

Improved Real-Time Multiplex Polymerase Chain Reaction Detection of Methylene tetrahydrofolate Reductase (*MTHFR*) 677C>T and 1298A>C Polymorphisms Using Nearest Neighbor Model-Based Probe Design

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The disorders of folate metabolism caused by methylenetetrahydrofolate reductase (*MTHFR*) gene polymorphisms may lead to several disease states including coronary heart disease, venous thrombosis, and several types of cancer. We have developed a real-time multiplex single-tube polymerase chain reaction procedure on the LightCycler for the detection of the two most commonly occurring variants, 677C>T and 1298A>C, in the *MTHFR* gene. An improved probe design, based on the nearest neighbor model for nucleic acid-probe duplex stability, resulted in a better separation ($\Delta T_m \sim 10^\circ\text{C}$) of melting peaks of the wild-type and mutant alleles than that by the existing method ($\Delta T_m \sim 3^\circ\text{C}$) for specimens heterozygous for the 1298A>C polymorphism. Of the 333 blood specimens analyzed by this procedure, we did not find any samples that gave ambiguous results. The specimens with homozygous mutation for one polymorphism were of the wild type for the other variant. The assay was validated by the comparison of the genotyping results of 50 blood specimens from the LightCycler polymerase chain reaction with the conventional restriction fragment length polymorphism procedures. There was 100% concordance of the test results obtained by the two techniques. This assay is reliable, economical, and can be performed by less trained technologists compared with the procedure performed by the conventional restriction fragment length polymorphism technique. (*J Mol Diagn* 2007, 9:345–350; DOI: 10.2353/jmoldx.2007.060035)

More than a dozen single nucleotide polymorphisms (SNPs) have been reported in the human methylenetetrahydrofolate reductase (*MTHFR*) gene, but the two most commonly occurring variants, 677C>T (refSNP ID: rs1801133, A222V) and 1298A>C (refSNP ID: rs1801131, E429A), have been linked to the disorders of folate metabolism. Mild hyperhomocysteinemia, caused by a reduction in the enzymatic activity of *MTHFR* as a result of these polymorphisms,^{1–10} may lead to arterial thrombosis, atherosclerosis, and coronary heart disease. These genetic defects have been linked to several types of cancer, namely, colorectal,¹¹ gastric,¹² esophageal,¹³ and cervical.¹⁴ Furthermore, 677C>T and 1298A>C polymorphisms have been reported to be associated with adverse drug interactions in patients treated with methotrexate, the most widely used drug for the treatment of rheumatoid arthritis¹⁵ and for the prevention of graft-versus-host disease.¹⁶ Hyperhomocysteinemia, caused by the 677C>T variant, has also been reported to increase neural tube defects.¹⁰ It has been shown that individuals with the homozygous mutation, 677TT or 1298CC, or compound heterozygotes are at a higher risk of folate metabolism disorders.^{8–10}

Several polymerase chain reaction (PCR)-based methods have been used for the detection of genetic abnormalities. The original protocols used restriction fragment length polymorphisms (RFLP) in which PCR products are digested with restriction enzymes followed by gel electrophoresis. Enzymes *HinfI* and *MboII* have been used for the detection of 677C>T⁶ and 1298A>C¹⁰ SNPs, respectively. The latter enzymatic digestion method suffers a drawback of specificity, and it has been reported^{8,9} that a silent mutation 1317T>C creates a restriction pattern almost identical

Accepted for publication January 11, 2007.

An algorithm for hybridization probe selection is patented to Drs. Ekkehard Schütz (Chronix Biomedical, Inc., Göttingen, Germany) and N.v.A. (USP no. 6,475,737).

