Proc. Natl. Acad. Sci. USA
Vol. 96, pp. 6301–6306, May 1999
Genetics

Direct genetic analysis by matrix-assisted laser desorption/ionization mass spectrometry

TIMOTHY J. GRIFFIN*, JEFF G. HALL†, JAMES R. PRUDENT†, AND LLOYD M. SMITH*†‡

*Department of Chemistry, University of Wisconsin–Madison, 1101 University Avenue, Madison, WI 53706; and †Third Wave Technologies, Inc., 502 South Rosa Road, Madison, WI 53719

Communicated by James E. Dahlberg, University of Wisconsin Medical School, Madison, WI, April 5, 1999 (received for review February 3, 1999)

ABSTRACT An approach to analyzing single-nucleotide polymorphisms (SNPs) found in the human genome has been developed that couples a recently developed invasive cleavage assay for nucleic acids with detection by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS). The invasive cleavage assay is a signal amplification method that enables the analysis of SNPs by MALDI-TOF MS directly from human genomic DNA without the need for initial target amplification by PCR. The results presented here show the successful genotyping by this approach of twelve SNPs located randomly throughout the human genome. Conventional Sanger sequencing of these SNP positions confirmed the accuracy of the MALDI-TOF MS analysis results. The ability to unambiguously detect both homozygous and heterozygous genotypes is clearly demonstrated. The elimination of the need for target amplification by PCR, combined with the inherently rapid and accurate nature of detection by MALDI-TOF MS, gives this approach unique and significant advantages in the high-throughput genotyping of large numbers of SNPs, useful for locating, identifying, and characterizing the function of specific genes.

Single-nucleotide polymorphisms (SNPs) are the most abundant type of variation found in the human genome, with an estimated frequency of one polymorphic nucleotide per kilobase, giving them utility as genetic markers in linkage and association studies aimed at identifying and characterizing genes involved in biological function and human disease (1–4). The biallelic nature of SNPs makes them less informative than other types of genetic variations, thus large numbers of known SNPs will need to be genotyped to map and identify gene functions of interest (3). This necessitates the development of rapid, robust, and automatable approaches to the high-throughput screening of SNPs (2, 4).

Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) (5, 6) is a promising technology for the high-throughput genotyping of SNPs, and to this end several MALDI-based approaches have been developed in recent years (7–11). The benefits conferred by MALDI-TOF MS to the analysis of biomolecules include (12): (*i*) speed, in that the ionization and detection of the analyte are done in milliseconds; (*ii*) measurement based on the intrinsic mass-to-charge ratio (m/z) of the analyte ions, which is not susceptible to effects of secondary structure; and (*iii*) amenability to automation. Taken together, these aspects of MALDI-TOF MS make it ideally suited to the problem of high-throughput SNP analysis.

Common to almost all existing methods of SNP analysis, MALDI-based or otherwise, is an initial target amplification step using PCR (13), followed by further hybridization or enzymatic manipulation of the resulting PCR amplicon (4, 7–11). Despite its widespread utility in basic research, PCR does have significant limitations when used in a high-throughput setting, including crossover contamination issues (14, 15) and variability of optimal reaction conditions and yields between different amplification targets (16, 17), as well as differential amplification yields of alleles in regions containing sequence polymorphisms (18). Given these inherent limitations to PCR-based high-throughput SNP analysis methods, it is clear that the development of simpler and more direct analysis approaches would be desirable.

We describe an approach to analyzing SNPs in human DNA that employs the Invader (Third Wave Technologies) assay, an isothermal, highly sequence-specific, signal amplification method for the analysis of DNA (19), which does not require an initial PCR amplification of the target sequence. We have developed a rapid, solid-phase sample preparation method facilitating the detection of the products from the Invader assay by MALDI-TOF MS. We show here the successful use of this approach in the analysis of 12 SNPs directly from human genomic DNA. Our results show that this approach is simple, robust, and accurate, and of great potential for the high-throughput analysis of SNPs.

MATERIALS AND METHODS

Invader Squared Reaction. All oligonucleotides used were synthesized by the University of Wisconsin Biotechnology Center (Madison, WI) or Integrated DNA Technologies (Coralville, IA). All probe oligonucleotides used in the primary Invader reaction were purified by PAGE. All other oligonucleotides were synthesized with the trityl group on and purified by using Sep-Pak C_{18} reverse-phase purification cartridges (Waters). Each primary Invader reaction mix consisted of 3 μl of nuclease-free water, 1 μl of 10× reaction buffer (Third Wave Technologies), 1 μl of 10 μM primary Invader oligonucleotide, and 2 μl of 0.5 μg/μl human genomic DNA in water. This reaction mix was incubated at 95°C for 5 min to denature the genomic DNA. The reaction mix was brought to 63°C, and immediately 3 μl of a solution containing 75 nmol of $MgCl_2$, 5 pmol of each of the two primary probe oligonucleotides, and 100 ng of the *Afu* FEN 1 enzyme (Third Wave Technologies) was added to give a final reaction volume of 10 μl. This primary reaction mix was incubated at 63°C for 2 hr. The reaction was then brought to 50°C and the secondary reaction mix (3 μl) was added, which contained 40 pmol of 2′-*O*-methyl-RNA arrestor oligonucleotide, 10 pmol of each secondary probe oligonucleotide and 0.5 pmol of each secondary target oligonucleotide. The secondary reaction was incubated at 50°C for 2 hr.

