

# Multiplex PCR-RFLP Assay for Detection of Factor V Leiden and Prothrombin G20210A

IBRAHIM BARIS,<sup>1</sup> VEDAT KOKSAL,<sup>2</sup> and OZDAL ETLIK<sup>2,3</sup>

## ABSTRACT

**Factor V Leiden and prothrombin G20210A are clinically relevant genetic risk factors for venous thrombosis. Molecular diagnostic testing for factor V Leiden and prothrombin G20210A is widespread, and laboratories use a variety of technical approaches. Here we introduce a multiplex polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) based on single (*Mnl*I) restriction endonuclease digestion. The assay was shown to simultaneously and accurately detect factor V Leiden and prothrombin G20210A mutations.**

## INTRODUCTION

**T**WO COAGULATION FACTOR POLYMORPHISMS, G1691A in the factor V gene and G20210A in the prothrombin gene, are currently the most common known genetic risk factors for venous thrombosis in Caucasian populations (Bertina *et al.*, 1994; Poort *et al.*, 1996). For diagnostic analyses and for scientific studies of large numbers of patients, fast and economic assays that can be performed with standard polymerase chain reaction (PCR) instruments are highly desirable. 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 (Poort *et al.*, 1997; Raoul *et al.*, 1997; Ripoll *et al.*, 1997; Linfert *et al.*, 1998; Keeney *et al.*, 1999; Lastrucci *et al.*, 1999; Mitterer *et al.*, 1999; Xu *et al.*, 1999; Huber *et al.*, 2000; Endler *et al.*, 2001; Lucotte and Champenois, 2003). In the current study, multiplex PCR-RFLP approaches in the medical literature were reviewed, and a modification of reported approaches was established based on multiplex 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 a second PCR-RFLP. We evaluated the multiplex method for its ability to detect factor V Leiden and prothrombin G20210A in a 408-pa-

tient base sample. We determined that the method performed robustly and accurately.

## MATERIALS AND METHODS

### *Samples*

We have screened 408 individuals who requested genetic diagnosis from our laboratory between May 2001 and August 2004. Written informed consent was obtained from all participants.

### *Primer design*

Primers were designed by using a web-based PRIMER 3.0 program (workbench.sdsc.edu). We used the BLAST program at [www.ncbi.nlm.nih.gov/blast](http://www.ncbi.nlm.nih.gov/blast) to check for the specificity of the primers. Hypothetical RFLP results were tested by using NEBcutter V2.0 ([tools.neb.com/NEBcutter2](http://tools.neb.com/NEBcutter2)).

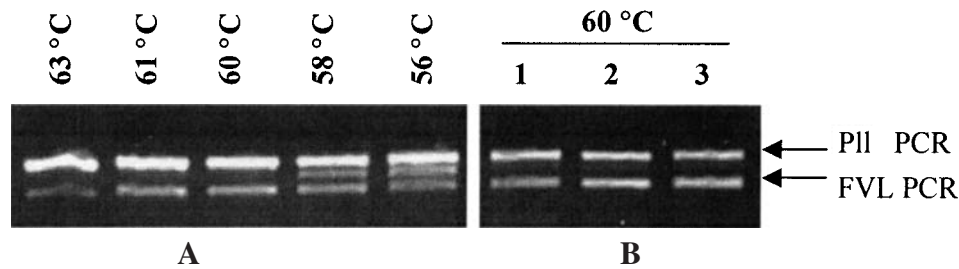
### *PCR method*

Genomic DNA was isolated from the peripheral blood samples according to a standard protocol. PCR reaction was performed in a total volume of 25  $\mu$ l containing approximately 100

<sup>1</sup>Department of Molecular Biology and Genetics, Bogazici University, 34342, Istanbul, Turkey.

<sup>2</sup>Burc Molecular Diagnostic Laboratory, 34365, Istanbul, Turkey.

<sup>3</sup>Ahenk Clinical Laboratory, 34365, Istanbul, Turkey.



