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Single nucleotide polymorphism determination using primer extension and time-of-flight mass spectrometry

The high frequency of single nucleotide polymorphisms (SNPs) in the human genome makes them a valuable source of genetic markers for identity testing, genome mapping, and medical diagnostics. Conventional technologies for detecting SNPs are laborious and time-consuming, often prohibiting large-scale analysis. A rapid, accurate, and cost-effective method is needed to meet the demands of a high-throughput DNA assay. We demonstrate here that analysis of these genetic markers can now be performed routinely in a rapid, automated, and high-throughput fashion using time-of-flight mass spectrometry and a primer extension assay with a novel cleavable primer. SNP genotyping by mass spectrometry involves detection of single-base extension products of a primer immediately adjacent to the SNP site. Measurement of the mass difference between the SNP primer and the extension peak reveals which nucleotide is present at the polymorphic site. The primer is designed such that its extension products can be purified and chemically released from the primer in an automated format. The reduction in size of the products as a result of this chemical cleavage allows more accurate identification of the polymorphic base, especially in samples from a heterozygotic population. All six possible heterozygotes are resolved unambiguously, including an A/T heterozygote with extension products differing by only 9 Da. Multiplex SNP determination is demonstrated by simultaneously probing multiple SNP sites from a single polymerase chain reaction (PCR) product as well as from multiplexed PCR amplicons. Samples are processed in parallel on a robotic workstation, and analyzed serially in an automated mass spectrometer with analysis times of only a few seconds per sample, making it possible to process thousands of samples per day.

Keywords: Single nucleotide polymorphism / Time-of-flight mass spectrometry / Heterozygote determination / Mitochondrial DNA / High-throughput DNA analysis EL 3381

1 Introduction

Single nucleotide polymorphisms (SNPs) are the most frequent form of DNA sequence variation in the human genome and, as such, are becoming increasingly popular genetic markers for genome mapping studies, medical diagnostics, and identity testing [1–4]. SNPs are typically biallelic with two possible nucleotides (alleles) having frequencies of > 1% throughout the human population at a particular site in the genome. Although SNPs are less polymorphic than the currently used microsatellite markers, SNPs are more abundant – occurring approximately every kilobase [5]. Conventional technologies for detecting and scoring SNPs (*e.g.*, direct sequencing) are laborious and time-consuming, prohibiting large-scale analysis without great effort and expense [6, 7]. Detection of SNPs is commonly performed using single-strand conformational polymorphism assays or denaturing gradient gel electrophoresis [7]. More recently, denaturing high-performance liquid chromatography has been used for identification of candidate SNP sites [8]. After an SNP site has been identified and confirmed through sequence analysis, scoring assays are performed to determine the nucleotide(s) present in the sample population of interest. A number of PCR-based strategies for scoring SNP markers have been developed recently – including restriction digestion [7], ligation assays [4, 7], allele-specific PCR [2, 7], primer extension assays [9–13], and hybridization assays using fluorescent probes with solution-phase [14, 15] and solid-phase detection formats [3]. Scoring methods must be rapid, accurate, and cost-effective to meet the demands of a high-throughput DNA assay. This report will focus on a new, high-throughput DNA analysis approach to scoring SNP markers using the rapid capabilities of time-of-flight mass spectrometry (TOF-MS).

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Abbreviations: ddN, dideoxynucleotide; **mtDNA**, mitochondrial DNA; **SAP**; shrimp-alkaline phosphatase; **SNP**, single nucleotide polymorphism

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In recent years, TOF-MS has been recognized as a technique with great potential for high-throughput DNA analysis [16, 17]. Speed, accuracy and capability for automation are appealing aspects of TOF-MS. Accurate data can be generated in only a few seconds per sample. In the past year, several groups have published reports using TOF-MS for SNP analysis. These reports include ligation analysis [28] and primer extension formats [13, 18–20] as well as hybridization approaches using peptide nucleic acids probes [21–23]. We describe here a novel approach to SNP analysis using mass spectrometry that is more cost-effective and flexible than previous approaches, as well as being fully automated, with a demonstrated capability for processing thousands of samples in a single day.

