We gratefully acknowledge the technical assistance of Francesca Stefini. This work was presented in part at the 51st AACC National Meeting, July 25–29, 1999, New Orleans, LA [Clin Chem 1999;45(Suppl 6):A56].

References

Multisite Study for Genotyping of the Factor II (Prothrombin) G20210A Mutation by the Invader Assay, Martin J. Hessner,1* Kenneth D. Friedman,1 Karl V. Voelkerding,2† Suzanne Huber,2 Daniel Ryan,3 Bonnie Nuccie,3 and Marlies Ledford4§

The factor II (prothrombin) G20210A mutation (PT20210) is recognized as a genetic risk factor for increased plasma prothrombin and venous thrombosis that is present in 6–7% of thrombosis patients and 1–2% of healthy individuals (1–3). The first occurrence of deep venous thrombosis is increased 1.4- to 5.6-fold in individuals carrying at least one mutant allele compared with the incidence in individuals without this mutation (4, 5).

In this report, we describe the novel application of the Factor II Invader® assay for the genotyping of patient specimens for the prothrombin G20210A mutation. The Invader assay is a sensitive, homogeneous, DNA-probe-based method for quantitatively detecting specific mutations directly from genomic DNA (6–8) as described previously by Hessner et al. (9) and Ledford et al. (10).

To evaluate the suitability of the Invader assay for detection of the prothrombin mutation, peripheral blood specimens from 528 patients (from four different sites) were collected in EDTA or sodium citrate tubes and genotyped. The four participating sites were (a) the University of Wisconsin Hospital and Clinics Molecular Pathology Laboratory (Madison, WI), (b) the Blood Center of Southeastern Wisconsin (Milwaukee, WI), (c) the University of Miami, Department of Pathology (Miami, FL), and (d) the University of Rochester, Department of Pathology and Molecular Laboratory (Rochester, NY).

Genomic DNA was isolated from 50–300 μL ofuffy coat or whole blood with the QiAamp® Blood Kit (QiAGEN, Inc.), the Epicentre MasterPure™ DNA Purification Kit (Epicentre, Inc.), or the Bio-Rad InstaGene™ Whole Blood Kit (Bio-Rad, Inc., Table 1). DNA was quantified by the PicoGreen® Assay (Molecular Probes) or by measuring absorbance at 260 nm. The DNA concentrations of all samples analyzed by the Invader assay were >7.0 ng/μL. Quantities of genomic DNA in the samples were 70–1320 ng. Six samples were excluded because they did not meet the minimum DNA concentration (>7.0 ng/μL). All samples were unlinked and tested anonymously.

Initial determination of factor II genotype was accomplished by subjecting samples to PCR restriction fragment length polymorphism (PCR-RFLP) analysis (sites 1, 3, and 4) (1) or allele-specific PCR (AS-PCR) (site 2) as described previously by Hessner et al. (11). Twenty-microliter samples of each amplified PCR product were analyzed by electrophoresis through 2% agarose gels stained with ethidium bromide (12).

All samples were genotyped with Invader Reagents (Third Wave Technologies, Inc.) as described previously for Factor V (Leiden) (9, 10). Sequences of synthetic oligonucleotides (oligos) used in the Invader assay are as follows (underlined bold letters represent single-nucleotide polymorphism sites): Invader Oligo, 5'-TATGTTCCAAATATGATCCTCAGCT-3'; wild-type primary probe, 5'-AACGAGCCGACAGGCTCAATGCTCC-3'; mutant primary probe, 5'-AACGAGCCGACAACTCCTCAATGCTCC-3'; synthetic wild-type target, 5'-TAGCAGCTGGAGCATTGAGGCT-3'; synthetic mutant target, 5'-TAGCAGGAGCATTGAGGCTGCTGAGACTCCTTATGGGAAACATAGTTTGAACAAATAAT-3'; and fluorescein resonance energy transfer cassette, 5'-F-CCCT-Q-GTC-TGGTTTTCCAGAGGAGGTTGCGGCTTGT-3'. Reaction plates were incubated at 63 °C for 4 h in a Perkin-Elmer GeneAmp® PCR System 9600 Thermal Cycler (PE-AEI), an MJ Research Thermocycler (96V) with Hot Bonnet (MJ Research),
or a water bath (Precision Scientific). Invader assay results were measured by stopping the reactions and transferring them to a new microtiter plate (stop-and-transfer method). Two sites, 2 and 3, also read plates by direct measurement (direct read) of fluorescence, which eliminated one addition and one transfer step without affecting assay performance.

All calculations were performed and interpreted as described previously by Hessner et al. (9). The net signal counts for the wild-type (20210G) and mutant (20210A) Invader reactions of each sample were determined by subtracting the “no-target-blank” (background) counts from the raw signal. In cases where net counts were equal to or less than zero, they were set equal to one to rank all ratios as positive numbers and eliminate division by zero.

