Anal. Chem. **2001,** *73,* 620−624

Genotyping of Two Mutations in the HFE Gene Using Single-Base Extension and High-Performance Liquid Chromatography

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Currently, a major focus of human genetics is the utilization of single-nucleotide polymorphisms for clinical diagnostics, whole-genome linkage disequilibrium screens to identify common disease genes such as Alzheimer disease, determination of the recent evolutionary history of a species, and the process of speciation. We have examined single-nucleotide extension coupled with high-performance liquid chromatography as a method to simultaneously genotype two SNPs occurring in the coding region of the HFE gene that produce clinical effects. This assay allows concurrent genotyping of the C282Y and H63D mutations in 11 min and is 100% concordant with current testing methods for both of these mutations.

The human genome project has advanced to a point where high-quality sequence information is available to provide unique opportunities for studying genetic variation. The most common type of human genetic variation is the single-nucleotide polymorphism (SNP), a base position at which two alternative bases occur at appreciable frequency (>1%) in the population.[1] It is estimated that the sequence analysis of the complete human genome will reveal at least 1 million SNPs in the nonrepetitive coding regions of genes, which includes both introns and promoters.[2] Linkage and association studies with large numbers of SNPs can be used to identify and characterize genes implicated in biological functions and diseases. Some authors have recently hypothesized that common SNPs occurring in coding regions (cSNPs) of genes may contribute significantly to genetic risk for common diseases.[3] In some cases, cSNPs have already been linked to diseases and are then referred to as mutations.

Hereditary hemochromatosis (HH) is a common autosomal recessive disease associated with the loss of regulation of dietary iron absorption and excessive iron deposition in major organs of the body. The HFE gene belongs to the major histocompatibility complex class I-related gene family and is located 4.5 Mb telomeric to the HLA-A gene on chromosome 6.[4] The clinical symptoms include cirrhosis of the liver, diabetes mellitus, cardiomyopathy, endocrine dysfunctions, and arthropathy.[5] HH affects between 1 in 200 and 1 in 400 persons of northern European descent[5] and has an estimated carrier frequency of 1 in 8 to 1 in 10 individuals.[6] Early diagnosis of HH can be followed by phlebotomy treatments to remove iron-rich red blood cells and deplete total body iron stores to prevent tissue damage and normalize life expectancy.

An optimal genotyping method that simultaneous determines the presence or absence of the two common missense mutations implicated in HH would greatly improve current diagnostic testing for this common disorder. The first missense mutation is a G-to-A transition that results in an amino acid change of cysteine to tyrosine at position 282 (C282Y mutation) in the HFE protein. This missense mutation was found to be homozygous in 83% of 178 HH patients within the Caucasian population.[6] Subsequent studies from the United States, Australia, France, and Italy showed homozygosity for the C282Y mutation in 64−100% of patients with hemochromatosis.[4] This mutation has an estimated allelic frequency of 0.04 in the Caucasian population.[4] The other missense mutation is a C-to-G transversion that results in an amino acid change of a histidine to asparagine at position 63 (H63D) in the HFE protein. The H63D mutation is heterozygous in 15−20% of the population and may contribute to increased hepatic iron concentrations, especially when combined with the C282Y mutation.[7] The H63D mutation has an estimated allelic frequency of 0.14 in the Caucasian population.[4] The two mutations (C282Y and

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10.1021/ac000912j CCC: $20.00
Published on Web 01/09/2001

H63D) segregate independently and have never been found on the same chromosome.[8]

We have developed a method to simultaneously genotype the HFE gene at both the C282Y and H63D positions utilizing single-base extension (SBE) or primer extension combined with high-performance liquid chromatography (HPLC).[9] SBE is a versatile method and has been combined with variety of techniques to detect the extension products including radiolabeling,[10] luminous detection,[11] gel-based fluorescent detection,[12] mass spectrometry,[13] capillary electrophoresis,[14] and HPLC.[9] After the polymerase chain reaction (PCR) amplification of a DNA fragment that contains a base change (mutation), an oligonucleotide primer is annealed downstream or upstream from the base change of interest in a 5′ to 3′ direction. We refer to the oligonucleotide primer as the SBE primer. In this method, a SBE primer anneals to the target nucleic acid immediately adjacent to the mutation locus. A DNA polymerase is used to specifically extend the 3′ end of the SBE primer with the 2′,3′-dideoxynucleotide that is the Watson−Crick complement of the base at the mutation site. The SBE primer is only extended one base because the dideoxynucleotide terminates further extension. After the SBE primer is extended with the nucleotide analogue, the resulting product is analyzed using ion-pair reversed-phase HPLC (IP-RP HPLC).

