Disease Markers 29 (2010) 243–249
DOI 10.3233/DMA-2010-0754
IOS Press

Rapid genotyping of the human renin (REN) gene by the LightCycler® instrument: Identification of unexpected nucleotide substitutions within the selected hybridization probe area

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Abstract. Preeclampsia is a serious disorder affecting nearly 3% of all pregnancies in the Western world. It is associated with hypertension and proteinuria, and several lines of evidence suggest that the renin-angiotensin system (RAS) may be involved in the development of hypertension at different stages of a preeclamptic pregnancy. In this study, we developed rapid genotyping assays on the LightCycler® instrument to allow the detection of genetic variants in the renin gene (*REN*) that may predispose to preeclampsia. The method is based on real-time PCR and allele-specific hybridization probes, followed by fluorescent melting curve analysis to expose a change in melting temperature (T_m). Ninety-two mother-father-child triads ($n = 276$) from preeclamptic pregnancies were genotyped for three haplotype-tagging single nucleotide polymorphisms (htSNPs) in *REN*. All three htSNPs (rs5705, rs1464816 and rs3795575) were successfully genotyped. Furthermore, two unexpected nucleotide substitutions (rs11571084 and rs61757041) were identified within the selected hybridization probe area of rs1464816 and rs3795575 due to aberrant melting peaks. In conclusion, genotyping on the LightCycler® instrument proved to be rapid and highly reproducible. The ability to uncover additional nucleotide substitutions is particularly important in that it allows the identification of potentially etiological variants that might otherwise be overlooked by other genotyping methods.

Keywords: Preeclampsia, renin, LightCycler, real-time PCR

1. Introduction

Preeclampsia is a heritable complication affecting nearly 3% of all pregnancies in Western populations [1]. Although the etiology remains to be elucidated, reduced placental perfusion, oxidative stress, activation of thrombosis and the renin-angiotensin system (RAS) are probably involved at different stages of a preeclamptic pregnancy [2–5]. Family studies have shown that genetic factors contribute to the development of preeclampsia, but the exact mechanism is still unknown despite extensive studies [6–8]. The presence of renin (REN) in the placenta indicates a local synthesis and suggests that REN may be involved in the regulation of maternal blood pressure [9,10]. Therefore,

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ISSN 0278-0240/10/$27.50

analysis of the gene coding for REN might contribute to an improved understanding of the underlying causes of preeclampsia.

A wide range of genotyping techniques have been described over the years, including gel-based restriction fragment length polymorphism (RFLP) [11,12], "closed tubes" assays such as the 5' fluorogenic nuclease assay (TaqMan®assay), single-base extension (minisequencing), Pyrosequencing™, and amplification refractory mutation system (ARMS) [12]. Some genotyping techniques combine real-time PCR with continuous fluorescence monitoring during amplification; an example is the LightCycler® instrument, which uses fluorescence resonance energy transfer (FRET) and real-time PCR. This technology quantifies an energy transfer between oligonucleotide fluorophores labeled with different dyes. The donor fluorophore is labeled at the 3'-end with Fluorescein (FL), while the other probe labeled at the 5'-end (LC-Red 640 or LC-Red 705) serves as an acceptor. If there is a mismatch between the target DNA and the hybridization probes, the melting temperature (T_m) will tend to decrease compared with a perfectly matched probe. Thus, this technology is also capable of detecting a particular SNP within the hybridization probe area as a result of a shift in T_m [13,14].

Here, we present three rapid and reliable genotyping assays based on this widely used method on the LightCycler® instrument. As a demonstration, DNA samples from 92 preeclamptic nuclear families ($n = 276$) were genotyped for three htSNPs in *REN*. We also report on the unexpected discovery of two additional nucleotide substitutions within the selected hybridization probe areas for two of the three htSNPs.

2. Materials and methods

2.1. Biological samples

Ninety-two women who delivered at the Stavanger University Hospital in Norway in the period January 1993 to December 1995 and who fulfilled the diagnostic criteria for preeclampsia were included in the study [11, 15]. Umbilical cord blood was collected from delivered neonates and peripheral blood was drawn from mothers and fathers post partum [11]. The study was approved by the Regional Committee for Ethics in Medical Research and the Norwegian Data Inspectorate. Written informed consent was obtained from the parents. The present work was undertaken as a part of a larger study examining genetic risk factors for preeclampsia [16].

