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C677T mutation of methylenetetrahydrofolate reductase gene determined in blood or plasma by multiple-injection capillary electrophoresis and laser-induced fluorescence detection

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We constructed an assay to detect the common C677T mutation in the methylenetetrahydrofolate reductase gene. The mutation creates a *HinfI* recognition site detected by restriction cleavage of a 198-bp fragment amplified in the polymerase chain reaction (PCR). Digested samples were subjected to capillary electrophoresis with laser-induced fluorescence detection (CE-LIF), with hydroxypropylmethylcellulose as the sieving matrix and SYBR Green I as the fluorescent dye. After amplification but before digestion, we added to the PCR mixture a fragment with the *HinfI* recognition site and a 15-bp truncation at the 3' end. Using this procedure, we could (a) verify completeness of digestion and monitor injection, (b) assign genotypes on the basis of pattern recognition, and (c) develop a multiple-injection mode with simultaneous separation of as many as eight samples. A seminested PCR protocol in combination with CE-LIF allowed genotyping of plasma/serum samples 20 years old.

INDEXING TERMS: restriction enzyme analysis • genotyping • homocysteine • hyperhomocysteinemia • risk factors • premature atherosclerosis • cardiovascular disease • heritable disorders • folate deficiency

Increased total homocysteine in the blood, "hyperhomocysteinemia," is an established risk factor for premature atherosclerotic disease in the coronary, cerebral, and peripheral arteries [1]. Hyperhomocysteinemia is caused by

acquired and genetic factors [2]. Frosst et al. [3]. We recently demonstrated that a common mutation (allele frequency ~35%) in the methylenetetrahydrofolate reductase (MTHFR) gene, characterized by a C to T substitution at nucleotide 677, creates a thermolabile enzyme variant.³ In its homozygous form, this mutation predisposes to hyperhomocysteinemia, especially in folate-deficient subjects [3-5]. Thermolabile MTHFR has itself been associated with increased incidence of early cardiovascular disease [6, 7].

Determination of the MTHFR genotype will probably become valuable for assessment of cardiovascular risk in large populations. Its effects will be investigated in future epidemiological studies of total homocysteine and cardiovascular disease, including prospective studies based on stored serum/plasma samples. Determination of genotype in large populations or in routine laboratories demands high assay throughput, and genotyping in residual blood cells in plasma/serum requires extensive amplification or sensitive detection systems.

The C677T mutation in MTHFR creates a *HinfI* restriction site that is detected by digestion of a polymerase chain reaction (PCR)-amplified fragment analyzed by conventional agarose gel electrophoresis [3]. Electrophoretic separation of DNA fragments by dynamic sieving in coated capillaries filled with entangled polymers (e.g., linear polyacrylamide or cellulose derivatives) has recently become an attractive alternative, characterized by short analysis time, automation, high resolution, and unsurpassed separation efficiency [8]. High sensitivity of detection can be obtained by staining the DNA with fluorescent dyes or fluorescent intercalators present in the

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³ Nonstandard abbreviations: MTHFR, methylenetetrahydrofolate reductase; PCR, polymerase chain reaction; LIF, laser-induced fluorescence; CE, capillary electrophoresis; F, forward; OF, outer forward; R, reverse; and CR, control reverse.

sieving medium, combined with laser-induced fluorescence (LIF) detection [8]. We have recently demonstrated the applicability of the novel monomeric dyes, SYBR Green I, YO-PRO-1, and Thiazole Orange, for the analysis of DNA fragments by capillary electrophoresis (CE) and LIF detection (CE-LIF) [9].

Here, we demonstrate a method for analyzing the C677T mutation in the MTHFR gene, in which the technique based on PCR amplification and *HinfI* restriction cleavage [3] has been modified and adapted to a CE-LIF format. The modifications include addition of a truncated fragment, which serves as a digestion control and time marker, and use of a nested PCR protocol, required when plasma or serum samples are the sources of DNA. Because commercial CE systems usually are equipped with a single capillary, we devised a multiple-injection mode that allows simultaneous separation of as many as eight samples, thereby increasing the sample throughput of the assay.

Materials and Methods

REAGENTS

The DNA Direct kit was obtained from Dynal (Oslo, Norway) and the QIAamp Blood Kit from Qiagen (Hilden, Germany). Reagents and reaction tubes (Thin Walled GeneAmp) for PCR were purchased from Perkin-Elmer (Norwalk, CT), except that the Taq polymerase (Supertaq) was obtained from HT Biotechnology (Cambridge, UK). The four primers referred to as forward (F), outer forward (OF), reverse (R), and control reverse (CR) were synthesized by Eurogentec (Seraing, Belgium). The F and R primers have been previously published [3]. The SF and CR primers were designed with the aid of the computer program Oligo (National Biosciences, Plymouth, MN). Their sequences were 5'-GGAGCTTTGAG-GCTGACC-TGAA-3' (F), 5'-TGAAGGAGAAGGTGTCT-GCGGGA-3' (OF), 5'-AGGACGGTGCGG-TGAGAGTG-3' (R), and 5'-GAGTGGGGTGGAGGGAGCTTAT-3' (CR). SYBR Green I (concentration not given), dissolved in dimethyl sulfoxide, was purchased from Molecular Probes (Eugene, OR), and working stock solution was prepared daily and stored in the dark at room temperature. Acrodisc polyvinylidene difluoride filters (0.45- μ m pore size) were from Gelman Sciences (Ann Arbor, MI).

