Development of a Homogeneous Assay to Measure Remnant Lipoprotein Cholesterol

Kazuhito Miyauchi,1 Norihiko Kayahara,2* Masato Ishigami,3 Hideyuki Kuwata,4 Hideharu Mori,4 Hiroyuki Sugiuchi,5 Tetsumi Irie,6 Akira Tanaka,7 Shizuya Yamashita,8 and Taku Yamamura3

Background: Quantification of triglyceride-rich lipoprotein (TRL) remnants is useful for risk assessment of coronary artery disease and the diagnosis of type III hyperlipoproteinemia. Although an immunoseparation procedure for remnant-like particle cholesterol has been evaluated extensively in recent years, available methods for measuring TRL remnants have not achieved wide use in routine laboratory practice, suggesting a need for a homogeneous assay that can measure TRL remnant cholesterol in serum or plasma without pretreatment.

Methods: We screened for suitable surfactants that exhibited favorable selectivity toward the VLDL remnant (VLDLR) fraction, including intermediate-density lipoproteins (IDLs). We investigated the principal characteristics of this assay by gel filtration of lipoproteins and their particle size distribution. We developed a simple assay and evaluated its performance with the Hitachi-7170 analyzer.

Results: Polyoxyethylene-polyoxybutylene block copolymer (POE-POB) exhibited favorable selectivity toward VLDLR and IDL fractions. POE-POB removed apolipoprotein (apo) E and apo C-III from IDL particles in the presence of cholesterol esterase (CHER), and the particle size distribution of IDLs became smaller after the reaction. These results revealed that IDL particles are specifically modified in the presence of CHER and POE-POB, making their component cholesterol available for enzymatic assay. Addition of phospholipase D improved the reactivity toward chylomicron remnants (CMRs). We found a high correlation \[ y = 1.018x - 0.01 \text{ mmol/L}, r = 0.962 (n = 160) \] between the proposed assay and the immunoseparation assay in serum from healthy individuals.

Conclusion: The homogeneous assay described in this report can measure TRL remnant cholesterol, including CMRs, VLDLRs, and IDLs, with high sensitivity and specificity.

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Previous clinical and experimental studies have suggested that triglyceride-rich lipoprotein (TRL)9 remnants play an important role in atherogenesis (1). It has also been reported that postprandial hyperlipidemia induces fat accumulation in arterial walls (2) and is strongly associated with the progression of coronary artery disease (CAD) (3). Many studies have shown a high correlation between postprandial hyperlipidemia and atherosclerosis (4, 5). In addition, the Montreal Heart Study showed an independent contribution of increased VLDL remnants (VLDLRs) plus intermediate-density lipoproteins (IDLs) to the progression of CAD and related clinical events (6). Furthermore, a report from the Framingham Heart Study found that remnant-like particle cholesterol (RLP-C),

1 Scientific and Technical Affairs Department, Kyowa Medex Co., Ltd., Tokyo, Japan.
2 Research and Development Department, Kyowa Medex Co., Ltd., Tokyo, Japan.
3 Division of Health Sciences, Osaka University Graduate School of Medicine, Osaka, Japan.
4 Research Laboratory Department, Kyowa Medex Co., Ltd., Shizuoka, Japan.
5 Kumamoto Health Science University, Kumamoto, Japan.
6 Faculty of Medical and Pharmaceutical Sciences, Kumamoto University, Kumamoto, Japan.
7 Laboratory of Clinical Nutrition and Medicine, Kagawa Nutrition University, Saitama, Japan.
8 Department of Cardiovascular Medicine, Osaka University Graduate School of Medicine, Osaka, Japan.
* Address correspondence to this author at: Research and Development Department, Kyowa Medex Co., Ltd., 1-8-10, Harumi, Chuo-ku, Tokyo 104-6004, Japan. Fax 81-3-6219-7614; e-mail norihiko.kayahara@kyowa.co.jp. Received May 21, 2007; accepted September 5, 2007.

*Nonstandard abbreviations: TRL, triglyceride-rich lipoprotein; CAD, coronary artery disease; VLDLR, VLDL remnant; IDL, intermediate-density lipoprotein; RLP-C, remnant-like particle cholesterol; apo, apolipoprotein; CM, chylomicron; LPL, lipoprotein lipase; CMR, CM remnant; TG, triglyceride; PL-D, phospholipase D; CHER, cholesterol esterase; CHOD, cholesterol oxidase; POE-POB, polyoxyethylene-polyoxybutylene block copolymer.
which provides an estimate of TRL remnants, is an independent risk factor for CAD in women (7).

