Clinical Chemistry 43:12
2262–2267 (1997)

Real-time fluorescence genotyping of factor V Leiden during rapid-cycle PCR

Marla J. Lay[1] and Carl T. Wittwer[2]*

A single-step method for factor V Leiden genotyping is presented that uses rapid-cycle PCR and simultaneous fluorescence analysis with resonance energy transfer probes. A fragment of the factor V gene containing the mutation is amplified asymmetrically through use of a primer labeled with Cy5™ in the presence of a 3′-fluorescein-labeled probe that covers the mutation site. When the fluorescein probe is annealed to the extension product of the Cy5-labeled primer, the fluorophores are brought into close enough contact for resonance energy transfer to occur. As the temperature increases, the probe melts from its target, decreasing the resonance energy transfer. When the probe is complementary to the product strand, it melts at 65 °C; if the single-base mutation is present, the probe melts at 57 °C. Concurrent amplification and analysis from genomic DNA takes 20–45 min and requires no sample manipulation after the fluorescence thermal cycler is loaded.

Factor V Leiden *[1, 2]* is a single-point mutation in the factor V gene (G1691A) that incorporates an arginine instead of glutamine at amino acid residue 506 (R506Q). This substitution prevents activated protein C from cleaving a peptide bond at amino acid 506 that would inactivate the coagulation factor. Patients both homozygous and heterozygous for this mutation have an increased risk for thromboembolic disease. As the most common cause of inherited thrombophilia, this mutation is a large-volume test for clinical molecular genetics laboratories.

The usual method for detecting factor V Leiden is to amplify by PCR a portion of the gene containing the mutation and then digest the products with a restriction enzyme that cuts only the wild-type sequence. Size separation of digested products by gel electrophoresis distinguishes wild-type from homozygous and heterozygous mutant genotypes. This process, not including DNA isolation, requires at least 4–6 h and involves several post-amplification handling steps, with the associated risks of end-product contamination, sample tracking errors, and incomplete enzyme digestion.

Fluorescent probes can be used to detect and monitor DNA amplification. When glass capillaries are used in rapid-cycle DNA amplification *[3, 4]*, the optically clear sample vessel serves as a cuvette for fluorescence analysis *[3, 5–7]*. Instruments that combine rapid-cycle PCR with fluorescence analysis for continuous monitoring during amplification are commercially available *[8]*. Various sequence-specific probes have been described that utilize fluorescence resonance energy transfer between two fluorophores to monitor product production *[5, 9, 10]*. Resonance energy transfer between adjacent fluorescein- and Cy5 (indodicarbocyanine)-labeled hybridization probes has been used as a measure of the amount of specific product generated during rapid-cycle DNA amplification *[5]*.

If PCR is carried out with a Cy5-labeled primer, a single fluorescein-labeled probe can also be used to monitor amplification *[6]*. When the probe is annealed to the extension product of the Cy5-labeled primer, the fluorophores are brought into close enough contact for resonance energy transfer to occur, increasing the fluorescence of the Cy5. If the fluorescein-labeled probe is placed across the mutation site, the presence of the mutation can be evaluated by monitoring fluorescence while the sample is being heated through the melting temperature of the probe. A single base change will cause the probe to melt at a lower temperature than if the probe is completely complementary. With asymmetric amplification, the strand formed from the labeled primer can be produced in excess, allowing probe hybridization without competition from the annealing of full-length strands.

As an alternative to sequential amplification, restriction enzyme digestion, and electrophoresis, we were able to genotype the factor V mutation by fluorescence melting-curve analysis during 45 cycles of asymmetric amplification.

[1] ARUP Institute for Clinical and Experimental Pathology, 500 Chipeta Way, Salt Lake City, UT 84108.
[2] Department of Pathology, University of Utah Medical Center, 50 N. Medical Dr., Salt Lake City, UT 84132. Fax 801-581-4517; e-mail Carl_Wittwer@hlthsci.med.utah.edu.
*Author for correspondence.
Received July 1, 1997; revision accepted August 29, 1997.

