



## **A NEW TOOL FOR THE AUTOMATED ANALYSIS OF LDL SUBFRACTION PATTERNS GENERATED BY THE LIPOPRINT™ LDL SYSTEM.**

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### **ABSTRACT**

Lipoproteins may be separated using the Lipoprint LDL System (Quantimetrix Corporation), a continuous polyacrylamide gel electrophoresis method, and then analyzed using a novel automated software program. The Lipoprint LDL System is capable of separating up to 12 lipoprotein fractions and subfractions in human serum or plasma samples. The resulting pattern consists of very low density lipoprotein (VLDL), 3 mid-bands, up to 7 low density lipoprotein (LDL) subfractions and a high density lipoprotein (HDL) fraction.

Following electrophoresis, the gel tubes are scanned by densitometry and the resulting data are exported to an external computer. Subsequently the scan is analyzed using a newly developed software program in conjunction with NIH Image (public domain software available from the National Institutes of Health). The program automatically introduces vertical lines at the appropriate Rf values for each fraction and calculates the corresponding fractional area under the curve. The computer-generated report contains the scan of the lipoprotein distribution along with percent area and cholesterol content for each of the fractions.

VLDL, LDL (including mid-band fractions) and HDL cholesterol values obtained with the Lipoprint System were compared to the corresponding cholesterol values obtained by the  $\beta$ -Quantification (ultracentrifugation) method. Pacific Biometrics Inc. (Seattle, WA), a laboratory associated with the Cholesterol Reference Method Laboratory Network (CRMLN), performed the  $\beta$ -Quantification. An inter-lab comparison using the Lipoprint System was conducted, resulting in good agreement between the labs for the following fractions: VLDL ( $r^2 = 0.909$ ), LDL/mid-bands ( $r^2 = 0.984$ ) and HDL ( $r^2 = 0.940$ ).

## INTRODUCTION

The relationship between plasma lipoproteins and coronary artery disease (CAD) has been well established. Apo B containing lipoproteins (LDL, IDL and VLDL) have been shown to be positively correlated with CAD risk, while HDL is negatively correlated with risk. The various lipoproteins are heterogeneous, consisting of multiple subclasses varying in particle size, density and chemical composition. Current evidence suggests that the LDL subfractions containing the smaller, more dense LDL particles may be associated with higher risk of CAD compared to larger, more buoyant LDL particles (1). These findings suggest that determining the relative amounts of the various LDL subclasses could lead to a more sensitive and specific assessment of CAD risk than is obtained from measurement of total cholesterol or LDL cholesterol concentrations.

Density gradient ultracentrifugation (2), non denaturing gradient gel electrophoresis (3) and nuclear magnetic resonance (4) have been used to determine LDL particle subfractions and particle sizes in patient samples. Typically, qualitative LDL particle profiles are based on the particle size of the predominant LDL subfraction in the patient sample. Patterns with the larger (buoyant) LDL are designated pattern A and those with small dense LDL as pattern B (5). The fact that these currently used methods are generally labor intensive, technically demanding, expensive and time consuming has prevented LDL subfractionation from becoming a routine clinical test.

Quantimetrix Corporation has developed the Lipoprint LDL (Polyacrylamide Gel Tube Electrophoresis System) for the separation and measurement of serum lipoproteins and their various subfractions. The resulting electropherogram resolves VLDL, three mid-bands, up to seven LDL subfractions and HDL. The procedure is simpler, more convenient and less expensive than the current methods of lipoprotein fractionation.

In order to facilitate the analysis of the Lipoprint LDL electropherograms, a software program has been developed. After completion of the electrophoresis, the gel tubes are densitometrically scanned. The data from the densitometer are exported to an external computer and graphically analyzed using a newly developed program within NIH Image, a public

domain software (National Institutes of Health). The program provides a graphical representation of the lipoproteins and the subfractions present in the sample and inserts boundaries at the appropriate R<sub>f</sub> value, partitioning all lipoprotein bands present. The graphical area in each band is calculated and multiplied by the total cholesterol of the sample to provide a quantitative value of cholesterol (mg/dL) in each lipoprotein subfraction. Average particle size for the LDL subfractions in the sample is estimated as well. The program automates analysis of multiple samples and provides a standardized, reproducible method for the analysis of the lipoprotein profiles. With this information a more specific assessment of the lipoprotein particle distribution of the patient can be established, providing a convenient valuable tool for the diagnosis and treatment of lipid disorders associated with CAD.