In this paper, we examine IP-RP HPLC as a rapid analytical method to simultaneously genotype two mutations linked to HH. The analysis time of 11 min/sample includes 7 min to genotype both mutations, the separation of a standard to normalize the results, and column cleanup and reequilibration. In addition, we examine the effect of oligonucleotide sequence composition on analysis using IP-RP to aid in the design of SBE assays.

EXPERIMENTAL SECTION

DNA Extraction. Samples were extracted from whole blood using the Nucleon I DNA extraction kit (Scotlab Bioscience, Glasgow, Scotland) according to the manufacturer's instructions. DNA samples representing all possible combinations of genotypes at the H63D and C282Y loci were tested along with 20 anonymous DNA specimens.

Multiplex PCR. Both loci were amplified in a single PCR reaction containing 50 ng of patient genomic DNA. PCR reactions were carried out in a total volume of 45 μL containing 12.5 pmol of each H63D primer and 9.4 pmol of each C282Y primer. Each reaction contained 5 μL of GeneAmp 10× buffer II, 3 μL of 25 mM $MgCl_2$, 1 μL of 10 mM dNTP, and 0.5 units of AmpliTaq Gold enzyme (PE Applied Biosystems, Foster City, CA). Amplification primers were as follows:

H63D forward 5′-TTCATGGGTGCC; TCAGAGCA-3′, H63D reverse; 5′-CTGGAAACCCATGGAGTTCG-3′, C282Y forward; 5′-TGGATGCCAAGGAGTTCGA-3′, C282Y reverse; 5′-ACCCCAGAT-CACAATGAGGG-3′.[15]

Samples were amplified under the following conditions in the PTC-200 thermal cycler (MJ Research, Woburn, MA): 96 °C for 10 min, 35 cycles consisting of denaturation at 94 °C for 1 min, annealing at 62 °C for 1 min, and extension at 72 °C for 1 min.

Preparation of PCR Product for Single-Base Extension. To eliminate the dNTPs and primers from PCR, 25 μL of PCR product was placed in a tube with 0.5 unit of shrimp alkaline phosphatase (SAP) and 10 units of exonuclease I (Amersham Pharmacia Biotech, Piscataway, NJ) in the SAP buffer supplied by the manufacturer and heated for 1 h at 37 °C followed by inactivation of the enzymes by heating at 80 °C for 15 min. The amount of DNA was quantified using the Beckman DU-640B spectrophotometer (Fullerton, CA), and samples were diluted to a final concentration of 10 ng/μL.

Single-Base Extension Reaction. The SBE primers were designed to allow for easy multiplexing to monitor the base at the H63D and C282Y positions by having the same melting temperature (T_m). T_m values were estimated using the nearest-neighbor thermodynamic parameters reported by SantaLucia.[16,17] A total strand concentration (C_T) of 10 μM and Na^+ counterion concentration of 0.115 M were used in T_m calculations. Potential intramolecular hairpin and intermolecular primer−dimer interactions were screened using the algorithm described by Rychlik and Rhoads.[18]

The SBE reactions were carried out in 16.5 μL containing 10 μM each SBE primer and 75 ng of DNA using the ThermoSequenase kit (Amersham Pharmacia Biotech). The SBE primer for the C282Y mutation (G → A) is 5′-GGGGCTCCACACGGC-GACTCTCAT-3′ (C282Y−SBE) and the SBE primer for the H63D mutation (C → G) is 5′-GGGGAAGAGCAGAGATATACGT-3′ (H63D SBE). The C282Y−SBE primer extends a "C" on the coding strand if the wild-type sequence is present and a "T" if the mutant sequence is present. The H63D−SBE primer extends a "G" on the coding strand if the wild-type sequence is present and a "C" if the mutant sequence is present. For our reactions, all four unlabeled ddNTPs (Amersham Pharmacia Biotech) were included. A premix consisting of 1 μL of H_2O, 2 μL of reaction buffer, 1 μL each ddNTP, and 2.5 units of enzyme was added to the primer and template.

