CRITICAL REVIEW
Termeh Ahmadraj and Anthony J. Killard
The evolution of selective analyses of HDL and LDL cholesterol in clinical and point of care testing
Cardiovascular disease is a leading cause of death worldwide and is caused by the build up of atherosclerotic plaques in the vasculature. It is now well established that the formation of these plaques is closely related to levels of both high density lipoprotein (HDL) and low density lipoprotein (LDL) cholesterol. Thus, the importance of the effective measurement of these is critical for the improved diagnosis and management of atherosclerosis. This review discusses the emergence of methodologies for the selective determination of both LDL and HDL cholesterol. It begins with an explanation of the first methodologies based on ultracentrifugation and precipitation techniques, the development of reference methods, through to the emergence of methodologies suitable for routine laboratory use, followed by the development of professional use, point of care technologies. Finally, the current status of selective tests for cholesterol based on biosensor methodologies is reviewed and the potential for application in consumer diagnostics is discussed.
Cholesterol (including cholesteryl esters), phospholipids (PLs), and triglycerides (TGs) are three major types of lipid present in the plasma. Cholesterol ([3β]-cholest-5-en-3-ol) is by far the most abundant member of a family of polycyclic compounds known as sterols. Cholesterol in plasma lipoprotein can be found in the free form, esterified to long-chain fatty acids (cholesteryl esters), and in other covalent and non-covalent linkages in animal tissues. The chemical structures of cholesterol and cholesteryl ester are shown in Fig. 1.

Since lipids are not readily soluble in water, only small amounts are present as unesterified cholesterol. To allow adequate transport of cholesterol and other lipids, lipoproteins form a coat around the lipids in order to suspend them in the plasma. Lipoproteins are particles composed of lipid and protein which are held together with noncovalent bonds. They consist of a nonpolar lipid core of mainly cholesteryl ester and triacylglycerols and an outer layer of phospholipids, unesterified cholesterol and proteins (Fig. 2). The function of the lipoprotein particle is to transport lipids such as cholesterol or triglycerides around the body via the blood stream. Based on the relative densities of these species, chylomicron (CM), very low-density lipoprotein (VLDL), low-density lipoprotein (LDL) and high-density lipoprotein (HDL) are the four major categories of lipoprotein.

Lipoproteins have unique physical and chemical characteristics, particularly with respect to their relative amount of lipids, protein–lipid ratio and specific protein species present (Table 1). Since lipoproteins vary in size and density, centrifugation techniques have been used to separate them and distinguish them from each other. LDL and HDL are the two major lipoproteins found in humans, and are responsible for carrying cholesterol in the blood. The lipid cores of HDL and LDL contain cholesteryl esters (CEs) and TGs surrounded by PLs, unesterified cholesterol and specialized proteins known as apolipoproteins (Fig. 2). The CEs are enriched in linoleate, reflecting their biosynthetic origin. Apolipoproteins are amphipathic in nature which can interact with lipid moieties of lipoproteins and the aqueous environment and are specialized to facilitate several biochemical steps associated with plasma lipid metabolism. Plasma apolipoprotein can be classified as the non-exchangeable apolipoproteins (e.g. apo B-100) and the exchangeable apolipoproteins (e.g. apo A-I, apo A-II).

LDL particles with a density range between 1.019 and 1.063 kg L⁻¹ carry 60–70% of the total serum cholesterol. A single apolipoprotein, apo B-100 is the only protein component of LDL, which is highly insoluble in aqueous solution and is the largest monomeric protein known. Since apo B-100 is highly insoluble in aqueous solution, it remains with the lipoprotein particle throughout its metabolism. LDL is the major atherogenic lipoprotein and has long been identified by the National Cholesterol Education Program (NCEP) as the primary target of cholesterol lowering therapy. The importance of reducing the risk of coronary heart disease (CHD) by lowering LDL-C has been shown by clinical trials.

HDL is the smallest lipoprotein, which normally carries 20–30% of the total serum cholesterol (HDL-C). Apo A-I and apo A-II are the two major apolipoproteins of HDL. They are both classified as exchangeable amphipathic apolipoproteins and are soluble in aqueous solutions. All the apolipoproteins, other than apo B-100, have a helical structure with a hydrophobic and a hydrophilic domain. The hydrophobic domain of the

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**Fig. 1** Chemical structure of (a) cholesterol, and (b) cholesteryl ester.

**Fig. 2** General structure of lipoproteins. Adapted from Griffin (2009).

**Fig. 3** LDL consensus model.
apolipoprotein interacts with the lipid, while the hydrophilic domain orients itself towards the aqueous phase. The main protein component of HDL is apo A-I, of which 70% is synthesized within the liver and the rest in the intestine. Fig. 4 illustrates that nascent HDL (HDL₃) is secreted by the liver and intestines and is transformed into mature HDL₂ by the action of the lecithin-cholesterol acyltransferase (LCAT). HDL₃ is discoid in structure and contains apo A-I, phospholipids and free cholesterol while HDL₂ is round, mature and contains esterified cholesterol.

Although HDL-C levels are inversely correlated with the risk of CHD, the value of treating low HDL-C is not as well established as treating high LDL-C. Most treatment options for lowering high LDL-C levels such as physical exercise, weight loss and even some of the cholesterol lowering drugs, have also demonstrated a beneficial effect on HDL-C concentration.

Importance of standardisation of measurement for HDL-C and LDL-C

Since low HDL-C and high LDL-C levels are linked to increased risk of heart attack, the importance of accurate measurement of both HDL-C and LDL-C has been emphasized by the National Cholesterol Education Program (NCEP). The NCEP is a program managed by the US National Heart, Lung and Blood Institute which established the laboratory standardisation panel on blood cholesterol measurement in order to assess the reliability of cholesterol measurement in clinical laboratories and improve the precision and accuracy of cholesterol testing. The main reason for standardisation is to ensure the agreement of reported results across measurement systems, laboratories and over time.

Table 2 shows the classification of serum total cholesterol (TC), LDL and HDL as summarised in the third report of the expert panel on detection, evaluation, and treatment of high Blood Cholesterol in Adults (Adult Treatment Panel III, or ATP III) presenting the NCEP’s updated recommendations for cholesterol testing and management. Based on the clinical need to reliably categorise patients, the NCEP established analytical performance goals for measurement of the total cholesterol, HDL-C and LDL-C (Table 3).

