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Simultaneous determination of methylenetetrahydrofolate reductase C677T and factor V G1691A genotypes by mutagenically separated PCR and multiple-injection capillary electrophoresis

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The C677T mutation of the methylenetetrahydrofolate reductase gene and the G1691A (Leiden) mutation of the factor V gene are established risk factors for thromboembolic disease. We here present an assay for the simultaneous genotyping of these common genetic variants. The assay involves a strategy based on multiplex mutagenically separated PCR performed in a single tube containing six primers. Separation of the resulting four PCR products (197, 207, 233, and 246 bp) was performed by capillary electrophoresis coupled with laser-induced fluorescence detection. The time for the automated electrophoresis was reduced to 2.5 min per sample by performing the capillary electrophoresis analysis in a multiple-injection mode.

The G1691A (Leiden) mutation in the factor V gene (J05368) is probably the most common genetic predisposition to venous thrombosis (allele frequency of 2–8% in Caucasians) *[1, 2]*, conferring a sevenfold increased risk in heterozygous individuals *[3]*, and has recently been identified as a strong risk factor for myocardial infarction in young women *[4]* .

The C677T mutation in methylenetetrahydrofolate reductase (MTHFR, U09806) is an even more common polymorphism with an allele frequency of 30–40% in most populations *[5, 6]*.[1] This mutation predisposes to a high concentration of total homocysteine (tHcy) in folate-deficient subjects *[7]*. Increased tHcy is a strong and independent risk factor for occlusive vascular disease, including venous thrombosis *[8]*, and the C677T mutation has been identified as a cardiovascular risk factor in some *[9–12]* but not all *[13]* study populations.

Thus, both the C677T MTHFR and G1691A factor V mutations are common genetic conditions associated with increased risk of occlusive vascular disease, and two reports suggest that these factors may interact *[14, 15]*. Notably, a recent study demonstrates that healthy men with coexisting G1691A factor V mutation and hyperhomocysteinemia are at a particularly high risk of developing venous thromboembolism *[16]*. Therefore, a rapid assay that includes both mutations may be useful in cardiovascular risk assessment.

Most published methods for determination of these genetic variants are based on gel electrophoresis of PCR products that have undergone postamplification modification. The MTHFR C677T mutation creates a *Hin*fI restriction site, and detection of the mutation is achieved by PCR followed by enzyme digestion and gel electrophoresis *[5]*. The factor V Leiden mutation has been detected by digestion of a PCR product with *Mnl*I restriction enzyme *[17]*, and, recently, with *Hin*dIII cleavage *[18]*, by PCR with sequence-specific primers (PCR-SSP) *[19, 20]*, or by oligonucleotide ligation *[21]*.

In the present work, we describe an assay for the simultaneous detection of MTHFR C677T, factor V G1691A, and the corresponding normal alleles. The construction of the assay combines three strategies: (*a*) A variant of PCR-SSP called mutagenically separated PCR (MS-PCR) makes it possible to determine biallelic polymorphisms in one-tube reactions *[22]*, and here we develop a multiplex version of this technique enabling the simultaneous analysis of several loci; (*b*) the allele-specific products from MS-PCR are then separated by capillary

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[1] Nonstandard abbreviations: MTHFR, methylenetetrahydrofolate reductase; tHcy, total homocysteine; SSP, sequence-specific primers; MS, mutagenically separated; and TBE, Tris–borate–EDTA buffer.

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electrophoresis and detected by laser-induced fluorescence, a separation technique characterized by high separation efficiency and sensitivity, small sample requirements, and automation *[23]*; and (*c*) a high assay throughput is obtained by using a multiple-injection mode for capillary electrophoresis recently developed in our laboratory *[24]* .

Materials and Methods

Reagents. The DNA Direct kit was obtained from Dynal. Taq polymerase (Supertaq) and reaction buffer were obtained from HT Biotechnology, dNTPs were from Pharmacia Biotech. PCR plates (96-well) were from Costar. The primers were designed with the computer program Oligo 4.0 (National Biosciences) and then synthesized by Eurogentec.

SYBR Green I (concentration not given) in dimethyl sulfoxide was purchased from Molecular Probes. Hydroxypropylmethylcellulose and other chemicals (analytical or molecular biology grade) were supplied by E. Merck and Sigma Chemical Co. Water, double distilled and purified on a MilliQ Plus Water Purification System (Millipore Corp.), was used for preparation of all aqueous solutions.