The reaction was run in an MJ Research PTC200 thermal cycler (MJ Research, Inc., Waltham, MA) under the following conditions: 96 °C for 5 min, followed by 50 cycles of denaturation at 96 °C for 30 s, SBE primer annealing at 54 °C for 30 s, and extension at 60 °C for 4 min.

Samples for Analysis of SBE Reaction. There are six possible genotype combinations for the H63D and C282Y loci. We used all six genotypes as controls for the SBE assay and to

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calculate normalized retention times. The first genotype is wild-type at the H63D locus (G allele) and wild-type at the C282Y locus (C allele), the second genotype is wild-type at the H63D locus and heterozygous at the C282Y locus, the third genotype is wild-type at the H63D locus and mutant at the C282Y locus (T allele), the fourth genotype is heterozygous at the H63D locus and wild-type at the C282Y locus, the fifth genotype is mutant at the H63D locus (C allele) and wild-type at the C282Y locus, and the sixth genotype is heterozygous at both the H63D and C282Y loci.

Preparation of the Mobile Phase. HPLC grade acetonitrile (ACN; Fisher, Pittsburgh, PA) and triethylammonium acetate (TEAA; Transgenomic, Inc., San Jose, CA) were used to constitute the mobile phase. The mobile phases consist of 0.1 M TEAA (solvent A) and 0.1 M TEAA−25% ACN (solvent B).

Instrumentation. The HPLC system used for analyzing oligonucleotides and SBE products is a Transgenomic Wave DNA fragment analysis system with a DNASep column that has a stationary phase consisting of 2-μm nonporous alkylated (C_{18}) poly(styrene−divinylbenzene) particles, a UV detector set at 260 nm, and an autosampler with the capacity to handle 96 samples.

HPLC Analysis of Oligonucleotides and SBE Products. To determine whether different bases at the 3′ ends changed the oligonucleotide elution time, HPLC was performed with a column temperature of 50 and 70 °C, a flow rate of 0.9 mL/min, UV detection at 260 nm, and a linear gradient from 18% B to 38% B over 25 min; following a wash with 500 μL of 75% ACN, the gradient conditions were returned to 18% B for 4 min. For SBE product analysis, the sample was denatured at 96 °C for 4 min and immediately placed at 4 °C. The gradient used for elution of the SBE products was 24% B to 30% B in 6 min, 30% B to 35% B in 1 min, and a hold at 35% B for 1 min with a 3-min equilibration at 44% B after the column was flushed with 500 μL of 75% ACN.

RESULTS AND DISCUSSION

Separation of Oligonucleotides of Different Sequences. IP-RP HPLC is a method that has been used extensively for the separation and purification of oligonucleotides.[19] The sequence of the oligonucleotide governs the retention on a column packed with 2-μm nonporous alkylated poly(styrene−divinylbenzene) particles and a mobile phase that consists of ACN and TEAA.[19] We examined how changes in the sequence at the 3′ end affect the retention of an oligonucleotide during IP-RP HPLC. In addition, analysis was performed at two column temperatures that have already been used to separate oligonucleotides using IP-RP HPLC (50 and 70 °C) to determine the optimal temperature for SBE product analysis.

Previous reports of oligonucleotide separation by IP-RP chromatography used a column temperature of 50 °C.[19] At a column temperature of 50 °C, heterooligonucleotides with each of the four individual bases (A, G, C, T) at the 3′ end were separately analyzed using the gradient conditions in the Experimental Section. The primers used for this study had a common sequence of 5′-CGT AGC TGA TCG TTC AAG C**X**-3′, where **X** is one of the following bases (A, G, C, T). For the analytes that differ at the 3′ end, the oligonucleotide with the cytosine (this oligonucleotide will be referred to as 3C throughout the rest of the paper) at 15.73 min, guanine (3G) at 15.89 min, adenine (3A) was retained the longest at 16.73 min followed by thymine (3T) at 16.64 min. The retention of an oligonucleotide using IP-RP is controlled by charge because the number of negatively charged phosphate groups on the DNA backbone determines the number of ion pairs that form with the TEAA. The number of individual bases within an oligonucleotide determines IP-RP HPLC separation of oligonucleotides. In addition, the hydrophobicity of the bases contributes to the retention time. Since hydrophobicity is base specific, overall hydrophobicity of a given oligonucleotide is the sum of the hydrophobicities of its individual bases.[20] The sequence of the oligonucleotides is identical except for the base at the 3′ end, and the retention times were in the order of 3C < 3G < 3T < 3A. This elution order is in agreement with the order of hydrophobicity reported for individual bases that increased in the order of C < G < T < A at neutral pH.[19]