**FIG. 1.** A 4% (w/v) agarose electrophoretic gel of the polymerase chain reaction (PCR) products of the 221-bp (prothrombin) and 169-bp (factor V) amplicons. The effects of the temperature (A) and primer concentration (B) the primer ratio of PII and FVL is 1:1.5 in lane 1; 1:2 in lane 2; 1:2.5 in lane 3.

ng DNA, 2.5  $\mu$ l of 10 $\times$  polymerase buffer (MBI Fermentas, Hanover, MD), 2.0 mmol/l MgCl<sub>2</sub>, 0.2 mmol/l dNTPs (MBI Fermentas), 0.4  $\mu$ mol/l of each prothrombin primer, 0.8  $\mu$ mol/l of each factor V primer and 1 unit of *Taq* polymerase (MBI Fermentas). The PCR program on PTC-150 Minicycler™ (MJ Research, Watertown, MA) thermal cycler was as follows: an initial denaturation step at 94°C for 4 min, followed by 33 cycles of 45 sec at 94°C, 30 sec at 60°C, 45 sec at 72°C, and a final extension step of 8 min at 72°C.

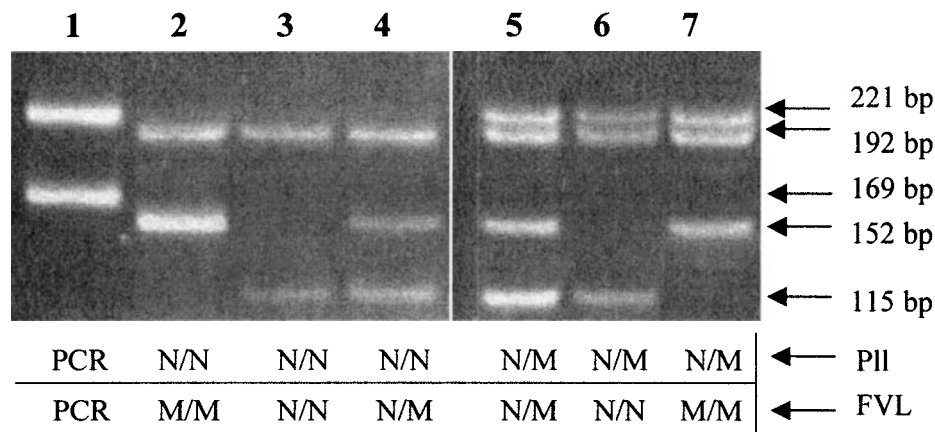
A 169-bp product from exon 10 of factor V (GenBank accession L32764) and a 221-bp product from the 3' untranslated region of the prothrombin gene (GenBank accession M17262) were coamplified using PCR. Factor V primers were as follows: forward, 5'-ACATCGCCTCTGGGCTAATA-3'; reverse, 5'-TTGAAGGAAATGCCCATTA-3'. Mismatched primer was used to amplify Prothrombin products. Prothrombin primers were as follows: forward, 5'-ATGGGGTGAAGGCTGTGACC-3'; reverse, 5'-AGCACTGGGAGCATTGAGCCT-3'. In the reverse primer, a single mismatched nucleotide (underlined) was

substituted so that amplification of a wild-type allele resulted in the generation of a new *Mnl* I restriction endonuclease site.

A total of 5  $\mu$ l from the PCR product was run on 2% agarose to check for any nonspecific bands. A total of 15  $\mu$ l of the PCR product was digested with 6 units of the *Mnl* I enzyme (MBI Fermentas) and 2  $\mu$ l of its 10 $\times$  reaction buffer in a 20  $\mu$ l reaction volume. The mixture was incubated at 37°C for 3 h. The digested products were electrophoresed on 4% (w/v) agarose or 6% acrylamide gels at 100 V for 30 min or 50 min, respectively. The gel and running buffers were 1 $\times$  TBE (0.89 M Tris-Base, 0.89 M boric Acid, 20 mM Na<sub>2</sub> ethylenediaminetetraacetic acid (EDTA) adjusted to pH 8.3). The fragments were visualized by ethidium bromide under UV transilluminator.

