Manual 8

Lipid and Lipoprotein Determinations

The National Heart, Lung, and Blood Institute
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ARIC Protocol

Manual 8

Lipid and Lipoprotein Determinations

Visit 2

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FOREWORD

This manual entitled, Lipids and Lipoprotein Determinations, is one of a series of protocols and manuals of operation for the Atherosclerosis Risk in Communities (ARIC) Study. The complexity of the ARIC Study requires that a sizeable number of procedures be described, thus this rather extensive set of materials has been organized into the set of manuals listed below. Manual 1 provides the background, organization, and general objectives of the ARIC Study. Manuals 2 and 3 describe the operation of the Cohort and Surveillance Components of the study. Detailed Manuals of Operation for specific procedures, including reading centers and central laboratories, make up Manuals 4 through 11. Manual 12 on Quality Assurance and Quality Control contains a general description of the study's approach to quality assurance as well as specific protocols for each of the study procedures.

The version status of each manual is printed on the title sheet. The first edition of each manual is Version 1.0. Subsequent modifications of Version 1 (pages updated, pages added, or pages deleted) are indicated as Versions 1.1, 1.2, and so on, and are described in detail in the Revision Log located immediately after the title page. When revisions are substantial enough to require a new printing of the manual, the version number will be updated (e.g., Version 2.0) on the title page.

ARIC Study Protocols and Manuals of Operation

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Manual 8: Lipid and Lipoprotein Determinations

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1.1 Introduction

A major objective of the Atherosclerosis Risk in Communities study (ARIC) is to measure associations of atherosclerotic manifestations and new coronary heart disease (CHD) events with plasma lipid, lipoprotein cholesterol, and plasma apolipoprotein levels known or suspected to be risk factors for CHD.

The primary function of the Central Lipid Laboratory within the entire project is to provide and implement lipid and apolipoprotein measurements that are (1) likely to assess the coronary risk of participants in cross-sectional and longitudinal studies; (2) feasible and practical in frozen plasma samples, in the large quantity required; and (3) accurate and precise enough to determine a relationship of atherosclerosis and its clinical manifestations to these factors in prospective studies.

Additional functions of the Central Lipid Laboratory are: (1) to provide storage space for aliquots of study specimens at conditions preserving the integrity of plasma components; (2) to collaborate with the Steering Committee in the design and implementation of tests on frozen specimens in case control studies of the cohort; and (3) to participate actively in the analysis and publication of study results.

1.2 Analytical Measurements

The measurements to be performed by the Central Lipid Laboratory may be divided into two categories: measurements performed in all plasma samples of the entire cohort and measurements performed in case control studies of the cohort. Measurements performed in cohort samples are: (1) plasma cholesterol; (2) plasma triglyceride; (3) HDL-cholesterol, HDL3-cholesterol, and calculation of HDL2-cholesterol, and calculation of LDL-cholesterol; (4) plasma apolipoprotein A-I (apoA-I); and (5) plasma apolipoprotein B (apoB). Measurements performed in case control samples of the cohort are: (1) plasma glycerol; (2) LDL-apoB, LDL-cholesterol, and calculation of the ratio of LDL-chol to LDL-apoB; (3) epitope expression of apoB; (4) phenotyping of apoE; (5) restriction fragment length polymorphism of apolipoprotein genes; (6)phenotyping of Lp[a]; and (7) measurement of postprandial lipemic response.

1.3 Justification of Measurements in Cohort Samples

1.3.1 Relation to Atherogenesis

Elevated plasma cholesterol levels are generally accepted as a risk factor in both the development of accelerated atherosclerosis and increased incidence of CHD (1-3). In some studies, elevation of both, plasma triglyceride (TG) and cholesterol, conferred the highest risk for CHD (4-5). Nevertheless, the evidence for elevated plasma TG levels as an independent risk factor is conflicting (6,7). The contribution of plasma cholesterol to the risk of CHD is determined by its partition among various lipoprotein fractions. A relatively large proportion of cholesterol carried in VLDL, chylomicron remnants, IDL, and LDL is "atherogenic", whereas a large proportion carried in HDL appears to be "protective" against the development of atherosclerotic manifestations (8-15). Among the factors that elevate plasma cholesterol levels and influence its transport by atherogenic lipoproteins are genetic abnormalities such as familial hypercholesterolemia (FH), dysbetalipoproteinemia, and familial combined hyperlipoproteinemia (16). These dyslipoproteinemias are results of changes in the rate of synthesis, postsecretory processing in plasma, and/or removal of lipoproteins from the circulation. Indeed, several mutations of genes regulating lipid metabolism have been described, which explain the bio-chemical abnormalities found in the plasma of various disorders of lipid transport (17). While major advances have been made in the understanding of the pathophysiology of dyslipoproteinemias, the mechanisms by which arterial wall damage occurs in these disorders are still not fully understood, though concepts have been presented (18-24). In FH, for instance, mutations in the LDL receptor gene lead to malfunctioning of the LDL-receptor pathway and accumulation of LDL in plasma (25,26). Increased residence time of LDL may lead to modification of these lipo-proteins, thus rendering them susceptible to endocytosis by macrophages and endothelial cells (27,28,29). Such a scavenger pathway may induce the development of lipid depositions and foam cells (30), typical for the atherosclerotic process (31). Although the dyslipoproteinemias mentioned confer greatly increased risk for CHD to affected individuals, only a minority of patients with clinical manifestations of atherosclerosis has these well-defined abnormalities of the lipid transport system (32). Thus, the impact of the classical dyslipoproteinemias on the overall incidence of CAD is relatively small. Other factors may be more important for the prevalence of atherosclerosis in the general population. Such factors may include more subtle genetic dispositions to abnormalities in lipid transport, and the inadequacy of lipid metabolism may only become apparent under certain environmental conditions.

Levels of HDL-cholesterol have been shown to be inversely correlated with the risk of CHD (11-15). Importantly, the predictive value of HDL-cholesterol levels was stronger than any of the other lipid measurements, and a wide range of HDL-cholesterol levels may be found in "healthy" subjects not affected by the classical dyslipoproteinemias (33). HDL represent one of the four families of plasma lipoproteins in humans, and they are heterogenous with respect to physical, chemical, and functional properties (34-41). HDL may exist in several subfractions (35-37,40). The most important are HDL2 and HDL3, which differ in density, particle size, lipid content, and apolipoprotein composition (38). The proportions of HDL2 and HDL3 in plasma may be determined by synthetic and removal rates of apolipoproteins, by LCAT activity (42-44), activities of lipoprotein lipase and hepatic lipase (45-48), and by lipid transfer factors (49-51). The mechanism(s) by which HDL "protect" against atherosclerosis may be related to the following observations: (1) HDL seems to be a major factor in the proliferation of vascular endothelial cells (52); (2) HDL reduces the uptake of LDL by several cell types (53-55); and (3) HDL enhances the net removal of cellular cholesterol from a variety of cells such as aortic smooth muscle cells and fibroblasts (56-59). Importantly, HDL increases the egress of cholesterol from macrophages preloaded with cholesterol by incubating them with chemically modified LDL (60). These observations support the hypothesis that HDL act as a reverse cholesterol transport mechanism (61); thus, HDL would serve as a scavenger of tissue cholesterol, including that of the arterial wall.

There is evidence to suggest that the protective effect of HDL is better reflected in the plasma levels of HDL2 than total HDL or HDL3. These conclusions can be drawn from correlations between the prevalence or incidence of CAD and HDL2 levels as observed in males and females (62-65), from clinicopathological studies (66), and from the inverse relationship of HDL2 with the magnitude of postprandial lipemia (67). These latter findings are compatible with the hypothesis that individuals with high levels of HDL2 are less prone to coronary disease due to their rapid clearance of the triglyceride-rich lipoproteins and their remnants from the circulation (22). Thus, HDL2 itself would not be protective for CHD, rather high levels of these lipoproteins would be the result of the phenomenon (the rapid clearance of TG-rich lipoproteins) which protects against atherosclerosis. The negative correlation between HDL2 levels and postprandial hyperlipidemia has been explained most recently (48). In the postprandial state, redistribution of triglyceride into HDL2 occurs at the expense of cholesteryl ester. These HDL2 particles because of their enrichment with triglyceride are substrates for hepatic lipase and can be transformed to the smaller HDL3 particles. When postprandial hyperlipidemia is extensive, a substantial amount of HDL2 may be converted to HDL3. If postprandial hyperlipidemia is mild due to effective clearance of triglyceride-rich

lipoproteins, HDL2 levels are maintained or even increased by transfer of surface components (47). This mechanism may be of quantitative importance in determining plasma levels of HDL2 and HDL3, because a major part of life is spent in the postprandial state.

The beneficial effects of high levels of HDL2 are also apparent in an autosomal dominant inherited condition, familial hyperalphacholesterolemia, in which elongation of life expectancy has been demonstrated (68, 69). In these subjects, most of the HDL-cholesterol is carried in the HDL2 fraction whereas concentrations of HDL3 are similar to normals (70).

Recently, the protein moieties of plasma lipoproteins have become the focus of much interest. These apolipoproteins appear to be the product of a multigene family dispersed in the genome (71,72), and knowledge about factors regulating the expression of apolipoprotein genes is beginning to emerge (73-76). Major advances have been made in our understanding of apolipoprotein structure and function (24,77,78). Apolipoproteins serve as: (1) structural components of lipoproteins thereby allowing the transport of purely water soluble lipids in the circulation; (2) activators of enzymes regulating lipid transport; and (3) recognition sites for cellular uptake of lipoproteins. Thus, the entire lipoprotein metabolism is regulated by apolipoproteins, and the spectrum or mosaic of apolipoproteins may determine the catabolism of individual lipoproteins (48,79-81). Based on the paramount importance of apolipoproteins in lipoprotein metabolism, one would expect the precise quantification of apolipoproteins to bring us closer to the understanding of the relation of lipoprotein metabolism with CHD. Yet, the predictive value of apolipoprotein levels in ascertaining the risk of CHD in populations is not fully established.

There was a surge of studies addressing the relation of plasma apolipoprotein levels to CAD in various populations (82-101). Except for ApoA-IV, data on all major apolipoproteins alone or in combination have been presented. With two exceptions (96,100), all these studies were case control studies. In the majority of reports, cases were defined as survivors of myocardial infarction. Some studies were based on angiographically defined CAD (66,88,90,97,99,101) in subjects with chest pain; the severity of disease was defined by the degree of stenosis or by an atherosclerotic score. In reviewing these publications, various criticisms may be raised related to poor definition of controls, comparability of apolipoprotein measurements, and statistical analysis. It is therefore not surprising that some of the conclusions derived by the investigators differ in key issues. For example, apoA-II discriminated cases from controls in one study (91), but not or only poorly in several others (89,66,96). Some studies claim the measurement of apoA-I to be superior to HDL-cholesterol in distinguishing cases from controls

(95), while other studies state the opposite (66). Despite these discrepancies, which may be related in part to the differences in study design and the populations studied, one pattern of consistent findings appears to emerge. In nine out of nine reports, apoB appeared to be elevated in cases when compared with controls. ApoA-I plasma levels were reduced in cases in all but one study. In contrast, apoD, apoE, and apoC did not discriminate between cases and controls when adjustments were made for the presence of dyslipoproteinemias. It should be pointed out that the number of studies measuring apoC, apoE, apoA-II, and apoD was much smaller when compared with investigations measuring apoA-I and apoB. More information is needed to estimate their value as predictors of CHD.

Plasma apolipoprotein levels were also measured in subjects with peripheral vascular disease and cerebrovascular disease (102-105). Here again, the pattern observed in CAD was present, i.e. cases were associated with elevation of apoB, reduction of apoA-I, or both.

Because of its potential role as a marker for CHD, lipoprotein(a), Lp(a), has attracted the interest of researchers. This lipoprotein bears a specific antigen on its surface that was originally discovered by Berg et al (106). Lp(a) exhibits prebeta-mobility on agarose gel electrophoresis, contains only apo(a) and apoB as apolipoproteins, and varies with respect to lipid composition, size, and apparent density (107). The frequency of Lp(a) positive individuals in healthy Caucasian populations was found by Berg et al to be around 35%. However, other investigators, using more sensitive techniques have detected the (a) antigen in 81 to 96% (108,109) of tested sera or in all individuals after concentration of sera (110). Autosomal inheritance is believed to control the levels of apo(a) in the plasma, and the effect of environmental factors on plasma levels of Lp(a) appears to be negligible (111). This contrasts with other lipoproteins, whose plasma levels can be altered by various perturbations including diet (112-114).

The principal interest in Lp(a) is associated with its possible linkage to accelerated atherosclerosis. Dahlen and Ericson reported a positive correlation between angina pectoris and the occurrence of a plasma lipoprotein fraction which exhibits slow prebeta-electrophoretic mobility on cellulose acetate and in 0.5% agarose gel (115). Subsequently, a highly significant association between the occurrence of this electrophoretic band and the presence of the Lp(a) antigen as detected by double immunodiffusion technique was described (116). Lp(a) detected either electrophoretically or immunochemically occurred more frequently in patients who had myocardial infarction than healthy controls (117). Levels of Lp(a) correlated with degree of coronary atherosclerosis, as determined by coronary angiography (118). In a small group of patients with intermittent

claudication, mean levels of Lp(a) were significantly higher than in age-matched controls (119).

These early observations of a significant association between high plasma Lp(a) levels and coronary heart disease have in the meantime been confirmed by several investigators (120,121). In a recent study, a significant correlation ($p < 0.002$) between Lp(a) levels and coronary score was observed in white patients undergoing diagnostic coronary arteriography (122). Among patients age 55 and younger, the Lp(a) level was the strongest risk factor, surpassing age, total cholesterol, and low levels of HDL-cholesterol. This study concluded that Lp(a) levels may be related to the degree of coronary sclerosis independent of other risk factors. A causal relationship between Lp(a) and CAD has, however, not been established, and the hypothesis that Lp(a) levels per se are related to CHD has been challenged recently. Plasma Lp(a) levels were quantified by electroimmunoassay in 105 black and 134 white healthy subjects. Study groups were similar with respect to body mass index, use of alcohol and nicotine, plasma cholesterol, and apoB. However, black subjects had levels of Lp(a) that averaged twice those of whites ($p < 0.001$). Among blacks, Lp(a) levels showed a bell-shaped frequency distribution, while among whites the distribution was strongly skewed, with the highest frequencies at low levels. Furthermore, the apoB levels correlated significantly, though weakly, with Lp(a). Regression analysis suggested that apoB associated with the Lp(a) lipoprotein could account for this correlation. Since coronary heart disease is not higher in blacks than in whites, the significance of Lp(a) levels in the development of CAD may vary among populations (123). Clearly, more studies in larger and well characterized populations are needed to understand the role of Lp(a) in atherogenesis.

Based on previous epidemiologic studies, the predictive value of Lp(a) levels in plasma may be independent of other lipid measurements (122).

No reference material is available for the measurement of Lp(a), but considerable knowledge and experience in the preparation and characterization of standard and the quantitation of Lp(a) in unknown plasma specimens has accumulated in the laboratory (107,166,176), assuring measurement of Lp(a) at the state of the art.

1.3.2 Feasibility to Perform Measurements in the Quantity Required

Including 5 percent of blinded samples, the program requires analysis of 125 plasma specimens per week. It is therefore essential to utilize procedures amenable for automatization. For

measurement of plasma lipids, automated enzymatic methods are used. The standard procedures for cholesterol and triglyceride measurements are the Monotest Cholesterol procedure (124) and the GPO Triglyceride procedure (125) of Boehringer Mannheim. Both these tests are adapted to automatic analysis using the Cobas-Bio Analyzer of Roche. By using these reagents and the instrumentation we have demonstrated that measurements meet the criteria of precision and accuracy set forth by CDC.

A variety of endogenous and exogenous substances has been tested for interference in the enzymatic determination of plasma lipids (126,127). Among common drugs, only ascorbic acid and '-methyldopa produced borderline negative interference at therapeutic plasma concentrations. In Trinder systems (128), bilirubin has been reported to interact in the peroxidase-catalyzed reaction by competing for hydrogen peroxide, which results in lower lipid levels (129). The inclusion of hexacyanoferrate (II) into the reagent mixture minimizes this chemical interference, and bilirubin concentrations of up to 15 mg/dL did not produce significant changes in results. Positive interference may be caused by hemoglobin, which acts as a pseudoperoxidase. However, no distortion of results was observed by concentrations of up to 2 g/dL.

Since the proper functioning of the Cobas-Bio is essential for the timely analysis of samples, a detailed maintenance program is in operation, which includes a service contract with 24 hour response time of a field engineer. Should the machine be out of operation, a back-up procedure will be effective. In this procedure, the same reagents are used, automated pipets are used for reagent and specimen delivery, and absorbance readings are done manually on a calibrated spectrophotometer. Results obtained are identical to the automated method.

In the unlikely event that the commercial reagent kits would deteriorate or would become unavailable, back-up procedures (130,131) will be set up which are, in essence, identical to the primary method, but differ in the source of reagents. The equivalence of a CDC-method to measure cholesterol (130) to the Monotest cholesterol of Boehringer Mannheim (124) has been established (132).

Methods to quantify plasma levels of HDL include various kinds of ultracentrifugation (133-135), electrophoretic techniques (136), and procedures which rely on precipitation of lipoproteins other than HDL (137). While the classical ultracentrifugation techniques are impractical for analyzing such large numbers of samples as required in this program, precipitation techniques have been proven useful in previous epidemiologic studies (14,15,138,139). In the majority of these studies, VLDL and LDL were precipitated by heparin and Mn2+, and the cholesterol of HDL remaining in solution was measured by a modified Lieberman

Burchard method. The use of heparin and Mn2+ is, however, inadequate when cholesterol is determined by enzymatic procedures, since Mn2+ interferes in the determination (140). To overcome this drawback, Mg2+ and dextran sulfate was used and evaluated to precipitate VLDL and LDL (137,141). The method was found highly reliable and reproducible, but revealed, on the average, 5% lower plasma levels of HDL-cholesterol, when compared with the LRC-procedure (141). The explanation for this negative bias may be that a more complete precipitation of apoB containing lipoproteins (141) and precipitation of apoE containing lipoproteins (142) had occurred.

The dextran sulfate Mg2+ technique is also suitable to measure cholesterol in HDL subfractions HDL2 and HDL3. The accuracy of this technique has been demonstrated by comparison with ultracentrifugal techniques (143). HDL2 determined by the precipitation technique was correlated with values obtained by analytical ($r = 0.94$) and preparative ultracentrifugation ($r = 0.96$). Respective correlation coefficients for HDL3 were 0.64 and 0.80 (143).

Quantitation of apolipoproteins in plasma is based on their immunological identity. While a number of immunological techniques have been described to measure apolipoproteins (144-155), radioimmunoassays (RIA) and enzyme linked immunoassays (ELISA) are the methods of choice, since these methods are amenable to automatization. For measurement of apoA-I and apoB, radioimmunoassays are used. Both assays are based on procedures developed by Schonfeld et al (144,152).