The development of routine methods for the measurement of HDL and LDL cholesterol

Given the fact that lipoproteins are defined by their density, one of the first separation methods used to differentiate between HDL-C and LDL-C and other lipoproteins was

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**Table 1** Classification of lipoproteins

<table>
<thead>
<tr>
<th>Class</th>
<th>Density (kg L⁻¹)</th>
<th>Diameter (nm)</th>
<th>Protein (%)</th>
<th>Free cholesterol (%)</th>
<th>Cholesteryl ester (%)</th>
<th>Phospholipids (%)</th>
<th>Triglycerides (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HDL</td>
<td>1.063–1.21</td>
<td>8–15</td>
<td>33</td>
<td>7</td>
<td>40</td>
<td>46</td>
<td>6</td>
</tr>
<tr>
<td>LDL</td>
<td>1.019–1.063</td>
<td>18–24</td>
<td>25</td>
<td>11</td>
<td>50</td>
<td>29</td>
<td>10</td>
</tr>
<tr>
<td>VLDL</td>
<td>0.95–1.006</td>
<td>30–52</td>
<td>10</td>
<td>7</td>
<td>18</td>
<td>20</td>
<td>55</td>
</tr>
<tr>
<td>CM</td>
<td>&lt;0.95</td>
<td>80–1200</td>
<td>&lt;2</td>
<td>2</td>
<td>3</td>
<td>8</td>
<td>85</td>
</tr>
</tbody>
</table>


**Table 2** ATP III Classification of total cholesterol, LDL Cholesterol and HDL cholesterol. Reproduced from ATP III report (2001)

<table>
<thead>
<tr>
<th>Analyte</th>
<th>Concentration (mg dL⁻¹)</th>
<th>Classification</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total cholesterol</td>
<td>&lt;200</td>
<td>Desirable</td>
</tr>
<tr>
<td></td>
<td>200–29</td>
<td>Borderline high</td>
</tr>
<tr>
<td></td>
<td>≥240</td>
<td>High</td>
</tr>
<tr>
<td>LDL cholesterol</td>
<td>&lt;100</td>
<td>Optimal</td>
</tr>
<tr>
<td></td>
<td>100–129</td>
<td>Near or above optimal</td>
</tr>
<tr>
<td></td>
<td>130–159</td>
<td>Borderline high</td>
</tr>
<tr>
<td></td>
<td>160–189</td>
<td>High</td>
</tr>
<tr>
<td></td>
<td>≥190</td>
<td>Very high</td>
</tr>
<tr>
<td>HDL cholesterol</td>
<td>&lt;40</td>
<td>Low</td>
</tr>
<tr>
<td></td>
<td>≥60</td>
<td>High</td>
</tr>
</tbody>
</table>

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Fig. 4 The metabolic origins of HDL. With permission from Rye and Barter (2012).
ultracentrifugation. However, since ultracentrifugation methods are tedious, time consuming and do not easily achieve complete and reproducible recovery, this approach is not considered practical for use in clinical laboratories.  

However, ultracentrifugation is still considered as the basis of reference methods for measuring HDL and LDL cholesterol.  

Since lipoproteins are a heterogeneous mixture of lipids and proteins which are not strictly defined, a significant overlap can exist in the physical properties of the major lipoprotein classes. Therefore, a primary reference method for HDL-C and LDL-C measurement has not been developed. The most common approach used to determine LDL-C in the clinical laboratory is the Friedewald calculation. The principle of the Friedewald calculation is as follows:

1. TC is distributed among the three major lipoprotein classes (HDL, LDL and VLDL).
2. VLDL carries most of the circulating TGs and therefore VLDL-cholesterol (VLDL-C) can be estimated reasonably well from measured total TGs (TG/5 for mg dL⁻¹ or TG/2.2 for mM units).
3. LDL-C is then calculated as:

\[
\text{LDL-C} = \text{TC} - \text{HDL-C} - \frac{\text{TG}}{5} \quad (\text{mg dL}^{-1})
\]

\[
\text{LDL-C} = \text{TC} - \text{HDL-C} - \frac{\text{TG}}{2.22} \quad (\text{mM})
\]

This method is the most commonly used method in the clinical laboratory and in large scale studies. Although the Friedewald method is widely used, the well-known limitation of this method increases the interest in improving the accuracy of LDL-C estimated by this equation. The Centers for Disease Control and Prevention (CDC) use a reference method (RM) based on the Lipid Research Clinic’s (LRC) beta-quantification procedure (BQ) for measuring LDL-cholesterol. In this method an aliquot of plasma is ultracentrifuged at density 1.006 kg L⁻¹ for at least 18 h at 105 000g to accumulate the VLDL as a floating layer. The amount of LDL-C is then calculated using eqn (3):

\[
\text{LDL-C} = \left[ d 1.006 \text{ kg L}^{-1} \right] \text{bottom fraction cholesterol} - \text{HDL-C}
\]

Accuracy in the HDL-C measurement has also been important for the calculation of LDL-C using the Friedewald formula. As recommended by NCEP, the CDC method is the current secondary reference method for HDL-C measurement. There are three key steps to this method:

1. Ultracentrifugation at a density of 1.006 kg L⁻¹ to isolate HDL and LDL from other lipoproteins.
2. Selective precipitation of LDL with heparin/MnCl₂.
3. Analysis of cholesterol in the HDL (supernatant) using the Abell-Kendall assay.

Since there are only a few laboratories capable of performing the ultracentrifugation steps necessary in the CDC method, and due to the high volume (greater than 5.0 mL) of sample required, the Cholesterol Reference Method Laboratory Network (CRMLN) also developed the Designated Comparison Method (DCM) based on a modified dextran sulphate procedure. This method uses 50 kDa dextran sulphate with MnCl₂ for the precipitation of non-HDL, followed by measurement of the cholesterol in the supernatant by the CDC reference method. Other than reference methods based on ultracentrifugation, laboratory based tests using electrophoresis, chromatography and spectrophotometry have been developed.