2.2. SNP selection and DNA extraction

A 40 kb-long region containing *REN* on chromosome 1q32 (NT 004487.18) was downloaded from HapMap (http://www.hapmap.org) and imported into Haploview v.3.0 [17] for the selection of htSNPs and the evaluation of marker metrics. Three htSNPs on chromosome 1 (rs5705, rs1464816, and rs3795575) were chosen using the "pairwise tagging" algorithm. All nucleotide positions are according to the National Center for Biotechnology Information (NCBI) assembly 34 of the human genome (Table 1). To capture the genetic variation encompassing *REN*, we used a minor allele frequency (MAF) cut-off of at least 10% and an r^2 greater than 0.8 for linkage disequilibrium within haplotype blocks. Genomic DNA from 276 samples was extracted using the QIAamp DNA Blood Mini Kit (Qiagen, Hilden, Germany).

2.3. PCR primers and hybridization probes

PCR primers and hybridization probes were designed and synthesized by TIB MOLBIOL (Berlin, Germany). Details are provided in Table 1.

2.4. Genotyping

To genotype rs5705, rs1464816 and rs3795575, three separate real-time PCR reactions were performed followed by fluorescent melting curve analyses on the LightCycler® 2.0 instrument (Roche Diagnostics, Mannheim, Germany). The reaction mixtures contained 2.5 μL of DNA (approximately 10–50 ng), 4 μL of LightCycler FastStart DNA Master^{PLUS} HybProbe (Roche Diagnostics), PCR primers, hybridization probes and sterile water to a final volume of 10 μL. Asymmetric PCR conditions were used. PCR primers and hybridization probes were added at the following concentrations: for rs5705, 0.2 μM RENe2S, 0.4 μM RENe2A, 0.2 μM sensor C, and 0.4 μM Anchor REN; for rs1464816, 0.2 μM RENi4S, 0.4 μM RENi4A, 0.2 μM Sensor wt and 0.4 μM of Anchor REN; and, finally, for rs3795575, 0.2 μM RENi8A, 0.5 μM RENi8S, 0.2 μM REN mut and 0.4 μM of REN LC. Cycling conditions were 95°C for 10 min, followed by 45 cycles of 95°C for 5 s, 52°C, 56°C and 58°C for 10 s for rs5705, rs1464816 and rs3795575 respectively, then 72°C for 15 s, with a ramp rate of 20°C/s. Melting curve analysis was performed as follows: 95°C for 10 s, cooling to 55°C and 45°C for 30 s each, 40°C for 2 min, then heating to 75°C (0.2°C/s). Thirty pa-

Table 1
PCR primers and hybridization probes used for genotyping *REN*

SNP	Nucleotide position*	Alleles	PCR primers (5'-3')	Hybridization probes (5'-3')**
rs5705	202397809	A/C	RENe2S: CAAGAGAATGCCCTCAATCC	Sensor C: ATGAAGAGGCTGACCCTTGGC-FL
			RENe2A: CAAGCACTCACGTCCATGTAG	Anchor REN: 640-ACACCACCTCCTCCGTGATCCTCACC-p
rs1464816	202395477	G/T	RENi4S: GCTTTTCTTTTGCTGCTTGG	Sensor wt: TGCAGGGTTGAGGCAATAC-FL
			RENi4A: TCCTTGGTTGGAATCTGGTC	Anchor REN: 640-CTTACCCCGATTTCTGTACCCTGGA-p
rs3795575	202391712	C/T	RENi8S: AAGAAGCCAAAGAGGGAAGG	REN mut: GCCTTCTTGAGTATGGAAGACATCTCAGC-FL
			RENi8A: GAAAGAGATGTCGGGGAGTG	REN LC: 640-GACAAGGAGTCCTGCGCTGGTGGC-p

*Nucleotide positions according to the National Center for Biotechnology Information (NCBI) human assembly 34.
**The position of the polymorphic site is underlined.

