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Technical Advance

Homogeneous Multiplex Genotyping of Hemochromatosis Mutations with Fluorescent Hybridization Probes

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Multiplex polymerase chain reaction amplification and genotyping by fluorescent probe melting temperature (T_m) was used to simultaneously detect multiple variants in the hereditary hemochromatosis gene. Homogenous real-time analysis by fluorescent melting curves has previously been used to genotype single base mismatches; however, the current method introduces a new probe design for fluorescence resonance energy transfer and demonstrates allele multiplexing by T_m for the first time. The new probe design uses a 3′-fluorescein-labeled probe and a 5′-Cy5-labeled probe that are in fluorescence energy transfer when hybridized to the same strand internal to an unlabeled primer set. Two hundred and fifty samples were genotyped for the C282Y and H63D hemochromatosis causing mutations by fluorescent melting curves. Multiplexing was performed by including two primer sets and two probe sets in a single tube. In clinically defined groups of 117 patients and 56 controls, the C282Y mutation was found in 87% (204/234) of patient chromosomes, and the relative penetrance of the H63D mutation was 2.4% of the homozygous C282Y mutation. Results were confirmed by restriction enzyme digestion and agarose gel electrophoresis. In addition, the probe covering the H63D mutation unexpectedly identified the A193T polymorphism in some samples. This method is amenable to multiplexing and has promise for scanning unknown mutations. *(Am J Pathol 1998, 153:1055–1061)*

As databases for polymorphic markers[1] and disease-causing mutations[2,3] continue to grow, the ability to simultaneously interrogate multiple DNA sites that may be physically separated by great distances becomes increasingly useful. Multiplex polymerase chain reaction (PCR) is a rapid, versatile, and cost-effective method for this type of analysis. Since the introduction of multiplex PCR in 1988 for identifying specific exon deletions,[4] the concept has continued to develop in both application and technique. We have developed a new homogeneous multiplex genotyping method and applied it to the simultaneous analysis of multiple mutations within two exons of the hemochromatosis gene (HFE).

Hereditary hemochromatosis is the most common genetic illness known in the Northern Hemisphere with more than 1 million Americans estimated to be at risk for the disease.[5] This autosomal recessive disorder of iron metabolism occurs with a frequency of approximately 0.5% in Caucasian populations.[6,7] The dysregulation of intestinal iron absorption can eventually lead to parenchymal cell damage and end-organ dysfunction. Long-term complications of iron overload include arthritis, cardiomyopathy, diabetes, cirrhosis, and hepatocellular cancer. The morbidity associated with iron overload is preventable through early diagnosis and treatment by phlebotomy.

A cysteine-to-tyrosine amino acid substitution, caused by a G845A transition at codon 282 (C282Y), is found on 85 to 100% of disease chromosomes from patients of northern European ancestry who meet well defined clinical criteria for iron overload.[8–11] Another mutation (H63D) is created by a C187G transversion. This substitution has an estimated penetrance between 0.44 and 1.5% of the homozygous C282Y genotype.[8–10]

Current methods for genotyping the C282Y and H63D hemochromatosis-causing mutations include oligonucleotide ligation,[8] allele-specific oligonucleotide hybridization,[9] and PCR restriction fragment length analysis.[10–12] All of these methods require multiple manual steps and

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are time consuming. An alternative is the use of fluorescent hybridization probes and rapid-cycle PCR, a technique that provides homogeneous amplification and genotyping in approximately 45 minutes.[13,14] This technique previously used an internally labeled Cy5 primer to asymmetrically amplify excess Cy5-labeled strand.[13,14] Biallelic variations at a single site were genotyped by a complementary 3′-fluorescein-labeled probe. This method requires manual synthesis of the Cy5 primer, and multiplex analysis is difficult because the primers must be near the mutation sites.