The apoA-I assay has been modified, in that delipidation of samples is omitted, but Tween 20 is included in the assay to unmask the antigeneic sites of apoA-I in plasma (155). The second antibody is replaced by formalin fixed staphylococcus aureus cells. In the apoB assay, LDL isolated by zonal ultracentrifugation (134) is used for preparation of the tracer and as primary standard. Again, staphylococcus aureus cells are used instead of the second antibody to separate antibody bound antigen from free antigen. Up to 100 unknowns are analyzed in a typical RIA. Lp(a) will be analyzed by a double antibody ELISA.

1.3.3 Feasibility to Perform Analyses in Frozen Plasma Specimens

The validity of cholesterol and triglyceride determination in frozen plasma is generally accepted (156). Freezing of plasma may change physiochemical properties of lipoproteins (157-159), but does not seem to affect quantitation of HDL-cholesterol by precipitation. Storage of plasma at constant temperature below -15°C did not alter HDL-cholesterol levels when cholesterol was

measured in heparin Mn2+ supernates by various methods (160,161). In another study, small increases of HDL-cholesterol upon freezing were reported (162). Mean HDL-cholesterol levels in frozen specimens differed by 1.7 - 4.2% of HDL-cholesterol levels in fresh samples. These differences would seem to be within the error of the method used to determine HDL-cholesterol. The slightly increased HDL-cholesterol levels noted in this study may be related to incomplete precipitation of frozen LDL with heparin Mn2+, because the efficiency of heparin Mn2+ to remove apoB containing lipoproteins from lipemic sera is low (163). Precipitation of VLDL and LDL by dextran sulfate and Mg2+ is more effective and should therefore minimize the quantitatively small changes in HDL-cholesterol that might occur in frozen specimens (141). The control material provided by CDC is also kept frozen, and stable results were obtained in numerous laboratories when storage conditions of less than -50°C were maintained. To evaluate the adequacy of the dextran sulfate Mg2 procedure for quantitation of HDL subfraction cholesterol in frozen specimens, we performed a local pilot study. HDL2 and HDL3 cholesterol was quantified in 95 fresh plasma specimens. Aliquots were stored at -70°C and assayed two weeks later. Upon storage at -70°C mean HDL-cholesterol decreased by 2.5% and mean HDL3 cholesterol decreased by 5.4%, while mean HDL2 cholesterol increased by 5.0%. Thus, the changes observed were minor and did not exceed the error of the method used for quantitation of cholesterol in HDL subfractions.

Several reports indicate stability of immunoreactivity of apoB and apoA-I in frozen plasma samples, at least within the limits of detection (164-166). Quantitation of apolipoproteins in specimens which had been frozen for several years was applied to examine the relationship of apolipoproteins and risk of CHD (96). Stability of apolipoprotein levels in frozen plasma samples can also be derived from data on quality control pools.

In addition, various conditions of blood collection (EDTA, EDTA plus aprotinin and epsilon-aminocaproic acid) and storage of plasma (storage of native plasma, addition of antiproteolytic and antibacterial agents with or without antioxidants) were evaluated with respect to quantitation of apoA-I and apoB in a local pilot study. Virtually none of these various pretreatments affected quantitation of apoA-I in specimens stored at -70°C for six weeks when compared with quantitation of apoA-I in fresh plasma. Apparent apoB content in samples stored at -70°C for six weeks declined by 7%. This decrease was not related to the content of apoB in fresh plasma specimens nor was this decrease prevented by any of the variables introduced during blood collection or storage of plasma. Furthermore, stable results on quality control pools indicate that freezing of plasma for up to one year does not alter the apparent apoB content.

1.3.4 Avoidance of Costly Redundancy in the Mix of Lipid Measurements

The majority of the proposed measurements including cholesterol, HDL-cholesterol, apoA-I, apoB, and Lp(a) has been shown to be of value in ascertaining the cardiovascular risk in epidemiologic studies. HDL2-cholesterol may be a negative predictor superior to HDL-cholesterol. Triglyceride measurements are necessary to calculate LDL-cholesterol (167), to define lipoprotein phenotypes of study subjects, and to allow data analysis controlling for hyperlipidemia. In addition, subsets of hypertriglyceridemic subjects with increased incidence of CHD may be defined, who may be characterized by a distinct pattern of lipoprotein lipids and apolipoproteins (168). This would clarify contradicting observations of previous epidemiologic studies (6,7).

The measurement of three HDL parameters, HDL2-chol, HDL3-chol, and apoA-I, offers the opportunity to determine and compare the value of each parameter in predicting risk of CHD. ApoA-I is the major apolipoprotein of HDL (169). While HDL2 particles contain one mole of apoA-I more than HDL3 particles, apoA-I makes up 65 to 70% of total apolipoprotein in both, HDL2 and HDL3 (170,171). In contrast, cholesterol content in HDL2 and HDL3 differs not only on a molar basis, but also when expressed as percentage of lipid. The ratio of cholesterol to apoA-I is twice as high in HDL2 when compared with HDL3 (170,172). Indices may therefore be derived, to address the importance of the number of HDL particles and the degree of lipid association of these particles with regard to risk of CHD. Such a distinction may bring us closer to an understanding of HDL function.

In normotriglyceridemic subjects, the majority of plasma apoB is associated with LDL. The contribution of VLDL-apoB to plasma apoB may be calculated from plasma triglyceride levels and from compositional data of VLDL (173). LDL-cholesterol can also be calculated. Thus, these measurements should allow to test current hypotheses regarding the predictive value of LDL-cholesterol and LDL-apoB. A more rigorous analytical approach to test this hypothesis is outlined in case control studies of the cohort.

1.3.5 Accuracy and Precision of Measurements

Accuracy of cholesterol, triglyceride, and HDL-cholesterol is traceable to reference material provided by CDC. Accuracy of HDL2- and HDL3- cholesterol is traceable to plasma pools in which these levels have been determined by zonal ultracentrifugation. Unlike for cholesterol there is no generally accepted reference method for HDL2 and HDL3 cholesterol, but zonal

ultracentrifugation affords an excellent separation of the two major HDL subfractions when compared with all other methods currently available. The agreement between HDL subfraction quantitation as obtained by precipitation and zonal ultracentrifugation was excellent. In 16 plasma specimens characterized by a wide range of HDL2 and HDL3 concentrations, correlation coefficients for HDL2 and HDL3 were 0.96 and 0.95, respectively.

Accuracy of apoA-I measurements is traceable to a well characterized apoA-I standard and apoA-I determinations in CDC pools for which consensus values have been developed by the laboratories participating in the CDC apolipoprotein standardization program. Accuracy of apoB measurements are traceable to zonally isolated LDL, which contains apoB as the sole apoprotein (174) and whose protein content is quantified by the Lowry procedure (175). In addition, analyses of apoB plasma pools provided by CDC are also available.

Based on previous experience, average intraassay coefficients of variation anticipated for this study would be 1.5 - 2.5% for plasma cholesterol, 2-3% for plasma triglycerides, 2-4 % for HDL-cholesterol, and 3-6% for HDL3-cholesterol. The average CV for replication of triplicates of unknowns in the apoA-I and apoB should be around 5 to 6% and in the Lp(a) assay between 5 and 10%. Average inter-assay coefficients of variations as determined by using overlapping plasma pools are expected to be 2% for plasma cholesterol, 2-3% for plasma triglycerides, below 6% for HDL-cholesterol, and 5-11% for HDL3-cholesterol. Unlike lipids, plasma apolipoprotein analyses have not been extended for time periods as this program dictates. A reasonable estimate of mean interassay variability of apoA-I and B is between 6 and 10%. Interassay variability of Lp(a) should be around 10 to 15%.

1.4 Justification for Case Control Studies of the Cohort

1.4.1 Measurement of Free Glycerol in Plasma

Free glycerol and glycerol esterified to fatty acids (monoglyceride, diglyceride, and triglyceride) are measured by the enzymatic method of triglyceride determination. However, the contribution of glycerol to total plasma glycerol is small. In one study, the mean contribution of free glycerol to triglyceride was 5.5 mg/dL (131). We have also performed a study on fresh plasma specimens (n=60). Glycerol content in these specimens ranged from 0.2 to 11 mg/dL when expressed as triglyceride. Free glycerol will therefore not be measured in all cohort samples,

but will be reserved for case control studies of the cohort, where a more complete workup is desirable.

1.4.2 Measurement of LDL-apoB and LDL-cholesterol

To understand the association of lipoprotein metabolism and atherogenesis knowledge about structure and function of lipoproteins is necessary. Interindividual differences do occur in the structure of LDL. Among individuals, LDL differs in size, density, and lipid content, while the amount of apoB per particle is fairly constant (177-179). With a few exceptions (180), LDL size and the proportion of lipid to protein does not change within subjects (181). A high cholesterol diet, for example, does not alter size and chemical composition of LDL, but increases the number of LDL particles in the circulation (182,183). LDL size may therefore be inherited (181), and one of the determinants of LDL size may be heterogeneity in the apoB sequence among subjects (184). A possible association between LDL structure and risk of CHD has been described recently. In subjects undergoing coronary arteriography, LDL-apoB separated cases from controls better than LDL-cholesterol (88). These observations were extended to hypertriglyceridemic patients in whom increased LDL-apoB levels were associated with a higher prevalence of atherosclerotic disease (168). Furthermore, the progression of atherosclerotic disease and the development of new lesions in bypass grafts was associated to a greater degree with LDL-apoB than with LDL-cholesterol (185). The importance of the level of LDL-apoB as a risk factor for atherosclerotic disease may be estimated by approximating LDL-apoB from measurements performed in all cohort samples (plasma apoB, plasma TG, plasma cholesterol, HDL-cholesterol). To test a possible relationship of LDL structure with progression of atherosclerosis more rigorously, direct quantitation of apoB and cholesterol in LDL is necessary. In case control studies, VLDL will be removed from plasma samples by ultracentrifugation, and cholesterol and apoB will be measured in the VLDL-free supernate. LDL will then be precipitated to quantitate the contribution of HDL to the cholesterol content in the VLDL-free fraction.

1.4.3 Epitope Expression of ApoB

ApoB is a major apolipoprotein of chylomicrons, VLDL, IDL, and the predominant or sole apoprotein of LDL (186). Major advances have been made recently in unraveling the amino acid sequence of apoB and the nucleic acid sequence of its mRNA (187,188). ApoB contains several antigenic sites, whose expression may differ among individuals. Immunochemical polymorphism of human apoB was originally demonstrated by Blumberg et al using antisera from

patients receiving multiple transfusions (189). Immunochemical heterogeneity of apoB among subjects may result from conformational effects of boundary lipids or true heterogeneity (difference in primary structure). While changes in surface exposure of apoB epitopes have been demonstrated (190,191), sequence heterogeneity of apoB among individuals is still a working hypothesis (184) until differences in primary structure are presented. Immunologic differences of apoB among subjects may be related to the development of atherosclerosis. Increased ratios of apoB to cholesterol in LDL were associated with higher prevalence of CHD (88). Increased ratios of apoB to cholesterol imply changes in the structural organization of LDL, which may result in altered epitope expression. Alternatively, differences in the primary structure of apoB may limit the amount of lipid associated with it, thereby determining the ratio of apoB to cholesterol in LDL. Moreover, animal studies revealed a strong association of certain apoB phenotypes with plaque formation in arteries (192).

The ability to determine the expression of epitopes of complex antigens such as apoB was greatly enhanced by the introduction of monoclonal antibodies (193). These reagents can be produced in unlimited quantities and are homogenous and each specific for a single immunogeneic locus or epitope.

Monoclonal antibodies directed against apoB differ widely in epitope recognition. Some antibodies bind to purified, lipid-free apoB, while others bind to apoB only when associated with lipids as in lipoproteins (194,195). A subset of antibodies reacts with apoB-48 (196), which is the only apoB subspecies produced by the intestine in humans (197). Binding of LDL to fibroblasts may also be inhibited by selected antibodies, indicating the presence of an immunogeneic locus in the vicinity of the LDL receptor binding domain of apoB (196,198). Quantitative studies of various human LDL preparations with a battery of antibodies indicated that expression of some apoB epitopes on LDL is correlated with the core lipid content of particles, whereas the expression of other epitopes correlated with the content of polar surface lipids (198,200). Schumaker and colleagues studied LDL from different individuals by using a set of monoclonal antibodies with closely related specificities (184). These researchers observed weak, intermediate, or strong binding of various LDL preparations to monoclonal antibodies. The division into three phenotypes is consistent with a genetic polymorphism resulting from two codominant alleles specifying apoB sequence. These findings support and extend conclusions derived by Fisher et al in studies on variability of LDL size among individuals (181).

While monoclonal antibodies have increased our knowledge about the structure function relationship of apoB, the utility of these reagents in clinical and epidemiologic studies has not been

demonstrated. The potential of these reagents for such studies is, however, obvious and the use of monoclonal antibodies in case control studies of the cohort represents a logical extension of studies on the importance of the ratio of apoB to cholesterol in LDL.

A battery of monoclonal antibodies will be used to measure apparent apoB content in plasma specimens by RIA technology. Apparent apoB content will be compared with a standard LDL preparation and related to the apoB content determined by polyclonal antisera.

1.4.4 ApoE Phenotyping

ApoE is a human apolipoprotein which mediates the uptake of lipoproteins by fibroblasts via the LDL receptor (201) and serves as a ligand for receptor-mediated endocytosis of lipoproteins in the liver (202). Based on amino acid sequence data, apoE has a molecular weight of 34,145 daltons (203). Plasma levels of apoE average 3 mg/dL in normal subjects but are elevated in hyperlipoproteinemic states (204-206). ApoE occurs in three major isoelectric variants designated apoE-4, apoE-3, and apoE-2. These isoforms differ in their arginine and cysteine content at positions 112 and 158 of the amino acid sequence (207). An additional apoE variant, designated apoE-2*, has been described in which the arginine residue at position 145 is substituted by cysteine (208). Minor, more acidic isoforms of apoE are the result of posttranslational glycosylation of the major isoforms (209).

In man, six apoE phenotypes are distinguished which probably result from three alleles (n2, n3, n4) at a single genetic locus. The n3 allele occurs at the highest frequency and was therefore designated as the wild type (210). Analysis of the nucleotide sequence of the n3 allele showed that the codons for residue 112 and 158 could undergo single base changes to account for the amino acid differences in the product of the n2 and n4 alleles (211).

The clinical and epidemiological importance of apoE phenotyping relates to the association of n2-homozygosity (n2/n2) with familial dyslipoproteinemia (type III hyperlipoproteinemia) (212,213). While the n2/n2 phenotype occurs in about 1% of unselected subjects, only a fraction of these individuals presents with familial dyslipoproteinemia (214). Thus, an additional not yet defined defect of lipoprotein metabolism must be present to precipitate dyslipoproteinemia. Homozygosity for the n2 allele without elevation of plasma lipids is not associated with an increased risk of CHD (215); rather, the n2 allele seemed to decrease the risk of CHD, because the n3/n2

phenotype was less prevalent in patients with CHD when compared with control subjects (215). This observation is consistent with reduced LDL-cholesterol levels found in subjects with the n3/n2 phenotype (215,216). The pathophysiologic significance of the n4-allele is less clear, but the prevalence of the n4-allele in patients with type V hyperlipoproteinemia seemed to be increased (217).

ApoE phenotyping in case control studies of the cohort will serve to: (1) identify subjects with familial dyslipoproteinemia, and (2) determine the association of apoE phenotype with atherogenesis.

The analytic method for apoE phenotyping includes the separation of apoE isoforms by isoelectric focusing of plasma, electrophoretic transfer of proteins to nitrocellulose paper (218), incubation of blots with anti-apoE IgG, and visualization of antigen antibody complexes on the paper by using a second antibody conjugated with horseradish-peroxidase (219).

1.4.5 Restriction Fragment Length Polymorphism (RFLP)

RFLP refers to differences in the size of gene fragments when genomic DNA from different subjects is digested with specific restriction endonucleases. These enzymes have specificities for defined sequences in DNA (220,221). An altered cleavage pattern can arise from mutations within the sequence recognized by the specific enzyme used or from more complex changes in nucleotide sequence such as insertion, deletion or rearrangement of DNA between two cutting sites. The diagnostic value of RFLP is becoming increasingly recognized. For example, RFLP on the 3′ flanking region of structural genes has been reported in sickle cell disease (222,223) and hereditary persistence of fetal hemoglobin (224). Altered phenylalanine hydroxylase genes associated with phenylketonuria may also be detected by this technique (225), and polymorphism in the 5′ flanking region of the insulin gene may provide a genetic marker for non-insulin dependent diabetes (226,227).

Polymorphism in the flanking regions of apolipoprotein genes has been described as well and, in some cases, was found to be associated with lipid disorders and/or premature atherosclerosis. Rees et al (228) reported a Sst-1 restriction site polymorphism in the 3′ flanking region of the human apoA-I gene. The frequency of the heterozygous state was much higher in hypertriglyceridemic subjects when compared with a normal population ($p < 0.001$). Homozygosity for the polymorphism was only found in hypertriglyceridemic patients. A polymorphism in the intergenic region of the apoA-I and apoC-III genes, apparent upon digestion of genomic DNA with Pst I, was much more prevalent

in CHD patients and in subjects with familial hypoalphalipoproteinemia when compared with normal subjects or patients with normal coronary arteries as judged by angiography (229).

Rearrangement of DNA sequences in the gene coding for apoA-I and apoC-III has been described in a rare form of apolipoprotein deficiency accompanied by premature atherosclerosis (230,231). Most recently, a MsP-I restriction site polymorphism within the Alu sequence of the 3' flanking region of the human apoA-II gene was reported (232). Homozygosity for this polymorphism was associated with increased levels of apoA-II but lower apoA-I/apoA-II ratios in plasma. Thus, this polymorphism was linked to retarded apoA-II catabolism or to increased apoA-II gene expression. These researchers reasoned that homozygosity for the polymorphism in the apoA-II gene may confer protection against atherosclerosis, since a negative correlation of apoA-II plasma levels with the prevalence of myocardial infarction (233) and peripheral vascular disease (234) was found. The predictive value of apoA-II plasma levels for CHD is, however, not generally accepted. ApoA-II levels were not discriminatory in some studies (235,236), apoA-I/apoA-II ratios tend to be lower in the plasma of patients with CHD (236-238), and decrease in lipoprotein particles containing apoA-I without apoA-II was strongly associated with the prevalence of CAD (239).

The potential of RFLP to define and recognize disease entities associated with atherosclerosis should develop rapidly with the increasing understand-ing of apoprotein gene structure, regulation of gene expression, and availability of suitable probes. The analytic procedure includes extraction of high molecular weight DNA (240) from peripheral blood cells, digestion of DNA with various restriction enzymes, agarose gel electrophoresis of DNA fragments, transfer of DNA to nitrocellulose paper (241), hybridization of blots with nick-translated cDNA probes (242) or synthesized oligonucleotides, and autoradiography of blots.

1.4.6 Determination of Lp[a] Phenotyping

Although the important correlation of plasma Lp[a] levels with heart disease was demonstrated more than 30 years ago, only recently have major advances in the structural properties of Lp[a] been made. In 1983, it was first established that Lp[a] contained one molecule of apoB disulfide-linked to one molecule of apo[a] (243,244), both of which are very high Mr proteins. The density heterogeneity of Lp[a] has been attributed to apo[a] species of differing molecular weights (245). This size polymorphism of apo[a] may originate from polypeptides of differing lengths and/or differing extents of glycosylation. The

polymorphic pattern of apo[a] is characteristic and constant for each individual (246). In several recent reports, Utermann et al.(247-249), has described the resolution of six Mr polymorphs of apo[a]; no individual possessed more than two Mr species, however, apo[a] polymorphs were totally absent from 44% of the population they tested. Their data suggested that apo[a] polymorphs are genetically controlled and are associated with Lp[a] levels.