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Table 1. Oligonucleotide Primers and Probes

Name	Sequence (5' to 3')	Source (reference)
Primer 677F	5'-CGAAGCAGGGAGCTTTGAGGCTG-3'	20
Primer 677R	5'-AGGACGGTGCGGTGAGAGTG-3'	6
Primer 1298F	5'-CTTTGGGGAGCTGAAGGACTACTAC-3'	10
Primer 1298R	5'-CACTTTGTGACCATTCCGGTTTG-3'	10
Probe 677-HP1	5'-TGACCTGAAGCACTGAAGGAGAAGGTGTC-3'-FLU	20
Probe 677-HP2	LCRed640-5'-CGGGAGCCGATTCATCAT-3'-PHO	20
Probe 1298-HP1	5'-GTGAAGCAAGTGTCTTTGAA-3'-FLU	This study
Probe 1298-HP2	LCRed705-5'-TCTTTGTTCTTTACCTCTCGGGAGAACC-3'-PHO	This study
Probe FLU1298	5'-AAGGAGGAGCTGCTGAAGATGTGGGGGAGGAGCT-3'-FLU	18
Probe LC1298	LCRed705-5'-ACCAGTGAAGAAAGTGTCTTTGA-3'-PHO	18

GenBank accession no. U09806.
 FLU, fluorescein; PHO, phosphate.

to that created by the variant 1298A>C. Digestion of PCR products, amplified with another set of primers with enzyme *Fnu4HI*, has been recommended⁸ to distinguish between the two possibilities. The recently reported¹⁷ multiplex PCR assay for the detection of 677C>T and 1298A>C polymorphisms in the *MTHFR* gene used RFLP technique similar to that used earlier.^{6,10} In addition to the time-consuming multiple steps after PCR in the above procedures, there may be technical problems caused by partial digestion with restriction enzymes that may make it difficult to interpret electrophoretic patterns.

The advent of the real-time PCR technology has enabled the detection of amplification products after each PCR cycle so that the time-consuming manipulations after PCR used in the conventional RFLP procedures could be eliminated. In the LightCycler (Roche, Indianapolis, IN) PCR used in this study, two fluorescently labeled oligonucleotide probes, one of which spans the mutation site (sensor or mutation probe), hybridize in tandem to an internal sequence of the amplified fragment resulting in fluorescence resonance energy transfer between the two fluorophores. In the successive PCR cycles, accumulation of the target sequence results in an increased fluorescent energy that is measured by the instrument. When the temperature of the reaction mixture is slowly increased, the sensor probe melts off and the two fluorescent dyes are no longer in close proximity, resulting in a decrease in fluorescence. The temperature at which the probe dissociates depends on whether a mismatch is present, with a mismatch yielding a lower melting temperature (T_m).

To our knowledge, only one report¹⁸ has described a multiplex LightCycler PCR for the simultaneous detection of 677C>T and 1298A>C polymorphisms in the *MTHFR* gene. However, when we tried to set up the assay in our laboratory using their protocol, a considerable overlap was observed in the melting peaks of the wild-type and homozygous mutant alleles for heterozygous specimens. This difficulty prompted the present study, and by redesigning hybridization probes, we were able to achieve an excellent separation of melting peaks that enabled a positive and reliable determination of the genotypes.

Materials and Methods

DNA Extraction

Genomic DNA was extracted from 200 μ l of peripheral blood by QIAamp DNA mini blood kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions except for the final elution volume (100 μ l instead of 200 μ l) with PCR-grade water prewarmed at 60°C. DNA concentrations were not routinely measured.^{19,20} This study was approved by the institutional review board at the Georgetown University.

PCR Primers and Probes

Oligonucleotide primers and hybridization probes were obtained from Sigma-Proligo (St. Louis, MO). The sequences of oligonucleotides are summarized in Table 1. The probes were designed using the MeltCalc software (MeltCalc Software, Göttingen, Germany).^{21,22} The software uses the nearest neighbor model and an algorithm to select probes that are most destabilized by the polymorphism of interest.