## **MATERIALS AND METHODS**

### **Lipoprint System**

The Quantimetrix Lipoprint System consists of the test kit, a tube rack with a fluorescent light source for photopolymerization of polyacrylamide, an electrophoresis chamber and a power supply. The test kit includes enough material (precast polyacrylamide tube gels, loading gel and buffer) to run 100 individual patient samples.

A fasting serum or plasma sample (up to seven days old if stored at 5 to 8° C) is recommended. A gel tube containing precast stacking and separation gel is placed into the tube rack. 25µl of sample and 200µl of loading gel (with lipid specific dye) are placed into the gel tube and mixed by inversion. The rack, with gel tube, is then placed in front of the fluorescent light and the loading gel is photopolymerized for 30 minutes. The gel tube is finally inserted into the silicone tube adapter of the upper chamber of the electrophoresis unit. An appropriate amount (~1L) of electrolyte buffer is placed into the lower chamber and the gel tubes are immersed into the solution while being suspended from the upper chamber. Electrolyte buffer (200mL) is then poured into the upper chamber and a lid, connected to the power source, is put into place. Electrophoresis is conducted at a constant current for 1 hour. Gel tubes are allowed to diffuse for 30 minutes and then scanned on a densitometer.

## **β-Quantification**

Pacific Biometrics Inc. (CRMNL member) performed the β-Quantification (ultracentrifugation) procedure. Lipoprotein fractions VLDL, LDL and HDL, differing in density as a function of their composition, are thus separated by a standardized ultracentrifugation procedure. Quantification of the individual lipoprotein classes is based on cholesterol content. Fasting serum samples, less than five days old and never frozen, were obtained from consenting subjects.

The samples were tested for total cholesterol, triglyceride and HDL-C. Total cholesterol was measured by an enzymatic method performed on the Hitachi® 911 analyzer. Triglyceride was measured using the Roche® Triglyceride (GB) method on the Hitachi® 911. HDL-C was measured using the dextran sulfate precipitation method in conjunction with an enzymatic method.

Samples were first centrifuged for 18 hours in a Beckman® Model L5-40 ultracentrifuge using 40.3 Ti rotor. Then the samples were adjusted to  $d = 1.025$  (by careful addition of KBr) and centrifuged at 40,000 rpm for 18 hours.

VLDL was the serum fraction with a density less than 1.006 ( $d < 1.006$ ). LDL was the fraction with a density of greater than 1.006 ( $d \geq 1.006$ ), minus the HDL fraction. The cholesterol content of VLDL and LDL fractions was determined using an enzymatic method.

## **DATA ANALYSIS**

Scan data is exported via the serial port from the densitometer to an external computer. The data is graphed and saved as a PICT file. The PICT files are subsequently downloaded directly into NIH Image (a public domain software program).

Quantimetrix software, which runs within NIH Image, automatically identifies the lipoprotein bands present in the sample, based on band migration distance ( $R_f$ ).

$R_f$  values for the various lipoprotein subfractions were determined based on lipoprotein band patterns of 125 patient samples. Each band was visualized on a Lipoprint tube gel and assigned a  $R_f$  value using  $R_f = 0$  for the VLDL band and  $R_f = 1$

for the HDL band (the farthest migrating band). Each band was assigned to one of twelve possible lipoprotein classes (VLDL through HDL). Within each of the twelve classes, the mean and standard deviation for the Rf values of the respective bands (measured to the center of the band) were calculated. Then the Rf ranges, based on 99% confidence intervals, were established for the various lipoprotein classes. Lipoprotein Rf ranges:

VLDL	-	0.00 to	0.06
Mid C	-		0.13
Mid B	-		0.22
Mid A	-		0.27
LDL 1	-		0.33
LDL 2	-		0.39
LDL 3	-		0.44
LDL 4	-		0.49
LDL 5	-		0.54
LDL 6	-		0.58
LDL 7	-		0.74
HDL	-	0.74 to	1.00

