Evolution of Methods for Measurement of HDL-Cholesterol: From Ultracentrifugation to Homogeneous Assays

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Background: Adoption of automated homogeneous assays for HDL-cholesterol (HDL-C) is increasing, driven by the need of clinical laboratories to cope with increasing workloads while containing costs. However, performance characteristics of homogeneous assays often differ in important aspects from those of the earlier precipitation methods. This review provides an overview of the new generation of homogeneous assays for HDL-C within the historical context of the evolution of methods and the efforts to standardize measurements of the lipoproteins.

Approach: This is a narrative review based on method evaluations conducted in the laboratories of the authors as well as on relevant publications, especially comparative evaluation studies, from the literature. Publications considered here have been collected by the authors over the past 30 years of involvement as methods for HDL-C made the transition from their early use in lipid research laboratories to clinical laboratories and the recent emergence of homogeneous assays.

Content: The presentation includes descriptions of methodologies, including homogeneous, precipitation, electrophoresis, and ultracentrifugation assays. Reference methods and recommended approaches for assessing accuracy are described. Accuracy and imprecision are summarized in the context of the National Cholesterol Education Program (NCEP) standards for analytical performance. The effects of interfering substances and preanalytical sources of variation are presented.

Summary: Homogeneous assays have been shown to be reasonably well suited for use in routine clinical laboratories, generally meeting the NCEP criteria for precision, accuracy, and total error. However, discrepant results compared with the reference methods have been observed with some of the assays, and the sources of discrepancies are not well characterized. Some homogeneous reagents have not been thoroughly evaluated. At least three of the reagents have experienced successive adjustments in formulation; hence, the reagents may not yet be fully optimized. For these reasons, the homogeneous assays cannot be confidently recommended for use in long-term clinical trials and other research applications without thorough validation.
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Well accepted now is the inverse association between HDL-cholesterol (HDL-C)[4] and risk of developing coronary artery disease (CAD), a relationship that persists after adjustment for other risk factors and is consistent with putative protective mechanisms (*1, 2*). The measurement of HDL-C in characterizing risk for CAD and managing treatment of dyslipidemia has become increasingly common in clinical laboratories. Until recently, laboratory methods for HDL-C measurement, adapted from research techniques, were among the more tedious of the common laboratory assays, requiring a manual separation step with precipitation reagents, followed by analysis of the cholesterol content, most often by an automated chemistry analyzer. The need for cost containment in the clinical laboratory as well as the suboptimal precision associated with precipitation methods created a

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[4] Nonstandard abbreviations: HDL-, LDL-, and VLDL-C, HDL-, LDL-, and VLDL-cholesterol; CAD, coronary artery disease; apo, apolipoprotein; TG, triglyceride; NCEP, National Cholesterol Education Program; RM, Reference Method; CRMLN, Cholesterol Reference Method Laboratory Network; PTA, phosphotungstic acid method; DCM, Designated Comparison Method; SPD, synthetic polymer method; PEG, polyethylene glycol-modified enzyme method; AB, antibody-based method; and CAT, catalase reagent method.

demand for more efficient methods. The consequence has been the development of a new generation of fully automated methods, the so-called homogeneous assays, that use specific reagents of various types to selectively expose and directly measure the cholesterol associated with HDL.

This review, drawing on two previous publications including the same authors (3, 4), critically examines the revolutionary new generation of homogeneous assays within the historical context of the evolution of HDL-C methods. Biochemical and physical characteristics of HDL that facilitate separations are discussed. Consensus opinions regarding the clinical utility of HDL-C are presented together with guidelines for use in characterizing and treating dyslipidemias. In addition, the characteristics of the various assay systems are summarized in relation to nationally established analytical performance criteria, and demonstration of traceability is summarized in relation to accepted targets for accuracy.

HDL

CHARACTERISTICS

The HDL class comprises a heterogeneous and polydisperse population of particles that are densest and generally smallest in size among the lipoproteins (5). Heterogeneity refers to the presence of several different subparticle classes within HDL, as many as 14 subfractions depending on the analytical technique used for separation (6). The term polydisperse indicates that each of the subclasses includes varying proportions of lipids and proteins. The proposed nomenclatures for the subclasses are based on either a physical property such as density, e.g., HDL_2, HDL_3, and so forth (7), or composition, e.g., HDL AI and HDL AI–AII (8). Of the lipoprotein classes, HDLs have the highest proportion of protein relative to lipid, containing slightly more than 50% protein (Table 1). The major proteins are apolipoproteins AI (apo AI) and AII. The C apolipoproteins (CI, CII, and CIII) and apo E, AIV, and D are also present in minute amounts. HDL particles do not contain apo B, a protein rich in positively charged arginine residues that is present in LDL, intermediate-density lipoprotein, and VLDL. Phospholipids are the principal lipid component of normal human HDL, comprising >50% of the total lipid, with lesser amounts of cholesterol esters (30%), unesterified cholesterol (10%), and triglycerides (TGs; 10%). Because cholesterol esters are hydrolyzed to unesterified or free cholesterol in most analytical procedures, the esterified portion is usually quantified as unesterified cholesterol. Of particular interest in the context of the homogeneous assays is one of the larger and less dense HDL subclasses that is rich in apo E, which like apo B contains a high proportion of positively charged arginine residues (9). The amount of this subclass varies and may or may not be included with HDL-C, depending on the method (discussed in more detail later).

Classically, HDL particles are defined in terms of hydrated density as the fraction with a density between 1.063 and 1.21 kg/L as obtained by preparative ultracentrifugation (10). Because there is little lipid-associated material with a density >1.21 kg/L, HDL is often measured as the cholesterol with a density >1.063 kg/L. Traditionally, the fraction corresponding to HDL as separated by electrophoresis is designated the α-lipoproteins. However, in common practice this and the fraction separated by chemical precipitation or chromatography are also referred to as HDL, although these fractions may not be exactly equivalent. Thus, HDLs are defined in terms of the analytical procedure used to isolate them and include a family of similar particles that vary in size and composition. By contrast, VLDL particles include a substantially greater proportion of lipids, especially TGs, with a density <1.006 kg/L. LDL particles, which carry most of the circulating cholesterol, range in density from 1.006 to 1.063 kg/L. These classes correspond to pre-β- and β-lipoproteins, respectively, when separated by electrophoresis.

METABOLISM

HDLs are secreted from the liver or intestine as disk-shaped nascent particles that consist mainly of phospholipids and apo AI (11). In the circulation, additional phospholipids, cholesterol, and the minor apolipoproteins are transferred from TG-rich particles to these small, dense HDL particles, designated HDL_3, converting them to larger and less dense spherical particles. Cholesteryl esters are produced from free cholesterol on the particle surface by the action of lecithin cholesterol acyltransferase in the presence of its cofactor apo AI and move to the core, contributing to enlargement of the HDL particle. These cholesteryl ester-rich particles, referred to as HDL_2, contain twice as many cholesterol molecules per unit of apolipoproteins compared with HDL_3. In vitro, HDL_2 can be converted back to HDL_3 in the presence of hepatic lipoprotein lipase (12).

The HDL cholesteryl esters are cleared through the liver by one of three mechanisms: (a) selective removal from HDL by the hepatic HDL receptors; (b) transfer from HDL to apo B-containing lipoproteins via the mediation of cholesterol ester transfer protein, then uptake by the

Table 1. Chemical composition of normal human HDL (5).

Lipid (~50% by weight)	
Cholesterol	20%
Esterified	15%
Unesterified	5%
Phospholipids	25%
TGs	5%
Protein (apolipoproteins ~50% by weight)	
Major components	
apo AI, AII	
Minor components	
apo CI, CII, CIII, E, AIV, D	

liver through receptors for these TG-rich lipoproteins; or (*c*) removal with apo E-rich HDL, which is recognized by the hepatic remnant receptors (*11*). These processes constitute the reverse cholesterol transport mechanism by which cellular and lipoprotein cholesterol is delivered back to the liver for reuse or disposal, a process that is considered to contribute in part to the cardioprotective role of HDL. Another presumed protective role of HDL is in resisting oxidation of LDL, a process that makes lipoproteins more atherogenic (*13*).

CLINICAL RELEVANCE

Many epidemiologic investigations have demonstrated the strong and independent inverse association of HDL, measured in terms of either its cholesterol or apo AI content, to risk of CAD [reviewed in (*14*)]. The risk of CAD increases 2–3% for every 10 mg/L decrease in HDL-C (*15*); thus, higher HDL-C concentrations are considered protective. Many factors, some modifiable, are known to affect HDL-C values (*16*), including age, gender, genetic factors, cigarette smoking, physical exercise, dietary intake, obesity, and certain drugs (Table 2).

Population distributions for HDL-C have been established in various studies with means of ~450 mg/L in men and ~550 mg/L in women. The Lipid Research Clinics Program Prevalence Study (*17*), a major North American study funded by the NIH, used the heparin/ $MnCl_2$ precipitation procedure with $MnCl_2$ at 0.046 mol/L in EDTA-plasma specimens. Today, a higher concentration of $MnCl_2$ (0.092 mol/L) would be recommended for EDTA plasma, giving slightly lower HDL-C values. The most recent large population data are from the US National Health and Nutrition Examination Survey III (*18*), which used a dextran sulfate (50 000 Da) with the $MgCl_2$ method.

