Technical reports

Rapid detection of the factor V Leiden (1691G>A) and haemochromatosis (845G>A) mutation by fluorescence resonance energy transfer (FRET) and real time PCR

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Abstract

A rapid method based on fluorescence resonance energy transfer (FRET) and real time polymerase chain reaction (PCR) was used to identify the haemochromatosis genotype in 112 individuals and the factor V genotype in 134 individuals. The results were compared with conventional methods based on restriction enzyme digestion of PCR products. The two methods agreed in 244 of the 246 individuals; for the other two individuals, sequencing showed that they had been incorrectly genotyped by the standard method but correctly genotyped by FRET. The simplicity, speed, and accuracy of real time PCR analysis using FRET probes make it the method of choice in the clinical laboratory for genotyping the haemochromatosis and factor V genes.

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Diagnostic tests for disease associated genetic polymorphisms typically use the polymerase chain reaction (PCR) to amplify the DNA segment of interest and subsequently analyse the products using techniques such as hybridisation with allele specific oligonucleotides or restriction enzyme digestion followed by electrophoresis. There is now a new generation of PCR instruments which monitor PCR product formation during thermal cycling so that "real time" analysis is possible. Using these instruments the products of PCR reactions can be analysed more quickly, simply, and cheaply than previously and the likelihood of product contamination of the work place is reduced.

Two clinically important inherited point mutations are the factor V Leiden (1691G>A) mutation,[1] which in the heterozygous or homozygous form is associated with thrombosis, and the 845G>A mutation of the HFE gene, which in the homozygous form is associated with genetic haemochromatosis.[2] Currently these mutations are usually detected by PCR followed by one of a variety of analytical methods, most commonly restriction enzyme digestion and electrophoresis. During the last two years, methods have been reported for detection of both the factor V Leiden mutation and the haemochromatosis mutation using an Idaho Technology "LightCycler" for real time PCR analysis. The factor V Leiden mutation has been detected by fluorescence resonance energy transfer (FRET) using an allele specific oligonucleotide (ASO) probe which hybridises to the mutant and wild-type sequences; the alleles are then distinguished by melting the products and monitoring the loss of fluorescence.[4] The HFE mutation has been detected by the same method[3] and also by using a peptide nucleic acid (PNA) probe which hybridises differentially to mutant and wild-type sequences at a critical annealing temperature and which therefore differentially blocks polymerase extension at mutant and wild-type alleles.[5]

We have used the former approach[4] to detect the factor V Leiden mutation and the same approach to detect the HFE gene mutation as well. For both mutation systems, the results obtained were compared with mutation detection by a conventional approach and we have demonstrated the value of real time PCR analysis for routine diagnosis.

Methods

CONVENTIONAL FACTOR V LEIDEN DETECTION

DNA was extracted from blood by a simple lysis method.[6]

The factor V Leiden mutation was detected by modification of the two stage PCR strategy of Beauchamp *et al*.[7] Briefly, in stage 1 primers were used to amplify a 161 base pair (bp) product for genotyping by Mnl I digestion and electrophoresis.[8] A seminested PCR using amplified product from stage 1 and primer FV-7[7] was then used to amplify a 74 bp product for stage 2 genotyping by Nla III digestion.

CONVENTIONAL HAEMOCHROMATOSIS MUTATION DETECTION

Detection of the HFE mutation was achieved using PCR followed by digestion of PCR product with the enzyme Rsa I.[9]

REAL TIME PCR

The Idaho Technology LightCycler (model LC32) enables fluorescent resonance energy transfer (FRET) to be monitored during the PCR.[10] In one strategy for achieving FRET, two fluorophores, Cy5 and fluorescein, are brought into close proximity by the attachment of the Cy5 molecule to an internal nucleotide of one of the primers and of the fluorescein molecule to the 3' end of a probe which specifically hybridises to sequence in the PCR product adjacent to the labelled primer. The PCR is asymmetrical to facilitate hybridisation of the probe.

The LightCycler also enables DNA melting curves to be determined, as melting results in loss of FRET owing to separation of the two fluorophores. Probes can be designed to bind preferentially to mutant or wild-type sequences and the genotype of a patient DNA sample can thus be assigned according to the melting temperature (Tm) at which the probe and complementary PCR product strand separate.

