Venous thrombosis is a serious disease that affects ~1 in 1000 individuals in the US (1). Although thrombophilia has many causes, among the most frequent is activated protein C resistance (2–4). Activated protein C resistance manifests as an abnormally reduced activated partial thromboplastin time (5). Among nonidiopathic mechanisms, the most common is genetic (6), in particular, a G-to-A substitution at nucleotide 1691 of the factor V gene on chromosome 1 (7). This transition, located in exon 10 of the gene, produces an Arg-to-Gln substitution at amino acid 506 of the protein. Individuals bearing this genetic risk factor for venous thrombosis comprise ~5% of the general population (8). Amino acid 506 is the less consensual of two important sites for proteolytic cleavage mediated by activated protein C (9). Mutant Va, the α-thrombin-activated membrane-bound cofactor product of the factor V Leiden (FVL) locus, is kinetically inactive at one-tenth the rate of wild-type Va (5). In addition, wild-type factor V is cleaved at another site, Arg 306 (8, 9), fully inactivating it (6). The combined effects of kinetically slowed inactivation and full inactivation through Arg 306 cleavage are believed to account for the FVL heterozygous effect (6). Screening for the FVL risk factor is considered an important part of a thrombophilia evaluation. Heterozygotes are 5- to 10-fold more likely to suffer a deep vein thrombosis than the general population, whereas individuals with a homozygote FVL genotype are estimated to be at 50- to 100-fold greater risk than those with two wild-type alleles (10–12).

Our aim was to accommodate testing several thousand samples per month at a 25% cost savings over conventional PCR-restriction fragment length polymorphism (RFLP). We developed a reverse allele-specific oligonucleotide (ASO) hybridization assay in a microwell plate format. Our method for genotyping is a major modification of that published by Zehnder et al. (13). In our assay, a different primer pair is used, oligonucleotide probes are covalently attached directly to the microwell plate surface, and biotinylated amplicons are visualized using a chromogenic amplification system (14).

Because the mutation responsible for FVL is located in close proximity to the intron-exon border, both our group and Bertina et al. (7) chose PCR forward and reverse primers in exon 10 and intron 10 regions, respectively. In our initial studies, a 265-bp fragment spanning the exon-intron junction of the factor V gene was PCR amplified from genomic DNA using primers described previously (7), except that the upstream exon 10 primer was shorter by two bases at the 5’ end. With these primers, observed amplification reaction yields were less than what we desired. In response, we decided to improve our results through the use of optimized amplification primers. The interrogated base is at position 1691; therefore, we used a pair of PCR primers, 5’-ATCAGAGCAGTCAACCAGG-3’ (upstream primer; bp 1490–1509 in GenBank Accession No. M14335.1 HUMVA) and 5’-ACAACCTAGACTTGCCTTCG-3’ (downstream primer; bp 62701–62720 in emb PAC86F14 Accession No. 99572.1 HS86F14), to amplify a 423-bp fragment encompassing the same junction. Amplification primers were biotinylated at their 5’ ends and synthesized using the cyanethylphosphoramidite method on a Perseptive Biosystems Expedite Nucleic Acid Synthesis System (Perkin-Elmer). With our new PCR primers, the amplifications were more robust and consistent.

The capture probes had the following sequences: wild-type probe (bp 1667–1684 in GenBank Accession No. M14335.1 HUMFVA), 5’-H2N-CTGGACAGGCGAAGATA-3’; mutant probe, 5’-H2N-CTGGACAGGGCAAGATA-3’. The probes differ from each other by a single base, the 11th base from the 5’ end. Oligonucleotide probes were synthesized using a C-12-amino-link phosphoramidite as the final ligand. Controls were synthetic single-stranded DNA fragments biotinylated at their 5’ ends and synthesized using the cyanethylphosphoramidite method on a Perseptive Biosystems Expedite Nucleic Acid Synthesis System (Perkin-Elmer). With our new PCR primers, the amplifications were more robust and consistent.