Consensus Guidelines and Medical Decision Points for Diagnosis and Treatment

The NIH-sponsored National Cholesterol Education Program (NCEP) in 1988 released consensus guidelines for the diagnosis and management of patients with hypercholesterolemia (*19*). The initial 1988 guidelines focused diagnosis and treatment efforts on LDL-cholesterol (LDL-C), the major atherogenic cholesterol-carrying particles that typically represent 75% of total cholesterol, and recommended measurement of HDL-C only in patients at increased risk for CAD because of increased cholesterol. After an NIH-sponsored consensus conference (*14*) that formally recognized HDL-C as an independent risk factor, new guidelines released in 1993 (*18, 20*) recommended measurement of HDL-C for all adults during the initial screen. Medical decision points were given for HDL-C with values <350 mg/L considered "high risk" and values ≥600 mg/L considered "protective". Subsequent consensus statements in 1997 from the American Heart Association for primary prevention (*21*) and from the American Heart Association and American College of

Table 2. Factors affecting HDL-C concentrations (*16*).[a]

Biological	
Genetics	⇕
Gender (female)	↑
Race	⇕
Age	⇕
Behavioral	
Diet	
High fat	↑
Alcohol	↑
Physical activity	↑
Smoking	↓
Obesity	↓
Clinical	
Acute infections	↓
Diabetes (type II)	↓
Myocardial infarction	↓
Renal disease	↓
Stress	↓
Pregnancy	↓
Thyroid status	⇕
Hospitalization	↓
Medications	
Primary	
Niacin	↑
Fibrates	↑
Statins	↑
Resins	↑
Probucol	↓
Secondary	
Corticosteroids	↓
Androgens	↓
Estrogens	↑
Progestins	↓
Diuretics	↓
Propanolol	↓
Sampling	
Nonfasting	⇕
Recumbent posture	↓
Standing posture	↑
Venous occlusion	↑

[a] ↑, increases HDL-C concentrations; ↓, decreases HDL-C concentrations.

Cardiologists for secondary prevention (*22*) reinforced the enhanced role of HDL-C. In 2001, the NCEP increased the high-risk medical decision point to <400 mg/L (*23*). The European Atherosclerosis Society and other international consensus panels have also issued guidelines (*24, 25*). Dissemination of these guidelines has enhanced the use of HDL-C in the diagnosis and management of patients at increased risk for coronary heart disease and led to increasing demand for measurements by clinical laboratories. Appropriate classification of patients based on the cutpoints mandates the use of accurate methods for HDL-C measurement.

Treatment decisions are primarily based on LDL-C because this atherogenic lipoprotein has well-established

interventions that have been shown in randomized clinical trials to be efficacious *(18, 20)*. Some patients will have total cholesterol and/or LDL-C values below the high-risk medical decision points but be at increased risk because of low HDL-C (<350 mg/L), in many cases but not always associated with increased TGs. The efficacy of treating low HDL-C values is not as well established but still considered worthwhile. Fortunately, many of the therapies appropriate for high LDL-C and TGs have a beneficial effect on HDL-C, e.g., increased physical exercise, weight loss, and smoking cessation (Table 2). More recently, some of the cholesterol-lowering drugs, specifically niacin, gemfibrozil, statins, and resins (in decreasing order of effectiveness), have been recognized for increasing HDL-C concentrations and thereby further contributing to overall risk reduction. Thus, measurement of HDL-C not only improves the identification of patients at increased risk for CAD, but also is useful in monitoring efficacy of treatment, especially with therapies likely to affect HDL-C. Some have advocated the use of ratios, either total cholesterol or LDL-C to HDL-C, as a better indicator of overall risk. However, the ratio is considered to have no physiologic significance, and NCEP guidelines focus on the individual values *(18)*.

Guidelines for Analytical Performance

In the late 1980s, the NCEP convened expert laboratory panels *(26, 27)* that emphasized the importance of accurate measurements of HDL-C and issued guidelines, including analytical performance goals (Table 3) similar to those for LDL-C, total cholesterol, and TGs. The panels recommended efforts to standardize the lipid and lipoprotein measurements, i.e., achieve traceability to the accepted reference methods (RMs) with consistent accuracy in both research and routine measurements. Accuracy in the HDL-C measurement has been important not only for reliable classification based on the established cutpoints, but also for the calculation of LDL-C using the Friedewald formula: LDL-C = total cholesterol − (HDL-C + TG/5) *(28)*. Inaccurate measurement of HDL-C leads to substantial misclassification because the risk associations of HDL-C and LDL-C are opposite and the error is reciprocal; erroneously decreased HDL-C leads to falsely increased LDL-C.

In developing guidelines for analytical performance, the expert panel considered the requisite analytical performance needed for reliable medical decisions in relation to biological variation and the capabilities of routine laboratories [for a more detailed explanation of the goal setting process, see Refs. *(26, 27)*]. The current primary goal is that total error (bias + 1.96CV) be within 13% of the true value. The total error term, combining the contributions of imprecision (random error) and inaccuracy or bias (systematic error), represents the maximum tolerable error in measurement of a single specimen to 95% tolerance limits, i.e., in 19 of every 20 measurements. The underlying specifications for imprecision and bias consistent with the total error goal are informative. Imprecision, in proportional units or CV, should be ≤4%. At low HDL-C values, this proportional target becomes difficult to achieve, e.g., at 250 mg/L the proportional goal would be 10 mg/L; therefore, the precision guideline shifts to an absolute or standard deviation target of 17 mg/L for HDL-C values <420 mg/L. The bias should be <5% from the true value, determined by the accepted RM. Thus, in this example the total error target of 13% is equal to 5% plus 1.96 times 4%.

The imprecision of a particular method can be assessed by replicate analysis. Results are most representative with actual patient specimens or pools prepared from serum freshly collected and frozen. Commercial control materials, because of changes in their analyte and matrix properties during the manufacturing process, may not truly depict performance on patient specimens. For reasons of practicality, such pools are generally lyophilized for long-term stability and often artificially supplemented with concentrated lipoproteins, sometimes of animal source, which can substantially alter their analyte and matrix characteristics *(29)*. Such artificial materials often give not only distorted indications of imprecision, but also unreliable estimations of bias. The term "commutable" refers to a material that is similar to patient specimens in measurement characteristics, often not achieved in even the best commercial control materials. Currently the only approach considered reliable for establishing the accuracy of an HDL-C assay is a direct comparison study on actual patient specimens with a RM. The CDC, in collaboration with the National Heart, Lung and Blood Institute, has established programs *(30)* for standardizing the lipid and lipoprotein analytes to defined RMs for total cholesterol, LDL- and HDL-C, and TGs. The CDC coordinates a network of reference laboratories, the Cholesterol Reference Method Laboratory Network (CRMLN), which offers direct comparison protocols using fresh patient specimens for reliable transfer of accuracy. The HDL RM and the CRMLN program will be described in detail after a general description of the analytical techniques used.

Evolution of Methods for Measurement of HDL-C

Unique physical and chemical characteristics of the lipoproteins have been exploited to achieve their separation. The presence of lighter lipids in varying proportions with the heavier protein facilitates separations by density, leading to the early use of ultracentrifugation. Other common research methods, such as electrophoresis and chromatography, separate lipoproteins based on differences in charge and the dramatic range in particle size.

Table 3. NCEP analytic performance goals for HDL-C *(26, 27)*.

Bias	Imprecision	Total error
≤5% RV[a]	SD ≤17 at <420 mg/L	≤13%
	CV ≤4% at ≥420 mg/L	

[a] RV, reference value.

Certain reagents, such as polyanions and divalent metals, were found to selectively and conveniently aggregate and precipitate lipoproteins with appropriate choices of reagents and conditions. Separation methods were developed for both preparative and analytical studies and subsequently, in the mid-1980s, adapted for routine separations in clinical laboratories.

Ultracentrifugation

Separations of lipoproteins by ultracentrifugation include both equilibrium and rate methods after adjustment of the specimen density with salts such as NaBr or KBr *(10)* to float or sediment particles based on differences in hydrated density. In the early years, HDL was defined as the particles with a density between 1.063 and 1.21 kg/L, i.e., the bottom fraction after ultracentrifugation at a density of 1.063 kg/L was readjusted to a density of 1.21 kg/L and again subjected to ultracentrifugation. Cholesterol recovered in this top fraction was taken as a measure of HDL. Because there is virtually no cholesterol in particles with a density >1.21 kg/L, in practice cholesterol was often simply measured in the 1.063 kg/L bottom fractions.

Ultracentrifugation as a separation technique is not only tedious and time-consuming, but the labile lipoproteins can be substantially altered by the high salt concentrations and centrifugal forces used. Furthermore, there is a plethora of different types of equipment, making conditions extremely difficult to reproduce from one laboratory to another, and separations are highly dependent on the skills of the technician. Achieving complete and reproducible recovery is difficult, even for experienced technologists, and fractions may be cross-contaminated. In addition, the fractions are heterogeneous and contain other functional lipoproteins. For example, the HDL fraction with a density between 1.063 and 1.210 kg/L may contain considerable amounts of the atherogenic apo B-containing lipoprotein(a). In summary, although ultracentrifugation has been highly useful in research and as a comparison method for validation of other methods, this approach is not considered practical for routine analytical measurements.

