Analytical Evaluation of Primer Engineered Multiplex Polymerase Chain Reaction–Restriction Fragment Length Polymorphism for Detection of Factor V Leiden and Prothrombin G20210A

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Factor V Leiden and prothrombin G20210A are clinically relevant genetic risk factors for venous thrombosis. Analysis for both mutations is increasingly being performed on patients exhibiting hypercoagulability. The goal of the current study was to evaluate the performance of primer-engineered multiplex polymerase chain reaction–restriction fragment length polymorphism (PCR-RFLP) for the simultaneous detection of factor V Leiden and prothrombin G20210A. Primer-engineered multiplex PCR-RFLP methods for the detection of factor V Leiden and prothrombin G20210A from the medical literature were reviewed. A modified method was optimized in which both mutations generate *Hind*III RFLPs and the prothrombin amplicon contains an invariant *Hind*III recognition site to assess the completeness of endonuclease digestion. Digested amplification products were analyzed by agarose gel electrophoresis in a single gel lane and visualized by ethidium bromide. Primer-engineered multiplex PCR-RFLP was used to analyze 205 human genomic DNA samples whose factor V Leiden genotypes had been previously determined by *Mnl*I PCR-RFLP. Complete concordance for factor V Leiden genotypes was observed between the two methods in the 205-sample cohort comprising 139 wild-type, 62 heterozygous mutant, and four homozygous mutant individuals. For prothrombin G20210A, primer-engineered multiplex PCR-RFLP identified 196 wild-type and nine heterozygous mutant individuals in the 205-sample cohort. To independently verify prothrombin genotypes, the nine heterozygous mutants and an additional 11 wild-type patient samples (representing 10% of patient samples) were subjected to DNA sequencing. Complete concordance was observed between DNA sequencing and primer-engineered multiplex PCR-RFLP results. In further validation, 123 of the DNA samples consisting of four heterozygous mutant and 119 wild type individuals were genotyped with the Invader Assay for Factor II (prothrombin

G20210A). Results showed 100% concordance between the Invader Assay™ and primer-engineered multiplex PCR-RFLP. A primer-engineered multiplex PCR-RFLP based on single restriction endonuclease digestion has been evaluated and shown to simultaneously and accurately detect factor V Leiden and prothrombin G20210A mutations. The method is robust and readily adaptable to the clinical molecular diagnostic laboratory. (*J Mol Diag* 2000, 2:153–157)

Genetic risk factor assessment has become an integral component of the diagnostic evaluation of patients presenting with signs and symptoms of venous thrombosis. The most common known genetic risk factor associated with venous thrombosis is resistance to activated protein C, which, in the majority of cases, is due to a single G-to-A nucleotide transition in exon 10 of the factor V gene that results in the replacement of arginine, at amino acid position 506, with glutamine (factor V Arg⁵⁰⁶Gln or factor V Leiden).^{1,2} The factor V Leiden mutation is found in Caucasians, and its prevalence varies from 2% to 13%, depending on geographic locale and ethnic composition. Heterozygotes for factor V Leiden have an approximate eightfold increased relative risk for the development of venous thrombosis, and homozygotes are estimated to have an approximately 90-fold increased relative risk.³

The identification of factor V Leiden in 1994 was followed in 1996 by the elucidation of a single G-to-A transition at position 20210 of the prothrombin gene. The G20210A mutation occurs in the 3' untranslated region of the prothrombin gene just before the site of polyadenylation. Heterozygous carriers of prothrombin G20210A exhibit higher mean plasma prothrombin concentrations, and increased prothrombin levels are hypothesized to be a risk factor for venous thrombosis. The prothrombin G20210A mutation is present in approximately 2% of

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healthy Caucasians and 6% of patients with a first episode of venous thrombosis. The prothrombin G20210A mutation increases the relative risk of venous thrombosis by approximately threefold.⁴

Molecular diagnostic testing for factor V Leiden is widespread, and laboratories use a variety of technical approaches. An increasing number of laboratories are additionally analyzing for prothrombin G20210A. Several authors have reported on multiplex polymerase chain reaction–restriction fragment length polymorphism (PCR-RFLP) approaches for the combined detection of both factor V Leiden and prothrombin G20210A.^{5–16} In the current study, multiplex PCR-RFLP approaches in the medical literature were reviewed, and a modification of reported approaches was established based on primer-engineered multiplex PCR-RFLP. We evaluated the multiplex method for its ability to detect factor V Leiden and prothrombin G20210A in a 205-patient base sample. We determined that the method performed robustly and accurately.