MALDI-TOF MS Sample Preparation. Solid-phase capture of the biotinylated signal molecules was achieved by adding 100 μg of Dynabeads M-280 streptavidin-coated magnetic beads

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

PNAS is available online at www.pnas.org.

Abbreviations: SNP, single-nucleotide polymorphism; MALDI-TOF MS, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry; αCHCA, α-cyano-4-hydroxycinnaminic acid.

‡To whom reprint requests should be addressed. e-mail: smith@chem.wisc.edu.

(Dynal, Oslo) contained in 120 μl of immobilization buffer (10 mM Tris·HCl/2 M NaCl, pH 7.0) to each completed Invader reaction. This solution was incubated at room temperature for 10 min with gentle shaking. The bead suspension was transferred to a 1.5-ml microcentrifuge tube and placed in a Dynal magnetic concentrator. The beads were then washed once with 125 μl of wash buffer 1 (10 mM diammonium citrate/0.1% SDS, pH 7.0) and then twice with 150 μl of wash buffer 2 (200 mM diammonium citrate). The beads were then resuspended in 150 μl of ultrapure deionized water, transferred to a clean 1.5-ml microcentrifuge tube, and washed three times with 150 μl of ultrapure water. The washed beads were then resuspended in 40 μl of freshly prepared elution buffer (1:1 CH_3OH/30% NH_4OH) and incubated at 60°C for 10 min (20). After this incubation, the microcentrifuge tube was immediately placed in the magnetic concentrator and the supernatant was removed and transferred to a clean tube, being careful to remove the magnetic beads as completely as possible. The volatile elution buffer was then completely removed by centrifugation under reduced pressure for about 15 min. The clean dry sample was then redissolved in 1 μl of 1:1 CH_3CN/ultrapure water.

MALDI-TOF MS Analysis. One microliter of MALDI matrix [1% α-cyano-4-hydroxycinnaminic acid (αCHCA) in 1:1 CH_3CN/ultrapure water] was spotted on the MALDI sample plate and allowed to air-dry (21). To the dried matrix crystals, the resuspended sample in 1 μl of 1:1 CH_3CN/ultrapure water was added and allowed to air dry. MALDI-TOF MS analysis was done on a PerSeptive Biosystems (Framingham, MA) Voyager DE-STR mass spectrometer using a nitrogen laser at 337 nm with an initial accelerating voltage of 20 kV and a delay time of 100 ns. The instrument was run in reflector mode using negative ion detection with external instrument calibration. All spectra acquired consisted of averaged signal from 50–100 laser shots, and the data were processed by using accompanying PerSeptive Biosystems mass spectrometry software.

DNA Sequencing. DNA sequencing of gel-purified PCR amplicons containing each SNP was done by using the Big Dye terminator cycle sequencing kit (Perkin–Elmer). Sequencing products were run on an Applied Biosystems Prism 377 DNA Sequencer at the University of Wisconsin Biotechnology Center. Data processing was done with Applied Biosystems sequencing software.

RESULTS

Mechanism of the Invader Assay. The Invader assay (19) involves the hybridization of two sequence-specific oligonucleotides, one termed the Invader oligonucleotide and the other termed the probe oligonucleotide, to a nucleic acid (in this case DNA) target of interest (Fig. 1*A*). These two oligonucleotides are designed so that the nucleotide on the 3′ end of the Invader oligonucleotide (nucleotide N in Fig. 1*A*) invades at least one nucleotide into the downstream duplex formed by the probe oligonucleotide and the target strand, forming a sequence overlap at that position. The Invader assay is based on the well described (22–25) ability of the 5′-nuclease domains of eubacterial Pol A DNA polymerases and structurally homologous DNA repair proteins called flap endonucleases (FENs) to specifically recognize and efficiently cleave the unpaired region on the 5′ end of the probe oligonucleotide, resulting in a 3′-hydroxyl terminating DNA cleavage product. Relative to a flap formed by simple noncomplementarity of the 5′ end of the probe oligonucleotide to the target, a flap that contains sequence overlap between the Invader and probe oligonucleotide is cleaved at a dramatically enhanced rate 3′ of the nucleotide located at the position of overlap (19) (indicated in boldface in the probe oligonucleotide in Fig. 1*A*). It has also been shown that while the nucleotide at the position of overlap

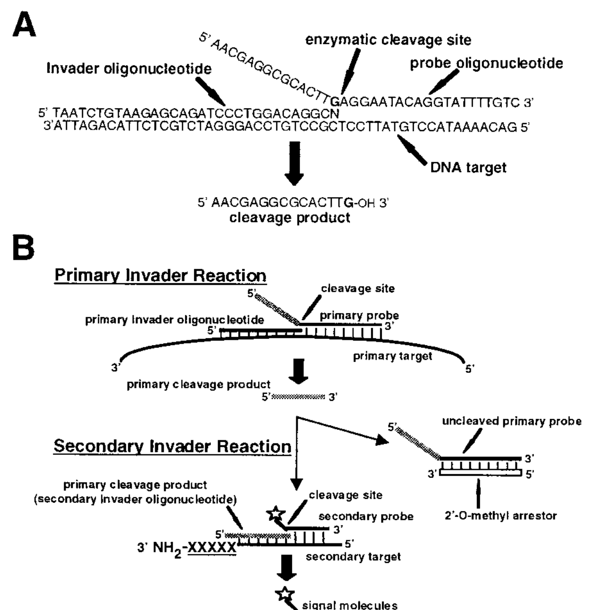

FIG. 1. Mechanism of the Invader assay (*A*) and the Invader squared assay (*B*).

