Optimization of Apolipoprotein(a) Genotyping with Pulsed Field Gel Electrophoresis

Pieter H. Griffioen,1 Louwerens Zwang,1 Ron H.N. van Schaik,1 Henk Engel,2 Jan Lindemans,1* and Christa M. Cobbaert3

Background: Increased lipoprotein(a) [Lp(a)]4 has been established as one of the major risk factors for atherosclerosis (1, 2). Lp(a) serum concentrations were shown to be inversely correlated with the size of the apolipoprotein (a) [apo(a)] component (3, 4), which is determined mainly by the number of Kringle IV repeats (5). apo(a) gene size polymorphisms can be detected with pulsed field gel electrophoresis (PFGE) (5). In currently described protocols, DNA is isolated from 10 mL (6) or 16 mL (5) of fresh EDTA blood, and the apo(a) genotype is determined using radioactive (5) or digoxigenin-labeled (6) probes. In ongoing clinical studies of the determinants of coronary artery disease, we wanted to use either tiny EDTA blood samples or frozen buffy coats as the starting material for the determination of apo(a) gene size. Established procedures were adjusted and subsequently tested for accuracy and reproducibility. Modifications were made with respect to the amount and type of starting material, the efficiency of digestion, the sensitivity of chemiluminescent detection, and the practicability. We verified the accuracy of the modified method using plugs containing DNA for which the apo(a) genotype had been established in advance by Trommsdorff et al. (6). We tested the reproducibility of the modified method by running a self-manufactured control sample on each gel throughout the study. We concluded that the proposed modifications enhance the sensitivity, reproducibility, and practicality of the technique for apo(a) genotyping.

Materials and Methods

DNA specimens and patient sera
Fresh EDTA blood and serum were collected from 24 unrelated healthy Dutch subjects. Fresh-frozen sera were

1 University Hospital Rotterdam Dijkzigt, Department of Clinical Chemistry, P.O. Box 2040, 3000 CA Rotterdam, The Netherlands.
2 St. Sophia Hospital, Department of Clinical Chemistry, P.O. Box 10400, 8000 GK Zwolle, The Netherlands.
3 Hospital De Baronie, Department of Clinical Chemistry, P.O. Box 90157, 4800 RL Breda, The Netherlands.
*Author for correspondence. Fax 31-10-4367894; e-mail lindemans@ckcl.azr.nl.
Received October 15, 1998; accepted March 16, 1999.

4 Nonstandard abbreviations: Lp(a), lipoprotein a; apo(a), apolipoprotein a; and PFGE, pulsed field gel electrophoresis.
stored at −70 °C for a maximum of 5 months, whereas fresh EDTA blood was used immediately for DNA isolation. Buffy coat samples and sera that had been stored frozen for 3–5 years at −20 °C and −70 °C, respectively, were collected from the Rotterdam Elderly Study (468 samples from unrelated Caucasians) (7). Low-melting point agarose plugs (n = 8) that had been genotyped for apo(a) by Trommsdorff et al. (6) were used for an accuracy check. Cells within these plugs had been isolated and digested with proteinase K, and the plugs were used immediately for PFGE. These plugs had been stored in 0.5 mol/L EDTA for 1.5 years prior to their use for PFGE.

SERUM apo(a) QUANTIFICATION
Serum apo(a) protein was measured using a commercially available kit (Mercodia). This method is a solid-phase two-site immunoradiometric assay for which serum samples were diluted 42-fold with a pretreatment solution. Subsequently, apo(a) was determined in duplicate, using monoclonal antibodies directed against separate antigenic determinants on the apo(a) molecule. The procedure was performed according to the instructions of the manufacturer.