Based upon experimental data gathered by us and other laboratories, it is clear that the apo[a] phenotypic pattern of an individual can be useful for assessing cardiovascular risk. For this reason, it seems appropriate that this pattern be determined in a significant fraction of the ARIC population.

1.4.7 Postprandial Lipemia

The major portion of a life time is spent in the postprandial state, which is the time period between food ingestion and the removal of alimentary lipids from the blood. However, the majority of studies examining the lipid transport system and its relationship to risk of coronary heart disease has been performed in the postabsorptive state. The fasting plasma represents an equilibrated state of the lipid transport system in the circulation. This equilibrated state is disrupted by the influx of triglyceride-rich lipoproteins, that result from the ingestion of a fatty meal, and postalimentary lipemia ensuses. The magnitude of the postprandial lipemia varies greatly among individuals, even if they are normolipemic, but is rather constant in an individual, provided that no major changes in lifestyle were made (67). In normolipidemic subjects, the magnitude of postprandial lipemia as observed after a standardized fat load shows a strong inverse correlation with plasma levels of HDL, especially HDL(2). Fasting levels of plasma triglycerides, apoB, and apoE are correlated with the extent of postprandial lipemia (67,250). For comparison of the magnitude of postprandial lipemia among subjects, standardization of the fat load is essential, and several methods have been used to quantify postprandial lipemia. Measurements made during the course of alimentary lipemia may include plasma triglycerides, optical density of plasma, radioactivity after ingestion of labelled triglycerides as part of the fatty meal, lipoprotein mass of triglyceride-rich lipoproteins, retinyl palmitate after ingestion of vitamin A, and the amounts and proportions of apoB-48 and apoB-100 in triglyceride-rich lipoproteins(251-261). Measurements of retinyl palmitate and apoB-48 reflect better the concentration of intestinally-derived lipoproteins than measurements of triglycerides and lipoprotein mass.

Since 1956, several studies compared indices of postalimentary lipemia in subjects with CHD and controls. The majority of studies showed higher levels of postprandial analytes in cases than in controls (251-261). The discriminatory power of tests applied depended on the population studied, the amount of fat ingested, and the type of measurements used. Specifically, smaller fat loads distinguished cases from controls only when markers of intestinalG lipoproteins were measured postprandially. Furthermore, time points late after the ingestion of the standardized meal seemed to have a greater discriminatory power. While there is little doubt, that parameters of postprandial lipemia are associated with CHD, the strength of this association and how it compares to the established risk factors in subsets of the population needs further definition. In addition, previous studies dealt with clear and/or advanced cases of CHD, and several variables introduced by dietary or drug intervention or lack of exercise could have affected the study outcome. ARIC has the unique opportunity, to study and compare indices of postprandial lipemia in subjects with no, early, or advanced atherosclerosis. Furthermore, associations with the coagulation system can be adequately explored.

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2. TECHNICAL PART

2.1 Specimen Processing in Field Centers, Shipment to Central Laboratory, Temporary and Long-term Storage

2.1.1 Specimen Processing in Field Centers

The details of blood drawing and processing in field centers are described in Manual 7. For the measurements to be performed at the Central Lipid Laboratory, important steps include collecting blood into EDTA containing tubes (lavender-stoppered tubes containing 15 mg EDTA per 10 mL tube), keeping the collected blood at 4°C, and separating plasma by centrifuga-tion at 4xC within one hour of blood collection. Two full 10 mL tubes are required for lipid measurements. Plasma is then separated and placed into 10 lavender sample tubes. The following aliquots are prepared:

1. Two 1.5 mL aliquots (for measurement of plasma, cholesterol, plasma TG, HDL-cholesterol, and HDL3 cholesterol, and repeats if necessary)

2. Four aliquots, each containing 0.3 mL plasma for measurement of apolipoproteins and repeats if necessary.

3. Four aliquots, each containing 1.0 - 1.5 mL of plasma for long-term storage. These aliquots may be prepared dividing the remaining plasma from the two lavender stoppered tubes into four equal aliquots.

4. Two aliquots, each containing 1.5 mL of the buffy coat of the two lavender stoppered tubes for DNA extraction.

The plasma aliquots are frozen at -70°C and placed into a prelabeled 3"x6" storage bag. Bags containing the aliquots of one plasma specimen are then placed into a shipping box kept at -70°C.

2.1.2 Shipment of Specimens and Receiving in the Central Lipid Laboratory

Shipping boxes are prepared according to the protocol outlined in Manual 7 and sent once a week (Monday or Tuesday) to the Central Hemostasis Laboratory which delivers a separate container to the Central Lipid Laboratory. Shipments generally contain a maximum of 30 bags. Shipments with over 30 bags require two shipping boxes. Two forms, a face sheet and a contents sheet, and a sample inventory diskette will accompany each shipping box. Upon receipt at the Central Lipid Laboratory, the time and date of

receipt at the Central Lipid Laboratory, the time and date of arrival in the laboratory will be entered in the space provided on the face sheet. The condition of the total shipment with respect to temperature will also be described on the face sheet. On the contents sheet, individual samples will be checked, and deficiencies with regard to volume, labelling, or temperature will be indicated. Both forms will be initialled by the laboratory technician who unpacked the shipments and maintained in the laboratory for inventory purposes.

2.1.3 Temporary and Long Term Freezing of Specimens

Upon arrival, the six aliquots of one plasma specimen, which are used for lipid and apolipoprotein analyses, are transferred into boxes, labelled with the shipment number, and stored at -70^{o}C in a freezer shelf assigned for this purpose.

The four aliquots assigned for long term storage will be placed as received by shipment into standardized freezer containers. Boxes will be labelled according to the shipment identification number assigned by the field center and stored at -70^{o}C in freezers dedicated solely for long term storage.

Documentation of specimens sent by each field center will be as follows. Specimen identification code will be logged in manually into the computer or transferred via diskettes accompanying each shipment. For each sample the sample identification code, arrival date, and a code for sample condition are entered. From these entries a data base is constructed to provide a record of samples received in each shipment, worksheets for analyses by technicians, and a file for interfacing of analytical instruments with the computer (direct data transfer). This data base also provides a file for reporting the data and cataloguing frozen samples.

2.2 Automated Determination of Cholesterol

Cholesteryl esters are hydrolyzed to free cholesterol and fatty acids by cholesteryl esterase. The free cholesterol produced and that already present in the sample is oxidized by cholesterol oxidase to form cholest-4-en-3-one and hydrogen peroxide. Peroxidase catalyzes the reaction of hydrogen peroxide, 4-aminoantipyrine, and phenol (or a substituted phenol) to produce a quinoneimine dye, with maximum absorbance at 500 nm. The absorbance readings at this wavelength are proportional to the concentration of total cholesterol in the samples (1).

2.2.1 Materials

2.2.1.1 Equipment and Supplies

- Gilson pipetman
- Sample cups
- Disposable rotors
- Reagent boats
- Reagent tips
- Sample needles

- Cobas-Bio analyzer: The Cobas-Bio is a compact self-contained centri-fugal analyzer utilizing the horizontal light path principle. The Cobas-Bio is equipped with the following modules:

1. Automatic pipetting station consisting of the sample dish, sample arms, sample needle, sample tubing, washtower, reagent arm, reagent head, reagent tips, a reagent drip cup, and sealable reagent trays. This pipettor is equipped with optical sample selection and is capable of loading one or two reagents with sample and diluent into a twenty-five-place disposable plastic cuvette-rotor. Each cuvette in the rotor has two compartments, one for reagent and one for sample, and a second reagent if desired. Reagents and samples are, thus, kept separate until the rotor accelerates and mixes by means of vibration and breaking.

2. Optical system. The instrument employs a high intensity xenon flashlight source in combination with a holographically inscribed grating monochromator to give linear measurements of absorbance up to 3.0 at 340 nm. The monochromator is capable of selecting any wavelength from 285 nm to 750 nm with a half bend pass of 5.5 nm at 500 nm. Absorbance measurements are made using a horizontal placement of the cuvettes relative to the light path.

3. Rotor including photometer arm, photometer lenses, and cuvette rotors.

4. Microprocessor. The instrument is equipped with a 64 K 8080A micro-processor into which the user may program 19 parameters for each of thirty tests via a keyboard and a display panel on the system. The programmed tests are retained in memory until changed by the operator. In the event of power failure, programs are protected by a battery backup within the system for a period of two weeks. Hard copy printouts are made by an alphanumeric printer. Each printout is identified with test name, number, abbreviated parameter list, and both disk and cup number (2).

2.2.1.2 Reagents

Two reagents are used: deionized water and a standard cholesterol reagent. Tap water is deionized by a Barnsted apparatus. The water is prepurified by being passed through an organic removal cartridge, a submicron filter assembly, and a reverse osmosis membrane. This prepurified water is then passed through four research grade ion exchange cartridges and a 0.2 micron filter cartridge. Conductivity is then measured by an online ohmmeter and is consistently 17 Mohms or greater.

Standard cholesterol reagent is purchased from Boehringer Manneheim Bio-chemical, Indianapolis (Cat No. 236691). The shelf life of this product is 2-3 years. Prepare the reagent by transferring the premeasured powder from the plastic shipping bottle to a 500 mL volumetric flask using a plastic funnel. Add approximately 400 mL of deionized water and dissolve the powder by using a magnetic stir bar. Remove the stir bar after 10 min and add water up to the 500 mL mark. Transfer the final solution, which is light pink in color, and store at 4°C. The reagent is stable for up to four weeks when stored at this temperature.

A backup procedure (3) is used if the sale of the commercially available reagent is stopped or if the quality of the reagent deteriorates.

1. For PIPES buffer, 50 mmol/L, pH 6.9: Add 8.84 g PIPES to 450 mL deionized water in a glass beaker. Stir for approximately 10 min until the solution clears. Equilibrate the solution to 37°C, and adjust, if necessary, to pH 6.9. Quantitatively transfer the solution to a 500 mL volumetric flask. After cooling to ambient temperature, adjust volume to 500 mL with deionized water.

2. For stock phenol reagent: Prepare from phenol stored in a desiccator at room temperature. Carefully but quickly weigh 1.43 g of phenol crystals into a 150 mL beaker, add 50 mL PIPES buffer and swirl to dissolve; transfer solution to a 250 mL volumetric flask, rinse beaker into flask several times with PIPES buffer, and adjust volume to 250 mL with buffer. This reagent may be stored for up to one month at 4°C in a tightly closed glass container.

3. For stock mixed reagent: Transfer to a 250 mL volumetric flask: i) 0.101 g 4-aminoantipyrine, ii) 0.65 g sodium cholate, and iii) 3.73 g KCL. Add approximately 200 mL PIPES buffer to a flask and swirl to dissolve; add 0.50 mL Triton X-100; adjust volume to 250 mL with buffer to avoid bubbles, and mix. This reagent is stable for at least one month at 4°C.

4. For working reagent: Mix 50 mL of the stock mixed reagent and 50 mL of the stock phenol reagent. Add 25 U of cholesterol oxidase, 25 U of cholesterol esterase, and 1250 U of peroxidase (based on the respective specific enzyme activities) either from solutions of the concentrated enzymes or by weighing the dry enzyme preparations.

2.2.1.3 Standard Solutions and Quality Control Material

Critical for the accuracy and precision of the automated cholesterol determination is the use of serum or plasma standards instead of cholesterol solutions. All reference material for cholesterol is prepared from human serum and is obtained from the Centers for Disease Control (CDC). Reference material will consist of serum calibrator (Sercal) to be used as the primary standard and Q-pools to be used for internal quality control purposes. The accuracy of CDC assigned values for cholesterol in Sercal and Q-pools is traceable to pure cholesterol (standard reference material) obtained from the National Bureau of Standards and to definitive analyses performed on CDC pools by the National Bureau of Standards. The target value for each lot of Sercal is established by CDC with the CDC reference method for cholesterol (4). Continuity of the cholesterol analysis is assured by overlapping the analyses of each new Sercal with that of the previous Sercal.

We have experimented with other primary standards containing cholesterol solubilized by detergents or by organic solvents. These preparations gave accurate and precise measurements when the analyses were done manually with the standard reagents and absorbance was read on a spectrophotometer. These standards were, however, unsatisfactory in the automated procedure. Major variations in samples located in proximity to the primary standard were observed in repetitive runs. Since standards and unknowns are aspirated by the same sample needle, poor results may be explained by pipetting errors due to differences in the physiocochemical composition of samples and standards.

2.2.2. Analytical Procedure

After performing the daily maintenance of the Cobas-Bio analyzer, transfer 20 mL of the standard cholesterol reagent to the large well of the reagent boat. Pipette two 0.5 mL aliquots of standard (Sercal) and 0.5 mL of deionized water used for measuring the reagent blank into the designated wells of the reagent boat. Cover the boat with a snap-on lid to prevent evaporation. Fill the sample tray, which has 25 positions, labeled CS and 1 through 24, with empty sample cups. Pipette 0.25 mL of unknown or control plasma samples using a Gilson pipetman and disposable tips into the sample cups. Tray

positions identified by CS, 5 and 15, 10 and 20 receive Sercal, Q-pool (low cholesterol), and Q-pool (high cholesterol), respectively. This leaves space for 20 unknown plasma samples to be analyzed (positions 1-4, 6-9, 11-14, 16-20, 21-24). The sample cups are closed and pushed down in the tray. Place sample tray and reagent boat in the appropriate spaces of the machine, load a clean cuvette-rotor into proper position, place a clean reagent tip onto the head of the reagent arm. Activate the program for cholesterol analysis by depressing the appropriately labeled button on the keyboard and start analysis. Sample volume, diluent volume, and reagent volumes are 4, 10, and 330 μL, respectively. Incubation temperature is 37°C, and running time is 6 min. Cholesterol content of samples is calculated by the microprocessor of the instrument using the Autoblank Endpoint Mode. A factor is derived by:

$$F = \frac{C_{standard}}{(A_n - A_o)stand - (A_n - A_o)blank}$$

where A_n and A_o are absorbance readings at 0 and 6 min at 500 nm. At the end of the run a data printout is generated which contains the factor derived, and results of unknowns expressed in the units of the standards. Absorbance readings may also be printed out when desired. The printout of the conversion factor provides a convenient check for the quality of the reagents. Identification numbers of samples are then transferred from the work sheet to the printout which is kept as a permanent record. The method is linear for up to 500 mg/dL. Measurements exceeding this value are flagged by the instrument. Analysis is repeated subsequent to diluting the plasma with an equal volume of saline.

2.2.2.1 Maintenance Program for the Cobas-Bio Analyzer

Each day laboratory personnel will check the sample needle and sample syringes, clean the optical lenses with methanol, and check the printer paper, the seating of sample tubing, the action of the pipettor arm, reagent delivery, and the uniformity of the diluent stream of the sample needle. Each week the staff will empty and refill the pipette reservoir, empty the waste reservoir, check the sample loop tubing, clean the reagent drip cup, perform precision checks for reagent and sample delivery, fill reagent boat and all sample cups with bichromate solution, activate the appropriate program on the keyboard and record coefficients of variation for sample delivery and reagent delivery, which must be below 2%. Each month the teflon syringe tips and sample needles will be replaced and the photometer check performed.

The field service engineer of Cobas-Bio will be responsible for perform-ing preventive maintenance every three months. In

addition, an emergency service (24-hour response time) is part of the service contract.

2.2.3 Manual Back-up Procedures in Case of Instrument Failure

In case of machine problems exceeding two working days, the manual backup procedures will be in effect. These procedures are: Add 20 μL of water (blank), standard, plasma, and control material to 10x75 mm glass tubes by using an automatic dispenser. Add 2.0 mL of standard cholesterol reagent or backup reagent by using the automatic dispenser to tubes. Vortex tubes and incubate for 12 min at room temperature. Read absorbance of blanks, standards, pools, and unknowns at 500 nm on a Gilford 250 spectrophotometer equipped with an automatic sampling device. Calculate cholesterol content of unknowns by the formula:

$$Conc_X = \frac{(A_X - A_{bl}) \times Conc._{st}}{(A_{st} - A_{bl})}$$

where A_X, A_{bl}, and A_{st} are the absorbances of the unknown, reagent blank, and standard, respectively.

2.3 Automated Determination of Plasma Triglycerides

Triglycerides are hydrolyzed to glycerol and fatty acids by lipase. The glycerol formed is then phosphorylated by glycerokinase in the presence of ATP. Oxidation of the resulting glycerol-3-phosphate to dihydroxyace-tone phosphate and hydrogen peroxide is catalyzed by glycerol phosphate oxidase. An intense red chromogen is produced by the peroxidase catalyzed coupling of 4-aminoantipyrine and sodium 2-hydroxy-3, 5-dichlorobenzene-sulfonate with hydrogen peroxide. The absorbance readings at 510 nm are proportional to the concentration of total glycerol content (free and esterified) in the samples (5).

2.3.1 Materials

2.3.1.1 Equipment and Supplies

- Gilson pipetman
- Sample cups
- Disposable rotors
- Reagent boats
- Reagent tips
- Sample needles
- Cobas-Bio analyzer.

2.3.1.2 Reagents

Deionized water and standard triglyceride reagent are used. Standard triglyceride reagent is purchased from Boehringer Mannheim Biochemical, Indianapolis (Cat. No. GPO 701912). Prepare the reagent in the same plastic bottle in which the premeasured solution is shipped. Using a pasteur pipette, transfer the solution to the vial containing a premeasured amount of reagent powder; dissolve powder to obtain a clear yellow solution. Transfer the clear yellow solution back to the plastic bottle and mix. Rinse the vial which contained the premeasured powder three times with the working solution for quantitative transfer. This reagent may then be stored in the original plastic bottle for up to two weeks at 4°C.

A backup procedure is used if the sale of the commercially available reagent is stopped or if the quality of the reagent deteriorates.

1. Stock-reagent buffer. Add 6.055 g Tris-HC1 to 900 mL distilled water in a 1000 mL beaker and stir to dissolve. Add 1.016 g magnesium chloride and 203 mg 4-aminoantipyrine and dissolve. Adjust pH to 7.6 with dilute hydrochloric acid and add 100 mg Triton X-100, rinse the weighing boat which contained Triton X-100 several times with buffer; stir for 10 min and transfer to 1000 mL volumetric flask; adjust to volume with distilled water. This reagent is stable for at least 1 month at 4°C.

2. HBDS-reagent. Weigh out 2.4 g 2-hydroxy-3,5 dichlorobenzene-sulfonate, transfer to a 50 mL glass beaker, add 100 mg Triton X-100, dissolve in 40 mL Tris-HC1 buffer (pH 7.8, 0.05 M). Transfer to a 50 mL volumetric flask, make volume up to 50 mL with Tris-HC1 buffer. This reagent is stable for at least one month at 4°C.

3. Working reagent. Weigh out the following enzymes and place in an iced beaker containing 50 mL stock reagent buffer: 10 kU lipase, 25 U glycerokinase, 400 U glycerol-3-phosphate: 02oxidoreductase, 1 KU peroxidase, 25.4 mg adenosine 5′-triphosphate. Mix well and quickly transfer to 100 mL volumetric flask. Add 1.0 mL of HBDS reagent and ake volume up to 100 mL with stock reagent buffer.

