paper on methodology

Characterization of a composite gradient gel for the electrophoretic separation of lipoproteins

David L. Rainwater,[1] Perry H. Moore, Jr., Wendy R. Shelledy, Thomas D. Dyer, and Susan H. Slifer

Department of Genetics, Southwest Foundation for Biomedical Research, San Antonio, TX 78245

Abstract We describe a protocol for making a new type of gradient gel, the Composite gradient gel, that was designed to resolve plasma lipoproteins using nondenaturing gradient gel electrophoresis. The new gel format allows analysis both of high density lipoproteins (HDLs) and low density lipoproteins (LDLs) on the same gel. The gel gave highly repeatable ($r^2 = 0.999$) size estimates. We compared lipoprotein phenotypes determined from the new gradient gel with those obtained using specialized HDL and LDL gradient gels. The comparisons indicated that the Composite gel gave lipoprotein particle size estimates for HDLs and LDLs that were virtually identical to those obtained, respectively, from the specialized HDL and LDL gradient gels. We measured median diameters, which reflect the distributions of absorbance, for LDLs and for HDLs and found that the Composite gel gave lipoprotein size distributions that were virtually identical to those measured using the specialized LDL and HDL gels. Finally, comparison of fractional absorbance for six lipoprotein size intervals obtained from the Composite and specialized gels revealed a close correlation ($r^2 = 0.828$). Thus, it appears that both LDL and HDL size phenotypes may be evaluated simultaneously using a single gradient gel format.—**Rainwater, D. L., P. H. Moore, Jr., W. R. Shelledy, T. D. Dyer, and S. H. Slifer.** Characterization of a composite gradient gel for the electrophoretic separation of lipoproteins. *J. Lipid Res.* 1997. **38:** 1261–1266.

Supplementary key words gradient gel electrophoresis • low density lipoprotein • high density lipoprotein • human • baboon

For many years, nondenaturing gradient gel electrophoresis (GGE) has been used to characterize lipoprotein particle size phenotype. Such characterizations have been performed commonly on high density lipoproteins (HDLs) and low density lipoproteins LDLs), and two specialized gradient gel formats have been devised for these studies. HDL gradient gels are available commercially and we have described a protocol for producing the equivalent gels in the laboratory (1). Similarly, LDL gradient gels are available commercially or can be made in the laboratory (2). However, some studies require that both HDL and LDL phenotypes be determined in the same sample. For example, we have recently found significant correlations between the size and density properties of LDL and Lp[a] and those of HDLs (2, 3) in the same samples. For these and other studies it would be desirable to analyze both types of lipoprotein on the same gel. Key reasons include *1*) conservation of sample (and, potentially, reduction in number of freeze-thaw cycles), *2*) consolidation of effort needed for electrophoresis, staining, and densitometry, and *3*) comparability of data (i.e., HDLs and β-lipoproteins would share the same staining conditions and calibrators). Thus, it is the purpose of this report to evaluate a new gradient gel format, the Composite gel, that enables characterization of both LDLs and HDLs in the same gel and that gives results comparable to those obtained from the specialized LDL and HDL gels.

MATERIALS AND METHODS

Materials

Blood samples were obtained from fasted baboons using procedures approved by the Institutional Animal Care and Use Committee. After clotting, serum was obtained by low-speed centrifugation and stored at −80°C as small aliquots, protected from oxidation and desiccation, in plastic tubing (4). Thus, each aliquot was subjected to a single freeze-thaw cycle. Human blood samples were drawn from fasted participants in the San Antonio Family Heart Study using a protocol approved by the Institutional Review Board of the University of Texas Health Science Center, San Antonio. Plasma was

Abbreviations: GGE, gradient gel electrophoresis; LDL, low density lipoprotein; HDL, high density lipoprotein.

[1]To whom correspondence should be addressed.

TABLE 1. Description of linear gradient segments that make up the Composite gradient gel for lipoprotein separations

Segment Number	% High Start	% High End	Duration
			min
1	0.0	2.5	2.0
2	2.5	5.7	2.0
3	5.7	9.6	2.0
4	9.6	13.8	2.0
5	13.8	18.7	2.0
6	18.7	24.6	2.0
7	24.6	31.4	2.0
8	31.4	41.9	2.0
9	41.9	59.3	2.0
10	59.3	100.0	2.0

Given are the percents of total flow that come from the high limit acrylamide solution at the start and end of each linear gradient segment. Flow rate is adjusted according to the individual characteristics of each casting chamber.

prepared by low-speed centrifugation and aliquots were stored at −80°C until use as described above.