The Lipoprint software analysis program automatically inserts vertical lines based on these predetermined parameters and calculates the corresponding area under the enclosed portion of the curve for each of the fractions. The area multiplied by the total cholesterol yields the cholesterol concentration of the fractions. A graphical report is generated detailing % area, cholesterol content and LDL particle size (Fig. 1). LDL peak particle diameter (PPD) is estimated based on the algorithm,  $PPD = (1.429 - RF) \times 25$ , developed by Kazumi et al. (6)

## RESULTS

### REPRODUCIBILITY

Intra-assay and inter-assay precision (Table 1) were assessed using a lyophilized control material, which was reconstituted fresh daily. The inter-assay testing was done over twelve separate days during a two month period. The data represent cholesterol concentrations (mg/dL) obtained by the Lipoprint System for all lipoprotein fractions. Intra-assay CVs were typically less than 10% for the larger fractions. The inter-assay CVs for small fractions (cholesterol concentration < 5 mg/dl) were larger than 10%.

Table 1. **Precision.** A freshly reconstituted lyophilized control was run in replicates of twelve in the same run (one chamber) for the evaluation of the intra-assay precision of the cholesterol concentrations for the various lipoprotein fractions. Inter-assay precision was established for the cholesterol concentrations of the various fractions by running a freshly reconstituted control sample on twelve separate days over a period of one month. Cholesterol values (mg/dL) for each of the lipoprotein fractions were determined with the automated analysis software in all instances.

<b>Intra-Assay (n=12)</b>												
cholesterol [mg/dL]	VLDL	mid C	mid B	mid A	LDL 1	LDL 2	LDL 3	LDL 4	LDL 5	LDL 6	LDL 7	HDL
Mean	18.7	12.3	8.5	10.9	38.0	28.4	8.8	2.2	-	-	-	42.3
SD	1.07	1.46	0.50	0.88	0.94	1.68	0.60	0.26	-	-	-	1.76
% C.V.	5.7	11.9	5.8	8.1	2.5	5.9	6.8	11.7	-	-	-	4.2

<b>Inter-Assay (n=12)</b>												
cholesterol [mg/dL]	VLDL	mid C	mid B	mid A	LDL 1	LDL 2	LDL 3	LDL 4	LDL 5	LDL 6	LDL 7	HDL
Mean	18.1	15.2	9.7	11.2	37.8	28.9	9.4	2.6	-	-	-	41.9
SD	1.24	1.64	0.62	0.83	1.34	1.83	0.88	0.49	-	-	-	1.00
% C.V.	6.9	10.8	6.3	7.5	3.5	6.3	9.4	18.8	-	-	-	2.4

An inter-laboratory comparison using the Lipoprint System was performed in conjunction with Pacific Biometrics Inc. (PBI). Fasting serum samples, less than five days old and never frozen, were obtained from consenting subjects (n=40) and split. Aliquots of the samples were shipped to Quantimetrix and retained at PBI. The samples were then tested on the Lipoprint System at both locations. All resulting lipoprotein fractions were grouped into three major classes, namely VLDL, LDL (including mid-bands) and HDL. The resulting data are displayed as a scatter plot (Figs. 2-4). Correlation for VLDL, LDL/mid-bands and HDL were ( $r^2 = 0.909$ ), ( $r^2 = 0.984$ ) and ( $r^2 = 0.940$ ) respectively.