The microtiter plate filtration unit MADV N65 was from Millipore (Bedford, MA). Hydroxypropylmethylcellulose (4000 cP at 25 °C, 20 g/L aqueous solution) and other chemicals (analytical- or molecular biology-grade) were obtained from E. Merck (Darmstadt, Germany) and Sigma Chemical Co. (St. Louis, MO). Solutions containing hydroxypropylmethylcellulose were prepared as described elsewhere [10]. Water, doubly distilled and purified with a MilliQ Plus Water Purification System (Millipore), was used to prepare all aqueous solutions.

CE INSTRUMENTATION

CE was performed on a Prince CE system from Prince Technologies (Emmen, The Netherlands), equipped with an in-house-built LIF detector. Laser excitation was at 488 nm (produced with a 20-mW argon laser from Uniphase, Herts, UK), and the light was focused 30 μ m below the end of the capillary that was placed in a rectangular sheath-flow cuvette. A sheath flow of electrophoresis buffer (without entangled polymer) was delivered at a rate of 30 μ L/h. The emitted light was collected through a 535-nm band-pass filter (no. 535DF35; Omega Optical, Brattleboro, VT).

We used a 50 μ m (i.d.)/192 μ m (o.d.) silica capillary (Polymicro Technologies, Phoenix, AZ) coated according to Hjertén [11]. Total capillary length, and the distance between the column inlet and detector window, was 42 cm.

Caesar software (Version 4.1) from Prince Technologies was used for data collection and processing. Electropherograms used to prepare the illustrations were transferred as text files to an Apple Macintosh computer and redrawn by Sigma Plot (Version 5.0; Jandel Scientific, Erkrath, Germany).

DNA EXTRACTION

Three different methods for DNA extraction were used, involving magnetic particles, filtration, or adsorption to silica.

Magnetic particle method. DNA was extracted from whole blood by using the DNA Direct kit and following the instructions provided by the manufacturer. Briefly, 5 μ L of whole-blood sample was mixed with 200 μ L of magnetic particles suspended in a buffer; this lysed the blood cells and allowed the DNA to adhere to the magnetic particles. The particles were washed twice and thereafter resuspended in 40 μ L of Tris-EDTA buffer (10 mmol/L Tris-HCl, 1 mmol/L EDTA, pH 8.0); 5 μ L of the particle suspension was used for PCR.

Filtration. Serum or plasma samples (100 μ L) were loaded on a microtiter plate filtration unit (MADV N65), and cell debris was trapped on the filters by centrifugation at 500g for 5 min. DNA was released from the filters by adding 40 μ L of ion-exchange purified water and heating at 95 °C for 2 min. We used 15 μ L of the crude DNA preparation for the first PCR reaction in the seminested protocol.

Adsorption to silica. Serum or plasma samples were purified with the QIAamp Blood Kit, according to the instructions of the manufacturer. Briefly, 200 μ L of plasma was treated with a lysis buffer containing proteinase K. Isopropanol was added to the solution, and the mixture was applied to a column containing an integrated silica membrane and centrifuged. The DNA adhered to the membrane, and impurities were removed by a washing step. Captured DNA was eluted in 50 μ L of 10 mmol/L Tris buffer, pH 9.0, and 15 μ L of the effluent was used for PCR.

PCR

The PCR reaction mixture contained 10 mmol/L Tris-HCl (pH 9.0), 50 mmol/L KCl, 1.5 mmol/L MgCl₂, 0.1 g/L gelatin, 1 mL/L Triton X-100, 125 μmol/L of each dNTP, 0.2 μmol/L of each primer, 0.2 U of Taq polymerase, and template DNA in a total volume of 100 μL. A Perkin-Elmer 480 thermocycler was used for a two-step thermocycling profile: denaturation at 94 °C for 15 s and annealing at 55 °C for 30 s, the temperature cycles being preceded by 2 min at 94 °C and concluded with 5 min at 72 °C.

A seminested protocol and a single-step protocol were constructed. The seminested protocol included the OF and R primers in the first reaction of 25 cycles; 1 μL of the first reaction was then transferred to the second reaction, which consisted of 40 cycles with the F and R primers. The single-step protocol was identical to the second reaction of the seminested protocol, except the template was 5 μL of purified DNA.

RESTRICTION ENZYME CLEAVAGE

The C677T substitution creates a *Hinf*I recognition sequence. A solution containing *Hinf*I, reaction buffer, and a 183-bp control fragment was pipetted in 15-μL aliquots, and 10 μL of PCR product was added. The final mixture contained 2 U of *Hinf*I in 1× Gibco buffer II (50 mmol/L Tris-HCl, pH 8.0, 10 mmol/L MgCl₂, and 50 mmol/L NaCl) and a 2:5 dilution of the PCR reaction buffer (see preceding section) in a total volume of 25 μL. Incubation was at 37 °C for 90 min.

CAPILLARY ELECTROPHORESIS

Electrophoresis buffer was 89 mmol/L Tris-borate, pH 8.3, containing 1 mmol/L EDTA. Separation buffer was electrophoresis buffer containing 6 g/L hydroxypropylmethylcellulose as sieving matrix. The separation buffer was passed through the Acrodisc filters before use. SYBR Green I (1:20 000 final dilution of stock solution) was added to the separation buffer after filtration (to avoid adsorption of dye to the filter matrix).

