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SHORT COMMUNICATION

Conditions of High Resolution Melting Analysis on the Cobas z480 Instrument for the Genotyping of *VKORC1* in The Clinical Routine Laboratory

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SUMMARY

Background: **High resolution melting (HRM) of amplicons is a simple method for genotyping of single nucleotide polymorphisms (SNPs). Albeit many applications reported, HRM seems to be rarely used in clinical laboratories. The suitability of HRM-PCR for the clinical laboratory was investigated for genotyping of SNPs of the vitamin K epoxide reductase complex unit 1 gene.**
Methods: **About 100 DNA samples were analyzed by two different HRM-PCRs on the Cobas z480 instrument and compared with a PCR with fluorescently labeled probes (HybProbe-PCR) on the LightCycler 2.0 instrument as reference.**
Results: **Reliable genotyping with 100% matching results was obtained, when the amplicon size was small (63 bp) and DNA input was limited by e.g., sample dilution with salt-free water.**
Conclusions: **DNA extracted by differing methods may be used for genotyping by HRM-PCR. Compared with HybProbe-PCR, HRM-PCR on the Cobas z480 instrument allows for higher through-put, however, at the cost of a higher degree of laboratory standardization and a slower turnaround.**
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KEY WORDS

genotyping, SNP, LightCycler; real-time PCR, FRET-probes, high resolution melting, HRM, *VKORC1*

INTRODUCTION

Genotyping of single nucleotide polymorphisms (SNP) in the clinical laboratory is usually performed by PCR followed by various methods of sequence analysis such as reverse dot blot hybridization, allele specific hybridization, sequencing and DNA melting [1-5]. DNA melting was initially used in real-time PCRs with fluorescence resonance energy transfer hybridization probes (HybProbes) [6]. Numerous HybProbe-PCR assays for SNP genotyping have been developed and they have become customary in clinical laboratories [7].

Further developments of the real-time PCR technique led up to high resolution melting (HRM) of amplicons or of unlabeled probes [8,9]. Thus, many HRM-PCR assays have been reported for the detection of SNPs in clinical laboratories [10]. Albeit allowing for simple and high through-put, the prevalence of HRM-PCRs in clinical laboratories seems not to match that of HybProbe-PCR assays.

We compared two published HRM-PCR assays for the genotyping of *VKORC1* with a matching HybProbe-PCR assay as reference [11-14]. The *VKORC1* gene encodes the vitamin K epoxide reductase complex unit 1. Polymorphisms within this gene have been associated with inter-individual differences to the inhibition of vitamin K-dependent blood clotting factors by coumarins [15,16]. Genotyping of *VKORC1* is usually performed by the analysis of the SNP either at -1639 A>G (rs9923231) or at 1173 C>T (rs9934438). These two SNPs represent the haplotype with increased sensitivity to coumarins and are in linkage disequilibrium [15]. We show conditions and limitations for the use of HRM-PCR for the *VKORC1* genotyping on the Cobas z480 instrument in the clinical laboratory.

MATERIALS AND METHODS

Upon approval of the local ethics committee 100 human DNA samples were randomly selected from -20°C storage. DNA had been prepared either by using the QIAamp DNA blood mini kit (Qiagen, Hilden, Germany) or the Total Nucleic Acid Isolation Kit (Roche Vienna, Austria). DNA concentrations were assessed by spectrophotometry (NanoPhotometer, Implen, Munich, Germany). The DNA samples were used neat and at 5 ng/µL per dilution with PCR-grade water.

For the HybProbe-PCR (*VKORC1* 1173 C>T (rs99344 38)) on the LightCycler 2.0 (Roche, Vienna, Austria) Fast Start DNA Master Hybridization Probes Mastermix (Roche) was supplemented to 2.5 mmol $MgCl_2$, 0.2 µmol/L primers each and 0.2 µmol/L hybridization probes each (Data Supplement Table 1) [13, 14]. PCR with 2 µL sample DNA was performed with 95°C for 10 minutes, 45 cycles with 5 seconds at 95°C, 10 seconds at 59°C, and 15 seconds at 72°C followed by melting for 1 minute at 95°C, 1 minute at 45°C, 2 minutes at 40°C and temperature ramping to 80°C at 0.2°/second. The cell lines Raji, HeLa, and OCI-AML1 (German Collection of Microorganisms and Cell Cultures, Braunschweig, Germany) were used as reference for wildtype, mutant heterozygous, and mutant homozygous genotypes after having defined their genotypes by the HybProbe-PCR and by sequencing of *VKORC1* at positions 1173 and -1639 (reference sequence, Gen Bank acc. number NG011564.1) (Data Supplement Figures 1 and 2). DNA from these three cell lines was only extracted with the QIAamp method and exclusively used at 5 ng/µL.