**Electrophoresis**

Due to the differences in the size and charge of various lipoproteins, isolation can be also achieved using electrophoresis techniques, with visualization achieved using lipophilic dyes. However, due to the fact that the lipophilic dyes are not specific for a class of lipid such as cholesterol, TGs or PLs, these techniques cannot be used for quantitative analysis, but can be used for qualitative analysis of lipoproteins. Visual presentation is a distinct advantage of the electrophoretic methods facilitating observation of atypical lipoproteins. For the routine clinical laboratory, both ultracentrifugation and electrophoresis have disadvantages especially when the workload is high.

In the last decade, there has been significant progress in the development of microsystems applied to separation techniques such as capillary electrophoresis. Ruecha et al. (2011) have proposed a method for rapid detection of cholesterol using poly(dimethylsiloxane) microchip capillary electrophoresis (PDMS MCE) based on the coupling of enzymatic assays and electrochemical detection. Such techniques, could, in the future, have the potential for selective determination of HDL-C and LDL-C at the point of care.

**Chromatography**

A variety of HPLC methods have been used to separate lipoproteins such as HDL, LDL, VLDL and CM, but this has been impeded by the poor stability of the columns used for separation. Even improved HPLC techniques which separate serum lipoproteins based on their size using two connected columns with subsequent determination of cholesterol concentration using an online enzymatic reaction cannot be used in the routine clinical laboratory.

Recently Dong et al. (2011) have established a new method for determination of HDL and LDL cholesterol using ultracentrifugation and HPLC. Ultracentrifugation is used to separate HDL and LDL subfractions and Lipoprotein (a). Cholesterol levels in the ultracentrifugal bottom fractions were analyzed by...
HPLC. While this method requires substantially less specimen volume when compared to other separation methods, it still requires the use of ultracentrifugation which is not applicable to clinical measurement.

Spectrophotometry

Due to its widespread adoption and simple methodology, many spectrophotometric methods have been developed to measure HDL-C and LDL-C. Initially, cholesterol was measured using non-enzymatic spectrophotometry in the form of the Liebermann–Burchard (L–B) and Killani–Zak assays. The L–B reaction is performed in an acetic acid mixture. 4AAP, phenol and HRP forms a quinoneimine dye, which can be measured at 500 nm by spectrophotometry. The selectivity of spectrophotometric methods was improved significantly by using enzymes such as cholesterol esterase (ChEs), cholesterol oxidase (ChOx) and horseradish peroxidase (HRP) (Fig. 5).

Where 4AAP is 4-aminantipyrine and Trinder's dye is an enhancer such as phenol. Measuring the amount of O2 consumed or the levels of H2O2 produced are the preferred methods of quantifying cholesterol spectrophotometrically. Due to the consumption of oxygen by other substances such as ascorbic acid in clinical samples, any method measuring the amount of oxygen consumed in Fig. 5 is not accurate. Therefore, measuring the amount of H2O2 produced was found to be a more accurate method of quantifying blood total cholesterol. In this method, the H2O2 generated in the presence of 4AAP, phenol and HRP forms a quinoneimine dye, which can be measured at 500 nm by spectrophotometry.

For the selective measurement of HDL-C or LDL-C, two additional aspects of the assay need to be employed. Firstly, the enzymes must gain effective access to the cholesterol associated with the lipoprotein fraction. Secondly, the enzymes must also only gain access to the cholesterol from the specific lipoprotein fraction to be measured and be preventing from catalysing cholesterol in other fractions. In the following sections, some of the approaches that have been used for measuring HDL-C and LDL-C will be reviewed.

Selective methods of HDL and LDL cholesterol measurement

Chemical precipitation methods

In chemical precipitation methods, lipoproteins other than the target, e.g., HDL-C are aggregated and rendered insoluble using polyanions in combination with divalent cations which can then be sedimented by low-speed centrifugation, while HDL remains soluble. The supernatant containing HDL-C can then be recovered for cholesterol analysis. The specific mechanism of lipoprotein precipitation by polyanions and divalent cations has not yet been fully elucidated. However, it is important to consider the interaction between negatively charged groups on the polyanions and positively charged groups on the protein moieties of the lipoproteins. Divalent metal ions interact with negatively charged groups (such as phospholipids) on the lipoproteins to facilitate formation of insoluble complexes. The larger, more lipid-rich lipoproteins such as VLDL and LDL, form insoluble complexes more readily than the smaller, protein-rich HDL. The insoluble complexes may either remain suspended in the solution or float to the surface in the presence of high concentrations of TG-rich lipoproteins. Heparin–Mn2+ has been a popular polyanion/divalent ion combination which has been used to assign target values to reference materials. Since commercial heparin showed some inconsistency in its properties for routine use, dextran sulphate–Mg2+ (50 kDa), phosphotungstic acid–Mg2+ (ref. 55) and polyethylene glycol (PEG) have been used as alternatives.

LDL particles can also be precipitated using certain reagents. The amount of LDL-C is determined by subtracting the cholesterol measured in the supernatant from the total cholesterol. Alternatively, the precipitate could be dissolved to measure the level of LDL-C directly. A number of reagent formulations have been used for the selective precipitation of LDL-C including: heparin at pH 5.12 in sodium citrate buffer; polyvinyl sulphate (PVS) in EDTA; PEG methyl ether; unspecified amphipatic polymers in imidazole buffer, at pH 6.10. These precipitation methods did not show noticeable advantages in precision and accuracy compared to the Friedewald calculation.

Homogenous methods

Homogeneous assays were a major step forward in improving the precision of earlier precipitation methods. Full automation eliminated manual pipetting, off-line pre-treatment, centrifugation and separation steps and improved assay precision, in line with recommended NCEP criteria. The development of such assays has been an area of intense commercial research and development dominated by several Japanese companies including Kyowa Medex, Seikisui Medical (formerly Daiichi Pure Chemicals Company), Deneka Seiken Co., Kokusai or Sysmex International Reagents (formerly International Reagents Corporation, IRC), Wako Chemicals, UMA and Serotec. The precise mechanisms involved in the interaction between the lipoproteins and the assay reagents used in these assays remain unclear. Fig. 6 illustrates a generalised approach to the selective detection of HDL-C in the presence of other lipoproteins using the homogeneous principle. A brief description of the principles of each of the homogeneous HDL-C assay methodologies is given in the following section and their reaction mechanisms and performance characteristics summarised in Table 4.