contained in the probe oligonucleotide has a strict requirement of complementarity to the target, the overlapped nucleotide (indicated as N) on the 3′ end of the Invader oligonucleotide does not have to be complementary to the target for efficient enzymatic cleavage of the 5′ flap (19). The use of thermostable variants of these flap endonucleases permits the reaction to be run near the melting temperature (T_m) of the duplex formed between the probe oligonucleotide and target, such that cleaved and uncleaved probe oligonucleotides will cycle off and on the target strand. Thus, with excess probe oligonucleotide present in solution, when a probe oligonucleotide is cleaved it is replaced by an uncleaved probe oligonucleotide, which is in turn cleaved and replaced and so on, resulting in a linear accumulation of cleavage product with respect to both time and target strand concentration. The ability of the Invader assay to discriminate with high specificity between DNA target sequences differing by a single nucleotide has been shown (19).

The Invader Squared Assay. A modification of the Invader assay, called the Invader squared assay, has also been developed (J.G.H., unpublished work), and it is detailed in Fig. 1*B*. The Invader squared assay is a two-step reaction, in which a primary invasive cleavage reaction is directed against a DNA target of interest, producing an oligonucleotide cleavage product as shown in Fig. 1*A*. This cleavage product in turn serves as an Invader oligonucleotide in a secondary invasive cleavage reaction directed against a target oligonucleotide and probe oligonucleotide that are externally introduced into the reaction mix, producing secondary cleavage products (signal molecules) that are then detected. The use of two sequential stages of cleavage reactions approximately squares the amount of amplification of cleavage products compared with a single-stage Invader reaction.

Along with the increased amplification of signal molecules when the Invader squared assay is used, there is also an increased potential for the presence of nonspecific background signal. One step taken to suppress this background potential is to add an excess of a 2′-*O*-methyl-RNA oligonucleotide to the secondary reaction mix, called the arrestor oligonucleotide, that is complementary to the target hybridization sequence of the primary probe oligonucleotide (J.G.H., unpublished work). This arrestor oligonucleotide anneals to the uncleaved primary probe oligonucleotide molecules present after the

primary reaction (Fig. 1*B*), rendering the 5′ cleavage product sequence still present on these probe molecules unavailable to undergo hybridization with the secondary target, which can lead to background signal accumulation if allowed to occur. 2′-*O*-methyl-RNA nucleotides are not recognized by the FEN 1 enzyme, ensuring that there is no additional enzymatic cleavage of the structure formed between the arrestor and the probe oligonucleotides. Another step taken to suppress background is to designate the last five nucleotides on the 3′ end of the secondary target as 2′-*O*-methyl-RNA (denoted as underlined Xs in Fig. 1*B*), and also to have a 3′-amino group (3′-amino-modifier C3, Glen Research, Sterling, VA), rendering this end of the target inert to the enzyme (J.G.H., unpublished results). Design of the secondary target in this way is necessary because the 3′ end of the relatively short target has the potential to wrap around and act as the Invader oligonucleotide with the secondary probe oligonucleotide, causing nonspecific cleavage and background accumulation of signal molecules. The Invader squared assay was used in this work to obtain signal at a level necessary for robust detection by MALDI-TOF MS.

Invader Squared Assay Design for SNP Genotyping by MALDI-TOF MS. Fig. 2 shows the design of the Invader squared assay employed here for the analysis of SNPs in human genomic DNA by MALDI-TOF MS. Fig. 2*A* details the general design of the primary reaction. For any SNP, two allele-specific probe oligonucleotides and one Invader oligonucleotide were designed. The probe oligonucleotides each contain an allele-specific hybridization portion (gray shaded region of probes in Fig. 2*A*), differing in sequence by only one nucleotide, at the SNP position of the target. These differing nucleotides are designated in Fig. 2A as X and Y, each being complementary to one of the two possible nucleotides at the SNP position. The nucleotide sequences 5′ of X and Y in the probe oligonucleotides (designated as arm 1 and arm 2) are not complementary to the target DNA, and they are designed specifically for use in the secondary Invader reaction. The primary Invader oligonucleotide is designed to be complementary to the target upstream of the probe oligonucleotide region, with one additional 3′ nucleotide (indicated as N in Fig. 2*A*) that overlaps the probe oligonucleotide sequence at the SNP position, so that enzymatic cleavage occurs immediately 3′ of nucleotide X or Y in the probe oligonucleotide. The nucleotide N of the primary Invader oligonucleotide is designed to be noncomplementary to either of the two possible nucleotides found at the SNP position. Previous studies (19) have shown that target specificity is optimal when the polymorphic nucleotide is located at the position of overlap between the probe oligonucleotide and the Invader oligonucleotide; this design confers threefold specificity for SNP detection: First, the Invader oligonucleotide must be complementary to the target and anneal to form the correct overlap structure with the correctly annealed probe oligonucleotide. Second, the endonuclease used in the Invader assay has a strict requirement of absolute complementarity between the target and the nucleotide that occurs at the overlap position in the probe oligonucleotide. Thus, nucleotides X or Y in the probe oligonucleotide must be perfectly complementary to the target at the SNP position in order for the enzyme to recognize the overlap structure and for cleavage to occur. Third, a mismatch at the polymorphic nucleotide between the probe oligonucleotide and the target is thermodynamically destabilizing when the reaction is run near the T_m of the duplex. This highly stringent threefold specificity results in the allele-specific accumulation of cleavage products. If the nucleotide complementary to the allele 1 probe is present, then arm 1 cleavage product will accumulate; if the allele 2 nucleotide is present, arm 2 will accumulate; in the case of a heterozygote, both cleavage products will accumulate at similar rates.