Materials and Methods

Genomic DNA was isolated using the Epicentre Master Pure Genomic DNA purification kit (Madison, WI). Use of residual human genomic DNAs in this study was approved by the University of Wisconsin Hospital and Clinics Institutional Review Board. A 241-bp product from exon 10 of factor V (GenBank accession L32764) and a 506-bp product from the 3' untranslated region of the prothrombin gene (GenBank accession M17262) were coamplified using PCR. Mismatched antisense primers were used to amplify both products. Factor V primers were as follows: sense, 5'-TCA GGC AGG AAC AAC ACC AT-3'; antisense, 5'-GGT TAC TTC AAG GAC AAA ATA CCT GTA AAG CT-3'.¹⁷ In the antisense primer, three mismatched nucleotides (underlined) were substituted so that amplification of a mutant allele resulted in the generation of a new *HindIII* restriction endonuclease site. Prothrombin primers were as follows: sense, 5'-GCA CAG ACG GCT GTT CTC TT-3'; antisense, 5'-ATA GCA CTG GGA GCA TTG AAG C-3'.⁸ Similarly, a single mismatched nucleotide (underlined) was replaced in the antisense primer, so that amplification of a mutant allele would result in the creation of a *HindIII* site. Genomic DNA (250 ng) was added to a PCR mix containing 1× Gene Amp Buffer (10 mmol/L Tris-HCl (pH 8.3), 50 mmol/L KCl, 1.5 mmol/L MgCl₂, 0.001% w/v gelatin; Roche Molecular Systems, Branchburg, NJ), 0.2 mmol/L (each) dNTP mix (Promega, Madison, WI), 1.2 μmol/L factor V primers, 0.1 μmol/L prothrombin primers, and 2.5 U AmpliTaq Gold (Roche Molecular Systems) in a final volume of 50 μL. DNA was amplified in a thermal cycling reaction consisting of a 10-minute enzyme activation at 95°C followed by 40 cycles of 94°C for 1 minute, 55°C for 1 minute, and 72°C for 1 minute with a final 5-minute extension at 72°C. A prothrombin G20210A heterozygote and a factor V Leiden heterozygote were used as controls in each run. Water (reagent) controls were included at the beginning and end of each run. Twenty-six microliters of

amplified products was then digested with 1 μL *HindIII* (60,000 U/mL; Promega) with 3 μL of 10× enzyme buffer at 37°C for either 2 hours or overnight. Digested and undigested products were separated in a 20 cm × 27 cm × 7 mm, 3% agarose gel containing 0.5 μg/mL ethidium bromide for 840 V-hours. Undigested products resulted in two products of 506 and 241 bps, representing prothrombin and factor V amplicons, respectively. *HindIII* digestion of factor V and prothrombin wild-type amplicons yielded fragments of 241 bp (factor V amplicon) and 407 bp and 99 bp (prothrombin amplicon); the 99-bp fragment was a result of an invariant *HindIII* site. Digestion of the factor V Leiden heterozygote resulted in fragments of 241 bp, 209 bp, and 32 bp, and the prothrombin G20210A heterozygote yielded fragments of 407 bp, 384 bp, 99 bp, and 23 bp. Factor V Leiden and prothrombin G20210A homozygotes digested with *HindIII* yielded fragments of 384 bp, 99 bp, and 23 bp (prothrombin amplicon) and 209 bp and 32 bp (factor V amplicon). For DNA sequencing of prothrombin alleles, a 392-bp product from exon 14 and the 3' UTR of the prothrombin gene that encompasses the G20210A mutation was amplified using the primers of Poort et al (5'-TCT AGA AAC AGT TGC CTG GC-3' and 5'-TCC AGT AGT ATT ACT GGC TC-3').¹⁸ This fragment was sequenced using the ABI Prism Dye terminator cycle sequencing ready kit (PE Applied Biosystems, Foster City, CA) and the Applied Biosystems 373 DNA Sequencer. Sequencing results were surveyed for heterozygosity with MT Navigator software (PE Applied Biosystems).