TRL remnants are metabolic intermediates formed when apolipoprotein (apo) B-48–containing chylomicrons (CMs) of intestinal origin and apo B-100–containing VLDL of hepatic origin are hydrolyzed by lipoprotein lipase (LPL) on the surface of vascular endothelium; they are referred to as CM remnants (CMRs) and VLDL remnants, respectively (8). VLDL remnants are found not only in TRL (d < 1.006 kg/L), but also in IDLs (1.006 to 1.019 kg/L). Therefore IDLs are also referred to as remnants (1). Compared with nascent VLDLs and CMs, TRL remnants and IDLs contain fewer triglycerides (TGs) and phospholipids, but relatively more esterified cholesterol and apo E (9–12). TRL remnants have been identified, isolated, and quantified in plasma on the basis of their density, charge, size, specific lipid components, apo composition, or apo immunospecificity (1, 13). Major methods include ultracentrifugation for IDLs (14, 15); agarose gel electrophoresis for β-VLDLs and slow pre-β VLDLs and PAGE for IDLs (presence or absence of a midband) (11, 14, 16); and immunoseparation for RLP-C as an unbound fraction not adsorbed by anti–apo A-I or anti–apo B-100 antibodies (17, 18). These methods require special equipment and are time-consuming; hence, they are not well suited as routine tests in hospitals and clinics. There is a need for a simple, rapid, automated method to quantify TRL remnants in serum or plasma.

Specific interaction of surfactants with lipoproteins has been successfully used for developing direct lipoprotein cholesterol assays, in which the surfactants are able to recognize differences in hydrated density, net charge, or size of the various lipoprotein fractions (17, 18). For separation of lipoprotein fractions, we used Superose 6 HR 10/30 columns (bed volume 24 mL; inner diameter 10 mm; length 30 cm; count 2; Pharmacia) (23, 24). Each lipoprotein fraction was eluted with 0.15 mol/L NaCl and 1 mmol/L EDTA (pH 7.4) at a flow rate of 0.3 mL/min. We collected the column effluent in 0.5-mL fractions, in which total cholesterol concentration was measured by enzymatic assay (Kyowa Medex) and apo E concentration was measured by immunoturbidimetric assay (Daichi Pure Chemicals).

**Materials and Methods**

**Materials**

We used cholesterol esterase (CHOD) (EC3.1.1.34: 134 kDa, *Pseudomonas* species) from Toyobo, cholesterol oxidase (CHOD) (EC1.1.3.6: 58 kDa, recombinant *Escherichia coli*) from Kyowa Hakko, PL-D (EC1.1.3.4: 46 kDa, *Streptomyces* species) from Asahi Kasei, Good’s buffer MOPS from Dojindo Laboratories, 4-aminantipyrine from Kanto Chemical, and N-ethyl-N-(3-methylphenyl)-N’-succinylethylendiamine manufactured by Kyowa Medex. Polyoxyethylene-polyoxybutylene block copolymer (POE-POB) was obtained from NOF Corporation, and the immunoseparation reagents for RLP-C measurements were obtained from Otsuka Pharmaceutical. Human serum samples were obtained from 160 healthy volunteers (100 men and 60 women, age 20 to 59 years) who had fasted for 12 h and from hyperlipidemic patients with the approval of ethics committees at Osaka University School of Medicine and Tokyo Medical and Dental University. We used hyperlipidemic sera for lipoprotein fractions isolated by ultracentrifugation to obtain VLDL (0.96–1.006 kg/L), IDL (1.006–1.019 kg/L), LDL (1.019–1.063 kg/L), and HDL (1.063–1.021 kg/L) fractions (15, 21, 22). Blood was collected in plastic serum tubes (Venoject II; inner diameter 12 mm; length 10 cm; with procoagulant agent; Terumo).