Before each series of injections, the capillary was flushed with the electrophoresis buffer for 30 s and then filled with separation buffer by applying a pressure of 200 kPa for 150 s. Samples were diluted 1:50 in distilled water and injected electrokinetically. Separation field strength was between 500 and 700 V/cm, and temperature was set at 20 °C.

Results and Discussion

PRIMERS AND PCR PRODUCTS

The PCR primers for amplification of the analysis product (denoted here as F and R) were the same as previously described [3]. A third primer, OF, was constructed, positioned 26-bp 5' to the F primer, and used in concert with the R primer to produce the first fragment in the seminested protocol. A fourth primer, CR, was constructed and positioned 15-bp 5' to the R primer. Primers CR and F,

along with template DNA from a subject homozygous for the C677T mutation, were used to produce the control fragment that was added to the PCR products before *Hinf*I digestion. One function of the control fragment was to verify complete digestion by the restriction enzyme (see below). Fig. 1 outlines the positions of the primers relative to the mutation and the sizes of the different products before and after treatment with restriction enzyme.

SINGLE-INJECTION ANALYSIS

DNA samples were PCR-amplified, digested with *Hinf*I after addition of the control fragment, and then subjected to CE-LIF. A sample from a heterozygous subject showed four major peaks: the primers, the digested control fragment, the fragment of the mutated allele (T), and the fragment of the normal allele (C) (Fig. 2).

The control fragment (f) provided a reference for the migration of C and T, and the combinations of f-T, f-C, or

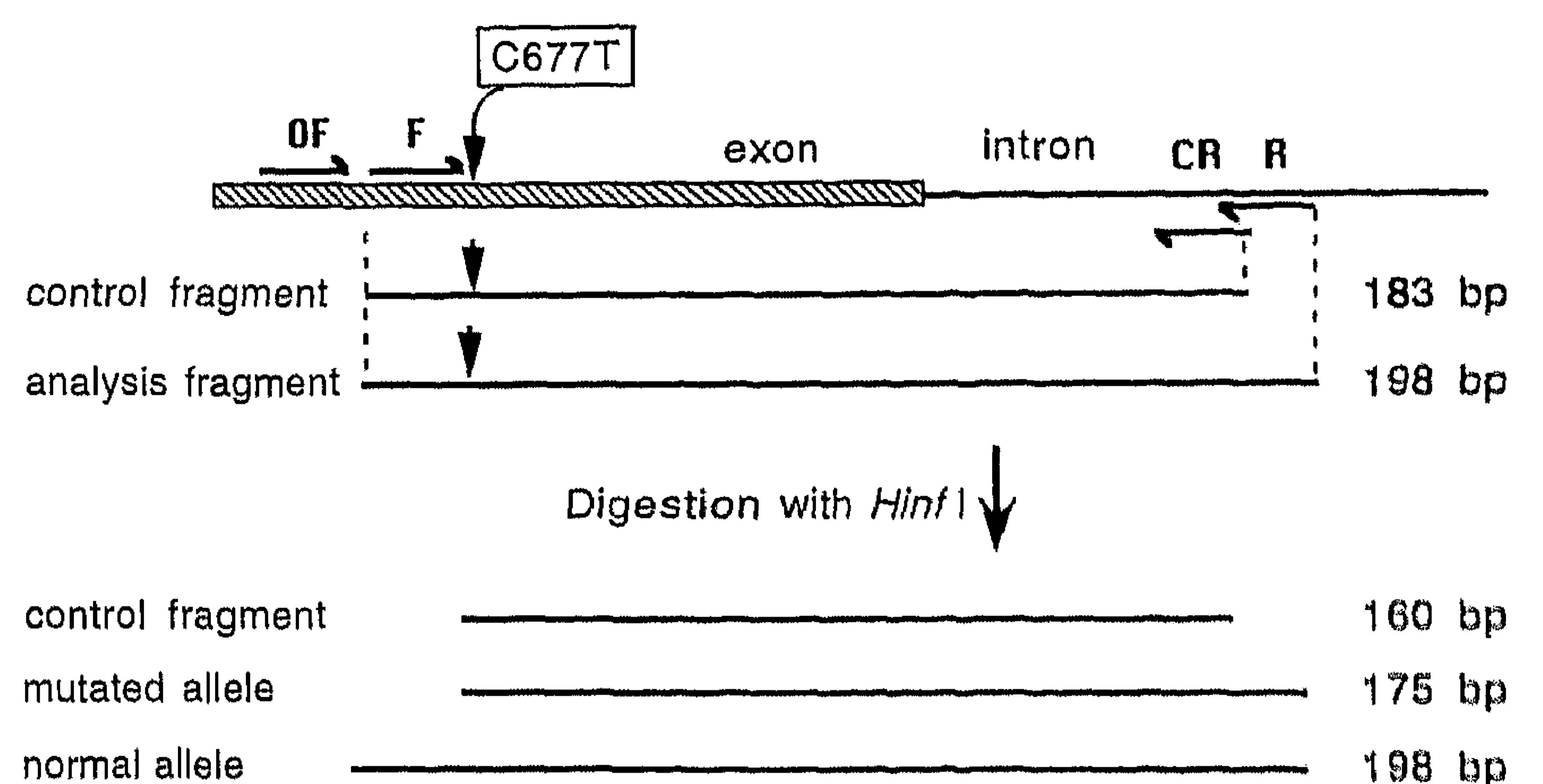


Fig. 1. Positions of primers—OF (outer forward), F (forward), R (reverse), and CR (control reverse)—relative to the mutation site (arrow) and an exon/intron boundary.

