Clinical Chemistry 50:2
391–402 (2004)

Automation and
Analytical Techniques

High-Level Multiplex Genotyping of Polymorphisms Involved in Folate or Homocysteine Metabolism by Matrix-Assisted Laser Desorption/Ionization Mass Spectrometry

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Background: **Increased plasma total homocysteine (tHcy), a risk factor for cardiovascular disease, is related to genetic, environmental, and nutritional factors, in particular folate status. Future large epidemiologic studies of the genetic basis of hyperhomocysteinemia will require high-throughput assays for polymorphisms of genes related to folate and Hcy metabolism.**

Method: **We developed a high-level multiplex genotyping method based on matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) for the detection of 12 polymorphisms in 8 genes involved in folate or Hcy metabolism. The assay includes methylenetetrahydrofolate reductase (*MTHFR*) 677C>T and 1298A>C, methionine synthase (*MTR*) 2756A>G, methionine synthase reductase (*MTRR*) 66A>G, cystathionine β-synthase (*CBS*) 844ins68 and 699C>T, transcobalamin II (*TCII*) 776C>G and 67A>G, reduced folate carrier-1 (*RFC1*) 80G>A, paraoxonase-1 (*PON1*) 575A>G and 163T>A, and betaine homocysteine methyltransferase (*BHMT*) 742G>A.**

Results: **The failure rate of the assay was ≤1.7% and was attributable to unsuccessful DNA purification, nanoliter dispensing, and spectrum calibration. Most errors were related to identification of heterozygotes as homozygotes. The mean error rate was 0.26%, and error rates differed for the various single-nucleotide polymorphisms. Identification of *CBS* 844ins68 was carried out by a semiquantitative approach. The throughput of the MALDI-TOF MS assay was 1152 genotypes within 20 min.**

Conclusions: **This high-level multiplex method is able to genotype 12 polymorphisms involved in folate or Hcy metabolism. The method is rapid and reproducible and could facilitate large-scale studies of the genetic basis of hyperhomocysteinemia and associated pathologies.**

Increased plasma total homocysteine (tHcy)[1] is an independent risk factor for cardiovascular disease *(1)*, venous thromboembolism *(2)*, impaired cognitive function *(3)*, Alzheimer disease *(4)*, and adverse pregnancy outcome *(5)*. Hyperhomocysteinemia is caused by low intake of folate and other B vitamins and by genetic factors *(6, 7)*, including polymorphisms of genes encoding enzymes involved in Hcy remethylation, such as methylenetetrahydrofolate reductase (*MTHFR*), methionine synthase (*MTR*), methionine synthase reductase (*MTRR*), and variants of cystathionine β-synthase (*CBS*), which catalyzes the irreversible step of the transsulfuration pathway (Fig. 1). Single-nucleotide polymorphisms (SNPs) with documented metabolic and biological effects include *MTHFR* 677C>T *(8)*, which is a strong determinant of tHcy in individuals with impaired folate status *(9)*. The 677TT genotype has been described as an independent risk factor for cardiovascular disease, colorectal neoplasias, neural tube defects, and pregnancy complications *(9)*. *MTHFR* 1298A>C *(10, 11)*, *MTR* 2756A>G *(12–14)*,

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Received September 1, 2003; accepted November 26, 2003.

Previously published online at DOI: 10.1373/clinchem.2003.026799

[1] Nonstandard abbreviations: Hcy, homocysteine; MTHFR, methylenetetrahydrofolate reductase; BHMT, betaine-homocysteine methyltransferase; MTR, methionine synthase; MTRR, methionine synthase reductase; CBS, cystathionine β-synthase; SNP, single-nucleotide polymorphism; TCII, transcobalamin II; RFC1, reduced folate carrier-1; PON1, paraoxonase-1; RFLP, restriction fragment-length polymorphism; MS, mass spectrometry; MALDI-TOF; matrix-assisted laser desorption/ionization time of flight; ddNTP, dideoxynucleotide triphosphate; and dNTP, deoxynucleotide triphosphate.

MTRR 66A>G *(15–17)*, and *CBS* 844ins68 *(18–21)* have been associated with hyperhomocysteinemia, neural tube defects, and colorectal cancer. Several other polymorphisms, including *CBS* 1080C>T and 699C>T *(22, 23)*, transcobalamin-II (*TCII*) 776C>G *(24–28)* and 67A>G *(29)*, reduced folate carrier-1 (*RFC1*) 80G>A *(30–33)*, betaine homocysteine methyltransferase (*BHMT*) 742G>A *(34, 35)*, and paraoxonase-1 (*PON1*) 575A>G and 163T>A *(36, 37)*, have been related to folate or Hcy status, and their possible associations with risk of cardiovascular disease and other diseases are under investigation.

Traditional methods for the identification of known polymorphisms are restriction fragment-length polymorphism (RFLP) analysis, oligonucleotide ligation assay, and minisequencing *(38–41)*. These techniques usually include separation of DNA fragments by gel electrophoresis, are labor-intensive, and provide low-throughput genotyping. Non-gel-based technologies have recently been developed for rapid genotyping of large study cohorts *(38–41)*. These methods achieve allelic differentiation by hybridization, mini- and pyrosequencing, oligonucleotide ligation assay, and cleavage of a flap probe. Homogeneous or solid-phase reaction formats are used in combination with fluorescent dyes or mass spectrometry (MS) for detection. Various high-density formats have been adapted to capillary electrophoresis *(42)*, DNA microarrays *(43)*, bead-based assays *(44)*, and MS *(45)* to obtain cost-effective genotyping by miniaturization and a high degree of multiplexing. Recent publications have described ultrahigh-throughput approaches that enable the identification of up to hundreds of thousands *(46)* or millions *(47)* of genotypes within 24h.