In their review, Warnick et al. (2001) described the methods of Kyowa Medex, Daiichi, Deneka, International Reagents Corporation (IRC) and Wako for HDL-C in detail and compared them with conventional assay methods. The authors reported that all five methods demonstrated acceptable accuracy, precision and total error by meeting the NCEP criteria, making them suitable for clinical application.
IRC was the first to publish a report of a HDL-C fully automatable homogeneous assay based on an immunological separation method in 1994. Based on this method, CM, VLDL and LDL were first aggregated using a reagent containing PEG and then protected with antibodies to apo B and apo C. In the next step, unprotected HDL-C underwent enzymatic reaction as described in Fig. 5. In the final step, guanidine salts were used to stop the enzymatic reaction and clear the reaction mixture. The final absorbance was measured at 600 and 700 nm. In spite of the fact that this assay showed reasonable precision, accuracy and specificity, the addition of four different reagents limited its application to a small number of automated analyzers.

Later on Kokusai (formerly IRC) developed a new reagent containing calixarene to produce a soluble complex of non-HDL–calixarene. In 1995, Kyowa Medex reported a homogeneous assay for HDL-C. Based on this method, the combination of PEG-modified enzymes with α-cyclodextrin sulphate provided selectivity for the determination of HDL C in serum in the presence of a small amount of dextran sulphate with no need for precipitation of lipoprotein aggregates. PEG-modified ChEs and ChOx showed selective catalytic activity toward lipoprotein fractions. The reactivity increased in the order LDL < VLDL = CM < HDL. Although the mechanism for the selectivity of the modified enzymes towards the lipoprotein fractions is not clear, it is suggested that the modified enzymes may be able to recognize differences in hydrated density, net charge, or size of the various lipoprotein fractions. Size-exclusion chromatography revealed that PEG-modified ChEs breaks up the lipoprotein particles more effectively than the native enzyme, probably because of the amphiphilic properties of the attached PEG molecules. Therefore, HDL should be more susceptible to the modified enzyme than LDL, explaining the observed differences in reactivities of cholesterol moieties of the lipoprotein fractions. H$_2$O$_2$ generated from the enzymatic reaction is measured spectrophotometrically. This method was evaluated in several studies including comparison with RM and DCM methods, which showed correlations of 0.993 and 0.996, respectively.

Daichi developed a homogeneous assay for HDL-C which employed a synthetic polymer together with a polyanion to block the non-HDL lipoproteins. Cholesterol in HDL was then exposed to the enzymes in the presence of a selective detergent which gives specificity for HDL-C. Kondo et al. (1999) visualized the formation of HDL–polymer complexes after the addition of polymer and polyanion (the first reaction) using electron microscopy. This showed that this complex breaks down in the presence of a detergent in the second reaction. It also showed that the polyanion in reagent 1 (phosphotungstate) caused the aggregation of almost all lipoprotein. However, the exact roles of the polyanion and synthetic polymer remain unknown. Commercial reagent sets included two reagent additions; the first with the polyanion and polymer blocking agents and the second with detergent, enzymes, and substrates. The specificity and analytical performance of this method was investigated and published in several studies.

Kurosaki et al. (2009) have reported that with the Daiichi method, free cholesterol in serum was eliminated in the first reaction, and free cholesterol in HDL was not measured. Later on in 2009, Daiichi published a patent regarding its modified...
In a first reaction, free cholesterol on the surface of only LDL and VLDL reacts with ChEs and ChOx. The H$_2$O$_2$ produced in the reaction is eliminated by peroxidase; thus no colour formation occurs in response to free cholesterol. In the second step, a special detergent causes only HDL to be dissolved to allow the reaction between HDL-C and the enzyme. As a detergent, a polyoxyethylene derivative such as Emulgen B-66 or Emulgen-90 (Kao Corporation, Japan), which directly hydrolyzes the HDL particles can be used. Using this methodology, HDL-C could be conveniently quantified without resort to the use of polyanions as in their original method.

Wako Chemicals introduced a more convenient immunoinhibition homogeneous assay using anti human-$\beta$-lipoproteins to produce soluble complexes of CM, VLDL and LDL with no reaction with enzymes involved in subsequent enzymatic cholesterol reaction. Only the cholesterol content of HDL is measured in the presence of enzymes in the second reagent.

The HDL-C method developed by Deneka Seiken Co. was based on the fact that non-HDL-C selectively reacts with the ChEs and ChOx in the first step without producing any colour. H$_2$O$_2$ produced in this step is scavenged by the enzyme catalase. In the next step, in the presence of a reagent containing a surfactant to solubilise HDL-C, an inhibitor of catalase, 4-AAP and N-(2-hydroxy-3-sulfopropyl)-3,5-dimethoxyaniline (HDAOS), colour is developed.

