Introduction

The factor V Leiden point mutation (Arg^506 Gln substitution), besides its well accepted relevance for thromboembolic events, is discussed as being associated with an increased risk for e.g., juvenile stroke, myocardial infarction in young women, and traumatic conditions. The prevalence of the factor V Leiden mutation Caucasian populations is relatively high in general, with figures ranging from 2 to 15% [1, 2]. Among individuals suffering initially from deep vein thrombosis or pulmonary embolism, the prevalence of factor V Leiden ranges from 12 to 50%, whereas less than 10% of subjects show hereditary deficiencies of antithrombin III, protein C or protein S [3]. Although many methods are described for the analysis of this mutation, there is a demand for automated assay procedures. Automation of this kind of analysis offers advantages for processing within a laboratory where there is sufficient demand for molecular genetic analysis and high sample throughput. Advantages include faster analysis and the standardization of both amplification and detection procedures. An interesting and promising new approach is the Roche Molecular Biochemicals LightCycler PCR analysis system based on rapid real-time PCR and fluorimetric DNA melting point analysis for the detection of single point mutations. To show the reliability of the LightCycler technology for our laboratory, we performed a preliminary comparison of factor V Leiden analysis using the allele-specific PCR (PCR-SSP) as a traditional analysis method and the LightCycler-Factor V Leiden Mutation Detection research kit for the LightCycler system from Roche Molecular Biochemicals. As a cost- and time-saving technique, PCR-SSP used to be employed as a screening method for factor V Leiden mutation in our laboratory before the LightCycler technology became available as an interesting alternative. The traditional PCR-method comprises simple and rapid detection possibilities for the mutation without subsequent digestion. Presence or absence of a specific PCR fragment is sufficient for allele assignment. No further post-amplification procedures, such as RFLP, single-strand conformation polymorphism analysis, or sequencing, are required for identification of the point mutation.

Material and Methods

Sample Material

Research samples of 57 individuals, 34 female and 23 male, who all had thromboembolic events before, were included in this methodological comparison. Samples were withdrawn by atraumatic venipuncture and collection in trisodium citrate tubes (ratio of blood to anticoagulant 9:1). They were then forwarded to the laboratory where they were centrifuged at 3000 rpm for 10 min.

DNA isolation

After centrifugation, the plasma supernatant was removed. Plasma and the cellular pellet were stored at -80°C. DNA was isolated from leukocytes according to standard procedures. DNA isolates were stored at 4°C.
Detection of the factor V Leiden mutation by allele-specific PCR (PCR-SSP)

Allele-specific PCR (PCR-SSP) was performed using the modified procedure of Blasczyk et al. [4]. One sense and two antisense primers were used in two separate primer mixes. FV1001 sense: 5’ TCT TCA GCC AGG AAC ACC ACC’s, FV1002 anti sense: 5’ GGA CAA AAT ACC TGT ATT CCT C3’; FV1004 antisense: 5’ AGG ACA AAA TAC CTG TAT TGC TCC TT3’. Each sample was investigated with two PCR amplifications, one amplifying the wildtype (FV1001 sense/FV1002 antisense) and the other amplifying the factor V point mutation (FV1001 sense/FV1004 antisense). In each PCR primer mix a primer pair was included that amplified a fragment of the human growth hormone gene as an internal positive amplification control, resulting in a 429 bp PCR fragment (ICF: 5’ GCC TTC CCA ACC ATT CCC TTA Tc 64°C; ICR: 5’ TCA CGG ATT TCT GTT GTG TTT C3’, Tm 82°C). The PCR mixture, amplifying the factor V wildtype together with the internal growth hormone amplification control, consisted of 35 µl H2O, 5.5 µl PCR buffer (100 mM Tris HCl, pH 8.3, 500 mM KCl), 6 µl 25 mM MgCl₂, 1 µl 10 mM dNTP, 0.6 µl of each specific (25 µM) and 0.3 µl of the internal control primers (25 µM) and 1.25 U AmpliTaq DNA Polymerase together with 1 µl genomic DNA. The factor V point mutation (Q506) was amplified together with the internal growth hormone amplification control, consisting of 37 µl H2O, 4.5 µl 10x PCR buffer (100 mM Tris HCl, pH 8.3, 500 mM KCl), 5 µl 25 mM MgCl₂, 1 µl 10 mM dNTP, 0.6 µl of each specific (25 µM) and 0.3 µl of the internal control primers (25 µM) and 1.25 U AmpliTaq DNA Polymerase together with 1 µl genomic DNA. The thermal cycle protocols are identical for both wildtype and mutant alleles: initial denaturation at 95°C for 5 min; denaturation at 95°C for 20 s; annealing and extension at 65°C for 40 s; repeated 10 times; followed by 21 cycles at 95°C for 20 s, 81°C for 20 s and 72°C for 20 s. Final extension was performed at 72°C for 8 min.

