Simultaneous Dual-Color Detection of the C282Y, H63D, and S65C Mutations in the Hemochromatosis Gene *HFE* by a LightCycler PCR Assay

Sven G. Gehrke

Department of Internal Medicine IV, University Hospital Heidelberg
Bergheimer Strasse 58, D-69115 Heidelberg, Germany

Dr. Sven G. Gehrke
University Hospital Heidelberg
Department of Internal Medicine IV
Bergheimer Strasse 58
D-69115 Heidelberg - Germany
Fax: 49-6221-564557
E-mail: sven.gehrke@med.uni-heidelberg.de
Hereditary hemochromatosis is an iron overload disease inherited as an autosomal recessive trait. Classical clinical features of advanced disease include cirrhosis of the liver, diabetes, cardiac failure, arthropathy, and hypogonadism. Approximately 3 to 5 per 1000 Caucasians are affected by the disease (1). Except in Italy (2), 83-100% of these patients present with a homozygous G-to-A-transition at nucleotide 845 in the hemochromatosis gene $HFE$ (3,4,5). The resulting cysteine-to-tyrosine substitution at amino acid position 282 (C282Y mutation) of HFE, resembling an MHC class I protein, disrupts the intramolecular disulfide bridging responsible for interaction with $\beta_2$-microglobulin. Since $\beta_2$-microglobulin is essential for export of HFE and its insertion in the cell membrane, the C282Y mutation would prevent surface presentation of HFE (6). Therefore, the interaction of HFE with the transferrin receptor as a key step of regulation of transferrin mediated iron uptake (7) is lost.

Two other frequent mutations in HFE have been identified at amino acid position 63 (H63D mutation, C-to-G-transition at nucleotide 187) and amino acid position 65 (S65C mutation, A-to-T-transition at nucleotide 193) (3,4,8). The H63D mutation may lead to a mild form of hemochromatosis if inherited together with the C282Y mutation. The resulting compound heterozygote state accounts for approximately 4-5% of hemochromatosis cases (3,4). In contrast, the S65C mutation is not obviously involved in the development of hemochromatosis. However, one recent study showed evidence that the S65C mutation is also implicated in mild form of hemochromatosis (8).

In general, detection of these mutations requires PCR-restriction fragment length polymorphism analysis or other time-consuming methods (4,5,8). Recently, assays for rapid simultaneous detection of the C282Y and H63D mutations (9) as well as H63D and S65C mutations (10) using fluorescently labeled oligonucleotide
hybridization probes on the LightCycler™ Instrument (Roche Diagnostics) have been described.

Detection of genetic polymorphisms using the LightCycler system is based on fluorescence monitoring of two adjacent hybridization probes (11). In such an approach, PCR is performed by rapid cycling with a set of primer. The reaction mixture also contains two fluorescently labeled hybridization probes recognizing adjacent sequences in the amplicon. The detection probe is labeled with fluorescein as donor fluorophore at the 3’ end and spans the polymorphic site. The anchor probe anneals at a distance of one to five bases 3’ from the detection probe. It is labeled with an acceptor fluorophore (LightCycler Red 640 or LightCycler Red 705) at its 5’ end and modified at the 3’ end by phosphorylation. During PCR real-time monitoring of the amplification process is provided by fluorescence resonance energy transfer (FRET) between the annealed hybridization probes. After completion of the PCR a melting curve is generated by increasing the temperature slowly. Depending on a mismatch between the PCR product and the detection probe, the hybrid is destabilized on different temperatures. The resulting melting curves reveal a characteristic melting profile for each genotype. Since fluorescence emitted from the available acceptor fluorophores LightCycler Red 640 and LightCycler Red 705 is monitored separately (channel 2 and channel 3), one-color or dual-color assays can be established. A dual-color assay may be necessary for genotyping two different mutation sites within a multiplex PCR (12).

Recently, Mangasser-Stephan et al. (9) published a LightCycler multiplex PCR assay containing two sets of unlabeled primers and two sets of hybridization probes for one-color detection of C282Y and H63D mutations with LightCycler Red 640 as acceptor fluorophore. Before using this method for genotyping 396 patients with
evidence for iron overload, the antisense primer for amplifying the C282Y mutation site (5´-CTC AGG CAC TCC TCT CAA CC) was replaced by 5´-TAC CTC CTC AGG CAC TCC TC. This modification was necessary in order to prevent incorrect results as described by Jeffrey et al. (13). In detail, the former antisense primer anneals at a frequent polymorphic site in intron 4 of HFE (IVS4+48G/A) which is associated with the wild-type allele at the C282Y mutation site. If the polymorphism in intron 4 is present, this specific allele may not be amplified effectively during the PCR process. As a consequence, a patient compound heterozygous for the C282Y mutation and the polymorphism in intron 4 of HFE may be misdiagnosed as homozygous for the C282Y mutation. In contrast, the latter antisense primer does not anneal at the polymorphic site within intron 4 and therefore prevents incorrect assignation of C282Y heterozygotes as homozygotes (13).