The Invader Assay™ for Factor II (prothrombin) was performed according to the manufacturer's instructions (Third Wave Technologies, Madison, WI).^{19,20} A total of 5 μL of Invader Reaction Mix (4 μL 16% polyethylene glycol/50 mmol/L 3-(N-morpholino)propanesulfonic acid and 1 μL 0.5 mmol/L Invader oligo of sequence 5'-TAT-GGTTCCCAATAAAAGTGACTCTCAGCT-3') was pipetted into each well of a 96-well microplate. Ten microliters of DNA sample (200 ng DNA) was added and mixed. The following synthetic oligonucleotide target controls were tested for both wild-type and mutant probes: no DNA target (for background determination), wild type, homozygous mutant, and heterozygous mutant. Fifteen microliters of Chill-out™ 14 (MJ Research, Watertown, MA) was overlaid on each well. The plate was heated in a PTC-100 thermal cycler (MJ Research) at 95°C for 5 minutes and cooled to 63°C. Then 5 μL Cleavase/Probe Reaction Mix (1 μL of 10 mmol/L wild-type probe or mutant probe, 2 μL 75 mmol/L MgCl₂, 1 μL 10 mmol/L 1-Piece FRET Oligo, and 1 μL 200 ng/mL cleavase VIII) was added, mixed, and incubated at 63°C for 4 hours. Probe sequences were as follows: factor II wild type, 5'-AAC-GAGGCGCACGAGCCTCAATGCTCCC-3'; factor II mutant, 5'-AACGAGGCGCACAGCCTCAATGCTCCC-3'; fluorescence resonance energy transfer (FRET) probe, 5'-F-CCTC-Q-GTCTCGGTTTTCCGAGACGAGGGTGCG-CCTCGTTT-3', where F is a fluorescein dye and Q is a quenching dye, Cy3. Reactions were stopped by adding 100 μL of 10 mmol/L EDTA. One hundred microliters of each reaction was transferred to a FluoroNunc microtiter plate, and the fluorescent emission was measured in a

Figure 1. Photograph of ethidium bromide-stained 3% agarose gel demonstrating primer-engineered PCR analysis for prothrombin G20210A and factor V Leiden. M denotes the molecular weight marker; W denotes water control. **Lane 1:** Undigested wild-type 506-bp (prothrombin) and 241-bp (factor V) amplicons. **Lane 2:** A prothrombin G20210/factor V wild type with digested products of 407, 241, and 99 bp. **Lane 3:** A prothrombin G20210 wild-type/factor V Leiden heterozygote with digestion products of 407, 241, 209, and 99 bp (32-bp product not shown). **Lane 4:** A prothrombin G20210 wild-type/factor V Leiden homozygote with digestion products of 407, 209, and 99 bp (32-bp product not shown). **Lane 5:** A prothrombin G20210A heterozygote/factor V Leiden heterozygote with digestion products of 407, 384, 241, 209, and 99 bp (32- and 23-bp products are not shown).

CytoFluor Series 4000 Fluorescence MultiWell Plate Reader (PerSeptive Biosystems, Framingham, MA) or a Dynex Fluorite 1000 Fluorescence MultiWell Plate Reader (Dynex Technologies, Chantilly, VA). Settings for the CytoFluor Series 4000 were excitation 485/20 nm (wavelength/bandwidth), emission 530/25 nm (wavelength/bandwidth), and gain 50, which yielded no target counts between 100 and 200. Settings for the Dynex Fluorite 1000 were excitation 485 nm, emission 530 nm, and gain 2.6–4.0, which yielded no target counts between 100 and 200.

Results

A modified primer-engineered multiplex PCR method was optimized to result in the coamplification of 241-bp factor V and 506-bp prothrombin amplicons that contain the Leiden and G20210A mutation sites, respectively. *Hind*III-digested amplification products were separated by electrophoresis on 3% agarose gels and visualized by ethidium bromide. A representative multiplex analysis is shown in Figure 1. To evaluate the multiplex method, 205 residual human genomic DNA samples that had previ-

ously been genotyped in our laboratory by *Mn*II PCR-RFLP were analyzed.²¹ Complete concordance for factor V Leiden genotypes was observed between the two methods in the sample cohort comprising 139 wild-type, 62 heterozygous mutant, and four homozygous mutant individuals. The multiplex analytical results indicated that of the 205 samples, nine were heterozygous mutant for prothrombin G20210A, and the remaining 196 samples were wild type. To independently verify prothrombin results, DNA sequencing was performed on the nine heterozygous mutants and an additional 11 wild-type samples. These 20 samples, representing 10% of the sample base, showed complete concordance between DNA sequencing and multiplex PCR results. In further validation, 123 of the DNA samples, consisting of four heterozygous mutant and 119 wild type, were genotyped using the Invader Assay for Factor II (prothrombin G20210A).^{19,20} Results showed 100% concordance between the Invader AssayTM and primer-engineered multiplex PCR-RFLP.