## RESULTS

Here we established a multiplex PCR-RFLP assay to detect the factor V Leiden and prothrombin G20210A mutations. The



**FIG. 2.** Photograph of ethidium bromide-stained 6% acrylamide gel demonstrating multiplex polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) analysis for prothrombin G20210A and factor V Leiden. Lane 1: Undigested wild-type 221-bp (prothrombin) and 169-bp (factor V) amplicons. Lane 2: A prothrombin G20210 wild-type/factor V Leiden homozygote with digestion products of 192, 152, 29, and 17 bp. Lane 3: A prothrombin G20210/factor V wild-type with digested products of 192, 115, 37, 29, and 17 bp. Lane 4: A prothrombin G20210 wild-type/factor V Leiden homozygote with digestion products of 192, 152, 115, 37, 29, and 17 bp. Lane 5: A prothrombin G20210A heterozygote/factor V Leiden heterozygote with digestion products of 221, 192, 152, 115, 37, 29, and 17 bp. Lane 6: A prothrombin G20210A heterozygote/factor V wild-type with digestion products of 221, 192, 115, 37, 29, and 17 bp. Lane 7: A prothrombin G20210 heterozygote/factor V wild-type with digestion products of 221, 192, 152, 29, and 17 bp. The 37, 29, and 17-bp products are not shown because they electrophoresed off the gel. N denotes the wild-type allele; M denotes mutant allele.

effects of several factors, including MgCl<sub>2</sub> concentration, primer concentration, and PCR cycling conditions, on PCR specificity and efficiency have been determined and optimized. 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 2:1 ratio of factor V to prothrombin primers, which resulted in approximately equal amplification of the two amplicons. The amplification conditions have been optimized as 2:1 primer concentrations (factor V:prothrombin) at 60°C (Fig. 1).

A representative multiplex analysis to detect factor V Leiden and prothrombin G20210A is shown in Figure 2. Undigested products resulted in two products of 221 and 169 bps, representing prothrombin and factor V amplicons, respectively. *MnII* digestion of prothrombin and factor V wild-type amplicons yielded fragments of 29 bp and 192 (prothrombin amplicon) and 17 bp, 37 bp and 115 bp (factor V amplicon); the 17-bp fragment was a result of an invariant *MnII* site. Digestion of the factor V Leiden homozygote resulted in fragments of 17 bp and 152 bp, and the prothrombin G20210A homozygote yielded fragments of 221 bp.

To evaluate the assay, we tested 408 known patients with recurrent venous thrombosis for whom we had previously determined the genotypes for the factor V Leiden (21 homozygote and 58 heterozygotes) and the prothrombin G20210A (19 heterozygote, 5 of them were also heterozygote for the FV Leiden) variants by PCR followed by restriction enzyme digestion as described (Gomez *et al.*, 1998; Endler *et al.*, 2001). We observed complete concordance between methods.

## DISCUSSION

Because the polymorphisms, G1691A in the factor V gene and G20210A in the prothrombin gene, are common mild risk factors for venous thrombosis, it would be useful to assay both polymorphisms for thrombotic patients and their family members. Multiplex PCR-based restriction enzyme analysis permits simultaneous determination of these two important polymorphisms as demonstrated here for analysis using *MnII* digestion that has been shown useful to identify each polymorphism individually.

Our multiplex assay presents at least three main advantages: first, the same restriction enzyme is used for the detection of both mutations; second, the disappearance of 169 bp and subsequent generation of a 152-bp and 17-bp band indicates that complete digestion has occurred because the 17-bp fragment results from an invariant *MnII* site; third, the electrophoretic patterns after *MnII* restriction can be easily identified regardless of combination of genotypes between both mutations. If the assays were done separately, the factor V amplicon has built-in control for completeness of digestion whereas the prothrombin amplicon does not have. In summary, the multiplex PCR-RFLP evaluated in this study performed robustly and accurately and is readily adaptable to the clinical molecular diagnostic laboratory setting without requiring expensive equipment that is not available to all laboratories.