Real-Time PCR Protocol

PCR reactions were performed in a final volume of 10 μ l containing 1 \times LightCycler master mix hybridization probes from Roche Diagnostics, magnesium chloride (3.5 mmol/L), 677-primer mix (677F/677R, 0.5 μ mol/L of each), 1298-primer mix (1298F/1298R, 0.5 μ mol/L of each), 677-probe mix (677-HP1/677HP-2, 0.2 μ mol/L each), and 1298-probe mix (1298-HP1/1298-HP2, 0.2 μ mol/L each). Water (2 μ l; Roche) was added to make up the volume to 8 μ l per assay. Template DNA (2 μ l, concentration as eluted from the extraction columns, see above) was pipetted into LightCycler capillaries (Roche) containing 8 μ l of master mix. Each test run included a reagent blank, in which template DNA was replaced with PCR-grade water. Capillaries were capped, centrifuged briefly at 1000 \times g and placed into the LightCycler instrument (maximum 32 capillaries per run) with the software version 3.5. The amplification protocol was as fol-

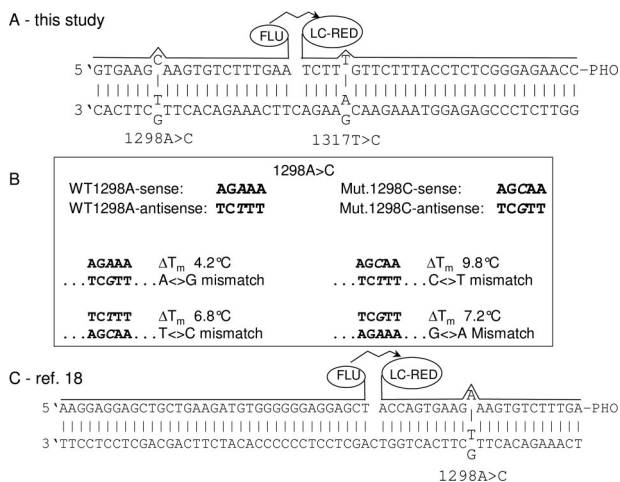


Figure 1. Probe hybridization schemes. **A:** *MTHFR* 1298A>C detection (this study) using the probe of the mutant sense strand sequence. The position of the rare *MTHFR* 1317T>C mutation is also shown. **B** (boxed): All four possibilities to detect the *MTHFR* 1298A>C polymorphism with hybridization probes are presented. The resulting mismatch and ΔT_m for the top ranking probe of each kind chosen by the MeltCalc software are also given. **C:** *MTHFR* 1298A>C detection¹⁸ using a probe of the wild-type sense strand sequence. The rare *MTHFR* 1317T>C is downstream of the probe position.

lows: one cycle of 30 seconds at 95°C followed by 45 cycles consisting of denaturation at 95°C for 0 seconds, annealing at 52°C for 10 seconds, and extension at 72°C for 10 seconds at a transition rate of 20°C/second. The emitted fluorescence was measured at the end of the annealing phase in each cycle. The amplification cycles were followed by a melting cycle, in which DNA was denatured at 95°C for 0 seconds, cooled to 38°C using a rate of 20°C/second, and held for 60 seconds. The temperature was then raised to 72°C with a ramp rate of 0.1°C/second during which the decline in fluorescence was continuously monitored. All analyses were performed with color compensation using reagents from Roche Diagnostics. The test results from the LightCycler multiplex PCR for both polymorphisms were confirmed by RFLP/PCR using restriction enzymes *HinfI* and *MbolI* (both from New England BioLabs, Boston, MA) as described previously.^{6,10}

Results

The process of the selection of hybridization probes for the detection of 1298A>C polymorphism is illustrated in Figure 1, A–C. The thermodynamic calculations based on the nearest neighbor model,²² showed that the probe 1298-HP2 when hybridized to the wild-type anti-sense strand (Figure 1A), resulted in a shift (ΔT_m) of 9.8°C between the melting peaks of the duplexes formed with the wild-type and mutant alleles. The values of the calculated ΔT_m , based on the other sequences considered, ranged from 4.2 to 7.2°C (Figure 1B). The probes FLU1298 and LC1298 (Figure 1C, Table 1) used earlier,¹⁸ corresponded to a ΔT_m of 3.1°C (Table 2). We also calculated the melting temperatures of the probe-target duplexes for the 1317T>C polymorphism (Figure 1A). The T_m values for the wild-type and the mutant alleles were 68.0°C and 65.5°C, respectively.