HRM-PCRs for *VKORC1* 1173 C>T and *VKORC1* -1639 G>A were performed as described [11,12]. High Resolution Melting Master Mix (Roche, Vienna, Austria) was complemented with 3 mM $MgCl_2$ and 0.4 µM forward and reverse primers each (Data Supplement Table 1). Amplification of 2 µL sample DNA was at 95°C for 10 minutes followed by 55 cycles at 95°C 10 seconds, 63°C 15 seconds and 72°C 15 seconds. HRM was performed at 95°C 60 seconds, 40°C 60 seconds, 70°C 1 second and ramping to 95°C at 0.02°C/second with 25 acquisitions/°C. The genotypic analysis of melting curves was carried out by using the Gene Scanning Software (version 1.5.1., Roche, Vienna Austria). After normalization (pre-melting 83 - 84°C, post-melting 88 - 89°C, HRM-PCR 1173 C>T; pre-melting 77 - 78°C, post-melting 84 - 85°C, HRM-PCR -1639 G>A) temperature-shifted curves were generated and samples were automatically clustered into groups (auto-grouping). Sensitivity was adjusted between 0.05 and 0.3, and the temperature shift threshold was set to 0. Normalized HRM curves were also manually grouped by inspection and by Tm calling (manual grouping). Initially PCR products were analyzed by agarose gel electrophoresis to ensure that only single amplification products were obtained (*VKORC1* 1173 C>T, 124 bp; *VKORC1* -1639 G>A, 63 bp). All assay runs were always accompanied by reagent controls and the three genotypic controls, which were always genotyped correctly.

The HRM-PCR results were dichotomized, compared with HybProbe-PCR results as reference, and analysed by the McNemar test (exact modification). A p-value of < 0.05 was considered significantly different. Calculations were performed with Stata 13.1 (Stata Corporation LP, College Station, TX, USA).

RESULTS

Genotyping of the 100 DNA samples neat by HybProbe-PCR as reference gave unequivocal results (Figure 1A). Thus, 39 wildtype, 41 mutant heterozygous, and 20 mutant homozygous genotypes were observed (Tables 1 and 2). With the HRM-PCR 1173 C>T PCR products and melting curves were obtained from all samples with median Tms for wildtype and mutant of 87.1°C (SD 0.08°C; range 86.9°C - 87.3°C) and 86.6°C (SD 0.07°C; range 86.5°C - 86.7°C). When the DNA was used neat and melting was analyzed by auto-grouping 27 samples were scored as wildtype, 27 as mutant heterozygous, 20 as mutant homozygous, and 26 samples were categorized as unknown (Table 1). Eight samples were incorrectly genotyped as mutant homozygous instead of wildtype compared to HybProbe-PCR. Thus, the success rate of HRM-PCR 1173 C>T with auto-grouping was only 66% ($p < 0.00001$, McNemar test). With manual grouping, 36 samples were scored as wildtype, 41 as mutant heterozygous, and 23 as mutant homozygous (Table 1). None of the samples were scored as unknown, but three samples were incorrectly geno-

Table 1. Genotyping of *VKORC1* by HRM-PCR 1173 C>T compared with Genotyping by HybProbe-PCR.

HRM-PCR *VKORC1* 1173 C>T	HybProbe-PCR		
Genotype	**Wildtype**	**Mutant heterozygous**	**Mutant homozygous**
Auto-grouping [a], DNA neat			
Wildtype	**27**	**0**	**0**
Mutant heterozygous	**0**	**27**	**0**
Mutant homozygous	**8 [c]**	**0**	**12**
Unknown	**4**	**14**	**8**
Manual grouping [b], DNA neat			
Wildtype	**36**	**0**	**0**
Mutant heterozygous	**0**	**41**	**0**
Mutant homozygous	**3 [c]**	**0**	**20**
Unknown	**0**	**0**	**0**
Auto-grouping [a], DNA 10 ng			
Wildtype	**38**	**0**	**0**
Mutant heterozygous	**0**	**41**	**0**
Mutant homozygous	**0**	**0**	**11**
Unknown	**1**	**0**	**9**
Manual grouping [b], DNA 10 ng			
Wildtype	**39**	**0**	**0**
Mutant heterozygous	**0**	**41**	**0**
Mutant homozygous	**0**	**0**	**20**
Unknown	**0**	**0**	**0**