2.3.1.3 Standard Solutions and Quality Control Material

Glycerol (0.210 g) dissolved in one liter of 0.9% NaC1 containing 0.5 g sodium azide is used as primary standard. Alternatively, glycerol standards are also available through commercial sources such as Boehringer Manneheim (Precimat Glycerol, Cat. No. 166 588, Boehringer Manneheim, Indianapolis, Indiana). Q-pools to be obtained from CDC are used for internal quality control purposes.

2.3.2 Analytic Procedure

Fill 20 mL of the standard or backup reagent into a new reagent boat. Pipette two 0.5 mL aliquots of primary standard and 0.5 mL of deionized water used for measuring the reagent blank into the designated wells of the reagent boat. Cover the boat with a snap-on lid. The sample tray prepared for the cholesterol determination is used without changes. Insert a new rotor, change the reagent tip, and activate the triglyceride program. Sample, diluent, and reagent volumes are 5, 40, and 350 µL. Incubation temperature is 30°C and running time is 5 min. Data are calculated by the Autoblank Endpoint Method as described under section 2.2.3. At the end of the run, transfer sample identification numbers to the printout which is kept as a permanent record. The measurements are linear up to 1000 mg/dL. Measurements exceeding this limit are repeated after dilution of samples with an equal volume of 0.9% saline.

2.3.2.1 Maintenance Program of the Cobas-Bio Analyzer

See section 2.2.2.1.

2.3.3 Manual Backup Procedure in Case of Instrument Failure

In case of machine problems exceeding two working days, a manual backup procedure will be in effect. Add 20 µL of water (blank), standard, plasma, and control material to 10x75 mm glass tubes by using an automatic dispenser. Add 2 mL of standard triglyceride reagent or backup reagent by using the automatic dispenser. Vortex tubes and incubate for 10 min at 37°C. Read absorbance at 510 nm within one hour. TG content of samples is calculated by the formula given in section 2.2.3.

2.4 Automated Determination of Glycerol

Quantification of free glycerol is based on the reaction mechanisms described for triglyceride determination with the exception that lipase is omitted in the reagent mixture. Thus, chromogen will only be derived from free glycerol.

2.4.1 Materials

2.4.1.1 Equipment and Supplies

See Section 2.2.1.1.

2.4.1.2 Reagents

Deionized water and a standard glycerol reagent are used. The enzymes for glycerol determination are obtained as a premeasured powder to provide 20 mL glycerol reagent (Fermco Diagnostics, Elk Grove Village, IL). One glass bottle contains 6.0 U glycerol kinase, 92 U L-a-glycerol-3-phosphate oxidase, 230 U horseradish peroxidase, 15 mmol ATP, 1 mmol Tris HCl to give a pH of 7.6 after reconstitution. The premeasured powder is stored at -20°C. To prepare the reconstituting solution, dissolve in a 200 mL volumetric flask 203 mg MgCl2.6H2O and 41 mg of aminoantipyrine in 150 mL of deionized water. Adjust pH to 7.2 with HCl. Add 20 uL Triton X-100 and fill up to 200 mL mark with deionized water. This solution is stable for up to one month when stored at 4xC. To prepare the working reagent, dissolve the premeasured powder containing the enzymes in 20 mL of reconstituting solution and add 8.6 mg sodium 2-hydroxy-3,5-dichlorobenzenesulfonate (HDCBS). The working reagent must be used within four hours.

2.4.1.3 Standard Solutions

Dissolve 1.045 g glycerol in one liter of 0.9% NaCl containing 1 g per liter sodium azide. This solution corresponds to 1000 mg/dL triolein. Dilute this solution with four parts (v/v) 0.9% NaCl containing sodium azide to prepare 200 mg/dL standard. This solution is further diluted with 19, 9, and 4 volumes of 0.9% NaCl to give solutions equivalent to 10, 20, and 40 mg/dL triolein, respectively.

2.4.2 Analytical Procedure

Transfer 20 mL of the glycerol reagent to the large well of the reagent boat of the Cobas-Bio analyzer. Pipette 0.5 mL aliquots of the 10, 20, and 40 mg/dL standard and 0.5 mL of deionized water used for measuring the reagent blank into the designated wells of the reagent boat. Cover the boat with a snap-on lid. Pipette 0.25 mL of sample or controls into the sample cups. Tray positions identified by CS and 10 receive MQ and the standard equivalent to 20 mg triolein. This leaves space for 23 unknown plasma specimens to be analyzed. Close sample cups and place sample tray and reagent boat in the appropriate space of the Cobas-Bio, load a clean cuvette rotor into proper position, place a clean reagent tip onto the head of the reagent arm. Activate the program for triglyceride blank by depressing the appropriately labelled button on the keyboard and start analysis. Sample volume, diluent volume, and reagent volumes are 10, 20, and 200 µL, respectively. Incubation temperature is 37°C and running time is 7 min. Absorbance readings are made at 510 nm. Glycerol content of samples is calculated by the Autoblank Endpoint Method. At the end of the run, transfer sample identification code on the data printout.

2.4.3 Manual Backup Procedure

Add 20 µL of water (blank) standard, plasma and control material to 10x75 mm glass tubes by using an automatic dispenser. Add 2 mL of glycerol working reagent, incubate tubes for 10 min at 37°C and read absorbance on spectrometer at 510 nm. Calculate glycerol content according to the formula shown in Section 2.2.3.

2.5 Determination of HDL-cholesterol and HDL3-cholesterol

Mixtures of dextran sulfate (molecular weight 50,000), an analog of heparin, and magnesium ions (Mg^{2+}) cause precipitation of low density lipoproteins (LDL) and very low density lipoproteins (VLDL) while the high density lipoproteins (HDL) remain in solution. Precipitated lipoproteins are separated by centrifugation, and the supernate containing HDL is analyzed for cholesterol content. An aliquot of the supernate is reprecipitated with additional dextran sulfate and increased amounts of Mg^{2+} ions, which results in the precipitation of HDL2. The precipitated HDL2 is separated by centrifugation, and the cholesterol content of the supernate containing HDL3 is determined. HDL2-cholesterol is then calculated by subtracting HDL3-cholesterol from total HDL-cholesterol (7).

2.5.1 Materials

2.5.1.1 Equipment and Supplies

- Gilson pipetman, 1 mL
- Gilson pipetman, 100 or 200 µL
- Polystyrene conical centrifuge tubes with caps
- Refrigerated centrifuge
- Disposable transfer pipettes
- Swinnex filter holders equipped with 0.22 mm Millipore filters (25 mm diam.)
- 12x 75 mm borosilicate glass test tubes
- Sample cups
- Reagent boats
- Reagent tips
- Sample needles
- Cobas-Bio analyzer.

2.5.1.2 Reagents

Preservative solution: Dissolve 5 g NaN_3, 0.1 g chloramphenicol, and 50 mg gentamicine sulfate in 100 mL deionized water.

Magnesium Chloride, 0.5 M: Dissolve 10.15 g $MgCl_2.6H_2O$ (stored in dessicator at room temperature) in 80 mL of deionized water in a 100 mL beaker; adjust to pH 7.0 with dilute NaOH solution and

add 1.0 mL of preservative solution. Transfer quantitatively to 100 mL volumetric flask and adjust volume to mark with deionized water.

Magnesium Chloride, 1.5 M: Dissolve 30.45 g $MgCl_2.6H_2O$ in 80 mL of deionized water in a beaker; adjust the pH to 7.0 with dilute NaOH solution and add 1.0 mL of preservative solution. Transfer quantitatively to 100 mL volumetric flask and adjust volume to mark with deionized water.

HDLT Reagent: Place 0.5 gm dextran sulfate in a 50 mL volumetric flask and dissolve in 0.5 M $MgCl_2$ solution.

HDL3 Reagent: Place 0.5 gm dextran sulfate in a 50 mL volumetric flask and dissolve in 1.5 M $MgCl_2$ solution. These reagents are stable for at least one month at 4xC.

Standard cholesterol reagent or cholesterol backup reagent:

See 2.2.1.2.

2.5.1.3 Standard Solutions and Quality Control Material

Since the mean HDL-cholesterol concentration of study samples is expected to be within 40 and 50 mg per dL, a primary standard with low total cholesterol concentration in the range of HDL-cholesterol must be used for analyses of cholesterol in HDL-containing fractions. For reasons discussed in section 2.2.1.3, a pool containing lipoprotein cholesterol rather than cholesterol solubilized by detergents or organic solvents is necessary for automated determination of cholesterol. The MQ series obtained from CDC will function as primary standards. These pools are prepared by diluting pooled serum with 0.9% NaCl to bring the concentration of cholesterol within the range usually found in HDL-cholesterol fractions. Target values for these pools are assigned by CDC and are based on the analyses of two vials in duplicate in a minimum of eight runs with the CDC reference method. To obtain long term stability of cholesterol measurement in the range of HDL concentrations, overlapping analyses of MQ pools is performed in the ARIC Central Lipid Laboratory. The mean cholesterol concentration of a newly introduced MQ pool is determined by duplicate analyses in 50 runs using the previous MQ pool as primary standard. This mean has to be within 5% of the target value assigned by CDC. The value assigned to this pool to be used as primary standard for low cholesterol determinations in future runs will then be the arithmetic average of the target value assigned by CDC and the mean established in 50 overlapping runs.

The pools for internal quality control of HDL-cholesterol measurement consist of the AQ series provided by CDC and locally prepared pools. The AQ pools are prepared at CDC by combining

units of human serum selected according to HDL-cholesterol concentrations to obtain the desired concentration of HDL-cholesterol in the final mixture. Target values for HDL-cholesterol are assigned by CDC in eight runs by the CDC reference method (ultracentrifugation at 1.006 g per mL density, treatment of the bottom fraction with 0.046 mol/L MnCl2 and heparin reagent and analysis for cholesterol by the CDC reference method). Only for HDL-cholesterol are target values assigned. Nevertheless, these pools are also used for quality control purposes of HDL2 and HDL3 measurements. The limits for these measurements are established in 50 runs in the ARIC Central Lipid Laboratory. A second pool prepared locally by combining human plasma of several subjects is used. In this pool the concentration of HDL2-cholesterol and HDL3-cholesterol are determined subsequent to separation of HDL2 and HDL3 by zonal ultracentrifugation (7) prior to freezing aliquots.

2.5.2 Analytical Procedure

2.5.2.1 Separation of HDL Containing Fractions

1. Allow specimens, control materials, and precipitation reagents to equilibrate to room temperature.

2. Pipette 1.0 mL of plasma specimens, standards, and controls into conical plastic tubes; add 0.1 mL of HDLT reagent; immediately after the addition of this reagent, mix the contents of each tube in sequence for at least three seconds with a vortex type mixture.

3. Allow tubes to stand at room temperature for 10 min; transfer tubes into refrigerated centrifuge and spin for 15 min at 2000 g.

4. Remove tubes from the centrifuge and inspect supernate for turbidity. Carefully remove the supernate and transfer into 10x75 mm glass tubes labelled HDLT.

5. Remove 0.5 mL of the HDLT supernate and place in another conical plastic tube labelled HDL3.

6. To the HDL3 labelled tube add 0.05 mL HDL3 reagent, vortex immediately and let stand 10 min at room temperature.

7. Centrifuge HDL3 tubes for 15 min at 2300 rpm in the refrigerated centrifuge.

8. Remove supernate and place into 10x75 mm glass tubes labelled HDL3.

Note: Any turbidity or cloudiness in the supernate indicates incomplete sedimentation of LDL/VLDL and, as a consequence contamination and overestimation of HDL-cholesterol. Turbidity is usually observed in specimens with high triglyceride content. To clear supernates from turbidity, use one of the following methods:

1. To the plastic separation tube, without separating the turbid supernate from the precipitate, add 1.0 mL of 0.9% NaCl solution (g/v) and another 0.1 mL of HDLT reagent. Mix thoroughly with a vortex-type mixer, then centrifuge as previously described. Obtain the clear supernate for further precipitation of HDL2 and estimation of cholesterol content. Make note of the dilution on work sheet to correct final results by factor of 2.

2. Alternatively, the turbidity can be removed by ultrafiltration. Assemble a 25-mm Swinnex filter holder with 0.22 fm Millipore filter, 25 mm diameter. Insert an AP15 and AP20 depth filter of 23 mm diameter within the O-ring over the filter; tighten the assembled filter and attach a 5 mL plastic syringe at the top fitting. Pour he turbid supernate solution in the syringe and force it through the filter with moderate pressure by using plunger of the syringe. The filtrate is usually clear and can be further processed (6).

2.5.2.2 Cholesterol Determination in HDL Fractions

Dextran sulfate magnesium supernates are analyzed by using the Cobas-Bio. The tray positions for control materials are CS and 15 (for MQ), 5 and 20 (for AQ series), and 10 and 24 for the local pool. Supernates labelled HDLT are analyzed in trays with odd numbers. Supernates labelled HDL3 are analyzed in trays with even numbers. HDL2- and HDL3-cholesterol content of the AQ series is determined per batch consisting of 39 unknown samples. Locally prepared HDL pool is analyzed once per working day.

Transfer 20 mL of the standard cholesterol reagent to the large well of the reagent boat. Pipette two 0.5 mL aliquots of standard (MQ) and 0.5 0.5 mL of deionized water (reagent blank) into the designated wells of the reagent boat; cover the boat with the snap-on lid. Pipette 0.25 mL of supernates containing total HDL into sample cups of tray one, three, five or seven. Pipette 0.25 mL of the HDL3 containing supernates into sample cups of tray two, four, six, and eight. Change the reagent tip and insert new rotor. Activate the program for HDL-cholesterol by depressing the appropriately labelled button on the keyboard of the instrument. Sample volume, diluent volume, and reagent volumes are 4, 10, and 200 mL, respectively. Data are calculated by the Autoblank Endpoint Method described in Section 2.2.2. At the end of the run transfer sample identification number to the printout. Multiply cholesterol values by the factor of 1.1 to

obtain HDLT-cholesterol concentration in plasma. Multiply cholesterol values of HDL3 by the factor of 1.21 to obtain HDL3-cholesterol concentration. HDL3-cholesterol represents the difference between HDLT-cholesterol and HDL3 cholesterol.

2.5.3 Manual Backup Procedure

Add 20 µL of water (blank), standard, supernates of specimens and control materials to 10x75 mm glass tubes by using an automatic dispenser. Add 1 mL of standard cholesterol reagent or backup cholesterol reagent; incubate tubes for 12 min at room temperature. Read absorbance at 500 nm on Gilford 250 spectrophotometer. Calculate cholesterol content of fractions according to the formula given in Section 2.2.3. Multiply values by 1.1 or 1.21 to obtain HDLT and HDL3 concentrations.

2.6 Determination of Apolipoprotein A-I (Apo-I) in Plasma by Radioimmunoassay

Determination of apoA-I by RIA is based on its immunological identity. Appropriate dilutions of specimens containing apoA-I are incubated with a fixed amount of iodinated apoA-I and limiting amounts of apoA-I antiserum. Antibody bound apoA-I is then separated from unbound apoA-I using formalin-fixed staphylococcus aureus cells which bind via protein A to the Fc region of IgG molecules. The amount of labelled apoA-I in the precipitate is compared with radioactivity in precipitates produced by known amounts of apoA-I, which allows the calculation of apoA-I in unknown specimens. In human plasma, most apoA-I is bound to lipids to form HDL, and the majority of antigeneic sites of apoA-I is masked in intact HDL, when polyclonal antibodies raised against apoA-I are used to measure apoA-I in plasma (8). Exposure of relevant antigeneic sites of apoA-I can be achieved by inclusion of Tween 20 into the incubation mixture (9).

2.6.1 Materials

2.6.1.1 Equipment and Supplies

- Refrigerated centrifuge
- Gilson pipetman: 200 µL, 1 mL, 5 mL.
- Dilution equipment: The Micromedic automatic pipette equipped with a 50 µL sampling pump and 5.0 mL dispensing pump is used for the preparation of plasma dilutions. Calibration checks to maintain stability are performed at regular intervals. The two pumps are checked separately. Distilled water at 20°C is dispensed into a tap container and accurately weighed with an analytical balance. The volume dispensed is calculated from the weight of water at which the temperature measurements are made (the density of water at 20°C is 0.998 gm per mL). To

increase the accuracy of weighing, dispense in 10 pump cycles 500 uL with the 50 µL sampling pump and 30 mL with the 5.0 mL dispensing pump set at a delivery of 60% of its total volume.

- Gamma counter: The Micromedic 4-600 is an automatic gamma counter with four detectors, counting four tubes at the same time. The instrument is equipped with an MACC assay data reduction system, designed to handle data generated by radioimmunassays. Data reduction may be achieved by the logit procedure or according to the four parameter method (10).
- Siliconized glass tubes: Soak glass tubes (borosilicate tubes, 10x75 mm, 12x75 mm, 13x100 mm) in a 1% solution of ProsilR28 in water (v/v) for several minutes, assuring that all surfaces to be coated come in contact with the solution. Remove tubes from solution and air dry for at least 24 hours.
- Two Gilson fraction collectors.
- Econo columns, 1x30 cm and 1.5x30 cm.

2.6.1.2 Reagents

- Sephadex G-50
- Sephadex G-75
- Anti apoA-I antisera: Antisera are raised locally either in rabbits or goats. These antisera are filtered and stored in aliquots at -70°C.

- Barbital buffer, 0.05 M, containing 1mM EDTA, pH 8.6: Weigh out 103 g sodium barbiturate, 20 g barbital, and 3.72 g EDTA-Na2, dissolve in 10 liters of deionized water.

- Bovine serum albumin, BSA, 10%: Weigh out 100 g of anhydrous BSA (Fraction V) and add slowly under constant stirring to a glass beaker containing 420 mL of deionized water. Stir until dissolved. Transfer solution into 500 mL volumetric flask and add deionized water up to the mark. Transfer solution into dialysis bags and dialyze at 4xC for two days against distilled water. Continue dialysis against 0.05 M barbital buffer, pH 8.6 for two days. Remove dialysis bags and adjust total volume to 1 liter in volumetric flask. Transfer the 10% albumin solution into 50 mL plastic tubes and store at -20°C.

- Barbital buffer containing 2% BSA: Mix two volumes of 10% BSA with eight volumes of barbital buffer.

- ^{125}I-apoA-I

1. Into a 10x75 mm glass tube add in this order:

   a) 0.5 mCi125I
   b) 30 µL 0.05 M phosphate buffer, pH 7.6
   c) 9 µL apoA-I (2.7 mg ApoA-I/mL in 3 M GuHCl)
   d) 15 µL lactoperoxidase (0.10 mg/dL in 0.05 M phosphate buffer, pH 7.6), and
   e) 3 µL of 0.22 mM H2O2 (this is a 1:40,000 dilution of the 30% solution).

2. Incubate for 2 min at room temperature.

3. Add 200 µL of 0.05 M phosphate buffer, pH 7.6 to quench the reaction.

4. Immediately load onto Sephadex G-50 column (1x30 cm) which has been equilibrated with 0.05 M barbital buffer, pH 8.6; elute with the same buffer and collect 50 fractions at 20 drops per fraction. Count each fraction for 0.1 min.