PRIMER AND PROBE DESIGN

Haemochromatosis

The primers used were 5' GGGATGGGACCTACCAGGGC 3' (sense, positions 779–798) and 5' GAGGGGCTGATCCAGGCCCTGGG 3' (antisense, positions 858–880) with the T at position 861 labelled with Cy5. A sense probe spanning the 845G>A mutation site and with homology to the mutant allele, 5' AGATATACGTACCAGGTGGAG 3' (positions 835–855), was fluoresceinated at the 3' end.

Factor V Leiden

The primer and probe design have been reported previously.[4] Briefly, an exon 10 primer, 5' TAATCTGTAAGAGCAGATCC 3' (sense, positions 1661–1680) with the T at position 1678 labelled with Cy5 was used with a downstream intron 10 primer, 5' TGTTATCACACTGGTGCTAA 3', to produce a product of 187 bp. An allele specific probe spanning the 1691G>A mutation site, 5' AATACCTGTATTCCTCGCCTGTC 3' (from position 1684 into the intron region) fluoresceinated at the 3' end was complementary to the wild-type coding sequence. For some studies, an alternative probe complementary to the Leiden mutation was used (5' AATACCTGTATTCCTTGCCTGTC 3').

PCR IN LIGHT CYCLER

The target strand to which the fluoresceinated probe bound was produced in excess in each system using an asymmetric PCR. A 10 µl reaction mix was made containing PCR buffer (Cetus Corporation), 3 mM $MgCl_2$, 200 µM dNTP (each), 1 mg bovine serum albumin (Boehringer Mannheim Biochemicals), 0.5 U Taq polymerase (Biotech International), 100 ng Cy5 labelled primer, 20 ng complementary strand primer, 20 ng fluorescein labelled ASO probe, and 10–100 ng of sample DNA. Four microlitres were pipetted into a LightCycler cuvette. Appropriate homozygous wild-type, mutant, heterozygous control, and water control were included in each amplification experiment.

PCR conditions were: 95°C for one second, 50°C for 10 seconds, 72°C for 10 seconds for 45 cycles, set on maximum ramp rates of 20°C/second. The ratio of fluorescein fluorescence (520–560 nm) to Cy5 fluorescence (655–695 nm) was monitored for 50 ms per sample towards the end of each annealing step. On completion, in order to establish the Tm of the annealed probe, the PCR products were subjected to a melting cycle of 95°C for one second, 40°C for 60 seconds, 50°C for 10 seconds at maximum ramp rates of 20°C/second, followed by heating at a ramp rate of 0.2°C/second to 75°C, with continuous monitoring of fluorescence ratio.

DATA ANALYSIS

The amplitude of FRET, which indicates the extent of hybridisation between fluoresceinated probe and PCR product, was measured as the ratio of Cy-5 fluorescence to fluorescein fluorescence. The plot of this ratio (F) against temperature (T) yielded a profile with one or two sigmoid components. The LightCycler software calculated the rate of change of fluorescence against temperature. Discrete maxima in this differential melting curve occurred at temperatures characteristic of the Tm of probe bound to a wild-type or mutant sequence.

Results

SPECIMEN RESULTS FROM THE LIGHTCYCLER

Haemochromatosis

Figure 1 shows typical differential melting curves obtained by FRET for individuals homozygous for the wild-type sequence, for the

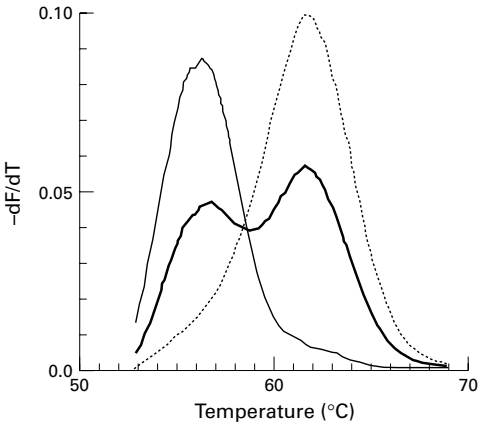

Figure 1 Genotyping at the haemochromatosis locus, as determined by melting curves using a fluorescent probe specific for the mutant allele. The rate of change of fluorescence with temperature (dF/dT) is plotted against temperature. The maxima indicate the Tms. ——, homozygous wild-type (Tm = 56.2°C); ·····, homozygous 845G>A mutation (Tm = 61.8°C); ——, heterozygote, with twin peaks showing melting temperatures of 56.7°C and 61.7°C.

Table 1 Haemochromatosis genotyping: comparison of PCR with restriction digestion and real time PCR with a FRET probe to the mutant sequence

	+/+	+/*mutant*	*mutant/mutant*
LightCycler (FRET)	62	23	27
Standard PCR	62	23	27

FRET, fluorescence resonance energy transfer; PCR, polymerase chain reaction.