The probe capture had the following sequences: wild-type probe (bp 1667–1684 in GenBank Accession No. M14335.1 HUMFVA), 5’-H2N-CTGGACAGGCGAAGATA-3’; mutant probe, 5’-H2N-CTGGACAGGGCAAGATA-3’. The probes differ from each other by a single base, the 11th base from the 5’ end. Oligonucleotide probes were synthesized using a C-12-amino-link phosphoramidite as the final ligand. Controls were synthetic single-stranded DNA fragments biotinylated at their 5’ ends and had the following nucleotide sequences: wild-type control, 5’-biotin-AGGACAAAATACCTGTATTCCTTGCTTGACAGGATCTGCTTCTACA-3’ (bp 62907–62954 in Z99572.1, which is PAC86F14 Accession No. 99572.1 HS86F14), to amplify a 423-bp fragment encompassing the same junction. Amplification primers were biotinylated at their 5’ ends and synthesized using the cyanethylphosphoramidite method on a Perseptive Biosystems Expedite Nucleic Acid Synthesis System (Perkin-Elmer). With our new PCR primers, the amplifications were more robust and consistent.
primary amines. Probes (50 pmol) were dissolved in phosphate buffer, pH 8.5 (100 μL/well), and added to the coated wells to allow specific coupling of amino-labeled oligonucleotides to the DNA-Bind plate during incubation for 18 h at 4°C. Unbound oligonucleotides were removed by washing three times with wash buffer (50 mmol/L Tris, 0.45 mol/L NaCl, 1 mL/L Tween 20, pH 7.7). The unreacted DNA-Bind active groups were blocked by incubating the plates for 2 h with wash buffer containing 10 g/L bovine serum albumin and 100 mg/L spermidine (both from Sigma). The microtiter plates were washed three times as described above and then were dried under reduced pressure and sealed in Mylar bags with packs of desiccant. Plates were stored at 4°C and protected from moisture and light.

Total genomic DNA was extracted from peripheral blood leukocytes using a Gentra Generation Capture Column Kit (Gentra Systems) and stored at 4°C in a buffer containing 10 mmol/L Tris-HCl, 1 mmol/L disodium EDTA, pH 8.0. The DNAs used in this study were archived anonymized samples. All studies adhered to our company’s policies and to those of the American College of Medical Genetics guidelines for research on archived DNA (15).

Samples containing 50 ng of genomic DNA were amplified in 100-μL reactions containing 10 mmol/L Tris-HCl, pH 8.3 (at 25°C), 0.5 μmol/L each PCR primer, 250 μmol/L each dNTP, 2.5 mmol/L MgCl2, 50 mmol/L KCl, and 5 U of AmpliTaq Gold DNA polymerase. All PCR reagents were obtained from Perkin-Elmer. Amplification was performed in a GeneAmp PCR System 9600 (Perkin-Elmer) using the following thermal profile: an initial hold at 95°C for 10 min; 35 cycles at 94°C for 1 min, 62°C for 30 s, and 72°C for 30 s; and a final extension hold at 72°C for 10 min.

Clinical specimens (n = 256) were used for validation. Anonymized and blinded samples were also assayed by PCR-RFLP: Samples were PCR amplified, digested with MnlI endonuclease, and electrophoresed on 6% polyacrylamide vertical gels, followed by ethidium bromide staining.

We modified the microtiter plate ASO hybridization assay for FVL detection described by Zehnder et al. (13) as follows: After PCR amplification, double-stranded DNA was melted to allow hybridization with the capture probe. A 50-μL aliquot of each PCR sample was mixed with an equal volume of denaturation solution (0.8 mol/L NaOH, 0.04 g/L m-cresol purple). A series of experiments demonstrated that complete ampiclon denaturation is a critical step in this assay (data not shown). Furthermore, we found that there was an inadequate description of the denaturation and neutralization conditions for our purposes in Zehnder et al. (13). Therefore, we conducted a series of experiments and instituted some controls (such as including the m-cresol purple as a pH indicator dye) to provide assurance that adequate denaturation followed by neutralization had been achieved (data not shown). Aliquots (25 μL) of the denatured, neutralized ampiclon were then combined with 100 μL of hybridization solu-

tion [1.5× standard saline citrate (1X = 0.15 mol/L NaCl and 0.015 mol/L sodium citrate), 1 g/L sodium dodecyl sulfate, and 150 mL/L deionized formamide in 0.5 mol/L sodium phosphate buffer, pH 4.2] in each of four wells coated with the two probes, in duplicate. Hybridization was allowed to proceed for 2 h at 37°C. Amplification products that did not hybridize were removed by washing the plates five times with wash buffer at room temperature.