Table 2
Genotype distribution of rs5705, rs1464816 and rs3795575 in the human renin gene (*REN*) among 92 preeclampsia mother-father-child triads

SNP	Genotype	Count (%)	$T_m^\circ C$ ($\pm$ SD)*	$\Delta T_m^\circ C$
rs5705	A/A	217 (78.6)	58.4 (0.24)	
	A/C	59 (21.4)	58.4 (0.24) and 66.4 (0.23)	8.2
	C/C	0		
rs1464816	G/G	112 (40.6)	63.0 (0.40)	
	G/T	139 (50.4)	63.0 (.040) and 53.1 (0.44)	10.1
	T/T	25 (9.0)	53.1 (0.44)	
rs3795575	C/C	212 (76.8)	65.0 (0.38)	
	C/T	64 (23.2)	65.0 (0.38) and 70.0 (0.27)	5.1
	T/T	0		

T_m, melting temperature; (ΔT_m; difference in T_m between the wild-type and mutant genotype).
*Mean $T_m \pm$ standard deviation.

tient samples, one positive (heterozygous target) and one negative (no-template) control ($n = 32$) were analyzed simultaneously. All samples (276 in total) were analyzed in duplicate. The wild-type and mutant genotypes were differentiated by their characteristic melting peaks, which were generated by plotting the negative derivative of the fluorescence signal with temperature versus temperature (T) [(*-dF*/*dT*) versus T].

2.5. Sequencing

To assess genotyping consistency, DNA from 30 randomly selected individuals was sequenced for each htSNP (PCR primers are provided in Table 1). Amplification products were electrophoresed on 2% agarose gel and visualized by ethidium bromide staining. PCR fragments were purified using QIAquick® PCR Purification Kit (Qiagen) followed by cycle sequencing using ABI PRISM® BigDye® Terminator v1.1 Cycle Sequencing Kit (AB Applied Biosystems, Foster City, CA, USA) according to the manufacturer's recommendations. Finally, the sequencing reactions were cleaned using Centri-Sep spin columns (Princeton Separations, Inc, Adelphia, NJ) according to the manufacturer's instructions and analyzed on an ABI PRISM®310 Genetic Analyzer instrument (AB Applied Biosystems). Resulting sequences were aligned in BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi) for confirmation.

3. Results

DNA samples from preeclamptic women, their partners and children (276 subjects in total) were genotyped for three htSNPs in *REN*. Each of the htSNPs showed a clear difference in T_m between the wild-type and mutant genotype (between 5.1°C and 10.1°C). All three genotyping assays were performed in duplicate and no inconsistencies between the genotypes were observed. The results are summarized in Table 2. When genotypes from the LightCycler® analysis were compared with the results of DNA sequencing in a random set of 30 patient samples, identical genotypes were obtained for each htSNP.

During fluorescent melting curve analysis, unusual melting peaks around rs1464816 (Fig. 1) and rs3795575 (Fig. 2) were observed. For rs1464816, seven of the patient samples revealed atypical melting