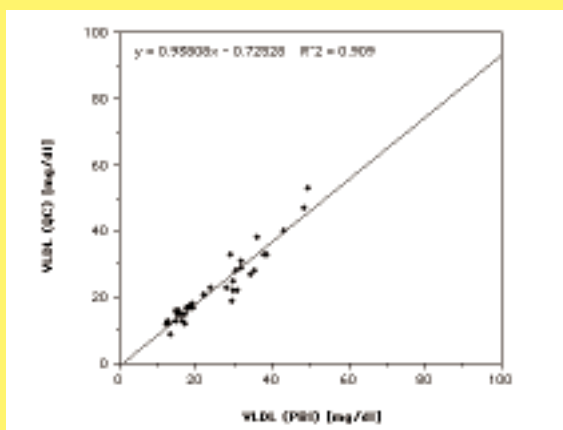


Figure 2. **Inter-Laboratory Comparison (VLDL).** Split samples were run at Quantimetrix Corporation (QC) and Pacific Biometrics Inc. (PBI) using the Lipoprint System. Very low density lipoprotein (VLDL) cholesterol results (mg/dL) from fasting serum samples (n=40) are compared.

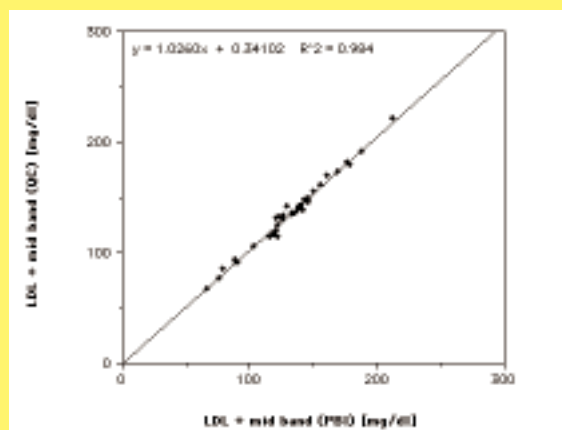


Figure 3. **Inter-Laboratory Comparison (LDL).** The combined LDL and mid-band fractions (cholesterol in mg/dL) as obtained with the Lipoprint System at Quantimetrix and PBI are compared (n=40 samples).

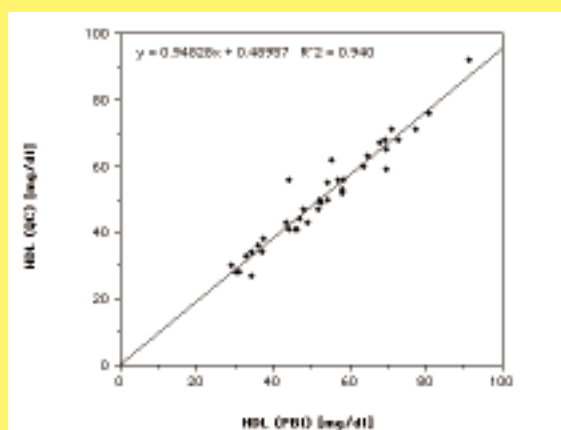


Figure 4. **Inter-Laboratory Comparison (HDL).** The high density lipoprotein (HDL) fractions (cholesterol in mg/dL) obtained with the Lipoprint System at Quantimetrix Corporation (QC) and Pacific Biometrics Inc. (PBI) are compared (n=40 samples).

## LINEARITY

A serum control was sequentially diluted in 10% increments to a final concentration of 10% with saline solution (8.5% NaCl, 7.0% human albumin). The data (actual versus expected cholesterol values) were plotted (Figs. 5-7). High positive correlation for VLDL ( $r^2= 0.925$ ), LDL/mid-band ( $r^2= 0.995$ ) and HDL ( $r^2= 0.993$ ) were obtained.

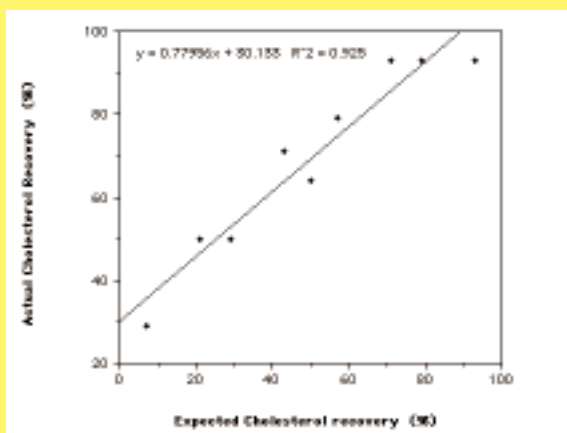


Figure 5. **VLDL Dilution Linearity.** A lyophilized control was serially diluted to 10% with normal saline. The recovered VLDL lipoprotein cholesterol fraction (mg/dL) obtained using the Lipoprint System (Actual) is plotted against a calculated (Expected) value for each dilution concentration ( $n = 9$ ).