[a] - Auto-group function was used for high resolution melting analysis.
[b] - High resolution melting analysis was performed manually.
[c] - Bold numbers represent misclassified genotypes.

typed as mutant homozygous instead of wildtype (success rate 97%; p = 0.25, McNemar test).
When, the HRM-PCR 1173 C>T was repeated with the DNA at 5 ng/µL the accuracy of genotyping was found to be improved. With auto-grouping, 90 samples were genotyped correctly (Table 1) and 10 samples were categorized as unknown. None of the samples were incorrectly genotyped (success rate 90%; p = 0.002, McNemar test). By manual grouping all samples yielded to matching genotypes compared with the reference assay (success rate 100%; p = 1.0, McNemar test) (Table 1). The accuracy of genotyping was markedly improved with the use of the HRM-PCR for *VKORC1* -1639 G>A. This HRM-PCR showed median Tms for wildtype and mutant of 82.2°C (SD 0.07°C; range 82.1°C - 82.3°C) and 81.6°C (SD 0.06°C; range 81.5°C - 81.7°C), respectively. With DNA neat and auto-grouping 96 of the 100 samples were correctly genotyped and only four samples were categorized as unknown (Table 2). None of the samples were incorrectly genotyped (the success rate 96%; p = 0.125, McNemar test). By manual grouping all genotyping results matched 100% to those which were obtained with the HybProbe-PCR as reference (success rate 100%; p = 1.0, McNemar test; Table 2). When HRM-PCR for *VKORC* -1639 G>A was repeated with the DNA at 5 ng/µL, genotyping by auto-grouping and by manual grouping completely matched the genotyping obtained with the HybProbe-PCR (success rate 100% each; p = 1.0, McNemar test; Table 2).

DISCUSSION

In our study we observed three factors that impacted on the accuracy of genotyping by HRM-PCR: i) DNA input, neat versus 5 ng/µL obtained by dilution with PCR-grade water, ii) size of amplicon, 124 bp versus 63 bp, and iii) auto-grouping versus manual grouping for HRM genotyping analysis. The standardization of DNA input to the PCR showed a marked improvement on genotyping accuracy. Since PCR product concentration has been reported to affect DNA melting, standardized input of DNA ought to have generated equal concentrations of PCR products leading to uniform DNA melting [17]. As the concentration of salts also influences DNA melting, the dilution of our DNA samples with salt-free wa-

Table 2. Genotyping of *VKORC1* by HRM-PCR -1639 A>G compared with Genotyping by HybProbe-PCR.

HRM-PCR *VKORC1* -1369 A>G	HybProbe-PCR		
Genotype	**Wildtype**	**Mutant heterozygous**	**Mutant homozygous**
Auto-grouping [a], DNA neat			
Wildtype	**38**	**0**	**0**
Mutant heterozygous	**0**	**40**	**0**
Mutant homozygous	**0**	**0**	**18**
Unknown	**1**	**1**	**2**
Manual grouping [b], DNA neat			
Wildtype	**39**	**0**	**0**
Mutant heterozygous	**0**	**41**	**0**
Mutant homozygous	**0**	**0**	**20**
Unknown	**0**	**0**	**0**
Auto-grouping [a], DNA 10 ng			
Wildtype	**39**	**0**	**0**
Mutant heterozygous	**0**	**41**	**0**
Mutant homozygous	**0**	**0**	**20**
Unknown	**0**	**0**	**0**
Manual grouping [b], DNA 10 ng			
Wildtype	**39**	**0**	**0**
Mutant heterozygous	**0**	**41**	**0**
Mutant homozygous	**0**	**0**	**20**
Unknown	**0**	**0**	**0**