Generally, genotyping by MS does not require labeled primers, and mass determination provides direct molecular information. Matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) MS in particular has become a powerful tool for detection of known SNPs and mutations *(48, 49)* as a result of recent advances in both sample preparation and instrument performance. Such advances include better sample desalting and choice of an appropriate matrix *(50–54)*. Introduction of nanoliter-volume handling *(45)*, hydrophilic anchors *(55)*, and chip hybridization *(56)* have enhanced sample throughput and reduced analysis costs. Delayed extraction has become a standard option in linear TOF instruments and offers enhanced resolution and high sensitivity *(57)*. Minisequencing is the convenient strategy for multiplex detection of known alleles by MALDI-TOF MS. The Pin-Point *(58)* and the PROBE *(59)* formats allow simultaneous detection of up to 12 alleles *(60)*, with use of dideoxynucleotide triphosphates (ddNTPs) or mixtures of deoxynucleotide triphosphates (dNTPs) and ddNTPs, respectively.

This report presents a fast and reliable Pin-Point assay for multiplex genotyping by MALDI-TOF MS of 12 polymorphisms related to folate or Hcy metabolism. The method detects *MTHFR* 677C>T, *MTHFR* 1298A>C, *MTR* 2756A>G, *MTRR* 66A>G, *CBS* 844ins68, *CBS* 699C>T, *BHMT* 742G>A, *RFC1* 80G>A, *TCII* 67A>G, *TCII* 776C>G, *PON1* 163T>A, and *PON1* 575A>G. The polymorphisms included in the MALDI-TOF MS assay and the role of the corresponding enzymes and transporters in folate or homocysteine metabolism are depicted in Fig. 1.

Material and Methods

DNA PURIFICATION AND MULTIPLEX PCR

Whole blood was obtained from 460 blood donors. DNA was purified from 30 μL of blood by use of the Geno M-96 workstation (Geno Vision). The yield was 200–400 ng and according to the manufacturer's description. The PCRs were multiplexed in two separate groups, as described in Table 1. PCR was performed in a total volume of 25 μL containing 12 ng of template DNA, 1.25 U of HotStarTaq™ polymerase (Qiagen), 4 mM $MgCl_2$, 1.2 μM dNTPs (Amersham Bioscience), and 0.25 μM each primer. Oligo 6.0 (Molecular Biology Insights) was used for design of primers, which were synthesized by Eurogentec. The cycling conditions (MJR Dynad thermal cycler) were 95 °C for 15 min followed by 40 cycles at 95 °C for 30 s, 66 °C (group 1) or 62 °C (group 2) for 30 s, and 72 °C for 1 min, with a final extension at 72 °C for 10 min.

Purification of PCR products, which removed primers and dNTPs, was carried out by incubation of 10 μL of PCR mixture with 3 μL of ExoSAP-IT™ (USB Corporation) at 37 °C for 15 min, followed by 15 min at 80 °C.

PRIMER EXTENSION REACTION

The multiplex primer extension reaction was carried out with purified PCR products as template and a mixture of 12 site-specific primers (Eurogentec; Table 2), which were extended by one matching ddNTP (Amersham Bioscience). The optimum primer concentrations were empirically determined and ranged from 1 to 26 pmol. The total reaction volume of 25 μL contained 5 μL of PCR product, 50 μM ddNTPs, and 4 U of ThermoSequenase™ (Amersham Bioscience). The cycling conditions (MJR Dynad thermal cycler) were 90 cycles at 94 °C for 30 s, 62 °C for 30 s, and 72 °C for 1 min. Liquid handling was performed in 96-sample format on a Biomek 2000 Workstation (Beckman Coulter).

COMPARISON METHODS

Two different techniques were used to verify the MALDI-TOF MS results. *MTHFR* 677C>T, *MTHFR* 1298A>C, *MTR* 2756A>G, and *MTRR* 66A>G were genotyped by a TaqMan real-time PCR assay, as described previously *(61)*. RFLP analysis was used to identify *BHMT* 742G>A *(35)*, *CBS* 699C>T *(22)*, *PON1* 163T>A, *PON1* 575A>G, *RFC1* 80G>A *(32)*, *TCII* 776C>G, and *TCII* 67A>G *(29)*. PCRs were performed in a total volume of 25 μL containing 12 ng of template DNA, 1.25 U of HotStarTaq polymerase, 1.5 mM $MgCl_2$, 0.48 μM dNTPs, and 0.5 μM each

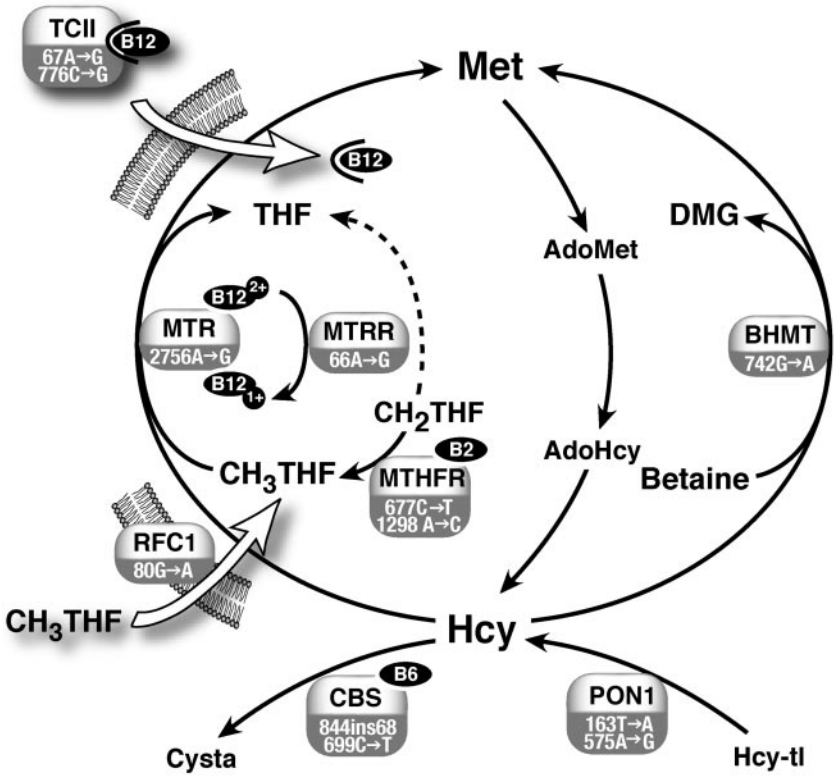

Fig. 1. Polyorphisms included in the MALDI-TOF MS assay, and the role of the corresponding enzymes and transporters in folate or Hcy metabolism.