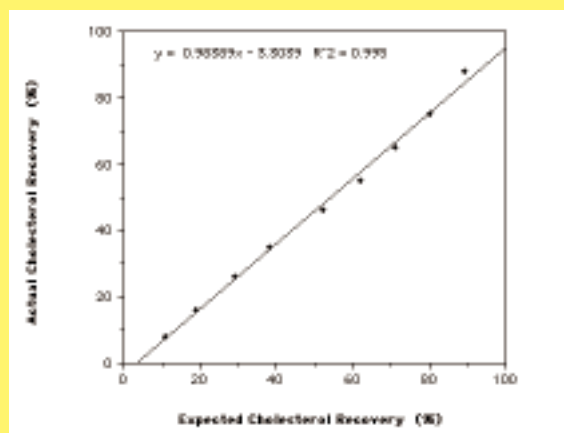


Figure 6. **LDL Dilution Linearity.** The recovered cholesterol concentrations (mg/dL) of the LDL bands of a serially diluted serum lipoprotein control (Actual) are plotted against the calculated (Expected).

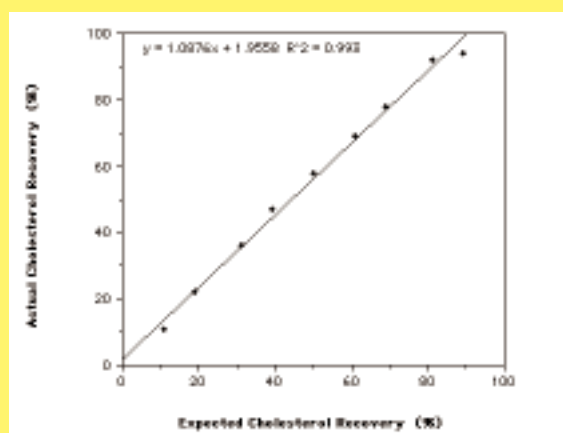


Figure 7. **HDL Dilution linearity.** The recovered (Actual) HDL cholesterol concentrations (mg/dL) of a serially diluted control serum are plotted against the calculated (Expected) values.

## STABILITY

Serum and Plasma with a wide range of lipoprotein values were obtained from volunteers and tested when first drawn (Day 0,  $n=17$ ). Subsequently the samples were stored refrigerated ( $5^{\circ}\text{C}$ ) and tested on day 3 and day 7. Correlation plots with all the individual lipoprotein fractions (between 7 and 14) for all the samples were established for day 0 and day 7 (Figs. 8,9). The correlation factors and the regression lines were virtually identical at 0 and 7 days. In addition, the stability of serum samples was evaluated. Serum samples were tested on days 0, 3 and 7 and all the resulting individual lipoprotein fractions (between 7 and 14 per sample) are displayed in a correlation plot of day 0 vs. day 7 (Fig 10). A high correlation was observed ( $r^2= 0.955$ ).

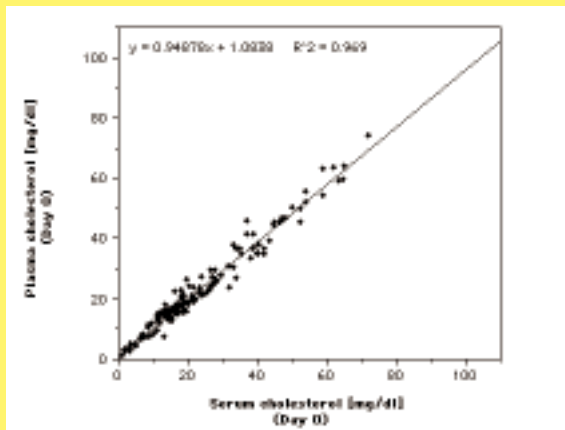


Figure 8. **Serum/Plasma Comparison.** Serum and Plasma was obtained from consenting subjects (n=17) and run on the Lipoprint System. Serum versus plasma cholesterol concentrations (mg/dL) for each lipoprotein fraction (up to twelve per sample) are plotted. Samples were run on the day drawn (Day 0).