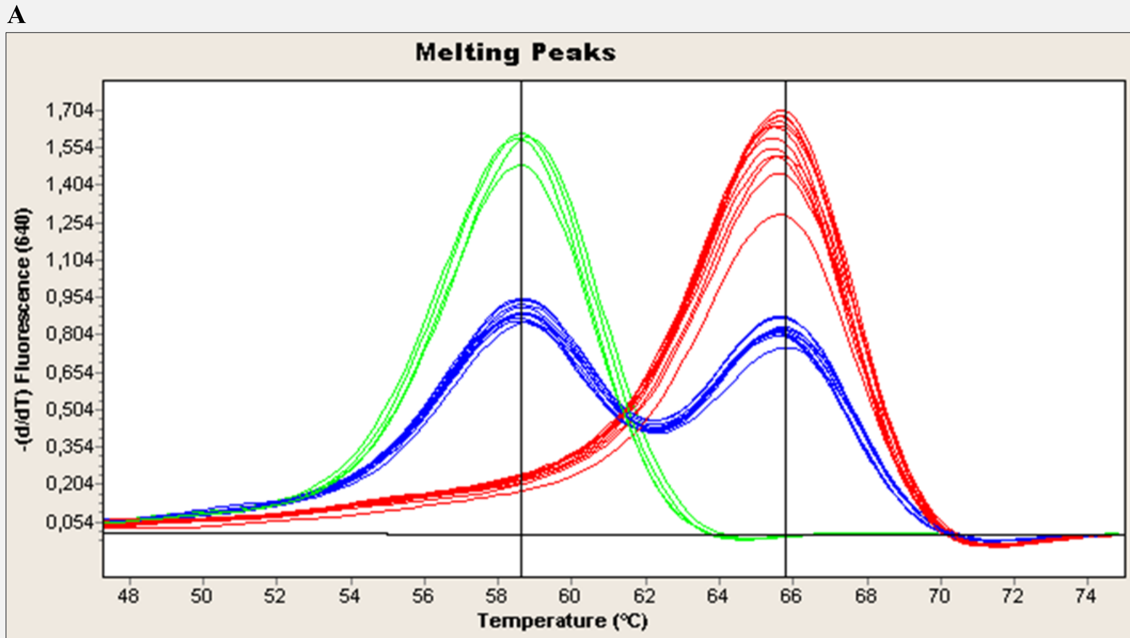
[a] - Auto-group function was used for high resolution melting analysis.
[b] - High resolution melting analysis was performed manually.

ter to arrive at 5 ng/µL should have also had a standardizing effect on salts in our HRM-PCRs, given that DNA samples from the two different preparation methods were blindly used in our study [17,18]. Our finding is in line with the study by Chen et al., who exclusively used 10 ng DNA per HRM-PCR on a Cobas z480 and found 100% concordant results with genotyping by sequencing [11]. In contrast, Liew and colleagues did not find the necessity to quantify DNA prior to HRM-PCR [8]. This discrepancy could be related to the fact that they used only one single DNA extraction method and, thus, salts were largely standardized in the DNA samples. In addition, they used glass capillaries for PCR and HRM, which exhibit greater temperature transmission than plastic ware which we have used on the Cobas z480. Others have used HRM-PCR with instruments such as a rotor-gene and also report standardization of DNA input [19].