**Separation of Lipoproteins by Ultracentrifugation and Gel Filtration**

The density of fasting serum obtained from a patient with type III hyperlipoproteinemia (apo E phenotype E2/2) was adjusted to 1.006 and 1.019 kg/L, and the treated serum was ultracentrifuged at 131,918g for 20 h (Beckman: 50.4 Ti rotor). We collected the supernatant fraction after tube-slicing with Pasteur pipette to obtain lipoprotein fractions with densities of <1.006 kg/L and <1.019 kg/L. For separation of lipoprotein fractions, we used Superose 6 HR 10/30 columns (bed volume 24 mL; inner diameter 10 mm; length 30 cm; count 2 columns; Pharmacia) (23, 24). Each lipoprotein fraction was eluted with 0.15 mol/L NaCl and 1 mmol/L EDTA (pH 7.4) at a flow rate of 0.3 mL/min. We collected the column effluent in 0.5-mL fractions, in which total cholesterol concentration was measured by enzymatic assay (Kyowa Medex) and apo E concentration was measured by immunoturbidimetric assay (Daichi Pure Chemicals).

**Characterization by Gel Filtration Analysis**

We added a mixture of POE-POB 12 g/L, CHER 1.5 kU/L, and MOPS buffer 20 mmol/L, pH 6.5, as the reaction reagent to the IDL, LDL, and HDL fractions obtained by ultracentrifugation at a 1:1 ratio, and mixtures were then incubated at 37 °C for 5 min. For gel filtration analysis of the reaction mixture, we used Superose 6 HR 10/30 columns (23, 24). With each sample, 0.2 mL was applied with 0.15 mol/L NaCl and 1 mmol/L EDTA (pH 7.4) at a flow rate of 0.5 mL/min, and 0.2-mL fractions were collected. Concentrations of total cholesterol and TGs in the fractions were measured by enzymatic assay (Kyowa Medex), and the concentrations of apo E, apo C-III, apo B, and apo A-I were measured by immunoturbidimetric assay (Daichi Pure Chemicals). For gel filtration analysis of serum samples obtained after feeding a high-fat meal, we used TSK gel Lipopropak XL columns (inner diameter 7.8 mm; length 30 cm; count 2; Tosoh) (24, 25). A postprandial serum sample was obtained from a single individual 10 h after a high-fat meal containing 100 g of fat and 75 g of alcohol. Of the serum sample, 0.2 mL was subjected to gel filtration with 0.15 mol/L NaCl and 1 mmol/L EDTA (pH 7.4) at a flow rate of 0.5 mL/min, and 0.2-mL fractions were collected. Concentrations of total cholesterol, TGs, apo E, apo C-III, and apo B were measured as described above, and apo B-48 was measured by chemiluminescence enzyme immunoassay (Fujirebio).
characterization by particle size distribution analysis
For analysis of lipoprotein size distribution, we used a fiber optic dynamic light-scattering photometer (particle size range from 3 nm to 3 μm; FDLS-5000, Otsuka Electronics). The VLDL, IDL, LDL, and HDL fractions obtained by ultracentrifugation were mixed with the reaction reagent at a 1:1 ratio, incubated at 37 °C for 0 to 9 min, and diluted 100-fold for testing.

analytical procedure
For the measurement of remnant lipoprotein cholesterol, the final formulation of the 1st reagent was N-ethyl-N-(3-methylphenyl)-N′-succinylhexenediamine (1.1 mmol/L), POE-POB (8 g/L), and MOPS buffer (20 mmol/L, pH 6.5); that of the 2nd reagent was PL-D (8 kU/L), CHER (1.5 kU/L), CHOD (3 kU/L), peroxidase (20 kU/L), 4-aminoantipyrine (2.5 mmol/L), and MOPS buffer (20 mmol/L, pH 6.8).

Serum samples were stored at 4 °C after collection and were subjected to testing (Hitachi-7170 autoanalyzer) within 2 days, within which time the values obtained by the assay were stable. To 3.8 μL of each serum sample, we added 180 μL of the 1st reagent and incubated the resulting solution at 37 °C for 5 min. Next, we added 60 μL of the 2nd reagent and incubated the reaction mixture at 37 °C for 5 min. The chromophore formed in a coupled reaction with peroxidase was measured spectrophotometrically at dual wavelengths of 600 nm (main) and 700 nm (subsidiary), and the end-point method was used for the calculation. A serum-base calibrator prepared from TG-rich human sera (Kyowa Medex, 0.592 mmol/L) was used to estimate remnant lipoprotein cholesterol. We performed the RLP-C immunoseparation method according to the package insert provided by Otsuka Pharmaceutical, and serum samples were stored at 4 °C after collection and tested within 2 days. For PAGEL, Lipophor (Jokoh) was used. The total imprecision (CV) of the methods was as follows: total cholesterol <1.2%, TG <1.1%, apo A1 <5%, apo CIII <5%, apo E <5%, apo B <5%, and apo B-48 <6.4%. The intraassay imprecision (CV) of the immunoseparation method was <15%.