Gel casting

Three different gel types were made: HDL, LDL, and the Composite gels (this study). All gels were made by methods described previously (1). Briefly, gel cassettes were placed in the Pharmacia GSC-8 Gel Slab Casting Apparatus and the gradient was pumped from the bottom at room temperature. A Wiz dual-pump gradient controller, consisting of two Wiz pumps, an Apple IIe computer, and the ChemInterface unit (ISCO, Lincoln, NE), was used to make the gradient which was mixed in an external mixing chamber prior to entering the casting chamber (1). The stock solutions used in this study were made in GGE buffer containing 90 mM Tris, 80 mM borate, and 2.5 mM EDTA, pH 8.3. Solutions and gradient characteristics for making the HDL gels (i.e., SFBR3/31) and the LDL gels have been described previously (1, 2). For the Composite gel gradient, the stock solutions were *1*) 292.95 g/L acrylamide and 17.05 g/L bis-acrylamide (i.e., 31% total and 5.5% cross-linker) and *2*) 28.8 g/L acrylamide and 1.2 g/L bisacrylamide (i.e., 3.0% total, 4% cross-linker). The high limit working solution was made of 1 L solution #1 plus 1.5 mL ammonium persulfate (100 g/L) and 0.25 mL 3-dimethylaminopropionitrile, while the low limit working solution was made of 1 L solution #2 plus 4.6 mL ammonium persulfate and 0.6 mL 3-dimethylaminopropionitrile. **Table 1** gives the characteristics of the Composite gel gradient. After polymerization, gels are stored individually with 2 mL of electrophoresis buffer in plastic bags. If kept refrigerated, gels may be used with no deterioration of performance for 2 or more months. However, we recommend adding sodium azide (20 mg/L) for storage periods longer than 2 months.

Gradient gel electrophoresis

Baboon serum and human plasma samples were made dense with sucrose and a volume containing 4 μL (baboon) or 6 μL (human) was subjected to electrophoresis in GGE buffer in Pharmacia GE-2/4 electrophoresis chambers by holding 125 V constant for 24 h (i.e., 3000 V · h). After electrophoresis, gels were removed from their glass cassettes and soaked in 100 g/L trichloroacetic acid to fix proteins. Lipoproteins in the gel were then stained by use of Sudan black B and proteins in the high molecular weight standards lane were stained with Coomassie brilliant blue R-250 as described (2). The protein stain was confined to the standard proteins by adding stain solution only to a narrow (2 mm wide) strip of filter paper centered on the standards line. After destaining, the original shape of each gel was restored by soaking in GGE buffer.

Gels were calibrated by use of: *1*) carboxylated polystyrene microspheres (38 nm diameter, Duke Scientific), *2*) a lyophilized plasma standard with two LDL bands (diameters 27.5 and 26.6 nm) (2), and *3*) Pharmacia high-molecular-weight standards containing thyroglobulin (17.0 nm diameter), ferritin (12.2 nm), lactate dehydrogenase (8.16 nm), and albumin (7.1 nm). Coating of the microspheres by proteins in the high-molecular-weight standards was avoided by loading the microspheres in the standards lane about 2 h after commencing electrophoresis. To calibrate the gels a quadratic equation in relative migration distance was fitted to the natural logarithms of the diameters of the standards (5).

Evaluation of lipoprotein size distributions

Densitometry was performed using an LKB-Ultroscan XL laser densitometer with GelScan XL software. Absorbance profiles were converted to ASCII files with Gelcon program (Pharmacia-LKB) and analyzed with software we developed (2, 6), which allows for gel calibration and baseline subtraction. Diameters of specific peaks in the absorbance profiles were determined using this program. In addition, we measured a variable termed median diameter. For a specific size interval, median diameter is the particle diameter at which half the absorbance in that interval is on larger and half on smaller particles. In contrast to predominant peak diameter, this variable contains information contributed by all peaks in a profile and is similar to particle score described previously (7, 8). For this study, median diameters were determined for particles within two broad size intervals: LDLs (21–35 nm, i.e., containing primarily LDLs) and HDLs (7.2–19 nm).