AdoHcy, *S*-adenosylhomocysteine; *AdoMet*, *S*-adenosylmethionine; *CH_2THF*, methylenetetrahydrofolate; *CH_3THF*, methyltetrahydrofolate; *DMG*, dimethylglycine; *Cysta*, cystathionine; *Hcy-tl*, homocysteine thiolactone; *THF*, tetrahydrofolate.

Table 1. PCR primers and products for genotyping by MALDI-TOF MS.

			Primers		
PCR[a]	Gene	Polymorphism	Type	Sequence, 5′–3′	Fragment size, bp
Group 1	*MTHFR*	677C>T	Sense	CCA AAG GCC ACC CCG AAG	180
			Antisense	GAA AGA TCC CGG GGA CGA TG	
	MTHFR	1298A>C	Sense	CTT TGG GGA GCT GAA GGA CTA CT	166
			Antisense	ACT CAC TTT GTG ACC ATT CCG GT	
	MTR	2756A>G	Sense	CAC GCC AGG CAG GAA TTA GCA C	272
			Antisense	CAA GCA AAA ATC TGT TTC TAC CAC TTA C	
	MTRR	66A>G	Sense	TGA TTT CTG AGC CAT GGA ATT AGA GTT	408
			Antisense	CGG TAA AAT CCA CTG TAA CGG CTC T	
	CBS	844ins68	Sense	GCA GTT GTT AAC GGC GGT ATT G	252/320
			Antisense	GCC GGG CTC TGG ACT CGA CCT A	
	CBS	699C>T	Sense	GGC GGC TGA AGA ACG AAA TC	456
			Antisense	GCC ATG CCC TGT GTT TGC TAT TA	
	TCII	776C>G	Sense	GGT CGG AGA CAA CGG ATC ACC	310
			Antisense	CAA AGC AAC CCT CGC CTT GA	
	BHMT	742G>A	Sense	GCC ACT TTG ACC CCA CCA TTA GT	155
			Antisense	TGG GAA TTC TGG GAG ATC GAT G	
Group 2	*TCII*	67A>G	Sense	TGG TTG CCT GGC AGT AGC ATC C	397
			Antisense	CCT GCA GAC TGG AGA GGC GTA G	
	RFC1	80G>A	Sense	TGT CAC CTT CGT CCC CTC	160
			Antisense	CGC CAT GAA GCC GTA GA	
	PON1	163T>A	Sense	AAT AAT CAT GGC TTT TGT ACG TTT T	479
			Antisense	TTT GAA AGT GGG CAT GGG TAT ACA G	
	PON1	575A>G	Sense	GTT CCA TTA TAG CTA GCA CGA AGG C	327
			Antisense	GGT GAA ATG TTG ATT CCA TTA GCA A	

[a] Annealing temperature was 66 °C for group 1 reaction and 62 °C for group 2 reaction.

Table 2. Extension primers for genotyping by MALDI-TOF MS.

			Primers				
Gene	Polymorphism	Type	Sequence, 5′–3′	Concentration,[a] pmol	Primer mass, Da	Extended primer mass, Da	Allele
RFC1	80G>A	Sense	CTC CGG TCC TGG CGG C	3	4850.19	5163.40	W[b]
						5147.40	M
PON1	163T>A	Sense	AAA CTG GCT CTG AAG AC	3	5203.48	5491.67	W
						5500.68	M
CBS	844ins68	Antisense	GCG GCT TCA GGG CTC AAG	3.5	5540.66	5853.87	W
						5813.85	M
TCII	67A>G	Antisense	ATG GCT GTC CAT CTC TGG TA	6.5	6099.03	6387.23	W
						6372.22	M
MTHFR	677C>T	Antisense	GCT GCG TGA TGA TGA AAT CG	7.5	6197.11	6510.32	W
						6494.32	M
MTHFR	1298A>C	Sense	GGG AGG AGC TGA CCA GTG AAG	12	6585.36	6882.57	W
						6858.55	M
BHMT	742G>A	Antisense	GGC TCA TCA GGT GAG CTT TCA GT	12	7070.66	7343.85	W
						7358.86	M
CBS	699C>T	Sense	TTT TTG CAA CCC CCT GGC TCA CTA	14.5	7214.76	7487.95	W
						7502.96	M
MTRR	66A>G	Sense	GGC AAA GGC CAT CGC AGA AGA AAT	12.5	7428.94	7726.15	W
						7742.15	M
MTR	2756A>G	Sense	GGA AGA ATA TGA AGA TAT TAG ACA GG	24	8140.42	8437.63	W
						8453.63	M
TCII	776C>G	Sense	CAC CAG TTC CTC ATG ACT TCC CCC ATG C	25	8380.42	8653.61	W
						8693.63	M
PON1	575A>G	Antisense	ACG CTA AAC CCA AAT ACA TCT CCC AGG AT	26	8783.83	9072.03	W
						9057.02	M

[a] Amount in 25-μL reaction volume.
[b] W, wild-type; M, mutant.

PCR primer. Assay conditions, including primers, are listed in Table 3. Because the sequence contexts of *BHMT* 742G>A, *CBS* 699C>T, and *TCII* 776C>G did not include a restriction site, primers were designed to introduce an artificial restriction site.

Each PCR product (10 μL) was digested with 10–15 U of restriction enzyme (Table 3) for 12 h and analyzed by gel electrophoresis. *CBS* 844ins68 was identified by gel electrophoresis without previous restriction enzyme cleavage.