DNA extraction and PCR. DNA was extracted from whole blood with the DNA Direct kit from Dynal, following the instructions provided by the manufacturer. Five microliters of whole blood was mixed with 200 μL of magnetic particles suspended in a buffer that lysed the blood cells and allowed the DNA to adhere to the magnetic particles. The particles were washed twice and thereafter resuspended in 40 μL of TE buffer (10 mmol/L Tris-HCl, 1 mmol/L EDTA, pH 8.0). Five microliters of particle suspension was used for PCR.

The PCR reaction mixture contained 10 mmol/L Tris-HCl pH 9.0, 50 mmol/L KCl, 1.5 mmol/L $MgCl_2$, 0.1 g/L gelatin, 1 mL/L Triton X-100, 125 μmol/L each dNTP, ~0.2 μmol/L of each primer (see *Results and Discussion*), 0.8 U of Taq polymerase, and template DNA in a total volume of 50 μL. The reaction mix was overlaid with 20 μL of mineral oil. A Hybaid Omn E thermocycler was used for 32 cycles of denaturation at 94 °C for 30 s, annealing at 60 °C for 1 min, and extension at 72 °C for 30 s. The temperature cycles were preceded by 3 min at 94 °C and ended with 5 min at 72 °C.

Extraction of DNA templates from whole blood and addition of PCR solutions were carried out in 96-well plates by the robot workstation BioMek 2000 (Beckman Instruments).

The time required for purification of 96 samples was approximately 3 h, set-up of PCR 15 min, and PCR approximately 2 h.

Electrophoresis instrumentation. Capillary electrophoresis was performed on a Prince capillary electrophoresis system (Prince Technologies) equipped with an in-house-built laser-induced fluorescence detector. Laser excitation was at 488 nm (argon laser, 20 mW, from Uniphase), and the light was focused 30 μm below the end of the capillary placed in a rectangular sheath-flow cuvette. A sheath flow of 1× TBE buffer (89 mmol/L Tris–borate, pH 8.3, 1 mmol/L EDTA) was delivered at a rate of 30 μL/h. The emitted light was collected through a 535-nm band-pass filter 535DF35 from Omega Optical.

A 50-μm (i.d.) and 192-μm (o.d.) silica capillary (Polymicro Technologies) coated according to Hjertén *[25]* was used. The total capillary length (the distance between the column inlet and detector window) was 40 cm.

Caesar software (version 4.1) from Prince Technologies was used for data collection and processing. Electropherograms for preparation of Figures were transferred as text files to an Apple Macintosh computer and redrawn by Sigma Plot (version 4.16; Jandel Scientific).

Capillary electrophoresis. Electrophoresis buffer was 1× TBE. Separation buffer was electrophoresis buffer containing 5 g/L hydroxypropylmethylcellulose as sieving matrix and the dye SYBR Green I (1:20 000 final dilution of a stock solution).

Before each injection or series of injections in the multiple-injection mode, the capillary was flushed with 1× TBE for 30 s and then filled with separation buffer by applying a pressure of 200 kPa for 150 s. The PCR samples were diluted 1:20 in distilled water containing a PCR-amplified marker fragment (225 bp), then injected electrokinetically at −10 kV for 6 s, and separated at a field strength of 450 V/cm. Temperature was set at 20 °C.

Results and Discussion

Design of the PCR primers. In MS-PCR, both alleles of a biallelic polymorphism can be amplified in the same reaction tube *[22]*. A set of three primers are designed to bind at a given locus, a common primer to one DNA strand and two allele-specific primers of different sizes to the opposite strand. Thus, different amplicon sizes for the two alleles are produced. In a multiplex setting, the primer set assigned to each locus must generate unique PCR product lengths. The main technical challenge of such an assay is to construct compatible primers with adequate selectivity and similar amplification efficiencies for the various alleles.

The sequence and characteristics of each primer are shown in Table 1, and Fig. 1 outlines their position and the length of the resulting PCR products. We positioned the 3′-end of the allele-specific primers at the allele-determining base. A mismatch is therefore generated when this primer hybridizes to the other allele. The selectivity of a primer depends on the specific mismatch created *[26]*. To increase selectivity, an additional mismatch was introduced 2 or 3 bases 5′ to the 3′-end *[22, 27]*. A different base substitution was made for the two allele-specific primers in a set *[22]*. The actual mismatches are shown in Table 1. Equal distance from the second

Table 1. Characteristics and nomenclature of primers.

