

error never exceeded 1%) but to real composition differences among the smallest samples.

- The total sum of the squares can be divided into the contributions attributable to experimental error, sampling site, and depth. The *F*-test at 5% probability applied to sampling and error variance gave a significant value only for needle biopsies divided into four parts (95th percentiles of the reference *F* distributions, $F_{17,17} = 2.29$; $F_{17,34} = 1.94$; and $F_{17,51} = 1.87$). On the other hand, the *F*-test relating to the effect of depth afforded clearly significant values (95th percentiles of the reference *F* distributions, $F_{1,17} = 4.46$; $F_{2,34} = 3.28$; and $F_{3,51} = 2.80$), highlighting that depth is the main factor determining variability among samples, as pointed out by the remarkable decrease in iron concentrations from the surface to the interior of the liver shown in Fig. 1B.

Our data clearly show that the iron concentration in a single liver needle biopsy, weighing ~5 mg and 2 cm long, may be considered representative of the mean HIC with a relative standard deviation of 15%. This value depends on the analytical procedure and the uneven iron distribution. Therefore, when only one portion of the needle biopsy core is used, the reduction in weight from 5 mg to 1 mg does not significantly increase the error attributable to the analytical procedure, although the measure is far less significant because the subcapsular portion of the needle biopsy contains much more iron than the inner part. In cases where the determination of HIC is important for monitoring iron chelation therapy, a random subdivision of the liver biopsy is to be avoided; if an entire liver biopsy is not available for chemical analyses, we suggest that the subcapsular and the deepest part of the biopsy be used as a unit to minimize the errors attributable to a casual choice of sample. Finally, we propose a consensus conference on the methods of trace element determination in needle biopsies. In our opinion, the standardization of these procedures may lead to measured HIC values more representative of the true HIC and much more useful for clinical purposes.

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References

1. Olivieri NF. The β -thalassemias. *N Engl J Med* 1999;341:99–109.
2. Cao A. 1993 William Allan Award Address. *Am J Hum Genet* 1994;54:397–402.
3. Ambu R, Crisponi G, Sciort R, Silvagni R, Nurchi VM, Faa G, et al. Uneven hepatic iron and phosphorous distribution in β -thalassemia. *J Hepatol* 1995;23:544–9.
4. Faa G, Sau F, Abbruzzese P, Silvagni R, Nurchi VM, Crisponi G, et al. Uneven cardiac iron distribution in β -thalassemia. *Cardiovasc Pathobiol* 1997;3/4:1–7.
5. Faa G, Crisponi G. Iron chelating agents in clinical practice. *Coord Chem Rev* 1999;184:291–310.
6. Crisponi G, Nurchi VM, Silvagni R, Faa G. Oral iron chelators for clinical use. *Polyhedron* 1999;18:3219–26.
7. Brittenham GM, Cohen AR, McLaren C, Martin MB, Griffith PM, Nienhuis AW,

- et al. Hepatic iron stores and plasma ferritin concentration in patients with sickle cell anemia and thalassemia major. *Am J Hematol* 1993;42:81–91.
8. Angelucci E, Baronciani D, Lucarelli G, Baldassarri M, Galimberti M, Giardini C, et al. Needle liver biopsy in thalassemia: analyses of diagnostic accuracy and safety in 1184 consecutive biopsies. *Br J Haematol* 1995;89:757–61.
 9. Tricta F, Spino M. Assessment of iron chelation in thalassemia: experience with Deferiprone. 9th International Conference on Oral Chelation, March 25–28, Hamburg, Germany, 1999:22.
 10. Galanello R, De Virgiliis S, Giagu N, Cao A, Mancosu MG, Faa G. Evaluation of iron overload in thalassemia intermedia. 9th International Conference on Oral Chelation, March 25–28, Hamburg, Germany, 1999:29.
 11. Bassett ML, Halliday JW, Powell LW. Value of hepatic iron measurements in early hemochromatosis and determination of the critical iron level associated with fibrosis. *Hepatology* 1986;6:24–9.
 12. Faa G, Nurchi V, Demelia L, Ambu R, Parodo G, Congiu T, et al. Uneven hepatic copper distribution in Wilson's disease. *J Hepatol* 1995;22:303–8.
 13. Aragoni MC, Crisponi G, Nurchi VM, Sciort R, Ambu R, Faa G, et al. Chemometric methods applied to an ICP-AES study of chemical element distributions in autopsy livers from subjects affected by Wilson and β -thalassemia. *J Trace Elem Med Biol* 1995;9:215–21.
 14. Faa G, Sciort R, Farci AMG, Callea F, Ambu R, Congiu T, et al. Iron concentration and distribution in the newborn liver. *Liver* 1994;14:193–9.
 15. Villeneuve JP, Bilodeau M, Lepage R, Cote J, Lefebvre M. Variability in hepatic iron concentration measurement from needle biopsy specimens. *J Hepatol* 1996;25:172–7.
 16. Emond MJ, Bronner MP, Carlson TH, Lin M, Labbe RF, Kowdley KV. Quantitative study of the variability of hepatic iron concentrations. *Clin Chem* 1999;45:340–6.
 17. Massart DL, Vandeginste BGM, Deming SN, Michette Y, Kaufman L. Evaluation of sources of variation in data. Analysis of variance. In: *Chemometrics: a textbook*. Amsterdam: Elsevier, 1988:59–74.
 18. Crisponi G, Nurchi VM, Silvagni R, Lubinu G, Ambu R, Faa G, et al. Critical evaluation of analytical procedures for trace-element determinations in human liver using ICP-AES. *Atom Spectrosc* 1995;16:73–8.

