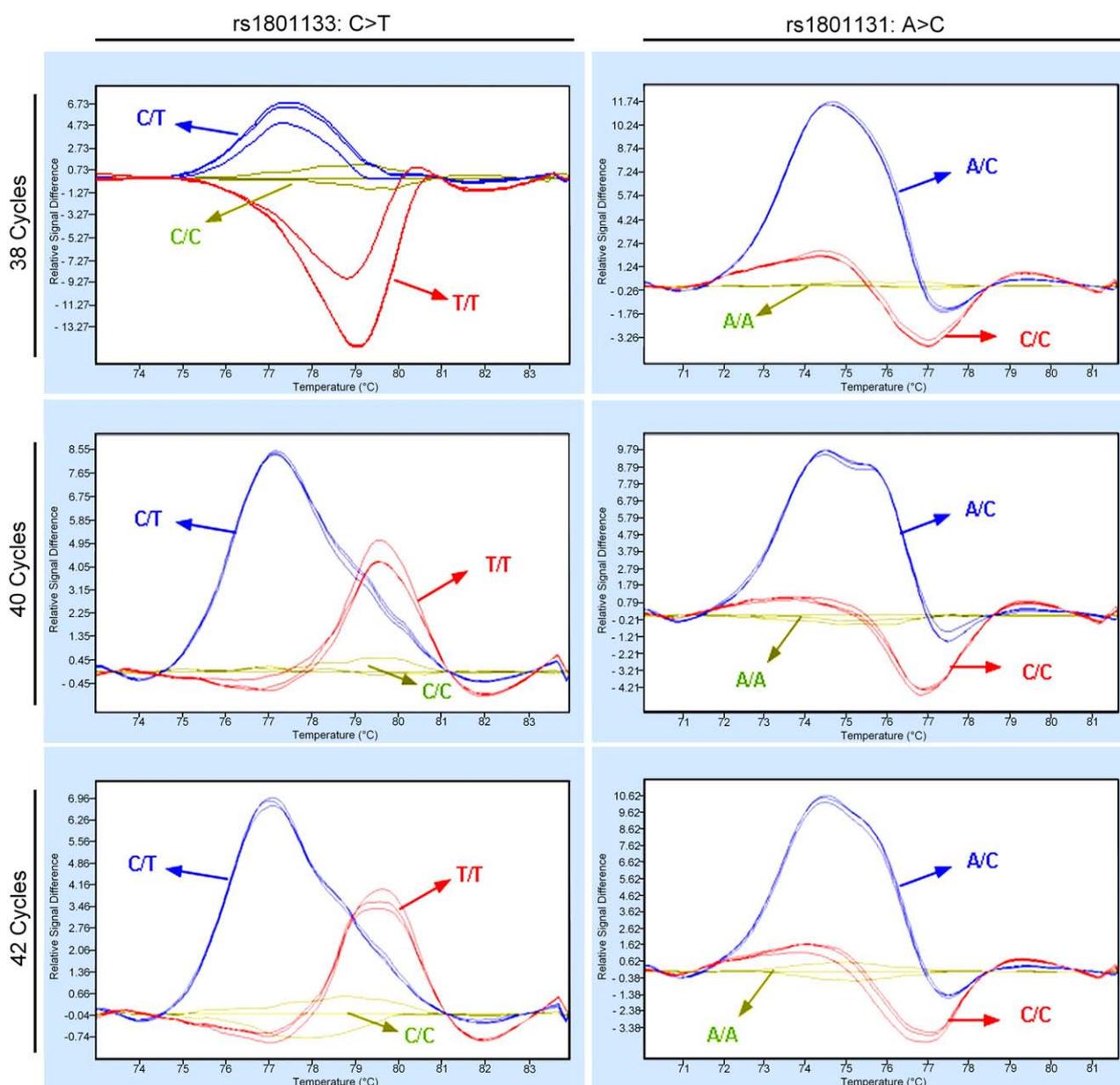


Fig. 5. Cycle number variation. Normalized and temperature shifted difference plots for the genotyping of rs1801133: C>T and rs1801131: A>C with  $\pm 2$  cycles variation from the normal PCR program (40 cycles) are shown. One sample from each genotype was run in triplicate; each genotype group is indicated by arrows.