5. Graph counts (y axis) versus fraction number (x axis). Pool peak fractions of the first peak.

6. Measure volume of pooled fractions and add half this volume as 10% BSA.

7. Rinse column with 200 mL of barbital buffer.

2.6.1.3 Standard Solution and Quality Control Material

2.6.1.3.1 Preparation of standard

Fresh pooled human plasma is adjusted to a solvent density of 1.063 g/mL by addition of potassium bromide and ultracentrifuged in a Beckman 50.2 rotor at 10°C for 24 hours at 44,000 rpm. The supernate is removed by aspiration and the infranate adjusted to a density of 1.21 g/mL by addition of solid potassium bromide. After 48 hours of ultracentrifuga-tion in a 50.2 rotor at 44,000 rpm, the HDL is removed from the top of each tube and exhaustively dialyzed against 50 mM NH4HCO3. The HDL is then lyophilized, and the apoHDL is isolated by exhaustive extraction of the lipid with absolute ethanol. After removal of ethanol by centrifuga-tion and decantation, the sample is solubilized in 3M guanidinium chloride (Gdm.Cl) and dialyzed against 50 mM NH4HCO3 to remove residual ethanol. The sample is made 3 M in Gdm.Cl and chromatographed on a 2.5 x 90 cm column of Sephadex G75 superfine. The column is eluted with a buffer containing 3 M Gdm.Cl, 100 mM NaCl, 20 mM Tris-HCl, pH 7.4, 0.01% EDTA. The major peaks are collected and pooled. The apoA-I containing fractions are rechromatographed on the same column. The apoA-I is identified by elution position during gel filtration, amino

acid composition and electrophoretic mobility in SDS polyacrylamide gel electrophoresis (11). Aliquots of apoA-I are stored at -70°C at protein concentration of about 3 mg/mL. Protein is determined by the Lowry procedure (12) using bovine serum albumin as standard.

2.6.1.3.2 Quality control material

Internal quality control of apoA-I determinations is maintained by analysis of overlapping pools. Pools are either prepared from single subjects or from a group of donors. In each assay, at least three plasma pools are analyzed that contain high (more than 150 mg/dL), medium (100-150 mg/dL), and low (below 100 mg/dL) concentrations of apoA-I. Selection of donors is based either on their HDL-cholesterol levels or on previous apoA-I measurements. Blood is collected into EDTA-containing vacutainer tubes (1.5 mg/mL). Plasma is obtained by immediate centrifugation at 4°C. Plasma is then kept on ice, filtered, and divided into aliquots of 100 uL each, and stored at -70°C. In these pools apoA-I content is determined at several dilutions. The range of the apoA-I assay is 5 to 20 ng per dose. Appropriate dilutions of pools are prepared to cover this assay range. Temporary limits of pools are established in 15 assays at a period of stable performance; permanent limits require 30 assays. Our previous experience with such pools indicates stability for at least 12 months, when pools are stored at -70°C. Long term stability is maintained by overlapping analysis of fresh pools in assays controlled by previous pools. Additional quality control material will be derived from CDC. The frequency of analysis of CDC specimens depends on the availability of control material.

2.6.2 Analytical Procedure

On the day before the assay, write assay protocol and label tubes. On the day of the assay, purify the apoA-I tracer, dilute plasma specimen, standard and the antiserum, prepare dilution of Tween-20, and run the assay.

2.6.2.1 Purification of ^{125}I-apoA-I:

1. Equilibrate Sephadex G75 column (Econo 1.5x30 cm) with BSA-barbital buffer.

2. Load 1.0 mL of 125I-apoA-I, elute with equilibration buffer. Collect 60 fractions at 34 drops per fraction. Count all fractions for 0.1 min.

3. Graph counts per fraction and pool peak fractions of the first major peak. Do not include into the pool the shoulder on the leading edge of the 125I-apoA-I peak if such a shoulder is present.

4. Dilute purified tracer for assay:

   a) Take 4 aliquots, 100 µL each, and count for 1 min.

   b) Dilute with BSA barbital buffer to give 15,000-16,000 cpm/100 µL.

5. Rinse column with 200 µL of barbital buffer containing 0.1% sodium azide, but no albumin.

2.6.2.2 Dilution of Control Pools and Plasma Specimens

Place the frozen control pools and the plasma specimens in a rack (in the order of the assay protocol) in a water bath of ambient temperature for 20 min and allow the specimens to equilibrate to room temperature. Dilute specimens 1:3,000 with 2% BSA-barbital in two steps.

1. First dilution: Aspirate 30 µL of sample with the sampling syringe of the dilution equipment (pump setting 60) and dispense with 2.970 mL of 2% BSA-barbital (this corresponds to a setting of the dispensing pump of 59.4%) into siliconized 12x75 mm glass tubes; let stand for at least 5 min.

2. Second dilution: Aspirate 50 µL of the first dilution and dispense with 1.450 mL of BSA-barbital into siliconized tubes (this corresponds to a 100% setting of the sampling syringe and a setting of 29% of the dispensing syringe).

2.6.2.3 Dilution of standard: Dilute standard with 2% BSA-barbital in siliconized tubes for a final concentration of 0.3 µg/mL. This is done by a two-step procedure similar to that described above for samples.

2.6.2.4 Dilution of anti-apoA-I antiserum: Dilute the antiserum which has been previously diluted 1:20 with BSA-barbital according to the specifications given for the particular antiserum.

2.6.2.5 Preparation of Tween 20 dilution: Weigh 0.75 g of Tween 20 and add 20 mL of BSA-barbital.

2.6.2.6 Preparation of ImmunoprecipitinR:

1. Centrifuge the commercial solution containing formalin-fixed Staphylococcus aureus cells at 3000 g for 10 min.

2. Aspirate supernate and suspend pellet in the original volume with phosphate buffered saline, pH 7.2, containing beta-mercaptoethanol (10%, w/v) and sodium dodecylsulfate (3%, w/v). Heat for 30 min at 95°C.

3. Centrifuge cells at 3000 g for 30 min. Aspirate supernate, and suspend cells in barbital buffer.

4. Centrifuge cells at 3000 g for 30 min, aspirate supernate, resuspend cells in barbital buffer, and store at 4°C.

2.6.2.7 Running the assay

1. Pipet in order of written protocol:

   a) Total tubes (tubes 1-6); these tubes receive only 100 µL of tracer and are not spun when harvesting the assay.

   b) Background tubes (tubes 7-9); these tubes receive 350 µL of BSA-barbital, 50 µL of diluted Tween 20, and 200 µL of tracer.

   c) Standard curve (tubes 10-42); volumes of 0, 5, 10, 15, 0, 30, 40, 50, 70, 100, and 150 µL of the standard containing 0.3 ng apoA-I/µL are pipetted in triplicates to give doses of 0, 1.5, 3, .5, 6, 9, 12, 15, 21, 30, and 45 ng/tube.

   d) Controls (tubes 43-60); pipette 25 µL of the low, medium, and high control pool for two separate aliquots per pool in triplicates.

   e) Unknown plasma samples (tubes 100-); pipette 25 µL of unknowns in triplicates.

2. Bring volume of all tubes except tubes 1-9 to 250 µL with 2% BSA-barbital.

3. Add 50 µL of diluted Tween 20 to all tubes except tubes 1-6.

4. Add 100 µL of diluted antibody to all tubes except tubes 1-9.

5. Add 100 µL diluted tracer to all tubes.

6. Incubate all tubes for 30-60 hours at 4°C.

7. Add to all tubes (except 1-6) 100 µL of ImmunoprecipitinR (which has been diluted five-fold with barbital buffer) and incubate assay for 15 min. at room temperature. Add 2 mL of barbital buffer to tubes and centrifuge tubes for 30 min at 3000 rpm.

8. Aspirate supernates and count tubes containing the pellets in the gamma counter for 1 min after selection of the appropriate program. Data reduction is performed by the instrument (both logit and four parameter data reduction is available).

9. Subsequent to counting the assay, precipitate tubes 1-6 with trichloroacetic acid. Add 100 µL of 2% BSA-barbital to tubes, add 1.5 mL of ice cold 10% TCA, vortex, and incubate for 10 min at 4xC. Spin at 1500 rpm at 4xC for 10 min. Siphon and count pellets.

10. Count the fraction of counts precipitated by TCA. Note: To convert results (ng/dose) into mg/dL, multiply results by 12.

11. The assay range is defined as 5 to 20 ng/dose. Unknowns reading outside this range are repeated in the next assay subsequent to two-fold higher or two-fold lower dilution of the specimen.

2.7 Determination of Apolipoprotein B (apoB) in Plasma by Radioimmunoassay

Determination of apoB by RIA is based on its immunological identity. The assay takes advantage of the fact that human LDL when isolated by zonal ultracentrifugation contains apoB as the sole apolipoprotein (13). Human LDL can therefore be used both as a tracer and as a standard. Appropriate dilutions of apoB-containing specimens are incubated with a fixed amount of iodinated apoB (in form of iodinated LDL) and limiting amount of anti-apoB antisera. Antibody bound apoB is then separated from unbound apoB by using formalin-fixed-Staphylococcus aureus cells which bind via protein A to the Fc region of IgG molecules. The amount of labeled apoB in the precipitate is then compared with the radioactivity in precipitates produced by known amounts of apoB (LDL) standard, which allows the calculation of apoB in unknown specimens (14).

2.7.1 Materials

2.7.1.1 Equipment and Supplies

These are the same as described in Section 2.6.1.1 for the apoA-I assays.

2.7.1.2 Reagents

- Sephadex G-50
- Bio-Gel A-5M
- Anti-apoB antisera: Antisera are raised locally either in rabbits or goats. These antisera are filtered, divided into aliquots, and stored at -70°C
- Barbital buffer, 0.05 M, containing 1 mM EDTA, pH 8.6
- Bovine serum albumin, BSA, 10%
- BSA (2%) - barbital buffer
- Phosphate buffer, 0.5 Mol, pH 7.6
- Phosphate buffer, 0.05 Mol, pH 7.6
- ^{125}I-ApoB (^{125}I-LDL).

1. Into a 10x75 glass tube in this order:
   a) 1.0 mC125_I,
   b) 9 µL LDL (3 µg LDL protein/µL),
   c) 16 µL of 0.5 M phosphate buffer, pH 7.6, and
   d) 10 µL chloramine T (0.066 mg/mL in 0.05 M phosphate buffer, pH 7.6).

2. Incubate for 3 min at room temperature.

3. Add 5 µL of sodium metabisulfite (0.2 mg/mL 0.05 M phosphate buffer, pH 7.6); add 0.5 mL barbital buffer, pH 8.6.

4. Immediately load onto Sephadex G50 column (Econo 1x30 cm) which has been equilibrated with barbital buffer; elute with the same buffer and collect 60 fractions at 20 drops per fraction. Count each fraction shielded for 0.1 min.

5. Graph counts versus fraction number and pool the 4 peak fraction of the first peak.

6. Measure volume of pool and add half of this volume as 10% BSA. Store at 4°C. Labelled LDL may be used as tracer for up to three weeks subsequent to iodination.

7. Rinse column with 200 mL barbital buffer.

2.7.1.3 Standard Solution and Quality Control Material

2.7.1.3.1 Preparation of standard

1. Fresh human plasma of 3-10 subjects is pooled to give a total volume of 30-50 mL. The density of the plasma is adjusted to 1.3 g/mL by addition of sodium bromide as described previously (15) and subjected to ultracentrifugation in a Ti 14 Beckman zonal rotor using a density gradient of sodium bromide of 1.0-1.3 gm/mL, which is linear with the rotor volume. Ultracentrifugation is performed at 42,000 rpm, 140 min, and 14°C (13). During unloading, the effluent volume of

the rotor is monitored continuously at 280 nm and 10 mL fractions are collected. The LDL peak typically eluting between 200 and 250 mL of the rotor effluent is pooled at half the peak height, and dialyzed against 0.9% sodium chloride containing 1 mM EDTA, pH 8.0 for one day. LDL is then removed from dialysis bags and ultracentrifuged in a 50.2 rotor for 16 hours at 40,000 rpm. The supernate of tubes is aspirated, and the yellow pellet is gently dissolved in 4 mL of EDTA saline at pH 8.0. LDL is filter sterilized by using 0.22 fm Millipore filters and stored in sterilized plastic tubes. The LDL standard is characterized by electrophoresis in 0.5% agarose and 3% polyacrylamide gels in the presence of 0.1% SDS (16). Protein content of LDL is determined by a modified Lowry procedure using bovine serum albumin as standard. Such LDL preparations maintain their immunological stability at least four weeks.

2. If zonal ultracentrifugation is not available, LDL can also be prepared by conventional ultracentrifugation in fixed angle head rotors. Plasma is ultracentrifuged in a 50.2 rotor at 45,000 rpm for 16 hours. The top layer containing VLDL is aspirated, and the bottom layers are mixed, pooled and adjusted to a density of 1.050 g/mL by addition of solid KBr. LDL is floated to the top of the tubes by ultracentrifugation in a 50.2 rotor at 45,000 rpm for 24 hours. The top layer containing LDL is recentrifuged under the same conditions. The top layer is collected and 0.9% saline is added to bring the solution to a density of 1.025 gm/mL.

   The volume of saline added is calculated by the formula:

$$V_{saline} = \frac{(1.050 - 1.025)}{(1.025 - 1.006)} \times Volume_{top\ layer}$$

   The resulting solution is ultracentrifuged for 16 hours at 40,000 rpm in a 50.2 rotor, the supernatant is aspirated, and the LDL containing pellet is processed as described above.

2.7.1.3.2 Quality Control Material

For internal quality control, 3 plasma pools containing low (below 65 mg/dL), medium (65-100 mg/dL), and high (above 100 mg/dL) pool levels of apoB are prepared. These pools are either prepared from individuals or from several donors. Selection of donors is based on plasma levels of LDL-cholesterol as estimated by the Friedewald formula (19). Plasma specimens are kept on ice, filtered and divided into 100 μL aliquots, which are frozen at -70^{o}C. As described in paragraph 2.6.1.3.2, limits of pools are determined in 30 assays during a period of stable performance. Limits of newly introduced pools are determined in

assays controlled by previous pools. Quality control material provided by CDC is analyzed according to CDC specifications.

2.7.2 Analytical Procedure

One day before assay write assay protocol, label tubes. On the day of the assay purify the apoB tracer, dilute plasma specimen, standard and antiserum, and run the assay.

2.7.2.1 Purification of ^{125}I-LDL

1. Equilibrate Biogel A-5m column (Econo 1.5x30 cm) with BSA-barbital buffer.

2. Load 1.0 mL of 125I-LDL, elute with equilibration buffer. Collect 50 fractions at 34 drops per fraction. Count all fractions for 0.1 min.

3. Graph counts per fraction and pool fractions of the first peak.

4. Dilute purified tracer for assay:
   a. Count 4 aliquots, 100 uL each, for 1 min.
   b. Dilute with BSA-barbital to give 15,000-16,000 cpm/μL.

5. Rinse column with 250 mL of barbital buffer containing no albumin, but 0.1% sodium azide.

2.7.2.2 Dilution of plasma specimens

Place quality control pools and plasma specimens in a rack in the order of the assay protocol. Dilute samples 1:1500 with 2% BSA barbital in two steps:

1. First dilution: Aspirate 30 μL of specimen with dispensing syringe and dispense with 2.970 μL of 2% BSA-barbital into siliconized tubes; let stand for 5 min.
2. Second dilution: Aspirate 50 μL of the first dilution with sampling syringe and dispense with 700 μL of BSA-barbital into siliconized tubes.

2.7.2.3 Dilution of standard: Dilute standard with BSA-barbital in siliconized tubes to give a final concentration of 0.5 μg/mL. This is done by a two-step procedure similar to that described above.

2.7.2.4 Dilution of anti-apoB antiserum: Dilute the antiserum, which has been diluted 1:20 previously, with BSA-barbital according to the specifications given for the antiserum in use.

2.7.2.5 Running the assay

1. Pipet in order of written protocol:

   a) Total tubes (tubes 1-6); these tubes receive only 100 µL of tracer and are not spun when the assays are harvested.

   b) Background tubes (tubes 7-9); these tubes receive 400 µL of buffer and 100 µL tracer.

   c) Standard curve (tubes 10-45); volumes of 0, 5, 10, 20, 30, 40, 50, 60, 80, 100, 150, and 200 µL of the standard containing 0.5 ng/µL are pipetted in triplicate to give doses of 0, 2.5, 5, 10, 15, 20, 25, 30, 40, 50, 75, and 100 ng/tube.

   d) Control pools (tubes 46-63); pipette 50 µL of the low medium and high pool in triplicates. Use two aliquots per pool.

   e) Samples; pipette 50 µL of samples in triplicates.

2. Bring volume of all tubes to 300 µL with BSA-barbital except tube 1-6.

3. Add 100 µL of diluted antibody to all tubes except tubes 1-9.

4. Add 100 µL of diluted tracer to all tubes.

5. Incubate the assay for 36-60 hours at 4xC.

6. Add to all tubes (except 1-6) 100 µL of ImmunoprecipitinR which has been prepared as described in 2.6.2.6 and diluted five-fold with barbital buffer, and incubate tubes for 15 min at room temperature. Add 2 mL of barbital buffer to tubes and centrifuge for 30 min at 3000 rpm at 4°C.

7. Aspirate supernatants and count tubes in gamma counter for 1 min. after selection of the appropriate program.

8. Precipitate tubes 1-6 with 10% trichloroacetic acid. Add 100 uL of 2% BSA-barbital to tubes, add 1.5 mL of 10% ice cold tricholoracetic acid, vortex, incubate for 20 min at 4°C and spin at 2500 rpm. Siphon and count pellet. Calculate the fraction of counts precipitated by TCA. Note: To obtain results in mg/dL multiply results (ng/50 of 1:1500 dilution) by 3.

2.8 Determination of Lipoprotein(a) by Double Antibody Elisa

Lp(a) is a lipoprotein that contains two protein antigens, apo(a) and apoB. Since LDL also contains apoB, this antigen cannot be used as a basis for measuring Lp(a). However, since apo(a) is found in no other lipoprotein than Lp(a), immunochemical measurements based on this antigen can be used to quantify the parent lipoprotein. Goat anti-human apo(a) (purified Ig-G) specific for the apo(a) antigen is bound to the surface of a plastic microtiter plate. When an Lp(a) sample is added to the plate, the lipoprotein binds to the solid-supported antibody. A second antibody, rabbit anti-human apo(a) (serum), is then added to the plate. This results in the formation of a sandwich: plate--goat Ab--Lp(a)--rabbit Ab. Finally, a peroxidase conjugated antibody, anti-rabbit Ig-G--peroxidase, is added. The peroxidase reduces hydrogen peroxide while oxidizing o-phenylenediamine to a colored compound which can be measured spectrophotometrically at 492 nm. The optical density at this wavelength is proportional to the amount of Lp(a) present in the triple antibody complex.

2.8.1 Materials

2.8.1.1 Equipment and Supplies

- Eight (8) channel pipettor
- Immulon I U-bottom microtiter 96-well plates
- Pipet tips
- Rotary shaker
- Flow lab plate reader.