Finally according to the phosphate complex inhibition method introduced by Serotec and UMA, HDL-C in the presence of a detergent and phosphate compound undergoes an enzymatic reaction. According to the homogeneous method for LDL-C, lipoproteins other than LDL such as VLDL, HDL and CM are removed in the first step using the first reagent described by each method. In the second stage, LDL-cholesterol undergoes an enzymatic reaction to produce hydrogen peroxide which is measured colorimetrically. Various physicochemical combinations of surfactants, polymeric complexes and specific binding molecules were also used in establishing homogeneous assays to selectively measure LDL-C. Although the mechanism that confers selectivity to LDL-C from a specific surfactant is also not well understood, the same general mechanism is thought to apply as for HDL-C in that the surfactant may be able to distinguish differences in hydrated density, net charge, or size of the various lipoprotein fractions. A brief description of the principles of each of the homogeneous LDL-C assay methodologies is given in Fig. 6.
<table>
<thead>
<tr>
<th>Assay kits</th>
<th>Performance and others</th>
</tr>
</thead>
</table>
| **Daiichi [accelerator selective detergent method]**  
First step:  
1. Non-HDLs + synthetic polymers + polyanions → soluble complexes of non-HDL  
Second step:  
2. HDL-C + selective detergent + ChEs + ChOx → cholesteneone + fatty acid + H₂O₂  
3. H₂O₂ + 4AAP/peroxidase + DSBmT → color development | Dynamic range: 1.5–150 mg dL⁻¹  
Not measurable in presence of abnormal lipoprotein caused by severe liver dysfunction.  
No interference by TG value at up to 1500 mg dL⁻¹  
No interference by hemoglobin at up to 500 mg dL⁻¹  
No interference by bilirubin at up to 50 mg dL⁻¹  
Recommended sample storage: one-week under refrigeration, sample can be frozen-thawed only once. |
| **Kyowa Medex (modified enzymatic method)**  
First step:  
1. Non-HDLs + α-cyclodextrin + MgCl → soluble complexes of non-HDL  
Second step:  
2. HDL-C + PEG modified ChEs and ChOx → cholesteneone + fatty acid + H₂O₂  
3. H₂O₂ + 4AAP/peroxidase + HDAOs → color development | Dynamic range: 0–120 mg dL⁻¹  
No interference by TG value at up to 1200 mg dL⁻¹  
No interference by hemoglobin at up to 1200 mg dL⁻¹  
No interference by bilirubin at up to 30 mg dL⁻¹ (conjugated form) and 70 mg dL⁻¹ free form  
Recommended sample storage: one-week under refrigeration, sample can be frozen-thawed only once. |
| **Kokusai or Sysmex (calixarene complex method)**  
First step:  
1. Non-HDLs + calixarene → non-HDL-calixarene soluble complex  
Second step:  
2. HDL-C + detergent + ChEs + ChOx + hydrazine + β-NAD → cholesteneone + fatty acid + H₂O₂  
★ ChEs from *Chromobacterium viscosum* cannot react to the non-HDL-calixarene soluble complex. | Dynamic range: 1.5–100 mg dL⁻¹  
Negative predictive value or the samples of liver dysfunction and positive value in presence of LDL fractions and apo E-rich serum  
No interference by TG value at up to 1500 mg dL⁻¹  
No interference by hemoglobin at up to 500 mg dL⁻¹  
No interference by bilirubin at up to 20 mg dL⁻¹  
Recommended sample storage: three days under refrigeration, sample can be frozen only once at −80 °C. |
| **Wako (immunoinhibition method)**  
First step:  
1. Non-HDLs + anti human-β-lipoprotein → soluble complexes of non-HDL  
Second step:  
2. HDL-C + detergent + ChEs + ChOx → cholesteneone + fatty acid + H₂O₂  
3. H₂O₂ + 4AAP/peroxidase + F-DAOS → color development | Dynamic range: 1–180 mg dL⁻¹  
Positive value in presence of LDL, VLDL and apo E-rich HDL  
No interference by TG value at up to 1200 mg dL⁻¹  
No interference by hemoglobin at up to 500 mg dL⁻¹  
No interference by bilirubin at up to 50 mg dL⁻¹  
Recommended sample storage: four days under refrigeration, sample can be frozen-thawed only once. |
| **Serotec and UMA (phosphate complex inhibition method)**  
First step:  
1. HDL-C + detergent and IP compound (inorganic/organic) + ChEs → free cholesterol + fatty acid  
Second step:  
2. Free cholesterol + ChOx → cholesteneone + fatty acid + H₂O₂  
3. H₂O₂ + 4AAP/peroxidase + F-DAOS → color development | Dynamic range: Up to 200 mg dL⁻¹  
Positive value in presence of VLDL fractions  
No interference by TG value at up to 1100 mg dL⁻¹  
No interference by hemoglobin at up to 500 mg dL⁻¹  
No interference by free bilirubin at up to 40 mg dL⁻¹ and −6% in the presence of 40 mg dL⁻¹ conjugated form  
Recommended sample storage: one-week under refrigeration, sample can be frozen-thawed only once at −20 °C or lower. |
| **Deneka Seiken (elimination method)**  
First step:  
1. Non-HDL-C + ChEs + ChOx → cholesteneone + fatty acid + H₂O₂  
2. H₂O₂ + catalase → 2H₂O + O₂  
Second step:  
3. HDL-C + detergent + ChEs + ChOx → cholesteneone + fatty acid + H₂O₂  
4. H₂O₂ + 4AAP/peroxidase + HDAOs → color development | Dynamic range: 1–150 mg dL⁻¹  
No interference by TG value at up to 1500 mg dL⁻¹  
No interference by hemoglobin at up to 500 mg dL⁻¹  
No interference by bilirubin at up to 30 mg dL⁻¹  
Recommended sample storage: one-week under refrigeration, sample can be frozen-thawed only once. |

a 4AAP: 4-aminoantipyrine, DSBmT: N,N-bis-(4-sulphobutyl)-m-toluidine, HDAOs: N-(2-hydroxy-3-sulphopropyl)-3,5-dimethoxyaniline, F-DAOS: N-Ethyl-N-(2-hydroxy-3-sulphopropyl)-3,5-dimethoxy-4-fluoroaniline.
the following section and their reaction mechanisms and performance characteristics summarised in Table 5.

Nauck et al. (2002) have reviewed homogeneous methods for LDL-C and compared them with conventional assay methods.19,26 The first LDL-C homogeneous assay was offered by Kyowa Medex in 1998 and distributed by Roche Diagnostics.76 To provide the required selectivity, the combination of polyoxyethylene–polyoxypropylene (POE–POP) block co-polymer with α-cyclodextrin sulphate was employed for the determination of LDL-C, followed by reaction with ChEs and ChOx. MgCl₂ and α-cyclodextrin sulphate are first used as a quencher for CMs and VLDL-C and the POE–POP acts as a quencher for HDL-C.62,76 Due to the limited specificity of POE–POP toward LDL-C, the LDL-C is selectively solubilised into mixed micelles and enzymatic reaction occurs. The level of selectivity toward LDL-C is directly dependent on the molecular mass and hydrophobicity of the POP blocks.62,76 This method was not found to be sufficiently specific for LDL-C, since LDL is only partially recovered and apo E-rich HDL and VLDL are not completely excluded.16,26