Despite changing the antisense primer for detection of the C282Y mutation, this method worked perfectly and 396 patients were genotyped for C282Y and H63D mutations. Twelve out of these patients demonstrated melting peaks indicating a homozygous H63D mutation and homozygous wild-type alleles within the C282Y mutation site (Fig. 1a). In two other patients an additional melting peak which could not be related to the typical melting profiles was observed (Fig. 1b). Initially, this additional peak was interpreted as a primer dimer and both patients were diagnosed as homozygous for the H63D mutation. Since these results were questionable, the C282Y and H63D mutation sites were sequenced in both patients using the ABI 310 Genetic Analyzer. Sequence analysis demonstrated that both patients were compound heterozygous for the H63D and S65C mutation in HFE. In fact, the additional melting peak resulted from the S65C mutation and both patients had been misdiagnosed.
The detection probe used in this assay anneals not only at the H63D mutation site (C187G) but also at the S65C mutation site (A193T). As shown in Fig. 1b, a compound heterozygous state for the H63D and S65C mutation results in a melting peak for the H63D mutation (187G allele) and in a loss of the melting peak for the wild-type allele at the H63D mutation site (C187 allele). Without taking into consideration that the results are influenced by the presence of the S65C mutation, this melting profile could be interpreted as homozygous for the H63D mutation. Another erroneous interpretation may result from the difference of only 2°C between the melting peaks for the C282Y and S65C mutations (845A and 193T allele; Fig. 1b). Therefore, a patient carrying the S65C mutation may be misdiagnosed as a carrier of the C282Y mutation.

Considering these observations we decided to develop a reliable multiplex PCR LightCycler assay using dual-color detection of all three mutations within one capillary. Based on the methods of Mangasser et al. (9) and Bollhalder et al. (10), primer and hybridization probes (obtained from TIB MOLBIOL, Germany) as described in Table 1 were used for genotyping the C282Y, H63D, and S65C mutations. Genotyping of the C282Y mutation was performed with a detection probe perfectly matching the 845A allele responsible for the C282Y mutation. Therefore, the melting peak indicating the C282Y mutation occurs at a higher temperature than the melting peak indicating the wild-type allele (G845 allele). For simultaneous detection of the H63D and S65C mutations we used a detection probe perfectly matching the 187G allele responsible for the H63D mutation. In this approach, the melting peak indicating the H63D mutation occurs at the highest temperature. The wild-type allele (C187/A193 allele) contains one mismatch compared with the detection probe. This mismatch results in a melting peak at a lower temperature. In
contrast, the 193T allele responsible for the S65C mutation contains two mismatches compared with the detection probe and leads to a melting peak at the lowest temperature. While the C282Y mutation is genotyped with an acceptor fluorophore labeled with LightCycler Red 640 (C282Y Anchor Probe), the H63D and S65C mutations are determined using an acceptor fluorophore labeled with LightCycler Red 705 (H63D/S65C Anchor Probe).

The PCR was performed in a total volume of 10 µL containing 1 µl genomic DNA isolated from 200 µL whole blood or PBMC using the QIAamp Blood Kit (Qiagen), 0.1 µl of 5 units/µl Platinum Taq Polymerase (Gibco), 0.5 µl of 10 mg/ml BSA (MBI Fermentas), 0.5 µl of 50 mM MgCl$_2$, 0.2 µl of 10 mM dNTP mixture (MBI Fermentas), 0.5 µl of DMSO 5%, and 1 µl of 10x PCR reaction buffer (Gibco). The reaction mixture contained each primer (C282Y Sense Primer, C282Y Antisense Primer, H63D/S65C Sense Primer, H63D/S65C Antisense Primer) at a concentration of 0.5 µmol/L. Hybridization probes were added to a final concentration of 0.5 µmol/L for C282Y Anchor Probe, 0.2 µmol/L for C282Y Detection Probe, 0.25 µmol/L for H63D/S65C Anchor Probe, and 0.25 µmol/L for H63D/S65C Detection Probe.

