multiple epitopes *(1)*. The cause of the increase in OVX1 on storage of blood at room temperature is uncertain, but one possibility is that cleavage of the antigen occurs, releasing smaller fragments, each with multiple OVX1 epitopes.

Different cutoffs at 7.2, 10.5, or 12.1 kilounits/L have been used in clinical studies *(2, 4)*. The present findings, therefore, are clinically relevant because the assay range is narrow and the effect we have noted can cause an increase of OVX1 concentrations above these cutoffs. The findings have important implications for the collection of samples for OVX1 analysis by radioimmunoassay and for our ongoing ovarian cancer screening trial. It is clear that samples must be collected in plain tubes and the serum either separated immediately or the sample kept at 4 °C until centrifugation is performed. Unfortunately, our screening trial is based on postal transport of blood samples and immediate centrifugation is not possible. We therefore will not be able to incorporate OVX1 measurement into the study protocol unless a method for stabilizing the antigen during transport at room temperature is identified. Fortunately, repetitive freezing at −20 °C and thawing did not significantly affect OVX1 concentrations. This result is reassuring and suggests that earlier OVX1 studies based on serum samples that were separated and stored immediately are likely to be valid even if the samples analyzed had been frozen and thawed on several occasions.

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Rapid Detection of Prothrombotic Mutations of *Prothrombin* (G20210A), *Factor V* (G1691A), and *Methylenetetrahydrofolate Reductase* (C677T) by Real-Time Fluorescence PCR with the LightCycler, *Nicolas von Ahsen,* Ekkehard Schütz, Victor William Armstrong, and Michael Oellerich* (Department of Clinical Chemistry, Georg-August-University, Robert Koch Strasse 40, 37075 Goettingen, Germany; * author for correspondence: fax 49-551-39-2955, e-mail nahsen@gwdg.de)

Analytical procedures for the identification of point mutations in genomic DNA are finding increasing application in the clinical laboratory. As the demand for these analyses grows, so does the need for rapid, reliable, and easy methods to detect known point mutations. Prothrombotic mutations can be found in almost 25% of unselected patients referred for thrombophilia workup *(1)*. These include the *factor V* (G1691A), *prothrombin* (G20210A), and *methylenetetrahydrofolate reductase (MTHFR;* C677T) mutations *(2–4)*. Recently, methods have been published for genotyping both the *factor V (5)* and the *MTHFR (6)* mutations by rapid cycle PCR using the LightCycler™ (Roche Molecular Biochemicals). We describe here a procedure for genotyping the *prothrombin* mutation using the LightCycler. In addition, we have modified the PCR methods to perform all three PCR analyses in parallel, using the same program on the LightCycler. When this method is combined with a rapid DNA extraction, results can be obtained within 60 min after a whole blood sample is received.

The appearance of a specific PCR product is monitored by adjacent hybridization probes (a labeled primer may also serve as a probe), that are usually designed to bind on one amplicon strand. The 3′ end of one probe is labeled with fluorescein (FLU), whereas the 5′ end of an adjacent probe is labeled with LC-Red640 (Roche Molecular Biochemicals) as the anchor probe. When both probes hybridize in close proximity, fluorescence resonance energy transfer (FRET) occurs, producing a specific fluorescence emission of LC-Red as a result of FLU excitation. Increasing the temperature during fluorescence reading yields a temperature/fluorescence curve from which the melting point of the probe can be derived. When the appropriate conditions are chosen, the mismatch under the detection probe caused by a single point mutation leads to a substantial decrease in the melting point of the probe.