Electrophoresis

Electrophoretic techniques separate the lipoproteins relatively quickly in a single operation, and the resulting bands, because of their lipid content, can be conveniently visualized and distinguished from other proteins by staining with lipophilic dyes. The lipoprotein bands have been named by comparison to mobilities of the major serum proteins: α (HDL), pre-β (VLDL), and β (LDL). Lipoproteins can be separated using a variety of electrophoretic media, such as paper, agarose gel, cellulose acetate membrane, and polyacrylamide gel, each used with various buffers *(31, 32)*. The common lipophilic stains are Oil Red O, Fat Red 7B, and Sudan Black.

Electrophoresis was long considered primarily useful for qualitative analysis because the lipophilic dyes are not specific for a particular class of lipid and because, with the varying composition of the lipoproteins, the staining may not be proportional to cholesterol content. A technique using precipitation of the bands with phosphotungstic acid after agarose gel electrophoresis was reported to allow more reliable quantification of lipoprotein cholesterol but gives discrepant results in samples with atypical lipoproteins *(33)*. Subsequent use of specific enzyme reagents, e.g., cholesterol esterase and oxidase with a peroxidase indicator, to stain electrophoretic plates for cholesterol improved the quantification *(34)*.

Even more promising is a recent modification that uses the enzyme cholesterol dehydrogenase rather than cholesterol oxidase, with the dye nitroblue tetrazolium chloride, which is insoluble and stable after reduction. Electrophoretic systems using this latter *(35, 36)* or another color system *(37)* have been reported to give acceptably accurate quantification of HDL-C and the other lipoproteins. A distinct advantage of the electrophoretic methods is the visual presentation that facilitates observation of atypical lipoproteins. Nevertheless, both ultracentrifugation and electrophoresis have disadvantages for use in the clinical laboratory, especially when the workload is high. The more practical chemical precipitation methods that had been used in the research laboratories were generally adopted as the method of choice when measurement of HDL-C moved into routine clinical laboratories.

Other Methods

Various HPLC methods have been used to fractionate lipoproteins, including HDL, but have been hindered by poor stability of the columns. An improved HPLC technique separates lipoproteins, including HDL and its subclasses, on the basis of size and quantifies the cholesterol with enzymatic reagent detection *(38)*. Another innovative method that uses nuclear magnetic resonance, which efficiently quantifies the lipoproteins, including HDL and its subclasses, might be suitable for use in high-workload environments *(39)*. These two methods, although sophisticated, have disadvantages for integrating HDL-C measurements with other testing in the routine clinical laboratory.

Chemical Precipitation (First-Generation) Methods for Separation of HDL

Burstein and co-workers *(40–43)* in France pioneered the precipitation methods to separate lipoproteins rapidly on a preparative scale. They and other groups later adapted the preparative reagents as a convenient means for research and then routine analytical separations. Chemical precipitation uses polyanions, sometimes combined with divalent cations, to selectively aggregate and render insoluble the lower density lipoproteins, leaving HDL in solution. The insoluble lipoproteins can then be sedimented by low-speed centrifugation. The resulting supernatant solution can be recovered by pipetting or decanting for cholesterol analysis as a measure of HDL-C.

Heparin with $MnCl_2$ was a popular early combination, used in pioneering lipoprotein research studies at the NIH (44). Subsequently, the CDC used this method in combination with ultracentrifugation to assign target values to reference materials (30). Because commercial heparin preparations were somewhat inconsistent in properties (45) and Mn^{2+} was observed to interfere with the early enzymatic assays, this combination was generally displaced for routine use by dextran sulfate or phosphotungstic acid, both used together with Mg^{2+}. A method using dextran sulfate (50 000 Da) and $MgCl_2$ at concentrations selected to minimize cross-contamination as determined by measurement of apolipoproteins in the fractions was credentialed as an AACC Selected Method (46) and became the most common precipitation procedure for HDL-C in the US. Methods using phosphotungstic acid (42) or polyethylene glycol (47) became more common in Europe. Table 4 lists several of the common reagents available for selective isolation of lipoproteins.

A major problem with the precipitation methods has been interference from increased TGs, which make aggregated lipoproteins lighter, preclude the sedimentation of the insoluble aggregates, and yield turbid supernates (48). The resulting cloudy supernatant is contaminated with lipoproteins other than HDL, leading to an overassessment of HDL-C. The extent of TG interference and the cutoff above which results are no longer reliable vary widely depending on the particular precipitation reagent and concentration. Turbid supernates can sometimes be cleared by centrifugation at higher g forces (49) or by filtration (50). Alternatively, specimens can be diluted (40), which decreases the background density of the solution, or the lightest TG-containing particles can be removed by ultracentrifugation before precipitation (51).

Each of the precipitation reagents evolved with modifications reported to change the selectivity or performance. For example, the heparin-$MnCl_2$ method (Table 4) was first described with 1.0 mol/L $MnCl_2$ (51). Subsequent studies (49) concluded that this concentration was appropriate for serum specimens but that a higher concentration, 2.0 mol/L, improved specificity in EDTA-plasma specimens, presumably to compensate for chelation of some of the divalent cation by EDTA and to avoid incomplete precipitation of apo B-containing lipoproteins. The higher concentration also decreased the extent of interference from high TGs. The original dextran sulfate-$MgCl_2$ precipitation method (Table 4), with a 500 000-Da material (52), was reported to give low HDL-C values, which was remedied by substituting a dextran sulfate of 50 000 Da (46). The phosphotungstic acid procedures (Table 4) have been described with and without $MgCl_2$, adjusted to different pH values, and at several concentrations (42, 53). A common version of this method (subsequently designated as PTA) has been reported to give lower values for HDL-C compared with heparin and dextran sulfate methods (54), an important observation in light of published evaluations of the third-generation methods. Similarly, a variety of polyethylene glycol molecular weights, concentrations, and pH conditions have been reported (47, 55). The accepted RM accuracy targets for HDL-C, which will be described in more detail subsequently, use heparin-$MnCl_2$ (30) and dextran sulfate (50 000 Da)-$MgCl_2$ (56) to achieve precipitation.

For more than a decade, quantification of HDL-C in routine laboratories was performed almost exclusively by one of the first-generation precipitation techniques. Although the separation step was performed manually, the required equipment was readily available and cholesterol in the supernate could be easily measured by various automated analyzers. Most laboratories relied on commercial reagents; early methods often used phosphotungstic acid (53), which is inexpensive and readily available. However, phosphotungstic acid reagents were found to be somewhat unstable with extended storage, and separations were reportedly less rugged, being sensitive to separation conditions and technique (54), which led to gradual replacement by the dextran sulfate reagent (Fig. 1) (46). The precipitation methods were capable of achieving precision consistent with the NCEP targets (Table 3), as demonstrated by their performance in lipid specialty laboratories (54), but in routine laboratories often did not (57), in part because of the need for manual pipetting and other manipulations.

With improvements in automated analyzers, specimen volume requirements progressively decreased and the large volume required for manual pipetting in the precipitation step was increasingly excessive in relation to blood volumes collected for other laboratory tests. The addi-

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Table 4. Methods for HDL separation/quantification.

Precipitation (first generation)
  Heparin-Mn^{2+} (HM)
    0.46 mmol/L (LRC[a] method) (51)
    0.92 mmol/L (recommended for EDTA plasma) (49)
  Dextran sulfate-Mg^{2+} (DS)
    500 000 Da (52)
    50 000 Da (AACC Selected Method and DCM) (46, 56)
  Phosphotungstate-Mg^{2+} (PTA) (53)
  Polyethylene glycol (PG) (55)
Facilitated separation (second generation)
  Magnetic with dextran sulfate-Mg^{2+} (MAG) (58)
Homogeneous (third generation)
  Antibody four-reagent method (IRC) (68)
    International Reagents Corporation
  Polyethylene glycol-modified enzymes with cyclodextrin (PEG) (69)
    Kyowa Medex
  Synthetic polymer/detergent (SPD) (94)
    Daiichi
  Antibody, two reagents (AB) (59)
    Wako
  Catalase (CAT) (79)
    Denka Seiken
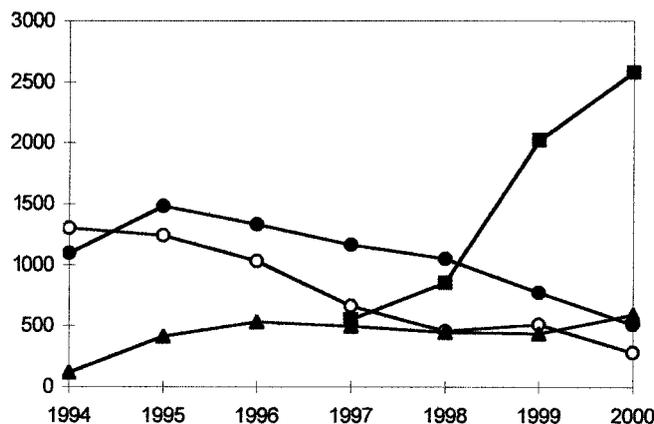
[a] LRC, Lipid Research Clinics.