Materials and Methods

PRIMER AND PROBE DESIGN

The primer GACCATACTACAGTGACGTG (sense, exon 10) and the previously described primer TGTTATCACACTGGTGCTAA (antisense, intron 10, *[1]*) were used to amplify a 250-bp fragment of the factor V gene for genotyping by *Mnl*1 digestion and electrophoresis. For fluorescence monitoring, the primer TAATCTGTAAGAGCAGATCC (sense, exon 10), 63 bp closer to the mutation site (Genbank locus HUMF510), was chosen and labeled with Cy5 on the base third from the 3′-end. The 3′-fluorescein probe AATACCTGTATTCCTCGCCTGTC-fluorescein (antisense, intron 10/exon 10 junction) includes the site of factor V Leiden, 8 bases from the 3′ end. The orientation of the Cy5-labeled primer, fluorescein-labeled probe, and mutation site is illustrated in Fig. 1.

PRIMER AND PROBE SYNTHESIS

Oligonucleotides were synthesized by phosphoramidite chemistry on a Gene Assembler Plus™ (Pharmacia Biotech). The 3′-fluorescein probe was synthesized on fluorescein controlled-pore glass (BioGenix, San Ramon, CA). With the final trityl group attached, failure sequences were removed with reversed-phase HPLC on a 4 × 250 mm C_{18} column (Hypersil ODS; Hewlett-Packard) in a 0.1 mol/L triethylammonium acetate mobile phase with a linear gradient with acetonitrile. At a flow rate of 1 mL/min, the desired fraction eluted at an acetonitrile content of 25% (250 mL/L). Detritylation was performed on a PolyPak column (Glen Research) and the oligonucleotide was eluted with an equivolume solution of acetonitrile in water. Two additional 3′-fluorescein probes of the same size were also synthesized, one with a 3-bp overlap with the primer (a shift of 6 bases from that shown in Fig. 1), and one complementary to the mutation instead of to the wild-type.

Synthesis of the Cy5-labeled primer required both automated and manual steps. First, automated synthesis of the oligonucleotide incorporated an amino-modifier C6dT (Glen Research) in place of the most 3′ thymidine residue. Next, the Cy5 moiety was attached as the monovalent *N*-hydroxysuccinimide ester of Cy5 (Cy5.29–OSu, Amersham; absorbance maximum 650 nm, absorptivity >200 000 L $mol^{-1}cm^{-1}$; see *[5]* for additional references), according to the manufacturer's instructions. The Cy5-labeled primer was purified by reversed-phase HPLC as described above and eluted at a mobile-phase acetonitrile content of ~22%. Primer and probe purity were assessed by analytical HPLC with tandem absorbance and fluorescence detectors (Waters Models 486 and 474, respectively). The ratio of the concentration of fluorescent label to that of oligonucleotide, as judged by absorbance measurements *[11]*, was 1.0 for the Cy5-labeled primer and 0.8–0.9 for the fluorescein-labeled probes.

SAMPLES AND CONTROLS

Human genomic DNA was obtained by phenol/chloroform extraction and ethanol precipitation *[12]* from 103 samples of EDTA-anticoagulated blood submitted to Associated Regional and University Pathologists reference laboratory for factor V Leiden testing. The DNA was resuspended to a concentration of 50 μg/mL in 10 mmol/L Tris, pH 8.0, containing 0.1 mmol/L EDTA. Three of these samples, with normal, decreased, and highly decreased activated protein C sensitivity ratios *[1]*, were used as controls and confirmed by direct DNA sequencing to be respectively wild-type, heterozygous, and homozygous for factor V Leiden.