For *prothrombin* (GenBank accession nos. M17262 and M33691) genotyping, a new primer set was constructed that gave superior amplification to previously published primers. The forward primer was Fac2for 5′-CCG CTG GTA TCA AAT GGG-3′, and the reverse primer was Fac2rev 5′-CCA GTA GTA TTA CTG GCT CTT CCT G-3′. The mutation site is covered by a wild-type complementary detection probe: Fac2wt 5′-CTC AGC GAG CCT CAA TG-3′FLU, labeled with fluorescein as indicated (Fig. 1A). The adjacent anchor probe Fac2anchor 5′-LC-Red640-TCC CAG TGC TAT TCA TGG GC-3′-PHO (Fig. 1A) is 5′ labeled with the LC-Red640 dye. If these probes lie adjacent to each other on a DNA strand, FRET occurs and the fluorescence is detected by the LightCycler. In this approach, PCR amplification and detection occur in the same closed tube in ~40 min.

Oligonucleotides were synthesized by standard phosphoramidite chemistry. The 3′ end of the Fac2anchor probe was phosphorylated to prevent probe elongation by the Taq polymerase. LC-Red640-*N*-hydroxysuccimide ester was linked with the respective oligonucleotide via an amino linker and purified by HPLC.

Factor V primers and probes were used and synthesized according to Lay and Wittwer *(5)*, with the only modifications being the reverse primer concentration and that LC-Red640 dye was used instead of Cy5. In brief, the

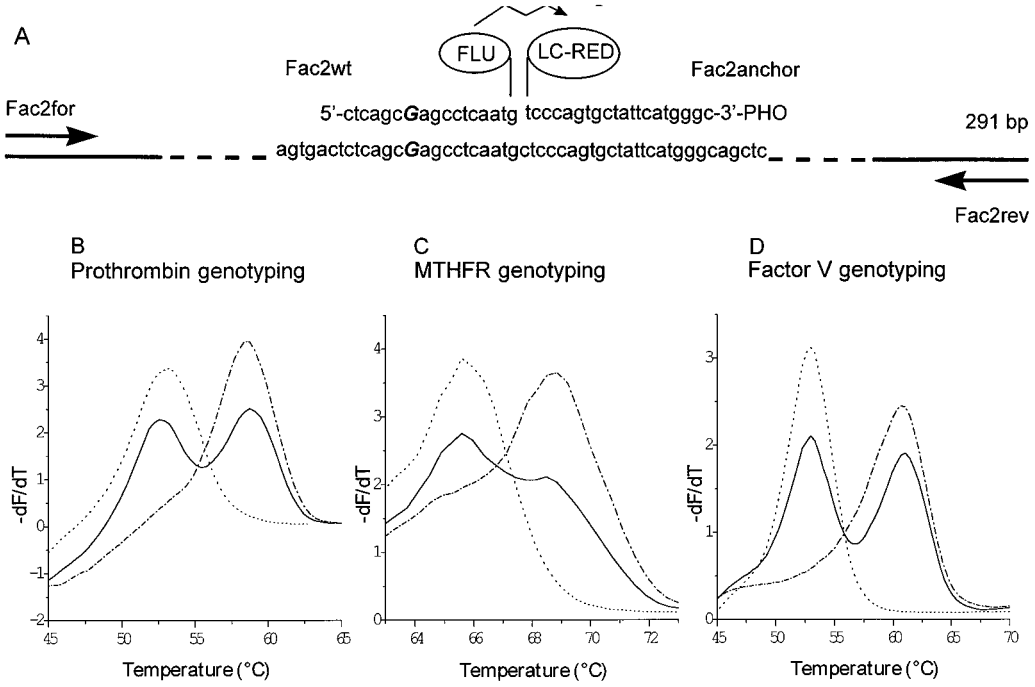

Fig. 1. Schematic of the amplified 291-bp product of the *prothrombin* gene (*A*) and melting curves for genotyping of *prothrombin* (*B*), *MTHFR* (*C*), and *factor V* (*D*).