Generation of colorimetric signal was achieved by adding to each well 100 μL of a 1:2 000 dilution of ExtrAvidin-Alkaline Phosphatase conjugate (Sigma) in wash buffer containing 10 g/L bovine serum albumin, 500 mg/L bovine γ-globulins, and 100 mg/L spermidine. After a 60-min incubation at 37°C, the unbound conjugate was washed five times as described above. The signal was detected using the ELISA Amplification System reagents (Life Technologies) according to the manufacturer's instructions. Absorbance was read at 490 nm in a ELx 800 spectrophotometer (BioTek Instruments). In our assay, four microwells were run for each sample: two wells (duplicates) were coated with the wild-type capture probe, and two with the mutant probe. The conditions of the assay were optimized for minimal hybridization between probe-target containing a mismatch, at the same time favoring efficient hybridization of fully complementary fragments. Arrays of hybridization buffer compositions were tried, including some with tetramethylammonium chloride, deionized formamide, and various salt conditions. Tetramethylammonium chloride was tried because of its known property of eliminating the dependence of melting point (Tm) on G-C content. In our work, the addition of this quaternary ammonium salt did not substantially improve the signal-to-noise ratio.

Through optimization, wild-type samples yielded a strong signal in the wells coated with the wild-type capture probe and a low, background signal after hybridizing with the mutant probe. The opposite was obtained for samples obtained from FVL homozygous individuals. Heterozygous samples yielded signals of similar intensity when hybridized with both allele-specific probes. A typical plate is shown in Fig. 1A. We analyzed 256 specimens with this reverse ASO hybridization assay and by PCR-RFLP. The results were 100% concordant. Ratios of the signal absorbance generated in the mutant probe well divided by the absorbance generated in the wild-type probe well were calculated for these same specimens. This is a simplified form of discriminant analysis. Of the patient samples included in statistical calculations, 187 were wild type, 26 were FVL homozygous, and 43 were heterozygous. Table 1 shows the mean values, the minimum and maximum ranges, and the standard deviations for ratios of each genotype observed. Visual inspection revealed no overlap between the genotypes called (see Fig. 1C). The reverse ASO hybridization assay can be interpreted visually or by use of a colorimetric microplate reader coupled to a computer with the appropriate software, producing a cutoff value that determines the genotype
computer-assisted quantitative interpretation produces a hard copy of spectrophotometric readings and decreases the likelihood of misinterpretation of results. Our computer program is similar to that of Zehnder et al. (13). Occasional differences in the colorimetric signals and ratios between microwells containing samples of identical genotypes were mostly attributable to differential amplification as can be seen on PCR-RFLP gels. To eliminate the need for microwell duplicates, pilot tests were conducted that called genotypes on the basis of one mutant-probe-coated microwell and one wild-type-probe-coated microwell. These experiments demonstrated that single wells worked as well as duplicate wells (data not shown). We used a relatively wide input range (40–300 ng) of extracted genomic DNA for simplicity because it is not practical to rigorously measure the DNA concentration of each sample. Input variability appeared to be smoothed through the use of a quantitative approach for genotype reporting.

Graphing corrected mutant to wild-type absorbance ratios and their multiplicative inverse allowed us to infer that there was differential hybridization between the PCR products and their ASOs (data not shown). We speculated that batch-to-batch variation in coating of microwell plates accounted for some fluctuation in signal intensity (shown graphically in Fig. 1C). To obtain similar colorimetric signals from different batches of plates and reagents, we designed and synthesized a set of controls. The wild-type and mutant controls were synthetic oligonucleotides, each with a length of 49 bp and biotinylated at the 5' end. The oligonucleotides behaved like denatured amplified patient samples in that the wild-type control hybridized specifically to the wild-type capture probe and the mutant control specifically hybridized to the mutant capture probe. We also created a heterozygous control by mixing equimolar solutions of wild-type and mutant controls. From these experiments, we confirmed that differential hybridization of the capture probes existed. Using equimolar solutions of controls should theoretically yield equal colorimetric signals for each capture probe. In reality, we obtained slightly lower signals with the wild-type capture probe than with the mutant capture probe. By adjusting the molar ratios of the wild-type and mutant controls, one can obtain a perfect signal and ratio for the heterozygote control.