RESTRICTION DIGEST PROTOCOL

PCR was performed by rapid-cycling techniques *[3, 4]* in a reaction volume of 10 μL with each dNTP at 200 μmol/L, each primer at 0.5 μmol/L, 3.0 mmol/L $MgCl_2$, 50 mmol/L Tris, pH 8.3, 500 mg/L bovine serum albumin, 20 g/L sucrose, 0.1 mmol/L cresol red, 50 ng of DNA, and 0.4 U of Taq polymerase. The samples were loaded into glass capillaries (no. 1705; Idaho Technology, Idaho Falls, ID) and sealed with a butane torch. The 250-bp product was amplified for 45 cycles in a rapid air thermal cycler (Rapidcycler™; Idaho Technology) with denaturation at 94 °C for 0 s, annealing at 55 °C for 2 s, and extension at 74 °C for 5 s. The amplification was completed in <20 min. The amplified products were dispensed into microcentrifuge tubes with 1 μL of *Mnl*1 (5000 U/mL) and 1 μL of buffer: 500 mmol/L NaCl, 100 mmol/L Tris-HCl, pH 7.9, 100 mmol/L $MgCl_2$, and 10 mmol/L dithiothreitol (NE Buffer 2; New England Biolabs.). After incubation for 2 h at 37 °C, samples were electrophoresed on 1.5% agarose gels at 5 V/cm for 60 min in the presence of 0.5 mg/L ethidium bromide. The DNA fragments were visualized with UV light, and the samples were genotyped by restriction fragment length

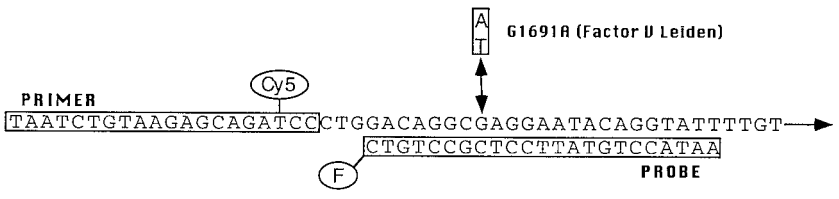

Fig. 1. Relative orientation of the Cy5-labeled primer, fluorescein-labeled probe, and factor V Leiden.

An amplification primer near the factor V Leiden mutation locus is labeled with Cy5 on the third base from the 3′ end. During amplification, extension of the labeled primer occurs, allowing hybridization to the 3′-fluorescein (F)-labeled probe, which includes the mutation site. The physical proximity of the Cy5 and fluorescein labels results in resonance energy transfer when the probe is hybridized. Probe melting is monitored as the temperature increases. The mismatch caused by factor V Leiden results in a decrease in the probe melting temperature in comparison with that for the wild-type.

polymorphism as previously described *[1]*. Samples homozygous for factor V Leiden were characterized by a band at 200 bp, wild-type samples by a band at 163 bp, and heterozygous samples by bands at both 200 and 163 bp.

FLUORESCENCE PROTOCOL

PCR was performed as above except that 0.5 μmol/L Cy5-labeled primer (Fig. 1), 0.2 μmol/L intron 10 primer (for a 187-bp product), and 0.1 μmol/L fluorescein-labeled probe (Fig. 1) were used without sucrose or cresol red. The samples were transferred to disposable capillary cuvettes (no. 1720; Idaho Technology) and centrifuged to place the sample at the capillary tip before capping. Amplification was performed for 45 cycles of denaturation (94 °C for 0 s), annealing (50 °C for 10 s), and extension (75 °C for 0 s) on a rapid-temperature cycler with integrated fluorescence monitoring (Lightcycler™; Idaho Technology). The ramp rates were programmed at 20 °C/s from denaturation to annealing, 1 °C/s from annealing to extension, and 20 °C/s from extension to denaturation. The epi-illumination fluorometer in the LightCycler uses a blue light-emitting diode to excite the capillary tips at 450–490 nm. Total internal reflection at the glass/air interface along the capillary axis increases the observed fluorescence after spectral filtering and focusing on photodiodes. The optical design is similar to that used in flow cytometers *[8]*.