The primary reaction was allowed to run for 2 hr at 63°C, resulting in the allele-specific accumulation of either or both arm 1 and arm 2 cleavage products. The reaction was then brought to 50°C and a secondary reaction mix was added that included two allele-specific secondary target oligonucleotides, two secondary probe oligonucleotides, and one arrestor oligonucleotide that anneals to the hybridization sequence common to each of the primary allele-specific probe oligonucleotides (gray shaded region of probes in Fig. 2*A*). The sequences of the secondary target and probe oligonucleotides were designed so that the cleavage products from the primary Invader reaction would anneal specifically to one of the secondary targets and act as the Invader oligonucleotide in the secondary reaction (Fig. 2*B*). The two allele-specific secondary systems were designed to produce biotinylated signal molecules of unique molecular weights, so that in the subsequent MALDI-TOF MS analysis the deprotonated, negative, singly charged molecular ion values detected ($[M - H]^-$ values) would be distinct from each other ($[M - H]^- = 1234$ for allele 1 product and 1538 for allele 2 product). The signal molecules contained a biotin group to enable their solid-phase

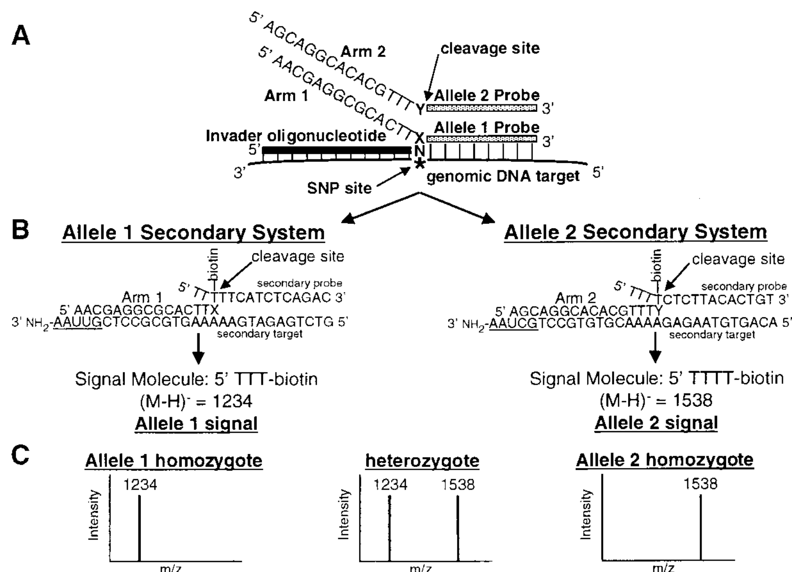

FIG. 2. SNP genotyping by the Invader squared assay and MALDI-TOF MS.

Table 1. SNP genotyping results

SNP	Chromosome	Sequence (5′ to 3′)	MALDI-TOF MS genotyping result
Factor V Leiden*	1	CCT[C/T]GCC	[C]
STS WI-867†	7	TCC[A/G]TTT	[G]
STS WI-921†	11	TCT[A/G]GGG	[A/G]
STS WI-1126†	7	TTT[T/C]GGA	[T]
STS WI-1325†	20	TCT[C/T]ACC	[T]
STS WI-1803†	4	GAT[A/G]AGT	[A/G]
STS WI-2026†	6	ATC[A/G]TTT	[A]
STS WI-2032†	9	TCA[G/C]CTT	[G/C]
STS WI-2529†	10	CAA[T/C]TCT	[T]
WIAF-1432‡	18	TTG[C/T]AGG	[T]
WIAF-123‡	16	TTT[A/T]ACA	[T]
WIAF-2057‡	22	ATT[G/C]AAT	[G/C]

The two possible polymorphic nucleotides are indicated in brackets within each SNP target sequence. A single nucleotide in the results column indicates a homozygote and two nucleotides indicate a heterozygote.
*This is a single-nucleotide substitution mutation (26).
†These SNPs have been previously described (27). STS, sequence-tagged site.
‡These SNPs are from the Whitehead Institute for Biomedical Research/MIT Center for Genome Research human SNP database available on-line at http://www.genome.wi.mit.edu/SNP/human/index.html.

purification, as described in *Materials and Methods*. The production of the signal molecules is contingent on the identity of the cleavage products produced in the allele-specific primary reaction, so that the peaks detected in the MALDI-TOF MS analysis are specific to the alleles present in the genomic DNA sample. Fig. 2*C* shows the three possible MALDI-TOF MS outputs from this Invader system, corresponding to two possible homozygous genotypes (a single peak at an m/z value of either 1234 or 1538) or a heterozygous genotype (peaks at both m/z values). The same two arm sequences shown in Fig. 2*A* were used in every pair of SNP-specific primary probe oligonucleotides, which enabled the use of the same secondary oligonucleotides and signal outputs for each unique SNP analyzed. The nucleotides X and Y do not have to be complementary to the secondary target, so primary cleavage products containing any of the four possible nucleotides at the X and Y positions can work as Invader oligonucleotides in the secondary reaction.