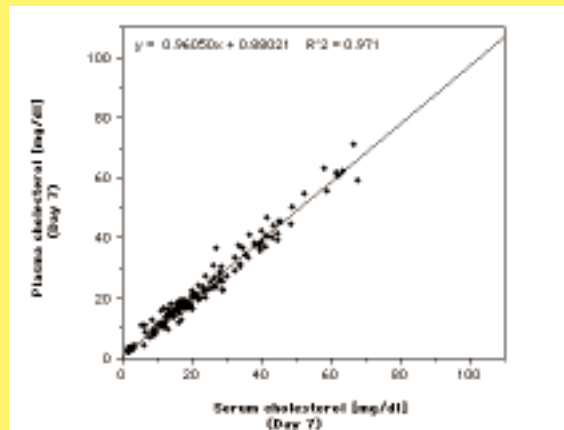


Figure 9. **Serum/Plasma Comparison.** Serum and plasma samples (n=17) were kept refrigerated (5°C) for seven days. Samples were then run on the Lipoprint System on day 7. The cholesterol concentrations (mg/dL) for each fraction were compared between serum and plasma.

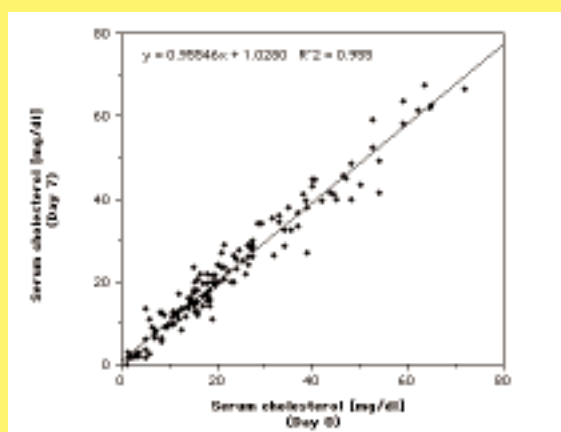


Figure 10. **Sample Stability (Serum).** Freshly drawn serum samples (n=17) were run on the Lipoprint System (Day 0). Samples were then stored refrigerated (5°C) and retested on day 7.

## ACCURACY

Samples (n=40) were tested by  $\beta$ -Quantification (ultracentrifugation), which is the most widely accepted procedure for the subfractionation of lipoproteins. The same samples were tested on the Lipoprint System and by  $\beta$ -Quantification. Figure 11 shows the agreement of LDL cholesterol in mg/dL obtained ( $r^2=0.917$ ). Similarly HDL ( $r^2=0.941$ ) and, to a lesser degree VLDL ( $r^2=0.789$ ), were found to correlate well between the two methods (Figs.12, 13). VLDL concentrations were generally found to be lower when tested by the Lipoprint System than by  $\beta$ -Quantification.

The cholesterol critical decision points for low and high-risk assessment of Coronary Artery Disease are 130, 160 mg/dL for LDL and 35, 60 mg/dL for HDL. From the correlation data we discovered the corresponding critical decision points obtained using the Lipoprint System to be 131, 159 mg/dL for LDL and 36, 59.6 mg/dL for HDL.