The ratio of fluorescein (520–560 nm) to Cy5 (655–695 nm) fluorescence was acquired during temperature cycling to monitor amplification and probe hybridization. The fluorescence at the end of each annealing step reflects the cumulative amount of product resulting from extension of the Cy5-labeled primer during asymmetric amplification. In some experiments, the dependence of probe hybridization on temperature was monitored continuously within a temperature cycle. After amplification was complete, a final melting curve was usually performed by cooling to 50 °C, holding at 50 °C for 1 min, and then heating slowly at 0.2 °C/s until 75 °C. Fluorescence was collected continuously during this heating to monitor the dissociation of the 3′-fluorescein-labeled probe. The fluorescence ratio (Cy5/fluorescein, F_R) was plotted against temperature (T) to give melting curves for each sample.

Melting curves were converted to derivative melting curves in two steps. First, the negative derivative of the fluorescence ratio with respect to temperature ($-dF_R/dT$) was plotted against temperature. The resulting derivative curves ($-dF_R/dT$ vs T) had a beginning baseline (before the melting transition) higher than the final baseline (after the melting transition). Each curve was then corrected by reducing the initial baseline to the level of the final baseline. Intermediate points within the melting transition of each curve were reduced in proportion to the fractional area under the final derivative curve.

Results

Fluorescence monitoring of product accumulation and factor V genotyping can be achieved during DNA amplification with resonance energy transfer. When fluorescence is observed at the end of each 10-s annealing phase, the ratio of Cy5/fluorescein fluorescence increases during each cycle as product accumulates. For the labeled primer and probe shown in Fig. 1, this ratio approximately doubles during 45 cycles of asymmetric amplification (Fig. 2). A majority of the ratio change (85–90%) comes from increased Cy5 fluorescence; only a little (10–15%) results from decreased fluorescein fluorescence (data not shown). No increase in the fluorescence ratio is observed during temperature cycling in the absence of template (Fig. 2). The observed fluorescence with the labels separated by 5 bases (Fig. 1) was more than twice that with an alternative probe in which the fluorescein was directly opposite the Cy5 with a 3-bp overlap between primer and probe (data not shown).

The resonance energy transfer signal develops in each cycle as the fluorescein-labeled probe hybridizes to the Cy5-labeled strand. By observing fluorescence throughout a cycle of PCR (Fig. 3), the process of hybridization and melting of the probe to target can be monitored. As the sample is cooled to 50 °C, the fluorescence signal increases and continues to increase as the sample is held at 50 °C for 10 s. When the sample is subsequently reheated, the probe melts from the target sequence, as evidenced by a decrease in fluorescence. The melting transitions of homozygous wild-type, heterozygous mutant, and homozygous mutant sequences at the factor V Leiden locus can be observed directly during cycling. Fig. 3 shows fluorescence vs temperature tracings during cycle 40 of amplification with a 1 °C/s temperature transition during melting. Melting of the amplified homozygous

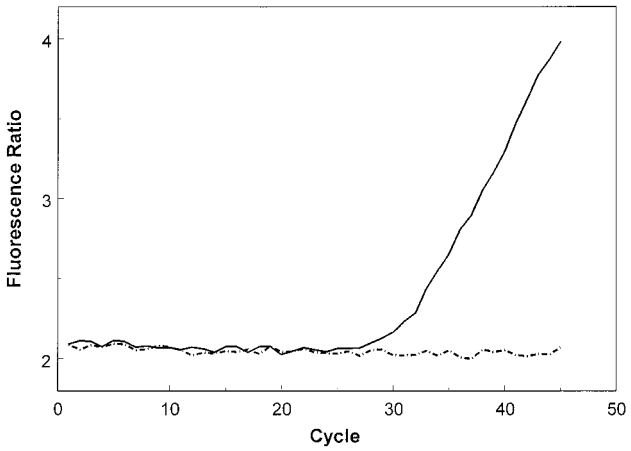

Fig. 2. Fluorescence ratio (Cy5/fluorescein) vs cycle number during asymmetric amplification of a DNA fragment than includes the factor V Leiden locus.