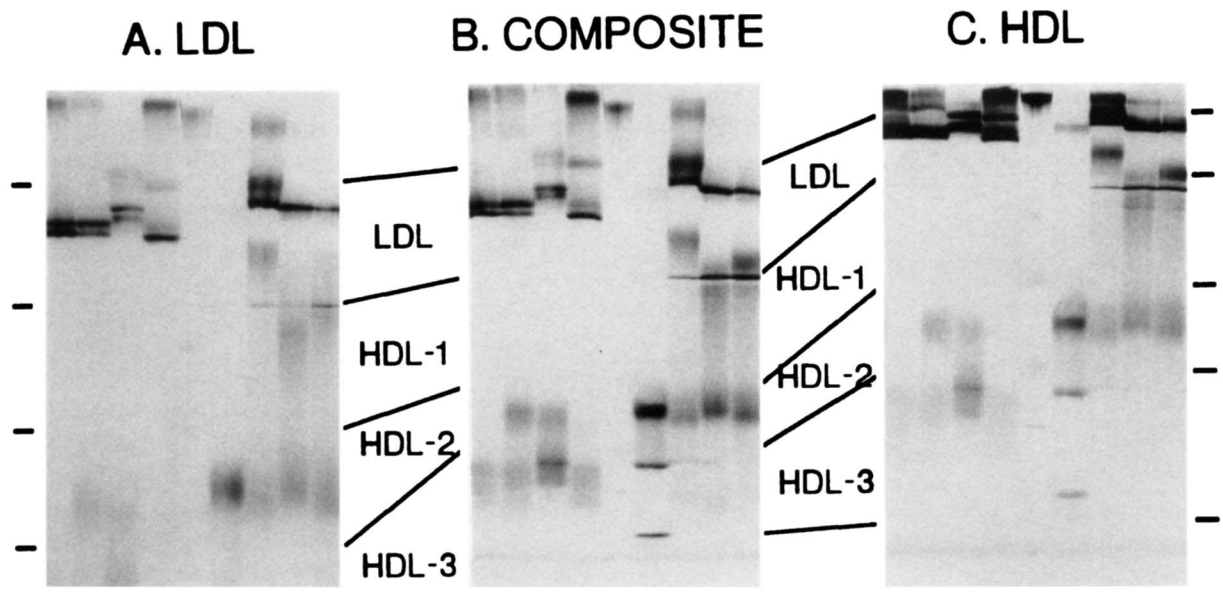

Fig. 1. Eight samples (4 human, 4 baboon) run on three types of gradient gel: LDL (panel A), Composite (panel B), and HDL (panel C). On the left side of each gel were loaded the same four human samples; the same four baboon samples were loaded on the right side of each gel; the center lane contains standards.

Statistical analyses were performed using a commercial statistics package (StatGraphics Plus, Manugistics).

RESULTS AND DISCUSSION

Separation of lipoproteins using different gradient gel formats

The Composite gradient gels were made by the same protocol as described previously (1). This procedure has been shown to repeatably produce nondenaturing gradient gels with consistent properties from lot to lot. Samples, both baboon and human, were selected to possess a diversity of lipoprotein phenotypes in order to evaluate the behavior of different lipoprotein species in the different gradient gel formats. **Figure 1** illustrates the relative migration distances of lipoprotein species in eight such samples subjected to electrophoresis in LDL (panel A), Composite (panel B), and HDL (panel C) gradient gels. To aid comparisons, the approximate locations of four major lipoprotein regions are indicated: LDL, HDL_1, HDL_2, and HDL_3. In Fig. 1, these four regions are delimited by lines which are drawn at 30, 20, 13, 9.7, and 7.2 nm and they approximate generally accepted intervals for the indicated human lipoprotein species (5, 9).

As we have described before (10), some baboons present high levels of very large HDL particles, actually larger than 20 nm in diameter. Baboon samples in the last three lanes were selected to display some variation in the unusual HDLs. These apoE-rich particles also contain apoA-I, but not apoB (10, 11). Also apparent in Fig. 1 is a refractile band caused by a commonly occurring protein approximately 20 nm in diameter (10). We find this refractile band in all serum and plasma samples studied (human, baboon, opossum), but it is not found in lipoproteins isolated by ultracentrifugation. Also suggesting it is not a lipoprotein, is the fact that the band is not stained for lipid unless there are nearby lipoproteins whose mobilities are altered somewhat by the co-occurrence of the protein (e.g., compare the refractile bands in the last three sample lanes with those barely detected in the other lanes).

Table 2 presents a comparison of migration distances for particles with a range of diameters run on the three gel types. Given are the measured migration distances for the standards and the calculated migration distances for other particles. To help indicate the repeatability of these migration distances, we give measured or calculated migration distances for the same particles run on replicate gels.