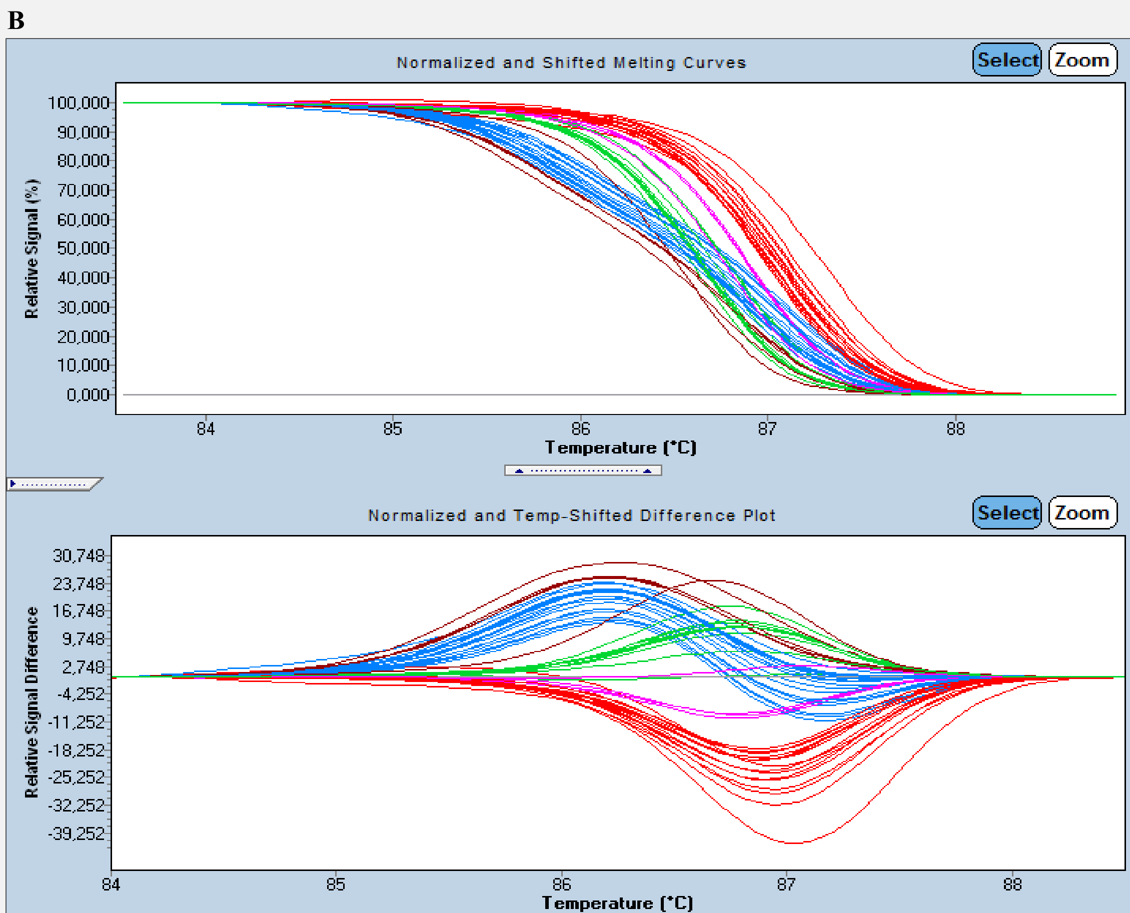
Amplicons from mutant heterozygous genotypes form mixtures of two heteroduplexes and two homoduplexes with differing melting temperatures that generate superimposing melting curves, which are quite distinct from melting curves of amplicons from wildtype or mutant homozygous genotypes [8]. Thus, all mutant heterozygous samples were either correctly genotyped or classified as unknown by both HRM-PCR assays. Particularly, misclassifications of mutant heterozygous samples were not observed in our study (Tables 1 - 2).

In contrast, amplicons from wildtype or from mutant homozygous genotypes melt in a single transition [8]. As the differences in melting temperatures are minute, discrimination of wildtype from mutant homozygous can be challenging [8]. The influence on melting temperature has been attributed to nearest-neighbor interaction involving bases next to the position of the SNPs and to amplicon size. As the ratio of one single differently paired base to total base pairing in an amplicon increases with decreasing amplicon size, one single different paired base on large amplicons exhibits a smaller effect on the melting temperature than on shorter amplicons [8,9]. Our results follow along these lines. In the two HRM-PCRs the mean melting temperature differences between wildtype and mutant were 0.5°C for the 124 bp amplicon (HRM-PCR 1173 C>T) and 0.6°C for the 63 bp amplicon (HRM-PCR -1639 G>A). Thus, with the HRM-PCR 1173 C>T wildtype and mutant ho-