A 187-bp fragment was amplified from 50 ng of human genomic DNA (——) or from a no-template control (- - -) in the presence of 0.1 μmol/L 3′-fluorescein-labeled probe and 0.5 μmol/L of a Cy5-labeled primer (Fig. 1). Fluorescence was acquired once per cycle at the end of annealing.

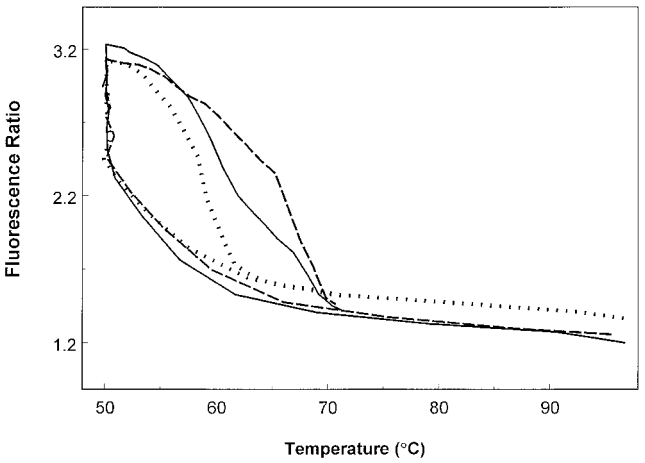

Fig. 3. Fluorescence ratio (Cy5/fluorescein) vs temperature plot during cycle 40 of asymmetric amplification of factor V wild-type (- - -), factor V Leiden homozygous (. . . .), and factor V Leiden heterozygous (——) human genomic DNA.

Fluorescence data were acquired continuously throughout cycle 40. Temperature programing was for 0 s at 94 °C, 10 s at 50 °C, and 0 s at 75 °C with a 1 °C/s transition between 50 and 75 °C. When displayed as fluorescence vs temperature, the data for each sample include cooling (*lower tracing* for each sample) and heating (*upper tracing*) segments.

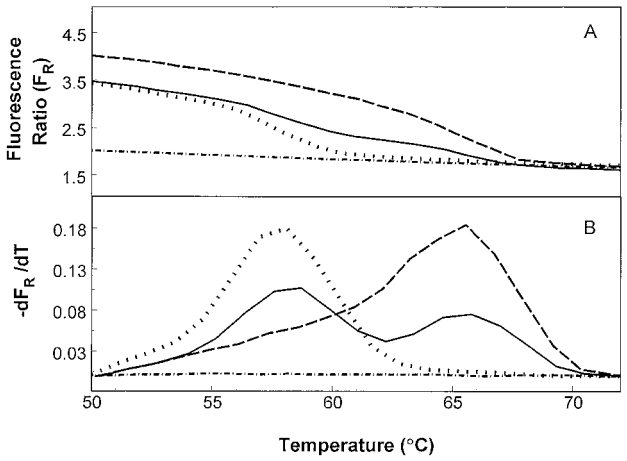

Fig. 4. Genotyping of factor V Leiden by melting-curve (*A*) and derivative melting-curve (*B*) plots.

A melting-curve plot of Cy5/fluorescein fluorescence (F_R) vs temperature is compared with a plot of $-dF_R/dT$ vs temperature. Data for both plots were obtained during the melting transition of a fluorescein-labeled probe from an asymmetrically amplified fragment of the human factor V Leiden locus. Genotypes were factor V wild-type (- - -), factor V Leiden homozygous (· · · ·), factor V Leiden heterozygous (——), and a no-template control (- · - ·). The temperature transition was programmed at 0.2 °C/s, with fluorescence acquisition at every 0.2 °C for each sample from 50 °C to 75 °C.

mutant sample shows a rapid decrease in fluorescence at 57–58 °C, whereas the wild-type transition occurs at 65–66 °C. The heterozygous mutant sample exhibits two distinct decreases in fluorescence, corresponding to both the mutation and the wild-type locuses. Change in the fluorescent signal between 75 °C and 94 °C is minimal, because all of the probe is dissociated from its target in that region.