In this paper, a more versatile fluorescence energy transfer probe design is introduced that uses adjacent fluorescent hybridization probes and allows multiple variants to be analyzed simultaneously. The 5′-Cy5-labeled and 3′-fluorescein-labeled probes can be made on automated synthesizers and are designed to hybridize to the same strand between unlabeled primers. Monitoring the fluorescence energy transfer between adjacent hybridization probes allows real-time detection of specific PCR product.[15] When one of the probes is positioned over an allele variant, the melting curve profile allows homogeneous genotyping in the same instrument.

The thermal stability of a DNA duplex relies on duplex length, GC content, and Watson-Crick base pairing.[16] Changes from Watson-Crick pairing destabilize a duplex by varying degrees depending on the length of the mismatched duplex, the particular mismatch, the position of the mismatch, and neighboring base pairs.[17,18] Considering these factors, two probe sets were designed for multiplex analysis of the two mutation sites in the hemochromatosis gene. Four different alleles could be identified simultaneously during melting curve analysis. In addition, a single probe identified three alleles, including an unexpected polymorphism (A193T), demonstrating the potential of adjacent fluorescent hybridization probes for scanning unknown mutations.

Materials and Methods

Samples

Genomic DNA from Caucasian individuals was collected over a 10-year period for studying hemochromatosis pedigrees in Utah and neighboring states.[19] Methods of collection were approved by the institutional review board at the University of Utah. Two clinically defined subject groups consisting of 117 patients and 56 controls were selected from 250 genotyped samples to determine the prevalence of the C282Y and H63D mutations in the HFE gene. Family-based controls had either married into a pedigree or had no HLA identity with the proband. Genotyped patients with ambiguous clinical histories were excluded from the subject group. Patients were selected through laboratory evidence of iron overload (transferrin saturation >55% and serum ferritin >600 μg/L), and liver biopsies were performed on most of these patients to determine the grade of liver siderosis. All controls had normal values for serum ferritin and percent transferrin saturation.

Genotyping

All samples were genotyped at the C282Y and H63D sites with adjacent fluorescent hybridization probes. Genotyping both sites simultaneously by multiplexing was performed on 70 samples. Reagent concentrations for multiplexing and single-site analysis were the same. However, multiplexed reactions contained two primer sets and two fluorescently labeled probe sets. Each 10-μl reaction contained 50 mmol/L Tris, pH 8.3 (25°C), 500 μg/ml bovine serum albumin, 0.2 mmol/L each deoxyribonucleoside triphosphate, 4 mmol/L $MgCl_2$, 0.5 μmol/L each primer, 0.1 μmol/L site-specific 3′-fluorescein-labeled probe, 0.2 μmol/L site-specific 5′-Cy5-labeled probe, 50 ng of genomic DNA, and 0.4 U of native *Taq* DNA polymerase. Samples were loaded into separate plastic/glass composite cuvettes, centrifuged, and capped. Homogeneous PCR and melting curve acquisition used a 24-sample rapid fluorescent thermal cycler (LightCycler LC24, Idaho Technology, Idaho Falls, ID).

Forty repeats of a two-temperature cycle were performed (94°C for 0 seconds and 62°C for 20 seconds with programmed transitions of 20°C/second). Fluorescence was acquired once each cycle for 50 milliseconds per sample at the end of the combined annealing/extension step. An appended analytical cycle after amplification allowed immediate genotyping by derivative melting curves. The genotyping protocol included denaturation at 94°C for 20 seconds; annealing for 20 seconds each at 65°C, 55°C, and 45°C (C282Y, S65C, and multiplexing) or 75°C, 65°C, and 55°C (H63D); and a high-resolution melting transition to 75°C at a rate of 0.1°C/second. Cy5 (655 to 695 nm) and fluorescein (520 to 560 nm) fluorescence were monitored for 50 milliseconds per sample at each 0.1°C temperature increment.

The data collected during the melting phase were used to genotype each sample. Melting curves were generated by plotting Cy5 fluorescence (*F*) *versus* temperature (*T*). Easily discriminated melting peaks were obtained by plotting the same data as $-dF/dT$ *versus* temperature.