Daiichi reported a method employing non-ionic surfactant to solubilise all non-LDL lipoproteins.26 Hydrogen peroxide enzymatically generated in this step is then catalysed by a HRP in the presence of 4-aminoantipyrine. No colour is produced in this step. A second specific detergent is used to solubilise LDL-C and generate hydrogen peroxide. The hydrogen peroxide in presence of N,N,N′,N′-bis-(4-sulphobutyl)-m-toluidine disodium salt, HRP and 4-aminoantipyrine then generates colour which is proportional to the level of LDL-C. The method is linear up to 1000 mg dL⁻¹, with the detection limit of 0.4 mg dL⁻¹ and seems to be affected by increased TGs and is not completely specific for LDL-C.16,26

The homogeneous LDL-C assay from Wako is based on first protecting LDL-C selectively from enzymatic reaction in the presence of polyanions and amphoteric surfactants. As a result, lipoprotein cholesterol other than LDL-C undergo enzymatic catalysis and generate hydrogen peroxide which is consumed by catalase. In the second step, a non-ionic surfactant as a deprotection agent then enables the LDL-C to react with ChEs and ChOx to produce hydrogen peroxide. The colour yield from the reaction of hydrogen peroxide and Trinder’s reagent and 4-aminoantipyrine is again measured spectrophotometrically.26 Total CVs at LDL-C concentrations between 103.4 and 219.6 mg dL⁻¹ were 1.2% and total error ranged from 2.6% to 5.6%.78 The method was found linear up to 300 mg dL⁻¹ with the detection limit of 1.0 mg dL⁻¹.26 In spite of linearity issues and TG interference, the assay seems to be relatively specific for LDL-C.75,78

Deneka Seiken reported a homogeneous LDL-C assay using a combination of two non-ionic surfactants (Emulgen B-66 and Emulgen A-90) with a hydrophile–lipophile balance (HLB) of 13.5 which can selectively remove non-LDL-C in the presence of MgCl₂. Based on this method, the reactivity of cholesterol in each lipoprotein depends on the HLB of the detergents.26,79 The evaluation of this assay has been very limited. However, available data suggests that it has high specificity for LDL-C.

The method developed by IRC for LDL-C uses a calixarene as a detergent which converts LDL to an LDL-calixarene soluble complex. In the presence of ChEs (from Chromobacterium viscosum), ChOx and hydrazine, cholesterol in the lipoproteins other than LDL converts to cholestene hydradrene. Finally the LDL-calixarene complex is broken down after the addition deoxycholate, ChEs, ChOx and NAD, yielding cholestene and NADH. NADH is then measured spectrophotometrically.26,80,81 Finally according to the Serotec and UMA methodology, LDL-C in presence of a selective detergent, phosphate compound and cholesterol esterase produce free cholesterol. The free cholesterol is determined according to Fig. 5.81

A multicentre evaluation of five direct assays of LDL-C (Daichi, Denka Seiken, Kyowa and Wako) was performed using 45 serum samples (TG below 3.1 mmol L⁻¹) in eight laboratories using different analysers.82 Inter-laboratory reproducibility was improved markedly compared to the Friedewald calculation for Daichi, Kyowa and Wako. All the above mentioned methods showed strong correlation in comparison with the BQ assay.

Miller et al. (2010) have compared some of the commercially available homogeneous methods for measurement of HDL-C and LDL-C with the RM for accuracy and total error. Specificity and imprecision were also estimated. Table 6 shows that 5 of 7 HDL-C homogeneous methods reached the 95% acceptance criteria for non-diseased individuals but deteriorated considerably for the samples from the diseased group.

Only the Kyowa and Seikisui methods met total error goals for measuring HDL-C in diseased individuals.83 None of the LDL-C homogeneous methods met the criteria for the diseased individuals and only four of them met the criteria for non-diseased individuals.83

Iizuka et al. (2012) also compared homogeneous assay kits for HDL and LDL cholesterol.85 The aim was to clarify the commutability of currently used homogeneous assays to measure HDL-C and LDL-C. It was shown that all of the above mentioned HDL-C assay kits are commutable. Wako was the only assay kit which showed discrepancy in the high bilirubin samples. All the LDL-C assay kit results were affected by the lipoprotein in the patient samples.83 Accuracy and precision issues for the measurement of LDL-C have been reviewed in several papers and the necessity of improving the accuracy of LDL-C measurement has been emphasised.81,84–89

Point of care testing (POCT) for HDL-C and LDL-C

In order to remove sample transport requirements, reduce processing and assay times and facilitate near patient testing, point of care devices that measure HDL-C and LDL-C directly are very attractive in biomedical diagnostics. In general, point of care testing needs relatively small volumes of whole blood directly from a fingerstick and test results are available soon after sampling, which is highly advantageous in self-management of hypercholesterolemia. There is no requirement for transportation of samples to a central laboratory which helps to reduce result turnaround time and transport costs. Earlier diagnosis and disease management as well as potential for improving patient satisfaction and cost-effectiveness are some other advantages offered by POCT.90–92

A number of both professional use and consumer point of care devices for measurement of cholesterol are commercially
Table 5 Schematic reaction mechanism for LDL-C Assay kits and their performance. Adapted from Nakamura et al. (2006) (ref. 61)*