The melting-curve analyses of the PCR products, after color compensation for the crosstalk caused by the simultaneous presence of the hybridization probes labeled with LCRed 640 and LCRed 705 in the same tube, showed that the melting peaks of heterozygous mutations for both polymorphisms were composites of the wild-type and mutant allele peaks, separated by ~8°C for 677C>T (Figure 2A) and ~10°C for 1298A>C (Figure 2B) polymorphisms. Melting peaks, generated by the hybridization probes used previously¹⁸ (Figure 2C) were separated by ~3°C, and there was a considerable overlap between the homozygous wild-type and homozygous mutant alleles. The values of the melting temperatures, observed and predicted from the nearest neighbor calculations,^{21,22} are summarized in Table 2.

Analysis of 50 random specimens by RFLP showed 100% concordance between the results obtained by the two techniques for both polymorphisms (data not shown). Although there was a possibility of generating false-positive results⁸ in the detection of 1298A>C polymorphism by the RFLP, we did not observe this difficulty in our patient population. The distributions of the *MTHFR* genotypes, determined by the multiplex LightCycler PCR procedure in 333 blood specimens are summarized in Table 3. Among the heterozygotes, 24 patients (7.2%) were

Table 2. Probe Characteristics: Comparison between New Genotyping Probes and the Old Probe Set¹⁸

Detection probe: hybridization to the sequence of <i>MTHFR</i> polymorphic alleles	T_m (SD, °C) Observed	T_m (°C) Predicted†	ΔT_m (°C) Observed	ΔT_m (°C) Predicted	Figure
677-HP2: wild type (677CC)	62.0 (0.2)	61.9			2A
677-HP2: mutant (677TT)	53.9 (0.2)	51.6	8.1	10.3	2A
1298-HP1: wild type (1298AA)	48.0 (0.3)	48.8			2B
1298-HP1: mutant (1298CC)	58.4 (0.3)	58.6	10.4	9.8	2B
LC1298: wild type (1298AA)	63.1*	62.1			2C
LC1298: mutant (1298CC)	60.2*	59.0	2.9	3.1	2C

T_m and SD values are mean of 10 test runs on different days. The sequences of the probes are given in Table 1.

*Sequences of probes used were from Nakamura and colleagues¹⁸; values of SD are not given because of the small number of samples analyzed.

†Nearest-neighbor calculations were performed using the MeltCalc software^{21,22} parameterized with 250 mmol/L sodium equivalents and 0.2 μ mol/L hybridization probe concentration.