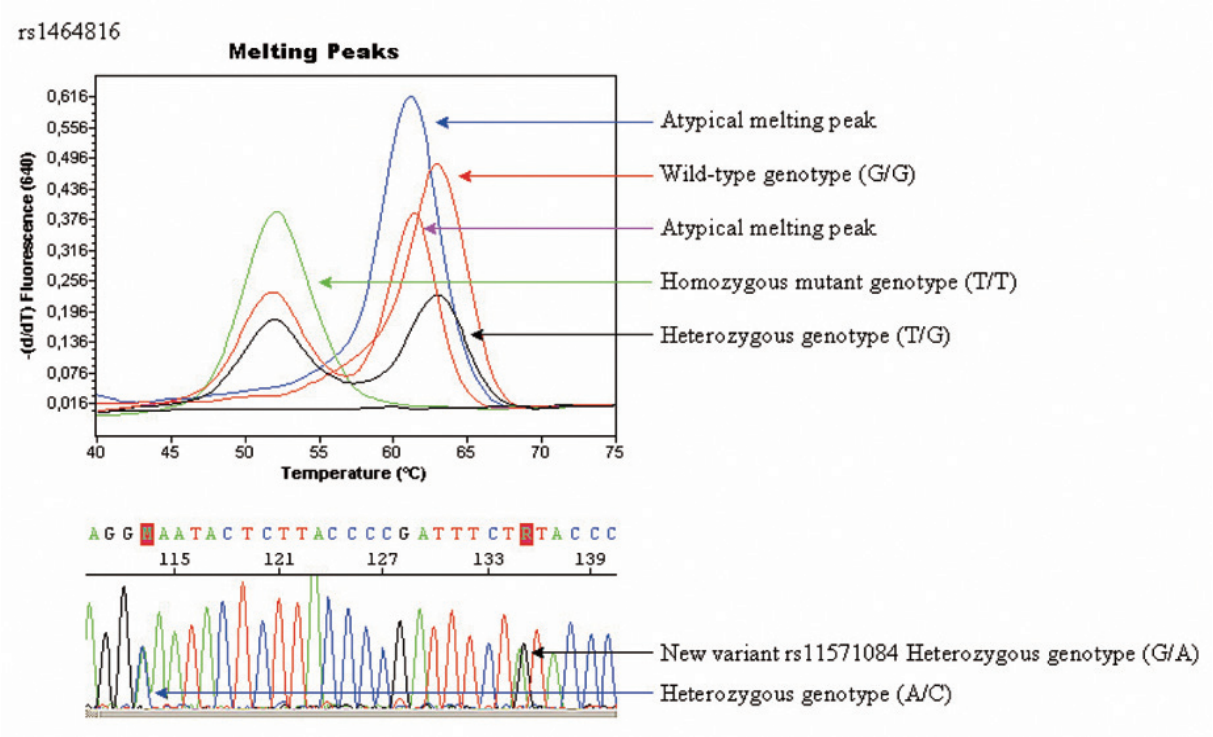

Fig. 1. Typical melting peaks obtained after genotyping rs1464816 by the LightCycler® instrument. Patients carrying the homozygous mutant T/T genotype revealed one single melting peak with a T_m of 53.1°C (green), whereas those with the wild-type G/G genotype revealed a single peak with a T_m of 63.0°C (red). Heterozygous carriers with the T/G genotype revealed two melting peaks (black), one at 53.1°C and the other at 63.0°C. Atypical melting peaks are shown in blue and pink, with a T_m of 61.1°C. A no-template control is shown in dark green. The DNA sequence (reverse direction) is from a patient who is heterozygous A/C for rs1464816 (blue arrow) and simultaneously heterozygous G/A for rs11571084 (highlighted by the black arrow).

peaks, with a T_m of 61.1°C, 1.9°C ± 0.12 (mean ± SD) lower than the T_m of the wild-type genotype G/G (Fig. 1). For rs3795575, 16 samples revealed atypical melting peaks, with a T_m of 59.8°C, 5.2°C ± 0.28 lower than the T_m of the wild-type genotype C/C (Fig. 2). To confirm that the atypical melting peaks were not due to error, we reanalyzed DNA from patients exhibiting atypical melting peaks. Identical results were observed. When we sequenced these samples, additional nucleotide substitutions were discovered in the hybridization probe areas; namely, rs11571084 [A/G] (Chr1: 202395455), 22 nucleotides upstream of rs1464816, and rs61757041 [A/G] (Chr1: 202391716), 4 nucleotides downstream of rs3795575, respectively.

4. Discussion

Detection of etiologically relevant SNPs is a powerful diagnostic test for inherited disorders. In this study, we developed genotyping assays for *REN* by allele-specific hybridization probes followed by fluorescent melting curve analysis on the LightCycler® instrument. The technique proved to be rapid and reliable. Thirty patient samples, together with positive and negative controls, were genotyped in less than an hour, and the results were highly reproducible when cross-checked against direct sequencing.

Genotyping on the LightCycler® instrument is particularly advantageous compared with other traditional genotyping techniques [11,12] in that it does not require any post-PCR sample processing, no hazardous reagents are involved, and the entire reaction takes place in a close-tube system, which minimizes the risk of contaminations. All these factors are important to consider in a moderate-sized lab-facility. Furthermore, the unanticipated detection of rs11571084 and rs61757041 within the hybridization probes areas designed for rs1464816 and rs3795575 demonstrates the ability of the technique to distinguish patient samples with additional nucleotide substitutions. Samples from patients carrying these unexpected nucleotide substitu-