Melting-curve analysis at the completion of 45 cycles of PCR is shown in Fig. 4. Fluorescence data are acquired as the sample is heated at 0.2 °C/s from 50 °C to 75 °C. A plot of the ratio of Cy5 to fluorescein fluorescence vs temperature (Fig. 4A) illustrates the melting properties of the fluorescein-labeled probe with each genotype. By plotting the negative derivative of the ratio with temperature vs temperature (Fig. 4B), the data show peaks for where the maximum melting occurs. When the wild-type probe melts from a wild-type target, the peak melting occurs at 66 °C. With a homozygous mutant sample, the A–C mismatch results in a melting peak 8 °C lower, at 58 °C. The heterozygous sample contains both types of target sites and generates both peaks. When a 3′-fluorescein probe was designed to match the mutation instead of the wild-type, the resulting shift in peak melting from the G–T mismatch was 4 °C (data not shown).

Evaluation of 100 human DNA samples for factor V Leiden by both the restriction digest and the fluorescence protocols yielded identical genotyping results by both methods: 60 samples were wild-type, 37 were heterozygous, and 3 were homozygous for the factor V Leiden mutation. These samples had been originally submitted for clinical factor V testing and hence were from patients already biased for thrombotic tendencies.

Discussion

Probe hybridization and resonance energy transfer were used to monitor product accumulation and to determine factor V genotype during rapid-cycle DNA amplification. In combination with rapid temperature cycling, fluorescence genotyping allows simultaneous amplification and analysis in ~30 min without any need for enzyme digestion or electrophoresis. In a closed system with no post-amplification processing, potential problems with sample tracking and end-product contamination are eliminated. Indeed, both fluorescence genotyping and conventional restriction enzyme digestion followed by electrophoresis gave identical results for the samples tested here.

When resonance energy transfer between two adjacent hybridization probes is monitored as the temperature changes during PCR, a sharp decrease in fluorescence is observed around the melting temperature of the probes *[5]*. If one fluorophore is on a primer and the other on an internal hybridization probe, the resonance energy transfer depends only on hybridization of the single internal probe *[6]*. This allows the melting characteristics of the internal probe to be monitored during amplification. Sequence alterations in the target at the probe site can be detected because the stability of the duplex and hence the melting temperature of the probe decrease when mismatches are present.

Single-base mismatches were first noted to lower the melting temperature of oligonucleotide probes by ~10 °C many years ago *[13]*. One study demonstrated that a C–A mismatch in the center of a 15-mer probe lowered the melting temperature by 8 °C *[14]*, the same amount observed here for the factor V Leiden C–A mismatch. The extent of destabilization depends on the specific mis-

match, the nearest neighbor environment, and the position of the mutation site relative to the probe. For example, the mismatch between the probe homologous to factor V Leiden and wild-type sequence (G–T) lowered the melting temperature by only 4 °C.

Melting-curve resolution is affected by the temperature transition rate. Conventional absorbance-based melting curves are usually obtained at very slow rates, ranging from 1 °C/min to 1 °C/h, to ensure equilibrium *[15]*. However, fluorescence melting curves of PCR products with SYBR™ Green I can be obtained at 0.1–1 °C/s *[7]*. As the rate is increased from 0.1 °C/s to 1 °C/s, the transition broadens and shifts ~1 °C to higher temperatures. When probe melting is monitored, genotyping is easily discerned at transition rates of 1 °C/s during amplification (Fig. 3). Higher-quality melting curves can be obtained by slowing the transition rate to 0.2 °C/s (Fig. 4). The time required for amplification and analysis depends on the type of monitoring desired and the melting-curve resolution needed. For example, 45 cycles of rapid-cycle amplification (94 °C for 0 s, 55 °C for 2 s, 74 °C for 5 s) that is not monitored at each cycle but is followed by a 0.2 °C/s melting curve can complete the genotyping in <20 min. Alternatively, monitoring the hybridization and melting for each cycle requires slower cycling and may take as long as 45 min. An interesting option for future instrumentation might use fluorescence feedback control to automatically terminate temperature cycling once a given amount of hybridization fluorescence is obtained and then initiate a melting-curve cycle for genotyping.