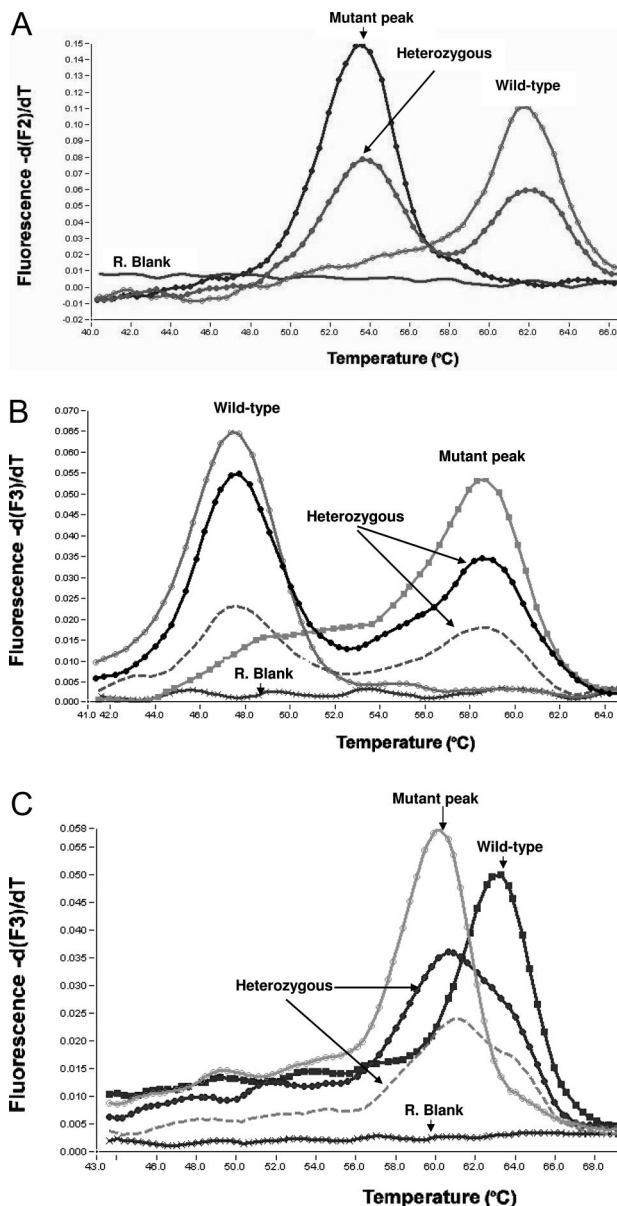


Figure 2. **A:** Melting-curve analysis for the determination of 677C>T polymorphism; primers and probes used: 677F, 677R, 677-HP1, and 677-HP2. **B:** Melting-curve analysis for the determination of 1298A>C polymorphism; primers and probes used: 1298F, 1298R, 1298-HP1, and 1298-HP2. **C:** Melting-curve analysis for the determination of 1298A>C polymorphism; primers and probes used: 1298F, 1298R, FLU1298, and LC1298.

heterozygous for both polymorphisms. We did not find any specimens that had a homozygous mutation for both polymorphisms. Specimens with a homozygous mutation for one polymorphism were of the wild type for the other variant.

Discussion

In this LightCycler multiplex PCR, two regions spanning the nucleotides 677 and 1298 of the *MTHFR* gene were amplified simultaneously by using two sets of primers in one tube. The sequence of the mutant sense strand for the detection probe for 1298A>C polymorphism, when hybridized to the wild-type anti-sense strand (Figure 1A), resulted in a greater destabilizing C<>T mismatch (ΔT_m 9.8°C) as compared with the other sequences resulting in A<>G, T<>C, and G<>A mismatches shown in Figure 1B. The most stable mismatch (A<>G) resulted (Table 2) when the sequence of the forward wild-type strand (Figure 1C) was chosen for the probe as was done earlier.¹⁸ Such mismatches cause a considerable overlap between the melting peaks of the wild-type and mutant alleles (Figure 2C) and are unsuitable for genotyping assays. A close agreement between the observed and the predicted melting temperatures (Table 2) showed that the theoretical model is reliable in the selection of the optimal choice of sequences of the hybridization probes that accounted for an improved resolution of the melting peaks (Figure 2B) in the present study.