Table 1. Resolution between Oligonucleotides with Different Bases on the 3′ End at a Column Temperature of 70 °C

oligonucleotide	3′ base	retention time, min	resolution
3T	T	11.15	
3A	A	10.97	0.55
3G	G	10.34	3.99
3C	C	10.07	2.14

Hoogendoorn and co-workers used a column temperature of 70 °C for SBE assays using IP-RP HPLC with great success.[9] Therefore, we repeated the experiments at a column temperature of 70 °C to determine whether the column temperature change would allow separation of the oligonucleotides with the four different bases at the 3′ end (3A, 3T, 3C, 3G). At 70 °C, the retention time of the oligonucleotides is reduced because the exothermic enthalpy changes associated with the transfer of solutes from the mobile to stationary phases dominate the retention process in most chromatographic systems.[21] Also, a change in temperature can have a profound effect on the efficiency of a separation because an increase in temperature will reduce viscosity and increase diffusion rates, thereby enhancing the mass-transfer rate.[21] The retention times for the analytes that differ at the 3′ end were 3C at 10.07 min, 3G at 10.34, 3A at 10.97 min, and 3T at 11.15 min. Therefore, the elution order of the oligonucleotides with the four different bases at the 3′ end was 3C < 3G < 3A < 3T. This differs from the elution order of the same oligonucleotides at 50 °C. However, this is in agreement with the results from a recent publication that found the same elution order (C < G < A < T) for 22-mer oligonucleotides that differed at the 3′ end by the four different bases at a column temperature at 80 °C.[23]

Each of the four oligonucleotides with a different 3′ end base was mixed in equal concentrations and separated at a column temperature of 70 °C. The resolution was calculated between all oligonucleotides (Table 1). Interestingly, the resolution is much higher between 3C and 3G than between 3A and 3T. This is due

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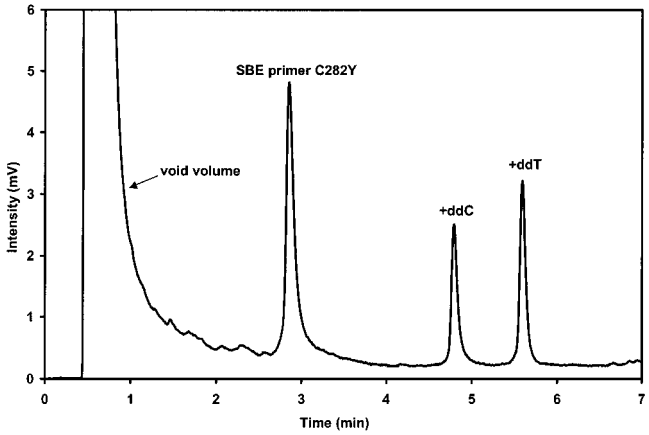

Figure 1. Chromatogram of the SBE reaction for the C282Y position. The gradient conditions are described in the Experimental Section. This sample is heterozygous, thus containing both alleles (C allele and T allele).

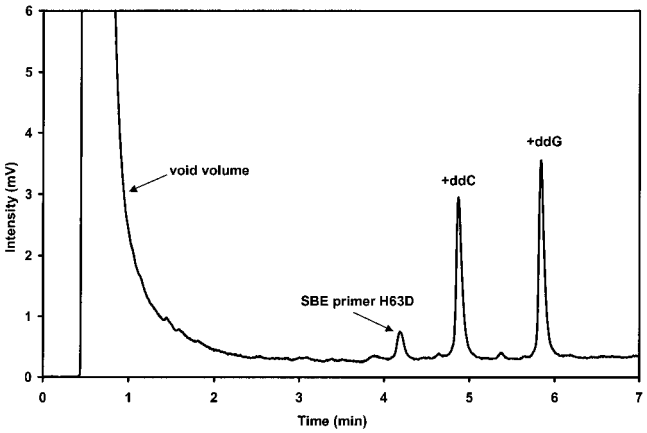

Figure 2. Chromatogram of the SBE reaction for the H63D position. The gradient conditions are described in the Experimental Section. This sample is heterozygous, thus containing both alleles (C allele and G allele).

to the hydrophobicity differences between the individual bases on the 3′ end of 3C, 3G, 3A, and 3T.