Agreement Study of Methods Based on the Elimination Principle for the Measurement of LDL- and HDL-Cholesterol Compared with Ultracentrifugation in Patients with Liver Cirrhosis, Frederic Gómez, Jordi Camps,* Josep M. Simó, Natàlia Ferré, and Jorge Joven (Centre de Recerca Biomèdica, Hospital Universitari de Sant Joan, 43201-Reus, Catalunya, Spain * address correspondence to this author at: C/. Sant Joan s/n, 43201-Reus, Catalunya, Spain: fax 34-977-312569, e-mail jcamp@grupsgs.com)

In recent years, several methods for the direct measurement of HDL-cholesterol (HDL-C) and LDL-cholesterol (LDL-C) have been introduced (1–5). These methods have been effective and inexpensive tools for the routine screening of large populations because they prevent the need for complex ultracentrifugation procedures. In addition, the direct methods for LDL-C are better than indirect calculation by the Friedewald formula because they can be used accurately in hypertriglyceridemic patients and in nonfasting subjects (4). However, it is still being debated whether these methods are also valuable under more challenging conditions than those found in the general population (4), i.e., pathological circumstances in which there are abnormalities in lipoprotein structure and/or composition.

In a recent study, we observed that three different homogeneous methods significantly undervalue HDL-C concentrations in patients with liver cirrhosis. We suggested that this could be related to the abnormal lipoprotein composition in these subjects (6, 7). A new type of

homogeneous method, based on the elimination principle (Randox Laboratories Ltd., Crumlin, UK), has been developed for measuring HDL-C and LDL-C. The manufacturer states that lipoproteins with abnormal compositions may be correctly identified and measured with these methods. The present study therefore aimed to compare the elimination methods for determining homogeneous HDL-C and LDL-C with single vertical-spin ultracentrifugation in a group of patients with liver cirrhosis.

The study was carried out on 44 control subjects and 55 cirrhotic patients. Control subjects were chosen randomly from the routine health and safety-at-work checks conducted in several companies in our area. Cirrhotic patients were diagnosed by liver biopsy and came from the outpatient clinics of the Hospital Universitari de Sant Joan de Reus. All procedures were in accordance with the ethics standards of our institution. Blood samples were drawn in the fasted state into glass tubes containing EDTA; the plasma was separated by centrifugation at 1500g for 25 min and stored at 4 °C for analytical determinations, which were performed in <3 days.

The homogeneous methods for HDL-C and LDL-C determination were used according to the manufacturer's instructions. They involved releasing the undesirable lipoprotein components by means of several components included in reagent 1 (selective ionic strength buffers, cholesterol esterase, cholesterol oxidase, and catalase). Subsequently, the desired lipoprotein fraction was released by specific surfactants in reagent 2, and cholesterol was measured by the CHOD/PAP method. The intra- and interassay CVs were <2.5% and <4.7%, respectively. Single vertical-spin ultracentrifugation was performed as published previously (8). This technique was chosen as a reference method because it minimizes *in vitro* apolipoprotein dissociation from lipoprotein particles (9), an aspect of crucial importance in patients with altered lipoprotein composition. Cholesterol in the lipoprotein fractions and in the patients' plasma was determined by standard methods (ITC Diagnostics). Plasma concentrations of apolipoprotein (apo) A-I and B were analyzed by immunoturbidimetry (Biokit) and calibrated according to the IFCC standard (10).