Whenever hybridization or restriction enzyme digestion is used to identify a particular sequence change, there is always some risk of other sequence alterations occurring at the recognition site. A silent A1692C transversion, resulting in false-positive genotyping of factor V Leiden by the restriction digest protocol, has been reported *[16]*. Although the frequency of this change is unknown, the correlation between functional and restriction digest assays suggests it is low *[17, 18]*. Most other genotyping methods for factor V Leiden would also be affected by this adjacent sequence change, including allele-specific amplification *[19]*, single-stranded conformational polymorphism *[20]*, oligonucleotide ligation *[21]*, and heteroduplex analysis *[22]*. In the fluorescence hybridization assay reported here, the resulting C–T mismatch of the A1692C transversion would lower the melting temperature of the 23-bp probe, although not necessarily to the same extent as the C–A mismatch arising from the factor V Leiden mutation. Different mismatches destabilize to different extents *[23]* and can often be distinguished. Sequencing could be used to rule out any chance of a false positive. As an alternative, a fluorescence probe designed to match the mutant sequence could be used. An apparently positive G1691A genotype with the wild-type probe could be reamplified with the mutant probe. The fully complementary G1691A allele would be easily distinguished from the A1692C allele that is mismatched to the mutant probe at 2 bases. Indeed, any base change in the region of the probe at positions other than 1691 would be mismatched at 2 bases with the mutant probe and should be easily distinguishable. A sequence change at position 1691 other than G1691A would result in single-base mismatches with both wild-type and mutant probes. Whether or not testing for potential false positives should be performed will depend on the frequency of A1692C and similar alleles. The extent of polymorphism in this area is not currently known. However, the persistence of a unique haplotype in individuals with factor V Leiden, as well as its focal Indo-European distribution, suggests that the mutation occurred only once and that the region is very conserved *[24]*.

This work was financially supported by an STTR grant from the NIH (GM51647), a Biomedical Engineering grant from the Whitaker Foundation, Idaho Technology, and Associated and Regional University Pathologists. Aspects of rapid temperature cycling and fluorescence analysis during amplification are licensed from the University of Utah to Idaho Technology pursuant to the STTR agreement. C.T.W. holds equity interest in Idaho Technology.

References

1. Bertina RM, Koeleman BPC, Koster T, Rosendaal FR, Dirven RJ, deRonde H, et al. Mutation in blood coagulation factor V associated with resistance to activated protein C. Nature 1994;369: 64–7.
2. Voorberg J, Roelse J, Koopman R, Buller H, Berends F, tenCate JW, et al. Association of idiopathic venous thromboembolism with single point-mutation at Arg^{506} of factor V. Lancet 1994;343: 1535–6.
3. Wittwer CT, Reed GB, Ririe KM. Rapid cycle DNA amplification. In: Mullis KB, Ferre F, Gibbs RA, eds. The polymerase chain reaction. Boston: Birkhauser, 1994:174–81.
4. Brown RA, Lay MJ, Wittwer CT. Rapid cycle DNA amplification for construction of competitive templates. In: Horton RM, Tait RC, eds. Genetic engineering with PCR. Wymondham, UK: Horizon Scientific Press, 1998: in press.
5. Wittwer CT, Herrmann MG, Moss AA, Rasmussen RP. Continuous fluorescence monitoring of rapid cycle DNA amplification. BioTechniques 1997;22:130–8.
6. Wittwer CT, Ririe K, Rasmussen RP. Fluorescence monitoring of rapid cycle PCR for quantification. In: Ferre F, ed. Gene quantification. Boston: Birkhauser, 1997, in press.
7. Ririe KM, Rasmussen RP, Wittwer CT. Product differentiation by analysis of DNA melting curves during the polymerase chain reaction. Anal Biochem 1997;245:154–60.
8. Wittwer CT, Ririe KM, Andrew RV, David DA, Gundry RA, Balis UJ. The Lightcycler™: a microvolume multisample fluorimeter with rapid temperature control. BioTechniques 1997;22:176–81.
9. Livak KJ, Flood SJA, Marmaro J, Giusti W, Deetz K. Oligonucleotides with fluorescent dyes at opposite ends provide a quenched probe system useful for detecting PCR product and nucleic acid hybridization. PCR Methods Appl 1995;4:357–62.
10. Kramer RK, Tyagi S. Molecular beacons: probes that fluoresce upon hybridization. Nature Biotechnol 1996;14:303–8.
11. Morrison LE. Detection of energy transfer and fluorescence