PREPARATION OF SAMPLES FOR MALDI-TOF MS

AG 50W-X8 resin (Bio-Rad) was activated by incubation in a solution of 1 mol/L ammonium acetate (Sigma Aldrich) and then washed five times with deionized water. Ammonia solution (ultrapure; Merck) was then used to adjust the pH of the resin to ~9. The products from minisequencing reactions were incubated for at least 1 h with resin to remove salt adducts. Homogeneous crystallization was achieved by dispensing nanoliter samples on silicon chips (SpectroChip™; Sequenom) prespotted with formulated MALDI matrix. The robot, equipped with four piezoelectric pipettes, dispensed 8 nL of sample at the positions of each hydrophilic anchor of the chip holding 96 samples.

MALDI-TOF MS

A Reflex™ III (Bruker Daltonik) was used for MALDI-TOF MS. The spectrometer was equipped with a nitrogen laser emitting at 337 nm with an 8 Hz pulse rate. The SCOUT™ 384 ion source was run in positive linear ion mode at 25 kV. To avoid detector saturation from matrix ions, masses <4000 Da were suppressed by detector gating. MALDI-TOF MS analyses were run fully automated by fuzzy-logic instrument control, and the numbers of shots per spectrum were 30 or 50 (2 × 25).

DATA PROCESSING

XMASS™ software (Bruker) was used to automate spectrum processing and genotype identification. Spectra were calibrated by linear regression using three oligo(A) nucleotides (15A, 23A, and 30A) as internal standards. Background subtraction and data smoothing preceded peak registration. For all detected peaks, intensities relative to the highest peak in the observed mass range were determined. Signal cutoff was set at a relative intensity of 0.15. The mass window used for signal identification was ±6 Da of the theoretical mass. If data processing detected a peak doublet, the ratio between the signals was calculated to distinguish heterozygous from homozygous samples. The threshold values for the ratio were determined

Table 3. PCR primers and restriction enzymes for the comparison method based on RFLP analysis.

Gene	Polymorphism	Restriction enzyme	Annealing *T*, °C	Primers	
				Type	Sequence, 5′–3′
RFC1	80G>A	*Hha*I	66	Sense	TGT CAC CTT CGT CCC CTC
				Antisense	CGC CAT GAA GCC GTA GA
PON1	163T>A	*Hsp*92II	62	Sense	AAT AAT CAT GGC TTT TGT ACG TTT T
				Antisense	TTT GAA AGT GGG CAT GGG TAT ACA G
PON1	575A>G	*Nde*II	64	Sense	GTT CCA TTA TAG CTA GCA CGA AGG C
				Antisense	GGT GAA ATG TTG ATT CCA TTA GCA A
TCII	67A>G	*Rsa*I	64	Sense	TGG TTG CCT GGC AGT AGC ATC C
				Antisense	CCT GCA GAC TGG AGA GGC GTA G
TCII	776C>G	*Mlu*I	64	Sense	CCA GTT CCT CAT GAC TTC CCC CAC GC[a]
				Antisense	CAA AGC AAC CCT CGC CTT GA
BHMT	742G>A	*Taq*I	64	Sense	CAT GAA GGA GGG CTT GGA GGC TGC TC[a]
				Antisense	TGG GAA TTC TGG GAG ATG GAT GAA TC[a]
CBS	699C>T	*Rsa*I	64	Sense	AAC GCC AGC AAC CCC CTG GCT CAG TA[a]
				Antisense	GCC ATG CCC TGT GTT TGC TAT TA
CBS	844ins68	No enzyme[b]	66	Sense	GCA GTT GTT AAC GGC GGT ATT G
				Antisense	GCC GGG CTC TGG ACT CGA CCT A

[a] Primers introduced an artificial restriction site.
[b] PCR product was analyzed without previous digestion.

empirically, and doublets were identified as heterozygous if the ratio was between 0.56 and 1.8.

ASSAY VALIDATION

The accuracy of the assay was evaluated in terms of failure rate and error rate, which were determined by no-calls and discrepant calls, respectively. Two experiments were carried out. In the first experiment, 368 samples were genotyped by MALDI-TOF MS, and the results for *MTHFR* 677C>T were verified by the real-time PCR assay. In the second experiment, all SNPs were identified in 92 samples by both the MALDI-TOF MS and by the comparison methods. Sample deposition on chips and MALDI-TOF MS analyses were repeated seven times to assess the performance of the MALDI-TOF procedure. In both experiments, we determined the failure and error rates attributable to each step in the protocol and for all SNPs.

IDENTIFICATION OF *CBS* 844ins68

The *CBS* 844ins68 polymorphism is a duplication of 68 bp from intron 7 and exon 8 into exon 8, with changes of 4 bases in the inserted sequence (http://www.uchsc.edu/sm/cbs/cbsdata/ins68.htm). The entire wild-type sequence is also present in the allele containing the insertion. Therefore, a semiquantitative approach was applied to achieve unambiguous allele determination. A unique site inside the insertion was used to distinguish between the wild-type sequence and insertion by probe extension. The ratios between insertion and origin were 0:2 in wild-type, 1:2 in heterozygous, and 2:2 in homozygous individuals.

Results

MALDI-TOF MS SPECTRA

The differences in masses of the four ddNTPs (ddA, 297.21 Da; ddC, 273.19 Da; ddG, 313.21 Da; ddT, 288.20 Da) are the molecular basis for identification of genotypes. Fig. 2 shows the spectra of the 12 extension primers and their single-base extension products. Most of the peaks could be assigned to extension products, and only two signals were unextended primers. The mass difference of the heterozygous signals varied between 9 (ddA/ddT) and 40 Da (ddC/ddG), depending of the incorporated base. The heterozygous extension products of *PON1* 163T>A had the only peak doublet, with a 9-Da difference, and was clearly distinguished. Sodium adducts and depurination products in the spectrum were minor and did not interfere with assignment of genotype.