For visualization purposes, 10 µl of the amplification product were subjected to electrophoresis on a 1.2% agarose gel prestained with ethidium bromide (0.15 µg/ml).

Detection of the factor V Leiden mutation by the LightCycler technology

The LightCycler system consists of a microvolume fluorimeter integrated with a thermal cycler that combines rapid-cycle PCR in glass capillaries heated with hot air with real-time fluorescence monitoring. Sequence-specific hybridization probes can be designed for the detection and analysis of PCR products without the need for any post-PCR sample manipulation, allowing high-throughput genotyping and product quantification. The wild-type can easily be discriminated from the mutant type by converting melting curves into melting peaks. Heterozygotes show two distinct melting peaks representing one mutant and one wildtype allele.

PCR was performed using the LightCycler – Factor V Leiden Mutation Detection Kit (Roche Molecular Biochemicals, Cat. No. 2 212 161). In brief, 2 µl of the LightCycler – Factor V Leiden Mutation Detection Mix, 2 µl of the LightCycler – Factor V Leiden Reaction Mix, and 11 µl of PCR-grade water supplied with the kit were combined with 5 µl of genomic DNA from human blood. Alternatively, 14 µl of PCR-grade water were combined with 2 µl of the LightCycler – Factor V Leiden Mutation Detection Mix, 2 µl of the LightCycler – Factor V Leiden Reaction Mix, and 11 µl of PCR-grade water supplied with the Control Template/PCR-grade water as the positive and negative controls, respectively. Samples were spun into glass capillary cuvettes, capped and placed in the LightCycler Instrument. For factor V genotyping, an initial denaturation step at 95°C (30 s, ramp rate 20°C/s) was followed by amplification for 45 cycles of denaturation (95°C, 0 s, ramp rate 20°C/s), annealing (55°C, 10 s, ramp rate 20°C/s), and extension (72°C, 10 s, ramp rate 20°C/s). After amplification, a melting
curves were generated at 95°C (0 s, ramp rate 20°C/s), 45°C (60 s, ramp rate 20°C/s), 75°C (0 s, ramp rate 0.1°C/s, acquisition mode: continuous). After a final cooling step at 40°C (30 s, ramp rate 20°C/s) the thermal chamber was again accessible. Fluorescence was recorded continuously during the slow temperature ramp. The fluorescence signal from LC-Red 640 was plotted against temperature to give melting curves for each sample. Melting curves were converted to melting peaks by plotting the negative derivative of the fluorescence with respect to temperature (–dF/dT) against temperature, thereby allowing easy distinction of the wildtype from the mutant.

**Comparison**

Comparison of both methods revealed a very good correlation. None of the results obtained in PCR-SSP differed from those obtained in the corresponding LightCycler run. Remarkably, the LightCycler technology was significantly less heparin-sensitive. Whereas PCR-SSP was inhibited by heparin, the LightCycler method was affected by heparin to a much lesser extent. Within the population of 57 individuals investigated in parallel for factor V Leiden mutation, 19 samples could not be analysed by PCR-SSP. In these cases the analysis had to be repeated either by a new DNA isolation from the same sample or by isolation from a new sample. In all 19 cases we were able to perform mutation analysis from the original DNA isolate using the LightCycler technology, confirming the results obtained by PCR-SSP using DNA isolated from a new blood sample from the same individual and re-submitting it to both tests. Genotypes from the same subjects were always identical when analyzed by the two methods of PCR-SSP and LightCycler detection. In the LightCycler method, positions and distances of melting peaks were identical in individuals with the same genotypes. Likewise, PCR-SSP showed identically reproducible results.