Fig. 1. Trends in use of HDL-C methods by laboratories participating in proficiency surveys of the College of American Pathologists.

Based on the third survey of each year for years 1994–2000. *Plots* illustrate number of laboratories, not number of assays. ●, first generation, dextran sulfate (50 kDa); ○, first generation, phosphotungstate; ▲, second generation, magnetic; ■, third generation, homogeneous.

tional manipulations required to clear specimens with high TGs interfered with laboratory workflow. As the HDL-C measurement became increasingly common in the 1990s, pressures to reduce operating costs in the laboratory gradually moved other methods toward full automation, and the labor-intensive manual steps became increasingly untenable. Some higher-volume laboratories attempted to streamline the precipitation methods with automated pipetting stations, but in most cases the pretreatment step continued to be performed manually, making HDL-C one of the more tedious common procedures in the clinical laboratory. A result was a second generation of more streamlined methods.

Second-Generation Methods

To eliminate the tedious centrifugation process, one method linked dextran sulfate to encapsulated iron beads (distributed by Polymedco Inc., Cortlandt Manor, NY) to facilitate separation of apo B-containing lipoproteins from the HDL fraction by a magnet (Table 4) *(58, 59)*. Small magnets could even be placed directly in each of the sample tray slots, allowing separation in conjunction with analysis on the automated analyzer. The specificity of separation was acceptable when fractions were characterized by electrophoresis and apolipoprotein content, suggesting no significant cross-contamination *(59)*. In comparisons with conventional precipitation methods, a small negative bias (−7 mg/L) was observed with conventional dextran sulfate and a positive bias with PTA (38 mg/L) *(58, 59)*. Interference from TGs became significant at concentrations >10 000 mg/L, as the bound TG-rich lipoprotein become too buoyant to pull down by magnetic force. Dilution with saline (9 g/L NaCl) was sufficient to clear most specimens. However, when TG in the form of VLDL was added at concentrations >5000 mg/L, HDL-C results were falsely decreased. Hemoglobin was shown to falsely increase the observed HDL-C concentrations, whereas

increased concentrations of free fatty acids did not interfere *(59)*.

A method developed for small and low-volume laboratories streamlined the separation with a plastic device (Spin-Pro) containing premeasured precipitation reagent *(60)*. The user simply poured the specimen in the top of the plastic tube and placed the tube in the centrifuge. During centrifugation, the device accurately measured the sample, which was mixed with the precipitation reagent, and the resulting precipitate sedimented. After centrifugation, the supernate rose into the central well, and the device could be placed directly in the sample tray of a chemistry analyzer. Highly sophisticated compact analyzers, developed for physicians' offices and other point-of-care sites, integrated HDL separation and quantification from plasma or whole blood applied to cassettes or reagent-impregnated strips, eliminating the need for separate pretreatment steps *(61, 62)*.

Accuracy Targets

CDC RM

The separation techniques described above were combined into the accepted accuracy target for HDL-C, which combines ultracentrifugation with a first-generation precipitation method using heparin and $MnCl_2$. As routine laboratories began to adopt the first-generation HDL-C methods in the mid-1980s and especially after the NCEP began recommending the measurement in characterizing patients, the need for a RM as the accuracy target for standardization became apparent. The NCEP Lipoprotein Panel, after considering various alternatives, recommended adoption of the method used by the CDC to assign target values to the reference materials that had been used in their Lipoprotein Standardization Program for lipid research laboratories *(26, 27)*. An ultracentrifugation step had been included *(51)* to remove TG-rich lipoproteins, avoiding their interference with sedimentation. Thus, the accepted RM for HDL-C combines the precipitation technique described above with ultracentrifugation in a three-step procedure *(30)*. After VLDL and chylomicrons are removed by ultracentrifugation, the remaining apo B-containing lipoproteins, primarily LDL, are precipitated with heparin and $MnCl_2$ (46 mmol/L), and the supernate containing HDL is recovered by low-speed centrifugation. Cholesterol in the supernate is quantified using a modification of the Abell-Kendall Cholesterol RM *(63, 64)*, which in contrast to the enzymatic assays does not experience interference from manganese. This RM was selected not necessarily because of its demonstrated accuracy or capability to best select the protective fraction of lipoproteins, but rather for historical reasons; adoption as the general accuracy target was expected to make patient classification consistent with the NCEP decision points derived from NIH-sponsored epidemiologic studies. Thus, the CDC RM, although not formally accredited through the National Reference Sys-

tem process, is now considered the appropriate accuracy target for assigning calibrator setpoints and verifying the accuracy of routine methods.

There is no definitive method for HDL-C; indeed, considering the complexity of the lipoprotein particles, it may be impossible to unambiguously define the fraction of interest and develop such a method. Cholesterol is a readily defined molecule, providing a basis for a definitive method. By contrast, lipoproteins are complex, heterogeneous, and polydisperse particles with overlapping properties that have not been fully characterized, and the various separation methods can obtain different populations of particles with varying associations with CAD risk. There is no compelling evidence that the particular fraction obtained by the CDC RM is a better indicator of CAD risk than the fraction obtained by any other method. Thus, the CDC method is accepted as the RM based on consensus in consideration of its use as the accuracy target for population studies from which decision points were taken and not because the method is based on an unambiguous definition and thoroughly validated and specific quantification of the most protective fraction of lipoprotein particles.

In fact, the current emphasis on the RM as the accuracy target, although necessary to achieve standardization of results, might actually lead to rejection of even more specific methods, which might actually better separate a risk-negative population of lipoprotein particles. Developers of new methods should be aware of this potential and consider that a new method, perhaps not in agreement with the RM because it fractionates a different population of lipoprotein particles, might need to be validated by demonstrating better prediction of risk in population studies, e.g., in cross-sectional retrospective studies. Accumulation of compelling evidence supporting better prediction by a different fractionation might necessitate redefining the RM.

DESIGNATED COMPARISON METHOD

Few laboratories have an ultracentrifuge and the skills required to perform the CDC RM for HDL-C. In addition, ultracentrifugation is expensive and requires a large sample volume, typically 5.0 mL, which complicates the process of obtaining specimens for comparison. Therefore, the CRMLN laboratories developed and validated a modified dextran sulfate (50 000 Da) procedure as the Designated Comparison Method (DCM) to provide results approximately equivalent to those of the CDC RM but without the need for ultracentrifugation (56). The MgCl$_2$ precipitation reagent concentration was decreased slightly from that used in the AACC Selected Method to make the HDL-C values slightly higher and more consistent with those of the CDC RM. Comparison studies with the less costly DCM, closely standardized to the CDC RM, are available through the CRMLN laboratories (30).

CRMLN

Because of matrix and analyte changes in processed control materials, judgments of accuracy based on such materials may be compromised. The approach considered most reliable for assuring accuracy is a direct comparison using actual representative patient specimens with a method of established accuracy (65). The CRMLN, coordinated by the CDC and consisting now of five experienced US laboratories and several international partner laboratories in Canada, Europe, Japan, and South America, all closely standardized to the CDC RM, offers a protocol, based on NCCLS guidelines (66), whereby diagnostic manufacturers can assure accuracy by completing a comparison study with the DCM (67). Agreement within specified limits (5% for HDL-C), as well as within other limits for total and LDL-cholesterol and expected in the near future for TG, qualifies the method for certification. On the basis of comparison results, appropriate adjustment of calibrator setpoints can be made to bring performance into agreement with the accuracy target. Diagnostics manufacturers as well as distributors and instrument partners are encouraged to certify at least every 2 years and to ensure that every production lot is calibrated to maintain accuracy consistent with the accuracy targets, which can be accomplished by ongoing participation in the CDC/CRMLN program. The website of the AACC (www.aacc.org/standards/cdc/cholesterolinfo.stm) provides details of the comparison studies, contact information for CDC/CRMLN, and a listing of the commercial methods that have qualified for certification. Clinical laboratories can promote standardization by encouraging suppliers of their reagent sets to participate in the CRMLN comparison process. In addition, clinical laboratories can also make arrangements with a CRMLN laboratory to perform their own direct comparisons, which are especially useful in the case of new or modified methods. Modest user fees support the CRMLN program.

Third-Generation Homogeneous Assays

The provision of a reliable standardization program was a factor in improving accuracy in HDL-C measurements, but imprecision remained a problem with the conventional precipitation methods. A major breakthrough was reported in 1994 (68), with the first of a series of so-called "homogeneous" methods capable of full automation and considered third generation (Table 4). The term homogeneous is based on a previous convention in clinical chemistry used to describe an immunoassay that does not require separation of free from bound label, i.e., an assay that can be fully automated in a single reaction vessel. Some have described the new assays with the term "direct", which implies that the analyte is measured directly. Because all existing methods, including those requiring pretreatment, measure HDL-C directly, the term homogeneous is considered more descriptive.