SNP Genotyping Results. Twelve unique SNPs were successfully genotyped directly from genomic DNA by using this method, and these results are presented in Table 1. For each SNP analyzed, the primary Invader reaction contained 1 μg of genomic DNA and was run at 63°C for 2 hr, followed by a 2-hr secondary Invader reaction run at 50°C. Eleven of these SNPs are located in sequence-tagged sites (STSs) (28). The position of the factor V Leiden single-nucleotide substitution mutation (26) was also analyzed successfully. PCR-amplified DNA from the same genomic sample was sequenced for each SNP analyzed to confirm the results obtained by the Invader assay and MALDI-TOF MS. Fig. 3 shows representative results from four different SNPs, where both the MALDI-TOF MS output and the conventional sequencing results of the relevant target region are shown.

DISCUSSION

All 12 of the SNPs analyzed by this approach gave unambiguous mass spectral results. Fig. 3 *A* and *B* show representative results for homozygous genotypes, where in each case a single peak at the correct m/z value is observed in the mass spectrum, and this result is confirmed by the sequencing trace. In the case of heterozygous genotypes, two peaks of roughly equal intensities are observed in the mass spectrum, corresponding to the presence of both alleles (Fig. 3 *C* and *D*); the corresponding sequencing traces show two overlapping peaks at the polymorphic nucleotide, confirming these results. It is also clear that this method effectively discriminates different types of SNPs (G-to-A transitions, G-to-C transversions, etc.). For all of the SNPs analyzed, negative controls were also run in which the Invader oligonucleotide in the primary reaction was omitted, but all other reaction components were added as normal, and in all cases these negative controls showed negligible background signal.

In all but one case, the MALDI-TOF MS genotyping results perfectly matched the sequencing results of PCR amplicons

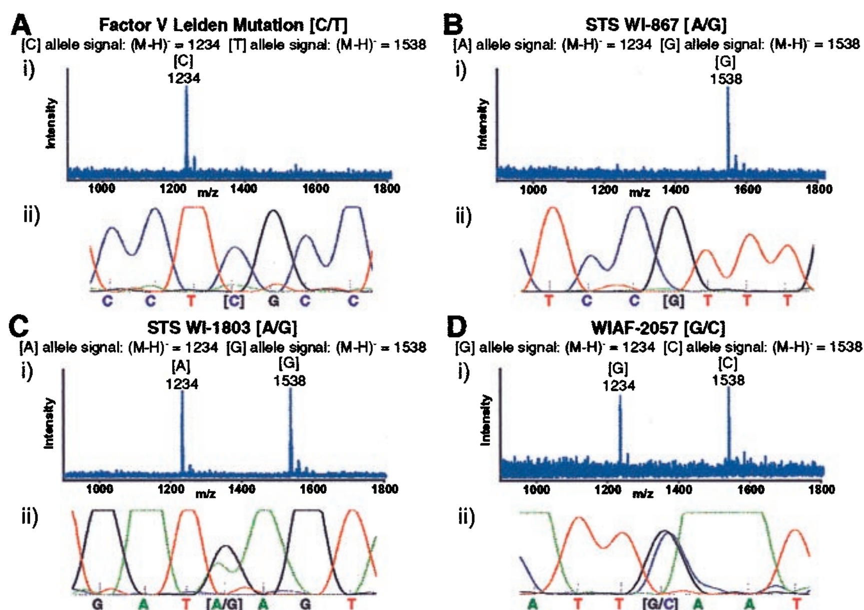

FIG. 3. Four representative genotyping results. The two possible nucleotides at the polymorphic position are given in brackets next to the name of each SNP analyzed. The expected signal molecule deprotonated, negative singly charged ion ($[M - H]^-$) m/z for each of the possible two alleles is also given, along with (*i*) the MALDI-TOF MS analysis result and (*ii*) the relevant portion of the four-color sequencing trace, where the polymorphic nucleotide position is given in brackets.