Statistics
We used Pearson correlation coefficient analysis and simple regression to assess the relation between the proposed assay and the immunoseparation assay. Statistical analysis was performed with Excel 2003 (Microsoft) with the add-in software Statcel 2 (26).

Results
Separation of lipoproteins by ultracentrifugation and gel filtration and selection of surfactant
To obtain the VLDLR fraction from the serum of a patient with type III hyperlipoproteinemia, we conducted ultracentrifugation and gel filtration. Fig. 1 shows the gel filtration profile of the lipoproteins from the patient serum. Whereas the main peak of the VLDL fraction is ordinarily detected at void volume (24), that of the d < 1.006 kg/L fraction from the patient was detected at void volume and at a smaller particle size. Compared with the void volume fraction, the smaller peak contained more apo E. Because the density of IDL is 1.006 to 1.019 kg/L, IDL elution can be identified by subtracting the concentration of the d < 1.006 kg/L fraction from that of the d < 1.019 kg/L fraction (Fig. 1). The smaller VLDL particle size area of the gel filtration (d < 1.006 kg/L) mostly overlapped with the elution profile of IDL. In the present study, the fractions 14 through 19 from the d < 1.019 kg/L fraction, as shown in Fig. 1, which were considered to contain VLDLR including IDL, were mixed to become the VLDLR fraction.

We examined 180 surfactants with diverse structures, with which selectivity toward cholesterol in VLDLRs was investigated. Of the surfactants tested, POE-POB of 10 042 Da (see Supplemental Data Fig. 1), which has relatively high solubility as well as high selectivity toward cholesterol in VLDLRs, was selected for further consideration.

Effects of POE-POB on gel filtration of lipoproteins and their size distribution
Using gel filtration and size distribution studies, we gained insight into the mechanism by which POE-POB exhibits its selectivity toward VLDLRs. Fig. 2A shows the profiles of cholesterol, TGs, apo B, apo E, and apo C-III after applying the IDL fraction to gel filtration obtained by ultracentrifugation. Total cholesterol, TGs, apo B,
apo E, and apo C-III were eluted at mostly the same retention time. Next, the IDL fraction was mixed with the reaction reagents at a 1:1 ratio, incubated at 37 °C for 5 min, and applied to gel filtration (Fig. 2B). When comparing the retention times of apos, apo B was eluted at approximately the same time as total cholesterol and TGs, but apo E and apo C-III were eluted with significant delay. When LDLs and HDLs were analyzed by the same methods, in contrast to IDLs, different apo peaks were not seen before and after the reaction reagent treatment.

We examined the effect of POE-POB on the size distribution of lipoprotein fractions by use of a fiber optic dynamic light-scattering photometer (FDLS-3000), which quantifies particle size based on light scattering. As shown in Fig. 2C, the particle size of the IDL fraction decreased with time after mixing with the reaction reagent. By contrast, changes in particle sizes of the VLDL, LDL, and HDL fractions were relatively small, particularly with LDL and HDL.

**Linearity study and lower limit of detection**

Five serum samples were diluted with 155 mmol/L NaCl, and linearity of the method was examined. The linearity was obtained up to 2.1 mmol/L cholesterol. The lower detection limit was 0.005 mmol/L.