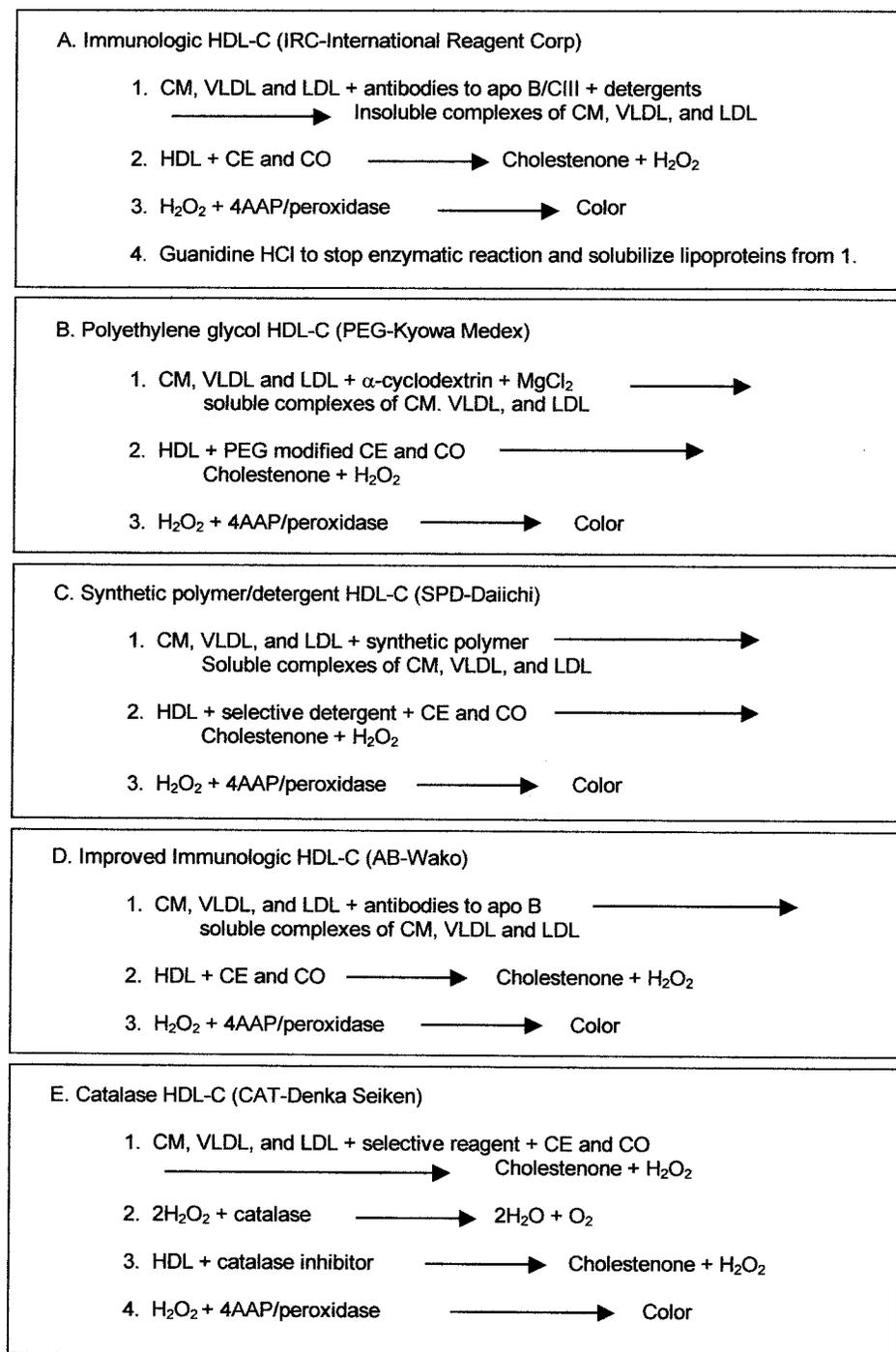The new-generation homogeneous assays do not re-

A. Immunologic HDL-C (IRC-International Reagent Corp)

1. CM, VLDL and LDL + antibodies to apo B/CIII + detergents
$\longrightarrow$ Insoluble complexes of CM, VLDL, and LDL

2. HDL + CE and CO $\longrightarrow$ Cholestenone + H_2O_2

3. H_2O_2 + 4AAP/peroxidase $\longrightarrow$ Color

4. Guanidine HCl to stop enzymatic reaction and solubilize lipoproteins from 1.

B. Polyethylene glycol HDL-C (PEG-Kyowa Medex)

1. CM, VLDL and LDL + α-cyclodextrin + $MgCl_2$ $\longrightarrow$
soluble complexes of CM. VLDL, and LDL

2. HDL + PEG modified CE and CO $\longrightarrow$
Cholestenone + H_2O_2

3. H_2O_2 + 4AAP/peroxidase $\longrightarrow$ Color

C. Synthetic polymer/detergent HDL-C (SPD-Daiichi)

1. CM, VLDL, and LDL + synthetic polymer $\longrightarrow$
Soluble complexes of CM, VLDL, and LDL

2. HDL + selective detergent + CE and CO $\longrightarrow$
Cholestenone + H_2O_2

3. H_2O_2 + 4AAP/peroxidase $\longrightarrow$ Color

D. Improved Immunologic HDL-C (AB-Wako)

1. CM, VLDL, and LDL + antibodies to apo B $\longrightarrow$
soluble complexes of CM, VLDL and LDL

2. HDL + CE and CO $\longrightarrow$ Cholestenone + H_2O_2

3. H_2O_2 + 4AAP/peroxidase $\longrightarrow$ Color

E. Catalase HDL-C (CAT-Denka Seiken)

1. CM, VLDL, and LDL + selective reagent + CE and CO
$\longrightarrow$ Cholestenone + H_2O_2

2. $2H_2O_2$ + catalase $\longrightarrow$ $2H_2O$ + O_2

3. HDL + catalase inhibitor $\longrightarrow$ Cholestenone + H_2O_2

4. H_2O_2 + 4AAP/peroxidase $\longrightarrow$ Color

Fig. 2. Schematic reaction mechanisms for each of the five homogeneous methods.

CM, chylomicron; *CE*, cholesterol esterase; *CO*, cholesterol oxidase; *4AAP*, 4-aminoantipyrine.

quire off-line pretreatment and separation, eliminating the manual pipetting, mixing, and centrifugation steps. This first homogeneous assay for HDL-C (Fig. 2A), described by International Reagents Corporation (Kobe, Japan), was highly innovative but limited in application with four successive reagent additions. The first reagent contained polyethylene glycol, which caused aggregation of the apo B-containing chylomicrons, VLDL, and LDL. The second reagent protected or blocked the aggregated lipoproteins with antibodies to apo B and apo C. The third

reagent included the cholesterol reaction enzymes (cholesterol esterase, cholesterol oxidase, and peroxidase), which reacted only with the unprotected HDL-C. A fourth and final reagent stopped the enzymatic color reaction and cleared the reaction mixture with guanidine salts, which solubilized the aggregates. HDL-C was quantified based on the final reaction absorbance, monitored at 600 and 700 nm. This breakthrough method, designated subsequently as IRC, introduced the possibility of full automation and set the stage for subsequent, two-reagent

homogeneous methods. Although not suitable for many analyzers because of the four reagent additions, the IRC method is still distributed in Japan.

In 1995, Kyowa Medex Co. (Tokyo, Japan) (69) introduced two clever innovations (Fig. 2B): (a) sulfated α-cyclodextrins together with Mg^{2+} were found to selectively block but not precipitate chylomicrons and VLDL, providing selectivity without the need for a clearing reagent; and (b) the specificities of the enzymes cholesterol esterase and cholesterol oxidase toward HDL-C were enhanced by covalently linking polyethylene glycol molecules to the enzymes, excluding cholesterol in the larger LDL particles. Polyethylene glycol 6000 Da in size was found to optimize the specificity at concentrations much lower than those used previously to precipitate lipoproteins, leading the developers to speculate that the modified enzymes were able to distinguish the lipoprotein classes based on their size and/or charge. These innovations facilitated a fully automated homogeneous assay with only two successive reagent additions. The commercial version of this reagent (designated subsequently as PEG) included in the first reagent α-cyclodextrin sulfate together with dextran sulfate, Mg^{2+}, and detergents with buffering to pH 7. The second reagent contained the modified enzymes and substrates. This two-reagent assay, now also distributed through Roche Diagnostics (Indianapolis IN; formerly Boehringer Mannheim Corporation), offered a practical homogeneous assay suitable for many of the common chemistry analyzers. The original commercial reagent sets included the second enzyme-containing reagent in lyophilized form, necessitating reconstitution. A second version (70) introduced in mid-1998 provides both reagents in liquid form. A third version with decreased Mg^{2+} concentration, apparently to reduce carryover effects, is currently in evaluation by one of the authors (M.A.N.). (Users should recognize that this and other homogeneous reagents have been successively modified; different versions with various measurement properties may be supplied by various distributors and instrument partners.)