containing each SNP position. For the SNP identified as WIAF-1432 in Table 1, analysis by the Invader assay and MALDI-TOF MS approach unambiguously and reproducibly identified the polymorphic nucleotide to be a T (C and T are the two possible nucleotides at this polymorphic position) for the genomic DNA sample analyzed. However, dye-terminator sequencing of the PCR product containing this SNP showed discordant results. In the sequencing of the forward strand, the polymorphic nucleotide was identified as a C; in the sequencing of the reverse strand, the nucleotide was identified as a T. Although the sequence analysis software was able to identify the polymorphic nucleotide position in both cases, the corresponding fluorescence peaks in the sequencing trace had low signal intensities and were poorly resolved in comparison to other peaks in the trace. Upon inspection of the local sequence around this SNP position, it was discovered that the sequence has the potential to form a hairpin structure, with the polymorphic nucleotide position contained in a three-nucleotide loop resulting from the formation of a seven-base-pair stem structure containing fairly stable G-T mismatch base pairs (29, 30). The poor quality sequencing data and resulting base identification discrepancy may be due to a gel compression caused by this secondary structure formation. The results from the Invader assay and MALDI-TOF MS analysis of this SNP were unambiguous and reproducible, suggesting that the elevated temperature of the primary Invader reaction (63°C), in conjunction with the specific hybridization requirement of the probe and Invader oligonucleotides, may make this assay less susceptible to problems resulting from secondary structures in the target DNA compared with other methods.

The MALDI-TOF mass spectrometric analysis of the signal molecules produced in the Invader squared assay was very rapid, taking only a matter of seconds per sample to obtain a high quality mass spectrum that gave unambiguous SNP genotyping results. The total allele-specific arm cleavage product produced in the primary reaction when starting with 1 μg (0.5 amol) of human genomic DNA and allowing the reaction to run for 2 hr is calculated by estimating the enzymatic turnover rate to be 10 cleavage events per minute, to give 10 turnovers per min $\times$ 120 min $\times$ 0.5 amol of target = 600 amol of total arm cleavage product. In the secondary reaction, the secondary target is in excess so that the output is limited by the amount of primary arm cleavage product that acts as the Invader oligonucleotide in the secondary step. Running the secondary reaction for 2 hr results in the production of approximately 720 fmol of signal molecules (10 turnovers per min $\times$ 120 min $\times$ 0.6 fmol of primary arm cleavage molecules), which are very easily detected after sample preparation by MALDI-TOF MS. This predicted yield of signal molecules produced in the Invader squared reaction was confirmed by comparison of MALDI-TOF MS signal intensities obtained in control experiments analyzing known amounts of synthetic signal molecule oligonucleotides, with intensities obtained for signal molecules produced in the Invader squared reaction (data not shown).

The signal molecules were designed to be composed entirely of deoxythymidine nucleotides because these oligonucleotides are most resistant to backbone fragmentation in the MALDI process (31, 32), and they are consequently detected with the greatest sensitivity compared with oligonucleotides of mixed nucleotide composition. Use of these stable homodeoxythymidine signal molecules also allowed for the use of αCHCA as the MALDI matrix, which cannot be used for the analysis of oligonucleotides containing mixed nucleotide sequences because of substantial backbone fragmentation of these oligonucleotides in this matrix (33). Use of αCHCA is advantageous because this matrix forms a more confluent, easily sampled, "lawn" of crystals on the MALDI probe tip than other MALDI matrices commonly used for DNA analysis. The dispersed crystals formed by other matrices may necessitate some searching on the probe tip to obtain satisfactory signal, which is detrimental to automated sampling in MALDI-TOF MS analyses. Another interesting point is that the sample preparation method employed captures not only the biotinylated signal molecules but also the biotinylated, uncleaved secondary probe oligonucleotides that are added in excess (10 pmol of each probe) in the secondary reaction (Fig. 2*B*). As slightly less than 10% of the secondary probes are cleaved in the secondary reaction, more than 9 pmol of each biotinylated, uncleaved probe oligonucleotide is present along with the cleaved signal molecules. Initial experiments (data not shown) showed that significant amounts of uncleaved probes are immobilized on the streptavidin-coated beads, eluted, and spotted on the MALDI probe tip along with the signal molecules. The presence of these longer probes in the MALDI-TOF MS analysis was a potential concern because of the fragmentation propensity of these oligonucleotides when αCHCA is used as the matrix (33). The resultant fragment ions could have m/z values similar to those of the signal molecules produced in the secondary Invader reaction, leading to increased background levels and possibly yielding false-positive results. However, these fragment ions were not evident in the MALDI-TOF mass spectra. The most likely explanation for this finding is that because long oligonucleotides require higher laser powers for MALDI-TOF MS analysis than short oligonucleotides (34), the laser power used to efficiently detect the short homodeoxythymidine signal molecules is not sufficient to produce signal (or fragmentation products) from the longer uncleaved probe oligonucleotides. This effectively results in a differential detection of the desired signal molecules without interference from the uncleaved probe oligonucleotides or their fragmentation products.

The design of the sequences of the oligonucleotides used in the Invader assay is straightforward, with the only design criterion being that the sequences have duplex thermal stabilities that enable them to be used at the desired reaction temperature (19). Standard nearest-neighbor methods for predicting DNA duplex stabilities in terms of melting temperatures (T_ms) under the enzymatic reaction conditions were used (35, 36) to design the oligonucleotides. This approach resulted in primary probe oligonucleotides with hybridization sequences (gray shaded region in Fig. 2*A*) that were 16–23 nucleotides in length, depending on the target sequence, and gave predicted T_m values 4–7°C above the reaction temperature of 63°C. The primary Invader oligonucleotides were designed to have a T_m about 15–20°C above the corresponding probe oligonucleotides, and were 30–40 nucleotides in length depending on the target sequence. The secondary reaction oligonucleotides, shown in Fig. 2*B*, were designed similarly to work at a reaction temperature of 50°C. All of the oligonucleotides designed for the SNP analyses shown worked on the first attempt. Therefore, the design of oligonucleotide sequences for use in the Invader assay is simple and robust, indicating that the Invader assay should be effective in analyzing the vast majority of SNPs found throughout the human genome.