In synopsis, the hydrophobicity differences of the four bases at the 3′ end of oligonucleotides are a gauge for the elution times when using IP-RP HPLC and the elution order is C < G < A < T at a column temperature of 70 °C.

Single-Base Extension Reaction Analysis. The purpose of this study is to develop a genotyping method for two common mutations in the HFE gene. The SBE primers for the analysis of the H63D and C282Y mutations were selected to facilitate optimization of the HPLC gradient. The SBE primer for the C282Y mutation is a 22-mer (C282Y−SBE) and the SBE primer for H63D mutation is a 24-mer (H63D−SBE). When the SBE primers were analyzed using an optimized gradient, the 22-mer eluted at 3.00 min and the 24-mer eluted at 4.30 min (data not shown).

With elution times known for the SBE primers, the elution time for the SBE products (SBE primer plus the extended ddNTP) were needed. Therefore, the SBE assays were run for each mutation in the HFE gene and analyzed independently. Using the optimized gradient conditions, the C282Y−SBE primer was added to a wild-type sample, a sample that is heterozygous at the C282Y position, and a sample that is homozygous mutant at the C282Y position. A chromatogram for sample 2 (Figure 1) shows extension products representing both alleles. The elution characteristics of the individual alleles were established by the analysis of the homozygous samples. The extended product for the T allele at position C282Y elutes at 5.59 min before the C allele extended product that elutes at 4.77 min. The SBE products elute differently because of the hydrophobicity of the base that is added at the 3′ end.

The same experiments were repeated for the H63D locus. The samples used for these experiments had the genotypes for the H63D position of a wild-type sample, a sample that is heterozygous at the H63D position, and a sample that is homozygous mutant at the H63D position. A chromatogram for the heterozygous sample is shown that represents extended products for both alleles (Figure 2). The C allele elutes at 4.87 min and the G allele elutes at 5.84 min. Previously, the use of HPLC IP-RP as a method to discriminate different alleles has worked for all mutations except for the G → C transversion.[23] However, the SBE method worked well for the G → C transversion at position H63D in the HFE gene in our study.

Table 2. NRT for SBE Products and Confidence Intervals (n = 15)

genotype	NRT	std dev of NRT	confid interval for NRT (99.0%)
C282Y, C allele (wild-type)	0.602	0.006	(0.586, 0.618)
C282Y, T allele (mutant)	0.702	0.004	(0.692, 0.712)
H63D, G allele (wild-type)	0.741	0.005	(0.727, 0.755)
H63D, C allele (mutant)	0.628	0.004	(0.619, 0.637)

In a routine clinical diagnostic setting, a clinical test must be rapid and accurate. A multiplex PCR was used to amplify both the C282Y locus and the H63D locus in one tube. The SBE primers were designed to have the same annealing temperatures for the SBE reaction (55 °C) with a Na^+ ion concentration equal to 0.115 M. Therefore, the SBE primers for both loci were added to the single tube and the SBE reaction was completed. In addition, the SBE primers were designed to be two bases apart to allow for rapid separation using IP-RP HPLC.

To genotype the samples for two common mutations in the HFE gene, a normalized retention time (NRT) method based on the work of Mitchell and Walsh was used.[23] The NRT is calculated using a normalizing peak, in this case a 25-mer that is noncomplimentary to the sequence of both PCR products, and the following equation:

$$\text{NRT} = \text{retention time for the SBE product} / \text{retention time for the normalizing peak} \quad (1)$$

Using NRT as a method to genotype allowed any variation in retention times of SBE products to have no effect on the accuracy of the test.