## REFERENCES

- BERTINA, R.M., KOELEMAN, B.P.C., KOSTER, T., *et al.* (1994). Mutation in blood coagulation factor V associated with resistance to activated protein C. *Nature* **369**, 64–67.
- ENDLER, G., KYRLE, P.A., EICHINGER, S., *et al.* (2001). Multiplexed mutagenically separated PCR: simultaneous single-tube detection of the factor V R506Q (G1691A), the prothrombin G20210A, and the methylenetetrahydrofolate reductase A223V (C677T) variants. *Clin. Chem.* **47**, 333–335.
- GOMEZ, E., VAN DER POEL, S.C., JANSEN, J.H., *et al.* (1998). Rapid simultaneous screening of factor V Leiden and G20210A prothrombin variant by multiplex polymerase chain reaction on whole blood. *Blood* **91**, 2208–2209.
- HUBER, S., MCMASTER, K.J., and VOELKERDING, K.V. (2000). Analytical Evaluation of Primer Engineered Multiplex Polymerase Chain Reaction–Restriction Fragment Length Polymorphism for Detection of Factor V Leiden and Prothrombin G20210A. *J. Mol. Diagn.* **2**, 153–157.
- KEENEY, S., SALDEN, A., HAY, C., *et al.* (1999). A whole blood, multiplex PCR detection method for factor V: Leiden and the prothrombin G20210A variant. *Thromb. Haemost.* **81**, 464–465.
- LASTRUCCI, R.M.D., DAWSON, D.A., BOWDEN, J.H., *et al.* (1999). Development of a simple multiplex polymerase chain reaction for the simultaneous detection of factor V Leiden and prothrombin 20210A mutations. *Mol. Diagn.* **4**, 247–250.
- LINFERT, D.R., REZUKE, W.N., and TSONGALIS, G.J. (1998). Rapid multiplex analysis for the factor V: Leiden and prothrombin G20210A mutations associated with hereditary thrombophilia. *Conn. Med.* **62**, 519–525.
- LUCOTTE, G., and CHAMPENOIS, T. (2003). Duplex PCR-RFLP for simultaneous detection of factor V Leiden and prothrombin G20210A. *Mol. Cell Probes* **17**, 267–269.
- MITTERER, M., LANTHALER, A.J., MAIR, W., *et al.* (1999). Simultaneous detection of FV Q506 and prothrombin 20210A variation by allele-specific PCR. *Haematologica* **84**, 204–207.
- POORT, S.R., BERTINA, R.M., VOS, H.L. (1997). Rapid detection of the prothrombin 20210 A variation by allele specific PCR. *Thromb. Haemost.* **78**, 1157–1158.
- POORT, S.R., ROSENDAAL, F.R., REITSMA, P.H., *et al.* (1996). A common genetic variant in the 39-untranslated region of the prothrombin gene is associated with elevated plasma prothrombin levels and an increase in venous thrombosis. *Blood* **88**, 3698–3703.
- RAOUL, M., MATHONNET, F., PELTIER, J.Y., *et al.* (1997). An improved method for the detection of the G20210A transition in the prothrombin gene. *Thromb. Res.* **88**, 441–443.
- RIPOLL, L., PAULIN, D., THOMAS, S., *et al.* (1997). Multiplex PCR-mediated site-directed mutagenesis for one-step determination of factor V Leiden and G20210A transition of the prothrombin gene. *Thromb. Haemost.* **78**, 960–961.
- XU, X., BAUER, K.A., and GRIFFIN, J.H. (1999). Two multiplex PCR-based DNA assays for the thrombosis risk factors prothrombin G20210A and coagulation factor V G1691A polymorphisms. *Thromb. Res.* **93**, 265–269.

Address reprint requests to:

Ibrahim Baris  
Department of Molecular Biology and Genetics  
Bogazici University  
Bebek, 34342  
Istanbul

E-mail: ibrahim.baris@boun.edu.tr