Gene	Mutation	Name of primer	Specificity of primer	Sequence (5′-3′)	T_m, °C	Length, bp
MTHFR	C677T	MTHFR-C	Wild type	CTCTCTCTCTGAAGGAGAAGGTGTCTGCGGTAGC (C A)	68.0	34
		MTHFR-T	Mutation	GAAGGAGAAGGTGTCTGCGGAAGT (C G)	68.0	24
		MTHFR-com	Common	AGGACGGTGCGGTGAGAGTG	71.9	20
Factor V	G1691A	Factor V-G	Wild type	AACAAGGACAAAATACCTGTATTCATC (G A)	61.0	27
		Factor V-A	Mutation	GTCTGTCTGTCTCTTCAAGGACAAAATACCTGTATTCTTT (G G)	62.8	40
		Factor V-com	Common	GGCAGGAACAACACCATGAT	66.6	20

Mismatched bases (including the 5′ length extensions) are underlined. The actual mismatches at the 3′-end are shown. T_m for the allele-specific primers are calculated for the length of bases from the second mismatch to the 5′-end, or, when present, to the length extension. Oligo 4.0 was used for calculations of T_ms at concentration of salt and nucleic acid of 80 mmol/L and 200 nmol/L respectively.

mismatch to the 3′-end was found to be important to obtain similar amplification efficiencies of the two allele-specific primers. Stability (T_m >60 °C) was obtained by adding 20–24 specific bases 5′ to the second mismatch, whereas sufficient difference in sizes of the PCR products was obtained by adding 10–13 nonspecific bases (at the 5′-end) to one of the two allele-specific primers. Stability computations were made with the primer selection software Oligo 4.0. One of the main selection criteria of this program is to avoid primers with a high hybridization stability in the 3′-end (i.e., the last five bases). Such primers have an increased tendency of initiating nonspecific synthesis. The hybridization stability of a small stretch of bases is highly dependent on the sequence. Thus, the deliberate base substitutions provided a means both to decrease the general hybridization stability of the 3′-end of the primers, and avoid primer sequences that interacted too strongly with the other primers.

Reaction conditions. Goodman reported that the relative amplification efficiency of different primers competing for the same position in DNA is not affected by the annealing temperature *[26]*. We therefore initially set and did not adjust the temperature and time for melting, annealing, and extension. We also selected and kept constant the concentrations of $MgCl_2$, dNTP, and enzyme, taking into account the final concentration, number, and size of the PCR products. The concentration of each primer was initially set at 0.2 μmol/L, and the subsequent adjustment was done by altering the primer ratios only.

The balancing of PCR product yield was done by a two-step procedure. First, the optimal molar ratio of the two allele-specific primers in a set, defined as giving equal signals for the two alleles when amplifying heterozygous DNA, was found in separate (three-primer) reactions. Then, a six-primer mix giving equally strong signals from the two genes was composed. This was obtained by adjusting the primer ratios between sets while maintaining the within-set ratios. The final primer concentrations producing balanced PCR products was 0.23 μmol/L for MTHFR-com, MTHFR-T, and factor V-G; 0.25 μmol/L for factor V-A; 0.20 μmol/L for factor V-com; and 0.18 μmol/L for MTHFR-C.

Identification of genotypes by capillary electrophoresis. Electropherograms of various genotype combinations are shown in Fig. 2. Before injection, the PCR samples were supplemented with a constant amount of a preamplified control fragment that migrated between the peaks of the two most common alleles, i.e., the MTHFR-C and factor V-G alleles. Thus, a sample with all four alleles contained five peaks eluting in the order MTHFR-T (197 bp), MTHFR-C (207 bp), control fragment (225 bp), factor V-G (233 bp), and factor V-A (246 bp). The size of the control fragment was adjusted so that it migrated closer to the factor V-G fragment. The resulting asymmetry in the elution profile guided the identification of the individual peaks. Moreover, the presence of the control fragment peak distinguished between failure of injection or detection (no

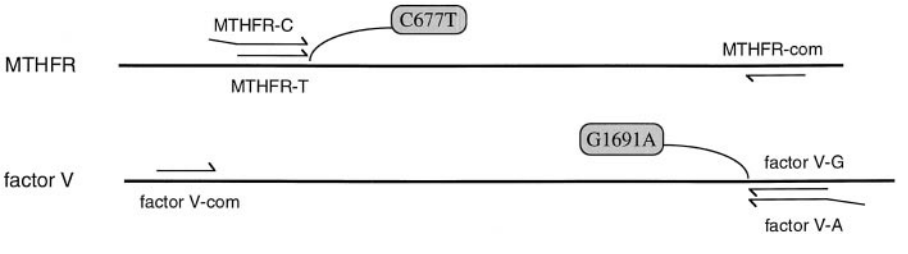

Fig. 1. Positions and directions of primers.
All lengths are drawn to scale.

