To the Editor:

The major histocompatibility complex class I-like gene, HLA-H, was recently reported as being the candidate gene for the autosomal recessive disorder hereditary hemochromatosis (HH). There is still no formal proof that HLA-H is the HH gene, but the evidence can be compelling, although it may not be the only gene involved. A single base mutation, Cys282Tyr, appears to have a causative role in HH and a second variant, His63Asp, may also have a role. Detection of these two mutations is certainly of value in diagnosing hemochromatosis individuals. As a result, polymerase chain reaction (PCR) has been used for rapid detection of the mutations and a further modification for elucidation of any codon 282 mutations was recently reported in Blood.

Beutler et al detect the presence of a mutation in their PCR product by time consuming allele-specific oligonucleotide hybridization. The original PCR-restriction fragment length polymorphism (PCR-RFLP) method of Jazwinska et al allows identification of zygosity at both alleles, but takes some time to complete and uses expensive restriction enzymes (SnaBI, £36/100 U; MboI, ~£43/200 U). Although the aim of Chang et al is rather to detect any mutation at codon 282 to emphasize the codon’s importance in HLA-H function, they also use protracted cycling and are tied to the use of the expensive restriction enzyme BclI. The His63Asp mutation appears to increase the risk of hemochromatosis when in conjunction with the Cys282Tyr mutation and should also be looked for until any causative role can be disproved. Jouanolle et al detect both mutations, but do not show their PCR cycling conditions and are still using MboI for codon 63 mutation detection. A rapid, robust, and less expensive method for PCR-RFLP detection of both HLA-H mutations has been developed for use in our routine laboratory.

Separate PCR reactions are conducted for the two mutations using the primers described by Feder et al for their oligonucleotide ligation assay (OLA; Table 1). DNA is extracted from 200 μL whole blood using a QIAamp Blood kit (Qiagen, Crawley, UK), eluting from the spin column with 200 μL buffer. A total PCR volume of 25 μL contains 100 ng of each primer, 1× manufacturer’s PCR buffer, 200 μmol/L each dNTP, 2 μL (~50 ng) DNA, and 0.4 U Dynazyme (Flowgen, Shenstone, UK). After 2 minutes of initial denaturation at 94°C, 35 cycles of just 1 minute at 94°C and 1 minute at 58°C are conducted in a Perkin Elmer 480 thermal cycler (Perkin Elmer, Warrington, UK). Restriction digests are performed directly in the PCR mixes by addition of 5 U RsaI (codon 282 reactions) or BclI (codon 63 reactions) and incubating for 2 hours at 37°C or 50°C, respectively. The products are electrophoresed on a 3% agarose gel (Fig 1).

Amplification with the primers for codon 282 produces a PCR product of 390 bp, which in the wild-type (Cys) digests to give fragments of 250 and 140 bp and in the mutant (Tyr) fragments of 250, 111, and 29 bp (Fig 1). (The 29-bp fragment is not always visible and is not shown here.) Amplification for codon 63 gives a 208-bp product that digests in the wild-type (His) to produce 138- and 70-bp pieces but does not digest in the mutant (Asp; Fig 1).

This PCR has also been conducted successfully in my laboratory using a Techne (Cambridge, UK) Genius thermocycler and other manufacturers’ DNA polymerase, including TaKaRa Taq (Severn Biotech, Kidderminster, UK) and AmpliTaq Gold (Perkin Elmer, Warrington, UK). The cycling conditions are rapid and the restriction enzymes used cheap, ie, approximately one tenth the cost of those used by Jazwinska et al (RsaI ~£34/1,000 U; BclI, ~£36/2,000 U).

The increased speed, reduced cost, and robust nature of this method make it the method of choice for the routine diagnostic laboratory.

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**Figure 1. Detection of the HLA-H Cys282Tyr and His63Asp mutations by PCR-RFLP.** The top panel shows the result of PCR with the codon 282 primers and restriction digest by RsaI. The bottom panel is PCR product obtained with the codon 63 primers and digested with BclI. The patients are as follows: track 1, normal; track 2, homozygous mutant, codon 282; normal, codon 63; track 3, heterozygous mutant, codon 282; normal codon 63; track 4, compound heterozygote; track 5, water negative control.

**Table 1. Primers for HH PCR**

<table>
<thead>
<tr>
<th>Codon</th>
<th>Forward Primer</th>
<th>Reverse Primer</th>
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<tr>
<td>282</td>
<td>5’-TGCCAAGGGTAAACAGATCC</td>
<td>5’-CTCAGGCACTCCTCTCAACC</td>
</tr>
<tr>
<td>63</td>
<td>5’-ACATGTTAAGGCTGTGTTG</td>
<td>5’-GCCACATCTGGCTTGAAATT</td>
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ACKNOWLEDGMENT
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REFERENCES

Neonatal Screening for the Hemochromatosis Defect

To the Editor:

Hereditary hemochromatosis (HC) is an autosomal recessive disorder of iron metabolism that is characterized by inappropriate iron absorption and storage of excess iron in the parenchymal cells of major organs, primarily the liver, pancreas, heart, pituitary, and joints. High levels of iron stored in these organs can lead to cirrhosis, hepatocellular carcinoma, cardiac dysfunction, diabetes, arthritis, hypogonadism, and premature death. However, patients can have a normal life expectancy if the disorder is diagnosed in the early stages and phlebotomy therapy is undertaken to remove the excess iron.

A novel major histocompatibility complex (MHC) class I-like gene termed HLA-H was recently identified telomerically of the classical MHC complex on the short arm of chromosome 6 and proposed as a candidate for HC. In this initial report, 83% of HC patients were found to be homozygous for a single missense mutation, causing an amino acid substitution of cysteine to tyrosine at residue 282. Subsequent studies have found 92.4% and 100% of a final step of 72°C for 5 minutes, followed by 35 cycles of 94°C for 50 seconds and then 72°C for 1 minute. Samples were then analyzed for 5 minutes, followed by 35 cycles of 94°C for 30 seconds, 55°C for 50 seconds, and 72°C for 50 seconds and then a final step of 72°C for 5 minutes. PCR was performed in a Perkin Elmer 9600 thermal cycler.

The presence of the C282Y mutation creates a Smal restriction site in the amplified product enabling detection of the mutation by enzyme digest. Twelve microliters of the amplified product was digested with 2 units of Smal (Promega, Madison, WI) for 2 hours at 37°C in a total volume of 20 µL. Samples were then analyzed on a 2% agarose gel. A control sample, known to be homozygous for C282Y, was included to confirm complete digestion of all samples.

Of the 1,660 samples analyzed, 8 (1 in 200 or 0.4%) were homozygous for the C282Y mutation and 186 (1 in 9 or 11.2%) were heterozygous. This gives a frequency for the HC allele of 0.061 in this predominantly Caucasian population. Previous estimates of the frequency of homozygotes for this disorder have been lower than this; however, all previous screening studies have relied on biochemical and/or pathological expression of HC for diagnosis. This indicates that phenotypic expression of HC might be prevented in approximately one third of individuals homozygous for the mutant allele, due to environmental factors such as dietary iron intake and physiological blood loss, as well as sex and age. In addition, a small percentage of patients heterozygous for the C282Y mutation have been shown to meet clinical diagnostic criteria for HC, further indicating the significance of environmental and possibly other genetic factors in expression.
A Cheaper and More Rapid Polymerase Chain Reaction-Restriction Fragment Length Polymorphism Method for the Detection of the HLA-H Gene Mutations Occurring in Hereditary Hemochromatosis

Caroline Lynas