Discussion

The aim of the current study was to establish in our molecular diagnostic laboratory a method for the simultaneous detection of factor V Leiden and prothrombin G20210A. In reviewing the literature, we elected to combine features of several methods to achieve a multiplex PCR approach based on primer-engineered RFLP detection of factor V Leiden and prothrombin G20210A.^{8,17} Central to the approach presented is the use of a single restriction endonuclease (*Hind*III) and incorporation of an invariant *Hind*III recognition site in the prothrombin amplicon to assess the completeness of endonuclease digestion. Presumably because of the three-base mismatch in the factor V antisense primer, we observed significantly less amplification of the factor V amplicon compared to the prothrombin amplicon when equivalent primer concentrations were used. We therefore titrated primer concentrations to arrive at a 12:1 ratio of factor V to prothrombin primers, which resulted in approximately equal amplification of the two amplicons.

For evaluation of the multiplex PCR method, 205 archived genomic DNA samples were analyzed. All samples had previously been genotyped for factor V Leiden in our laboratory, using a PCR-RFLP method based on *Mn*II recognition.²¹ We observed complete concordance between methods. Analysis for prothrombin G20210A identified nine heterozygotes in the 205-patient sample cohort; the remainder exhibited wild-type genotypes. We elected to sequence the nine heterozygotes and an additional 11 wild-type samples and observed complete concordance. Although we did not sequence all 205 samples for prothrombin G20210A, our survey of 10% of the samples, including those identified as heterozygotes, provides an independent measure of assay accuracy. In further validation, 123 of the DNA samples consisting of four heterozygous mutant and 119 wild type were genotyped with the Invader AssayTM for Factor II (prothrombin G20210A). Results showed 100% concordance

between the Invader Assay™ and primer-engineered multiplex PCR-RFLP.

The current multiplex PCR relies on the generation of novel *HindIII* recognition sites for demonstration of the Leiden and G20210A mutations. One can envision a scenario in which a second mutation or polymorphism at the site of primer hybridization prevents the generation of the *HindIII* site, a situation that may yield a false negative result. An additional possibility is that a polymorphism at the site of primer hybridization could result in nonamplification of an allele. This latter possibility would likely be most relevant to the factor V Leiden portion of the assay because of the inherent instability of the 3-bp mismatched antisense primer. Two apparently rare polymorphisms flanking the G1691A Leiden mutation have been reported (G1689A and A1692C).²²⁻²⁴

The former polymorphism would not affect the current multiplex PCR. The A1692C polymorphism would potentially affect the multiplex PCR. First, A1692C would cause a noncontiguous 4-bp mismatch (comprising of the 3-bp mismatch of the factor V antisense primer and that of the A1692C polymorphism). This degree of mismatch might result in loss or reduction of allele amplification. Second, if allele amplification were to proceed in the presence of A1692C, the polymorphism would likely preclude generation of a *HindIII* site in the presence of factor V Leiden. These two effects of the A1692C polymorphism could generate either false positive or false negative results, depending on the Leiden genotype of the individual.

The frequency of A1692C has been estimated at less than 0.025%.^{23,24} Although this represents a relatively rare polymorphism, it should be considered to be a limitation of the multiplex PCR. Interestingly, A1692C generates a false positive result for factor V Leiden in the widely used *MnlI*-based PCR-RFLP.

The multiplex method will allow us to provide genotypic information on the prothrombin locus at no additional cost or labor over that required for factor V Leiden genotyping by *MnlI* PCR-RFLP. A modest cost savings is realized because of the lower cost of *HindIII* compared to *MnlI*. The multiplex method generates genotypic information for two distinct loci. This raises complex issues pertaining to the generation of genetic information and specifically how to operationalize multiplex genetic assays when diagnostic information is sought for only one of the loci interrogated by the assay. In the setting of hypercoagulability assessment, current clinical practice is to evaluate multiple risk factors. In this regard, testing for both Leiden and prothrombin mutations is increasing. As the effort and cost of validation as described in the current study are considerable, we determined that referring physicians were interested in knowing the status of both Leiden and prothrombin mutations in their patients being evaluated for hypercoagulability. However, this approach may not be desired by all institutions and would have to be individually addressed by each laboratory.

In summary, the primer-engineered multiplex PCR-RFLP evaluated in this study performed robustly and accurately and is readily adaptable to the clinical molecular diagnostic laboratory setting.

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