Genotyping performed in the LightCycler was compared with conventional PCR restriction fragment length analysis. PCR restriction fragment length analysis was performed on all samples for the C282Y mutation and on 40 random samples for the H63D mutation. Amplification for the PCR restriction fragment length method was performed in an air thermal cycler (RapidCycler, Idaho Technology) using the same primers, $MgCl_2$ concentration, and temperature parameters as that used in the LightCycler. Probes were not added for PCR restriction fragment length analysis. Samples were restriction digested at C282Y or H63D by adding 1 μl of either *Sna*BI (4 U/μl; New England Biolabs, Beverly, MA) or *Bcl*I (10 U/μl; New England Biolabs), respectively, to 1 μl of the recommended digestion buffer and 8 μl of amplicon. The samples were incubated for 2 hours at either 37°C (*Sna*BI) or 50°C (*Bcl*I), and products were visualized by ethidium bromide staining after separation on a 1.5% agarose gel at 5 V/cm for 60 minutes.

Table 1. Primer and Probe Sequences Used for Genotyping the HFE Gene

Codon	Forward primers	Reverse primers	Fluorescent probes
C282Y	TGGCAAGGGTAAACAGATCC	CTCAGGCACTCCTCTCAACC	AGATATACGTACCAGGTGGAG-fluorescein Cy5-CCCAGGCCTGGATCAGCCCCTCATTGT-GATCTGGG-P
H63D	CACATGGTTAAGGCCTGTTG	GATCCCACCCTTTCAGACTC	CGTGTTCTATGATGATGAGAGTCGCCG-fluorescein Cy5-GGAGCCCCGAACTCCATGGGTTTCCAG-TAGAATTTCAAGCCAGAT-P

Nucleotide bases involved in duplex mismatch formation are underlined. P indicates the addition of a phosphate group.

Sequencing

Four samples were sequenced for identification of the A193T polymorphism. PCR products were sequenced (model 377, Perkin-Elmer, Foster City, CA) from TOPO TA plasmid vectors (TOPO TA Cloning, Invitrogen, San Diego, CA).

Primer/Probe Synthesis

Primers for the C282Y codon[8] and the H63D codon were synthesized by standard phosphoramidite chemistry (Pharmacia Biotech Gene Assembler Plus, Piscataway, NJ). The 3′-fluorescein-labeled probes were synthesized on fluorescein-controlled pore glass cassettes (BioGenex, San Ramon, CA). A 5′-trityl group was retained on the fluorescein-labeled probes for purification of full-length sequences. Detritylation was performed on a Polypack column (Glen Research, Sterling, VA), and the labeled oligo was eluted with 50% acetonitrile. The 5′-Cy5 probes were synthesized using a Cy5 phosphoramidite (Pharmacia Biotech) and a chemical phosphorylation reagent (Glen Research) to prevent extension from the 3′ end of the probe.

Purity of probe synthesis was determined by calculating the ratio of fluorophore concentration to oligonucleotide concentration.[20] Probes with ratios outside 0.8 to 1.2 were further purified by reverse-phase C18 high-pressure liquid chromatography. Labeled oligonucleotides were passed through a 4× 250-mm Hypersil ODS column (Hewlett Packard, Fullerton, CA) using 0.1 mol/L triethylammonium acetate, pH 7.0, and a 20 to 60% (fluorescein probe) or 40 to 80% (Cy5 probe) gradient of acetonitrile (1 ml/minute). The eluate was monitored with tandem absorbance and fluorescence detectors (Waters 486 and 474, Milford, MA). Fractions with both A_{260} and fluorescence peaks were collected.