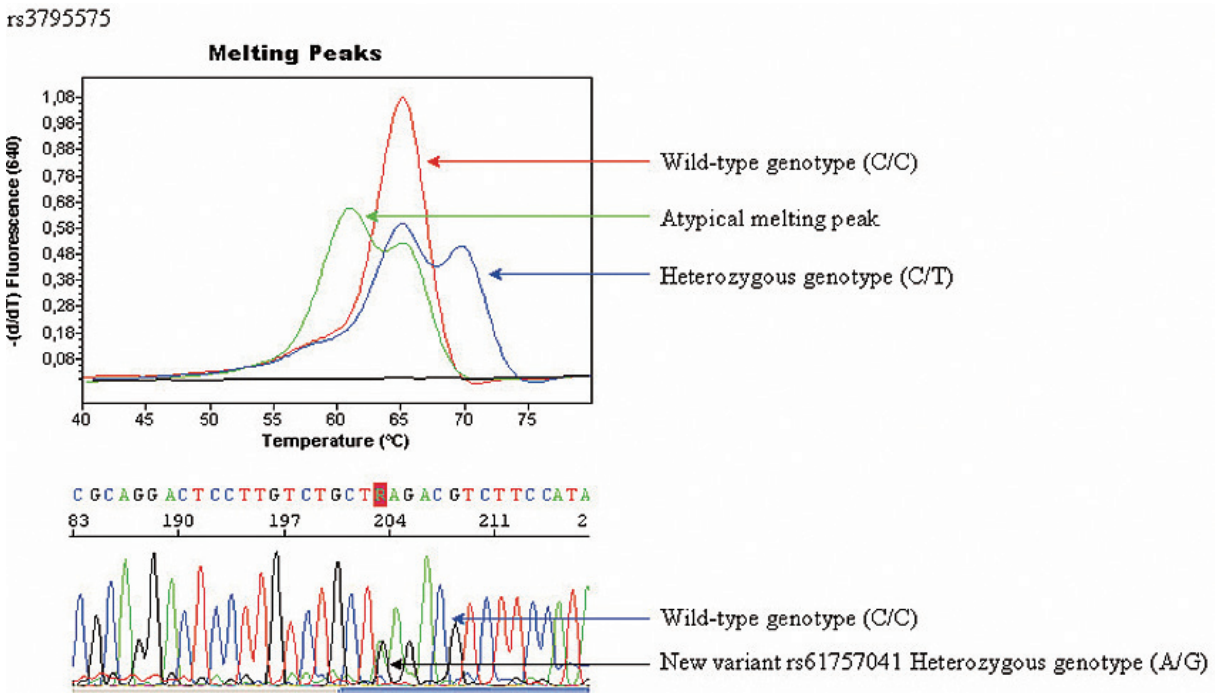

Fig. 2. Typical melting peaks obtained after genotyping rs3795575 by the LightCycler® instrument. Patients with the wild-type C/C genotype revealed a single melting peak with a T_m at 65.0°C (red) and patients with the heterozygous C/T genotype revealed two melting peaks, one at 65.0°C and the other at 70.0°C (blue). An atypical melting peak with T_m at 61.1°C is shown in green, and the no-template control is shown in black. The DNA sequence (forward direction) is from a patient who is homozygous wild-type C/C for rs3795575 (blue arrow) and simultaneously heterozygous A/G for rs61757041 (highlighted by the black arrow).

tions (rs11571084 and rs61757041) affected our genotyping assays by generating atypical melting peaks.

It is possible to accommodate known nucleotide substitutions located close to the actual SNP in the assay design by modifying the location or the size of the hybridization probe sequence [18,19]. Alternatively, this can be achieved by using a "masking technique", in which hybridization probes are designed as a "mask" over a non-target sequence. That is, an artificial mismatch with all possible alleles adjacent to the mutations is created as described by Margraf and co-workers [19]. In our case, however, all three genotyping assays were designed without prior knowledge of rs11571084 and rs61757041.

The impact of these two nucleotide substitutions on the development of preeclampsia is unknown and future studies are warranted to determine the frequency and association of these variants with disease development and progression. The sample size clearly needs to be increased in order to gauge the impact of these additional nucleotide substitutions on the risk of preeclampsia. According to the dbSNP database (www.ncbi.nlm.nih.gov/sites/entrez), two other SNPs (rs11571116 and rs61757042) have recently been reported in the vicinity of rs1464816 and rs3795575. rs11571116 has not been typed in the CEPH sample of Northern-European origin, while data for rs61757042 have not yet been released in all the HapMap test populations (www.hapmap.org).