LightCycler PCR assay was performed with the following protocol:

Denaturation of the template DNA and activation of the hot start Taq Polymerase for 1 cycle at 95°C for 90 s with a transition rate of 20°C/s;

Amplification of the target DNA for 35 cycles of 95°C for 0 s, 54°C for 10 s, and 72°C for 16 s, each with a transition rate of 20°C/s;

Melting curve analysis for 1 cycle of 95°C for 0 s and 45°C for 30 s with a transition rate of 20°C/s, and then ramping to 85°C for 0 s with a transition rate of 0.1°C/s;
Fluorescence parameters were set as followed: display mode F2/1 or F3/1 single at 54°C for amplification; display mode F2/1 or F3/1 continuous for melting curve analysis; fluorescence gains were F1/1, F2/15, F3/30;
For melting curve interpretation fluorescence parameters were set F2/F1 for detection of the C282Y mutation and F3/F1 for detection of the H63D and S65C mutations;
Color compensation was used for monitoring the PCR process and for melting curve interpretation.

Under these experimental conditions destabilization of hybrids within the C282Y and H63D/S65C mutation sites is monitored in different channels of the LightCycler instrument. Melting profiles of the C282Y and H63D/S65C mutation sites can be interpreted independently on different analysis screens (Fig. 1c, 1d, and 1e). The T_m differences (Table 1) allowed a clear distinction between each specific genotype.
In order to proof this method, DNA samples from 261 patients were genotyped. Melting curves were clearly interpretable without any questionable results in all patients including those two patients misdiagnosed before. The following genotypes were found: 17 patients homozygous for the C282Y mutation; 11 patients homozygous for the H63D mutation; 13 patients compound heterozygous for the C282Y and H63D mutations; 3 patients compound heterozygous for the C282Y and S65C mutations; 5 patients compound heterozygous for the H63D and S65C mutations; 45 patients heterozygous for the C282Y mutation and wild-type at the H63D and S65C mutation sites; 87 patients heterozygous for the H63D mutation and wild-type at the C282Y and S65C mutation sites; 8 patients heterozygous for the S65C mutation and wild-type at the C282Y and H63D mutation sites; 72 patients
wild-type at all three mutation sites. All nine genotypes were controlled by direct sequencing using the ABI 310 Genetic analyzer. Taken together, this method was tested successfully in nine of ten possible $HFE$ genotypes. The only genotype not found in our series of patients was the rare homozygosity for the S65C mutation. This LightCycler assay provides for the first time rapid detection of all three major $HFE$ mutations within one multiplex PCR. Regarding cost-efficiency the small reaction volume of 10 µL and the high speed of the PCR reaction (45 min) provides a clear advantage above all other available methods. In addition, we demonstrate a most reliable method for $HFE$ genotyping. Erroneous genotyping as a result of the IVS4+48G/A polymorphism in $HFE$ (13) is completely avoided. Compared with one-color detection of the C282Y and H63D mutations (9), this dual-color LightCycler assay prevents erroneous interpretation due to melting peaks from S65C mutations in $HFE$. 
**Figure caption and subcaption**

Fig. 1. Melting peaks for *HFE* genotyping using a one-color (A,B) and a dual-color assay (C-E).

DNAs from an individual homozygous for the H63D mutation (A) and an individual compound heterozygous for the H63D and S65C mutations (B) in *HFE* were genotyped according to the method of Mangasser et al. (9). This method was modified by changing the antisense primer for detection of the C282Y as described by Jeffrey et al. (13). The reaction mixture contained a hot start Taq polymerase as used for the dual-color assay. Both melting profiles were compared with those from an individual compound heterozygous for the C282Y and H63D mutations (melting peaks for all four alleles are present). Except an additional melting peak at approximately 61°C, the individual compound heterozygous for the H63D and S65C mutations (B) shows a melting profile similar to that of the individual homozygous for the H63D mutation (A). Dual-color detection of the C282Y mutation using LightCycler Red 640 as acceptor fluorophore (C) and the H63D/S65C mutations using LightCycler Red 705 as acceptor fluorophore (D,E) resulted in clearly interpretable melting profiles for each genotype. In order to generate the melting peaks, fluorescence data were converted by plotting the negative derivative of the fluorescence with respect to temperature against temperature (dF/dT vs T). Melting profiles for the C282Y and H63D/S65C mutation sites were monitored in channel 2 (F2) and channel 3 (F3), respectively. Color compensation was used for the dual-color assay (C-E).
References


