Genetic Hemochromatosis, to Detect the Use of Denaturing HPLC and a Heteroduplex Generator (Transgenomic) for genotyping of individuals with HH are homozygous for a 845G→A missense mutation (C282Y) in the HFE gene that encodes a MHC class I-like protein (1–5). One in 300 adults of Caucasian descent has this genotype (3, 6). The disease is characterized by increased absorption of dietary iron by the gastrointestinal tract and the progressive deposition of iron in the parenchymal cells. It produces a wide range of clinical complications, including hepatic fibrosis, cirrhosis, hepatocellular carcinoma, diabetes mellitus, hypopituitarism, hypogonadism, arthritis, and cardiomyopathy (7). These complications can be entirely prevented with regular iron removal by phlebotomy. The availability of this effective treatment highlights the value of screening appropriate populations for the C282Y mutation to detect HH before irreversible complications occur (8). Another frequent mutation in the HFE gene, H63D, has been described (1), but the relationship between this mutation and iron overload is less clear. Although compound heterozygotes C282Y/H63D (1) and H63D homozygotes (9) may show some degree of iron overload, the penetrance associated with these genotypes is very low, and clinical features of hemochromatosis, especially liver disease, are rare. In opposition to C282Y, screening the population for H63D is therefore not indicated.

An important issue is the development of a cost-effective methodology suitable for genotyping large numbers of samples for the C282Y mutation. Many methods have been used to detect C282Y, including oligonucleotide ligation assays (1), fragment length polymorphism analyses of PCR products after restriction enzyme digestion (5), allele-specific oligonucleotide hybridization assays (2), mutagenically separated PCR assays (10), primer extension assays (11, 12), and allele refractory mutation systems (13, 14). All of these assays are time-consuming. In addition, they rely on complicated multistep procedures involving radioisotopes or requiring manipulation of the PCR products before electrophoretic analysis, which makes them inadequate for large-scale screening purposes. Other methods, such as the TaqMan technology (15), are more suitable for large-scale analysis but require expensive probes.

The aim of this work was to assess the WAVE™ DNA Fragment Analysis System (Transgenomic) for genotyping the HFE C282Y mutation. The ability of the WAVE system to resolve heteroduplexes from homoduplexes by denaturing HPLC (DHPLC) in minutes makes it a powerful tool in the field of mutation detection. However, use of DHPLC in screening for an autosomal recessive disease such as hemochromatosis is limited by the fact that, although homo- and heteroduplexes are clearly resolved, it is not possible to distinguish between wild-type individuals and C282Y homozygotes unless each PCR product is mixed with DNA from a wild-type individual in a second step and run again (16, 17), which considerably slows down the genotyping process. To circumvent this problem, we constructed a heteroduplex generator by site-directed mutagenesis, as suggested by Jackson et al. (18), and coamplified this heteroduplex generator with each DNA sample. This approach was applied to the genotyping of 1011 unrelated individuals.

The 392-bp heteroduplex generator is homologous to a 395-bp wild-type sequence spanning the C282Y mutation site but contains a three-nucleotide deletion corresponding to the codon immediately 5′ to the codon containing the mutation. It was constructed using the QuickChange site-directed mutagenesis reagent set (Stratagene) and a supercoiled double-stranded DNA vector with a 395-bp insert corresponding to HFE exon 4. Use of the QuickChange reagent set is straightforward and requires only basic molecular biology techniques. Briefly, two oligonucleotide primers with the three-nucleotide deletion, 5′-GAA GAG CAG AGA TAT TGC CAG GTG GAG CAC-3′ and 5′-GTG CTC CAC CTG GCA ATA TCT CTG CTC TTC-3′, each complementary to opposite strands of the vector, were extended during temperature cycling with Pfu Turbo DNA polymerase. After temperature cycling, the product was treated with DpnI to digest the parental DNA template and to select for mutation-containing synthesized DNA. The vector DNA incorporating the three-nucleotide deletion was then transformed into XLI-Blue supercompetent cells, and the presence of the deletion in the heteroduplex generator was confirmed by sequencing. A plasmid preparation from a 200-ml culture produced ~500 µg of heteroduplex generator, a quantity large enough to genotype millions of individuals. Each DNA sample (5 µL at 10 ng/µL) was coamplified with 1 µL of a suitable dilution of heteroduplex generator (~0.1 ng/µL). PCR amplifications (total volume, 25 µL) were performed in PCR Master Mix 1X (Promega), with 0.5 µM each of the forward (5′-TGG CAA GGG TAA ACA GAT CC-3′) and reverse (5′-TAC CTC CTC AGG CAC TCC TC-3′) primers. The PCR conditions were 35 cycles of 95 °C for 30 s, 55 °C for 30 s, and 72 °C for 30 s. Heteroduplex formation was induced by heat denaturation of PCR products at 94 °C for 5 min, followed by gradual reannealing from 94 °C to 20 °C over 45 min.