The perfect agreement of the genotyping results of 50 random samples by the multiplex LightCycler PCR and conventional RFLP procedures showed that the real-time PCR technique with fluorescence resonance energy transfer probes could be used with confidence for the determination of the *MTHFR* gene polymorphisms. Whenever restriction enzymes or hybridization probes are used to identify a particular sequence change, there is always some risk of other sequence alterations occurring at the recognition site that may misidentify polymorphisms as mutations that are not related to disease states (false positives).^{8,23,24} In the LightCycler PCR assays, different mismatches between the target and detection probe sequences would destabilize the target-probe duplex to different extents, depending on the nearest neighbor environment and the position of the mutation site relative to the probe.²² Thus, melting-curve analysis of PCR products can detect other gene variants within the region of the probe-binding site that may not be detected by the traditional RFLP/PCR protocols.^{23,24} The National Center for Biotechnology Information reference SNP cluster report (<http://www.ncbi.nlm.nih.gov/projects/SNP/>) demonstrated the presence of *MTHFR* 1317T>C SNP in African and Hispanic populations. It seems to be absent in a population of European descent. This silent genetic variant also creates an *MbolI* restriction site with an electrophoretic pattern almost identical to *MTHFR* 1298A>C genotyping⁸ that can lead to erroneous hybridization probe-based genotyping if it significantly affects the

Table 3. Distribution of *MTHFR* Genotypes in 333 Blood Specimens

Wild type	Heterozygotes	Homozygotes	Wild type	Heterozygotes	Homozygotes
677CC	677CT	677TT	1298AA	1298AC	1298CC
184	124	25	180	129	24
(55.2%)	(37.2%)	(7.5%)	(54.0%)	(38.7%)	(7.2%)

binding of the probes. The thermodynamic calculations allowed us to conclude that this variant would not affect our assay because the anchor probe will only be destabilized from the calculated T_m of 68.0 to 65.5°C. This provided an adequate margin to ensure that the anchor probe binding occurred during the sense probe melting because it has a calculated T_m of 58.6°C (Table 2). However, the results from both techniques, especially RFLP, should be interpreted with caution to eliminate erroneous test results that may cause a misdiagnosis of patients.

LightCycler PCR results of 333 blood specimens showed that individuals with a homozygous mutation for one of the above polymorphisms had the wild type for the other variant. This is supported by previous studies,^{10,25} which indicated that the 677C>T and 1298A>C mutations occur in *trans* positions in the *MTHFR* gene, although in rare cases the genotype combinations 677TT/1298AC and 677CT/1298CC have been reported.^{8,26}

In multiplex PCR methods, more than one target is amplified in one tube, which allows the performance of several assays simultaneously in a single PCR run. These assays are economical and faster than conventional single PCR assays and are highly desirable for use in clinical laboratories.¹⁹ The development of real-time PCR techniques has made these assays even more valuable because the results of multiple assays can be monitored simultaneously in multichannel instruments.

Although genotyping by RFLP is reliable, the procedure is time-consuming because of numerous manual steps with an increased risk of errors in every step. Interpretation of results on gels may sometimes be impaired by technical problems, such as incomplete digestion of PCR products with restriction enzymes and the occurrence of nonspecific electrophoretic bands. Furthermore, this technique is generally unsuitable for multiplex PCR assays because of the complex or interfering electrophoretic patterns of more than one restriction enzyme used in the same digestion mixture. In the LightCycler method, amplification and genotyping by melting-curve analysis are performed in one sealed capillary tube eliminating the need for restriction enzyme digestion and gel electrophoresis. The risk of technologist-related problems, including the risk of contamination, is considerably reduced and reliable test results can be obtained. The capillary reaction vessels used in the LightCycler are made of plastic and glass and can break during insertion into the sample carousel. To overcome this difficulty, the capillaries were inserted into the sample carousel and then the master mix and DNA samples were added with the subsequent centrifugation step. Because of the high surface-to-volume ratio, heat transfer is rapid, and the delay during the temperature change (increase from 72 to 94°C) is sufficient for denaturation. Only 10 seconds are required for annealing and extension steps in the LightCycler PCR cycles. Analysis of 20 blood specimens by LightCycler PCR can be accomplished in ~1 hour compared with 6 hours required by the conventional RFLP technique. This leads to a substantial reduction in the turn-around time. In a detailed cost analysis including reagent costs, consumables, and labor, the LightCycler

PCR compares favorably to the RFLP procedure because the high reagent costs are offset by the savings in the technician time.