The relative sizes of the PCR products are shown before and after restriction enzyme cleavage. All lengths are drawn to scale.

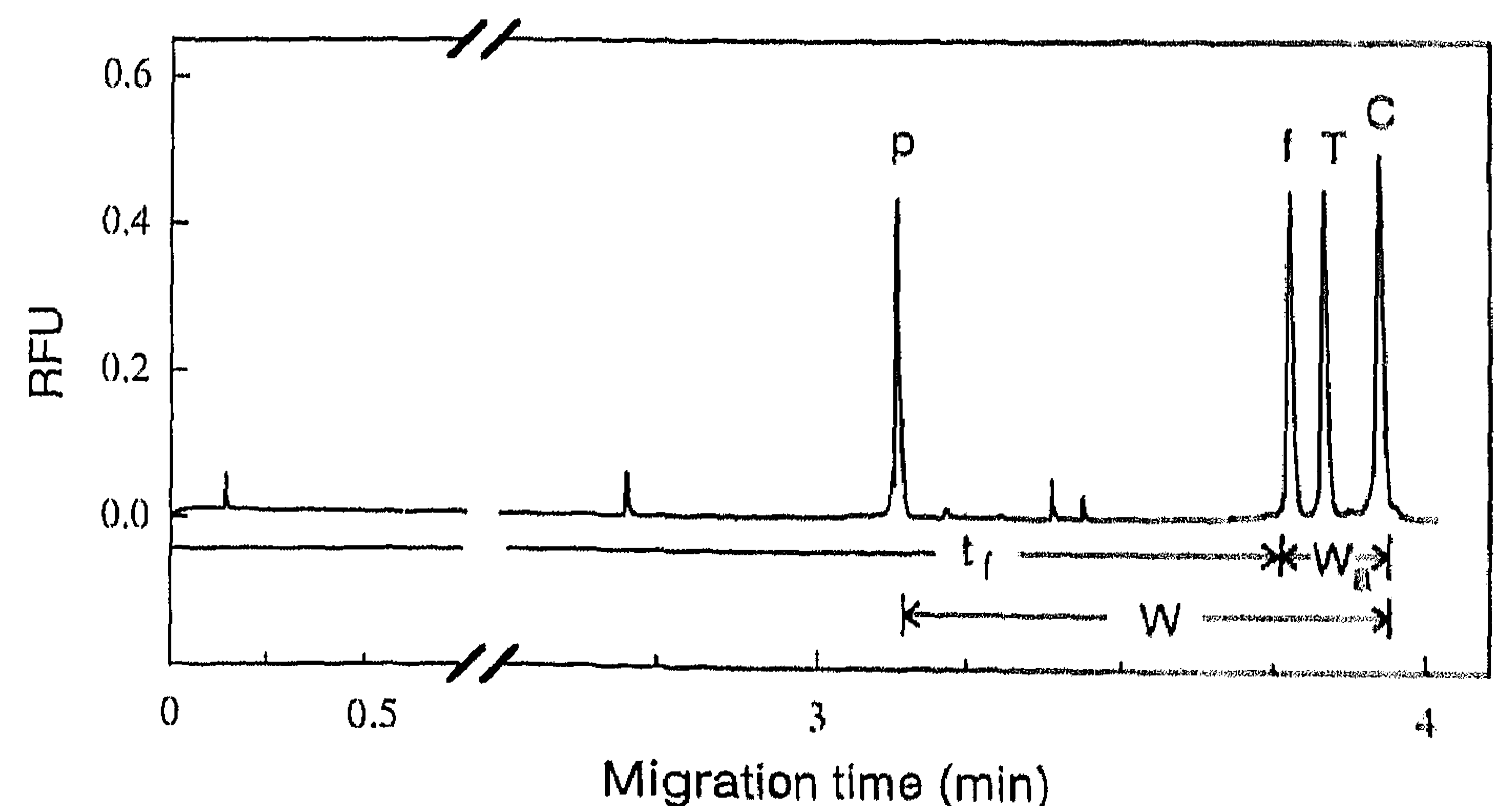


Fig. 2. Electropherogram of a single heterozygous (CT) sample, illustrating peaks for the primer (p), control fragment (f), mutated allele (T), and normal allele (C).

For W_a (the analysis window), W (a window spanning W_a and the primers), and t_f (the time at which the control fragment elutes at the detector), the actual values were 0.18, 0.80, and 3.77 min, respectively. Injection was at -15 kV for 6 s, and separation field strength was 660 V/cm (constant voltage). Other conditions were as described in *Materials and Methods*.

f-T-C peaks formed characteristic patterns for each of the three possible genotypes (TT, CC, and CT, respectively). The digestion control was built into the pattern recognition design, in that an incomplete cleavage was revealed by the appearance of the intact control fragment at a position between the mutated (T) and the normal (C) fragment. Remnants of uncleaved control fragment are indicated with arrows in Fig. 3. A further function of the control fragment was to differentiate between a failed PCR amplification (only control fragment was detected after amplification) and inadequate sample injection (no fragments were detected).