Primer/Probe Design

The HFE cDNA sequence was used for selection of primers and probes (Genbank accession M31944). Primers and probes were chosen using Primer Designer for Windows (Scientific and Educational Software, State Line, PA). Primers for both mutation sites were selected with similar melting temperature (T_m) values and GC content to allow multiplexing. The longer 5′-Cy5-labeled probes were designed with at least a 15°C higher T_m than the 3′-fluorescein-labeled probes that span the area targeted for mutation detection. In this way a Cy5-labeled probe acts as an anchor and remains annealed to the single-stranded amplicon while the fluorescein-labeled probe is heated through the characteristic T_m for that allele. The fluorescein-labeled probes were designed to have T_m values that would allow differentiation of all four alleles by melting peak analysis. The primer and probe sequences are shown in Table 1. Empirical melting temperatures for the 3′-fluorescein probe/allele duplexes are shown in Table 2.

Statistical Analysis

The statistical significance of the H63D mutation among the non-ancestral chromosomes was determined using a one-tailed Z test.[21]

Results

Genotyping with Adjacent Fluorescent Hybridization Probes

A schematic representation of the adjacent fluorescent hybridization probes used for genotyping the HFE locus is shown in Figure 1. The 3′-fluorescein-labeled probes spanning the C282Y and H63D sites were 21 and 27 bp long, respectively. These probes were designed so that during hybridization each probe formed a mismatch with the wild-type allele extended by the downstream primer. Table 1 shows the positions of the potential probe mismatches.

Amplification and genotyping of the C282Y site is illustrated in Figure 2. The 21-mer fluorescein probe formed an A:C mismatch with the wild-type sequence, lowering the T_m of the probe by 7°C from the completely complementary duplex. The wild-type allele shows no rise in fluorescence above background during amplification as the annealing temperature of the A:C mismatched duplex

Table 2. Empirical Melting Temperatures for Fluorescein Probe/Allele Duplexes

Codon	Allele	Duplex T_m
C282Y	G845	53°C
	845A	60°C
H63D	C187	63°C
	187G	68.5°C
	C187/193T	58.5°C

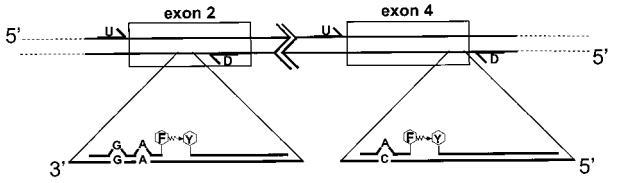

Figure 1. A schematic representation showing primer and probe placement for multiplex amplification and genotyping of HFE. Upstream (U) and downstream (D) primers are illustrated with respect to exon boundaries. Regions of exon 2 and exon 4 were amplified for analysis of the H63D (C187G) and C282Y (G845A) mutations, respectively. The fluorescein (F)-labeled probes are in fluorescence resonance energy transfer with the more thermally stable Cy5 (Y)-labeled probes. The fluorescein-labeled probes form a single mismatch when hybridizing to the single-stranded wild-type allele. The probe hybridizing within exon 2 forms two mismatches when hybridizing to a wild-type (C187) allele containing the S65C (193T) polymorphism.

was below the temperature at which fluorescence was acquired each cycle.

Amplification and genotype analysis for the H63D site is shown in Figure 3. The G:G mismatch formed at the center of the 27-mer fluorescein probe created a ΔT_m of 5.5°C from the completely Watson-Crick paired duplex. During amplification, both the wild-type allele and the allele with the H63D mutation were annealed to the probe at the fluorescence acquisition temperature of 62°C.

Genotyping most samples for the H63D mutation was done with a melting protocol that began at 55°C to observe melting transitions at 63°C and 68.5°C. However,