Samples with all the possible genotypes for C282Y and H63D were used to establish NRTs for the SBE product peaks (Table 2). Figure 3 shows the chromatogram for the multiplexed SBE reaction. Each SBE product was analyzed 15 times over a period of two weeks and eq 1 was used to calculate the NRT for all the SBE reaction products. The standard deviation for the NRTs of

Table 3. Genotyping of SBE Products from the HFE Gene

sample	NRT of SBE products	genotype for the C282Y position	genotype for the H63D position
1	0.692, 0.730	T allele	G allele
2	0.611, 0.700, 0.739	C allele	G allele, C allele
3	0.611, 0.742	C allele	G allele
4	0.608, 0.627 0.743	C allele	G allele, C allele
5	0.610, 0.742	C allele	G allele
6	0.693, 0.734	T allele	G allele
7	0.600, 0.730	C allele	G allele
8	0.599, 0.734	C allele	G allele
9	0.702, 0.742	T allele	G allele
10	0.614, 0.704, 0.745	C allele, T allele	G allele
11	0.611, 0.628	C allele	C allele
12	0.599, 0.734	C allele	G allele
13	0.595, 0.694, 0.728	C allele, T allele	G allele
14	0.598, 0.731	C allele	G allele
15	0.614, 0.704, 0.746	C allele, T allele	G allele
16	0.614, 0745	C allele	G allele
17	0.611, 0.700, 0.741	C allele, T allele	G allele
18	0.610, 0.746	C allele	G allele
19	0.598, 0.735	C allele	G allele
20	0.612, 0.704, 0.743, 0.630	C allele, T allele	G allele, C allele

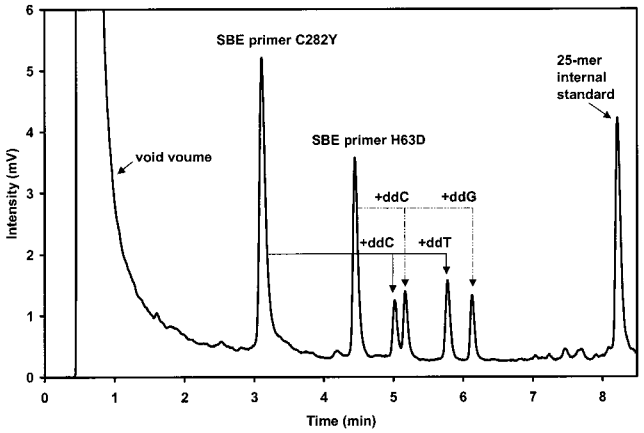

Figure 3. Chromatogram of the multiplex SBE reaction for the genotyping of both the C282Y position and the H63D position. The gradient conditions are described in the Experimental Section. This sample is heterozygous at both the C282Y and H63D positions.

the SBE products was less than or equal to a value of 0.006 for all the different genotypes. From the calculated means and standard deviations for the different SBE products, a 99.0% confidence interval was calculated for each SBE product's NRT. When unknowns are analyzed, the NRT for the SBE products can be compared to Table 2 for genotyping.

Using the NRT time confidence intervals from Table 2, 20 previously characterized samples were genotyped in a blind analysis using the normalization procedure. DNA isolated from human liver tissue from individuals who had undergone liver transplantation due to conditions other than hereditary hemochromatosis obtained from the Liver Tissue Procurement and Distribution System, University of Minnesota.[24] The samples were analyzed and an NRT was calculated for each SBE product (Table 3). The NRTs were compared to Table 3 to determine a genotype for the sample. The 20 samples analyzed produced the following genotypes: three samples had a homozygous mutation at the C282Y position and a wild-type genotype at the H63D position; four samples were heterozygous at the C282Y position and had a wild-type genotype at the H63D position; 10 samples had a wild-type genotype at both the C282Y and H63D positions; two samples were wild-type at the C282Y position and heterozygous at the H63D position; and one sample was heterozygous at both the H63D and C282Y positions. The genotypes of the samples matched the previous results of these samples using DHPLC and restriction enzyme analysis.[24] Genotyping using a normalizing peak allows for rapid (analysis time of less than 11 min), reproducible (average standard deviation for NRTs of the SBE products was 0.005), and accurate results (100% concordance with previous results).

This study shows the usefulness of IP-RP HPLC as a method to genotype two different mutations in the HFE gene using SBE. With the genotyping of these two mutations, a proper course of treatment can be provided for an affected individual. Currently, we are increasing the number of SBE reactions that can be multiplexed and analyzed. The multiplexing of the SBE reaction can be used to develop disease specific assays. For example, monitor numerous mutation hotspots in the p53 gene. We hope to reach simultaneous analysis and monitoring of 10−50 different SNPs throughout the genome.

Received for review August 3, 2000. Accepted November 28, 2000.

AC000912J

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