(*A*), probes (with the fluorophores attached) compatible with the wild-type DNA sequence are drawn above the sense strand. The site of the G20210A mutation is indicated by ***G***. When both probes are hybridized, FRET occurs over the 1-bp gap. The primer and probe set used is novel. (*B*), *prothrombin* genotyping shows a homozygous wild type and a heterozygous and a homozygous mutation. (*C*), *MTHFR* genotyping shows a homozygous wild type and a heterozygous and a homozygous mutation. (*D*), *factor V* genotyping shows a homozygous wild type and a heterozygous and a homozygous mutation. Contamination and water controls were negative in all examples (data not shown). The empirical melting points in the respective PCRs are ~59 °C for the *prothrombin* wild type and 53 °C for the mutation; 69 °C for the *MTHFR* wild type and 66 °C for the mutation; and 62 °C for the *factor V* wild type and 53 °C for the mutation. (————), heterozygous mutation; (– – – – –), homozygous mutation; (– - – - – - –), wild type.

sequences were: F5for, 5′-TAA TCT GTA AGA GCA GAT CC-3′; F5rev, 5′-TGT TAT CAC ACT GGT GCT AA-3′; and F5wt-probe, 5′-AAT ACC TGT ATT CCT CGC CTG TC-3′-FLU. For *MTHFR* genotyping, the following primers and probes were used *(6)*: MTHFR-for, 5′-TGA AGG AGA AGG TGT CTG CGG G A-3′; MTHFR-rev, 5′-AGG ACG GTG CGG TGA GAG TG-3′; and MTHFR-probe, 5′-AGC TGC GTG ATG ATG AAA TCG GCT CC-3′-FLU. The underlined T indicates the position of a thymidine amino modifier to which the LC-Red640 dye is linked, and FLU indicates the 3′ fluorescein.

Genomic DNA was extracted in <10 min by a simple method according to Rudbeck and Dissing *(7)*. In brief, 5 μL of anticoagulated blood was added to 1 mL of PCR-grade water in a 1.5-mL microcentrifuge tube. The tube was centrifuged immediately (12 000*g* for 1 min), and the supernatant was removed. The remaining leukocyte pellet was lysed by the addition of 20 μL of 0.2 mol/L NaOH, vortex-mixed thoroughly, and incubated for 5 min. Neutral pH was restored by the addition of 180 μL of 0.04 mol/L Tris buffer, pH 7.5. The DNA solution could be stored at 4 °C for at least 4 weeks. Neither the choice of anticoagulant (EDTA, heparin, or citrate) nor the freezing of samples before DNA isolation affected the method or inhibited later PCR amplification.

PCR reactions were performed in a final volume of 10 μL in the LightCycler glass capillaries. The reaction mixture consisted of 1 μL of DNA solution, 0.5 U of Taq DNA polymerase (Life Technologies), 1 μL of 10× PCR buffer (Life Technologies), 0.2 mmol/L each dNTP (Boehringer Mannheim), 2.5 mmol/L $MgCl_2$, 500 mg/L bovine serum albumin (New England BioLabs), and 50 mL/L dimethyl sulfoxide (Sigma). Primers were added at the following concentrations: for the *prothrombin* PCR, 0.5 μmol/L Fac2for, 0.5 μmol/L Fac2rev, 0.1 μmol/L Fac2wt, and 0.3 μmol/L Fac2anchor; for the *factor V* PCR, 0.5 μmol/L F5for, 0.5 μmol/L F5rev, and 0.1 μmol/L F5wt-probe; and for the *MTHFR* PCR, 0.5 μmol/L MTHFR-for, 0.1 μmol/L MTHFR-rev, and 0.1 μmol/L MTHFR-probe. PCR-grade water was added to a final volume of 10 μL. Because of the small volumes, we worked with master mixes. The reaction mixtures can be prepared in larger quantities and frozen in aliquots.

Each run included a heterozygous DNA control, a contamination control from the DNA preparation, and a water control. The genotyping of control DNA was performed by restriction fragment length polymorphism methods *(2–4)*. The cycling conditions are shown in Table 1 and allow all three PCRs to be carried out in parallel in one analytical run.

Fig. 1, B–D show typical results for genotyping with these methods. All samples were genotyped in the same assay. Successful amplification is evident from the appearance of specific fluorescence and the display of de-

Table 1. PCR program for parallel amplification of *prothrombin*, *factor V*, and *MTHFR* fragments with consecutive melting curve analysis for mutation detection.

