Rapid Detection of the Factor V Leiden Mutation by Real-Time PCR with TaqMan Minor Groove Binder Probes

To the Editor:
Individuals heterozygous for the factor V Leiden mutation have a sevenfold higher risk to develop venous thrombosis than wild-type (WT) individuals (1). The allele frequency of factor V Leiden in Europeans is approximately 4.4%, and the mutation is currently considered as the most important genetic risk factor for venous thrombosis (2). Various methods have been published to detect the factor V Leiden mutation by real-time PCR using hybridization probes (3–5) and TaqMan probes (6, 7). Here we describe a robust and convenient procedure for the detection of the factor V Leiden mutation that uses TaqMan probes conjugated to a minor groove binder (MGB) group.

TaqMan probes are degraded during amplification by the 5′→3′ exonuclease activity of the polymerase. During degradation of the probe, the fluorescent group is separated from the quencher, leading to increased fluorescence. For allelic discrimination, probes matching the WT and the mutant allele are conjugated to a different fluorescent group. Discrimination between alleles is based on the difference in melting temperature ($\Delta T_m$) between the differently labeled matched and mismatch probes. For TaqMan assays, relatively long probes are required that remain annealed during the extension phase of the PCR. A 1-bp mismatch in such a probe may lead to a small $\Delta T_m$ and subsequent difficult allelic discrimination. DNA probes conjugated to a MGB group form hyperstabilized duplexes with complementary DNA by folding of the MGB group into the duplex, giving a higher $T_m$(8, 9). The conjugation of a MGB group therefore allows the design of shorter probes with an increased specificity attributable to increased mismatch discrimination (9).

For detection of the factor V Leiden mutation, we designed MGB probes with a length of 17 bases, which is >25% shorter than the length (23–24 bases) of previously published TaqMan probes for factor V Leiden detection (6, 7) without a MGB group. In addition, the probes were designed with the mutation site located in the MGB region to achieve the maximum ability to discriminate alleles (9).

The following primer and probe sequences were designed with Primer Express software (Applied Biosystems) and synthesized by Applied Biosystems: forward primer, 5′-GCC TCT GGG CTA ATA GGA CTA CTT C-3′; reverse primer, 5′-TTT CTG AAA GGT TAC TTC AAG GAC AA-3′; WT factor V probe, FAM-ACC TGT ATT CCT CGC CT- nqf-MGB, and factor V Leiden probe, VIC®-ACC TGT ATT CCT TGC CT- nqf-MGB. The primers generate a 97-bp PCR product. The 5′-fluorescently labeled probes [6-carboxyfluorescein (FAM) or VIC] were conjugated to a nonfluorescent quencher (nFq) and an MGB group at the 3′ end.

DNA was isolated from 200 μL of EDTA-treated blood with the QIAamp DNA Mini Kit (Qiagen) according to the instructions of the manufacturer. The DNA was eluted in 200 μL of elution buffer and stored at $-20^\circ$C. PCR was performed in a final reaction volume of 25 μL, which contained 12.5 μL of 2× TaqMan Universal PCR Master Mix (Applied Biosystems), 450 nM each primer, 200 nM each probe, and 5 μL of DNA solution. PCR was performed in MicroAmp optical 96-well plates with optical adhesive covers (Applied Biosystems). Amplification and detection were carried out with an ABI prism 7000 sequence detection system (Applied Biosystems). The amplification conditions were 2 min at 50 °C for AmpEraser uracil-glycosylase activity and 10 min at 95 °C for AmpliTaq Gold activation, followed by 40 cycles of 15 s at 95 °C for denaturation and 1 min at 60 °C for annealing and extension. After amplification, endpoint detection of fluorescence was performed at 60 °C. The fluorescence data were analyzed with the allelic discrimination software of the ABI prism 7000 instrument.

The genotypes of 60 clinical samples were analyzed retrospectively. These samples were submitted to the laboratory for factor V Leiden mutation detection, and the genotype was initially established by PCR followed by restriction fragment analysis based on the method described by Koeleman et al. (10). Real-time factor V Leiden mutation detection was performed in duplicate. After amplification, four fluorescence patterns are expected. In samples from heterozygous WT factor V patients, FAM fluorescence attributable to degradation of the WT probe will be present. In samples from patients homozygous for factor V Leiden, VIC fluorescence attributable to degradation of the factor V Leiden probe will be present. In samples from heterozygous patients, both FAM and VIC fluorescence will be detected. In PCRs to which no DNA was added, no fluorescence is expected except for the background signal. Fig. 1 shows that these four groups are
clearly detected without overlap. The established genotypes were 100% identical to the results obtained with the method of Koeleman et al. (10).

The amount of isolated DNA added to the PCR will vary with the amount of leukocytes in the blood. To investigate the effect of various amounts of DNA on the performance of the assay, we made dilution series of DNA from all genotypes and added the DNA to the PCR in amounts ranging from 9.4 to 300 ng. A standard reaction is expected to contain ~150 ng of DNA. Although there was some variation in the amount of fluorescence, allelic discrimination was reliable with all amounts of input DNA (results not shown).

In conclusion, this real-time PCR method with TaqMan MGB probes is a robust and convenient way to determine the presence of the factor V Leiden mutation. The use of TaqMan MGB probes gives clear discrimination of genotypes independent of the amount of input DNA. In addition to unambiguous discrimination, the real-time assay with MGB probes has the advantages of other real-time assays, such as decreased assay time and contamination risks. Furthermore, the absence of ambiguous results eliminates the need for repetitive analysis of a sample. Designing primers and probes with Primer Express software (Applied Biosystems) makes it possible to run PCRs under universal conditions, meaning that other single-nucleotide polymorphisms can be detected in the same run. The combination of TaqMan MGB probes with universal assay conditions allows an efficient workflow when the number of different single-nucleotide polymorphisms that are detected in the clinical laboratory increases.

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References

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