<table>
<thead>
<tr>
<th>Assay kits</th>
<th>Performance and others</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Daichi (liquid selective detergent method)</strong></td>
<td>Dynamic range: 1–450 mg dL⁻¹ No interference by TG value at up to 1500 mg dL⁻¹ No interference by bilirubin at up to 39 mg dL⁻¹ (conjugated form) and 70 mg dL⁻¹ free form Recommended sample storage: one-week under refrigeration, sample can be frozen-thawed only once.</td>
</tr>
<tr>
<td>First step:</td>
<td>1. Surfactant 1 + ChEs/ChOx + CM, VLDL and HDL-C → cholesteneone + fatty acid + H₂O₂ 2. 2H₂O₂ + catalase → 2H₂O + O₂</td>
</tr>
<tr>
<td>Second step:</td>
<td>3. LDL-C + surfactant 2 + ChEs/ChOx/peroxidase + DSBmT → color development</td>
</tr>
<tr>
<td><strong>Kyowa Medex (selective solubilization method)</strong></td>
<td>Dynamic range: 0–550 mg dL⁻¹ No interference by TG value at up to 1200 mg dL⁻¹ No interference by hemoglobin at up to 1500 mg dL⁻¹ No interference by bilirubin at up to 20 mg dL⁻¹ Recommended sample storage: three days under refrigeration, sample can be frozen only once at −80 °C.</td>
</tr>
<tr>
<td>First step:</td>
<td>1. Non-LDLs are blocked by surfactant and sugar compounds</td>
</tr>
<tr>
<td>Second step:</td>
<td>2. LDL-C + surfactant + ChEs/ChOx → cholesteneone + fatty acid + H₂O₂ 3. H₂O₂ + 4AAP/peroxidase + HDAOS → color development</td>
</tr>
<tr>
<td><strong>Kokusai or Sysmex (calixarene complex method)</strong></td>
<td>Dynamic range: 1–350 mg dL⁻¹ Positive predictive value in the presence of LDL fractions, apo E-rich HDL and samples of liver dysfunction No interference by TG value at up to 1500 mg dL⁻¹ No interference by hemoglobin at up to 500 mg dL⁻¹ No interference by bilirubin at up to 20 mg dL⁻¹ Recommended sample storage: one-week under refrigeration, sample can be frozen-thawed only once.</td>
</tr>
<tr>
<td>First step:</td>
<td>1. LDL + calixarene → LDL-calixarene soluble complex</td>
</tr>
<tr>
<td>Second step:</td>
<td>2. CM, VLDL and HDL-C + ChEs(1)/ChOx + Hydrazine → cholesteneone hydrazone ★ ChEs (1) (from Chromobacterium), can not react with LDL-calixarene soluble complex 3. LDL-calixarene soluble complex + ChEs(2)/ChOx + hydrazine + β-NAD + deoxycholate → cholesteneone hydrazone + β-NADH ★ ChEs (2) (from Pseudomonas species)</td>
</tr>
<tr>
<td><strong>Wako (enzyme selective protecting method)</strong></td>
<td>Dynamic range: 1.0–400 mg dL⁻¹ Positive predictive value in presence of VLDL fractions No interference by TG value at up to 1000 mg dL⁻¹ No interference by hemoglobin at up to 500 mg dL⁻¹ No interference by bilirubin at up to 50 mg dL⁻¹ Recommended sample storage: one-week refrigeration, sample can be frozen-thawed only once.</td>
</tr>
<tr>
<td>First step: (elimination of non-LDL cholesterol)</td>
<td>1. LDL + protecting reagent → LDL-protecting reagent 2. CM, VLDL and HDL-C + ChEs/ChOx → H₂O₂ + Catalase → H₂O</td>
</tr>
<tr>
<td>Second step:</td>
<td>3. LDL-protecting reagent → deprotecting reagent → LDL 4. LDL-C + ChEs/ChOx → cholesteneone + H₂O₂ 5. H₂O₂ + 4AAP/peroxidase + HDAOS → color development</td>
</tr>
<tr>
<td><strong>Serotec and UMA (phosphate complex inhibition method)</strong></td>
<td>Dynamic range: Up to 500 mg dL⁻¹ Positive value in presence of VLDL and LDL fractions No interference by TG value at up to 1100 mg dL⁻¹ No interference by hemoglobin at up to 500 mg dL⁻¹ No interference by bilirubin at up to 40 mg dL⁻¹ Recommended sample storage: one-week under refrigeration, sample can be frozen-thawed only once at −20 °C or lower.</td>
</tr>
<tr>
<td>First step:</td>
<td>1. LDL-C + detergent and IP compound (inorganic/organic) + ChEs → free cholesterol + fatty acid</td>
</tr>
<tr>
<td>Second step:</td>
<td>2. Free cholesterol + ChOx → cholesteneone + fatty acid + H₂O₂ 3. H₂O₂ + 4AAP/peroxidase + HDAOS → Color development</td>
</tr>
<tr>
<td><strong>Deneka Seiken (elimination method)</strong></td>
<td>Dynamic range: 1–800 mg dL⁻¹ No interference by TG value at up to 1000 mg dL⁻¹ No interference by hemoglobin at up to 500 mg dL⁻¹ No interference by bilirubin at up to 30 mg dL⁻¹ Recommended sample storage: one-week under refrigeration, sample can be frozen-thawed only once at −80 °C.</td>
</tr>
<tr>
<td>First step:</td>
<td>1. Non-LDL-C + surfactant combination 1 + ChEs/ChOx → cholesteneone + fatty acid + H₂O₂ 2. 2H₂O₂ + catalase → 2H₂O + O₂</td>
</tr>
<tr>
<td>Second step:</td>
<td>3. LDL-C + surfactant combination 2 + ChEs/ChOx → cholesteneone + fatty acid + H₂O₂ 4. H₂O₂ + 4AAP/peroxidase + HDAOS → color development</td>
</tr>
</tbody>
</table>

*4AAP: 4-aminoantipyrine, DSBmT: N,N-bis-(4-sulphobutyl)-m-toluidine, HDAOS: N-(2-hydroxy-3-sulphopropyl)-3,5-dimethoxyaniline, F-DAOS: N-Ethyl-N-(2-hydroxy-3-sulphopropyl)-3,5-dimethoxy-4-fluoroaniline.
Table 6 Percentage total error results of a single measurement based on the first result (no replication) for five homogeneous HDL-C and LDL-C assay methods.