Comparison of lipoproteins separated using different gradient gel formats

As can be seen in Fig. 1, the Composite gradient gel format was designed to give mobilities and separations for LDL species very similar to those obtained using the specialized LDL gel format (compare panels A and B). However, smaller HDLs and the albumin standard were

TABLE 2. Comparison of migration distances for equivalent particles separated on two gels from each of three gradient gel formats

Diameter[a]	Migration Distance		
	LDL	Composite	HDL
nm		*mm*	
38 (microspheres)	4.2, 3.9	3.8, 4.9	3.7, 2.7
(30)	15.3, 15.1	13.7, 14.8	ND
27.5 (large LDL)	19.5, 19.1	17.8, 19.1	8.4, 9.0
26.6 (small LDL)	21.0, 21.0	19.3, 20.6	9.2, 10.0
(24)	25.7, 25.5	22.1, 23.1	ND
(22)	29.7, 29.7	25.1, 26.4	ND
(20)	34.2, 33.7	29.1, 30.2	ND
(18)	38.8, 38.2	33.0, 33.0	ND
17 (thyroglobulin)	41.4, 40.8	38.2, 36.4	22.6, 23.5
(16)	ND	36.3, 39.1	23.4, 25.0
(14)	ND	43.5, 44.3	27.8, 28.8
12.2 (ferritin)	54.5, 53.6	47.0, 46.8	32.7, 33.5
(10)	ND	57.5, 58.5	42.5, 42.3
8.16 (LDH)	ND	64.0, 65.8	55.6, 54.6
7.1 (albumin)	ND	75.7, 75.1	75.4, 74.9

ND, not determined because these particles fell outside the standard curve or were not retained by the gel.

[a]Migration distances for diameters in parentheses were estimated from the standard curve.

not retained by the LDL gel, although both were retained by the Composite gel. The HDL region in the Composite gel format is compressed when compared to the specialized HDL gel format (compare panels B and C). Compression was particularly evident in the HDL_3 size interval and this was necessary to retain albumin in the gel because this standard anchors the lower end of the calibration curve for HDL analyses.

Repeatability of particle diameter estimates using the Composite gel format

The eight samples presented in Fig. 1 were run on three different Composite gels in order to estimate repeatability of particle size estimation. We measured diameters in triplicate for each of 25 different peaks that spanned the HDL and LDL size regions in these samples. The average coefficient of variation for the 25 peaks was 0.96% (range 0.2 to 2.6%) and repeatability of the estimate was 0.999.

Comparison of lipoprotein particle diameters

A total of 48 samples obtained from baboons and humans was subjected to electrophoresis in each of the three gel formats. From these samples, we identified 146 lipoprotein species that gave distinct peaks in the absorbance profiles. **Figure 2** compares peak particle diameters for 92 distinct β-lipoprotein peaks that were measured on LDL gels and Composite gels (panel A) and for 54 distinct HDL peaks that were measured on HDL gels and Composite gels (panel B). For the comparison of LDL peaks, the regression line had a slope of 0.98 and the regression line for the HDL peak comparison had a slope of 1.00. Thus, molecular diameters estimated on the Composite gel format were very similar to those measured using the specialized gel formats designed to analyze HDLs and LDLs.

Comparison of lipoprotein size distributions

To compare lipoprotein patterns, we determined median diameters for LDLs and HDLs in the 48 samples run on each of the three gel formats. Median diameter