Liver-related tests were also measured in the plasma of all subjects by standard techniques (ITC Diagnostics). Total protein, albumin, bilirubin, alanine aminotransferase, alkaline phosphatase, and γ -glutamyltransferase were also measured in the plasma of all subjects by standard techniques. All measurements were performed on an ILab[®] 900 automatic analyzer (Instrumentation Laboratories). Results are presented as the mean \pm SD. The presence of lipoprotein X in the plasma of cirrhotic patients was tested qualitatively by agarose gel electrophoresis (11). The agreement between the homogeneous and the ultracentrifugation methods was estimated by the Bland-Altman graphical procedure (12). Statistical significance was set at $P < 0.05$.

The results of the analytical determinations are shown in Table 1. Cirrhotic patients presented with a significant decrease in total plasma cholesterol as well as in LDL-C,

intermediate-density lipoprotein-cholesterol (IDL-C), and VLDL-cholesterol (VLDL-C). These alterations were similar to those reported previously in this type of patient (13). As expected, several hepatic function indices were significantly altered.

The analytical results suggested that the cirrhosis was mainly hepatocellular because although the increase in esterified bilirubin in some patients may suggest cholestasis, alkaline phosphatase activity was never more than twice the upper reference limit and lipoprotein X was not detected in any of the patients' plasma.

Correlation and Bland-Altman plots for the elimination and ultracentrifugation methods for HDL-C and LDL-C are shown in Fig. 1. The mean difference for HDL-C measurements was 0.13 ± 0.14 mmol/L in control subjects and 0.18 ± 0.22 mmol/L in patients with cirrhosis. These differences were not related to the average HDL-C concentration. The limits of agreement were quite high, i.e., values showed a considerable degree of dispersion above or below the mean; however, the elimination method was superior to the previously published homogeneous assays, in which biases of -2.0 mmol/L were frequently observed in cirrhotic patients (7).

The mean difference for LDL-C determinations was -1.18 ± 0.46 mmol/L in control subjects, which indicates a high negative bias for the elimination method. Several authors have already observed a marked lack of agreement between the homogeneous assays and ultracentrifugation procedures in LDL-C determination. The reported biases are sometimes positive (14, 15), but they are more frequently negative (4, 5, 16–18). Some of the biases

Table 1. Summary of results.^a

	Control subjects, mean (SD)	Cirrhotic patients, mean (SD)	P
Cholesterol	5.52 (0.84)	3.97 (1.33)	<0.001
Triglycerides	1.46 (0.75)	1.22 (0.49)	NS ^b
apo A-I	1.48 (0.15)	1.35 (0.49)	NS
apo B	1.09 (0.15)	0.93 (0.25)	NS
Total protein	70 (5)	70 (8)	NS
Albumin	43 (8)	35 (9)	<0.001
ALT	0.35 (0.18)	0.85 (0.68)	<0.001
ALP	3.12 (0.72)	4.57 (1.79)	NS
GGT	0.75 (0.32)	1.71 (2.62)	NS
Total bilirubin	11 (3)	72 (7)	<0.001
Esterified bilirubin	5 (2)	28 (27)	<0.001
HDL-C (E)	1.29 (0.30)	1.22 (0.53)	NS
LDL-C (E)	2.98 (0.78)	2.09 (1.11)	<0.001
HDL-C (UC)	1.16 (0.24)	1.01 (0.50)	NS
LDL-C (UC)	3.90 (0.73)	2.12 (0.84)	<0.001
IDL-C (UC)	0.17 (0.07)	0.28 (0.28)	<0.05
VLDL-C (UC)	0.36 (0.26)	0.25 (0.25)	<0.05

^a All results are expressed in mmol/L, except for total protein, albumin, apolipoproteins (g/L); bilirubin (μ mol/L); and enzymatic activities (μ mol \cdot L⁻¹ \cdot s⁻¹).

^b NS, not significant; ALT, alanine aminotransferase; ALP, alkaline phosphatase; GGT, γ -glutamyl transferase; E, elimination method; UC, ultracentrifugation.

are very high and similar to those found in the present investigation (16, 17). Studies comparing homogeneous assays with the β -quantification of LDL have suggested an explanation of overestimation of the ultracentrifugation procedure because it includes IDL-C together with LDL-C (5, 16–18). This was not the case in the present investigation because our ultracentrifugation procedure efficiently separated IDL from LDL.