ASSAY VALIDATION AND PERFORMANCE

Analysis of the MTHFR 677C>T genotypes. In the first experiments, 368 samples were genotyped for the *MTHFR* 677C>T polymorphism by MALDI-TOF MS and real-time PCR. The total failure rate of the MALDI-TOF MS assay was 1.7%. Failures were related to preparation of DNA (1.1%), nanoliter dispensing (0.3%), and mass calibration (0.3%). PCR, primer extension, and spectrum acquisition were without failures (Table 4).

The three variants of the *MTHFR* 677C>T polymorphism were clearly distinguished by MALDI-TOF MS as shown in the scatter plot of allele signal intensities in Fig. 3. The mean ratio of the 677CT cluster was 0.85, which demonstrates that allele signals were not equal. For a few homozygous calls, a minor signal corresponding to the

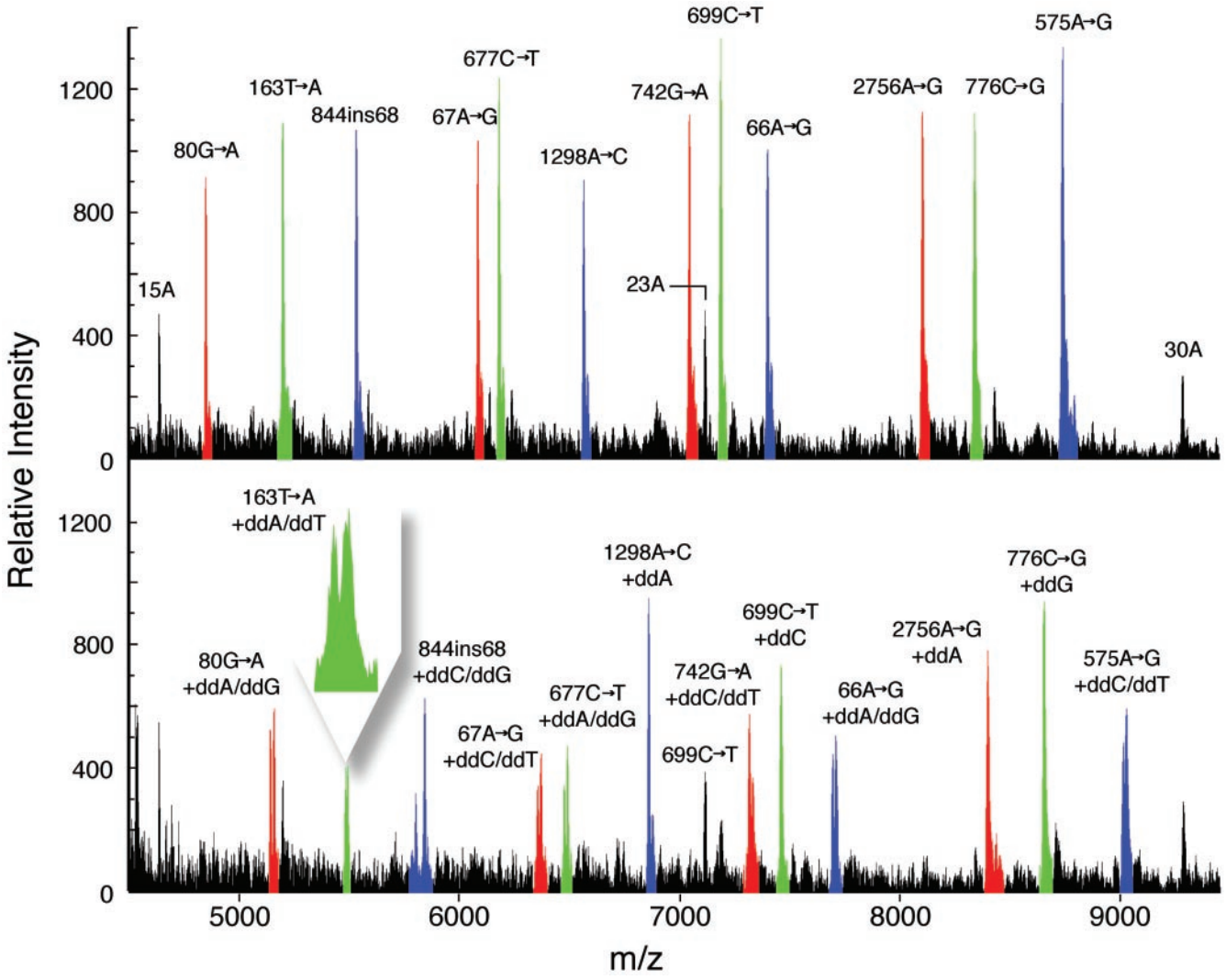

Fig. 2. MALDI-TOF MS analysis of 12 polymorphisms.

The *top panel* shows a spectrum of the extension primers before reaction. Twelve probes were distributed over a mass range of 4500–9500 Da. The concentrations were adjusted to achieve similar signal intensities over the entire mass range. Three oligo(A) nucleotides were included for internal mass calibration. The *bottom panel* shows the extended primers obtained by genotyping a real sample. Nearly all primers were consumed. The *inset* shows the peak of the *PON1* 163TA doublet corresponding to a mass difference of 9 Da. Spectra were acquired in positive linear ion mode.

other allele was detected, and such spurious signals were observed both in the TT and CC sectors (Fig. 3). Furthermore, one homozygous wild-type call by the MALDI-TOF MS assay was identified as heterozygous by the real-time PCR assay.

Replicate analysis of 11 SNPs by MALDI-TOF MS. In the second experiment, all SNPs were identified in 92 samples by comparison methods. DNA purification, PCR, and primer extension worked without failure (Table 4). All 92 samples were carried through the final steps, including the nanoliter dispensing and MALDI-TOF MS analyses, seven times to get an accurate assessment of the performance of the MALDI-TOF procedure. These experiments demonstrated that spectrum acquisition was without failure. The nanoliter dispensing and mass calibration showed 9 failure rates (0.3%) equal to those found in the first experiment (Table 4). Thus, the total failure rate was 0.6%.