## Discussion

Genotyping by HRM of small amplicons is a technique associated with high sensitivity and specificity [14,35–37]. We were able to prove these observations by discriminating between homozygous wild type and homozygous mutant melting profiles on the model of two common *MTHFR* variants. Moreover, the advantage of this approach is that it is carried out in a closed tube environment. There is no need to add an extra oligonucleotide probe for genotyping as in the “original” form of HRM gene scanning [38,39] and even after a  $T_m$  shift we can still clearly distinguish both homozygous genotypes. We only used two flanking primers, a proprietary saturating DNA fluorescence dye and ready-to-use commercial master mix. Costs per analysis could be further decreased by preparing an “in house” master mix. These features make this technique very simple, customizable and fast, thus useful for a routine diagnostic setting [10,11,35].

Although previous studies analyzed all types of HRM from the point of view of sensitivity and specificity [8,10,11,22–25], none of them subjected HRM of small amplicons to diagnostic validation as required by OECD guidelines or ISO 151989 [15,16]. In this study, we utilized suggested methodology and evaluated selected analytical validation-related parameters which are particularly relevant in DNA diagnostics [18–21]. Assessment of sensitivity and specificity alone is not sufficient enough to cover all aspects of the diagnostic use of a given technique [40], since these do not account for possible variability of scenarios encountered at DNA diagnostic laboratories (i.e. uneven DNA template quality, imprecise DNA template concentration, change of personnel, different laboratory devices, variations in ambient temperature etc). In addition, due to the small master mix volume (10  $\mu$ L) this method is particularly prone to pipetting inaccuracies. Therefore, it is necessary to include additional parameters, such as precision and robustness [18–21].

Monitoring of the progress of amplification by real-time PCR enabled us to exclude poor quality or insufficiently amplified template DNA samples. These quality measures contributed to high sensitivity and specificity [41] and our observations are in accordance with other studies [8,10,11,20–25].

Nevertheless, despite strictly applied quality measures the analytical failure rate was within the range of 1.6 to 1.9% for the tested variants. These samples cannot be assessed as “false negatives” since the assessor cannot infer any conclusions when the proprietary software discards unreliable data acquisitions due to its internal, quality-based algorithms, as is the case in LightCycler® 480 Real-Time PCR System. The only plausible technical explanation is that failure could likely result from inaccurate pipette handling of very small reaction master mix volumes, demonstrating that there is still a space for further improvements. When we repeated the analysis for the second time correct genotyping was achieved.

Since we were using the same reagents and DNA dilution conditions (i.e. same reaction chemistry), the precision (repeatability and reproducibility) of HRM was very high and we are able to use the same normalization settings between runs made on different days. This observation supports the

high value of this technique for reliable genotyping in routine diagnostics.

Minor modifications of the technique do not generally affect its performance. Nevertheless, a shortening of the PCR by 2 cycles, as was the case in rs1801133: C>T, might produce different melting profiles in instances when PCR has not reached its plateau. This can be avoided by monitoring amplification in real-time and stopping the PCR after its plateau has been reached, as is possible when using the LightCycler 480 system. This step can be done manually, but we recommend to evaluate this issue at the optimization stage to set up the correct number of PCR cycles prior to future analyses. The use of larger template DNA amounts impedes HRM due to altering ds-DNA/amplicon/proprietary saturating dye ratio, thereby leading to less precise melting curves [42].

In conclusion, we have successfully performed diagnostic validation of High Resolution Melting of small amplicons for the genotyping of rs1801133: C>T and rs1801131: A>C *MTHFR* variants according to OECD and ISO guidelines [15,16] by using parameters and approaches pertinent to a diagnostic setting. In addition to accurate genotyping HRM of small amplicons, altered melting profiles could signal another mutation within the analyzed sequence. As is the case with all mutation scanning-based techniques direct sequencing will elucidate the reason for altered melting profiles. Shortening of amplicons in HRM further decreases the false negativity rate due to poor discrimination of homozygous sequences. This feature substantially decreases the necessity to implement sequencing in samples where the estimated clinical risk is highly discordant with a “negative” test result.

Finally, we believe that our approach could be of general use for diagnostic validations of other methods.

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