2 Materials and methods

2.1 Materials

Human genomic DNA from the K562 cell line (Promega, Madison, WI) was used as a standard template to test each new SNP marker. Additional DNA samples were provided by Dr. Peter Oefner (Standord University Department of Genetics) and the Monsanto Company to demonstrate and validate this technology. Oligonucleotides used for the PCR and the SNP extension reactions were obtained from Biosource/Keystone (Foster City, CA) or synthesized in-house. The SNP primer was biotinylated at the 5′-end and contained a cleavable nucleotide near the 3′-end. The polymerase and buffers were obtained from PE Applied Biosystems (Foster City, CA) and the dNTP and ddNTP mixtures and shrimp-alkaline phosphatase (SAP) were purchased from Amersham Life Sciences (Cleveland, OH).

2.2 Sample preparation

Each 20 μL PCR reaction contained 1 μM of both forward and reverse primers, 5–20 ng DNA template, 1 U AmpliTaq Gold, 1 × PCR buffer II, 1.5 –2.5 mM $MgCl_2$, and 200 μM each dNTP. PCR was performed using an MJ Research DNA Engine (Watertown, MA). The typical thermal cycling conditions were 95°C for 10 min, 35 cycles at 94°C for 10 s, 55°C for 30 s, 72°C for 30 s, and 72°C for 5 min. For the mitochondrial DNA work, the primer sequences were the same as those described by Piggee *et al.* [11] and Tully *et al.* [12]. Following PCR, the amplicons were treated with shrimp-alkaline phosphatase to hydrolyze the unincorporated dNTPs. Typically, 1 U SAP was added to each 20 μL PCR reaction and then incubated at 37°C for 60 min followed by heating at 75°C for 15 min. The SNP extension reaction consisted of a 5 μL aliquot of the SAP-treated PCR product, 1 × *Taq*FS buffer, 1.2–2.4 U *Taq*FS, 12.5 μM ddNTP mix, and 0.5 μM biotinylated SNP primer in a 20 μL volume. For multiplex analysis, SNP primer concentrations were balanced empirically, typically in the range of 0.3–1.5 μM. The SNP extension reaction was performed in a thermal cycler at 94°C for 1 min, and 25–35 cycles at 94°C for 10 s, 45–60 °C (depending on the annealing temperature of the SNP primer) for 10 s, and 70° for 10 s. These universal conditions worked well under a wide variety of SNP loci tested. A purification procedure involving solid-phase capture and release from streptavidin-coated magnetic beads was utilized to purify the DNA samples prior to mass spectral analysis [24, 27]. Parallel sample preparation was conducted on a robotic workdeck operated with a 96-tip pipet head developed at GeneTrace. The entire sample process from PCR setup to mass spectrometric sample preparation has been automated on multiple robotic workstations and will be described in a future communication.

2.3 Mass spectrometry

The DNA samples, consisting of the primer and the extension product(s), were spotted with 1–2 μL of a 3-hydroxypicolinic acid matrix solution [25]. A GeneTrace-designed linear TOF mass spectrometer was used as previously described with ultraviolet laser pulses [26]. Sample analysis has been fully automated with this mass spectrometer so that sample spots may be located and a particular mass range searched without operator intervention. For SNP analysis, mass calibration was performed with two oligonucleotide mass markers: a 4-mer (1272 Da) and a 15-mer (4507 Da).

2.4 Data analysis

In-house automated SNP analysis software was developed and used to determine the genotype for each SNP marker. The software accepts as input an expected primer mass and, after locating the pertinent primer, searches for the four possible extension products. The mass between the primer and the extension product can then be correlated to the incorporated nucleotide at the SNP site. In the case of a heterozygote at the SNP site, two extension products exist and are called by the software.

3 Results

3.1 Design aspects for SNP assays

The approach to SNP determination described here has essentially three steps: (i) PCR amplification, (ii) phosphatase digestion, and (iii) SNP primer extension. Either strand of DNA may be probed in this SNP primer exten-