Cluster analysis was carried out on the absorbance called for each sample. Computer-assisted quantitative interpretation produces a hard copy of spectrophotometric readings and decreases the likelihood of misinterpretation of results. Our computer program is similar to that of Zehnder et al. (13).

<table>
<thead>
<tr>
<th>Specimen</th>
<th>n</th>
<th>Minimum</th>
<th>Maximum</th>
<th>Mean</th>
<th>SD</th>
</tr>
</thead>
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<tr>
<td>Wild type</td>
<td>187</td>
<td>0.0196</td>
<td>0.2618</td>
<td>0.1278</td>
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<td>1.0580</td>
<td>0.6997</td>
<td>0.1499</td>
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<tr>
<td>Homozygote</td>
<td>26</td>
<td>1.5720</td>
<td>6.0610</td>
<td>3.0840</td>
<td>1.2840</td>
</tr>
</tbody>
</table>

The ratios were determined by subtracting the absorbance at 490 nm from the absorbance in the negative control wells (no-DNA template). Ratios are expressed as the Leiden probe signal divided by the wild-type probe signal. In theory, the Leiden/wild-type ratios should approach 0 for the wild type, ~1 for the heterozygotes, and infinity for the homozygotes.
values graphed in Fig. 1C after log_{10} transformation. Gaussian distribution of values within each given cluster was observed. Parsimonious construction of a similarity tree through the join procedure in SYSTAT (SPSS, Inc.), using several quantitative algorithms, correctly revealed the existence of four data clusters: (a) no DNA added, (b) wild type, (c) heterozygous, and (d) homozygous mutant for FVL. Kmeans analysis demonstrated adequate cluster separation to distinguish 97.6% of the 256 results (P <0.01); the remaining 6 samples were not outliers in a repeat PCR and visualization. We believe this demonstrates the accuracy and laboratory utility of this micro-well plate-based genotyping method.

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Tables showing (a) cluster analysis, (b) cost analysis, and (c) a FORTRAN program to analyze data are available as a supplement at the Clinical Chemistry Online Web site (http://www.clinchem.org/content/vol46/issue8/).

References


Ischemic Exercise Testing in Suspected McArdle Disease, Zahur Zaman1* and Stefana De Raedt2 (1 Department of Laboratory Medicine, University Hospitals Leuven, Herestraat 49, B-3000 Leuven, Belgium; 2 Clinical Laboratory, St. Elizabeth Hospital, B-2300 Turnhout, Belgium; * author for correspondence: fax 32-16-34-70-42, e-mail zahur.zaman@uz.kuleuven.ac.be)

Ischemic exercise testing is used in evaluation of patients with suspected McArdle disease, also known as glycogen storage disease type V (1). Lack of an increase in the blood lactate concentration during exercise is indicative of a defect in conversion of glycogen (or glucose) to lactate, consistent with the deficiency of skeletal muscle phosphorylase in this disease. Other glycogen storage diseases, such as deficiencies of phosphofructokinase and debranking enzyme, would also yield an abnormal ischemic exercise response.

In anticipation of performing the ischemic exercise test on a suspected case, one of us (S.D.R.) carried out the test on a healthy subject according to the method of Threatte and Henry (2), who describe the procedure as follows: “Laboratory diagnosis of McArdle disease is made by applying a blood pressure cuff on an exercising forearm and sampling blood lactate one minute after the exercise has begun”. Thus, after a butterfly catheter was inserted into the antecubital vein, a pre-exercise blood specimen was drawn and the blood pressure cuff was inflated to 50 mmHg above the systolic pressure. The arm was exercised vigorously by opening and closing the fist for 1 min, and a blood sample was taken for lactate measurement. With the cuff still inflated, additional blood samples were taken 2 and 3 min after cessation of the exercise.

The pre-exercise lactate was 1.5 mmol/L, and the postexercise concentrations were 1.4 mmol/L at 1 min, 1.5 mmol/L after 2 min, and 1.5 mmol/L after 3 min. The test was repeated on two healthy subjects and gave similar results. According to these results, all three healthy test subjects had a glycogen or glucose metabolism defect. This was patently false. Therefore, the test was repeated on other healthy subjects with the modification that after