All discrepancies were related to misinterpretation of heterozygous signals. The errors were attributable to detection of only one peak of the doublets, resulting from insufficient peak resolution; low mass accuracy; or suppression of signal from one allele (Table 5). We observed 18 discrepant calls, corresponding to an overall error rate of 0.26%. Of these, 14 (78%) were attributable to insufficient peak quality (mass resolution and accuracy) and 4 (22%) to unequal allele signals (Table 5).

Insufficient peak resolution and mass accuracy of the heterozygous signals were essentially confined to two SNPs, *PON1* 163T>A and *PON1* 575A>G. *PON1* 163TA produced four and *PON1* 575AG produced six discrepant

Table 4. Failures attributable to sample preparation and MS.

	Failures, n (%)	
	Experiment 1[a]	Experiment 2[b]
Samples, n	368	92
Sample preparation		
DNA purification	4 (1.1%)	0
PCR	0	0
Primer extension	0	0
Nanoliter dispensing	1 (0.3%)	2 (0.3%)
MS		
Spectrum acquisition	0	0
Mass calibration	1 (0.3%)	2 (0.3%)
Total failures	6 (1.7%)	4 (0.6%)

[a] Experiment 1 involved determination of the *MTHFR* 677C>T genotypes in 368 samples by MALDI-TOF MS and the comparison methods.

[b] In experiment 2, all 11 SNPs were identified in 92 samples by MALDI-TOF MS and the comparison methods, and the failure attributable to sample preparation is based on this number. For these samples, the dispensing and MALDI-TOF MS were repeated seven times, and assessment of the failure rate attributable to these components of the procedure is based on 644 spectra.

calls, which were related to insufficient resolution of one peak within each doublet (Table 5).

The standard variation of the allele signal ratio for seven replicates of one sample was determined for the *MTHFR* 677CT genotype and was 15%.

We analyzed 92 samples by MALDI-TOF MS, applying 50 shots (2 × 25 shots) and 30 shots per sample. Errors were again caused by underestimation of heterozygotes, and the rates were 0.4% at 50 shots and 1.5% at 30 shots. At 50 shots per spectrum, peak broadening by spectrum summation led to insufficient mass resolution, which explained most discrepant calls. The 30-shot spectra suffered from lower signal intensities, which caused both insufficient mass resolution and unequal signal intensities for heterozygous doublets (data not shown).

Spectrum acquisition of one chip (holding 96 samples) required ~20 min at 50 shots per sample and 10 min at 30 shots per sample.

IDENTIFICATION OF *CBS* 844ins68

The theoretical peak-intensity ratio of the mutant to wild-type alleles by MALDI-TOF MS is 0.5 for heterozygotes and 1 for homozygotes (Fig. 4A). Three experiments were carried out to determine the actual threshold for the peak-intensity ratios allowing differentiation of the three genotypes by MALDI-TOF MS.

We first genotyped 92 samples by both the conventional comparison methods and MALDI-TOF MS. A threshold of 0.25 for the ratio of mutant to wild-type alleles distinguished heterozygous from homozygous wild-type samples. The observed error rate was 1.2%.

We then analyzed 368 samples by MALDI-TOF MS; 92 samples had peak-intensity ratios above the threshold of 0.25. Of these samples, 2 (2.1%) were identified as homozygous wild type, 2 as homozygous mutant, and 88 as heterozygous by the comparison method. All heterozygous samples had peak intensity ratios <0.8, whereas the 2 homozygotes had ratios >0.8 (Fig. 4B).

In a last experiment, we analyzed the 2 homozygous and 20 of the heterozygous samples seven times by MALDI-TOF MS. The peak ratios are plotted in the right-hand panel of Fig. 4B. A signal ratio of 0.8 was again found to differentiate between heterozygotes and homozygotes.

Discussion

We report the development and application of a MALDI-TOF MS assay based on multiplex primer extension for the simultaneous determination of 12 polymorphisms in genes encoding enzymes involved in folate or Hcy metabolism. The method is rapid and allows unambiguous identification of 11 SNPs. *CBS* 844ins68 was successfully determined by a semiquantitative approach.

PCR AND PRIMER EXTENSION REACTION

The high level of multiplexing in PCR and primer extension required appropriate primer analysis software and empirical optimization to obtain primer compatibility and

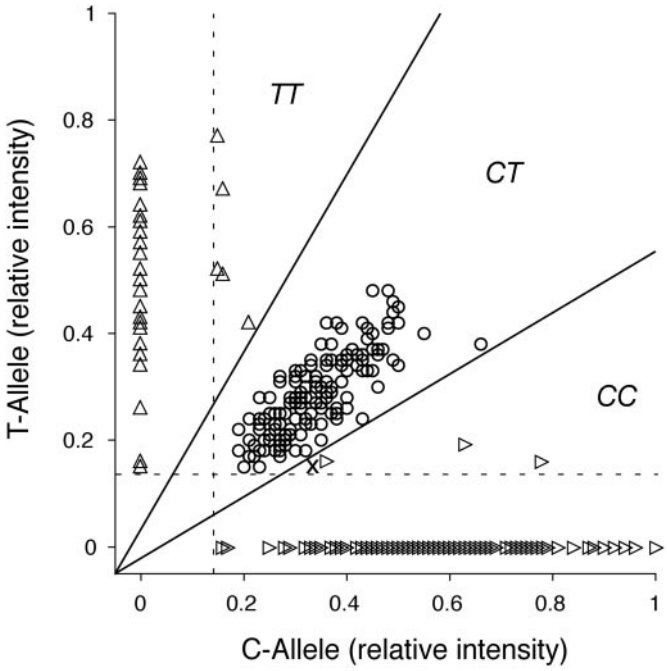

Fig. 3. Relative allele signal intensities of the *MTHFR* 677C>T polymorphism by MALDI-TOF MS.