Subsequently, other innovative homogeneous reagents were developed, also in Japan. Daiichi Pure Chemicals Company (Tokyo, Japan) offered a homogeneous assay (Fig. 2C) using a synthetic polymer together with a polyanion to block the non-HDL lipoproteins (59, 60, 71). A detergent then exposed only cholesterol in HDL to the enzymes, giving specificity for HDL-C. Commercial reagent sets, also distributed by Genzyme Corporation (Cambridge, MA) and other distributors and instrument partners, include two reagent additions, the first with the polyanion and polymer blocking agents and the second with detergent, enzymes, and substrates. The first commercial version required reconstitution of the lyophilized enzyme reagent. In mid-1998, a second version was introduced with both reagents as liquids (72, 73). A subsequent evaluation (74) indicated that the fully liquid formulation was substantially modified (Fig. 2) to im-

prove specificity and decrease potential interference. A third version of this reagent without Mg^{2+} was described in poster presentations from Daiichi Pure Chemicals (Tokyo, Japan) at the 2000 AACC Annual Meeting (75, 76).

A fourth homogeneous method (59, 77, 78), involving immunoinhibition (Fig. 2D), was introduced by Wako Pure Chemicals Industry (Osaka, Japan). Antibody to human apo B in the first reagent reacts with the apo B-containing lipoproteins, chylomicrons, VLDL, and LDL, blocking their reaction with the enzymes added in a second reagent and giving specificity for HDL-C. The original reagent set required reconstitution of the enzyme reagent; however, both reagents in the second version introduced in mid-1998 are liquids.

A fifth homogeneous method was introduced by Denka Seiken Co. (Niigata, Japan) (78, 79) and was also distributed through Polymedco Inc. (Cortlandt Manor, NY) and by Randox Laboratories Limited (Crumlin, UK). This method (Fig. 2E) reportedly uses a selective reagent with cholesterol esterase and oxidase to generate peroxidase from the cholesterol in lipoproteins other than HDL. The initial non-HDL-derived peroxidase is scavenged by the enzyme catalase. A second reagent includes an inhibitor of catalase and a surfactant to release specifically HDL-C, which develops color through the usual peroxidase sequence.

These commercial homogeneous methods better conform to the workflow patterns in the modern clinical laboratory; sample handling can be as simple as placing a bar-coded specimen tube on the automated analyzer. Elimination of manual steps can provide cost savings; higher reagent costs are generally offset by decreased labor costs. Additionally, these newer methods improve precision through more consistent pipetting of smaller specimen volumes as well as precise temperature control and reaction timing, which facilitate achieving the NCEP analytical performance goals. Clinical laboratories are rapidly adopting the homogeneous methods (Fig. 1), although questions regarding specificity, especially on specimens with unusual lipoprotein composition, have been raised. Performance characteristics of each of the common commercially available homogeneous assays as well as some of the potential problems are described in detail subsequently.

POLYETHYLENE GLYCOL-MODIFIED ENZYME METHOD (PEG)

The PEG assay has been extensively evaluated in a variety of studies, including CRMLN certification (www.aacc.org/standards/cdc/cholesterolinfo.stm), comparisons with the RM and DCM, and multicenter studies (Table 5).

Imprecision. Most of the published evaluation studies report total CVs <3.1% (Table 6).

Dynamic range. The dynamics range spans from ~30 mg/L up to at least 1500 mg/L.

Table 5. Validation of the commercial homogeneous methods available in the US.

Method	CRMLN certification	Published comparison		Published multicenter study	TG interference[a]
		RM	DCM		
IRC (International Reagents)	√	√			
PEG (Kyowa Medex)	√	√	√	√	√
SPD (Daiichi)	√	√	√	√	√
AB (Wako)	√				√
CAT (Denka Seiken)	√				√

[a] Reported to be free of interference up to 10 000 mg/L TG.

Specificity. Okamoto et al. *(80)*, using gel chromatography to separate lipoproteins by size, demonstrated that the PEG assay includes the apo E-containing HDL particles with the rest of HDL-C, in contrast to a PTA method that precipitated this population, consequently yielding HDL-C results ~10% lower. Because the apo E-containing HDL particles are also included by the CDC RM, the homogeneous assay was considered more accurate. Okazaki et al. *(81)* supported these findings by demonstrating close agreement of the PEG method with a HPLC procedure: PEG = 1.026HPLC − 7.5 mg/L (*r* = 0.998). Another study similarly observed correlation with a conventional PTA precipitation procedure in normo- (*r* = 0.987) and hypertriglyceridemic (*r* = 0.953) samples; but supporting the previous studies, that *y*-intercept (~5–70 mg/L) was consistent with a negative bias by PTA *(82)*. apo E-containing particles are known to account for ~10% of the total HDL-C, consistent with the bias observed in these studies and also consistent with a previously observed negative bias of the PTA method in comparison with dextran sulfate and heparin precipitation methods *(9, 83)*.

Accuracy. The homogeneous PEG HDL-C assay was compared with the CDC RM (PEG = 1.068RM − 17 mg/L; *r* = 0.993; mean bias, 2.2%) and the DCM (PEG = 1.037DCM + 4 mg/L; *r* = 0.996; mean bias, 4.5%) in specimens with

Table 6. Imprecision in direct comparisons between conventional and homogeneous HDL-C methods.

	Total CV, %			
		Homogeneous method		
Reference[a]	Precipitation pretreatment	SPD (Daiichi)	PEG (Kyowa Medex)	AB (Wako)
81	2.0		0.6	
59	3.7	1.6	2.3	1.8
	3.6	1.3	1.8	1.6
	3.1	1.1	1.1	1.4
72	5.5	2.4	3.1	
	3.3	2.7	2.8	
60	3.7	2.6		
	3.0	2.6		

[a] One, two, or three control materials were evaluated in each study.

TGs <2000 mg/L *(84)*. In a multicenter study performed before the commercial release of the reagent by six laboratories in Europe, comparisons with a PTA precipitation method gave results with correlation coefficients of 0.96–0.99, but slopes that ranged from 0.95 to 1.08. In this study, which used a calibrator with the distributor's preliminary HDL-C setpoint, the PEG assay was negatively biased on average by 3% compared with the CDC RM. The distributor adjusted the calibrator by the suggested 3% to make results consistent with the RM *(84, 85)*, an adjustment expected to bring total error to ≤7.5%, within the 13% NCEP target. An earlier evaluation of this assay using a calibrator from the manufacturer (Kyowa Medex) gave a slope of 0.87 with a negligible *y*-intercept that produced negative total errors of 15–21% *(58)* compared with another CDC standardized method.

The first commercial reagents introduced to the market did not reproduce the encouraging results of the multicenter study with the subsequent calibrator adjustment. Correlation coefficients were acceptable (*r* = 0.987), but the slope was 12% high with a *y*-intercept of only 10 mg/L *(59)*. At the clinical decision cutpoints of 350 and 600 mg/L, total errors of 16.2% and 14.5%, respectively, were observed. However, subsequent calibrator lots yielded results as expected from the reference standardization (PEG = 0.994RM + 21 mg/L; *r* = 0.997) *(72, 86)*. Other investigators observed similar agreement *(77)*. These studies illustrate the challenges in appropriately assigning target values to calibrators. The concentration of the calibrator for the PEG assay now appears to be appropriately assigned, which is supported by a US proficiency survey *(87)*. Roche, a distributor of the PEG method, now has each new reagent lot routinely validated for accuracy by the CRMLN laboratory in Rotterdam. Users of all HDL methods should be aware of the lot-to-lot calibration issue and are advised to confirm the accuracy of each new lot by overlap studies and/or by direct comparison studies with one of the CRMLN laboratories.

The second, fully liquid PEG reagent version, introduced in 1998, was evaluated in another multicenter study and gave similar performance characteristics *(88)*. The two generations were correlated in seven different laboratories (*r* = 0.989) with a mean deviation of only 2 mg/L. A comparison with the DCM (PEG = 0.968DCM + 24.9 mg/L; *r* = 0.996) produced total errors at the decision points (350 and 600 mg/L) of 7.3% and 3.4%, respectively *(84)*.

Interferences. The PEG assay is free of TG interference up to ~10 000 mg/L regardless of whether the TGs are in chylomicrons or VLDL. With the fully liquid reagent, hypertriglyceridemic samples should be diluted with saline (9 g/L NaCl) to bring TG concentrations below 18 000 mg/L *(88)*. LDL-C concentrations of 3000−000 mg/L did not cause a significant bias, whereas LDL-C concentrations >6000 mg/L produced falsely increased HDL-C values *(88)*. Hemoglobin up to 10 g/L did not interfere,

but at higher concentrations a slight negative bias was observed. Bilirubin concentrations >100 mg/L yielded discrepant results when the homogeneous PEG assay was compared with precipitation procedures, but the studies did not make clear which assay was most affected. However, interferences from hemoglobin and bilirubin seem much less pronounced in this homogeneous assay compared with conventional precipitation procedures. Increased free fatty acids did not interfere, whereas paraproteinemias gave discrepant results in some samples compared with the PTA procedure. Heparin as anticoagulant does not interfere, but results with EDTA plasma should be multiplied by 1.06 to compensate for dilution caused by osmotic shifting of water from the red cells (26).