The strategy presented here describes a 2-fold multiplexed system (two alleles analyzed per one Invader squared reaction). The efficacy of 24-fold multiplex analysis of 12 separate SNPs by MALDI-TOF MS has been recently shown in a PCR-based assay (37). Further multiplexing would greatly increase the power of our approach. As evidenced by the results presented here, the Invader assay has the ability to specifically analyze different SNP positions under the same reaction conditions, giving it great potential for multiplex SNP analysis. In a multiplex format, a unique secondary oligonucleotide system would be designed for each different SNP allele being analyzed in the same reaction tube. By designing each allele-specific signal molecule to have a unique mass, the signal molecules from several different multiplex reactions

could be pooled after the Invader reaction for purification and simultaneous detection by MALDI-TOF MS. In this way it may be possible to analyze up to hundreds of SNP alleles per sample spot on the MALDI sample plate. Multiplexing at such a high level would nevertheless still be an ambitious undertaking, as the sequences of the secondary oligonucleotides would have to be chosen carefully so as to not cause cross-interference with each other and consequent accumulation of nonspecific background signals. Furthermore, purification methods may need to be developed to remove the many uncleaved secondary probe oligonucleotides and prevent signal suppression in the MALDI-TOF MS analysis. Reaction conditions that eliminate the need for the arrestor oligonucleotide would help to simplify the assay.

The 1 μg of genomic DNA per SNP currently used in this approach could be reduced by increasing the sensitivity of the MALDI-TOF MS detection of the signal molecules. Miniaturization of the crystalline matrix sample spot on the MALDI sample plate can substantially decrease the detection limit by effectively increasing the concentration of analyte within the volume of matrix crystals that is irradiated by each laser pulse, thus giving a significant boost in the detection sensitivity observed by MALDI-TOF MS. Low femtomole sensitivities for mixed sequence oligonucleotides have been achieved by using nanoliter sample volumes deposited by a piezoelectric device (38). Simpler methods employing MALDI sample plates coated with hydrophobic materials such as paraffin (39) or Teflon (40) cause aqueous matrix solutions to concentrate into substantially smaller volumes than on metal sample plates when the conventional "dried droplet" method (5) is used for sample deposition. As robust detection of DNA present in amounts as low as 25 fmol has been shown with these simple miniaturization techniques (40), there is hope that a similar increase in MALDI-TOF MS sensitivity can be achieved that would allow the amount of genomic DNA required in the Invader reaction to be decreased to low nanogram levels competitive with PCR-based assays (13).

In conclusion, integrating the inherent benefits of the Invader assay (highly specific, direct signal amplification without the need for target amplification by PCR) with those conferred by MALDI-TOF MS (extremely rapid and accurate signal detection) represents a significant advance in the development of approaches for the high-throughput genotyping of SNPs. The Invader reaction, sample preparation step, and MALDI-TOF MS analysis can be completed in a total of about 5 hr; by comparison, SNP genotyping by sequencing PCR products, which entails PCR amplification and sample clean-up, followed by enzymatic sequencing and sample clean-up, and finally separation and detection of the sequencing products by gel electrophoresis, takes more than a full day in total to complete. Finally, the relatively simple, isothermal Invader assay and the solid-phase sample preparation procedure lend themselves nicely to automated sample handling, which, combined with the advantages outlined above, make this a powerful approach to the high-throughput genotyping of SNPs for genetic analysis.

We thank David Arco, Stephanie Vavra, Tamara Sander, and David Marshall of Third Wave Technologies for their assistance with this work, and also Mary Ann Brow of Third Wave Technologies for her help in preparing this manuscript. T.J.G. was supported in part by Grant DE-FG02-91ER61130 from the U.S. Department of Energy and by a Proctor and Gamble predoctoral fellowship. J. E. Dahlberg and L.M.S. each have a financial interest in Third Wave Technologies.