Surprisingly, the situation in patients with liver cirrhosis was very different. In these subjects, the bias was not as strongly negative as in the control group and was clearly positive at high average LDL-C concentrations. There was a direct relationship between bias and LDL-C concentrations in cirrhotic patients ($r = 0.61$; $P < 0.001$) but not in the control subjects ($r = 0.08$; P , not significant). This difference between the two groups was also observed in the correlation study, in which the regression

lines were different in control and cirrhotic subjects. The reason for this disagreement is unknown but may be related to the abnormal synthesis of lipoproteins in these patients (19). Liver diseases are known to alter lipoprotein structure and composition. VLDL and HDL are synthesized in the liver as nascent lipoproteins. Nascent VLDL is rich in apo B and poor in apo C and E. On the other hand, nascent HDL is rich in apo C and E and poor in apo A. In blood, nascent VLDL is converted into the mature lipoprotein by lecithin:cholesterol acyl transferase, an enzyme also synthesized by the liver. This enzyme transfers apo C and E from HDL to VLDL. In liver diseases, however, lecithin:cholesterol acyl transferase deficiency means that apo C and E are not transferred to VLDL, which leads to the accumulation of nascent VLDL. A misidentification of apo B-rich nascent VLDL as LDL by

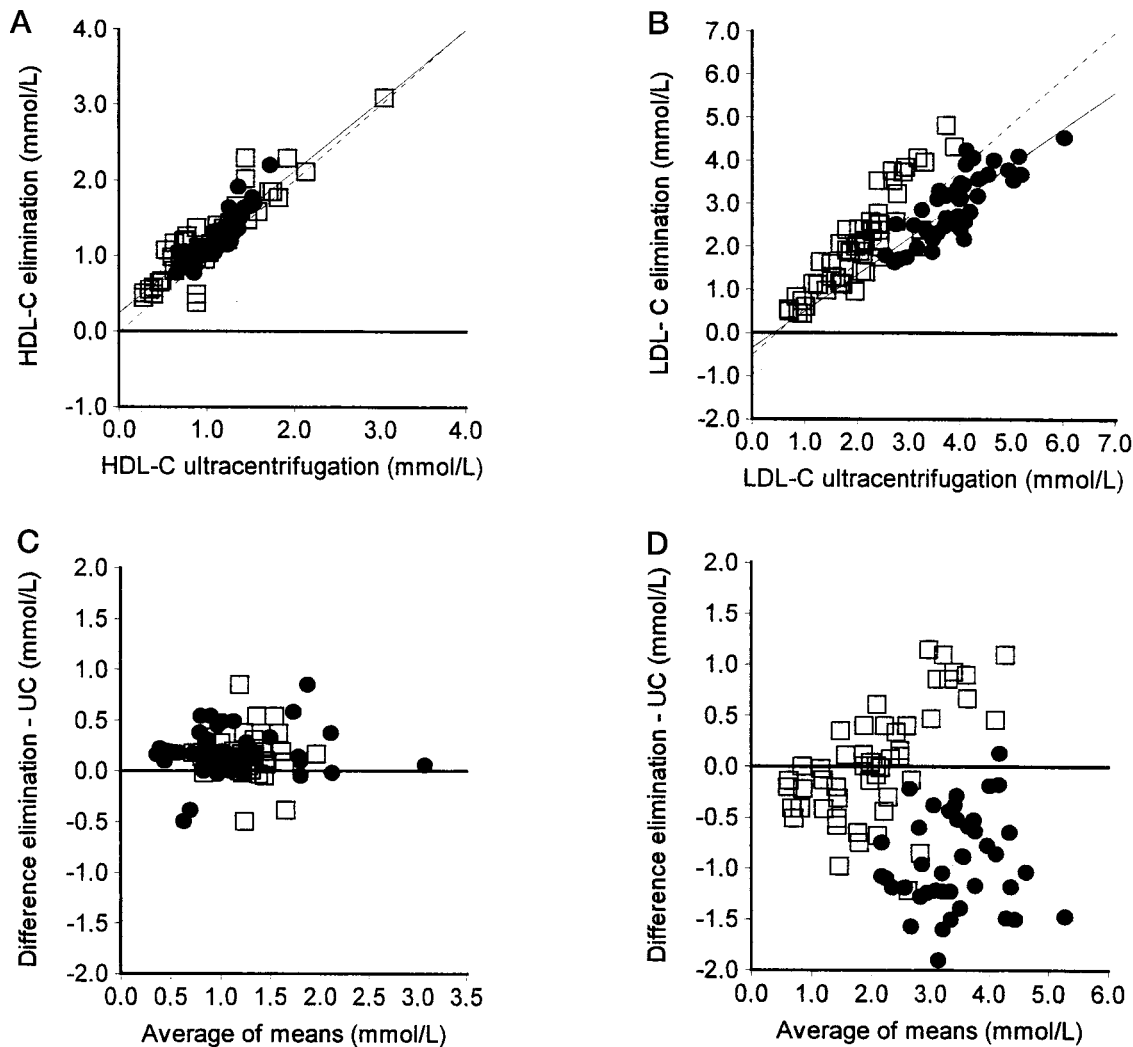


Fig. 1. Correlation (A and B) and Bland-Altman (C and D) plots for HDL-C (A and C) and LDL-C (B and D) in control subjects (●) and cirrhotic patients (□).

The correlation equations for the correlation plots are as follows: (A), $r = 0.90$; $y = 0.94x + 0.24$ for control subjects (solid line); $r = 0.90$; $y = 1.12x + 0.02$ for cirrhotic patients (dashed line). (B), $r = 0.80$; $y = 0.85x - 0.35$ for control subjects (solid line); $r = 0.88$; $y = 1.26x - 0.51$ for cirrhotic patients (dashed line). UC, ultracentrifugation.

reagent 1 is likely to account for the positive bias observed with the elimination method.

Therefore, although homogeneous LDL assays have demonstrated that they are useful in routine analyses, they fail to measure LDL-C accurately in extreme pathological situations in which profound structural and/or compositional lipoprotein alterations are likely to be observed.

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References

1. Sugiuchi H, Uji Y, Okabe H, Irie T, Uekama K, Kayahara N, Miyauchi K. Direct measurement of high-density lipoprotein cholesterol in serum with polyethylene glycol modified enzymes and sulfated α -cyclodextrin. *Clin Chem* 1995;41:717–23.
2. Harris N, Galpochian V, Rifai N. Three routine methods for measuring high-density lipoprotein cholesterol compared with the reference method. *Clin Chem* 1996;42:738–43.
3. Huang YC, Kao JT, Tsai KS. Evaluation of two homogeneous methods for measuring high-density lipoprotein cholesterol. *Clin Chem* 1997;43:1048–55.
4. McNamara JR, Cole TG, Contois JH, Ferguson CA, Ordovas JM, Schaefer EJ. Immunoseparation method for measuring low-density lipoprotein cholesterol directly from serum evaluated. *Clin Chem* 1995;41:232–40.