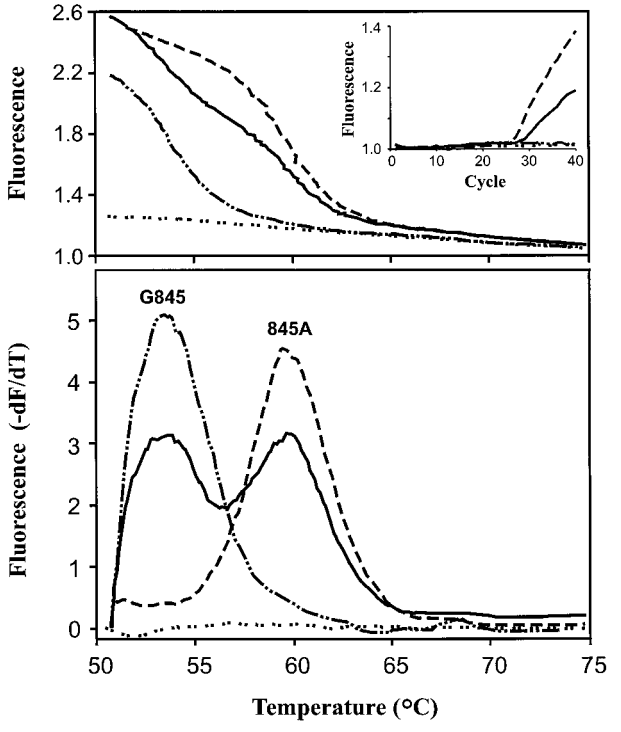

Figure 2. Real-time amplification and genotyping of the C282Y site. The **inset** shows amplification (Cy5 fluorescence *versus* cycle number) of the three genotypes: homozygous wild type (— - - —), heterozygous C282Y (———), and homozygous C282Y (– – –). A no-template control (- - - - -) is also included. Data for amplification and melting curve analysis were normalized to baseline for each sample by dividing each fluorescence value by the minimum fluorescence signal for that sample. Melting curve plots (**top**) of Cy5 fluorescence (*F*) *versus* temperature (*T*) are transformed into melting peaks (**bottom**) by plotting $-dF/dT$ *versus* temperature. Both amplification and genotyping analysis are completed within 45 minutes.

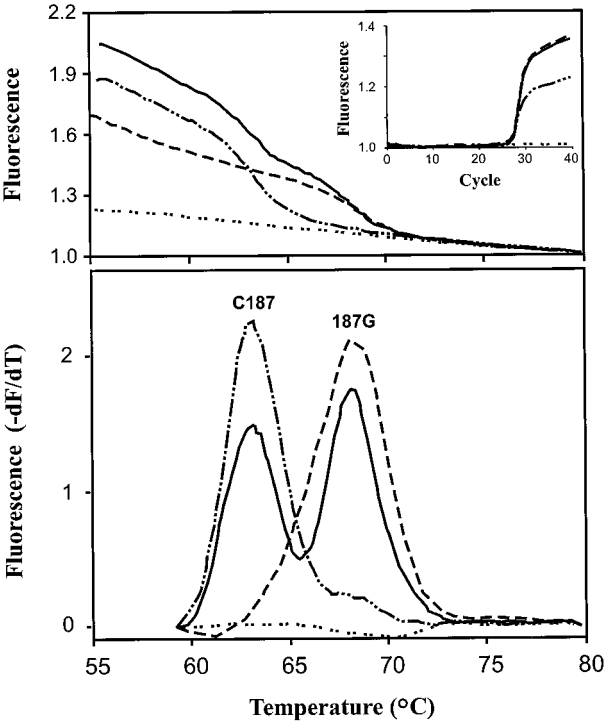

Figure 3. Real-time amplification and genotyping of the H63D site. The **inset** shows amplification (Cy5 fluorescence *versus* cycle number) of the three genotypes: homozygous wild type (— - - —), heterozygous H63D (———), and homozygous H63D (– – –). A no-template control (- - - - -) is also included. Data for amplification and melting curve analysis were normalized to baseline for each sample by dividing each fluorescence value by the minimum fluorescence signal for that sample. Melting curve plots (**top**) of Cy5 fluorescence (*F*) *versus* temperature (*T*) are transformed into melting peaks (**bottom**) by plotting $-dF/dT$ *versus* temperature. Both amplification and genotyping analysis are completed within 45 minutes.