Several diagnostic assays have been developed on the LightCycler® instrument, enabling the identification of genotypes by characteristic melting peaks and T_m shifts [20–22]. Some of these well-established diagnostic assays have reported atypical melting peaks due to the presence of new nucleotide substitutions in the hybridization probe area beside those the assays were originally designed for. These additional nucleotide substitutions have shown varying clinical effects [23–26]. Atypical melting peaks are usually detected by T_m shifts of 5–6°C or higher and may therefore be easily overlooked if the T_m shifts are less than 1°C, or if there is an overlap in T_m [27,28]. However, if the hybridization probe area is particularly ambiguous or has not been reported in the public databases, validation with an alternative method and/or direct sequencing of patient samples in diverse populations is warranted.

In conclusion, genotyping using the LightCycler® instrument allows highly specific detection of SNPs in

the targeted DNA sequence. In particular, the discovery of new nucleotide substitutions within the hybridization probe area represents a significant advance over other conventional genotyping platforms. This, coupled with relatively low cost and minimum hands-on time, makes the LightCycler® technique particularly attractive for cost-effective and rapid genotyping in smaller lab-facilities.

Acknowledgements

This work was supported from the Western Norwegian Health Authorities and by The Norwegian Society of Engineers and Technologists (NITO).

Conflict of interest

There are no conflicts of interest.