sion assay. PCR primers are designed to generate an amplicon that includes one or more SNP sites. The initial PCR reaction is performed with standard (unlabeled) primers. A phosphatase is added following PCR to remove all remaining dNTPs so that they will not interfere with the single-base extension reaction involving dideoxynucleotides. These reactions can all be performed in the same tube or well in a sample tray. A portion of the phosphatase-treated PCR product is then used for the primer extension assay. In our SNP primer extension assay, a special primer containing a biotin moiety at the 5′-end and cleavable nucleotide near the 3′-end hybridizes upstream of the SNP site with the 3′-end immediately adjacent to the SNP polymorphic site (Fig. 1). The biotin permits solid-phase capture for sample purification prior to MS analysis, and the cleavable nucleotide allows the 3′-end of the primer to be released from the immobilized portion as well as reducing the overall mass of the measured DNA molecule [27]. The complementary nucleotide(s) to the nucleotide(s) present at the SNP site are inserted during the extension reaction. In the case of a heterozygote, two extension products result. Only a single base is added to the primer during this process because only ddNTPs are used and the dNTPs left over from PCR are hydrolyzed with the phosphatase digestion step. If the extension reaction is not driven to completion (where the primer would be totally consumed), then both primer and extension product (*i.e.*, primer plus single nucleotide) are present after the primer extension reaction. The mass difference between these two DNA oligomers is used to determine the nucleotide present at the SNP site. As will be described below, we have used this approach to reliably determine all four possible SNP homozygotes and six possible heterozygotes.

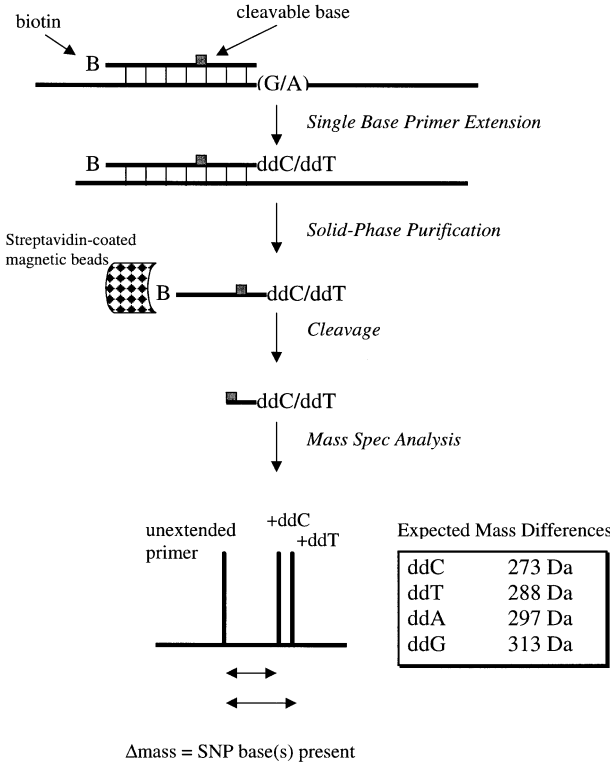

Figure 1. Schematic of SNP assay using a cleavable primer approach. An SNP primer hybridizes to the target DNA immediately upstream of the polymorphic site and a polymerase incorporates the complementary dideoxynucleotide in a single-base extension reaction. The sample then undergoes a solid-phase purification *via* a capture and release protocol to prepare it for mass spectrometry analysis. The mass difference between the primer and the extension product(s) indicates the nucleotide(s) present at the SNP site.

3.2 Analytical results

A high degree of precision and accuracy in making a mass difference measurement is required to correctly call the SNP nucleotide. We balance the primer extension reaction so that some of the primer remains to act as an internal standard. Thus, an absolute mass for the extension product(s) is not as crucial as the mass difference between the primer and the extension product(s). Figure 2 illustrates the precision and accuracy of 50 SNP meas-

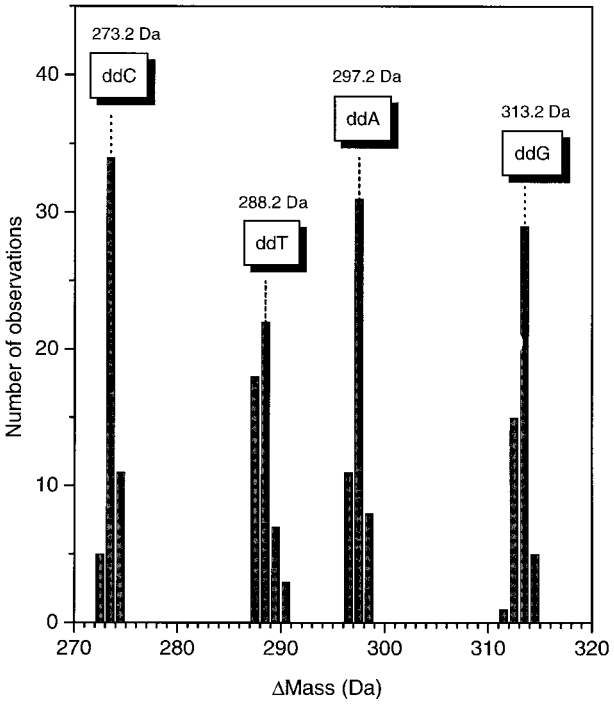

Figure 2. Histogram of mass difference measurements for 200 samples (50 for each ddN). Expected masses for the dideoxynucleotides are 273.2 Da for ddC, 288.2 Da for ddT, 297.2 Da for ddA, and 313.2 Da for ddG.