apo(a) GENOTYPING WITH PFGE
Isolation of genomic DNA from fresh blood samples and frozen buffy coats. The procedure was based on the method described by Siraganian and Hook (8). A 3-mL aliquot of fresh EDTA blood, 0.75 mL of dextran/glucose [0.77 mmol/L dextran (M, 77 800; Sigma Chemical Co.) and 166.5 mmol/L glucose in 9 g/L NaCl], and 60 μL of 0.5 mol/L EDTA, pH 8.0, were mixed in a 10-mL sterile conical tube (Greiner Laborteknik). The tube was placed in a diagonal position of 45 degrees for 15 min and subsequently upright for 60 min for sedimentation of erythrocytes. The leukocytes remained floating in the plasma. The plasma was transferred to a new sterile 10-mL tube and centrifuged (415 g for 15 min) at room temperature. The supernatant was decanted, and the pellet was suspended in 10 mL of phosphate-buffered saline, pH 7.4. After the sample was centrifuged at 223g for 7 min at room temperature, the leukocytes were resuspended in 3 mL of phosphate-buffered saline and a cell count (Sysmex NE-8000; TOA Medical Electronics) was performed. After recentrifugation at 223g for 7 min at room temperature, the leukocytes were resuspended in 25 mmol/L EDTA in 9 g/L NaCl to a concentration of 3 × 10⁷ cells/mL. The suspension was mixed with two volumes of 10 g/L low-melting point agarose (Incert agarose; FMC Bioproducts), and the mixture was aliquoted into disposable plug molds (Bio-Rad). One plug contained ~7.5 × 10⁶ cells.

For the frozen buffy coats, 250 μL of each sample was transferred into a 10-mL sterile conical tube (Greiner), and 10 mL of lysis buffer (20 mmol/L Tris, 10 mmol/L EDTA, pH 8) was added. The tubes were centrifuged at 3000g for 5 min at room temperature, and the supernatant was decanted. The sediment was resuspended in the remaining lysis buffer, 200 μL of 10 g/L low-melting point agarose was added, and the mixture was poured into disposable plug molds.

After isolation, the plugs were treated with 0.5 g/L proteinase K (Merck) at 55 °C for 48 h, treated with phenylmethylsulfonyl fluoride, and stored in 0.5 mol/L EDTA, pH 8.0, according to the protocol of Trommsdorff et al. (6).

Digestion and PFGE. Plugs were washed twice with Tris-EDTA buffer (10 mmol/L Tris, 1 mmol/L EDTA, pH 7.5) and once with Kpn1 digestion buffer (10 mmol/L Tris, 10 mmol/L MgCl₂, pH 7.5) for 30 min. Digestion was carried out with 2 × 60 U of Kpn1 in a buffer volume of 75 μL in the presence of bovine serum albumin (4 h at 37 °C in 20 g/L DNA-quality albumin; Boehringer Mannheim). DNA was size separated (PFGE) with a fragment separation range of 50–175 kb, using the machine-calculated autotreshold according to the protocol of Trommsdorff et al. (6). Further electrophoresis was performed with the gel submerged in buffer (50 mmol/L Tris, 50 mmol/L boric acid, 1 mmol/L EDTA) at 14 °C, and the run time was set at 24 h. As a control, a λ 48.5-kb DNA ladder was used (CHEF Mapper PFGE system, CHEF DNA size standards, Lambda ladder; Bio-Rad). The gel was stained with ethidium bromide (10 g/L), photographed, and incubated ~2 min in 10 mL of 4 mol/L NaOH to denature the DNA. The DNA fragments were blotted (in 0.4 mol/L NaOH overnight at room temperature) onto a nylon membrane (Boehringer Mannheim) by reversed capillary blotting using a Turboblotter system (Schleicher & Schuell). The DNA was fixed by baking the nylon membrane for 30 min at 120 °C.

Probe manufacturing, hybridization, and chemiluminescent detection. The probes used for hybridization, directed against either λ DNA or apo(a) Kringle IV type 2 repeats, are listed in Table 1. All probes were prepared with a PCR-digoxigenin-labeling kit (Boehringer Mannheim). Template DNA for the apo(a) probes was prepared by PCR reaction on genomic DNA with all four apo(a) primer sets. PCR was performed in a 110-μL reaction volume with 10 μL of buffer (0.5 mol/L KCl, 0.1 mol/L Tris, 25 mmol/L MgCl₂, pH 9.0, containing 1 g/L gelatin and 10 mL/L Triton X-100), 20 μL of 1 mmol/L dNTP, 1.2 μL of primer (50 pmol/μL Lp1–Lp4), 0.3 μL of Promega Taq (5 U/μL), 67 μL of sterile water, and 10 μL of genomic DNA. The reaction mixture was overlaid with mineral oil. The PCR conditions were as follows: first denaturation step, 7 min at 95 °C; followed by 40 cycles of denaturation for 45 s at 95 °C, annealing for 1 min at 60 °C, and elongation for 2 min at 72 °C. The final elongation step was 7 min at 72 °C.
Table 1. Primer sequences and locations.^[a]