Patients with type III hyperlipoproteinemia, characterized by cholesterol enrichment of the VLDL because of impaired clearance of remnant lipoproteins, were observed to give erroneous values with the PEG assay, one falsely high and one low (89, 90), apparently because of remnant particles reacting atypically with the PEG assay. In contrast, the PEG assay overestimated HDL-C by 72% in hyperlipidemic (C57BL/6) mice with accumulation of cholesterol-rich remnant particles (91), made so by a high-fat, high-cholesterol diet. In apo E-deficient mice, which also accumulate remnant lipoproteins, the overestimation was 228%. The discordance seemed to be related to the degree of hyperlipidemia because the apo E-deficient mice had extremely high VLDL-cholesterol (VLDL-C) and LDL-C concentrations. By contrast, mice with normal cholesterol and TG concentrations demonstrated good agreement between the PEG assay and PTA precipitation. On the basis of these studies, users would be prudent to validate the PEG and other homogeneous assays before use in studies of animals and humans expected to have unusual lipoprotein characteristics such as increased remnants.

A recent evaluation of this method in patients with diabetes inferred but did not explicitly state minimal interference (92). Whether liver disease interferes with the PEG assay, as has been observed with other homogeneous assays, is not currently known.

synthetic polymer/detergent (spd) method
The earliest version of this reagent, which included dextran sulfate and a lyophilized enzyme reagent, demonstrated poor specificity for HDL-C (59, 60, 82, 93). Moderate increases of VLDL-C and LDL-C caused falsely high HDL-C values. On the other hand, only ~70% of HDL (isolated by ultracentrifugation) added to specimens in addition experiments was recovered. Although the specificity of this reagent seemed adequate in studies using electron microscopy (94), in comparisons with a method combining ultracentrifugation with dextran sulfate precipitation, results were biased whether the TG concentration was low or high (TGs <4000 mg/L: SPD = 0.96RM + 8 mg/L; TGs ≥4000 mg/L: SPD = 0.85RM + 108 mg/L),

but correlations were good, $r = 0.96$ in both comparisons (60). The observed lack of specificity made the first version of this assay of questionable value (59, 82), especially in hypertriglyceridemic samples (60) or in patients with liver cirrhosis (93). The 1998 modification, which includes both liquid reagents with a more specific detergent, has been certified by the CRMLN (www.aacc.org/standards/cdc/cholesterolinfo.stm) and reportedly demonstrates improved performance (74).

Imprecision. Between-run and total CVs over a wide range of HDL-C concentrations were <2.1%.

Dynamic range. The detection limit of the SPD method was 3–40 mg/L with linearity to at least 2000 mg/L (74).

Accuracy. A multisite comparison of the newer liquid reagent with the DCM (SPD = 1.015DCM + 10 mg/L; $r = 0.993$) showed mean biases of 4.2% and 3.1% and total errors of 7.7% and 6.6%, respectively, at the HDL-C decision points, well within current NCEP targets (74). Comparison with the CDC RM on samples with TGs >2 g/L (SPD = 1.020RM − 2 mg/L; $r = 0.985$) gave total errors of 5.1% and 5.3%, respectively (79). Two independent comparisons with a PTA assay yielded comparable results with excellent correlation coefficients of ~0.99 and mean biases of ~30 mg/L (74, 86).

Interferences. Addition experiments with lipoproteins isolated by ultracentrifugation revealed no significant interference at LDL-C concentrations up to 7000 mg/L and VLDL-TG up to 19 000 mg/L. Interference was <5% in addition experiments with bilirubin up to 300 mg/L, free hemoglobin up to 6 g/L, ascorbic acid up to 500 mg/L, and γ-globulin up to 10 000 mg/L. Sera with high concentrations of IgG or IgM paraproteins produced similar results after dilution with saline (9 g/L NaCl), suggesting minimal interference. In contrast to the earlier lyophilized version, the second reagent version did not demonstrate interference from increased free fatty acids (59). Comparison of samples from 10 subjects drawn in both the fasting and postprandial state revealed mean HDL-C concentrations of 446.5 and 439.5 mg/L, respectively (74), suggesting that results were not significantly different in the nonfasting state.

In their study using hyperlipidemic mice, Escolà-Gil et al. (91) observed that the SPD assay overestimated HDL-C by 101%. In contrast to the PEG assay, interference in humans with overt type III hyperlipoproteinemia has not been reported, although the mouse studies suggest caution. Surprisingly, no difference was observed with the SPD assay when EDTA plasma was used instead of serum, despite the fact that EDTA has an osmotic effect that is expected to produce a dilution of ~3–6% (95, 96). On the other hand, heparinized plasma gave values that, on average, were slightly but statistically significantly higher (2%) for unknown reasons (74).

In an evaluation of the earlier reagent version, Simó et al. *(93)* observed that the concordance of the SPD HDL-C assay with a polyethylene glycol precipitation method was dependent on the type of patients. Good agreement was observed in healthy controls and in elderly people, whereas the scatter increased in patients with myocardial infarction, nephrotic syndrome, and liver cirrhosis. The current assay version has not been evaluated in such patient groups. A reagent that appears to have the same formulation (Olympus Diagnostica, Hamburg, Germany) was reported to be free of interference in hemodialysis patients with moderately increased TG values *(97)*. The third version of this reagent, without Mg^{2+}, reportedly demonstrates less interference from TGs and immunoglobulins *(76)*.

IMMUNOLOGIC METHOD (AB)

An evaluation *(98)* of the first reported immunoinhibition (IRC) method *(68)* observed acceptable imprecision, accuracy, and specificity but concluded that the four successive reagent additions made the method less desirable commercially. The IRC method has recently qualified for CRMLN certification (Table 5). Subsequently, Wako Chemicals USA, Inc. reported a more convenient method (AB) that uses antibodies to achieve specificity *(59)* with the following performance characteristics.

Imprecision. Within-day, among-day, and total CVs were ≤1.8%.

Dynamic range. According to the manufacturer, the lower limit of detection is 10 g/L, and the method is linear up to at least 1800 mg/L; an independent evaluation confirmed linearity up to at least 1000 mg/L *(59)*.

Accuracy. An early comparison *(59)* with a PTA precipitation method, with approximately one-half of the samples measured by the PTA method after ultracentrifugation to remove TG-rich particles, observed high correlation but an apparent positive bias (mean, 414 mg/L for the AB method vs 336 mg/L for PTA). This bias gave somewhat high total error values (22.3% and 15.6%) at the clinical decision cutpoints of 350 and 600 mg/L. Even recognizing that the PTA method is considered negatively biased by ~100 mg/L, these data suggest that the calibrator set point was too high at the time of this study. The AB assay was recently certified for accuracy through the CRMLN program (www.aacc.org/standards/cdc/cholesterolinfo.stm) with biases of ~1% and total errors of ≤5%, according to comparison results supplied by the manufacturer.

Interferences. In an independent study, LDL-C and VLDL-TG did not interfere significantly up to 6000 and 9000 mg/L, respectively *(59)*. Antibody concentrations appeared to be sufficient to complex all of the non-HDL particles. Hemoglobin did not interfere up to 2 g/L. The manufacturer claims freedom from LDL-C and VLDL-TG interference up to 10 g/L, hemoglobin to 5 g/L, and bilirubin and ascorbic acid to 500 mg/L. Free fatty acids at high concentrations were reported to cause overestimation of HDL-C in the earlier lyophilized version *(59)*, but this potential interference has not been reported for the current reagent.

CATALASE REAGENT METHOD (CAT)

Imprecision. The CAT method meets NCEP targets with CVs ~2%.

Dynamic range. The assay is reported to be linear at 90–1490 mg/L *(78, 79)*.

Accuracy. the method qualified for CRMLN certification (www.aacc.org/standards/cdc/cholesterolinfo.stm), although comparisons with the RM or DCM have apparently not been published. The relationship by linear regression to the PEG method was reported to be good: CAT = 1.09PEG − 49 mg/L; r = 0.966 *(57)*.

Interferences. Interference was reportedly insignificant from TGs <17 000 mg/L, bilirubin to 250 mg/L, ascorbic acid to 500 mg/L, and hemoglobin to 5 g/L. According to the manufacturer's package insert, the method is suitable for use with either EDTA or heparinized plasma. A recent study suggested that this method is less subject to interference from TGs and immunoglobulins than other homogeneous assays *(99)*. Another recent report evaluating the same reagent from Randox Laboratories in cirrhotic patients concluded that this reagent was less subject to interference from liver disease compared with other homogeneous methods *(100)*.

Performance Summary of Homogeneous Assays

IMPRECISION

In summary, evaluation studies of the homogeneous assays clearly confirm the expected improvements in precision over the earlier precipitation methods, as seen in Table 6, which shows imprecision observed on one to three quality-control materials in direct comparison studies that included at least one homogeneous assay and a conventional assay *(59, 60, 72, 81)*. The homogeneous assays typically demonstrated within-run imprecision of ~1% and between-run and total imprecision of 2–3%. Conventional precipitation methods generally gave CVs approximately twice as high, in the range of 2–6%.