MULTIPLE-INJECTION ANALYSIS

When adapting the single-injection analysis to a multiple-injection mode, the essential requirements were (a) to avoid coelution of primers from one sample with the digested PCR fragments from another sample, and (b) to avoid injection during the time interval when the PCR fragments pass the detector. The analytical parameters used for designing an optimized injection protocol that would meet these requirements are defined in Fig. 2: the analysis window W_a ; the time interval W , which spans W_a and the primers; and t_f , the time at which the control fragment elutes at the detector.

The injections were performed with alternating time intervals Δ_s (small interval) and Δ_L (long interval). Two injections separated by Δ_s defined a group. Δ_s had to be $>W_a$ to avoid overlap between analysis windows, and maximal separation was obtained when $\Delta_s = W/2$. Thus, an optimal Δ_s (balancing analysis throughput and separation) would be within the interval $W_a < \Delta_s < W/2$.

To fulfill the second requirement above (b), g groups were distributed in the capillary (by means of varying Δ_L) so that elution of a group at the detector was followed by injection of a group at the capillary inlet, and vice versa. This put some limitations on Δ_L , which were determined

by W_a , t_f , Δ_s , and g as shown by the expression:

$$[t_f + W_a - (g - 1)\Delta_s]/g < \Delta_L < (t_f - g\Delta_s)/(g - 1) \quad (1)$$

From requirement (b) it also follows that Δ_L had to be larger than one group, that is $\Delta_L > \Delta_s + W_a$. To avoid coelution between PCR fragments and primers (requirement a), a stronger restriction was $\Delta_L > W$. By substituting W for Δ_L in expression 1, we obtained the following expression for g_{\max} : $g_{\max} < (t_f + W)/(\Delta_s + W)$.

Using the values of t_f , W , and W_a from Fig. 2, we determined a g_{\max} of 4 (i.e., 4×2 samples migrating simultaneously). Fig. 4 depicts schematically the distribution of eight injections ($g = 4$) in the capillary at the boundary conditions for Δ_L (A and B). Expression 1 and the expression for g_{\max} can be derived from this Fig. 4.

Reduction of g from its maximal value increases the separation between the peaks of successive injections, thereby allowing for some variability in system performance that affects parameters W_a , W , and t_f . Thus, we routinely used $g = 3$ for calculating the appropriate Δ_L . Fig. 3 shows the last six injections in a run with 10 injections ($g = 3$). Mean analysis time was 1.5 min per sample.

The above discussion is based on grouping two injections together, separated by a single Δ_s . Conceivably, each group could comprise more than two injections. The grouping of the injections depends on the analytical parameters (e.g., W_a , W , and t_f), the relative values of which can be modified by changing the position of the PCR primers that determine the sizes of the analysis products and control fragment(s). In practice, the difference between the cut and uncut fragments should be as small as possible. This would decrease W_a and make the small part of the cut fragment elute close to the primers, which would allow several injections to be grouped within the time period W . The parameters also reflect the resolution properties of the system, which in turn are