urements at each of the four possible nucleotides. The observed masses for each dideoxynucleotide (ddN) were 273.5 ± 0.5 Da for ddC, 288.3 ± 0.7 for ddT, 297.2 ± 0.5 Da for ddA, and 313.2 ± 0.5 Da for ddG. The average mass difference measurement for each ddN came within 0.3 Da of the expected masses, which are ddC = 273.2 Da, ddT = 288.2 Da, ddA = 297.2 Da, and ddG = 313.2 Da. In addition, the standard deviation for each ddN was less than 1 Da.

In terms of resolution, the most difficult bases to distinguish from one another are adenine and thymine, which differ by only 9 Da. As seen in Fig. 3, a T/A heterozygote can be resolved with our cleavable primer approach. A heterozygote containing C and G bases is the easiest to resolve as these two nucleotides differ by 40 Da. Table 1 describes the results from these heterozygous samples. The SNP scoring approach described here works well over a wide range of PCR product amounts. No prequantitation of PCR product is required to obtain an SNP result. We have reliably called the SNP nucleotide from as little as 10 fmol PCR product although we typically work in the high femtomole to low picomole range.

3.3 Multiplexing SNP markers

Examining multiple SNP markers in the same reaction reduces time, labor, and cost compared to single reactions. Multiplexing SNP markers may be achieved by using primers that are resolvable on a mass scale. With the approach described here, compatible primers with similar annealing temperatures may be used and cleavage sites may be placed at different positions in each primer. For example, one primer could have the cleavage site five bases from the 3′-end and another primer could have eight bases between the cleavage site and the 3′-