A



B



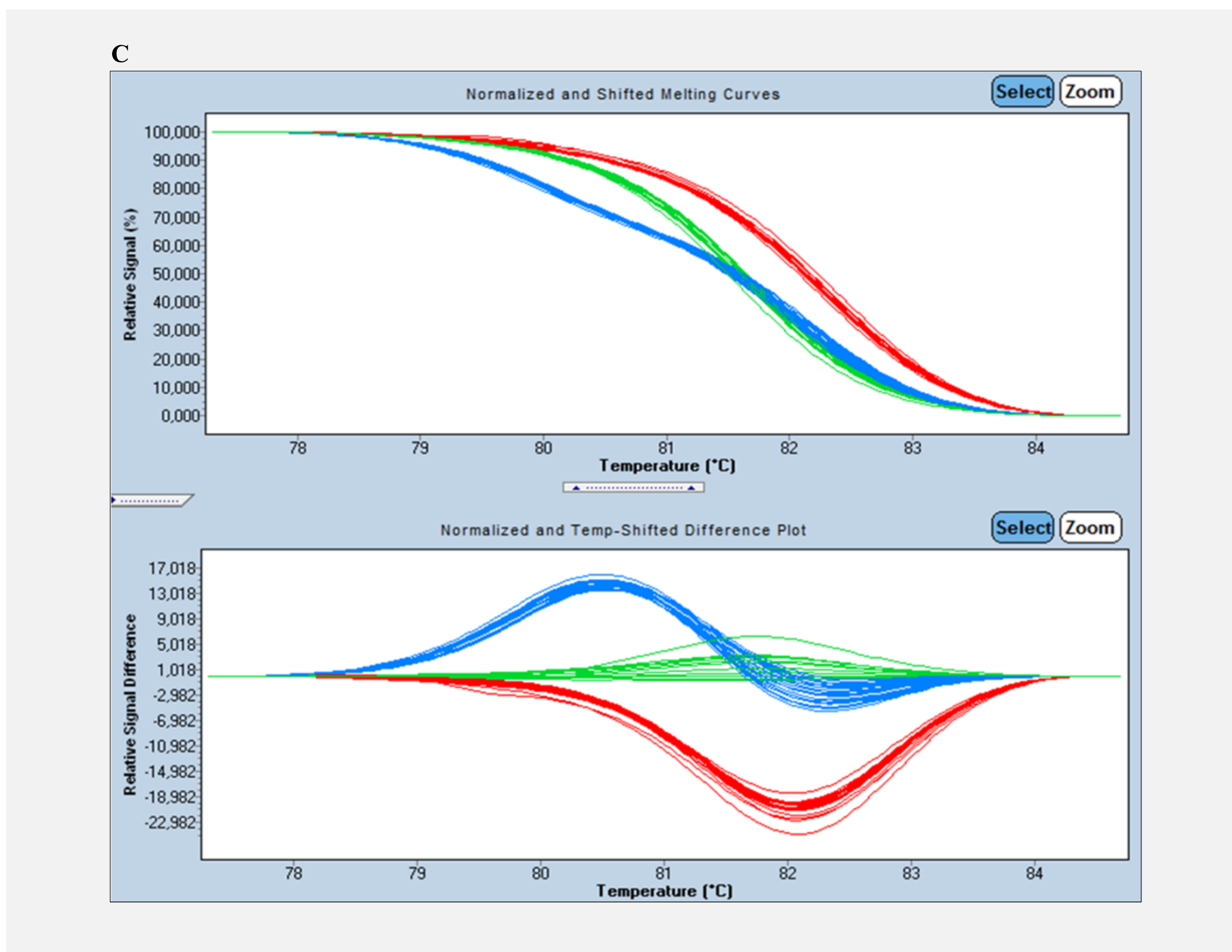


Figure 1.

A. Melting curves generated by HybProbe-PCR for *VKORC1* 1173 C>T. The screenshot shows the melting curves of 25 samples and the three genotypic controls and a reagent control. Red, wildtype; blue, mutant heterozygous; green, mutant homozygous, black, reagent control. Vertical lines indicate approximate melting temperatures (TMs) for wildtype (C) and mutant (T) alleles. The Tms for wildtype and mutant alleles of the 100 DNA samples showed a median of 65.8°C ± 0.2°C (range 65.4°C - 66.3°C) and of 58.8°C ± 0.2°C (range 58.5°C - 59.2°C), respectively, and were significantly different ($p < 0.01$).
B, C. Normalized and temperature shifted melting curves generated by the HRM-PCRs for *VKORC1* 1173 C>T (B) and *VKORC1* -1639 G>A (C). The screenshots each show the analysis of 50 DNA samples by auto-grouping. (B) DNA samples were used neat except for the three genotypic controls (10 ng/reaction). Red, wildtype; blue, mutant heterozygous; green, mutant homozygous, burgundy, unknown; pink, non-matching genotypes compared with HybProbe-PCR. (C) DNA samples including the three genotypic controls were used at 10 ng/reaction. Red, wildtype; blue, mutant heterozygous; green, mutant homozygous. Note, none of the samples were grouped as unknown, and all genotypes completely matched those obtained by HybProbe-PCR.

mozygous samples were found either correctly genotyped, or classified as unknown, or misclassified. In contrast, with the HRM-PCR -1639 G>A all wildtype and all mutant homozygous samples were either correctly genotyped or classified as unknown, not, however, misclassified.
The gene scanning software for the HRM analysis slipped at instances described above, especially, when auto-grouping was used. When the HRM analysis was performed manually and with inspection of the melting curves, a higher degree of accuracy in the genotyping was observed. For the routine clinical application of HRM-PCR this finding suggests to have HRM curves always carefully inspected by the operator after HRM analysis by auto-grouping. This may minimize misclassification and will assist in the decision which sample(s) to sequence for confirmation.