Table 2 Factor V genotyping: comparison of standard PCR with restriction digestion and real time PCR with a FRET probe having either the wild-type sequence (A) or Leiden mutation sequence (B)

	+/+	+/*Leiden*	*Leiden/Leiden*
LightCycler (A)	61	69	4
Standard PCR	59*	71	4
LightCycler (B)	45	41	4
Standard PCR	43*	43	4

*Discordance occurred in the same two individuals with both probes.
FRET, fluorescence resonance energy transfer; PCR, polymerase chain reaction.

mutant sequence, and for heterozygotes. The probe, which is complementary to the mutant sequence, melted from the wild-type target at 56.6°C (SD 0.56°C, n = 105 alleles), whereas it melted from the mutant target at 62.2°C (SD 0.5°C, n = 51 alleles). Heterozygous targets produced two clearly distinct peaks corresponding to the wild-type and mutant allele (fig 1). The genotype of each sample was thus readily distinguished.

Factor V Leiden

Differential melting profiles using a fluoresceinated factor V probe complementary to the wild-type sequence have been reported.[4] Using the same probe in our studies, the mean melting temperature for the wild-type allele was 65.6°C (SD 0.29°C, n = 195 alleles) and for the mutant allele, 57.5°C (SD 0.46°C, n = 77 alleles).

A probe complementary to the mutant sequence showed a smaller difference in the melting temperature between mutant and wild-type sequences. A part of the differential melting curve for the mutant allele showed a small shoulder overlapping the location of the peak associated with the wild-type allele. Fewer samples were analysed with this probe. However, there was no difficulty in distinguishing the status of each sample, given that there was no significant difference in amplification of each allele in the heterozygous state and that peak heights in the differential melting profile were approximately equal in heterozygotes.

RESULTS IN PATIENTS

Haemochromatosis

Table 1 shows a comparison for 112 patients of the standard test and that by FRET in the LightCycler. These tests were completely concordant.

Factor V Leiden

Table 2 shows a comparison between genotype assignment using the standard factor V test and the LightCycler analysis for 134 individuals, using the probe complementary to the wild-type sequence. With the exception of two individuals, there was complete concordance between methods. These two individuals were classified by the standard test as being heterozygous but were classified by the LightCycler as being homozygous wild-type. Gene sequencing confirmed that the results obtained by the LightCycler were correct, but a retrospective analysis did not reveal the factor responsible for the incorrect results from the standard method. When a fluoresceinated probe complementary to the mutant sequence was used to genotype 90 individuals from the same population, there was again complete concordance between the two methods and the two probes with the exception of the same two individuals (table 2).

Discussion

Various methods have been developed for detection of point mutations based on analysis of PCR amplified product. All have limitations, both the specific limitations of each method and the general limitation that a second step is required for analysis of PCR products. Real time PCR analysis expands the repertoire of available methods. In the two systems we have used, discrimination of wild-type and normal sequences by the use of melting curves was found to be highly reproducible and discriminatory. For haemochromatosis the results from the LightCycler were in complete agreement with those obtained from the standard method involving restriction enzyme digestion of PCR products and electrophoretic analysis. For detection of the factor V Leiden mutation the results were in agreement except for two patients, for whom the LightCycler gave the correct result while the standard method was erroneous for unknown reasons.

Real time PCR analysis has a number of advantages over conventional methods for detection of the haemochromatosis and factor V Leiden mutations. On a per sample basis, reagent costs are much the same. The direct labour time which is saved by real time PCR is 30 to 60 minutes a batch, but owing to the fewer steps involved the improvement in work flow leads in practice to a somewhat greater saving of time. The total time required from receipt of a sample to generation of a result is four hours, which enables the test to be performed and reported on the same day. There is no manipulation of PCR products after amplification and the potential for contamination of the working environment is therefore reduced. The procedure involves fewer manipulations, thus decreasing the likelihood of transfer and transcriptional errors; PCR problems can be rapidly identified and corrected as a consequence of real time monitoring; and, with correct choice of probes, there is unambiguous assignment of genotypes. Although the LightCycler is relatively expensive, it performs a PCR very quickly. As a result, the diagnostic tests for the haemochromatosis and factor V Leiden mutations use only a small proportion of instrument time.

The component of test cost attributable to instrument amortisation is therefore small.

For these reasons, we now use the LightCycler for routine genotyping for genetic haemochromatosis and the factor V Leiden mutation.

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