Conclusions

The newly developed multiplex LightCycler PCR procedure, for the genotyping of 677C>T and 1298A>C *MTHFR* polymorphisms is rapid, reliable, and economical. Because it is a primarily automated assay, less hands-on time is required compared with the conventional RFLP assays. Our findings emphasize that the use of a well-discriminating detection probe (high ΔT_m) is essential for the design of robust assays. Nearest neighbor calculations facilitate the detection of the possible influence of other polymorphisms in the probe hybridization region on the assay performance.

Acknowledgments

We thank Sandra Hartung for excellent technical assistance and Dr. Victor W. Armstrong for his assistance in revising the manuscript.

References

- Keijzer MB, den Heijer M, Blom HJ, Bos GM, Willems HP, Gerrits WB, Rosendaal FR: Interaction between mutated *MTHFR* and inherited thrombophilic factors in recurrent venous thrombosis. *Thromb Haemost* 2002, 88:723–728
- Den Heijer M, Lewington S, Clarke R: Homocysteine, *MTHFR* and risk of venous thrombosis: a meta-analysis of published epidemiological studies. *J Thromb Haemost* 2005, 3:292–299
- Frederiksen J, Juul K, Grande P, Jensen GB, Schroeder TV, Tybjaerg-Hansen, Nordestgaard BG: Methylene tetrahydrofolate reductase polymorphism (C677T), hyperhomocysteinemia, and risk of ischemic cardiovascular disease and venous thromboembolism: prospective and case-control studies from the Copenhagen City Heart Study. *Blood* 2004, 104:3046–3051
- Abu-Amero KK, Wyngaard CA, Dzimirri ND: Prevalence and role of methylenetetrahydrofolate reductase 677C→T and 1298A→C polymorphisms in coronary artery disease in Arabs. *Arch Pathol Lab Med* 2003, 127:1349–1352
- Wald DS, Law M, Morris JK: Homocysteine and cardiovascular disease: evidence on causality from a meta-analysis. *BMJ* 2002, 325:1202–1206
- Frosst P, Blom HJ, Milos R, Goyette P, Sheppard CA, Mathews RG, Boers GJH, den Heijer M, Kluijtmans LAJ, van den Heuvel LP, Rozen R: A candidate genetic risk factor for vascular disease: a common mutation in methylenetetrahydrofolate reductase. *Nat Genet* 1995, 10:111–113
- Goyette P, Christensen B, Rosenblatt DS, Rozen R: Severe and mild mutations in cis for *MTHFR* gene, and description of five novel mutations in *MTHFR*. *Am J Hum Genet* 1996, 59:1268–1275
- Weisberg IS, Tran P, Christensen B, Sibani S, Rozen RA: Second genetic polymorphism in *MTHFR* associated with decreased enzyme activity. *Mol Genet Metab* 1998, 64:169–172
- Weisberg IS, Jacques PF, Selhub J, Bostom AG, Chen Z, Ellison RC, Eckfeldt JH, Rozen R: The 1298A>C polymorphism in *MTHFR*: in vitro expression and association with homocysteine. *Atherosclerosis* 2001, 156:409–415
- van der Put NM, Gabreels F, Stevens EM, Smeitink JA, Trijbels FJ, Eskes TK, van den Heuvel LP, Blom HJ: A second mutation in the *MTHFR* gene: an additional risk factor for neural-tube defects? *Am J Hum Genet* 1998, 62:1044–1051
- Slattery ML, Potter JD, Samowitz W, Schaffer D, Leppert M: Methyl-