2.8.1.2 Reagents

- Deionized water.
- Phosphate buffered saline (PBS) - 75 mM phosphate, 75 mM NaCl, pH 7.2: Accurately weigh out 2.485 g $NaH_2PO_4.H_2O$, 8.09 g Na_2HPO_4, and 4.38 g NaCl. Transfer these salts to a 1 L volumetric flask and add 900 mL distilled water. Stir magnetically until all salts are dissolved, then adjust pH to 7.2 with 1 M HCl. Dilute the mixture to 1L and store at 4°C until needed.

- Phosphate buffered saline + bovine serum albumin (PBS + 0.5% BSA): Weigh out 5 g of anhydrous albumin and add portionwise to one liter of vigorously stirred PBS. After complete dissolution, adjust pH to 7.2 with 1 M HCl if required. Store tightly closed at 4°C until needed.

- Phosphate buffered saline + bovine serum albumin + Tween 20 (PBS + 0.5% BSA + 0.10% Tween 20): Weigh out one-half gram Tween 20 and transfer 1 liter of PBS + BSA. Stir the mixture gently to avoid frothing. Store tightly sealed at 4°C until used.

- 0.1 M citrate solution: Weigh out 21 g sodium citrate monohydrate and dissolve in 1 liter distilled water.

- 0.2 M phosphate solution: Weigh out 28.4 g Na_2HPO_4 and dissolve in 1 L distilled water.

- O-phenylenediamine substrate solution: Into a 25 mL volumetric flask pipet 6.0 mL of 0.1 M citrate solution, and 6.5 mL of 0.2 M phosphate solution. Add 10 mg O-phenylenediamine and stir mixture magnetically until all solid is dissolved. Dilute the mixture with distilled water to the 25 mL mark. Add 10 µL of 48% hydrogen peroxide. This will provide sufficient substrate solution for 1 microtitre plate. Store in brown bottle and protect from light.

- Goat and rabbit anti-human apo(a): Anti-human apo(a) is purified by immunoaffinity chromatography of anti-human Lp(a) over LDL which has been covaletly coupled to Sepharose (LDL-Seph) as described by Gaubatz et al (18). This purified Ig-G fraction is diluted 1:1000 in PBS before use.

- Peroxidase conjugate of goat anti-rabbit Ig-G: This commercial reagent is used at each dilution of 1:4000 in PBS-BSA-Tween 20.

2.8.1.3 Standard Solution and Quality Control Material

Purified Lp(a) prepared by the method of Gaubatz et al (19) is used as the primary standard. Criteria of purity include (1) absence of all apolipoproteins except apoB and apo(a) as judged by polyacrylamide electrophoresis and Western blotting with cognate antibodies, and (2) absence of LDL as judged by double-decker immunoelectrophoresis (no precipitin line in the upper anti-LDL deck). This primary standard is stored in the presence of proteolytic inhibitors (Trasylol and PMSF) and is prepared fresh on a monthly basis. It cannot be frozen without undergoing some precipitation and/or delipidation. However, in frozen plasma, Lp(a) does retain most of its immunoreactivity over an extended period. Accordingly, the plasma from six subjects with low, moderate, or high levels of Lp(a) have been aliquoted into separate vials and frozen for assaying at periodic intervals for retention or loss of immunoreactivity. Pooled plasmas will not be used since Lp(a) is known to occur as several different particle populations (20) (whose relative abundance varies with the donor) which may exhibit different stabilities when stored frozen over extended times. The secondary standard will be prepared in a series of dilutions falling within the sensitivity range of the assay. Six wells are designated for standard curve samples. Nine wells will be designated for internal quality control. Results obtained from samples run in these wells will be used to determine the acceptability of the data obtained from the remaining sample wells on the same plate.

2.8.2 Analytical Procedures

1. Coat microtiter plates with purified goat anti-human apo(a) (pure IgG fraction). Perform all incubation steps (e.g., 1, 2, 4, 7, 9, 11) using a rotating shaker. Dilute the IgG fraction 1:1000 in PBS buffer (10 μL of antibody in 10 mL of PBS). Pipet 100 μL of the above dilution into each well of the plate and let the plate sit in room temperature for two hours. After initial incubation at room temperature, place the plates in the cold at 4^{o}C for overnight incubation.

2. Next day, remove plates and reagents from the cold and allow them to equilibrate at room temperature. Wash plates with PBS-BSA (100 μL) several times. Add 200 μL PBS-BSA and incubate for 2-4 hours at room temperature to block non-specific sites.

3. While plates are incubating in step 2, prepare dilutions of Lp(a) samples to be assayed using PBS-BSA Tween buffer as dilutent. Wash plates with 2x100 μL of PBS-BSA Tween buffer just prior to dilution of samples.

4. Add 75 μL of sample per well and incubate at room temperature for two hours, then at 4^{o}C overnight.

5. Next day, bring plates and reagents to room temperature and then wash each well with 100 μL of PBS-BSA-Tween 20 three times, followed by 200 μL/well one time.

6. Make 1:750 dilution of rabbit anti-human apo(a), in PBS-BSA-Tween 20 (20 μL of Ab/15 ml PBS-BSA-Tween).

7. Add 100 μL of this antibody to each well on the plate and incubate at room temperature for two hours.

8. Next, wash each well with PBS-BSA-Tween as in 2.8.2.5.

9. Prepare the third antibody (goat anti-rabbit IgG, peroxidase conjugate). Make a 1:2500 dilution of this antibody by adding 10 μL to 25 mL of PBS-BSA-Tween. Add 100 μL of the above antibody dilution to each well. Incubate at room temperature for two hours.

10. Next, wash plates with PBS-BSA-Tween 3x100 μL, 2x200 μL, followed by 2x100 μL with PBS alone.

11. Transfer 160 μL of the above substrate reagent into each well of the ELISA plate. Cover the plate with aluminum foil immediately and allow plates to incubate at room temperature for 30 min.

12. Remove foil and stop reaction by adding 40 μL of 5N sulfuric acid.

13. Read optical density of each well on the plate reader at 492 nm.

14. Calculate results from standard curve of concentration vs. optical density. If optical density > 1.6, sample is diluted appropriately and rerun.

2.9 Measurement of LDL-apoB and LDL-cholesterol

VLDL-free plasma is obtained by ultracentrifugation of plasma which has been stored at -70°C. The VLDL-free infranate is analyzed for contents of cholesterol and apoB. In an aliquot of the supernate, LDL is precipitat-ed by increased concentration of dextran sulfate and Mg2+, to obtain a VLDL- and LDL-free supernate. Cholesterol content in this supernate represents HDL-cholesterol, which is subtracted from the cholesterol content in the VLDL-free supernate, to give LDL-cholesterol. Since apoB is found only in VLDL and LDL, analysis of apoB by RIA in the VLDL-free plasma fraction provides a measure of the LDL-associated apoB.

A pilot study confirmed that ultracentrifugation of plasma stored at -70°C will separate VLDL. A pilot project is under consideration to optimize this method for ARIC sample plasma volumes.

2.10 Expression of ApoB Epitopes

ApoB is a major apolipoprotein of VLDL and LDL, and the sole apoprotein of LDL. ApoB contains several antigeneic sites. Interindividual dif-ferences in the expression of these antigeneic sites have been described (21,22). Monoclonal antibodies are reagents recognizing single immuno-geneic sites or epitopes. Several monoclonal antibodies with differing epitope specificities are used to compare apparent apoB content of plasma with a LDL standard or control pools in solid phase RIA.

2.10.1 Materials

2.10.1.1 Equipment and Supplies

- Titertek pipettor_(50-200 µL)
- Eppendorf repeater pipette with 2.5 mL combitips
- Gilson pipetman (20 µL, 200 µL, 1 mL)
- Disposable 5 mL polystyrene beakers
- Pasteur pipette connected to a vacuum flask
- Immulon 2 Removawell strips with flat bottoms and holders
- Micromedic 4/600 gamma counter
- 16x125 mm borosilicate glass tubes for dilution of antibodies
- 13x100 mm borosilicate glass tubes for dilution of plasma control and LDL standard.

2.10.1.2 Reagents

- Bovine serum albumin, BSA, fraction V
- 125I-Na
- Sephadex G-25
- Anti-LDL affinity purified monoclonal antibodies: Monoclonal antibodies were prepared locally by immunizing Balb/C mice with LDL. Immune mouse lymphocytes were fused with the nonimmunoglobulin secreting mouse myeloma cell line P3x63-Ag.653. Stable clones were propagated, characterized, and injected into Balb/C mice. Monoclonal antibodies directed against apoB were prepared by immunoaffinity chromatography on LDL-sepharose. Antibodies were characterized in cotitration experiments and by immunoblotting to peptides produced by treatment of LDL with Staphylococcus aureus V8 protease.
- Sheep anti-LDL polyclonal antisera
- Phosphate buffered saline, pH 7.4 (PBS)

- PBS containing 1.0% BSA and 0.02% NaN3
- PBS containing 3.0% BSA and 0.02% NaN3
- PBS containing 0.25% BSA and 0.02% NaN3
- 125I-LDL.

1. Equilibrate the Sephadex G-25 minicolumn (1.5x5 cm) with two volumes of 20 mM Tris-buffer containing 150 mM NaCl and 0.3 mM EDTA.

2. Add 10 µL of iodine monochloride (1 M, ICL) to 990 µL of a solution of 1 N glycine which has been adjusted to pH 10 with 1 N NaOH.

3. Dilute ICL further 1:10 with glycine-NaOH.

4. Add to a 10x75 mm tube in the following order:

   a) 0.5 mL of LDL (6 mg/mL 20 mM Tris, 0.15 M NaCl, 0.3 mM EDTA, pH 7.4).
   b) 0.25 mL of 1 N glycine-NaOH, pH 10.0.
   c) 1 mC 125I-Na.
   d) 25 µL of diluted ICl.

5. Mix content of tube and apply to the Sephadex column. Collect 12 fractions each 1 ml.

6. Remove 1 µL from each tube and count for 0.1 min.

7. Pool the first peak of radioactivity, which contains the 125I-LDL.

8. Dialyze the labeled LDL three times against 600 mL of 20 mM Tris, 0.15 M NaCl, 0.3 mM EDTA, 0.02% NaN_3.

9. Determine specific activity of labeled LDL by measuring radioactivity and protein content. Typically, the specific activity is between 300 and 400 cpm/ng protein.

2.10.1.3 Standard Solution and Quality Control Material

The preparation of standard and quality control material is described in Section 2.7.1.3.

2.10.2 Analytical Procedure

2.10.2.1 Time schedule

Day 1: Prepare antibody solutions and bind antibodies to plates. Prepare the washing and blocking solutions and keep in cold overnight.

Day 2: 1. Remove excess antibody and wash plates. Apply blocking solution to plates and leave on plates for two hours. Prepare dilutions of tracer, LDL-standard, unknowns, and controls.

2. Dispense the various solutions into the appropriate wells. Incubate plates overnight.

3. Remove unbound radioactivity, wash plates, and count the assay.

2.10.2.2 Binding of antibodies to plates

1. Assemble as many RIA plates with Immulon 2 Removawell strips as you have antibodies to test. Provide one extra plate to hold strips for determining the total activity per well.

2. Dilute each monoclonal antibody to a final concentration of 25 μg/mL with 0.5% BSA-PBS. Dilute polyclonal sheep antibody to a final concentration of 50 μg/mL.

3. Dispense 50 μL aliquots of the various diluted antisera into all but three wells of the Immulon 2 plates using a Titertek pipettor. The three empty wells situated in the upper left corner of the plate are used to measure nonspecific binding (blank).

4. Cover each plate with Parafilm and shake gently overnight at $4^{o}C$.

5. Remove unbound antibody by suction and wash all wells three times with 200 μL aliquots of 0.25% BSA-PBS solution. Use a 12-channel dispenser to apply washing solutions.

6. Block nonspecific binding sites by adding 200 μL of the 3% BSA-PBS with the Titertek pipettor to each well.

7. Cover plates with Parafilm and shake for two hours at room temperature.

2.10.2.3 Dilution of 125I-LDL: Adjust the labeled LDL to a final concentration of 3 μg/mL by adding 1% BSA-PBS.

2.10.2.4 Dilution of LDL standard: Dilute standard LDL to 50 μg/mL with 1% BSA-PBS. Prepare serial dilutions of the stock dilution. Add equal volumes of the 125I-LDL to the various dilutions. Thus, tubes contain 25, 12.5, 6.25, 3.125, 1.562, and 0.781 μg/mL LDL standard, and 1.5 μg/mL 125I-LDL.

2.10.2.5 Dilution of control material and unknowns: Dilute each plasma specimen 1:100 and 1:200 with 1% BSA-PBS. Add to each dilution an equal volume of diluted tracer to obtain plasma dilutions of 1:200 and 1:400 which contain 1.5 µg/mL 125I-LDL.

2.10.2.6 Running the assay

For each plate, 3 wells are assigned for blanks, 3 wells for references, and 12 wells for 6 LDL-dilutions to be assayed in duplicate. The remaining wells are for controls and unknowns to be assayed at two dilutions in triplicate. Wells used to determine total radioactivity added per well are located on a separate plate.

To avoid excessive drying of sample wells, remove the blocking solution immediately prior to addition of sample to well. This is conveniently done by using a pasteur pipette connected to a vacuum flask. Apply 50 µL aliquots of specimen to well. Cover plates with Parafilm and incubate plates on a shaker overnight at 4xC. On next morning, remove unbound antigen with a 12-channel suction manifold. Wash each well six times with 200 µL of 0.25% BSA-PBS. Remove individual wells from each plate and place them in 12x75 mm glass tubes for counting. Place glass tubes in counting racks, select appropriate program for logit/log data reduction. For each control or unknown, calculate the arithmetic mean of the two dilutions assayed in triplicates.

2.11 ApoE Phenotyping

ApoE occurs in three major isoelectric variants designated apoE-4, apoE-3, and apoE-2. These isoforms differ in their arginine and cysteine content at positions 112 and 158 of the amino acid sequence. ApoE phenotyping will serve to: i) identify subjects with familial dyslipoproteinemia, and ii) determine the association of apoE phenotype with atherogenesis.

2.11.1 Materials

2.11.1.1 Equipment and Supplies

- Microcentrifuge
- Biorad Mini PROTEAN II Electrophoresis Cell
- Biorad Trans Blot Cell - Power Supply
- Gilson pipetman, 20uL, 200uL, 1 mL

2.11.1.2. Reagents

- Tris-HCL Buffer: 0.02M Tris-HCL, 0.1M NaCl, 0.017M Sodium Citrate, pH 7.7
- Ethanol:Ether (v/v,3:1): 300 mL ethanol and 100 mL petroleum ether

- IEF Polyacrylamide Gel: For two gels: Dissolve 6g urea in 10 mL deionized water. Add 0.4 mL of LKB Ampholine 5-7 and 0.4 mL of Servalyt 4-6. Add 5.0 mL of Acrylamide Gel stock solution (30% acrylamide and 0.8% bis-acrylamide) and add deionized water to make 20 mL. Add 0.012 mL of TEMED (Biorad) and 0.13 mL of 10% ammonium persulfate. Mix well and pour immediately into the casting stand.

- Sample Buffer : Take 1 mL of a 0.1M Tris-HCL solution at pH 8. Add 0.1g of decylsodium sulfate (Kodak, cat. num. 10374), 250uL LKB ampholine 5-7 and 250uL Servalyt ampholine 4-6. Add deionized water to 10 mL and mix well. Store frozen.

- Sample Overlay Solution: Combine 4 mL of 20% sucrose, 0.25 mL LKB Ampholine 5-7, and 0.25 mL Servalyt 4-6. Add deionized water to 10 mL and mix well. Store frozen.

- 20% Sucrose Solution: Dissolve 20g sucrose in 100 mL deionized water. Store frozen.

- 0.02M NaOH (Upper Chamber Buffer Solution): To 2.0 mL of 10N NaOH add enough deionized water to make 1 liter.

- 0.01M H2PO4 (Lower Chamber Buffer Solution): To 1.15 mL of 85% stock phosphoric acid add enough deionized water to make 1 liter.

- Gel Fixing Solution: Dissolve 17.25g sulphosalicylic acid and 57.5g trichloroacetic Acid in 500 mL deionized water.

- Gel Staining Solution: Combine 500 mL methanol, 120 mL glacial acetic acid and 380 mL deionized water. Add 0.01g Coomassie Brilliant Blue R-250 (Biorad Laboratories).

- De-staining Solution: Combine 500 mL methanol, 120 mL glacial acetic acid and 1380 mL deionized water.

- Transfer Buffer:

  Stock Solution: 0.6g Tris-HCl, 288.4g glycine in 2000 mL water, pH to 8.3 with HCL.

  Transfer Buffer: To 400 mL of Stock solution add deionized water to make 4 liters.

- Blotting Buffer: To 40 mL of Transfer Stock solution, add 80 mL methanol and deionized water to make 400 mL.

- Wash Buffer:

  Stock Solution: 24.3g Tris, 180g NaCl in 2000 mL water, pH to 7.4 with HCl.

Wash Buffer: To 100 mL stock solution add deionized water to make 1 liter. Add 500 uL Tween-20.

- Blocking Buffer: Dissolve 3.0g Carnation Instant Non-fat Milk in 10 mL of wash stock solution. Add water to make 100 mL. Add 59 uL of Tween-20.

- Goat-anti apoE antisera:

  BSA Buffer Stock Solution: Add 30g BSA to 100 mL of Wash stock solution. Add water to make 1000 mL. Add 500 uL Tween-20. Use this buffer to dilute both antibodies.

  Goat-anti apoE antisera: Gently mix 60 uL of antibody in 20 mL of BSA buffer. Store in cold room until ready to use.

- Rabbit anti-Goat Alkaline Phosphatase Conjugate: Gently mix 100uL of frozen antibody in 50 mL of BSA Stock solution. Store in cold room until ready to use.

- Alkaline Phosphatase Blotting Substrate: From Biorad Kit: Up to 30 minutes before use, mix together one part solution A, one part solution B, and ten parts solution C.

2.11.2 Procedure

2.11.2.1 Preparation of VLDL

1. To 1.5 mL of fresh plasma, add 5 uL of 0.2M PMSF.
2. Add 75 uL of 5% heparin and 75 uL of 2M MgCl2 and vortex.
3. Incubate at room temperature for 30 minutes.
4. Centrifuge in Microcentrifuge for 8 minutes and remove supernatant to obtain pellet.
5. Resuspend pellet in 0.5 mL of Tris-HCL buffer.
6. Add 25 uL of 2M MgCl2, precipitate at room temperature for 30 minutes.
7. Centrifuge in microcentrifuge for 8 minutes, obtain pellet and resuspend in 200 uL of saline.
8. Delipidate sample by incubating at -20^{o}C in 10 mL of ethanol:ether overnight.
9. Centrifuge at 3000rpm at 4^{o}C for 20 minutes and obtain pellet.
10. Re-extract pellet with ethanol-ether. Incubate at -20^{o}C for 2 hours. Centrifuge and obtain pellet.
11. Suspend in 5 mL ether. Incubate at -20^{o}C for 30 minutes. Centrifuge and obtain pellet.
12. Dry pellet under nitrogen gas of at room temperature.
13. Store delipidated VLDL in -20^{o}C freezer.