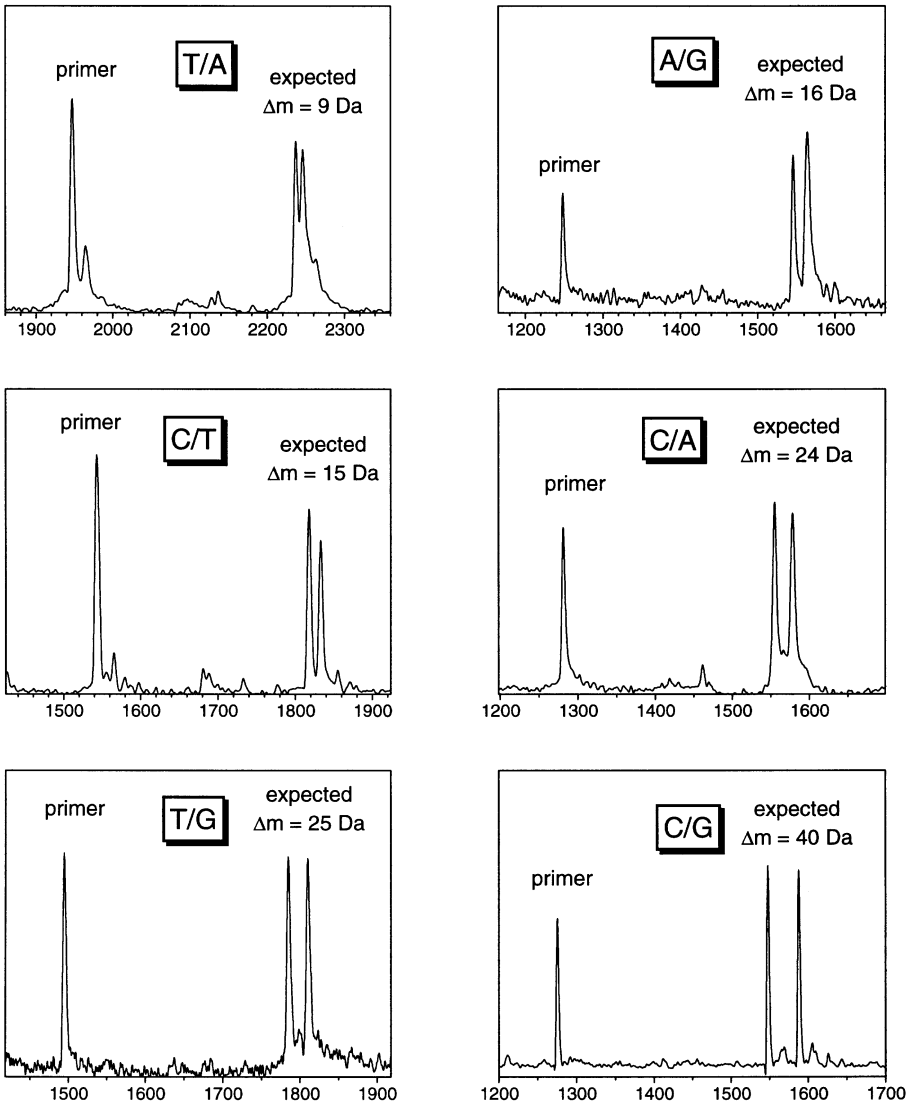

Figure 3. Mass spectra of heterozygous SNP samples. The six possible combinations of dideoxynucleotides are shown here (T/A, C/T, A/G, C/A, T/G, and C/G) with the expected mass differences between them. The SNP genotype is determined for each sample by measuring the mass difference between the primer and the extension products. Table 1 contains the measured mass information for these heterozygous samples.

Table 1. Heterozygote SNP detection results

Primer sequence[a)]	Expected primer mass (Da)	Primer mass (Da)	Extension masses (Da)	Mass difference (Da)	SNP site	Δ Mass between extension products (Da)
GACGGG	1937	1942.0	2231.0	289.0	T/A	9.1
			2240.1	298.1		
CAAA	1247	1244.7	1541.7	297.0	A/G	18.4
			1560.1	315.3		
ACACA	1536	1538.6	1812.9	274.3	C/T	15.1
			1828.0	289.4		
CGAG	1279	1277.7	1550.8	273.1	C/A	23.2
			1574.0	296.3		
TTGTC	1485	1490.5	1780.4	289.9	T/G	25.0
			1805.4	314.9		
GAAT	1278	1275.2	1547.8	272.6	C/G	39.9
			1587.7	312.5		

a) Sequence listed is from cleavage point to the 3′-end of the SNP primer
Mass spectra may be seen in Fig. 3