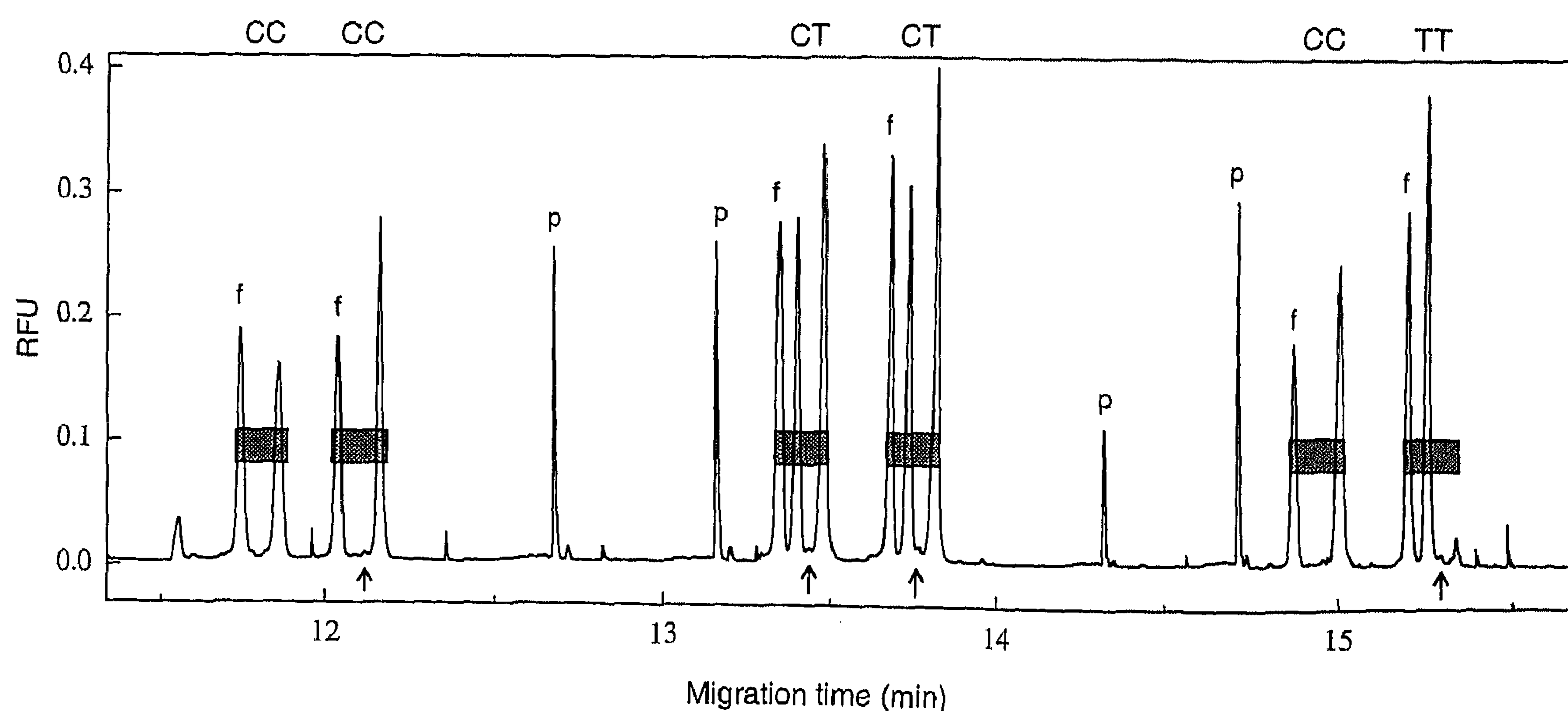


Fig. 3. Electropherogram of a multiple-injection run, showing the last six samples of a run with 10 injections.

Shaded boxes emphasize the size and distribution of the analysis windows. The genotypes are two homozygous normal (CC), two heterozygous (CT), one homozygous normal (CC), and a homozygous mutated (TT), in that order. Remnants of the undigested control fragment are indicated with arrows. p , f , and electrophoretic conditions are as in Fig. 2.

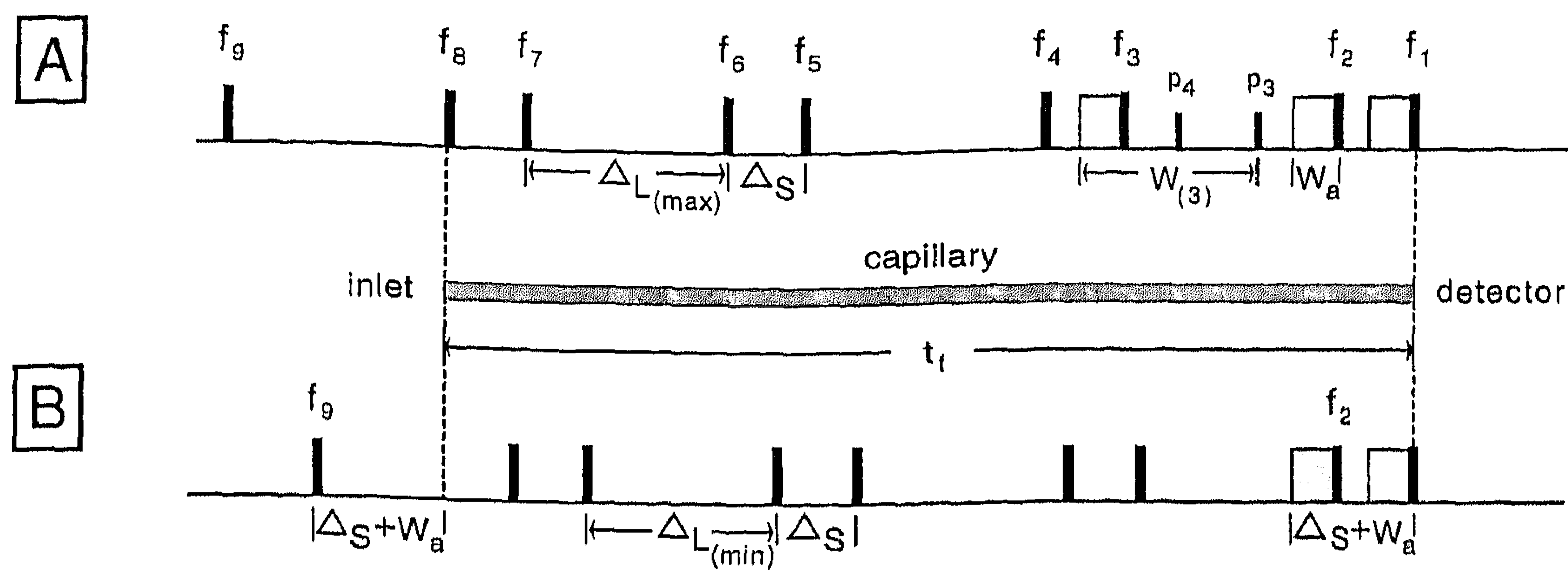


Fig. 4. Schematic presentation of multiple-injection assay, wherein the distribution of injections ($g = 4$) is represented by the migration of the respective control fragments f_1 through f_8 (f_9 will be the next injection).

Because the control fragments move with constant velocity, their separation in time is proportional to their separation in distance, and can be drawn on the same axis. The time intervals Δ_L and Δ_S are indicated, and the variables W_g and W (for injections f_2 and f_3 , respectively) are drawn at the size they would have at elution. Panels A and B show the distribution at $\Delta_{L(\max)}$ and $\Delta_{L(\min)}$, respectively.

related to type and concentration of the sieving matrix and fluorescent dye, temperature, and field strength [8].