- enetetrahydrofolate reductase, diet, and risk of colon cancer. *Cancer Epidemiol Biomarkers Prev* 1999, 8:513–518
12. Shen H, Zheng Y, Qien RY, Qin Y, Wang X, Spitz MR, Wei Q: Polymorphism of the 5,10 methylenetetrahydrofolate reductase and risk of gastric cancer in a Chinese population: a case control study. *Int J Cancer* 2001, 95:332–336
 13. Song CY, Xing DY, Tan W, Wei QY, Lin DX: Methylenetetrahydrofolate reductase polymorphisms increase risk of esophageal squamous cell carcinoma in a Chinese population. *Cancer Res* 2001, 61:3272–3275
 14. Piyathilake CJ, Macaluso M, Johanning GL, Whiteside M, Heimbürger DC, Giuliano A: Methylenetetrahydrofolate reductase polymorphism increases the risk of cervical intraepithelial neoplasia. *Anticancer Res* 2000, 20:1751–1757
 15. Evans WE: Differing effects of MTHFR single nucleotide polymorphisms on methotrexate efficacy and toxicity in rheumatoid arthritis. *Pharmacogenetics* 2002, 12:181–182
 16. Ulrich CM, Yasui Y, Storb R, Schubert MM, Wagner JL, Bigler J, Ariail KS, Keener CL, Li S, Liu H, Frain FM, Potter JD: Pharmacogenetics of methotrexate: toxicity among marrow transplantation patients varies with the MTHFR C677T polymorphism. *Blood* 2001, 98:231–234
 17. Yi P, Pogribny IP, James SJ: Multiplex PCR for simultaneous detection of C677T and A1298C polymorphisms in MTHFR gene for population studies of cancer risk. *Cancer Lett* 2002, 181:209–213
 18. Nakamura S, Aoshima T, Ikeda M, Sekido Y, Shimokata K, Niwa T: Simultaneous detection of MTHFR gene polymorphisms, C677T and A1298C, by melting curve analysis with LightCycler. *Anal Biochem* 2002, 306:340–343
 19. von Ahsen N, Oellerich M, Schutz E: Use of two reporter dyes without interference in a single-tube rapid-cycle PCR: α_1 -antitrypsin genotyping by multiplex real-time fluorescence PCR with the LightCycler. *Clin Chem* 2000, 46:156–161
 20. Aslanidis C, Schmitz G: High-speed methylenetetrahydrofolate reductase C→T 677 mutation detection on the LightCycler. *Rapid Cycle Real-Time PCR, Methods and Applications*. Edited by S Meuer, C Wittwer, K Nakagawara. New York, Springer, 2001, pp 83–89
 21. Schütz E, von Ahsen N: Spreadsheet software for thermodynamic melting point prediction of oligonucleotide hybridization with and without mismatches. *BioTechniques* 1999, 27:1218–1224
 22. von Ahsen N, Oellerich M, Armstrong VW, Schutz E: Application of a thermodynamic nearest-neighbor model to estimate nucleic acid stability and optimize probe design: prediction of melting points of different mutations of apolipoprotein B 3500 and factor V with hybridization probe genotyping assay on the LightCycler. *Clin Chem* 1999, 45:2094–2101
 23. Mahadevan MS, Benson PV: Factor V null mutation affecting the Roche LightCycler factor V Leiden assay. *Clin Chem* 2005, 51:1533–1535
 24. Lyon E: Discovering rare variants by use of melting temperature shifts seen in melting curve analysis. *Clin Chem* 2005, 51:1331–1332
 25. Meisel C, Cascorbi I, Gerloff T, Stangl V, Laule M, Müller JM, Wernecke KD, Baumann G, Roots I, Stangl K: Identification of six methylenetetrahydrofolate reductase (MTHFR) genotypes resulting from common polymorphisms: impact on plasma homocysteine levels and development of coronary artery disease. *Atherosclerosis* 2001, 154:651–658
 26. Hanson NQ, Aras O, Yang F, Tsai MY: C677T and A1298C polymorphisms of the methylenetetrahydrofolate reductase gene: incidence and effect of combined genotypes on plasma fasting and post-methionine load homocysteine in vascular disease. *Clin Chem* 2001, 47:661–666