For DHPLC analysis, PCR products (10 µL/sample) were eluted at a flow rate of 0.9 mL/min with a linear acetonitrile gradient. The buffer gradient (buffer A, 0.1 mol/L triethylammonium acetate; buffer B, 0.1 mol/L triethylammonium acetate containing 250 mL/L acetonitrile), start and endpoints of the gradient, and melting temperature predictions were determined by the WaveMaker software (Transgenomic). A run temperature of 61 °C allowed best discrimination between the different profiles. Data analysis was based on visual inspection of the chromatograms and comparison with three control
samples harboring a homozygous wild-type, a homozygous mutated, and a heterozygous sequence, respectively. As shown on Fig. 1A, heteroduplexes always resulted from the mixed hybridization between the DNA sample to be genotyped and the heteroduplex generator but had different retention times, depending on whether the wild-type (WT) G nucleotide or the mutated A nucleotide was present. The two heteroduplex species were formed by C282Y heterozygous samples. The profiles corresponding to the three possible genotypes (WT/WT, WT/C282Y, and C282Y/C282Y) were clearly distinguished, although with this approach, genetic diagnosis was reduced to a single PCR reaction per sample.

DHPLC analysis with the heteroduplex generator was first validated on 94 HH patients previously genotyped by allele-specific oligonucleotide hybridization (2). All C282Y heterozygous samples. The profiles corresponding to the three possible genotypes (WT/WT, WT/C282Y, and C282Y/C282Y) were clearly distinguished, although with this approach, genetic diagnosis was reduced to a single PCR reaction per sample.

DHPLC analysis with the heteroduplex generator was first validated on 94 HH patients previously genotyped by allele-specific oligonucleotide hybridization (2). All
individuals had given informed consent, as mandated by the French Committee for the Protection of Human Subjects. There was perfect agreement between the results obtained with the two genotyping methods, but in four individuals with a GG genotype, a variant profile was obtained. The DNA from these four individuals was reamplified with the original set of primers, and the amplicon was sequenced in both directions by means of ABI PRISM BigDye Terminator v3.0 Ready Reaction Sequencing reagent set on an ABI 3100 DNA sequencer (Applied Biosystems). All four were heterozygous for the previously described IVS4 + 48G→A polymorphism in intron 4 of the HFE gene (19).

To test the adequacy of this genotyping method for large-scale screening purposes, we genotyped 1011 unrelated individuals. Additional profiles were observed (Fig. 1B), which were shown by sequencing to correspond to different genotypic combinations at the two mutation sites (C282Y and IVS4 + 48G→A). The distribution of the observed genotypes in the 1011 unrelated individuals is given in Table 1. The allele frequency of the C282Y mutation was 4.6%, which was comparable to that previously described (2).

In this French population, the IVS4 + 48G→A variant has an allele frequency of 9.8%, which confirms that it is a fairly prevalent polymorphism, as observed by Jeffrey et al. (20) in a population of voluntary blood donors in Canada.

The recently described association between hemochromatosis and the C282Y mutation in HFE has prompted the need for a simple and rapid genetic test suitable for population screening. Various PCR-based methods have been used for the detection of the C282Y mutation, but not all are appropriate for the study of large numbers of samples. We have shown that heteroduplex analysis with the WAVE System provides a simple, rapid, and effective means of detecting the hemochromatosis-specific C282Y mutation and distinguishing the three possible genotypes when DNA samples are amplified with a heteroduplex generator. It is entirely automated, uses standard PCR buffer and DNA polymerase and regular primers (i.e., no fluorescent labeling or terminal modification), does not need restriction endonuclease digestion or other treatment after PCR, and allows rapid processing of large sample numbers. It can also detect additional mutations present in the targeted PCR product, as demonstrated here for the IVS4 + 48G→A polymorphism. Of interest, this technique, which allows differentiation of homozygous mutant from homozygous wild-type genotypes, could be applied more widely to the study of autosomal recessive diseases characterized by a few highly prevalent mutations.

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References


Table 1. Genotypes of 1011 unrelated French individuals obtained by DHPLC analysis.

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<thead>
<tr>
<th>Genotype</th>
<th>Observed number of individuals</th>
<th>Percentage</th>
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<tbody>
<tr>
<td>WT/WT</td>
<td>G/G</td>
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<tr>
<td>WT/WT</td>
<td>G/A</td>
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<tr>
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</tr>
<tr>
<td>C282Y/C282Y</td>
<td>G/G</td>
<td>3</td>
</tr>
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