50 samples were analyzed for the H63D mutation with melting curves beginning 10°C lower at 45°C. Using this melting protocol, four samples were identified that had a melting peak at 58.5°C, 4.5°C lower than the melting peak for the wild-type allele. Sequencing all four samples revealed an A-to-T transversion at nucleotide 193 of the open reading frame. This transversion is a recently reported polymorphism that results in a serine-to-cysteine amino acid substitution at codon 65 (S65C).[22] The S65C polymorphism creates an A:A mismatch located 8 bp in from the 3′ end of the 27-mer probe (see Figure 1 and Table 1). When the probe hybridizes to an allele that is wild type at the H63D site but contains the S65C polymorphism, two mismatches are present. This destabilizes the probe by 10°C compared with the completely complementary duplex. Samples heterozygous at the C282Y, H63D, and S65C sites are shown in Figure 4.

Comparison of Genotyping Methods

All cases genotyped by adjacent fluorescent hybridization probes agreed with PCR restriction fragment length analysis. Primers used for analysis of the C282Y site produced a 389-bp amplicon that was cleaved by *Sna*BI into fragments of 276 and 113 bp in the presence of the C282Y mutation. The PCR product for the H63D site was

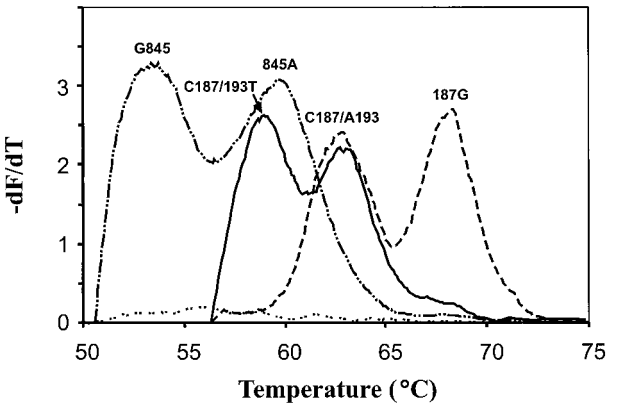

Figure 4. Derivative melting curves showing three heterozygous genotypes. The 3′-fluorescein-labeled probe spanning the region amplified within exon 2 (Figure 1) reveals the heterozygous C187G genotype (– – –) at the H63D site and the heterozygous A193T genotype (———) at the S65C site. The probe spanning the C282Y site identifies the heterozygous G845A genotype (— – – —).

241 bp long. The H63D mutation destroyed a *Bcl*I restriction site that upon restriction digestion of the wild-type allele yielded fragments of 138 and 103 bp. The run time alone required for PCR and genotype analysis by restriction digestion and gel electrophoresis was approximately 3 hours and 30 minutes.

In comparison, genotyping with adjacent fluorescent hybridization probes was faster and easier. Amplification and analysis of 24 samples independently at both sites required 45 minutes. No manual manipulation between amplification and genotyping was required. Analysis by multiplexing allowed twice as many samples to be genotyped at both sites in the same amount of time. Genotype analysis by multiplexing with adjacent fluorescent hybridization probes is shown in Figure 5.

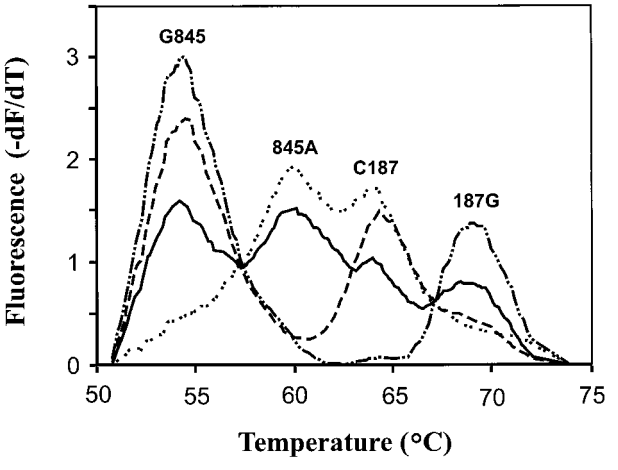

Figure 5. Homogeneous multiplex genotyping by derivative melting curves for four alleles. Shown are four samples with different C282Y/H63D genotypes: homozygous G845/homozygous C187 (– – –), homozygous G845/homozygous 187G (— – —), homozygous 845A/homozygous C187 (– – – – –), and heterozygous G845A/heterozygous C187G (———).