DURABILITY OF THE SEPARATION MATRIX

One multiple-injection run was usually programmed to have 10 or 20 injections, but as many as 30 injections could be performed without replacing the separation matrix. There were minor changes in plate number and resolution during the course of 10–20 multiple injections, but a progressive decrease in W was observed (i.e., the primers eluted closer to the PCR fragments).

With low concentration of SYBR Green I (dilution 1:100 000) in the sieving medium, we observed a decrease in resolution and migration time after the separation of

several samples with high DNA content. Based on previous observations [9], this can probably be explained by extraction of dye by the DNA. The electrophoretic performance was stabilized by increasing the SYBR Green I concentration to dilution 1:20 000 in the present protocol.

DNA PURIFICATION, AMPLIFICATION, AND VALIDATION OF CE-LIF ASSAY

The CE-LIF assay described here was developed to analyze the MTHFR C677T mutation in the clinical setting but also to genotype large populations as part of prospective studies based on stored samples. In the routine laboratory, DNA is obtained from whole blood, whereas stored sample collections often contain only serum or plasma.

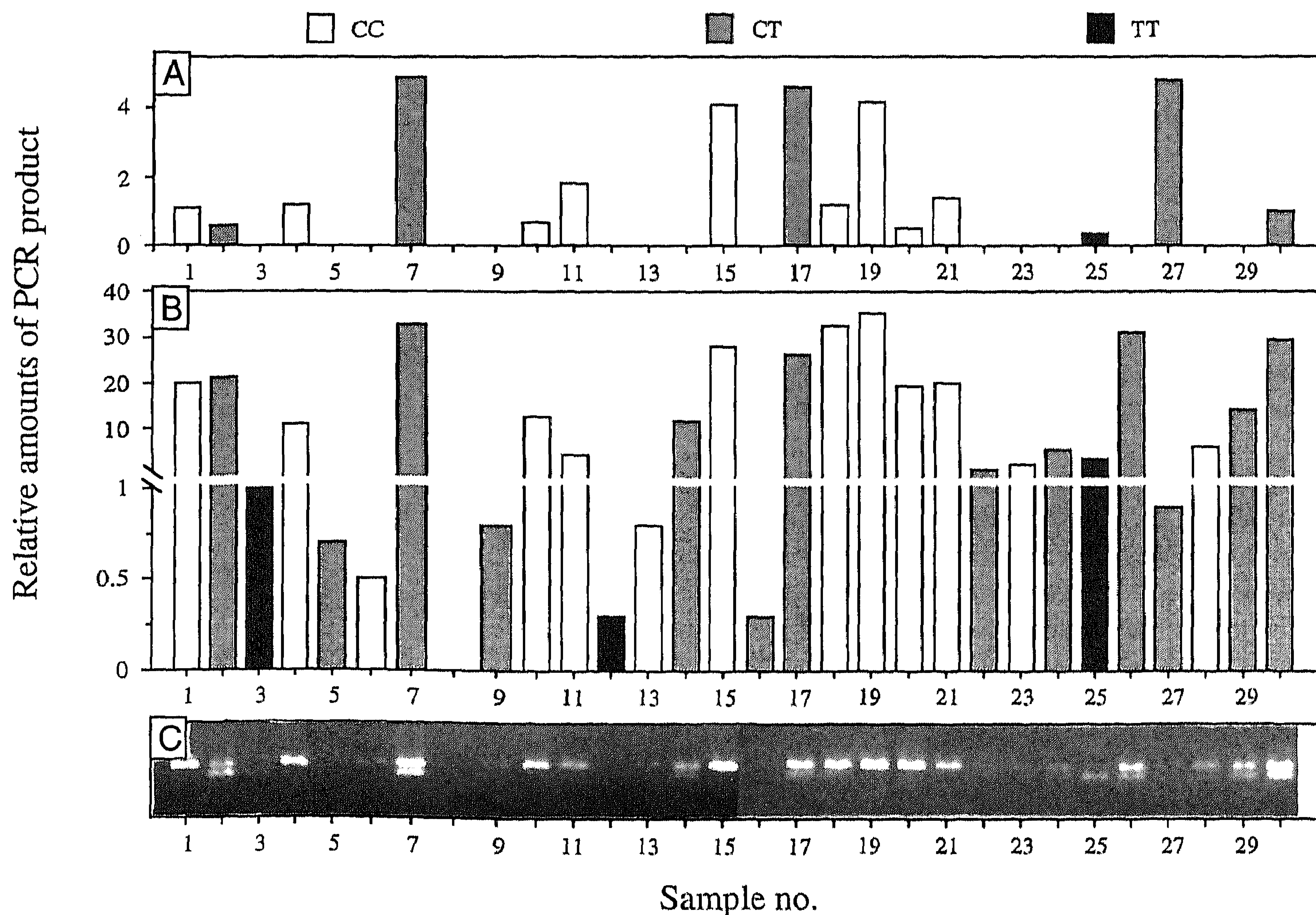


Fig. 5. Evaluation of single vs nested PCR and of CE-LIF vs agarose gel electrophoresis for genotyping 30 plasma samples for *MTHFR* gene.

All samples were purified by the Qiagen method, PCR-amplified, and digested by *Hinf*I: (A) single-step PCR and CE-LIF analysis, (B) seminested PCR and CE-LIF analysis, (C) seminested PCR in combination with conventional agarose gel electrophoresis and ethidium bromide staining. Samples in C are the same as in B, except that the control fragment was not added. The column heights indicate the relative amounts of PCR products, obtained by dividing the area of the allele peak(s) by the area of the control fragment peak.