**Improved reactivity to CMRs**

With the reagent containing POE-POB (8 g/L), which exhibited a high selectivity toward VLDLR in the presence of CHER (1.5 kU/L) and CHOD (3 kU/L), the correlation with the immunoseparation method for RLP-C was investigated using serum samples, including those with high TG concentrations. The results showed poor correlations with nonfasting samples having high TG concentrations. To clarify the cause of the differences, detailed reactivity was compared using lipoproteins isolated by gel filtration (24, 25). A serum sample collected from an individual after a high-fat meal was subjected to gel filtration. Fig. 3A shows gel filtration profiles of lipids and apos. Large CMs were eluted in the void volume (25), but apo B-48 and apo E were observed mostly in smaller particles, thus suggesting the presence of CMRs. Next, in the presence of CHER (1.5 kU/L) and CHOD (3 kU/L), the reactivity of reagent containing POE-POB (8 g/L) toward CMRs was examined, but the reactivity was very low in the region corresponding to CMRs. This low reactivity toward CMRs was thought to be the cause of the differences with the immunoseparation method, which detects all particles containing apo B-48. Therefore, PL-D (5 kU/L) was added to the reagent containing POE-POB (8 g/L) in the presence of CHER (1.5 kU/L) and CHOD (3 kU/L), and the reactivity of the fractions containing CMR significantly improved (Fig. 3A). As shown in Fig. 3B, the reagent containing POE-POB and PL-D in the presence of CHER and CHOD exhibited the required selectivity toward cholesterol in VLDLRs, including IDL, when a serum from a patient with type III hyperlipoproteinemia was used. The above results confirmed that the proposed assay made it possible to detect cholesterol in CMRs, VLDLRs, and IDLs.
The within-run imprecision (CV) of the method was 1.25% at 0.100 mmol/L, 0.96% at 0.317 mmol/L, and 1.8% at 0.412 mmol/L. The run-to-run imprecision (CV) of the method was 2.75% at 0.111 mmol/L, 1.54% at 0.199 mmol/L, and 1.59% at 0.499 mmol/L.

EFFECTS OF INTERFERING SUBSTANCES
To pooled serum samples, we added conjugated bilirubin (up to 0.68 mmol/L), free bilirubin (up to 0.68 mmol/L), hemoglobin (up to 4.9 g/L), and Intrafat (up to 10%) (Nihon Seiyaku Kogyo). None of these compounds produced more than a 5% error in the assay result.

COMPARISON WITH THE RLP-C IMMUNOSEPARATION METHOD
The proposed assay was compared with the immunoseparation method in fasting serum samples from 160 healthy volunteers. As shown in Fig. 4A, the regression equation for the comparison was \( y = 1.018x - 0.01 \) (\( r = 0.96 \)). The mean (SD) of 160 sera was 0.18 (0.15) mmol/L for the immunoseparation method and 0.17 (0.16) mmol/L for the proposed method, respectively.

The relation with the immunoseparation method was also examined by use of sera from diabetic patients, which showed that the proposed method occasionally exhibited higher concentrations of remnant lipoprotein cholesterol than the immunoseparation method (Fig. 4B). When these samples were analyzed by PAGE, a large amount of midband (14) was detected, indicating increased concentrations of IDLs.

Discussion
Various methods have been developed to detect remnant lipoproteins in recent years. These methods are not well suited for use in regular laboratory practice because they are time-consuming and require special equipment. We used a special surfactant, POE-POB, and an enzyme, PL-D, to develop a homogenous assay that conveniently measures TRL remnant cholesterol in serum or plasma. The proposed assay does not require any sample pretreatment and uses 3.8 \( \mu \)L of serum, with the assay taking only 10 min on a regular autoanalyzer.

The proposed assay measures cholesterol in CMRs, VLDLRs, and IDLs by selectively modifying these 3 remnant lipoproteins with POE-POB and PL-D in the presence of CHER and CHOD. Polyoxamers including POE-POP have been successfully used as surface modifiers for improving the stability of latex particles (27) and as vehicles for transdermal drug delivery (28). Furthermore, POE-POP, with a molecular weight of 3850 Da and a high polyoxypropylene content, was found to be LDL selective, and it has been used in assays for LDL cholesterol (29).

POE-POB, with a molecular weight of 10 042 Da, was selected for this proposed assay. When the IDL fraction (VLDLRs) incubated with POE-POB in the presence of CHER was applied to gel filtration, apo E and apo C-III were eluted from gel filtration columns with significant delay compared with the retention time of total cholesterol, TGs, and apo B (Fig. 2, A and B). This result indicates that apo E and apo C-III were removed from the...
lipoprotein surfaces in the presence of CHER with POE-POB and formed isolated small particles. When treated with the reaction reagent, the elution time for IDL particles themselves was reduced. However, the size of IDLs treated with the reaction reagent became progressively smaller with time (Fig. 2C), suggesting that the interaction between the reaction reagent and IDL particles may affect the shape of IDL particles, thus increasing their apparent size. In addition, with the LDL and HDL fractions, small changes of elution time after reaction reagent addition also suggested changes in particle shape, but apo B in LDLs and apo A-I and apo E in HDLs were not removed from the respective lipoprotein particles.

