Genotyping of HFE mutations responsible for hereditary hemochromatosis using isothermal Helicase Dependent Amplification (HDA) technology

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Introduction

Hereditary Hemochromatosis (HH), characterized by increased iron absorption and deposition, is one of the most common genetic diseases in people of European ancestry. Two mutations on the HFE gene were discovered to be primarily responsible for HH. The first mutation results in a change of cysteine at position 282 to tyrosine (C282Y); the second results in a change of histidine at position 63 to aspartate (H63D). Homozygosity for the C282Y mutation is found to be the most common cause for HH, with a clinical penetrance of about 80%. The H63D mutation is more prevalent than the C282Y mutation. However, the clinical penetrance of H63D homozygous and C282Y/H63D compound heterozygous is relatively low. Early diagnosis of HH by genotyping the HFE gene for C282Y and H63D mutations is essential for the administration of effective treatments.

We have developed two platforms for genotyping tests based on our Helicase Dependent Amplification (HDA) system. The HDA system amplifies the target fragment isothermally. In one platform, the "HDA-Inside" platform, the amplification is carried out in the presence of a fluorescently labeled probe. A subsequent melting curve analysis determines the genotype. This method can be easily carried out on a real-time PCR machine and is suitable for handling high volume tests.

Our second test platform, the "HDA-on Demand" platform, is designed for low volume, random access tests. This platform uses a lateral-flow device for genotype determination following allele specific HDA amplification of target fragments. The isothermal nature of the HDA system eliminates the need for a thermo-cycling machine and makes it possible to perform the test in a physician's office.

Materials and Methods

"HDA-Inside"

For probe based melting curve analysis, two pairs of primers were designed each to amplify a 87 base pair fragment surrounding the C282Y position (nucleotide 845) or a 120 base pair fragment surrounding the H63D position (nucleotide 187). Asymmetric HDA reactions were performed using 10 ng of purified human genomic DNA as input template, with primer concentrations of 75 nM and 300 nM, in the presence of 200 nM SimpleProbe® (Roche) or 50 nM of TaqMan MGB (ABI) probe designed to target each mutation. Reactions were incubated at 65ºC for 2 hours. Melting curve analyses were performed on a LightCycler480 instrument (Roche) or an ABI7300 real-time PCR system.

"IsoAmp On Demand"

For IsoAmp On Demand genotyping analysis, two allele specific primers are designed, each for the wild-type and mutant sequences, and labeled with FITC or DIG, respectively. A common probe is labeled with Biotin. Allele specific primer extension is performed at 65ºC for 1 hour in the presence of 50 nM of probe and 10 ng of of purified human genomic DNA. After reaction, the samples are applied directly to the Biohelix Express Strip (BESt™) cassettes for genotype determination.

Results

Helicase Dependent Amplification (HDA)

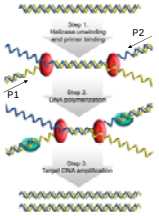
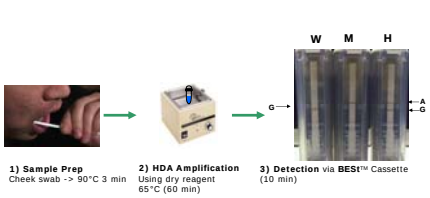


Figure 1: Schematic illustration of the HDA mechanism. Red oval: helicase; Blue oval: polymerase; Black arrows: primers.