We used the DNA Direct Kit for extraction of DNA from whole blood. This method is based on capture of the DNA in lysed blood onto magnetic beads. The extraction is carried out manually, but we are currently automating it, using the BioMek 2000 robot from Beckman Instruments (Fullerton, CA). Using this technique in combination with single-step PCR, we could determine the genotype of ~100% of the whole-blood samples. For plasma/serum samples, however, this DNA extraction procedure was inadequate. Moreover, combining this extraction approach with the seminested PCR technique described in *Materials and Methods* correctly identified the genotype of <10% of the serum/plasma samples.

Methods for the extraction of DNA from serum [12, 13] and plasma [13] have been reported before. In the present work, we established and demonstrated the applicability of two alternative methods. Collecting cell debris by filtration in a multiwell unit in combination with the seminested PCR protocol was successful with serum samples (29 of 30 were genotyped) but not with plasmas (5 of 30 were genotyped).

The silica adsorption method (QIAamp) was used to extract DNA remaining in 30 plasma samples that had been stored at -20°C for 20 years. Fig. 5 shows the genotypes and the relative amounts of PCR products. Signals from 15 of the 30 samples could be detected by CE-LIF after the single-step PCR protocol (Fig. 5A), whereas 29 of 30 were positive after PCR amplification with the seminested protocol (Fig. 5B). We also compared the sensitivity of CE-LIF (Fig. 5B) and a conventional agarose gel stained with ethidium bromide (Fig. 5C) for determining the genotypes of the samples amplified by seminested PCR. Some (5–7) of the samples genotyped by CE-LIF could not be determined by conventional agarose gel electrophoresis.

The versatility of the CE-LIF method is demonstrated by reliable genotyping based on highly variable amounts of PCR products. In the experiments depicted in Fig. 5, the ratio of the largest to the smallest amount was 117.

In conclusion, this work demonstrates a high-throughput method for analyzing the MTHFR C677T mutation based on CE-LIF. The method can be used with either rich sources of DNA (e.g., whole blood) or, coupled with a seminested PCR protocol, poor sources of DNA (e.g., serum or plasma). The built-in control features of the method make the genotyping reliable and robust. The principles of multiple injection and digestion control by inclusion of a control fragment should be widely applicable to mutation analyses based on restriction enzyme cleavage.

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References

1. Boushey CJ, Beresford SAA, Omenn GS, Motulsky AG. A quantitative assessment of plasma homocysteine as a risk factor for vascular disease: probable benefits of increasing folic acid intakes. *JAMA* 1995;274:1049–57.
2. Ueland PM, Refsum H, Stabler SP, Malinow MR, Andersson A, Allen RH. Total homocysteine in plasma or serum: methods and clinical applications [Review]. *Clin Chem* 1993;39:1764–79.
3. Frosst P, Blom HJ, Milos R, Goyette P, Sheppard CA, Matthews RG, et al. Identification of a candidate genetic risk factor for vascular disease: a common mutation at the methylenetetrahydrofolate reductase locus. *Nat Genet* 1995;10:111–3.
4. Jacques PF, Bostom AG, Williams RR, Ellison RC, Eckfeldt JH, Rosenberg IH, et al. Relation between folate status, a common mutation in methylenetetrahydrofolate reductase, and plasma homocysteine concentrations. *Circulation* 1996;93:7–9.
5. Kluijtmans LAJ, Van den Heuvel LPWJ, Boers GHJ, Frosst P, Van Oost BA, den Heijer M, et al. Molecular genetic analysis in mild hyperhomocysteinemia: a common mutation in the methylenetetrahydrofolate reductase gene is a genetic risk factor for cardiovascular disease. *Am J Hum Genet* 1996;58:35–41.
6. Kang S-S, Wong PWK, Susmano A, Sora J, Norusis M, Ruggie N. Thermolabile methylenetetrahydrofolate reductase: an inherited risk factor for coronary artery disease. *Am J Hum Genet* 1991;48:536–45.
7. Kang SS, Passen EL, Ruggie N, Wong PWK, Sora H. Thermolabile defect of methylenetetrahydrofolate reductase in coronary artery disease. *Circulation* 1993;88:1463–9.
8. Karger BL, Chu YH, Foret F. Capillary electrophoresis of proteins and nucleic acids. *Annu Rev Biophys Biomol Struct* 1995;24:579–610.
9. Skeidsvoll J, Ueland PM. Analysis of double-stranded DNA by capillary electrophoresis with laser-induced fluorescence detection using the monomeric dye SYBR Green I. *Anal Biochem* 1995;231:359–65.
10. Baba Y, Ishimaru N, Samata K, Tshako M. High-resolution separation of DNA fragments by capillary electrophoresis in cellulose derivative solutions. *J Chromatogr* 1993;653:329–35.
11. Hjertén S. High-performance electrophoresis. Elimination of electroendosmosis and solute adsorption. *J Chromatogr* 1985;347:191–8.
12. Martin M, Carrington M, Mann D. A method for using serum or plasma as a source of DNA for HLA typing. *Hum Immunol* 1992;33:108–13.
13. Fowke KR, Plummer FA, Neil Simonsen J. Genetic analysis of human DNA recovered from minute amounts of serum and plasma. *J Immunol Methods* 1995;180:45–51.