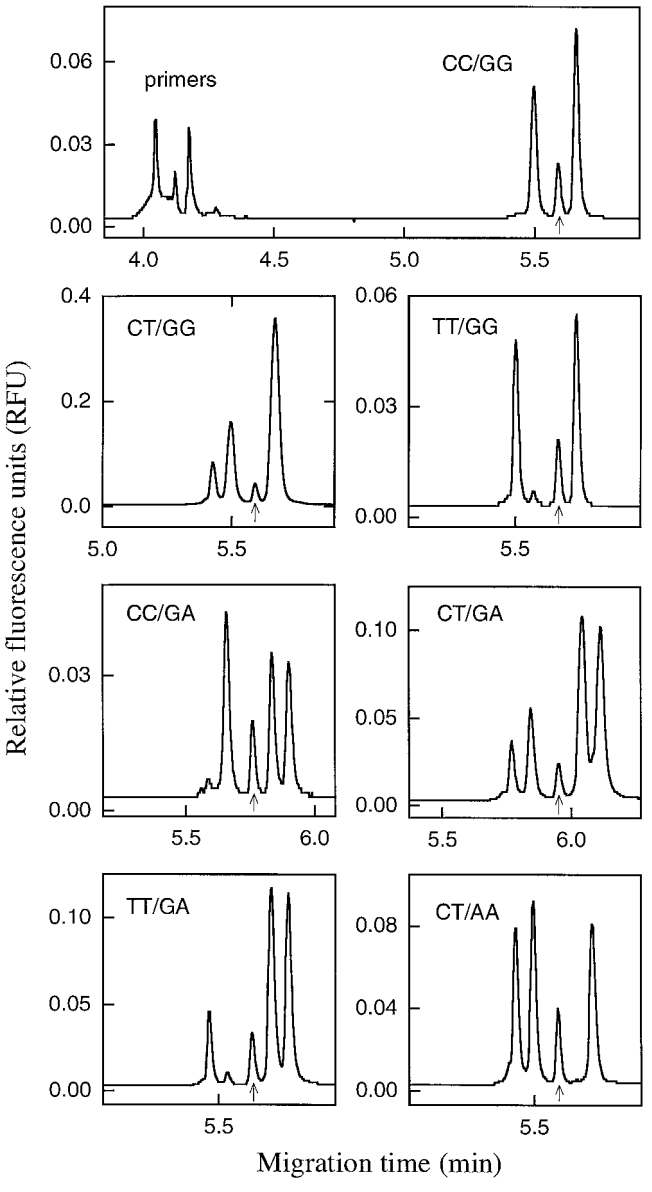

Fig. 2. Single-injection electropherograms of MS-PCR products.

Seven of the nine different possible genotypes are shown. The MTHFR genotypes are marked CC, CT, and TT, and the factor V genotypes GG, GA, and AA. The marker fragment is indicated with an *arrow*. Electrophoretic conditions were as described in *Materials and Methods*.

peaks) and failed PCR product formation (only control peak eluting).

Multiple-injection capillary electrophoresis. We adopted a multiple-injection format previously developed for the analysis by capillary electrophoresis of *Hin*fI-digested PCR products of the MTHFR gene *[24]*. The samples were injected electrokinetically at regular intervals without replacement of separation matrix. Optimal time intervals between injections were determined by using a mathematical algorithm *[24]* that includes the criteria of no interference between analytes from consecutive samples, and no injection concomitant with elution of analytes at the detector window.

Figure 3 demonstrates the electropherogram from a multiple-injection run. The time intervals between injections alternated between 0.7 and 4.0 min, with up to four samples migrating in the capillary simultaneously. The sieving matrix was replaced after each eight injections. Thus, this procedure greatly enhances sample throughput. It also prolongs column life, probably by reducing deterioration of capillary coating related to the pressure-driven replacement of separation matrix. The assay throughput was one sample each 3.1 min, which included time for replacement of the separation matrix.