Study of the C282Y and H63D Mutations

One hundred and seventeen patients who met clinical criteria for iron overload and fifty-six normal controls were selected for analysis of the C282Y and H63D mutations in the HFE gene. Both groups were Caucasian Americans from Utah and neighboring states. The results of this study are summarized in Table 3.

Ninety-eight (83.8%) of the patients and none of the controls were homozygous for the C282Y mutation. The C282Y mutation was found in 87% of patient chromosomes and 6.3% of chromosomes from normal controls. The H63D mutation was found in 11% of control chromosomes and only 4.3% of patient chromosomes. There were eight patients and seven normal controls heterozygous for the C282Y mutation. One-half of the C282Y heterozygous patients carried the H63D mutation, whereas there were no compound heterozygous genotypes among the controls.

The S65C polymorphism was found in 4 of 50 samples. Three of these samples were from the normal control group. The allelic frequency for the S65C variant was 5.5% among the control chromosomes and 2.8% among the patient chromosomes.

Discussion

Multiplex technology continues to advance both research and routine diagnostics. Sensitive methods of multiplex analysis combined with improved methods of DNA preparation increase the density of information obtained from small amounts of whole blood.[23,24] This reduces the cost and invasiveness of sample collection and is useful in the

Table 3. Analysis of the C282Y and H63D Mutations within the Utah Population

Genotype*		Patients with iron overload		Controls	
C282Y	H63D	*n*	%	*n*	%
hh	HH	98	83.8	0	0
Hh	HH	4	3.4	7	12.5
Hh	Hh	4	3.4	0	0
HH	HH	6	5.1	38	67.9
HH	Hh	4	3.4	10	17.9
HH	hh	1	0.9	1	1.8
Total		117		56	

* hh designates homozygosity for the mutation; Hh designates heterozygosity; HH designates homozygous wild type.

Allele frequencies for the C282Y genotype were 204/234 (87.1%) for patients with iron overload and 7/112 (6.3%) for controls. For the H63D genotype, allele frequencies were 10/234 (4.3%) for patients with iron overload and 12/112 (10.7%) for controls.

analysis of rare samples. Population screening for presymptomatic diagnosis of hereditary hemochromatosis by DNA analysis has been proposed since the first report of the hemochromatrosis gene.[8,9,25,26] As of November 1997, the American Medical Association has resolved to establish guidelines for screening for this genetic disease. Given that DNA testing is invariant and there is diverse etiology for elevated blood iron levels,[25,27] it seems prudent that genetic testing should be included in algorithms for the diagnosis of hemochromatosis. Such large-scale diagnostic tests will become more time efficient and cost effective through multiplexing.

Although many PCR-based fluorescent multiplex techniques are being developed,[28–32] few are homogeneous and none genotype by allele/probe duplex T_m. Several methods multiplex with target-specific oligonucleotides labeled with different colored fluorophores.[28–30] For example, hairpin and exonuclease probes use oligonucleotides dually labeled with quencher and reporter dyes to genotype during real-time PCR. Targets are distinguished by multiplexing allele-specific probes each labeled with a different colored reporter. The probe that is completely complementary to the template shows a rise in reporter fluorescence as it is cleaved during the extension of *Taq* polymerase. Multiplexing using a completely complementary probe for each allele provides a positive control for amplification, and variants can be genotyped in a homogeneous reaction.