2.11.3 IEF Gel Procedure

1. Cast a MiniProtean IEF polyacrylamide gel. Store at 4°C in the dark before use.
2. Prepare samples by dissolving a nitrogen-dried sample in 15 uL of 8M urea.
3. Add 10 uL of sample buffer and 1.5 uL of mercaptoethanol.
4. Incubate at room temperature for 30 minutes.
5. Add 10 uL of the 20% sucrose solution and load samples on gel.
6. Overlay with 20-30 uL of the overlay solution.
7. Overlay with 0.02M NaOH.
8. Fill upper buffer chamber with 0.02M NaOH.
9. Fill lower buffer chamber with 0.01M phosphoric acid. Add a stirring bar.
10. Place gel apparatus in an ice water bath on a stirring plate.
11. Run gel at 300V for 30 minutes and 400V for 2 hours.
12. Place each gel in the fixing solution for at least one hour. Stain in 60°C oven for 1 hour. Destain until bands appear.
13. Place second gel in blotting buffer for 30 min.

2.11.4 Gel Transfer Procedure

1. Prepare nitrocellulose paper by soaking it in transfer buffer for 10 minutes.
2. Load gel and paper into transfer apparatus in the following order:
    1. Mesh packing material
    2. Whatman filter paper
    3. Nitrocellulose paper
    4. Acrylamide gel
    5. Whatman filter paper
    6. Mesh packing material
3. Place gel in Trans-Blot appartus, making sure the paper is towards the positive pole.
4. Run at 400mA for 2 hours.
5. Label gel edges and lanes on paper with a ball point pen.
6. Soak nitrocellulose paper in Blocking solution at 4°C overnight.
7. Wash in washing buffer, 3 times for 15 minutes on a shaker.
8. Incubate in goat anti-apoE antisera overnight at 4°C.
9. Wash in washing buffer three times and incubate in rabbit anti-goat IgG alkaline phosphatase conjugate solution 1 hour at room temp.
10. Wash in washing buffer three more times and develop in alkaline phosphatase blotting substrate at room temperature for 15 minutes.
11. When color has developed, rinse paper in deionized water to stop the reaction.

12. Dry paper and store in the dark.

2.11.5 Sources of Material

Goat anti-ApoE antibody, titer 0.41, Cat no. 008SHJ, Daiichi,Japan.

Rabbit anti-Goat IgG Alkaline Phosphatase Conjugate, Cat no. 172-1037, Biorad Laboratories, Richmond CA.

Alkaline Phosphatase Blotting substrate Kit, Cat. no. 170-6549, Biorad Laboratories, Richmond CA.

2.12 Restriction Fragment Length Polymorphism (RFLP)

RFLP refers to differences in the size of gene fragments when genomic DNA from different subjects is digested with restriction endonucleases. The procedure includes extraction of high molecular weight DNA from peripheral leucocytes, digestion of DNA with suitable restriction endonucleases, electrophoresis of the digested DNA in agarose, transfer of the digested NDA to nitrocellulose paper, hybridization of the paper with a labeled probe specific for the genes to be studied, and visualization of the hybridization products by autoradiography.

2.12.1 Sample Preparation (in field centers)

After removal of plasma in the lavender tubes, pipette with a Pasteur pipet 1.5 mL/per 10 mL tube of the top of the formed blood elements and transfer into labeled specimen tube. Since two lavender tubes are collected per participant, 3 mL of buffy coat are obtained per participant. Freeze at -70^{o}C and send to Central Lipid Laboratory with regular shipment.

2.12.2 Extraction of DNA from leucocytes

2.12.2.1 Materials

2.12.2.1.1 Equipment and Supplies

- High-speed refrigerated centrifuge.
- Constant temperature water bath.

2.12.2.1.2 Reagents

- STE buffer: 0.1 M NaCl, 0.05 M Tris-HCl, pH 7.5, 1 mM EDTA, pH 7.4.
- 10% sodium dodecylsulfate.
- Phenol/chloroform: 25 volume redistilled phenol, 24 volumes chloroform, 1 volume isoamylalkohol.
- 2 M Na Acetate.

- TE buffer: 10 mM Tris-HCL, pH 7.5, 1 mM EDTA, pH 7.4.
- Proteinase K: 10 mg proteinase K/ml in 10 mM Tris-HCl, pH 7.5. Store at -20°C.
- Ribonuclease: 10 mg/mL in 10 mM Tris-HCl, pH 7.5. Boil solution for 10 min in water bath. Store at -20°C.
- 95% ethanol: Store at -20°C. All solution with the exception of SDS, ribonuclease, proteinase K, and the phenol mix are autoclaved.

2.12.2.3 Procedure

1. Transfer buffy coat to sterile plastic tube, and bring volume up to 20 mL with STE. Add 220 µL of proteinase K solution and 1 mL of the 10% SDS solution.

2. Incubate for 3-16 hours in water bath at 50-55°C.

3. Add equal volume of phenol mix and shake gently for 10 min. Place tube on ice for another 10 min.

4. Spin at 4000 rpm for 10 min. Collect upper phase.

5. Adjust upper phase to 0.2 M Na-acetate. Slowly add 2 volumes of cold 95% ethanol. Place tubes at -20°C for two hours. Pellet DNA by centrifugation at 5000 rpm for 15 min.

6. Wash pellet with 70% EtOH. Drain EtOH from DNA clump by touching DNA to the side of the sterile tube.

7. Dissolve DNA in 2 mL of TE.

8. Add ribonuclease to a final concentration of 100 µg/mL.

9. Incubate at 37°C for one hour. Gentle shaking optional.

10. Add SDS to a final concentration of 0.5% and proteinase K to a final concentration of 100 µg/mL.

11. Incubate at 50-55°C for one hour.

12. Add an equal volume of the phenol mix and shake gently for 10 min. Then place tube for 10-20 min in ice bucket to allow SDS to precipitate.

13. Spin 4000 rpm for 10 min. Collect upper phase.

14. Add Na-acetate to 0.2 M and 2 volumes of cold 95% EtOH. Place tube at -20°C for two hours.

15. Spin at 5000 rpm for 15 min to pellet DNA.

16. Wash the DNA pellet with 70% EtOH. Drain EtOH and air dry DNA for a few minutes.

17. Dissolve DNA in 1 mL TE. Quantify DNA by measuring absorbance at 260 nm. Take 5 µL of DNA solution and add 1 mL of distilled water. Mix well and read absorbance. Calculate DNA concentration based on 1 OD at 260 nm = 50 µg DNA. A DNA solution, free of protein, should have a 260/280 value of 1.7-2.0. Check size of DNA by electrophoresis in 0.8% agarose.

2.13 Lp[a] phenotyping

The procedures will be provided following their acceptance for publication.

2.14. Postprandial Lipemia

Program development is ongoing.

2.15 References

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3. Data Transmission

Specimens contained in each shipment from each field center are accompanied by the ARIC batch shipping log and a diskette describing the number of specimens. Subsequent to the processing of the samples from each batch shipment, the following information is recorded: ARIC batch number; data received; time period of sample collection within that particular batch; number of samples; assigned Central Lipid Laboratory number; designated long term storage area and transport condition of the shipment. The following code is recorded for the samples in each shipment.

00 = arrived frozen and unbroken
01 = arrived frozen, but vial broken
02 = thawed in transit, but vial not broken
03 = thawed in transit, and vial broken

If any discrepancy is noted with regards to sample identification or number of donor specimens, the person processing the ARIC shipment contacts the appropriate field center and resolves the problem. All such interchanges are logged. The ARIC batch shipment information can either be manually entered into the data base or acquired from the diskette received from each field center with each batch.

Q-Pro 4 of Quic-N-Easi Products, Inc. is the data base used for the recording, processing, and reporting of data from the field centers to the Coordinating Center. This program is installed on the Compupro CPM operating system in the Central Lipid Laboratory.

Information recorded with the ARIC batch shipments received that week is the basis of the data base for storing data and generating daily work sheets or technicians. It has been decided to assign to each ARIC sample its own unique sequential Central Lipid Laboratory number. This facilitates sample processing and data production. When using work sheets, these tubes receive numbers between 0001 and 9999, with a prefix of A for the ARIC study. Such numbers are easily written and identified by technicians and supervisors. A sample work sheet includes the Central Lipid Laboratory specimen number, the corresponding ARIC sample number, tube numbers in the assay, assay date, and test being determined. The following assays are performed on each specimen: plasma cholesterol, plasma triglyceride, HDL-cholesterol, HDL3-cholesterol, plasma apoA-I, plasma apoB, and plasma Lp(a).

Following each assay, the results generated for each sample are recorded in the Central Lipid Laboratory data base. Each

specimen record is generated containing the following pieces of information or fields within the record. The preliminary list of fields for each analyte data point for each specimen is listed below.

1. Central Lipid Laboratory sample identification number.
2. ARIC specimen number.
3. Sample condition: The following code is used to record the appearance of the sample at the time of analysis:

   00 = color normal
   01 = hemolyzed
   02 = icteric
   03 = lipemic
   04 = hemolyzed and lipemic
   05 = icteric and lipemic
   06 = utilized long-term storage aliquots
   07 = QNS - used all 10 aliquots
   08 = QNS - not enough from field centers

4. Run code: The code from each assay allows the supervisor to identify the date, time of the assay, and the technician responsible for these data.
5. Raw data.
6. Reported data: If there are dilution factors to be accounted for in data analysis, the raw data are transformed and reported here.
7. Data status code

A medical alert level for triglyceride of 1000 mg/dL has been established. The field centers are notified by telephone or electronic mail if ARIC specimens equal or exceed the alert status.

At the present time, data for ARIC specimens are generated by three instruments: the Micromedics Gamma counter for apoA-I and apoB determina-tions, and the Cobas-Bio for plasma lipid and lipoprotein lipid determina-tions, and an ELISA plate reader for Lp(a) measurements. These instruments are equipped to collect and reduce data. Each instrument meets the interface specifications necessary to transmit data, raw, and reduced, to the main central processing unit (CPU). These direct data transfers can be performed in ASCII via RS 232C connectors for these instruments.

The raw data from a sample assay are transmitted to the CPU and be stored in a designated disk area. A software program has been developed to retrieve only the data needed for the final data

base. For example with the apoA-I assay, the sample identification code, the run code, and the mean of these replicates are forwarded to the main data base. Structured within this software program is the ability to flag those samples which fall out of control limits. The raw data can be stored and down-loaded to 5.25 inch disks or to the 10 megabyte magnetic tape backup which is installed in the CPU. A hard copy of raw data is available which is printed by the analytical instruments. In the event of a communications problem between instruments and computer, all the information for the ARIC study can be entered manually until the difficulty is corrected. The organization of shipments, tests, and results in form of a data base will provide the framework for the orderly retrieval of data for reporting. From the updated data base, reports in the form of diskettes will be generated for and mailed to the Coordinating Center on a weekly basis. A post card from the Coordinating Center will confirm that the data have been received. Immediate problems will be handled by telephone calls.

4. Quality Control Procedures

The success of the project depends on the accuracy and precision of measurements performed over the entire study period. Such a goal can only be achieved by the implementation of rigorous quality control procedures. For lipid measurements, guidelines have been established by the LRC Program, and CDC continues to provide quality control material, which facilitates the operation of a laboratory within the recommendations set forth by CDC. Analogous quality control procedures are also applied for quality control of apolipoprotein measurements.

Key components of the quality control program are the use of internal quality control material and the analysis of external surveillance samples. These two components assure that the accuracy of measurements is traceable to reference material. Precision of measurements is estimated from the analytical error obtained in analysis of quality control material, which is assumed to represent the error obtained in the analysis of participant samples.

4.1 Quality Control of Plasma Lipid Determinations

4.1.1 Internal Quality Control

4.1.1.1 Quality Control Pools

The internal quality control material for measurement of plasma cholesterol and triglycerides includes two plasma pools which are provided by CDC. Lipid values assigned to these pools by CDC are traceable to standard reference material obtained by the National Bureau of Standards. Plasma cholesterol and triglyceride concentration in these pools corresponds to low-normal or high plasma levels of these two analytes when compared with their distribution in American populations (1).

Control pools are treated like regular samples. For every 18 plasma samples - this is the number of samples analyzed in one run using the Cobas-Bio analyzer - the two pools are analyzed in duplicate. Since at least two runs are performed per working day, a minimum of four analyses per pool per working day is done. Before a control pool is depleted, replacement pools are analyzed in overlapping fashion, to assure longitudinal stability of measurements. During periods of overlapping analyses, preliminary control limits for the new pools are derived from analyses in 20 working days, and final limits are calculated from 50 analyses performed on different days.

4.1.1.2 Calculation of Internal Quality Control Limits
The daily mean, $\bar{x}$, for each control pool, is calculated according to the formula:

$$\bar{x} = \frac{\Sigma x}{n}$$

where n is 4 or more for the Q-pools (pools for the low-normal and high cholesterol and triglyceride) and 2 or more for the serum calibrator to be used as primary standard of plasma cholesterol measurements. The mean of the daily means, the overall mean, x, is calculated by the formula:

$$\bar{\bar{x}} = \frac{\Sigma \bar{x}}{N}$$

where N is 20 days for preliminary limits and 50 days for permanent limits. $S\bar{x}$ is calculated by the formula:

$$S\bar{x} = \sqrt{\frac{\Sigma(x - \bar{x})^2}{N - 1}}$$

where $S\bar{x}$ is one standard deviation of the daily mean.

Control limits for the daily mean charts are:

Upper limit: $\bar{\bar{x}} + 3Sx$
Lower limit: $\bar{\bar{x}} - 3Sx$

Warning limits for the daily mean control charts are:

Upper warning limit: $\bar{\bar{x}} + 2Sx$
Lower warning limit: $\bar{\bar{x}} - 2Sx$

Apart from the x chart, a range chart is constructed and maintained, to monitor precision. The range, R, for control pools is the difference between the highest and the lowest value obtained in one day:

$$\bar{R} = x_{high} - x_{low}$$

The average range, $\bar{R}$, is then:

$$\bar{R} = \frac{\Sigma R}{N}$$

Where N is 20 days and 50 days for preliminary and definite limits,respectively. Limits for the range are obtained by multiplication of R by the appropriate probability factor which,

for four within-day values, is 1.855 for warning limits and 2.280 for control limits, respectively. In the determination of the definite limits, to be used in the daily charts, outliers will be excluded. An outlier is defined as any value of x or R outside the control limits. Limits are then recalculated.

Additional guidelines for the evaluation of internal quality control limits are based on the target value (TV) provided by CDC. The purpose of these additional rules is to place constraints on both, systematic and random errors. A large relative deviation of x from the TV must be accompanied by a small day-to-day variability to meet the criteria. Conversely, a smaller deviation of the x from the TV allows a larger between-day variability. These guidelines are:

Q-pools	Chol	$\bar{\bar{x}} \pm 2.5S_{\bar{x}}$ within TV $\pm$ 5%
	TG	$\bar{\bar{x}} \pm 3S_{\bar{x}}$ within TV $\pm$ 10%
	Chol, TG	$\bar{\bar{x}}$ within TV $\pm$ CDC limits

CDC limits are assigned by CDC to the quality control pools used for part III of the CDC-NHLBI Lipid Standardization Program. These limits are communicated to laboratories upon participation in this program.

4.1.1.3 Use of Internal Quality Control Limits

Warning signs that will alert the analyst and laboratory supervisor to prepare for corrective actions are:

1. $\bar{x}$ falls between $2S_{\bar{\bar{x}}}$ and $3S_{\bar{\bar{x}}}$.
2. Shifts and trends on the $\bar{x}$ chart, including seven successive values above or below $\bar{\bar{x}}$ and seven successive values in a pattern that indicates an abrupt or progressive change in one direction.
3. Excessive variability, such as seven R values above $\bar{R}$.
4. R exceeds the warning limit.

Runs are repeated - subsequent to taking corrective actions - when:

1. $\bar{x}$ of any control pool falls outside any of the control limits defined in Section 4.1.1.2.
2. Two successive $\bar{x}$ values fall outside the warning limits, or
3. R exceeds the control limit.

4.1.2 External Quality Control

4.1.2.1 Participation in Part III of CDC-NHLBI Lipid Standardization Program

The Central Lipid Laboratory has passed part I and II of this standardization program. Phase III of this program serves to maintain the performance of the laboratory by CDC. Surveillance samples sent by CDC usually cover the range of triglycerides and cholesterol, observed in more than 90% of the population to be studied. Surveillance samples are analyzed in duplicates at weekly intervals, and reported to CDC.

4.1.2.2 Analyses of Blinded Duplicates of Participant Samples

As part of the quality control of lipid measurements, 5% of samples consist of blinded duplicates prepared in the ARIC field centers to monitor intra- and interassay variability.

4.1.2.3 Calculation of Means or Medians of Plasma Lipids or Participants in 3-monthly Intervals

Long term drifts in laboratory measurements can be monitored by calculating means or medians in participant samples stratified according to sex and age. A truncated algorithm (e.g., the central 80%) may be used. While it is possible that plasma lipid profiles do change over the six years of the study, even when age and sex are kept constant in the group to be used, such a measure provides an additional tool to monitor long term drifts in laboratory performance.

4.1.2.4 Participation in Proficiency Testing of the College of American Pathologists (CAP)

The Central Lipid Laboratory is accredited by CAP and will participate in the proficiency testing by CAP.

4.2 Quality Control of HDL-Cholesterol and HDL3-Cholesterol Measurements

4.2.1 Internal Quality Control

Three types of pools are used for quality control of HDL-cholesterol measurements. One type of pool, the MQ-series from CDC, serves to assure precision and accuracy of cholesterol measurements in the range where HDL-cholesterol measurements of study subjects are to be expected. Target values for MQ are provided by CDC. In one run, MQ is analyzed in duplicate. Since at least two runs are performed in a typical working day, at least four values are obtained for this pool. The second type of pool belongs to the AQ series, which is also provided by CDC. This pool is carried through the precipitation procedure like a regular plasma sample. Per batch of 39 sampleided by CDC. This pool is carried through the precipitation procedure like a regular plasma sample. Per batch of 39 samples, one AQ sample is co-precipitated. The supernate of one AQ is analyzed for cholesterol in duplicate. Target values for the AQ pool series are assigned by CDC in eight runs according to the CDC reference method. This method consists of ultracentrifugation at d 1.006 g/mL, precipitation of LDL in the infranate by heparin and MnCl2+, and analysis of cholesterol by the CD reference method in the LDL-free supernate. Several reports indicate that the dextran sulfate Mg2+ method gives about 5% lower HDL-cholesterol

values when compared with the heparin Mn2+ procedure (2,3), which is also our experience. For quality control purposes, CDC target values will therefore be multiplied by a factor of 0.95 to overcome the systemic bias between the two procedures. CDC does not provide target values for HDL2-cholesterol or HDL3-cholesterol in the AQ series. Nevertheless, the AQ series will be used to monitor stability of quantification of cholesterol in HDL subfractions, since our past experience indicates stability in HDL2- and HDL3-cholesterol in this pool. In addition to the AQ series, performance of cholesterol measurements of HDL subfractions is monitored by using a locally prepared pool, in which the content of HDL2-cholesterol and HDL3-cholesterol has been determined subsequent to separation of HDL3 and HDL3 by zonal ultracentrifugation. One aliquot of this locally prepared pool is analyzed in duplicate per working day. Longitudinal stability is assured by overlapping analysis of pools as described in Section 4.1.1.1.