The relative intensities of the *MTHFR* 677C>T allele signals were plotted for 368 DNA samples. The *x axis* depicts the intensities of the C allele, and the *y axis* the intensities of the T allele. Three sectors were empirically defined for classification of the variants. The cluster of the heterozygous 677CT genotype had a mean signal ratio of 0.85. One sample in the sector of 677CC (marked *X*) was identified as heterozygous by the comparison method and was a discrepant call. Signals with relative intensities below a threshold of 0.15 (*dashed lines*) were recorded as no signal.

Table 5. Discrepant calls of heterozygous genotypes (error rate) attributable to quality of spectrum obtained by MALDI-TOF MS.[a]

		Source of error			
Gene	SNP	Insufficient mass resolution,[b] n	Insufficient mass accuracy,[c] n	Inequality of signal intensities,[d] n	Total errors, n
RFC1	80G>A	0	0	1	1
PON1	163T>A	4	0	1	5
TCII	67A>G	0	0	0	0
MTHFR	677C>T	0	0	0	0
MTHFR	1298A>C	0	1	0	1
BHMT	742G>A	0	0	0	0
CBS	699C>T	0	0	1	1
MTRR	66A>G	0	0	1	1
MTR	2756A>G	1	0	0	1
TCII	776C>G	0	1	0	1
PON1	575A>G	6	1	0	7
Total errors, n		11	3	4	18 (0.26%)[a]

[a] Assessment of error rate was based on 634 acquired spectra, each covering all 11 SNPs.
[b] Heterozygotes were identified as homozygotes because of insufficient resolution of one of the peaks.
[c] Heterozygotes were identified as homozygotes because of insufficient mass accuracy (>6 Da) of one of the peaks.
[d] Heterozygotes were identified as homozygotes because the signal intensity ratios were <0.56 or >1.8.

equal annealing temperatures. In the present assay, the multiplex PCR had to be divided into two separate reactions, whereas the extension primers worked satisfactorily in a single reaction.

The useful mass range of 4000–9000 Da of the TOF MS impacts the design and concentration of minisequencing primers. Fine adjustment of primer concentrations was necessary to obtain similar signal intensities over the entire mass range because sensitivity decreases with mass in TOF MS. The resolving power also decreases with increasing mass; therefore, extension primers creating a heterozygous doublet with a small mass difference should be placed in the lower mass range. This is the case for *PON1* 163T>A, which produced a doublet with a difference of 9 Da (difference between A and T). The extension primer for this SNP was assigned a mass of ~5500 Da (Fig. 1).

Multiplexing >12 SNPs would be desirable in clinical and population studies. However, a higher degree of multiplexing is restricted by the design of site-specific extension probes, which must be arranged in a limited mass range. Expanding the mass range beyond 4000–10 000 Da is essentially impossible, because shorter primers with <15 bases lead to lower priming efficiency, whereas longer primers are detected with decreased sensitivity and resolution in MALDI-TOF MS.

PREPARATION OF SAMPLES FOR MALDI-TOF MS

Analysis of oligonucleotides by MALDI-TOF MS is sensitive to the presence of metal contamination *(62)*. The presence of metal cations, e.g., Na^+, produces adducts, leading to peak broadening, reduced resolution, and low sensitivity. Various sample desalting procedures have been established for DNA analyses by MALDI-TOF MS *(54, 63, 64)*. We used cation-exchange beads as an effective and inexpensive means for desalting low-molecular-weight oligonucleotides. Incubation for 1 h was sufficient to obtain spectra with essentially no cation adducts. Depurination of oligonucleotides is also a potential problem and occurs most frequently at low pH *(65)*. Therefore, resin beads were washed repeatedly before use to remove excess ammonium acetate, but depurination products were still observed occasionally in some MALDI-TOF spectra. A final adjustment of the beads to pH 9 prevented depurination in all analyses.

3-Hydroxypicolinic acid is a widely used matrix for DNA analysis because it provides limited fragmentation *(50)*. However, nonhomogeneous crystallization is obtained with the classic dried droplet preparation, and a search for "hot" spots is required *(45)*. Moreover, mass resolution and accuracy are related to topography of large matrix crystals with a size in the millimeter range. Chip-based nanoliter dispensing on hydrophilic anchors allowed robust and controlled, high-density formation of small single crystals. This miniaturized sample preparation combined with fuzzy-logic-controlled MALDI analysis provided reproducible spectra of high quality.

FAILURE RATES

The maximum failure rate is given in Table 4. Summation of the highest rates observed in both experiments leads to a failure of ≤1.4% attributable to unsuccessful DNA preparation and nanoliter dispensing. Wrong mass calibration increased the failure rate to ≤1.7%, but spectra were easily recalibrated. Multiplex PCR, multiplex primer extension, and spectrum acquisition were without failure and, therefore, the most robust steps of the assay.