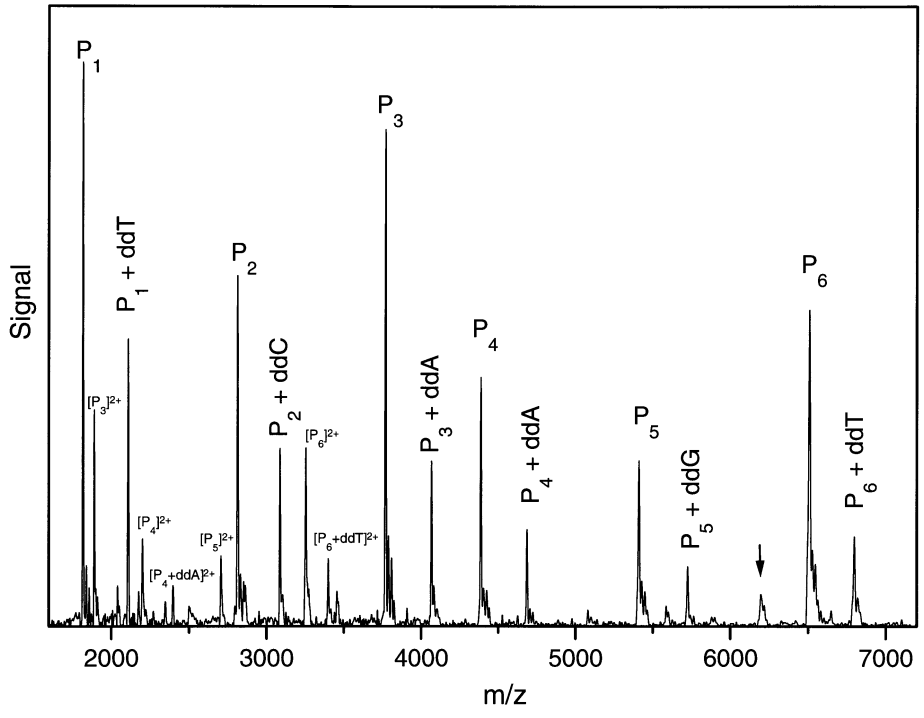

Figure 4. Mass spectrum of a multiplex SNP analysis involving six polymorphic sites within a single 1021 bp PCR product from the human mitochondrial DNA control region. All four bases are simultaneously determined from both strands of the PCR product. Primers were designed to avoid overlap between singly and doubly charged ions from the DNA products produced in this assay. The peak at 6200 Da indicated by the arrow is an impurity (n-1) from P_6 synthesis. Primer masses and SNP sites are described in Table 2.

end. Thus, after performing the SNP extension reaction and cleaving the primers, each primer and extension product can be easily resolved in the mass spectrometer.

Figures 4 and 5 illustrate multiplex SNP results from human mitochondrial DNA polymorphic sites used in human identity testing [12] and point mutations that are diagnositic for Leber's hereditary optic neuropathy [11]. Figure 4 is the mass spectrum of a 6-plex reaction from a single PCR product that demonstrates all four SNP bases being detected simultaneously. Additionally, both strands of the mitochondrial DNA PCR product are being probed in this example. Primers 2 through 5 probe the so-called heavy strand at positions H00247, H00152, H16311, and H16069, respectively, while primers 1 and 6 examine positions L00146 and L00195 on the light strand. In Fig. 5, three PCR products are multiplexed at the PCR level as well as in the SNP extension reaction portion. Table 2 describes the results from these human mitochondrial DNA SNP markers. DNA sequence analysis for these PCR templates confirmed the mitochondrial DNA control region SNP results listed in Table 2 (data not shown).

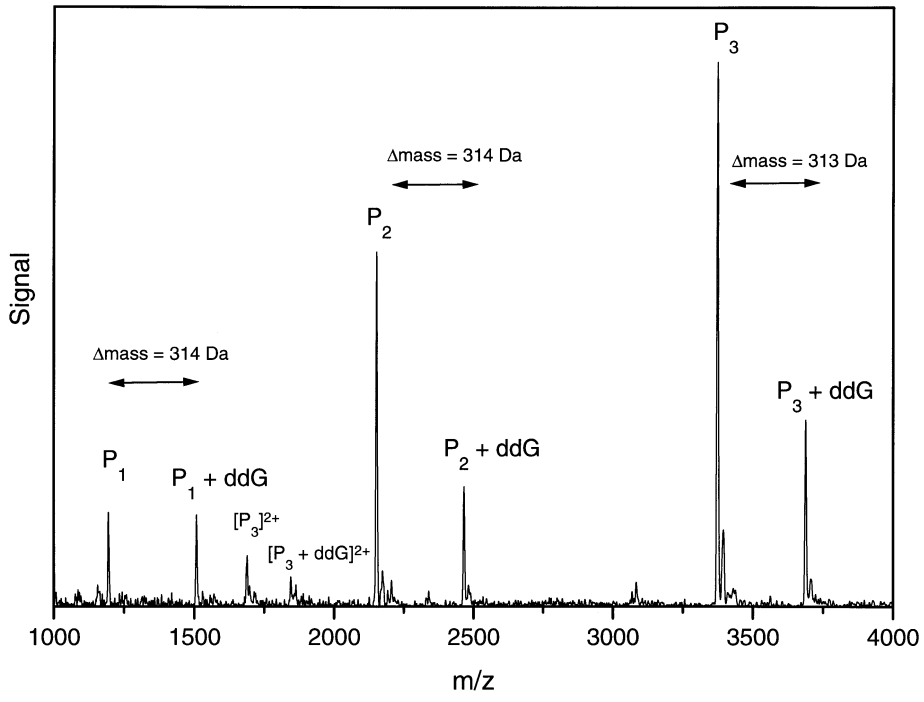

Figure 5. Mass spectrum of a multiplex SNP analysis involving three known mutations associated with Leber's hereditary optic neurophathy. The PCR products resulted from multiplex PCR of amplicons containing the three SNP sites. Some doubly charged ions from the larger DNA products may be seen between the first extension product (P_1+ddG) and the second primer (P_2). Primer masses and SNP sites are described in Table 2.