4.2.1.1. Calculation of Internal Quality Control Limits

The daily mean, x, for MQ, AQ, and local pool is calculated according to the formula given in Section 4.1.1.2. The number of analyses is 4 or more for MQ and 2 or more for AQ and local pool. Calculation of x, Sx, temporary and permanent warning and control limits is described in 4.1.1.2 as well. Precision is monitored by using a range chart. Limits for the range chart are obtained by multiplication of R by the appropriate probability factors. For AQ and local pool (two within-day values), the range warning limit is 2.46 R, and the range control limit is 3.27 R. For MQ (four within-day values), the range warning limit is 1.855 R, while the range control limit is 2.280 R.

Additional guidelines for HDL-cholesterol determinations are:

MQ	Chol	$\bar{\bar{x}}$ within 5% of TV or within 3 mg/ml of TV
AQ	Chol	$\bar{\bar{x}} - 3S_{\bar{x}}$ limits within TV ± 25%
MQ	Chol	$x - 3S_{\bar{x}}$ limits within TV ± 15%

The TV of AQ is defined as 95% of the TV provided by CDC, when assignment of TV was based on precipitation by heparin and Mn2+. Warning signs which will trigger a review of procedures are the same as described in Section 4.1.1.3.

Cholesterol analyses in HDL-containing supernates are repeated when:

1. $\bar{x}$ of MQ falls outside any of the control limits,
2. Two successive $\bar{x}$ values of MQ fall outside the warning limits, or
3. R of MQ exceeds the control limit.

The HDL precipitation procedure is repeated when:

1. $\bar{x}$ of AQ or local pool falls outside any of the control limits,
2. Two successive $\bar{x}$ values for AQ or local pool fall outside the warning limits, or 3. R of AQ or local pool exceeds the control limit.

4.2.2 External Quality Control

The external quality control for HDL2- and HDL3-cholesterol are similar as described in Section 4.1.2 for plasma lipids. CDC samples are analyzed for total HDL-cholesterol only, since CDC currently provides no surveillance samples for cholesterol in HDL subfractions. However, HDL subfraction cholesterol are monitored in blind duplicates provided by the field centers, and long term drifts are monitored by calculation of means for participants in 3-monthly intervals.

4.3 Quality Control of Apolipoprotein ApoA-I and ApoB Measurements

Despite the progress made in the characterization of apolipoproteins and in the understanding of apolipoprotein function, maintaining accuracy and precision in their quantitation is still a major challenge for a laboratory. Major variables in the quantitation of apolipoproteins by RIA are the standards, tracer, and antisera. It is therefore important to keep these sources of variation as constant as possible. Thus, one of the principles in maintaining long term stability is to avoid changing more than one of these variables within a reasonable period of time. There is no need to change the method of labeling apolipoproteins. ApoA-I standard is prepared in a quantity to last for the entire study period provided that this standard retains immunologic stability. ApoB standard (human LDL) may need to be prepared in monthly intervals because of instability of LDL. To avoid possible effects of interindividual differences in LDL on apoB quantitation, LDL is prepared from fresh plasma obtained from several subjects. With regard to antisera, we intend to use one batch of antiserum per analyte for the duration of this study. However, it has been our experience that identical results can be obtained with different antisera. Should a change in antiserum become necessary, equivalence of results with the previous assays will be established by using internal quality control pools.

4.3.1 Internal Quality Control

4.3.1.1 Quality Control Pools

Three plasma pools are prepared that contain high, medium, and low levels of the apolipoprotein to be measured. As a guideline for the preparation of pools, low, medium, and high plasma levels of apoA-I are defined to be less than 100 mg/dL, 100-150 mg/dL, and above 150 mg/dL. Low, medium, or high levels of plasma apoB are defined to be less than 65 mg/dL, 65-100 mg/dL, and above 100 mg/dL. Aliquots of pools are stored at -70^{o}C. The range of the apoA-I assay is defined as 5-20 ng/dose, which corresponds to plasma apoA-I values of 60 to 240 mg/dL when the dilution of samples is taken into account. More than 95% of specimens analyzed should fall in this range. The range of the apoB assay is defined as 10-70 ng/dose corresponding to plasma apoB values of 30 to 210 mg/dL. Again, more than 95% of unknowns should fall within this range. Analyses of unknowns falling outside the range specified will be repeated. Twice the usual amount of plasma will be analyzed in samples falling below the cutoff point, and half the usual volume of plasma will be analyzed in samples whose apparent apolipoprotein content is above the upper limit of the assay range.

Control pools are treated like regular samples. In each assay, two aliquots of each of the three control pools are analyzed in triplicate. One aliquot of unknown samples is analyzed in triplicate.

Calculation of control limits for accuracy:

The daily mean, $\bar{x}$, for each control pool is defined as the mean of the two aliquots, analyzed in triplicate. The overall daily mean, $\bar{\bar{x}}$, is calculated by the formula:

$$\bar{\bar{x}} = \frac{\Sigma \bar{x}}{N}$$

where N is 15 or 30 days for preliminary or definite limits. Sx is calculated by the formula:

$$S_{\bar{x}} = \frac{\Sigma (x - \bar{x})^2}{N - 1}$$

Warning limits for the daily mean chart are:

$$\text{Upper limit: } \bar{\bar{x}} + 2S_{\bar{x}}$$
$$\text{Lower limit: } \bar{\bar{x}} - 2S_{\bar{x}}$$

Control limits for the daily mean chart are:

$$\text{Upper limit: } \bar{\bar{x}} + 3S_{\bar{x}}$$
$$\text{Lower limit: } \bar{\bar{x}} - 3S_{\bar{x}}$$

Precision of analyses is monitored in two ways. Each sample is analyzed in triplicate, which provides a mean and CV for each sample. In addition two aliquots of each pool are analyzed in triplicate, which provides additional information on errors produced in preparing the dilutions of plasma lipids.

A range chart is constructed to monitor intra-assay variability in a strict sense, i.e., taking into account all dilutions. The range, R, for control pools is the difference between the two means of the triplicate analyses of aliquots per pool. In calculating the mean, outliers will be omitted. These are defined as single values differing by 40 or more percent of the mean of the two remaining replicates, should the CV of the two remaining replicates be less than 10%. The average range, R, is then calculated by:

$$\bar{R} = \frac{\Sigma R}{N}$$

Where N is 15 or 30 days for preliminary or definite limits. Limits for the range are obtained by multiplication of R with the probability factor of 2.46 (warning limits) or 3.27 (control limits).

An entire assay will be declared out of control and repeated, when:

1. $\bar{x}$ for two of the three control pools falls outside the control limits.
2. $\bar{x}$ values of two pools fall outside the warning limits on three successive days, or
3. R of two pools exceed the control limit.

An assay will be repeated in part, i.e., only those samples whose apparent apolipoprotein content falls in the range of the respective pool, when:

1. $\bar{x}$ of the respective pool falls outside the control limits.
2. $\bar{x}$ values of the respective pool fall outside the warning limits on three successive days, or
3. R of the respective pool exceeds the control limits.

Individual samples will be repeated when the CV of a given sample exceeds 18% of its mean value.

To maintain long term stability of analyses, overlapping analysis of newly prepared pools is performed in assays being controlled by the outgoing pools. To be prepared for changes in immunoreactivity of the primary pools upon storage, complimentary pools are stored. Temporary limits of these pools are established. Should there be any indication that immunoreactivity has changed within a given pool (i.e., drifts or shifts of pools, two subsequent analyses outside of the $2S_{\bar{x}}$ limits), the backup pool covering the complementary range is analyzed in the next assay together with the questionable pool.

4.3.1.2 Calculation of mean lipid to apolipoprotein ratios

The majority of apoA-I in plasma is associated with HDL, while the majority of apoB in normotriglyceridemic plasma is associated with LDL. While the ratio of cholesterol to apoA-I may vary among individuals, mean ratios for a group of 100 participants - the approximate number of unknowns analyzed per assay - should be fairly constant. Similarly, mean ratios of apoB to LDL-cholesterol should be relatively constant, provided that the group of samples does not include a substantial portion of hypertriglyceridemic specimens. Since quantitation of lipoprotein lipids is more established and can be better controlled by external quality control material, these ratios should provide a guide for the quality of apoprotein determinations. Thus, mean ratios for apoA-I/HDL-cholesterol and apoB/LDL-cholesterol will be calculated for each RIA. Such a measure requires adequate computing capabilities by means of a data base and will only be exercised after development and installment of adequate software. Limits of mean ratios will be established in 15 assays. An overall mean ratio, $\bar{\bar{x}}$, and $3S_{\bar{x}}$ will be calculated to monitor apolipoprotein quantitation. Assays in which ratios are outside the limits will be reviewed and repeated. (We would like to stress, however, that we have not yet exercised this kind of quality control and new definitions may become necessary dependent on the feasibility of such a procedure.)

4.3.2 External Quality Control

As part of the quality control of apolipoprotein measurements, 5% of samples consist of blinded duplicates prepared in the field centers. The means or medians of apolipoprotein levels of participants are calculated for 3-month intervals. As described in Section 4.1.2.3, this procedure serves to permit recognition of long term drifts of measurements. CDC samples are analyzed according to the specifications of the apolipoprotein standardization program of CDC.

4.4 Quality Control of Lp(a) Measurements

4.4.1 Screening and Dilution Selection

Three individual non-pooled plasmas are prepared that contain low (less than 75 μg/mL), medium (75-150 μg/mL), and high (more than 150 μg/mL) levels of apoLp(a) to be used as internal standards. Aliquots of pools are stored at -20°C. The range of the apoLp(a) assay is 0.075-.525 ng/dose. Because plasma levels of Lp(a) vary so widely among different individuals (5-700 μg/mL), no single dilution can be selected so that more than 50% of the unknowns will fall in the range of the assay. Therefore, a screening assay using the electroimmunoassay (EIA) is initially run to obtain a rough estimate. Upon the basis of this estimate, an appropriate dilution can be chosen for the more sensitive ELISA measurement. When this approach is used, >95% of the unknowns should fall within the assay range. Analysis of unknowns falling outside the range specified will be repeated. For samples whose Lp(a) concentration is below (above) the cutoff point of the EIA, a 3X larger (smaller) sample will be used for the ELISA. All internal standards are assayed at the same dilution so that they will fall at the high, medium, and low parts of the standard curve. On each plate, the three quality control standards are analyzed in triplicate as are the unknown samples.

Calculation of control limits for accuracy:

$$\bar{x} = \frac{\Sigma x}{N}$$

The daily mean, $\bar{x}$, for each control standard is defined as the mean of the standards from all microplates used, analyzed in triplicate. The overall mean, the mean of the daily means, x, is calculated from the formula:

$$\bar{\bar{x}} = \frac{\Sigma x}{N}$$

where N is 15 or 30 for preliminary or definite limits. Sx is calculated by the formula:

$$S_x = \frac{\Sigma (x - \bar{x})^2}{N - 1}$$

Warning limits for the daily mean chart are:

Upper limit: $\bar{\bar{x}} + 2S_x$
Lower limit: $\bar{\bar{x}} - 2S_x$

Control limits for the daily mean chart are:

$$\text{Upper limit:} \quad \bar{\bar{x}} + 3S_{\bar{x}}$$
$$\text{Lower limit:} \quad \bar{\bar{x}} - 3S_{\bar{x}}$$

Precision of analysis is monitored by calculation of the coefficient of variation (CV) for each sample from the triplicates. In each calculation of the mean, outliers will be omitted. Outliers are defined as single values differing by >40% from the mean of the two other replicates provided the CV of these two is <10%.

An assay is declared out of control and repeated when: 1) $\bar{x}$'s for 2 of 3 control standards fall outside the limits, and 2) three successive $\bar{x}$ values of two pools fall outside the warning limit. An assay is repeated in part (i.e., for only those samples whose dose falls in the range of the respective control standard) when:

1) $\bar{x}$ of the respective standard falls outside the control limits, and
2) three successive $\bar{x}$ values of the respective standard fall outside the warning limits.

Individual samples are repeated when CV_i exceeds 15%, where

$$Cv_i = \frac{\left[\frac{\Sigma (x_{ij} - x_j)^2}{2}\right]^{1/2}}{\bar{x}} \times 100$$

To maintain long term stability of the assay, overlapping analysis of newly prepared standards is performed in assays being controlled by the outgoing standards.

4.4.2 Effect of Freezing upon Lp(a) Measurement by ELISA

By the ELISA methodology, Lp(a) was measured in 23 plasma samples frozen at -20°C. There were no systematic changes in the concentrations measured over a one month time period. Albers and Hazzard also demonstrated for their immunoassay that plasma samples stored at -20°C or at 4°C did not change in immunoreactivity significantly over a four-week period (4). Therefore, we conclude that the effects of freezing on Lp(a) immunoreactivity are minimal.

4.5 Quality Control of Postprandial Lipemia Response measurements

Procedures are under development to insure quality assuarance of the procedures involved in this study. Currently a quality control system involving stored aliquots of known quantities of retinyl palmitate standards for the HPLC analysis exists. Other quality control materials and procedures will be instituted. Routine lipid analyses, such as plasma cholesterol and triglyceride, will be governed by existing quality control programs.

4.6 References

1. The Lipid Research Clinics. 1980. Population Studies Data Book, Vol. I, The Prevalence Study. NIH Publication No. 80-1527.

2. Warnick, G.R., J. Benderson, and W.V. Brown. 1984. Clin. Chem. 28, 1379-1388.

3. Gibson, J.C., A. Rubinstein, and M.W. Brown. 1984. Arteriosclerosis 4, 564a.

4. Albers, J.J. and W. Hazzard. 1974. Lipids 9, 15-26.

Appendix A

1. Sources of Materials for Automated Determination of Cholesterol

- Pipetman: Rainin Instrument Corporation, Woburn, Mass 01801.
- Sample cups, reagent tips, reagent boats, reagent covers, disposable rotors: Roche Diagnostic, 11 Franklin, Belleville, NJ 07109.
- Cleaning company (for disposable rotors): Retech Reconvergent Technology Incorporated, Suite A-I, 9150 Rumsey Road, Columbia, MD 21045.
- Test tubes (10x75 mm): American Scientific Products Co., 4660 Pine Timber Lane, Suite 100, Houston, TX 77041.
- Microbial cholesterol esterase: (E.C. 3.1.1.13) Cat. No. 129-046, Boehringer-Mannheim Biochemicals, Indianapolis.
- Microbial cholesterol oxidase: (E.C. 1.1.3.6) Cat. No. Beckman Microbics, Carlsbad, CA or Cat. No. 393-924 Boehringer Mannheim, Biochemical, Indianapolis.
- PIPES buffer: Cat. No. 0704, Research Organics, Cleveland, OH.
- Horseradish peroxidase, Type VI: (E.C. 1.11.1.7) Cat. No. P8375; 4-aminoantipyrine, Cat. No. A4382; cholic acid, sodium salt, Cat. No. C-1254, Sigma Chemicals, St. Louis, MO.
- Phenol, J.T. Baker Chemical Company.
- Triton X-100: Rohm A. Haas, Philadelphia, PA.

2. Sources of Materials for Automated Determinations of Plasma Triglycerides

The common sources of cholesterol and triglyceride determinations are described in Appendix A.

1. Lipase (from Chromobacterium viscosum; glycerol ester hydrolase; EC 3.1.1.3) and L-alpha-glycerophosphate oxidase (from Aerococcus viridans; glycerol-3-phosphate: O2-oxidoreductase; E.C. 1.1.3-): Fermco Biochemicals, Inc., Elk Grove Village, IL.

2. Glycerokinase (from Escherichia coli; glycero-3-phosphotransferase EC 2.7.1.30),peroxidase (from horseradish; hydrogen peroxide oxidoreductase; EC 1.11.1.7), adenosine 5'-triphosphate (ATP), 4-aminoantipyrine, and glycerol: Sigma Chemical Co., St. Louis, MO.

3. Triton X-100: Rohm and Hass, Philadelphia, PA.

4. Sodium 2-hydroxy-3,5-dichlorobenzenesulfonate: Research Organics, Inc., Cleveland, OH.

3. Sources of Materials for Automated Determinations of Glycerol

The premeasured powder containing the enzymes is from Fermco Biochemicals, Inc., Elk Grove Village, IL. The other reagents have been described under 2.2.4 and 2.3.4.

4. Sources of Materials for Determination of HDL-cholesterol and HDL3-cholesterol

- Dextran sulfate, Dextralip 50R, molecular weight 50,000: Sochibo, SA, Boulogne, France 92100.

- Magnesium chloride 6 H2O: Cat. NR. 2444, Baker Chem. Co.

- Polystyrene conical centrifuge tubes with caps: Sarsted Inc., P.O. Box 4090, NJ.

- Swinnex filter holders and filters: Millipore Corp., Bedford, Mass.

5. Sources of Material for Determination of Apolipoprotein A-I in Plasma by Radioimmunoassay

- Sodium iodide (125I) for protein iodination, 100 mCi/mL: Amersham.

- Bovine serum albumin, Fraction V: Cat. No. A-4503. Sigma Chemical Co., St. Louis, MO.

- Lactoperoxidase: Cat. Num. 1-2005, Sigma Chemical Co., St. Louis, Mo.

- Hydrogen Peroxide, 30% (v/v): Cat. No. H-325, Fisher Scientific Co., Houston, TX.

- Prosil R-28: Speciality Chemicals, Gainesville, FL.
- ImmunoprecipitinR: Bethesda Res. Lab., Gaithersburg, MD.

- Sephadex G-50: Cat. Num. 17-0042-01; and Sephadex-75, Cat. No. 17-0050-01, Pharmacia Fine Chemicals, Piscataway, NJ.

6. Sources of Material for Determination of Apolipoprotein B(apoB) in Plasma by Radioimmunoassay

- Chloromine T: Fisher Scientific, Pittsburgh, PA
- Biogel A-5M: BioRad, Richmond, CA

Sources of other regents are described under Section 2.6.3

7. Sources of Materials for Determination of Lipoprotein(a) by Double Antibody ELISA

- Eight (8) channel pipeter: Flow Laboratories, Ltd., Ayshire, Scotland.
- Microtiter plates: Dynatech Labs, Inc., Alexandria, VA.
- Plate reader: Flow Laboratories, Ltd., Ayshire, Scotland.
- Microcomputer: Apple Computer Corp., Cupertino, CA.
- NaCl, NaH2PO4.H2O, Na2HPO4, Na citrate.H2O: Fisher Scientific Co., Pittsburgh, PA.
- Bovine serum albumin, Sepharose 4B, and Tween 20: Sigma Chemical Co., St. Louis, MO.
- O-phenylenediamine: Aldrich Chemical Co., Milwaukee, WI.
- Peroxidase conjugate of goat anti-rabbit Ig-G: Cappel Labs, Cochranville, PA.

8. Sources of Material for Expression of ApoB Epitopes

- Titertek pipettor: Flow Laboratories, Inc.
- Eppendorf repeater pipette: Brinkmann Instruments, Inc.
- Immulon 1 Removawell strips and holders: Dynatech Laboratories, Inc.

- Bovine serum albumin, fraction V: Boehringer Mannheim Biochem.
- 125I-Na: Iso-Tex Diagnostics, Inc., Friendswood, Texas.