Instrument-free, "IsoAmp On Demand" genotyping using the BESt™ Cassette



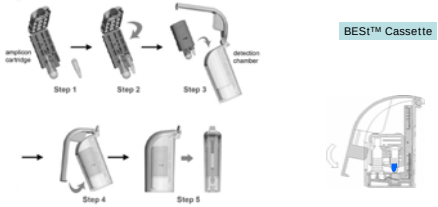
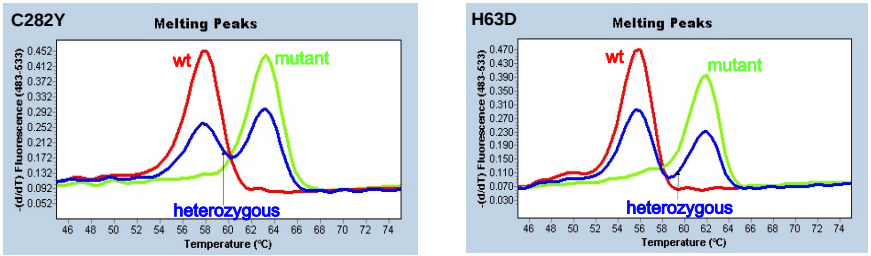


Figure 3: Schematic illustration of the workflow of "IsoAmp On Demand" Factor V Leiden genotyping assay. 1) Simple sample-prep using BuccalQuick™ kit (TrimGen). 2) Isothermal amplification using allele-specific HDA reaction, in which, a 121-bp target fragment is amplified using one common primer and two allele specific primers: one for the wild type G (labeled with FITC) and the other for the mutant A (labeled with DIG). 3) Rapid detection using the BESt™ Cassette.

"HDA – Inside" platform

Melting curve analysis using SimpleProbe®



Melting curve analysis using TaqMan MGB probe

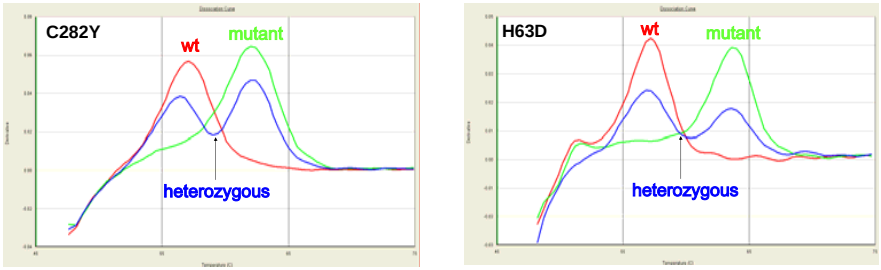
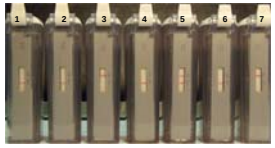


Figure 2: Genotyping results using the "HDA-Inside" platform. The top two panels show melting curves of SimpleProbe®s performed on Roche LightCycler480 and the bottom two panels show melting curves of TaqMan MGB probes performed on an ABI7300 instrument. In all experiments, the probes are designed to match the mutant sequences.



Sample #	Confirmed Genotype	BESt™ Result
1	Wild type	Wild type
2	Wild type	Wild type
3	Mutant	Mutant
4	Wild type	Wild type
5	Heterozygous	Heterozygous
6	Wild type	Wild type
7	Mutant	Mutant

Figure 4: Factor V Genotyping Results. A Wild-type allele (W) will show a visible T line and Mutant allele (M) will show a colored C line. Both T and C lines will be visible for a heterozygous genotype (H). 7 clinical samples from Lahey Clinic have been tested so far and 100% correlations were observed.

Conclusions

The "HDA-Inside" and "IsoAmp On Demand" platforms for genotyping have both been tested using six fully characterized clinical samples and all of them showed 100% correlation with known genotypes. The "HDA-Inside" platform is readily applied to various real-time PCR instruments and is capable for large volume tests. The "IsoAmp On Demand" platform requires no instruments other than a water bath or heat block. This platform is particularly useful when "Point of Care" genetic tests are desirable.

References

1. Vincent M, Xu Y, Kong, H. Helicase-dependent isothermal DNA amplification. EMBO Rep. 2004;5(8):795-800

2. Beutler E. Hemochromatosis: genetics and pathophysiology. Annu. Rev. Med. 2006;57:331-47

Acknowledgements

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