In a similar way, hybridization probes can be used to genotype in real time as shown by the amplification of the C282Y site. When fluorescence is acquired above the T_m for the mismatched probe/allele duplex, only the allele that is completely complementary to the probe shows a change in fluorescence during amplification. Heterozygotes yield one-half the maximal fluorescence compared with the homozygous perfect match. Similar to exonuclease probes, multiplexing with a probe set of a different color would be necessary for a positive control. However, multiple colors are not necessary for homogeneous genotyping with melting curves because multiple alleles can be distinguished with a single probe. Moreover, melting curves prevent false negative results as all alleles are represented and are equally effected by inhibitors within the reaction.

Multiplexing with fluorescent hybridization probes provides rapid and sensitive analysis of multiple alleles. Amplification and genotyping of the HFE gene mutations were performed in approximately 45 minutes. The current rate-limiting step in this assay is DNA extraction. Although the organic extraction method used here provided reliable amplification and fluorescent genotyping, more recent DNA extraction methods yield equivalent amounts of DNA from whole blood while being safer, cheaper, and requiring less time.[19,24] For example, several DNA extraction kits now available can provide approximately 2 μg of DNA from 25 μl of whole blood within 1 to 2 hours.[24] Thus, a small blood donation obtained by only a finger or heel stick can be rapidly processed and analyzed multiple times.

Genotyping with multiplexed hybridization probes has technical challenges and limitations in addition to the optimizations often necessary for multiplexing primer sets.[33] For example, the melting temperatures of the probe/template duplexes must allow differentiation of all alleles by derivative melting curve analysis. The number of mutations that can be analyzed within a single reaction is limited by the number of melting peaks that can be distinguished over a range of probe melting temperatures. The HFE assay demonstrated here showed four different alleles differentiated over a 15°C temperature range.

Another limitation of multiplexing hybridization probes is that unexpected variants may cause erroneous interpretation. When the A193T (S65C) variant was analyzed with the probe designed for the H63D site, the duplex T_m was shifted near the T_m of the probe covering the C282Y mutation. Hence, there is some risk of a C282Y false positive when multiplexing. However, the 1.5°C difference in T_m between S65C and C282Y is distinguishable, and the samples were correctly genotyped at the C282Y codon as verified by restriction enzyme digestion.

The C282Y substitution is a founding mutation for hemochromatosis.[34] It has been known for over 20 years to be associated with the HLA A3, B7 haplotype, and it appears to have occurred relatively recently in Celtic history.[34–37] Accordingly, studies from populations of northern European descent, including this study, show that between 82 and 100% of hemochromatosis patients are homozygous for this ancestral mutation.[8–11] However, an increase in the heterogeneity of the disease is observed in populations outside of northern Europe.[38,39]

In comparison, the role that the H63D substitution plays in the development of iron overload disease has been controversial. Concerns include 1) that the mutation occurs with similar frequencies among patients and controls, 2) that homozygotes for the H63D mutation are rare among patients, and 3) that compound heterozygotes (C282Y/H63D) within patient populations are found at a lower frequency than predicted.[40]

As the H63D mutation has never been found on the same chromosome as the C282Y mutation, only the nonancestral (non-C282Y) haplotypes are at risk for the H63D mutation. In light of this observation, a compelling review of the data has shown that 74% of chromosomes from iron overloaded patients heterozygous at the C282Y site carried the H63D mutation.[40] This suggests that the C282Y/H63D genotype confers a risk for the development of iron overload disease.[8,9] Our study found that the penetrance of the H63D mutation was slightly higher than previously reported values.[8–10]

Adjacent fluorescent hybridization probes are versatile and amenable to multiplexing. The C282Y and H63D mutation sites were co-amplified with different primer sets and simultaneously genotyped by the melting temperatures of multiplexed fluorescent probes. Moreover, the probe spanning the C187G (H63D) mutation could also be used for genotyping the A193T (S65C) polymorphism after the variant was detected by an aberrant melting curve and confirmed by sequencing. The identification of this polymorphism demonstrates genotyping of multiple alleles by a single probe and suggests a potential for

fluorescent hybridization probes in scanning for unknown variants.

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