5. Maitra A, Hirany SV, Jialal I. Comparison of two assays for measuring LDL cholesterol. *Clin Chem* 1997;43:1040–7.
6. Simó JM, Castellano I, Ferré N, Joven J, Camps J. Evaluation of a homogeneous assay for high-density lipoprotein cholesterol: limitations in patients with cardiovascular, renal, and hepatic disorders. *Clin Chem* 1998;44:1233–41.
7. Camps J, Simó JM, Guaita S, Ferré N, Joven J. Altered composition of lipoproteins in liver cirrhosis compromises three homogeneous methods for HDL-cholesterol. *Clin Chem* 1999;45:685–8.
8. Matas C, Joven J, Vilella E, Civillé X, Cabré M, Prats E, Camps J. Lipoprotein alterations in liver cirrhosis: a possible contribution to changes in plasma oncotic pressure and viscosity. *J Hepatol* 1997;27:639–44.
9. Chung BH, Segrest JP, Ray MJ, Brunzell JD, Hokanson HE, Krauss RM, et al. In: Segrest JP, Albers, JJ, eds. *Methods in enzymology*, Vol. 128. Plasma lipoproteins. Part A. Preparation, structure and molecular biology. Orlando, FL: Academic Press, 1986:181–209.
10. Marcovina SM, Albers JJ, Dati F, Ledue T, Ritchie RF. International Federation of Clinical Chemistry standardization project for measurements of apolipoproteins A-I and B. *Clin Chem* 1991;37:1676–82.
11. Seidel D, Weiland H, Ruppert C. Improved techniques for assessment of plasma lipoprotein patterns. I. Precipitation in gels after electrophoresis with polyanionic compounds. *Clin Chem* 1973;19:737–9.
12. Bland JM, Altman DG. Statistical methods for assessing agreement between two methods of clinical measurement. *Lancet* 1986;i:307–10.
13. Camps J, Pizarro I, Prats E, LaVillle A, Turner PR, Masana L, Joven J. Plasma lipoprotein alterations in patients with chronic hepatocellular liver disease resulting from alcohol abuse: effects of alcohol intake cessation. *J Hepatol* 1994;20:704–9.
14. Schectman G, Patsches M, Sasse EA. Variability in cholesterol measurements: comparison of calculated and direct LDL cholesterol determinations. *Clin Chem* 1996;42:732–7.
15. Krause BR, Schork NJ, Kieft KA, Smith MP, Maciejko JJ. High correlation but lack of agreement between direct high-performance gel chromatography analysis and conventional indirect methods for determining lipoprotein cholesterol. *Clin Chem* 1996;42:1996–2001.
16. Harris N, Neufeld EJ, Newburger JW, Ticho B, Baker A, Ginsburg GS, et al. Analytical performance and clinical utility of a direct LDL-cholesterol assay in a hyperlipidemic pediatric population. *Clin Chem* 1996;42:1182–8.
17. Vrga L, Contacos C, Li SCH, Sullivan DR. Comparison of methods for measurement of apolipoprotein B and cholesterol in low-density lipoproteins. *Clin Chem* 1997;43:390–3.
18. Rifai N, Ianotti E, DeAngelis K, Law T. Analytical and clinical performance of a homogeneous enzymatic LDL-cholesterol assay compared with the ultracentrifugation-dextran sulfate-Mg²⁺ method. *Clin Chem* 1998;44:1242–50.
19. Sabesin S. Lipid and lipoprotein abnormalities in alcoholic liver disease. *Circulation* 1981;64(Suppl III):72–84.