CONCLUSION

For the genotyping of *VKORC1* we have shown that HRM-PCR on the Cobas z480 instrument may reliably be used in the clinical laboratory, if amplicon size is short, DNA input is limited by e.g., dilution with salt-free water, single amplicons are generated by PCR, and auto-grouping is reviewed by manual grouping. Then, DNA from differing extraction procedures may be used. Compared with HybProbe-PCR on a LightCycler 2.0, HRM-PCR on the Cobas z480 instrument allows for higher through-put, however, at the cost of a higher degree of laboratory standardization and a slower turn-around.

Declaration of Interest:
The authors declare that they have no competing interests.

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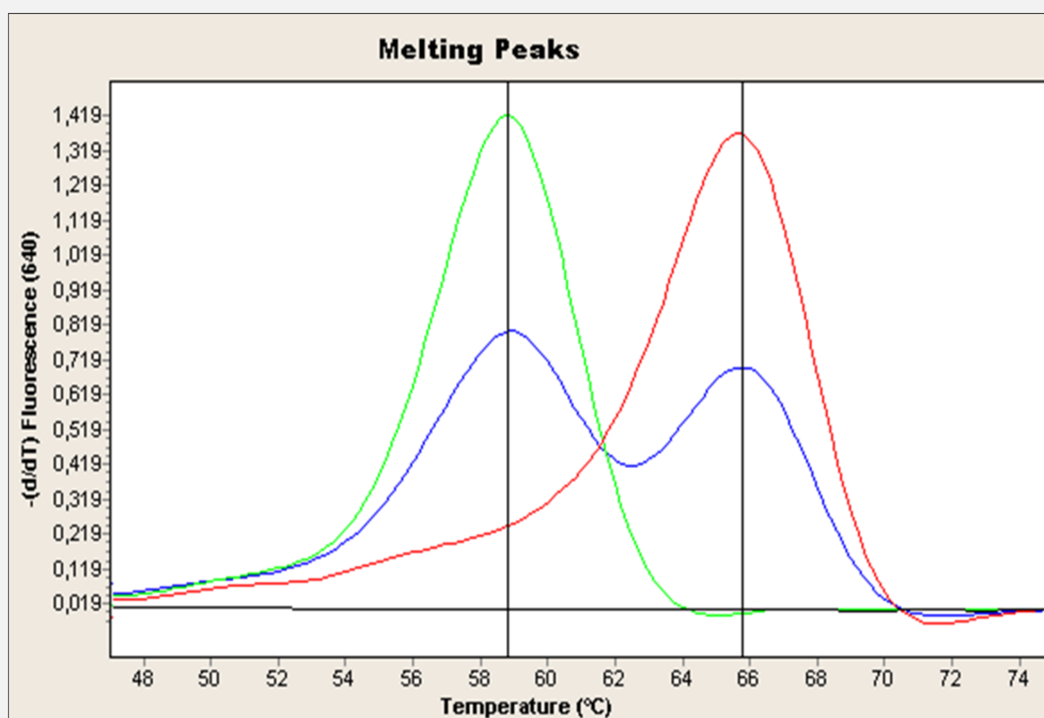
Data Supplement Table 1. Oligonucleotides used for the three different *VOKRC1* genotyping PCR$_s$.