ACCURACY

Making valid judgments about overall accuracy or bias from published evaluations can be difficult. A complicating factor is reagent modifications; for example, the conversion in 1998 of homogeneous assays, including PEG, SPD, and AB, from lyophilized to fully liquid format with other concurrent modifications in reagent formulation gave substantial changes in performance characteris-

tics (74). Details of reagent modifications may not be fully disclosed for proprietary reasons, and other modifications might have been made in the past or may be made in the future. Several versions of the same reagent might be in different segments of the market at any one time. Various distributors and instrument applications of a primary reagent may establish calibration by different means, and calibrations may be intentionally different from country to country to accommodate local accuracy targets. Accuracy may vary with lot-to-lot calibration changes, as mentioned specifically above in connection with the PEG and AB methods (59, 85). Published performance data thus may not be indicative of current or local performance. In addition, comparison methods in some studies were not standardized or validated by comparison with the RM, DCM, or another method traceable to the accepted RM. Therefore, caution must be exercised in generalizing conclusions about the accuracy of a particular method from the literature. All of the primary homogeneous methods now in distribution (Table 5) have current CRMLN certification (www.aacc.org/standards/cdc/cholesterolinfo.stm), but this demonstration of accuracy is specific for a particular instrument and calibrator and cannot be considered universally applicable to all distributor versions, instrument applications, and lots. Users can check the certification list and/or request that their reagent suppliers provide evidence of accuracy, best demonstrated by CRMLN certification, for their specific reagents, instrument application, and calibrator.

SPECIFICITY AND INTERFERENCE
Of equal concern is the accuracy of measuring HDL-C in each individual specimen. This is a function not only of bias or overall inaccuracy, but also of the ruggedness of the assays, i.e., specificity for HDL-C and absence of interference by other lipoproteins and constituents of the specimen matrix. This is an extremely important issue for the homogeneous methods, especially for laboratories supporting research studies and lipid clinics where a high proportion of specimens may have atypical lipoprotein characteristics. The published evaluations in which accuracy was assessed were often conducted using samples from relatively healthy subjects. Most studies did not determine performance in samples from patients with extreme hyperlipidemia, such as type III, or other conditions, such as liver and kidney disease, which often produce unusual lipoproteins with atypical separation characteristics. Since the introduction of homogeneous methods, there have been continuing anecdotal reports of discrepant results, which are supported by the systematic human and animal studies (91–93, 100) described above. Considering the importance of this issue, interference studies to date have been relatively modest, although generally encouraging (59, 70, 71, 74, 79, 84). Evaluations indicate that TGs <9000 mg/L do not appreciably interfere with any of the methods, and some methods are reportedly free of interference even up to 17 000 (CAT) or 19 000 mg/L (SPD). Hemoglobin <2 g/L does not seem to affect any homogeneous method, and the PEG method is not affected by hemoglobin up to 10 g/L. Bilirubin values <100 mg/L, depending on the homogeneous assay and the protocol, do not interfere appreciably. The SPD (74) and CAT methods (79) reportedly can be used with plasma, either EDTA or heparin, whereas the PEG method (59, 70) is acceptable with heparin plasma but may experience interference from EDTA, likely because of osmotic effects. The earliest version of the SPD assay (74) was validated in specimens collected postprandially.

Reports of significant discrepancies in atypical specimens deserve additional studies. Laboratories performing long-term studies with conventional methods and considering substitution of one of the homogeneous assays might perform studies on normal specimens and observe good agreement, yet encounter compromised results on atypical specimens. Laboratories supporting lipid clinics with a high proportion of specimens with atypical lipoproteins could observe discrepant results on certain specimens that might confound treatment decisions. Before the homogeneous assays can be recommended without qualification for such laboratories, they must be evaluated thoroughly in atypical specimens.

EFFECTS OF STORAGE AND FREEZING
HDL is relatively labile, and the freezing-thawing process has been clearly shown to affect some of the precipitation methods (29, 101, 102). However, the specific effects of prolonged storage and freezing have not been reported in detail for the homogeneous assays, although there is preliminary indication of significant effects (103). Changes in pH, bacterial action, enzyme and transfer protein activities, and other factors can affect HDL composition and separation characteristics. The NCEP Lipoprotein Panel report (26, 27) recommended that HDL separations be completed within 1–2 days of collection. Specimens stored for longer periods should be frozen, preferably at temperatures below −50 °C, at which specimens are considered reasonably stable for 1–2 years. Thawed specimens are not uniform in composition and must be thoroughly but gently mixed before sampling. Because the homogeneous methods have been shown to be somewhat sensitive to analyte and matrix factors, effects of storage and freezing should be determined for each of the reagent versions.

Considerations in Selecting a Homogeneous Method
Clinical laboratories must consider a variety of factors, technical and economic, in selecting the most suitable method for their particular situation (104). Two reports (58, 74) concluded that the homogeneous reagents decrease overall assay costs by ~20%, an important consideration. Although homogeneous reagents tend to be more expensive than conventional precipitation reagents, sav-

ings in labor costs more than compensate where labor costs are high. Capability for full automation and consolidation of HDL-C with other common tests on a single workstation are also important factors. In some cases, external factors such as personnel cuts and the need for other cost reductions force the choice; laboratories have simply needed to adopt a homogeneous assay to cope. In other cases, laboratories must carefully weigh their unique situation in making a choice based on tradeoffs. The potential for reducing labor and other costs and consolidation of workstations plus the improved imprecision and decreased specimen volumes must be weighed against the possibilities of introducing biases and discrepant results in certain types of specimens. Laboratories supporting long-term clinical trials must consider that future reagent modifications by manufacturers might cause bias shifts overall or in certain types of specimens, e.g., a modified reagent might be validated in normal specimens but produce biases in unusual specimens. Therefore, laboratories performing research investigations and long-term clinical trials and those supporting lipid clinics are advised to be more cautious in adopting the homogeneous methods.

Need for Additional Studies

The manufacturers of the homogeneous assays are encouraged to continue participation in the CRMLN certification program at least every other year or when systems are modified (for information, see www.aacc.org/standards/cdc/cholesterolinfo.stm) to assure consistent accuracy. Instrument partners and distributors might need to complete additional certification studies when their applications/calibrations differ from those of the primary manufacturer. In addition, current reagent formulations should be evaluated on a broad cross-section of unusual specimens, those at the extremes of lipoprotein distributions, and those from subjects with conditions likely to alter lipoprotein characteristics (e.g., advanced age, pregnancy, diabetes, liver and kidney disease). The homogeneous methods should also be compared with the RM or a well-characterized precipitation method in samples from a wide variety of patients before and after treatment to assure that results track the treatment effects appropriately. HDL-C measurements in these studies must be performed in sufficient replicates to distinguish random from systematic error and identify any matrix-related problems. Finally, the specimens giving highly discrepant results should be fully characterized to understand the cause of the differences, which could guide future improvements in the assay formulations. Developers should recognize that a method giving discrepant results in comparison with the RM could actually demonstrate improved prediction of CAD risk, which could be established in a valid population study, as mentioned above. The effects of prolonged storage and freezing

should also be established for each of the homogeneous methods.

Trends in Use of Major Conventional and Homogeneous Methods

The homogeneous methods for the quantification of HDL-C seem to be increasingly accepted by US clinical laboratories (Fig. 1), steadily replacing the conventional precipitation methods. Data gleaned from the proficiency testing reports of the College of American Pathologists indicate that the homogeneous assays first appeared as an identified class in 1997, with 550 laboratories reporting their use in the third cycle. One year later, the number of laboratories reporting use of homogeneous assays had increased to 853, and by the year 2000, the number had increased dramatically to 2578. The dextran sulfate (50 000 Da with Mg^{2+}) method peaked in 1995 at 1479 laboratories, and by year 2000 had decreased to 510. The PTA assay, which was most common in 1994 with ~1300 laboratories, decreased steadily to 281 by the end of the year 2000. A second-generation method with dextran sulfate linked to magnetic beads increased sharply from 117 laboratories in 1994 to 530 in 1996, declined moderately through 1999, and then increased slightly to 591 by the end of year 2000. In the last survey of year 2000, of the laboratories reporting use of homogeneous methods, 1599 used the SPD (Daiichi), 683 the PEG (Kyowa Medex), 144 the AB (Wako), and 120 the CAT (Denka Seiken) assays. These patterns clearly indicate the preference for fully automated, convenient, and labor-saving methods. The data also suggest that the homogeneous assays enabled more laboratories to perform the HDL-C assay with an overall increase in the number of laboratories measuring HDL-C in the proficiency surveys.

Conclusions

The new homogeneous assays for HDL-C represent a remarkable technologic breakthrough in achieving full automation of previously highly tedious manual pretreatment methods. The homogeneous assays reduce operating costs while improving workflow and making the results more likely to conform to NCEP targets for analytical performance. The assays appear reasonably specific and free from the effects of major endogenous interferences, although areas of concern still exist. Of the common interferences, TGs are especially important, and at least three methods can accommodate TG concentrations up to 10 000 mg/L. However, TG concentrations >20 000 mg/L will likely interfere with all of the methods. Many routine laboratories, for which factors such as full automation and cost reduction are paramount, have already adopted a homogeneous assay. Lipid research laboratories and laboratories supporting lipid clinics with a high proportion of atypical specimens will likely want additional validation. Laboratories supporting long-term trials may not adopt the methods until the reagents have become optimized and modifications are no longer likely.

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