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
<th>Location</th>
<th>Fragment size, kb</th>
</tr>
</thead>
<tbody>
<tr>
<td>apo(a) gene</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lp2 F</td>
<td>5’-GAA CTA CTG CAG GAA TCC-AGA TGC TGT GGC-3’</td>
<td>4148-4177</td>
<td>217</td>
</tr>
<tr>
<td>Lp2 R</td>
<td>5’-CAT CCC AGC ATC GAA GCG TGT AGA TGT CTG-3’</td>
<td>4335-4364</td>
<td></td>
</tr>
<tr>
<td>Lp3 F</td>
<td>5’-GCT TCC ACT TCC TGA AGG TGG CTT TGT CCT-3’</td>
<td>501-530</td>
<td>480</td>
</tr>
<tr>
<td>Lp3 R</td>
<td>5’-AAG ATG GCT ACA GTC TGC TGG CTC CCA ACT-3’</td>
<td>951-980</td>
<td></td>
</tr>
<tr>
<td>Lp4 F</td>
<td>5’-CTA AGT CCA TTG CTT CCT CAG CTA GTC GGT ATC-3’</td>
<td>1481-1510</td>
<td>480</td>
</tr>
<tr>
<td>Lp4 R</td>
<td>5’-GTG TGT AGT AGA ACT TGT GAG TTT CTG GCG-3’</td>
<td>1931-1960</td>
<td></td>
</tr>
<tr>
<td>Lp5 F</td>
<td>5’-CCA TGG CAT GAT AAT GCA TAT TGT GCA CGT-3’</td>
<td>2461-2490</td>
<td>480</td>
</tr>
<tr>
<td>Lp5 R</td>
<td>5’-TAA TAC CCA AAA CCT CAG AGT TCA AAC CTT-3’</td>
<td>2911-2940</td>
<td></td>
</tr>
<tr>
<td>Lambda gene</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lambda F</td>
<td>5’-GCG TCA CAG TAA TTA CGG TGC TGC GCT GGA-3’</td>
<td>4996-5025</td>
<td>225</td>
</tr>
<tr>
<td>Lambda R</td>
<td>5’-ACT GCA GCC TCG GTA TCC AGC ACA ACC TGC-3’</td>
<td>5191-5220</td>
<td></td>
</tr>
</tbody>
</table>

* The derived probes are mixed together in one hybridization mixture.

Template DNA was purified using microspin columns (QIAquickspin PCR Purification kit; Qiagen) according to the manufacturer’s protocol. With this template solution, a PCR reaction was performed in a 110-μL reaction mixture containing 1.5 digoxigenin-dUTP: dTTP; 10 μL of 0.5 mol/L KCl in 0.1 mol/L Tris, pH 8.3; 5 μL of synthesis mix (2 mmol/L dATP/dCTP/dGTP, 1.3 mmol/L dTTP, 0.7 mmol/L digoxigenin-11-dUTP), 5 μL of 2 mmol/L dNTP stock solution, 1.2 μL of primer (50 pmol/μL), 1.5 μL of Taq expand mix (1 U/μL), 76 μL of water, and 10 μL of template DNA. The reaction mixture was overlaid with mineral oil. The cycle conditions were as follows: a first denaturation step for 2 min at 95°C; followed by 10 cycles of denaturation for 10 s at 95°C, annealing for 30 s at 60°C, and elongation for 2 min at 72°C; and 20 cycles of denaturation for 10 s at 95°C, annealing for 30 s at 60°C, and elongation for 2 min at 72°C, with a 20-s time extension for the elongation step in each cycle of the last 20 cycles. The final elongation step was for 7 min at 72°C. After electrophoresis of 10 min after addition of the substrate). Three exposures were made of each gel, with exposure times of approximately 15, 30, and 45 min.

**Reproducibility and precision.** We prepared a control sample for imprecision studies by combining leukocytes (fresh samples) of four selected heterozygous volunteers, which yielded eight apo(a) fragments that covered the entire fragment size range of 72–233 kb. The combined cell count of the control sample was increased to 2 × 10^7/mL in the plugs to ensure adequate detection signals for all eight apo(a) fragments. Plugs were digested with proteinase K and stored (4°C) in 0.5 mol/L EDTA, pH 8. DNA digestion was performed with 2 × 120 U of Kpnl before electrophoresis.