Rapid Single-Tube Genotyping of the Factor V Leiden and Prothrombin Mutations by Real-Time PCR Using Dual-Color Detection, Frank A.J.T.M. van den Bergh,* Arletta M. van Oeveren-Dybcz, and Michelle A.M. Bon (Department of Clinical Chemistry, Medisch Spectrum Twente, Hospital Group, PO Box 50000, 7500 KA Enschede, The Netherlands; *author for correspondence: fax 31-53-487-3075, e-mail labmst@euronet.nl)

Patients carrying the G1691A mutation in the *factor V* gene (factor V Leiden) have been demonstrated to be at risk for venous thromboembolism. A second polymorphism also associated with hereditary thrombophilia was identified in the *prothrombin* gene (G20210A). Because of the high prevalence of these two mutations (5–10% for G1691A and 2–4% for G20210A) in the Caucasian population, there is growing demand for rapid, reliable, and simple methods for combined detection of both point mutations. Numerous PCR-based assays have been described for the detection of each of the mutations separately (1–4) as well as in combination by multiplex PCR analysis (5–10) and other single-tube alternatives [see, for example, Ref. (11)]. All of these methods, however, are time-consuming and require multiple manual steps such as restriction length polymorphism analysis, electrophoresis, and hybridization with specific oligonucleotide probes.

Recently, a new detection methodology was introduced on high-speed thermal cyclers based on real-time PCR analysis followed by hybridization of amplicon-specific oligonucleotides with adjacent fluorophores capable of fluorescence resonance energy transfer (LightCyclerTM; Roche Molecular Biochemicals). Probes, labeled with two different fluorescent molecules, hybridize next to each other on the target DNA molecule. The first fluorescent dye, the donor dye fluorescein, is excited at 470 nm by the light source of the LightCycler. Instead of emitting light at 530 nm, the fluorescein can transfer its energy in a nonfluorescent manner to a reporter dye. The reporter dye emits light of a longer wavelength, e.g., 640 nm. This process, called fluorescence resonance energy transfer (FRET), enables real-time detection of the specific PCR product followed by melting curve analysis, which monitors the temperature-dependent hybridization with fluorescent oligonucleotide probes to single-stranded DNA. The success of this new technology in detecting single nucleotide polymorphisms has been demonstrated by several recently published applications (12–17). The use of melting curve analysis for the detection of single nucleotide polymorphisms is rapid and convenient. Manual manipulation is minimized, as is the concomitant risk of contamination. Recently, the range of possible applications has been expanded by the availability of two fluorescent reporter dyes, each of which emits light at a different wavelength (640 and 705 nm, respectively). As described for the first time by Bernard et al. (18), this allows simultaneous measurement of two independent target sequences in one capillary. Here we report the genotyping of two different point mutations, G1691A and G20210A, in a single capillary on the LightCycler system.