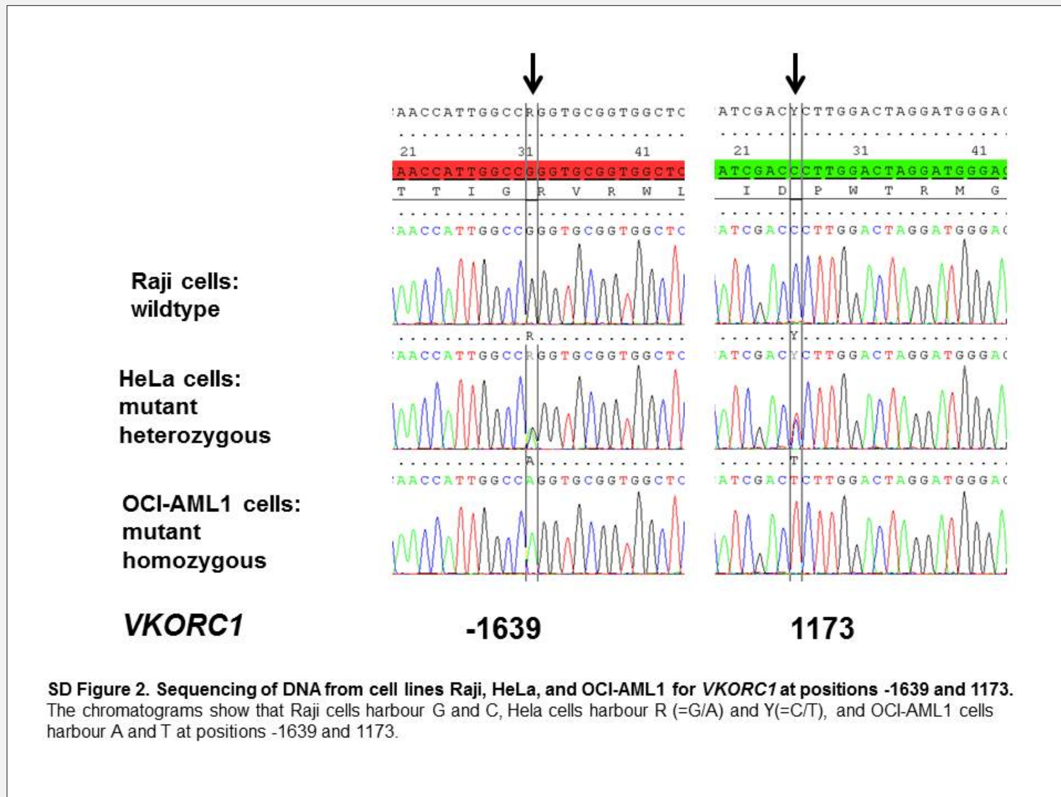
Name	Sequence	Nucleotide position [a]	Reference [b]
VKORC1 for	5'-AGA GAC TTA CTT AAG GTC TAA GAT GAA-3'	6256-6282	14
VKORC1 rev	5'-GCC CGA GAA AGG TGA TTT CC-3'	6452-6470	13
HybProbe *VKORC1* sens [c]	5'-AGA TCA TCG ACC CTT GGA CTA GG-Fluo-3'	6388-6410	14
HybProbe *VKORC1* anch [d]	5' LC640-GGG AGG TCG GGG AAC AGA GGA T-Pho-3'	6413-6434	14
HRM *VKORC1* 1173-F	5'-CCG AGA AAG GTG ATT TCC AA-3'	6346-6365	11
HRM *VKORC1* 1173-R	5'-TGA CAT GGA ATC CTG ACG TG-3'	6450-6469	11
HRM *VKORC1* -1639-F	5'-CAA GAG AAG ACC TGA AAA ACA ACC ATT-3'	3557-3583	12
HRM *VKORC1* -1639-R	5' TGC TAG GAT TAT AGG CGT GAG CC-3'	3597-3619	12

[a] - GenBank accession number: NG_011564.1.
[b] - Numbers refer to the list of references in the text.
[c] - Oligonucleotide is labelled with Fluorescein at the 3' terminus.
[d] - Oligonucleotide is labelled with LC-Red640 at the 5' terminus and phosphorylated at the 3' terminus.



Data Supplement Figure 1. Melting curves of HybProbe PCR for *VKORC1* 1173 C>T generated from genotypic controls of cell lines.

Red, Raji cell DNA representing wildtype; blue, HeLa cell DNA representing mutant heterozygous; green, OCI-AML1 cell DNA representing mutant homozygous. The three controls exhibited melting temperatures (Tm) for wildtype and mutant alleles at 66°C (median 66.0°C; SD 0.3°C; range 65.6°C - 66.3°C) and 59°C (median 59.0°C; SD 0.2°C; range 58.8°C - 59.3°C) in 8 PCR runs, and Tms were found significantly different by Student's *t*-test ($p < 0.01$).



SD Figure 2. Sequencing of DNA from cell lines Raji, HeLa, and OCI-AML1 for *VKORC1* at positions -1639 and 1173.
The chromatograms show that Raji cells harbour G and C, Hela cells harbour R (=G/A) and Y(=C/T), and OCI-AML1 cells harbour A and T at positions -1639 and 1173.

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