<table>
<thead>
<tr>
<th>Method</th>
<th>% HDL-C results non-diseased</th>
<th>% HDL-C results diseased</th>
<th>% LDL-C results non-diseased</th>
<th>% LDL-C results diseased</th>
</tr>
</thead>
<tbody>
<tr>
<td>Seikisui</td>
<td>100</td>
<td>96.4</td>
<td>100</td>
<td>91.1</td>
</tr>
<tr>
<td>Kyowa Medex</td>
<td>97.3</td>
<td>95.6</td>
<td>94.6</td>
<td>85.9</td>
</tr>
<tr>
<td>Deneka</td>
<td>100</td>
<td>92.7</td>
<td>89.2</td>
<td>85.2</td>
</tr>
<tr>
<td>Wako</td>
<td>100</td>
<td>74.5</td>
<td>97.3</td>
<td>87.4</td>
</tr>
<tr>
<td>Sysmex</td>
<td>100</td>
<td>89.8</td>
<td>86.5</td>
<td>71.9</td>
</tr>
<tr>
<td>UMA</td>
<td>91.9</td>
<td>83.9</td>
<td>97.3</td>
<td>75.6</td>
</tr>
<tr>
<td>Serotec</td>
<td>94.6</td>
<td>86.9</td>
<td>97.3</td>
<td>53.3</td>
</tr>
</tbody>
</table>

available. The Cardiocheck PA and Cholestech LDX are two systems that support the UK NHS health check vascular risk assessment. The Cardiocheck PA is based on a spectrophotometric method and the measurement of the light reflected off a disposable test strip that has changed colour after applying blood sample. The analyzer converts this reading into an HDL result and displays it. The Cholestech LDX system combines the enzymatic methodology and solid-phase technology to measure the quantity of TC, HDL-C, TGs, glucose, and others in the blood (capillary or venous), serum or plasma. The sample is applied to a Cholestech LDX cassette (Fig. 7). The cassette is then placed into the Cholestech LDX analyzer that can measure the resultant colour by reflectance photometry. The LDX uses the Friedewald equation to calculate LDL-C while the Cardiocheck PA measures LDL-C directly. A comparison between Cholestech LDX POC and hospital reference laboratory validates the use of the Cholestech LDX analyser for point of care lipid measurements in clinical practice under well-trained operators. A comparison between the performances of these two point-of-care analyzers and clinical diagnostic laboratory methods for the measurement of TC, HDL-C and LDL-C has been reported. Both devices meet NCEP guidelines for all analytes at two clinical cut-off points. Both of them were found to have acceptable performance, which offers healthcare professionals a rapid POC method for the measurement of cholesterol in specific lipoproteins. Moreover, determination of the accuracy and precision of TC, TG and HDL cholesterol measures by a nurse on capillary blood using the Cardiocheck system suggested that this approach was appropriate for predicting CHD risk and provided reliable fractionated lipid information which was consistent with traditional clinical chemistry platforms. The evolution of point of care tests from professional use instruments towards low cost, consumer diagnostics has been exemplified by the development of glucose biosensors. The progression from optical to electrochemical measurement methods is a natural evolution for many diagnostic devices as they progress from laboratory tests typically based on spectrophotometry, to electrochemical devices which allow lower cost instrumentation and system simplification. Cholesterol testing is also going through this evolution. Several devices are available which can measure free cholesterol using disposable electrochemical test strips. As with glucose, cholesterol lends itself effectively to electrochemical measurement as many of the transduction principles employed are transferrable, such as the use of electron transfer mediators or hydrogen peroxide to measure the oxidation of cholesterol. However, despite the growing importance of the selective determination of LDL-C and HDL-C for monitoring and managing hypercholesterolaemia, coupled with the suitability of electrochemical assay methodologies to translate well to cholesterol testing, there are still relatively few examples of electrochemical biosensors capable of the selective determination of LDL-C and HDL-C.

Several sensor techniques such as quartz crystal microbalance (QCM), surface plasmon resonance (SPR), cyclic voltammetry (CV) and electrochemical impedance spectroscopy (EIS) have been employed to measure LDL quantitatively or qualitatively. Piezoelectric devices have been extensively investigated as the basis for sensing due to their small size, small sample requirements and high sensitivity. A piezoelectric LDL biosensor was developed which was based on capturing and detecting apo B-100 using interactions between its lysine rich residues and immobilised components of the extracellular matrix such as collagens and proteoglycans. However, this was used to explore potential interactions between LDL and the vasculature, rather than to quantify LDL-C levels. In addition, although QCM biosensors have been found to be very useful as laboratory-based investigative tools, their cost has...
prohibited them from becoming the basis of consumer diagnostic devices.

Yan et al. (2008) reported a label-free electrochemical immunosensor for LDL with a detection limit of 0.34 pg mL\(^{-1}\). The electrode was fabricated using an antibody to apo-B-100 adsorbed onto silver chloride–polyaniline (PANI) core–shell nanocomposites (AgCl–PANI) at gold nanoparticle-modified glassy carbon electrodes. The specific antibody–antigen reaction facilitated the binding of a single apo-B-100 present in each LDL to the electrode surface. The biosensor showed a highly sensitive response to LDL with a detection limit of 0.34 pg mL\(^{-1}\).

Due to the major focus in cholesterol testing on decreasing LDL-C levels, there has only been a single report for the amperometric determination of HDL-C. A peroxidise-entrapped and ferrocene-embedded carbon paste electrode was used to detect H\(_2\)O\(_2\) at levels as low as 10 nM. The electrode was capable of measuring HDL-C in a very small volume (1–2 \(\mu\)L) using PEG-modified ChEs and ChEs in a manner analogous to the Kyowa Medex assay. The PEG-modified enzymes exhibited a selective activity toward HDL-C in the presence of dextran sulphate and MgCl\(_2\) to generate H\(_2\)O\(_2\). A comparison with the data obtained by the equivalent spectrophotometric method as well as a conventional precipitation method showed good correlation.

**Conclusion**

The importance of the selective determination of both LDL-C and HDL-C in determining atherogenic risk as well as monitoring the effectiveness of treatment for hypercholesterolaemia is now well-established. The implementation of effective testing and monitoring regimes has been hampered by the availability of suitable methods for their measurement. However, complex methods based on centrifugation have been extensively replaced with simple homogeneous methods that can be performed without the requirement for costly equipment and skilled personnel. A number of point of care instruments are also available for point of care testing of cholesterol levels. However, while there are a number of consumer-based devices for measuring free cholesterol, there are only a few examples of sensor-based devices for performing selective measurements of HDL-C and LDL-C, and none are available commercially. Given the effective development of homogeneous methodologies based on spectrophotometric measurement, it seems inevitable that such methodologies will soon migrate onto electrochemical biosensor platforms suitable for consumer use. This will, no doubt, improve the diagnosis and management of hypercholesterolaemia in a manner analogous to that achieved for the management of diabetes with glucose test strips.

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**Notes and references**