Verification and application of the assay. We verified the assay by genotyping blood samples from 325 healthy subjects and 67 individuals with high concentrations of plasma tHcy, i.e., hyperhomocysteinemia (Table 2). The C677T mutation of MTHFR was previously determined in these samples by PCR followed by *Hin*fI digestion *[7]*. The distribution of MTHFR alleles were in complete accordance with the previously published data. The factor V component of the assay was verified by genotyping 23 blood samples (10 of the GA and 13 of the GG genotype) by an established technique based on PCR amplification and *Mnl*I digestion *[28]*. This was carried out blindly in the laboratory of B. Dahlbäck (Malmø, Sweden), and showed complete agreement with the MS-PCR method. Furthermore, the frequency of the heterozygous factor V AG genotype (Table 2) was in line with that published for other Caucasian populations *[2]* .

Assay characteristics and performance. The relative signal strengths of the two peaks from a heterozygous MTHFR or a heterozygous factor V sample were reproducible. However, some variability in the signals for the MTHFR alleles relative to that of the factor V alleles was observed. This was especially noticeable in a few samples with high template concentration. Under these conditions, the MTHFR signals tended to dominate. This was overcome by diluting the samples 1:50 in water, or by reducing the cycle number or enzyme concentration.

When analyzing the MTHFR TT genotype, a minor peak at the position of the C allele (<10% of the peak area of the T allele) often appeared (Figs. 2 and 3). The mismatches (T/G and A/C) at the 3′-end impose poor selectivity *[26]*; hence, the minor peak may be related to a slight priming of the T allele by the C primer. This was not observed for the factor V genotypes, possibly due to a more favorable base sequence context and (or) the shorter distance to the second mismatch (2 bp for factor V vs 3 bp for the MTHFR primers).

In conclusion, we have demonstrated the successful combination of a multiplex MS-PCR assay with a high-throughput automated capillary electrophoresis procedure for the simultaneous genotyping of two loci. The

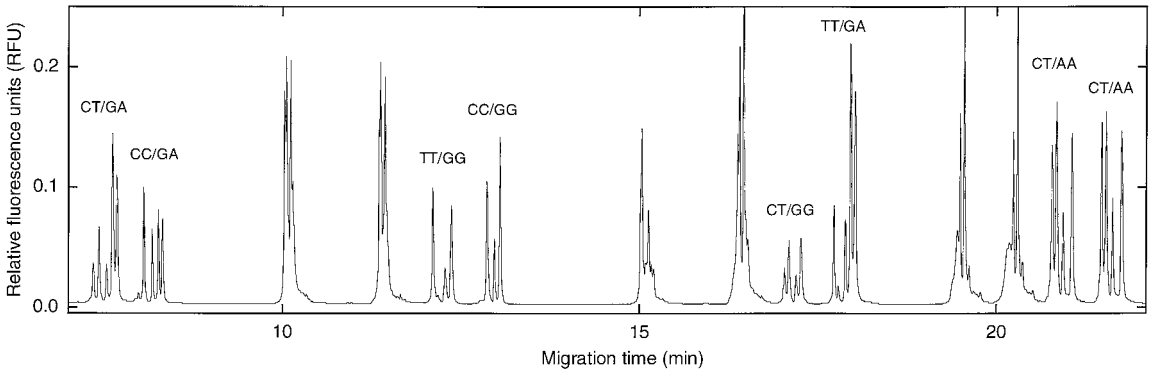

Fig. 3. Electropherogram of a multiple-injection run of eight samples.

The principles of multiple injection are described in the text. The peaks without a genotype designation are the primers of the different injections. Samples and electrophoretic conditions were the same as in Fig. 2.

Table 2. Frequencies of MTHFR and factor V genotypes in 325 healthy subjects and 67 subjects with hyperhomocysteinemia.

	Healthy subjects		Hyperhomocysteinemic subjects	
Genotype	**n**	**%**	**n**	**%**
MTHFR				
CC	170	52.3	5	7.5
CT	120	36.9	14	20.9
TT	35	10.8	48	71.6
Factor V				
GG	297	91.4	59	88.1
GA	28	8.6	8	11.9
AA	0	0.0	0	0.0

strategy for designing primers and adjustment of primer ratios may facilitate the inclusion of additional gene loci into the assay, the number of which may be limited by the ability to design compatible primers, the quantitative capacity of the PCR, or the resolving power of the capillary electrophoresis system.

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