Table 2. Multiplexed SNP markers examined from human mitochondrial K562 cell line DNA

SNP marker (mtDNA[d] position)	Expected bases (wild-type listed first)	Primer mass (Da)	Extension mass (Da)	Mass difference (Da)	Calculated SNP base
Control region polymorphisms[a]			Fig. 4		
L00146[b]	T or C	P_1 = 1819.7	2109.8	290.1	T
H00247	C or T	P_2 = 2813.7	3087.2	273.5	C
H00152	A or G	P_3 = 3770.0	4.068.6	298.6	A
H16311	A or G	P_4 = 4387.9	4686.8	298.9	A
H16069	G or A	P_5 = 5411.3	5725.9	314.6	G
L00195	T or C	P_6 = 6509.4	6798.4	289.0	T
LHON point mutations[c]			Fig. 5		
L03460	G or A	P_1 = 1194.1	1508.2	314.1	G
L11778	G or A	P_2 = 2152.4	2466.5	314.1	G
L14459	G or A	P_3 = 3374.6	3688.3	313.7	G

a) SNP primers same as Tully *et al.* [12] without poly-T tail
b) L, light strand; H, heavy strand
c) PCR and SNP primers same as Piggee *et al.* [11]
d) mtDNA, mitochondrial DNA
Mass spectra may be seen in Figs. 4 and 5.

4 Discussion

The cleavable primer approach for SNP analysis described here has a number of advantages over other existing technologies in terms of the molecular biology and the ability to be automated as well as the MS detection. One feature of using a cleavable primer is the ability to probe both DNA strands simultaneously and still perform a solid-phase purification after a single PCR reaction (see Fig. 4). Because the 3′-end of the primer can be released following cleavage, the primer probe rather than the template strand may be biotinylated. Other primer extension methods, such as solid-phase minisequencing [12] and Genetic Bit Analysis [9], require that all of the primers used for probing SNP sites be on the same DNA strand because the template strand is captured and then

made single-stranded through denaturation washes or digestion reactions. All four possible nucleotides can be probed simultaneously with our SNP approach (see Fig. 4), which permits multiplexing any SNP marker as well as permitting clear determination of heterozygotes (see Fig. 3) since all four bases are processed in the same reaction. Furthermore, as in all primer extension reactions, the specificity of the SNP determination is improved with primer hybridization and polymerase incorporation over just a hybridization event [9].

The capability of using different cleavage points along the SNP primer allows a greater flexibility for multiplexing several primers than using a poly-T tail for mass or electrophoretic mobility shifting as described by Haff *et al.* [19] and Tully *et al.* [12], respectively. Furthermore, the poly-T tail approach shifts the SNP primers to higher mass, where it more difficult to resolve closely spaced heterozygotes. The primary requirement for designing a multiplex assay is that primers and extension products from each SNP marker are resolvable in terms of mass. Avoiding overlap of doubly charged ions from higher mass primers is also important, but straightforward under the high-resolution conditions demonstrated here (see Fig. 4). With our cleavable primer approach, it is conceivable that 20 or more SNP markers could be simultaneously detected in a fairly narrow mass window at a rate of only a few seconds per sample. The cleavable nucleotide can be placed anywhere within the primer without disrupting normal hybridization (*i.e.*, Watson-Crick hydrogen bonding). We have tested hundreds of primers containing cleavable nucleotides and found them to hybridize to target DNA just as well as regular oligonucleotides.

An advantage on the mass spectrometry side of the assay is the reduced size of the detected oligonucleotide, a fact that directly leads to higher sensitivity and resolution. Challenging heterozygotes that contain T and A can be readily resolved in the lower mass range. Previous mass spectrometry efforts with T/A heterozygotes required more elaborate and expensive instrumentation than TOF mass spectrometry [18] or mass-tagging one of the dideoxynucleotides to shift the mass of its extension product away from the other extension product in the heterozygote [20]. Both of these approaches are more expensive and are thus less desirable in a high-throughput DNA operation. In addition, the reliability of making an SNP genotype determination is increased by having the primer present to act as an internal standard. Mass difference measurements are frequently more accurate in a mass spectrometer than absolute mass measurements by themselves. Most importantly, automated sample processing has been implemented on a robotic workstation so that hundreds to thousands of samples may be processed in parallel each day per instrument.

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