**Table 1. Primer and hybridization probes for dual-color detection of the C282Y, H63D, and S65C mutations in HFE**

<table>
<thead>
<tr>
<th>Oligonucleotides and Alleles</th>
<th>Sequence(^a)</th>
<th>Orientation</th>
<th>(T_m) (SD)(^b)</th>
</tr>
</thead>
<tbody>
<tr>
<td>C282Y Sense Primer</td>
<td>5’-TGG CAA GGG TAA ACA GAT CC-3’</td>
<td>Sense</td>
<td></td>
</tr>
<tr>
<td>C282Y Antisense Primer</td>
<td>5’-TAC CTC CTC AGG CAC TCC CC-3’</td>
<td>Antisense</td>
<td></td>
</tr>
<tr>
<td>C282Y Anchor Probe</td>
<td>5’-LC Red 640-CCC AGG CCT GGA TCA GCC CCT CAT TGT GAT CTG GG-p</td>
<td>Sense</td>
<td></td>
</tr>
<tr>
<td>C282Y Detection Probe</td>
<td>5’-AGA TAT ACG TAC CAG GTG GAG-fluorescein</td>
<td>Sense</td>
<td></td>
</tr>
<tr>
<td>845A Allele (282Y)</td>
<td>5’-AGA TAT ACG TAC CAG GTG GAG-3’</td>
<td>Sense</td>
<td>60.5°C (0.5)</td>
</tr>
<tr>
<td>G845 Allele (C282)</td>
<td>5’-AGA TAT ACG TGC CAG GTG GAG-3’</td>
<td>Sense</td>
<td>54.9°C (0.6)</td>
</tr>
<tr>
<td>H63D/S65C Sense Primer</td>
<td>5’-GCT CTG TCT CCA GGT TCA CAC TC-3’</td>
<td>Sense</td>
<td></td>
</tr>
<tr>
<td>H63D/S65C Antisense Primer</td>
<td>5’-CCC TCT CCA CAT ACC CTT GC-3’</td>
<td>Antisense</td>
<td></td>
</tr>
<tr>
<td>H63D/S65C Anchor Probe</td>
<td>5’-LC Red 705-CTG GTC ATC CAC GTA GCC CAA AGC TTC AA-p</td>
<td>Antisense</td>
<td></td>
</tr>
<tr>
<td>H63D/S65C Detection Probe</td>
<td>5’-CGG CGA CTG TCA TCA TAG AAC ACG AAC A-fluorescein</td>
<td>Antisense</td>
<td></td>
</tr>
<tr>
<td>187G Allele (63D/S65)</td>
<td>5’-CGG CGA CTG TCA TCA TAG AAC ACG AAC A-3’</td>
<td>Antisense</td>
<td>70.7°C (0.5)</td>
</tr>
<tr>
<td>C187/A193 Allele (H63/S65)</td>
<td>5’-CGG CGA CTG TCA TCA TAG AAC ACG AAC A-3’</td>
<td>Antisense</td>
<td>64.4°C (0.7)</td>
</tr>
<tr>
<td>193T Allele (H63/65C)</td>
<td>5’-CGG CGA CAG TCA TCA TAG AAC ACG AAC A-3’</td>
<td>Antisense</td>
<td>61.2°C (0.7)</td>
</tr>
</tbody>
</table>

\(^a\) Mismatches between detection probes and particular alleles are underlined

\(^b\) Empirical \(T_m\) (\(n=699\)) for detection probe / allele duplexes (standard deviation)
Fluorescence $-\frac{d(F2/F1)}{dT}$

- **C282Y** $-/-$
- **H63D** $+/+$
- **S65C** $-/-$

- **C282Y** $+/-$
- **H63D** $+/-$
- **S65C** $-/-$

**G845 Allele**

**845A Allele**

**C187 Allele**

**187G Allele**

Temperature ($^\circ$C)
B

Temperature (°C)

Fluorescence $-d(F_2/F_1)/dT$

C282Y -/- H63D +/- S65C +/-

C282Y +/- H63D +/- S65C -/-

G845 Allele

845A Allele

C187 Allele

187G Allele

Additional Melting Peak (193T Allele)
C

G845 Allele

845A Allele

Fluorescence $\frac{d(F_2/F_1)}{dT}$

- - C282Y $\sim$ /-
- - - C282Y $\sim$ +/-
- C282Y $\sim$ +/+
Fluorescence $-d(F_3/F_1)/dT$

193T Allele
C187/A193 Allele
187G Allele

Temperature (°C)

H63D+/- S65C+/-
H63D+/- S65C-/-