**Calculation of the apo(a) genotype**

**Kpnl digestion** of genomic DNA yields apo(a) fragments containing all Kringle IV (type 2) repeats as well as four nonrepeating Kringle types (1, 3, 4, and 5). The size of the fragments after these four nonrepeating Kringle (29,932 kb) are subtracted equals the number of Kringle IV type 2 repeats. Five Kri[ngle IV repeats (types 6–10) remain in the genomic DNA; therefore, the apo(a) genotype is calculated by adding the repeating type 2 Kringle to all nonrepeating Kringles (nine Kringles). The films were scanned, and final quantification was performed using Gelscompar 3.0/4.0 (Applied Maths BVBA). The λ DNA marker (48.5-kb Lambda ladder; Bio-Rad) was used as a reference for fragment size calculation as well as for correction of gel-to-gel variation by the Gelscompar normalization program. All Kpnl fragments were identified by interpolation of migration distances, using an exponential curve fitting. Calculations of the apo(a) genotype were based on the work by Lackner et al. (10) and the information presented by the National Center for Biotechnology Information GenBank database (GenBank name, HUMAPOAKIV; GenBank accession no. L14005; se-
The formulas are shown below, with one Kringle IV repeat representing 5.533 kb:

\[
\text{Kringle IV (type 2) repeats} = \left(\frac{\text{fragment size (kb)}}{5.533 \text{ (kb)}}\right) - 29.932 \text{ (kb)}
\]

\[
\text{apo(a) genotype} = \text{Kringle IV (type 2) repeats} + 9
\]

**Results**

**Optimization of the apo(a) Genotyping Procedure**

The dextran procedure enables the isolation of all leukocytes from fresh blood samples; the isolation of all leukocytes from frozen buffy coats was accomplished by a centrifugation method. The DNA yield of both isolation methods allowed for comparable analytical sensitivity (Fig. 1). When the centrifugation method was used on frozen EDTA whole blood, a less intense detection signal was found despite the fact that a comparable number of leukocytes \((7.5 \times 10^9)\) were present in the plug (Fig. 1A, lane 7). An extra wash step with digestion buffer was introduced before \(Kpn1\) digestion of isolated DNA. DNA samples digested according to existing protocols (Fig. 2A) were compared with the modified method (Fig. 2B). Digestion efficiency was increased in the modified method, as visualized by the disappearance of the high-molecular weight DNA band in the non-resolution zone. Reversed capillary blotting was used for transferring DNA from the gel to a nylon membrane. Visibly empty gels were obtained, indicating complete DNA transfer. Increased sensitivity was further accomplished by the use of four different apo(a) probes. Improved accuracy and practicality were obtained by the addition of a \(\lambda\) marker.
probe in the same hybridization mix, enabling detection on a single film (see Fig. 1; λ markers are in the outer lanes).

VALIDATION
The reproducibility of the modified PFGE method was determined with the control sample, obtained by combining leukocytes from four volunteers (as described in Materials and Methods). Kpn1 fragment size, as determined over 34 separate electrophoretic runs, was reproducible to within one or fewer Kringle IV repeats, with an SD ≤ 2.5 kb and a CV ≤ 3%. To ensure accuracy, the results were accepted only when the apo(a) genotype determination of the eight control fragments was within one Kringle IV repeat of the established value (within first electrophoretic run, n = 3). Furthermore, the accuracy of the method in relation to the absolute number of Kringle IV repeats was verified on DNA samples that were genotyped by Trommsdorff et al. (6). One of the eight samples obtained could not be genotyped because of denaturation of the DNA, and a smear was seen in the gel and on the blot. All 14 genotyped apo(a) fragments differed by no more than one Kringle IV from the results of Trommsdorff et al. (6).

RELATIONSHIP BETWEEN apo(a) GENOTYPE AND SERUM Lp(a) CONCENTRATIONS
An inverse correlation between serum Lp(a) concentrations and the number of Kringle IV repeats in either allele was found. When serum Lp(a) concentrations were plotted against the sum of the Kringle IV repeats in both alleles, a negative correlation was found (y = −22.757x + 1553.3; r = −0.442). From the frozen Buffy coat samples tested (n = 468), 93% were found to be heterozygous for the apo(a) Kringle IV repeat size.