quenching. In: Kricka LJ, ed. Nonisotopic DNA probe techniques. San Diego: Academic Press, 1992:311–52.

12. Thomas SM, Moreno RF, Tilzer LL. DNA extraction with organic solvents in gel barrier tubes. Nucleic Acids Res 1989;17:5411.

13. Wallace RB, Shaffer J, Murphy RF, Bonner J, Hirose T, Itakura K. Hybridization of synthetic oligodeoxyribonucleotides to phiX174 DNA: the effect of single base pair mismatch. Nucleic Acids Res 1979;6:3543–57.

14. Zhen G, Qinghua L, Smith LM. Enhanced discrimination of single nucleotide polymorphisms by artificial mismatch hybridization. Nature Biotechnol 1997;15:331–5.

15. Hillen W, Goodman TC, Wells RD. Salt dependence and thermodynamic interpretation of the thermal denaturation of small DNA restriction fragments. Nucleic Acids Res 1981;9:415–36.

16. Liebman HA, Sutherland D, Bacon R, McGehee W. Evaluation of a tissue factor dependent factor V assay to detect factor V Leiden: demonstration of high sensitivity and specificity for a generally applicable assay for activated protein C resistance. Br J Haematol 1996;95:550–3.

17. Voelkerding KV, Lambert WU, Williams EC, Hoffman SM, Sabatini LM, Borcherding WR, et al. Optimization, comparison with functional testing for resistance to activated protein C, and establishment of cell line controls. Am J Clin Pathol 1996;106:100–6.

18. Zehnder JL, Benson RC. Sensitivity and specificity of the APC resistance assay in detection of individuals with factor V Leiden. Am J Clin Pathol 1996;106:107–11.

19. Blasczyk R, Ritter M, Thiede C, Wehling J, Hintz G, Neubauer A, Riess H. Simple and rapid detection of factor V Leiden by allele-specific PCR amplification. Thromb Haemost 1996;75: 757–9.

20. Corral J, Iniesta JA, Gonzalez-Conejero R, Vicente V. Detection of factor V Leiden from a drop of blood by PCR-SSCP. Thromb Haemost 1996;76:735–7.

21. Zotz RB, Maruhn-Debowski B, Scharf RE. Mutation in the gene coding for coagulation factor V and resistance to activated protein C: detection of the genetic mutation by oligonucleotide ligation assay using a semi-automated system. Thromb Haemost 1996; 76:53–5.

22. Bowen DJ, Standen GR, Granville S, Bowley S, Wood NAP, Bidwell J. Genetic diagnosis of factor V Leiden using heteroduplex technology. Thromb Haemost 1997;77:119–22.

23. Ikuta S, Takagi K, Wallace BR, Itakura K. Dissociation kinetics of 19 base-paired oligonucleotide-DNA duplexes containing different single mismatched base pairs. Nucleic Acids Res 1987;15:797–811.

24. Cox MJ, Rees DC, Martinson JJ, Clegg JB. Evidence for a single origin of factor V Leiden. Br J Haematol 1996;92:1022–5.