**Table 1:** Factor V Leiden melting peak analysis in a representative LightCycler run.

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Tm (mutant allele)</th>
<th>Tm (wildtype allele)</th>
<th>ΔT between melting peaks</th>
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<tbody>
<tr>
<td>factor V wildtype</td>
<td>–</td>
<td>66°C</td>
<td>–</td>
</tr>
<tr>
<td>factor V heterozygous</td>
<td>59°C</td>
<td>66°C</td>
<td>7°C</td>
</tr>
<tr>
<td>factor V homozygous</td>
<td>59°C</td>
<td>–</td>
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**Results**

**Factor V genotyping**

Research samples of 57 subjects, who were selected because of their thromboembolic history, were screened for factor V mutation both by PCR-SSP and fluorescence-monitored PCR using the LightCycler technology (Figures 1 and 2). Figure 1 shows a 1.2% agarose gel, pre-stained with ethidium bromide. Lanes 1–3 represent homozygous, heterozygous, and wildtype controls. Lanes 4–8 represent research samples, lane 9 shows the negative water control and lane 10 the 100 bp ladder. In the upper part of the gel the internal growth hormone control (a) can be seen together with the wildtype allele (b) in the lower part of the gel together with the mutant allele (c). Figure 2 shows a representative LightCycler run for the different genotypes when using the LightCycler-Factor V Leiden Mutation Detection Kit. The different melting temperatures revealed by the LightCycler analysis are presented in Table 1. According to the manufacturer of the kit, the values for the respective melting temperatures may vary by ± 2.5°C between different experiments. This was confirmed by our own results. The difference in melting temperature between the wildtype and mutant allele may vary by 0.75°C.

**Figure 2:** Factor V melting peak analysis using the LightCycler-Factor V Leiden Mutation Detection Kit on the LightCycler Instrument. Negative control (water), homozygous factor V (Q506R) mutation, heterozygous mutation, and factor V wildtype.
**Faster analysis**

One of the main advantages of the LightCycler technology, apart from the possibility of a one-tube PCR and one-tube-mutation detection, is the much faster analysis with this method. Whereas PCR-SSP is already much superior in this respect to other methods, e.g. restriction fragment length polymorphism (RFLP), single-strand conformation polymorphism analysis or sequencing, analysis using the LightCycler technology is even faster. The process of DNA isolation prior to the analysis is identical. PCR and detection take about 2.5 h with PCR-SSP. The number of samples to be analyzed is restricted to the lanes available per gel. High speed LightCycler technology takes just 30 min per run. Sample numbers are 32 per run, including controls.

**Discussion**

Compared to traditional analytical methods the LightCycler technology offers a wide range of advantages. Significantly faster analysis can be obtained thanks to the high degree of automation and the unique technology that employs air heating of glass capillaries instead of the heating of plastic amplification cups in a thermal cycler metal block. Since detection is performed in the same reaction cup directly after the PCR, no time-consuming additional electrophoresis is necessary. This represents a further improvement over PCR-SSP, which itself was developed with the aim of saving time compared to complex, in some cases multiple, enzymatic reactions requiring restriction meth-
ods. This one-tube-technology also minimizes the risk of PCR contamination due to sample handling, allowing PCR and detection to be performed at the same location, thus minimizing laboratory space requirements for analyses of gene mutations. The results obtained by LightCycler analysis were identical to those from traditional PCR methods such as allele-specific PCR-SSP. Additionally, we have found the LightCycler system to be less susceptible to inhibition by heparin. In conclusion, factor V Leiden mutation analysis performed by Roche Molecular Biochemicals LightCycler real-time PCR with the LightCycler – Factor V Leiden Mutation Detection Kit is a reliable, high-speed method that is particularly suitable for large laboratories with high sample throughput.

**Acknowledgements**

We should like to thank Mrs. H. Schlawatzki-Martens for her skillful technical assistance.

**References**


**Order**

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<td>32 reactions</td>
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<tr>
<td>LightCycler – Red 640-NHS ester</td>
<td>2 015 161</td>
<td>1 mg</td>
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<tr>
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