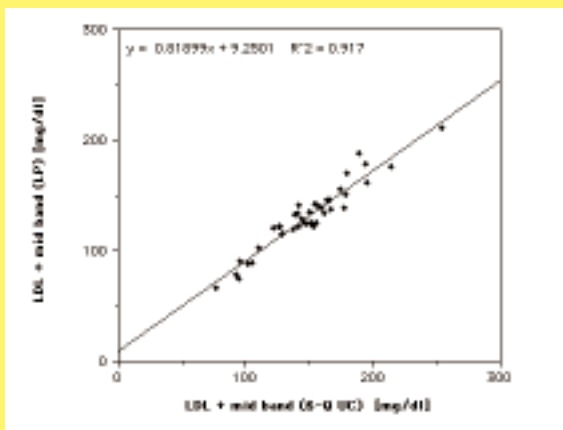


Figure 11. **LDL Accuracy Comparison.** Identical samples (n=40) were tested on the Lipoprint System at Quantimetrix Corporation, as well as with the  $\beta$ -Quantification (ultracentrifugation) method at Pacific Biometrics Inc. The cholesterol concentrations (mg/dL) from LDL lipoprotein fractions (LP) are compared between the methods. The  $\beta$ -Quant ( $\beta$ -Q UC) LDL cholesterol concentration (mg/dl) was determined from fractions with a density greater than 1.006 ( $d \geq 1.006$ ), minus the HDL fraction.

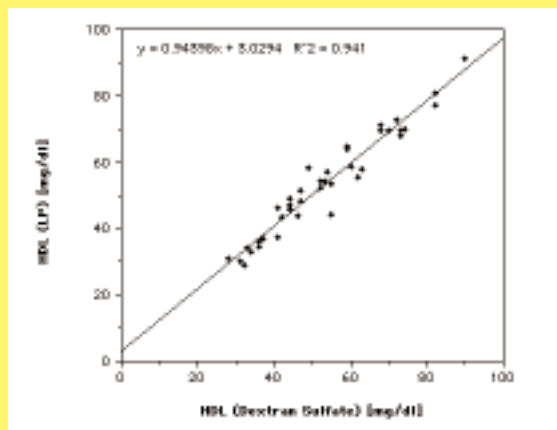


Figure 12. **HDL Accuracy Comparison.** HDL cholesterol concentrations (mg/dL) from fasting serum samples (n=40) run on the Lipoprint System (LP) are compared to dextran sulfate precipitated HDL cholesterol (mg/dl) values obtained at Pacific Biometrics Inc. ( $\beta$ -Q UC).

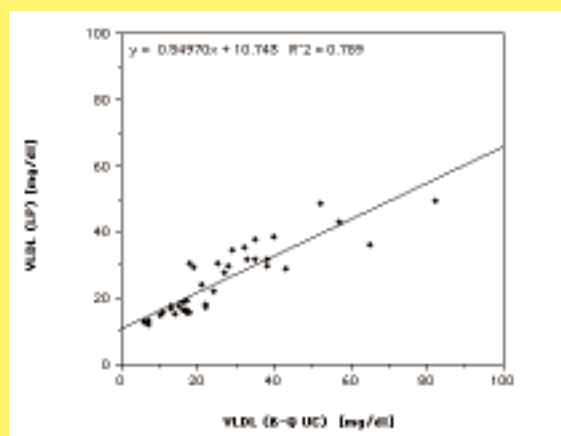


Figure 13. **VLDL Accuracy Comparison.** VLDL lipoprotein fractions (cholesterol in mg/dL) as obtained via the Lipoprint System (LP) are compared to VLDL fractions from the  $\beta$ -Quantification (ultracentrifugation).