1. Wang, D. G., Fan, J.-B., Siao, C.-J., Berno, A., Young, P., Sapolsky, R., Ghandour, G., Perkins, N., Winchester, E., Spencer, J., *et al.* (1998) *Science* **280,** 1077–1082.
2. Schafer, A. J. & Hawkins, J. R. (1998) *Nat. Biotechnol.* **16,** 33–39.
3. Kruglyak, L. (1997) *Nat. Genet.* **17,** 21–24.
4. Landegren, U., Nilsson, M. & Kwok, P.-Y. (1998) *Genome Res.* **8,** 769–776.
5. Karas, M. & Hillenkamp, F. (1988) *Anal. Chem.* **60,** 2299–2303.
6. Hillenkamp, F., Karas, M., Beavis, R. C. & Chait, B. T. (1991) *Anal. Chem.* **63,** 1193A–1202A.
7. Griffin, T. J., Tang, W. & Smith, L. M. (1997) *Nat. Biotechnol.* **15,** 1368–1372.
8. Ross, P. L., Lee, K. & Belgrader, P. (1997) *Anal. Chem.* **69,** 4197–4202.
9. Haff, L. A. & Smirnov, I. P. (1997) *Genome Res.* **7,** 378–388.
10. Higgins, G. S., Little, D. P. & Koster, H. (1997) *BioTechniques* **23,** 710–714.
11. Fei, Z., Ono, T. & Smith, L. M. (1998) *Nucleic Acids Res.* **26,** 2827–2828.
12. Smith, L. M. (1996) *Nat. Biotechnol.* **14,** 1084, 1087.
13. Saiki, R. K., Gelfand, D. H., Stoffel, S., Scharf, S. J., Higuchi, R., Horn, G. T., Mullis, K. B. & Erlich, H. A. (1988) *Science* **239,** 487–491.
14. Erlich, G. D. (1994) in *PCR-Based Diagnostics in Infectious Disease*, eds. Erlich, G. D. & Greenberg, S. J. (Blackwell Scientific, Oxford), pp. 3–18.
15. Kwok, S. & Higuchi, R. (1989) *Nature (London)* **339,** 237–238.
16. Farrell, R. E. (1997) *Immunol. Invest.* **26,** 3–7.
17. Vaneechoutte, M. & Van Eldere, J. (1997) *J. Med. Microbiol.* **46,** 188–194.
18. Barnard, R., Futo, V., Pecheniuk, N., Slattery, M. & Walsh, T. (1998) *BioTechniques* **25,** 684–691.
19. Lyamichev, V., Mast, A. L., Hall, J. G., Prudent, J. R., Kaiser, M. W., Takova, T., Kwiatkowski, R., Sander, T., de Arruda, M., Arco, D., *et al.* (1999) *Nat. Biotechnol.* **17,** 292–296.
20. Jurinke, C., Van den Boom, D., Collazo, V., Luchow, A., Jacob, A. & Koster, H. (1997) *Anal. Chem.* **69,** 904–910.
21. Gut, I. V., Jeffery, W. A., Pappin, D. J. & Beck, S. (1997) *Rapid Commun. Mass Spectrom.* **11,** 43–50.
22. Lindquist, R. C. & Olivera, B. M. (1982) *Cell* **31,** 53–60.
23. Lyamichev, V., Brow, M. A. & Dahlberg, J. E. (1993) *Science* **260,** 778–783.
24. Harrington, J. J. & Lieber, M. R. (1997) *Genes Dev.* **8,** 1344–1355.
25. Lieber, M. R. (1997) *BioEssays* **19,** 233–240.
26. Bertina, R. M., Koeleman, B. P., Koster, T., Rosendaal, F. R., Dirven, R. J., de Ronde, H., van der Velden, P. A. & Reitsma, P. H. (1994) *Nature (London)* **369,** 64–67.
27. Kwok, P.-Y., Deng., Q., Zakeri, H., Taylor, S. L. & Nickerson, D. A. (1995) *Genomics* **31,** 123–126.
28. Hudson, T. J., Stein, L. D., Gerety, S. S., Ma, J., Castle, A. B., Silva, J., Slonim, D. K., Baptista, R., Kruglyak, L., Xu, S. H., *et al.* (1995) *Science* **270,** 1945–1954.
29. Aboula-ela, F., Koh, D., Tinoco, I. Jr., & Martin, F. H. (1985) *Nucleic Acids Res.* **13,** 4811–4824.
30. Ikuta, S., Takagi, K., Wallace, R. B. & Itakura, K. (1987) *Nucleic Acids Res.* **15,** 797–811.
31. Zhu, L., Parr, G. P., Fitzgerald, M. C., Nelson, C. M. & Smith, L. M. (1995) *J. Am. Chem. Soc.* **117,** 6048–6056.
32. Tang, W., Nelson, C. M., Zhu, L. & Smith, L. M. (1997) *J. Am. Soc. Mass Spectrom.* **8,** 218–224.
33. Zhu, Y. F., Tarenenko, N. I., Allman, S. L., Martin, S. A., Haff, L. & Chen, C. H. (1996) *Rapid Commun. Mass Spectrom.* **10,** 1591–1596.
34. Wu, K. J., Shaler, T. A. & Becker, C. H. (1994) *Anal. Chem.* **66,** 1637–1645.
35. Breslauer, K. J., Frank, R., Blocker, H. & Marky, L. A. (1986) *Proc. Natl. Acad. Sci. USA* **83,** 3746–3750.
36. Wetmur, J. G. (1991) *Crit. Rev. Biochem. Mol. Biol.* **26,** 227–259.
37. Ross, P., Hall, L., Smirnov, I. & Haff, L. (1998) *Nat. Biotechnol.* **16,** 1347–1351.
38. Little, D. P., Cornish, T. J., O'Donnell, M. J., Braun, A., Cotter, R. J. & Koster, H. (1997) *Anal. Chem.* **69,** 4540–4546.
39. Hung, K. C., Rashidzadeh, H., Wang, Y. & Guo, B. (1998) *Anal. Chem.* **70,** 3088–3093.
40. Hung, K. C., Ding, H. & Guo, B. (1999) *Anal. Chem.* **71,** 518–521.