Reaction reagent treatment also made IDLs smaller with time, thus clarifying that IDL modification was enhanced in the presence of CHER and POE-POB. The above findings suggested that, in the presence of CHER with POE-POB, apo E and apo C-III—which are relatively hydrophilic, of low molecular weight, and rich in VLDLR—were removed, and this phenomenon triggers the modification of VLDLR.

Using postprandial sera with high TGs, there were substantial differences between the immunoseparation method and the reagent with POE-POB in the presence of CHER and CHOD. To determine the cause of discrepancies, postprandial serum was collected from an individual after a high-fat meal. Whereas large CMs were eluted in the void volume during gel filtration by use of a serum sample collected from this individual, lipoprotein fractions containing apo B-48 together with apo E and apo C-III were detected in smaller particles. Although CMs of varying size are secreted postprandially (1), the smaller particles include some CMRs, in which TGs and some phospholipids have been hydrolyzed by LPLs. In the presence of CHER and CHOD, the reactivity of POE-POB toward the fractions containing such CMRs was found to be very weak, and this reaction was evidently the cause of the discrepancies. Therefore, in an attempt to improve the reactivity toward CMRs, PL-D was added to the reagent with CHER, CHOD, and POE-POB. The results showed no changes in the reactivity to total cholesterol in lipoproteins other than the fractions containing CMRs, but the reactivity toward cholesterol in these fractions improved significantly. PL-D acts on the phosphodiester bond of the polar area of phospholipids to catalyze hydrolysis (30).

In the absence of POE-POB, PL-D increased the reactivity of the assay for all lipoprotein fractions, probably through the hydrolysis of phospholipids on the surfaces of all the lipoprotein particles, thereby triggering favorable modification of the particles, allowing detection of its cholesterol in the presence of CHER and CHOD. On the other hand, POE-POB interfered with the ability of lipoprotein cholesterol, with the exception of TRL remnant cholesterol, to participate in the subsequent enzymatic reactions. Therefore, the combination of PL-D and POE-POB seems to increase the reactivity of the assay selectively for CMRs (Fig. 3A).

Lipoproteins are essentially microemulsions in which a hydrophobic core containing nonpolar lipids, such as TG and cholesterol esters, is surrounded by a monolayer of apolipoproteins and polar lipids, such as phospholipids and free cholesterol (31). Remnant lipoproteins are produced when CM and VLDL lipids are hydrolyzed by LPLs. It has been reported that hydrolysis of TGs by LPLs occurs at the same time as removal of polar components on TRL.
surfaces (10). In other words, the surface structures of remnant lipoproteins differ from those of other lipoproteins and are thought to be modified by LPLs. Because there are no fundamental differences in the overall structure of CMs, VLDLs, CMRs, and VLDLRs, however, it has been difficult to differentiate these lipoproteins. By releasing apo E, apo C-III, and phospholipids from TRL remnants, the reagent containing CHER, CHOD, POE-POB, and PL-D may promote cholesterol reactivity specifically in these modified particles.

Although a high correlation was observed between the immunoseparation and proposed assay methods in healthy individuals, there were cases with discrepancies among diabetic patients, who displayed increased concentrations of IDLs. These sera were analyzed by Lipophor, in which IDLs were detected as a “midband” (14). We found that such sera contained more IDLs rather than large VLDLs. This result suggested the possibility that the proposed assay can quantify IDLs with high sensitivity.

There is a spectrum of sizes for CMRs and VLDLRs depending on their degree of lipolysis. Therefore, further studies need to be performed to determine the specificity of their assays to the various-sized remnant particles produced in the postprandial state.

The above findings indicate that this method detects not only TG-rich large particle size lipoproteins, such as CMRs, but also smaller remnants, such as VLDLRs and IDLs, with high sensitivity. Therefore, the proposed assay can quantify the VLDLR and IDL fractions, which reflect CAD progression, as reported in the Montreal Heart Study (6). Furthermore, the proposed assay does not require any sample pretreatment and can be performed in a short period of time with the use of an autoanalyzer. It may therefore be useful for risk assessment of CAD and the diagnosis of type III hyperlipoproteinemia and other dyslipidemias characterized by accumulation of TRL remnants.

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