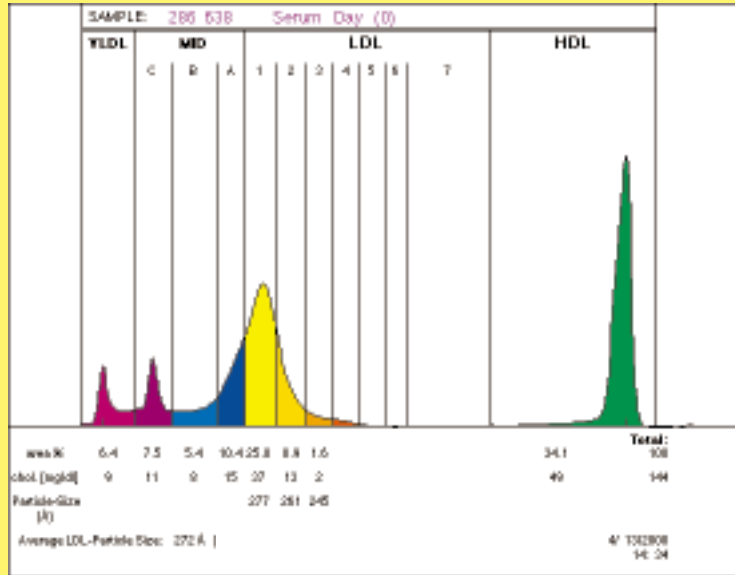
## CONCLUSION

- Comparable results were found with Lipoprint and  $\beta$ -Quantification. The corresponding Lipoprint values at the critical decision points for LDL and HDL were virtually identical to those performed by ultracentrifugation.
- Good precision, intra-assay and inter-assay, is achieved for the Lipoprint LDL in conjunction with the automated analysis program.
- The separation of lipoproteins by  $\beta$ -Quantification and Lipoprint are based on completely different physical principles, flotation for the  $\beta$ -Quant versus separation based on size and charge for the Lipoprint. These factors affect differences in VLDL quantification the most. However, VLDL typically constitutes a small percentage of the total lipoprotein present.
- The automated analysis program contributes to the standardization of lipoprotein subfractions.

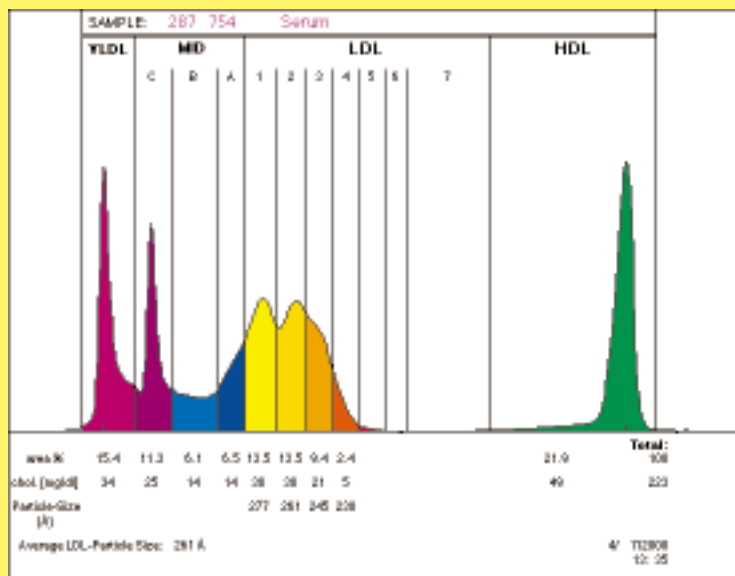
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(a)



(b)



(c)

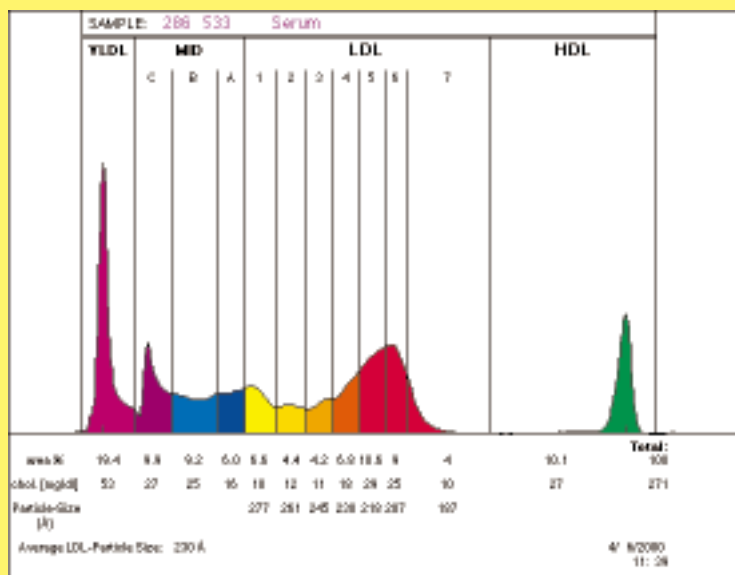


Figure 1. **LDL Profiles.** Representative sample patterns of Large (a), Intermediate (b) and Small (c) LDL subfractions as generated by the Lipoprint Analysis Program.