Determination of sample genotype was based on the ratio of the adjusted net, wild-type counts to the adjusted net, mutant counts as follows: wild type, ≥5.0; equivocal, ≥3 to <5; heterozygous, ≥0.25 to <3; and homozygous mutant, <0.25.

Sample inclusion and assay exclusion criteria were applied as described previously by Ledford et al. (10). Six specimens were excluded because their DNA concentration was below the minimum threshold required for Invader analysis. Two assay runs were initially invalid because the wild-type controls were incorrectly determined to be heterozygotes. When they were repeated, both sets of controls were called correctly and genotypes were accurately assigned for each sample. Sample data from invalid runs were not included in the analysis. Any sample that yielded a ratio value within the equivocal range was repeated. Two samples (0.4%) yielded equivocal readings; the correct genotype was assigned after the

<table>
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<th>Site</th>
<th>Stop and transfer method</th>
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<th>Direct</th>
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</table>

a ND, not determined.  
b Epicentre MasterPure DNA Purification Kit.  
c Qiagen QIAamp Blood Kit.  
d Bio-Rad InstaGene Whole Blood Kit.

Table 1. Concordance rate listed by site and genotype and protocol variables listed by site.

![Distribution of Ratios](https://academic.oup.com/clinchem/article/47/11/2048/5639236/fig1.png)

Fig. 1. Ratio distribution of all samples by genotype. The sample is identified on the x axis, and the wild-type/mutant signal ratio is plotted along the y axis. Solid horizontal lines indicate boundaries between the zones, including the equivocal zone. The data plotted were obtained by the transfer and read method. ♦, wild-type (≥5.0); ○, heterozygous (0.3–2.99); ▲, mutant (<0.25).
The Invader assay was rerun on these samples. Variability of background signal influenced by sample preparation methods and the type of sample caused the two samples to yield equivocal results.

The overall concordance with previously obtained genotype data was 100%. The mean ratios for wild-type, heterozygous, and homozygous samples were 510, 0.98, and 0.004, respectively. An equivocal ratio range (≥3 to <5) was used to avoid any ambiguity between heterozygote and wild-type genotypes (Fig. 1). In this study, two samples (0.4%) were classified as equivocal and, after retesting, generated ratios of >5, and therefore were correctly typed as wild type (20210G/G). Table 1 details genotype results, concordance data, DNA extraction method, PCR method, and type of fluorescence reader used.

In this study, we used the net wild-type/mutant allele signal ratio to establish genotypes. We found that the Invader assay clearly differentiates factor II 20210G/G, 20210G/A, and 20210A/A genotypes with no overlap in net signal ratios. Moreover, the majority of wild-type samples gave signal ratios far above the equivocal range border. The allele frequency in this population, 4% (42A of 1044 total alleles), was comparable to frequencies reported for thrombosis patients (3.2%) (11). Individuals heterozygous for this allele comprised 6.5% of the total, and the frequency of homozygous mutant individuals was 0.8%, findings that are comparable to previously reported carrier frequencies in thrombosis patients (1–3, 11). These results were consistent with a population at increased risk for thrombosis.

We conclude that the Factor II Invader assay is a suitable method for the analysis of the factor II (prothrombin) G20210A mutation in the clinical setting. Unlike existing molecular assays for the prothrombin mutation, the Invader assay can be carried out directly on DNA extracted from blood samples without the need for PCR amplification. In addition to providing complete concordance with results obtained through PCR-based mutation detection methods, the method is rapid and simple to perform. DNA can be extracted from buffy coat or whole blood with any of several commercially available extraction methods and then added directly to the Invader assay. The assay functions well over a wide range of DNA concentrations. We recommend using >100 ng per reaction. Reactions require only two reagent additions and a single 4-h incubation, and reactions are carried out directly in microtiter plates without the need for physical separation of work areas, as is required for PCR. The reactions can be read on a fluorescence plate reader directly from the microtiter plate after incubation, or reactions can be terminated and then read. Additionally, reactions can be held in the thermocycler at 4 °C overnight and read the following day by either readout method. Total “hands-on” time for the Invader assay is ~1–1.25 h compared with ~2 h for allele-specific PCR and PCR restriction fragment length polymorphism analyses. In routine practice, results are available from Invader assays within 1 working day.

The accuracy demonstrated by the Invader assay in studies such as ours suggests that this technique is a promising method for clinical laboratories to evaluate patients for venous thrombotic risk factors.

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References


Single Nucleotide Polymorphism (SNP) Genotyping in Unprocessed Whole Blood and Serum by Real-Time PCR: Application to SNPs Affecting Homocysteine and Folate Metabolism, Arve Ulvik and Per Magne Ueland (LOCUS for Homocysteine and Related Vitamins, University of Bergen, Armauer Hansens hus, N-5021 Bergen, Norway, *author for correspondence: fax 47-5597-4605, e-mail arve.ulvik@farm.uib.no)

Several single nucleotide polymorphisms (SNPs) have been identified that affect folate and homocysteine metabolism, which in turn are implicated in the pathogenesis of cardiovascular disease (1), neural tube defects (2), and...