Cycles	Temperature, °C	Time, s	Ramp rate, °C/s	Acquisition
1	95	30	20	None
55×	94	0	20	None
	50	5	20	Single[a]
	72	5	20	None
1	94	15	20	None
1	40	20	20	None
1	75	0	0.1	Continuous

[a] This acquisition may be omitted if no online PCR is wanted.

rived melting curves. The typical melting curve pattern with a probe compatible with the wild-type DNA sequence is a single melting peak at a characteristically high temperature. In cases with homozygous mutations, there is a mismatch under the wild-type DNA-compatible probe, which leads to strand instability and consecutive earlier melting. The result is a single melting peak at a characteristically lower temperature. Accordingly, patients with heterozygous mutations show two melting peaks. Our approach produces melting points 3 to 4 °C lower for the *factor V* product than those reported by Lay and Wittwer *(5)*. This might be caused by the inclusion of 50 mL/L dimethyl sulfoxide in our amplification mixture. Neither the use of LC-Red640 instead of Cy5 dye nor our amplification mixture had a comparable effect on the melting behavior of the *MTHFR* product.

All mutation detection methods not based on sequencing may give misleading results if there are other base exchanges in the vicinity of the mutation of interest. With this hybridization-based method, any other mutation under the wild-type-compatible probe will also produce a mismatch with a typical melting curve. Such mutations are extremely rare, and it is unlikely that a melting curve of such a base exchange will be identical to that of the one being analyzed or the wild type. This issue has been discussed *(5)* for a rare silent A1692C mutation in the *factor V* gene *(8)*. Lay and Wittwer *(5)* reasoned that the A-C mismatch is likely to be distinguished from the G-A Leiden mutation because it does not destabilize the strand to the same extent. All cases found to have unusual melting curves with this approach should, therefore, be further clarified either by sequencing or by use of a mutation complementary probe.

In conclusion, the LightCycler offers the possibility of parallel detection of three mutations that are often screened in cases with thrombophilia. This approach is a very rapid and convenient, and therefore economic, alternative to other methods described for the detection of prothrombotic mutations.

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High-Throughput Measurement of Oxidizability of Low-Density Lipoproteins Suitable for Use in Clinical Trials,[1] *Thomas G. Cole* and Nilima Parikh* (Core Laboratory for Clinical Studies, Department of Medicine, Washington University School of Medicine, Box 8046, 660 S. Euclid Ave., St. Louis, MO 63110; * author for correspondence: fax 314-362-4782, e-mail Thom@imgate.wustl.edu)

The susceptibility of lipoproteins to oxidation is thought to be a critical step in a complex process culminating in arterial lipid deposition; however, the role of the oxidizability of lipoproteins in atherosclerosis has not been demonstrated in any large-scale clinical trials *(1–4)*. Although several measurements of the oxidizability of LDL have been developed, the most commonly used method is the measurement of the rate of formation of conjugated hydroperoxides of polyunsaturated fatty acids in response to exposure to copper ions, which leads to the formation of conjugated dienes (CDs) *(5)*. Three major parameters are used to describe the oxidizability of LDL by the CD assay: (*a*) the lag time (LT) during which lipoprotein-associated antioxidants are consumed, thereby sparing the LDL lipids from oxidation; (*b*) the maximum rate of CD formation (Max V); and (*c*) the total amount of CD formed (ΔCD).

In practice, the CD assay requires the sequential measurement of absorbance at 234 nm in a spectrophotometer over a prolonged period of time, often >3 h. Because the automated cell carrier of most spectrophotometers holds a maximum of only six cuvettes, the analysis of large numbers of specimens, such as for large-scale clinical trials, is difficult and time-consuming. In addition, the analysis of replicate specimens or the inclusion of quality-control materials generally is precluded by the small number of specimens that can be analyzed simulta-

[1] This work was presented previously in abstract form: Cole TG, Parikh N. High-throughput analysis of oxidizability of LDL suitable for use in clinical studies [Abstract]. Clin Chem 1998;44:A84.