Discussion
The rationale of this study was to improve both the sensitivity and practicality of currently described apo(a) genotyping procedures. Overall sensitivity of the procedure can be increased by optimization of each step in the procedure, starting with the leukocyte isolation. The availability of tiny blood samples or frozen Buffy coats persuaded us to search for a modified isolation procedure in which a high DNA yield allowed the reduction of sample volume. We selected a dextran method and a centrifugation method, which isolated all leukocytes from the blood samples and Buffy coats, respectively. In contrast to the isolation of mononuclear cells, this modification theoretically produces a threefold amplification of the DNA yield. Dextran accelerates sedimentation of erythrocytes by “rouleau” formation (11), and increased plasma density keeps leukocytes floating in the plasma layer. The recovery of leukocytes in the dextran isolation method was not fully reproducible between samples. It was necessary to perform a cell count after the dextran isolation to ensure the presence of 7.5 × 10⁶ cells in the plugs. Because the freezing process damages and clusters white blood cells (and DNA), a cell count could not be performed when the frozen Buffy coats were used. We started with the same sample size for all frozen Buffy coats and concentrated the isolated leukocytes (DNA) into the plugs as much as possible, which produced a certain variation in detection signal. Notwithstanding, the proposed procedure enabled us to determine apo(a) genotypes in Buffy coats that had been kept frozen at −20 °C for 3–5 years.

Traces of EDTA, which were present in the plugs after the Tris-EDTA buffer wash, captured magnesium ions, an essential cofactor of Kpn1. For complete digestion by Kpn1, it was necessary to introduce an extra wash with digestion buffer to saturate the remaining traces of EDTA with magnesium. With this modification, an optimal magnesium concentration in the Kpn1 digestion reaction was ensured and led to efficient cutting of the genomic DNA. A higher yield of each separated apo(a) fragment was obtained, and a stronger detection signal and improved sensitivity were found.

Reversed capillary blotting enabled easy-to-perform, time-saving stack-building, which yielded highly reproducible results.

The final step taken to increase the detection signal was the combination of four different apo(a) probes, which enhanced the detection signal. The probes were designed to hybridize within one Kringle IV type 2A repeat. Hybridization sites are distributed over the length of the two introns and two exons. However, the detection signal may increase only proportionally because the increase in the number of apo(a) probes that can be hybridized is limited by steric hindrance from the incorporated digoxigenin label in the probes. Integrated detection of λ markers and unknown samples was accomplished by the addition of a labeled λ marker probe to the hybridization mixture of the apo(a) probes. This procedure allowed for single film detection, which improved accuracy and practicality because markers and unknown samples were visible on the same film. The sensitivity of detection (with all probes) was influenced most strongly by the amount of digoxigenin label incorporated in the probes by PCR labeling. From a standardization viewpoint, it is therefore essential that the amount of labeled probe administered to the hybridization mix is kept at a constant concentration throughout the entire investigation.

Published procedures (5, 6) indicate that discrimination should be possible for single apo(a) Kringle IV repeats. This is in agreement with our findings. Furthermore, the modified method was reproducible to within one or fewer Kringle IV repeats. If fragments of the control sample differ by more than one Kringle IV repeat from the assigned value, repeated analysis of the samples is required. This happened only once throughout the whole investigation.

Analogously to Lackner et al. (5), we found an inverse relationship between serum Lp(a) concentration and the
number of Kringle IV repeats in either allele. In our epidemiological study, a heterozygosity index of 93% was found, which is comparable to the 94% of Lackner et al. (10). The results of our epidemiological study of Caucasians, in which we used buffy coats stored frozen at −20 °C for several years, were comparable to the results of the studies using fresh EDTA blood samples (5, 10).

In summary, the proposed modifications improved the apo(a) genotyping procedures described to date and made them suitable for repeated analysis with small blood sample volumes and with frozen buffy coats. Because the DNA isolation, Kpn1 digestion, hybridization, and detection steps were optimized, a difference of one or fewer Kringle IV repeats in apo(a) could be detected. When 3 mL of EDTA blood or 250 µL of buffy coat is used as the starting material, adequate sensitivity can be achieved with the use of four different apo(a) probes. These modifications, when used together, make the apo(a) genotyping procedure suitable and practical for large-scale apo(a) genotyping studies such as large epidemiological projects and clinical trials.

This work was funded by the Dutch Heart Foundation (Grant 94.032). We thank Dr. Y.Y. van der Hoek for arranging the exchange of the DNA plugs with defined apo(a) genotypes. We thank Prof. Dr. G. Utermann and Prof. Dr. H.G. Kraft of the Institute for Medical Biology and Human Genetics, Innsbruck, Austria, for the apo(a) genotyping of the plugs and for allowing us to use the data for the accuracy check.

References