References

[1] C.W. Redman and I.L. Sargent, Latest advances in understanding preeclampsia, *Science* **308** (2005), 1592–1594.
[2] R. Arngrimsson, Epigenetics of hypertension in pregnancy, *Nature Genetics* **37** (2005), 460–461.
[3] G.A. Dekker and B.M. Sibai, Etiology and pathogenesis of preeclampsia: Current concepts, *American Journal of Obstetrics and Gynecology* **179** (1998), 1359–1375.
[4] P. Parham, NK cells and trophoblasts: partners in pregnancy, *Journal of Experimental Medicine* **200** (2004), 951–955.
[5] J.M. Roberts and D.W. Cooper, Pathogenesis and genetics of pre-eclampsia, *Lancet* **357** (2001), 53–56.
[6] A.M.A. Lachmeijer, G.A. dekker, G. Pals, J.G. Aarnoudse, L.P. ten Kate and R. Arngrimsson, searching for preeclampsia genes: the current position, *European Journal of Obstetrics and Gynecology an Reproductive Biology* **105** (2002), 94–113.
[7] E. Nilsson, H.S. Ros, S. Cnattingius and P. Lichtenstein, The importance of genetic and environmental effects for pre-eclampsia and gestational hypertension: a family study, *BJOG: an International Journal of Obstetrics and Gynaecology* **111** (2004), 200–206.
[8] R. Skjærven, L.J. Vatten, A.J. Wilcox, T.Rønning, L.M. Irgens and R.T. Lie, Recurrence of pre-eclampsia across generations: exploring fetal and maternal genetic components in a population based cohort, *British Medical Journal* **331** (2005), 877.
[9] C. Li, R. Ansari, Z. Yu and D. Shah, Definitive molecular evidence of renin-angiotensi system in human uterine decidual cells, *Hypertension* **36** (2000), 159–164.
[10] E. Takimoto, J. Ishida, F. Sugiyama, H. Horiguchi, K. Murakami and A. Fukamizu, Hypertension induced in pregnant mice by placental renin and maternal angiotensinogen, *Science* **274** (1996), 995–997.
[11] H. Vefring, R.T. Lie, R. Ødegrd, M.A. Mansoor and S.T. Nilsen, Maternal and fetal variants of genetic thrombophilias and the risk of preeclampsia, *Epidemiology* **15** (2004), 317–322.
[12] C.D. Mamotte, Genotyping of single nucleotide substitutions, *Clinical Biochemist Reviews* **27** (2006), 63–75.
[13] P.S. Bernard, G.H. Pritham and C.T. Wittwer, Color multiplexing hybridization probes using the apolipoprotein E locus as a model system for genotyping, *Analytical Biochemistry* **278** (1999), 221–228.
[14] C.T. Wittwer, K.M. Ririe, R.V. Andrew, D.A. David, R.A. Gundry and U.J. Balis UJ, The LightCycler^{TM}: A microvolume multisample fluorimeter with rapid temperature control, *BioTechniques* **22** (1997), 176–181.
[15] CLASP (Collaborative Low-dose Aspirin Study in Pregnancy) Collaborative Group, CLASP: a randomized trial of low-dose aspirin for the prevention and treatment of preeclampsia among 9364 pregnant women, *Lancet* **343** (1994), 893–901.
[16] H.K. Vefring, L. Wee, A.Jugessur, H.K. Gjessing, S.T.Nilsen and R.T. Lie, Maternal anigitensinogen (*AGT*) haplotypes, fetal rennin (*REN*) haplotypes and risk of preeclampsia; estimation of gene-gene interaction from family-triad data, accepted in *BMC Medical genetics* **11** (2010).
[17] J.C. Barrett, B. Fry, J. Maller and M.J. Daly, Haploview: analysis and visualization of LD and haplotype maps, *Bioinformatics* **21** (2005), 263–265.
[18] C.T. Wittwer, M.G. herrmann, C.N. Gundry and K.S.J. Elentitoba-Johnson, Real-time multiplex PCR assays, *Methods* **25** (2001), 430–442.
[19] R.L. Margraf, R. Mao and C.T. Wittwer, Masking selected sequences variation by incorporating mismatches into melting analysis probes, *Human mutation* **27** (2006), 269–278.
[20] N. von Ahsen, E. Schütz, V.W. Armstrong and M. Oellerich, Rapid detection of prothrombotic mutations of prothrombin (G20210A), factor V (G1691A), and methylenetetrahydrofolate reductase (C677) by real-time fluorescence PCR with the LightCycler, *Clinical Chemistry* **45** (1999), 694–696.
[21] V. Bach, M.J. Barceló, A. Altés, A. Remacha, J. Félez and J. Baiget, Genotyping the HFE gene by melting point analysis with the LightCycler system: *Pros and Cons, Blood Cells, Molecules, and Diseases* **36** (2006), 288–291.
[22] G. Bodlaj, M. Stöcher, P. Hufnagl, R. Hubmann, G. Biesenbach, H. Stekel and J. Berg, Genotyping of the lactase-phlorizin hydrolase -13910 polymorphism by LightCycler PCR and implications for the diagnosis of lactose intolerance, *Clinical Chemistry* **52** (2006), 148–151.
[23] C.G Tag, M.C. Schifflers, M. Mohnen, A.M. Gressner and R.Weiskirchen, A novel proximal -13914G > A base replacement in the vicinity of the common-13910T/C lactase gene variation results in an atypical LightCycler melting curve in testing with the MutaREAL Lactase test, *Clinical Chemistry* **53** (2007), 146–148.
[24] M. Wylenzek, C. Geisen, L. Stapenhorst, K. Wielckens and K.R. Klingler, A novel point mutation in the 3' region of the prothrombin gene at position 20221 in a Lebanese/Syrian family, *Thrombosis and haemostasis* **85** (2001), 943–944.
[25] I. Warshawsky, C. Hren, L. Sercia, B. Shadrach, S.R. Deitcher, E. Newton and K. Kottke-Marchant, Detection of a novel point mutation of the prothrombin gene at position 20209, *Diagnostic Molecular Pathology* **11** (2002), 152–156.
[26] C.G. Tag, M.C. Schifflers, M. Mohnen, A.M. Gressner and R. Weiskirchen, Atypical melting curve resulting from genetic variation in the 3' untranslated region at position 20218 in the prothrombin gene analyzed with the LightCycler factor II

(prothrombin) G20210A assay, *Clinical Chemistry* **51** (2005), 1560–1561.

[27] E. Lyon, A. Millson, T. Phan and C.T. Wittwer, Detection and Identification of Base Alterations Within the Region of Factor V Leiden by Fluorescent Melting Curves, *Molecular Diagnosis* **3** (1998), 203–209.

[28] C.G. Tag, C. Oberkanins, G. Kriegshauser, C.J. Ingram, D.M. Swallow, A.M. Gressner, M. Ledochowski and R. Weiskirchen, Evaluation of a novel reverse-hybridization StripAssay for typing DNA variants useful in diagnosis of adult-type hypolactasia, *Clinica Chimica Acta* **392** (2008), 58–62.