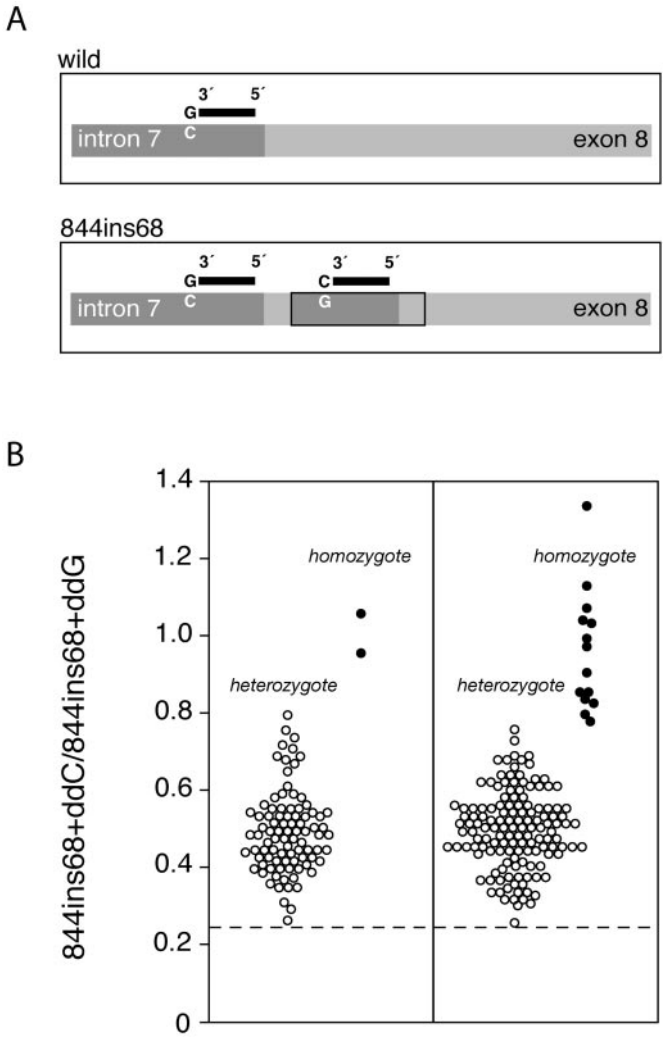

Fig. 4. Determination of *CBS* 844ins68 by quantitative MALDI-TOF MS.

(*A*), graphic presentation of the positions of the 68-bp insertion and extension primers. (*B*), ratios between the signal intensities for the two *CBS* alleles in heterozygous (n = 88) and homozygous (n = 2) samples identified by genotyping 368 samples by RFLP analysis. The signal-intensity ratios obtained from these 368 DNA samples by MALDI-TOF MS were plotted in the *left panel*. Both homozygotes were clearly resolved from the heterozygotes. The *right panel* shows the allele signal ratios of the 2 homozygous and 20 heterozygous samples subjected to seven repetitive analyses. Differentiation between the two variants was again obtained at a signal-ratio threshold of ~0.8. Samples with relative signal intensities below a threshold of 0.25 (*dashed lines*) were classified as wild type.

ERROR RATES

All discrepant calls were caused by incorrect identification of heterozygotes as homozygotes. Low peak quality (insufficient mass resolution, low mass accuracy) occasionally hampered the detection of one of the alleles within a heterozygous doublet, and such mistakes explained the majority (14 of 18) of all errors. In particular, *PON1* 163TA and 575AG gave more discrepant calls than the other SNPs (Table 5). Low resolution of the *PON1* 163TA doublet hindered identification in spite of the primer being placed in the lower mass range. In addition, *PON1* 575AG gave insufficient peak resolution, which was related to the high mass of the extension primer.

A second explanation for incorrect identification of heterozygotes is the inequality of allele signals within the same SNP, which explained 22% (4 of 18) of errors (Table 5). This unbalance has a statistical and systematic component. Repetitive analyses of *MTHFR* 677CT demonstrated a variation in the peak ratio of 15%, which may be related to nonhomogeneous distribution of the two extended primers. A systematic disparity was also observed for *MTHFR* 677C>T (Fig. 3). The mean peak ratio in a heterozygous sample was 0.85 and may be related to preferential incorporation of one dideoxynucleotide and/or allele-specific differences in desorption/ionization probabilities.

SUCCESS RATE

The maximum failure rate (≤1.7%) and the mean error rate (0.26%) give a total success rate of >98%, which demonstrates accurate SNP genotyping by the MALDI-TOF MS method described here. The performance is comparable to or better than that of MALDI-TOF MS SNP analyses published by others *(66–68)*. Duplicate analysis and comparison of simultaneously prepared chips are recommended to identify discrepant calls and may further improve the performance of the assay.

CBS 844ins68 POLYMORPHISM

The *CBS* 844ins68 polymorphism consists of the insertion of 68 bp from intron 7 and exon 8 into exon 8 *(69)*. The wild-type sequence is present in all alleles. Therefore, unambiguous differentiation of heterozygotes from homozygotes by primer extension required a semiquantitative approach. This further demonstrates the quantitative ability of MALDI-TOF MS, which previously has been exploited for the assessment of SNP prevalences in pooled samples *(70, 71)*.

The number of samples homozygous for 844ins68 was too low to determine the exact measurement error, but at this stage, this approach is potentially useful for identifying samples for confirmatory genotyping by conventional methods. Additional work is necessary to determine the accuracy of the MALDI-TOF MS assay for 844ins68.

THROUGHPUT

Acquisition of MALDI-TOF MS spectra for one sample takes <15 s. At a rate of 50 shots/sample, the mean sampling time was 12 s/sample. A lower shot number to increase throughput is not recommended because the error rate would increase significantly. Upgrading the MALDI-TOF MS to a higher laser pulse rate would enhance the throughput without decreasing accuracy. The overall assay throughput of two 96-microtiter plates per day could be further increased by implementing a fully automated 384-sample format for sample preparation.

In conclusion, we have developed an assay for polymorphisms, based on MALDI-TOF MS. The assay is based on allele-specific extension to achieve allelic differentiation

and is characterized by a high degree of multiplexing. Eleven SNPs (*MTHFR* 677C>T, *MTHFR* 1298A>C, *MTR* 2756A>G, *MTRR* 66A>G, *CBS* 699C>T, *BHMT* 742G>A, *RFC1* 80G>A, *TCII* 67A>G, *TCII* 776C>G, *PON1* 163T>A, and *PON1* 575A>G) and 1 insertion polymorphism (*CBS* 844ins68) are included. The assay thus exploits the multiplexing potential, the resolving power, and the quantitative features of the MALDI-TOF platform. All of the polymorphisms measured are involved in folate or Hcy metabolism. Therefore, the assay represents a potentially valuable tool for large population-based studies on the biological role of polymorphisms related to one-carbon metabolism.

We are grateful to Rita Skålevik for excellent technical assistance and Arve Ulvik for valuable discussions. This work was supported by the Foundation to Promote Research into Functional Vitamin B_{12} Deficiency.

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