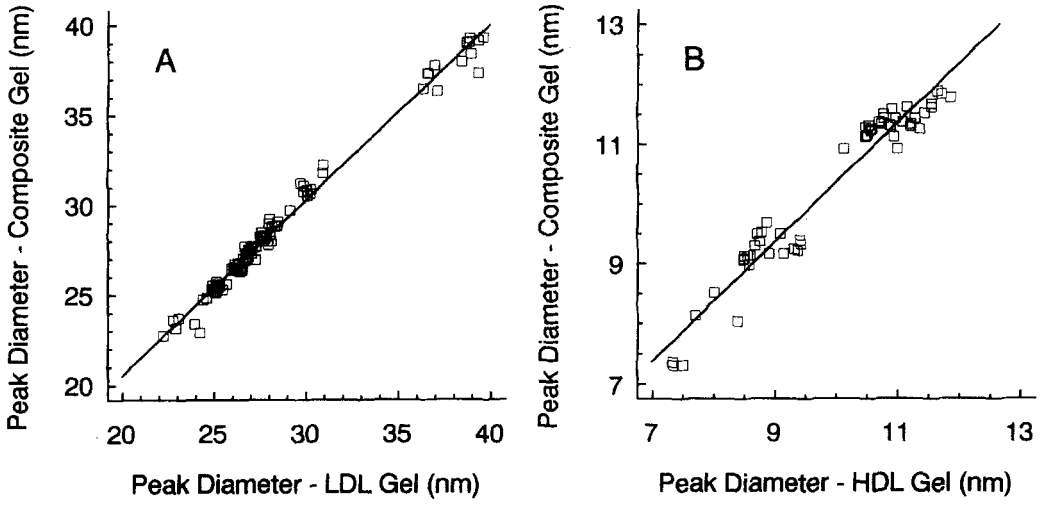

Fig. 2. Comparison of peak particle diameter estimates for β-lipoproteins run on LDL and Composite gels (panel A) and for HDLs run on HDL and Composite gels (panel B). The lines represent the least squares fit of data for 92 β-lipoprotein peaks (slope was 0.98 ± 0.01 and Y-intercept was 0.97 ± 0.34, $r^2 = 0.987$) and for 54 HDL peaks (slope was 1.00 ± 0.03 and Y-intercept was 0.40 ± 0.35, $r^2 = 0.941$).

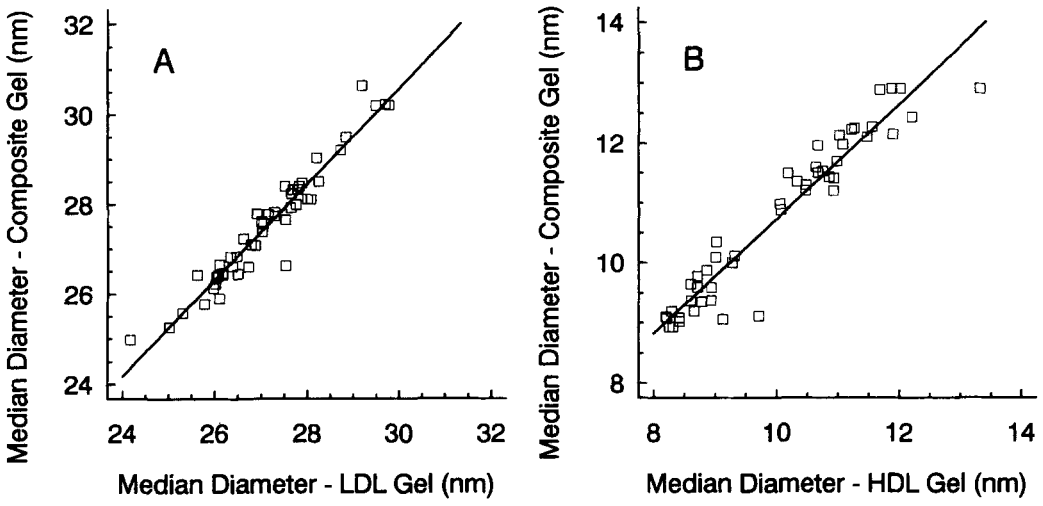

Fig. 3. Comparison of median diameter estimates. Panel A: LDL median diameters were estimated for 48 samples run on LDL and Composite gels (slope was 1.07 ± 0.04 and Y-intercept was −1.39 ± 1.15, $r^2 = 0.932$). Panel B: HDL median diameters were estimated for 48 samples run on HDL and Composite gels (slope was 0.95 ± 0.04 and Y-intercept was 1.18 ± 0.43, $r^2 = 0.917$). Median diameters were estimated as described in the text.

is defined as particle diameter at which half the LDL (or HDL) absorbance is on larger and half on smaller particles. Thus, median diameter is a measure of the overall absorbance profile that reflects the size distribution of lipoprotein particles within the defined size interval. Estimates of median diameters will be sensitive to variation in lipoprotein separation and stain uptake, in addition to gel calibration (which also affects peak diameter estimates). We estimated median diameters for LDLs (i.e., 21–35 nm) of 48 samples run on LDL and Composite gels. Using the same absorbance profiles obtained from the Composite gels, we estimated median diameters for HDLs (i.e., 7.2–19 nm) and these were compared with those derived from HDL gels. **Figure 3** shows that both LDL and HDL median diameters estimated from the Composite gels were very similar to those estimates made using the specialized gel formats. The regression lines had slopes of 1.07 and 0.95, respectively; neither slope was significantly different from the expectation of one. Using the same experiment described above (i.e., eight samples run three times), we found the estimates of LDL and HDL median diameters were highly repeatable (repeatability was 0.999, n = 48) and the average coefficient of variation was 1.46% (range 0.1 to 6.6%).

We calculated fractional absorbance for each of six lipoprotein size intervals spanning from intermediate density lipoproteins (IDL) to HDL_3. Comparison of fractional absorbance obtained from Composite gels and from the dedicated gels revealed a strong correspondence ($r^2 = 0.828$) for the relationship (**Fig. 4**).

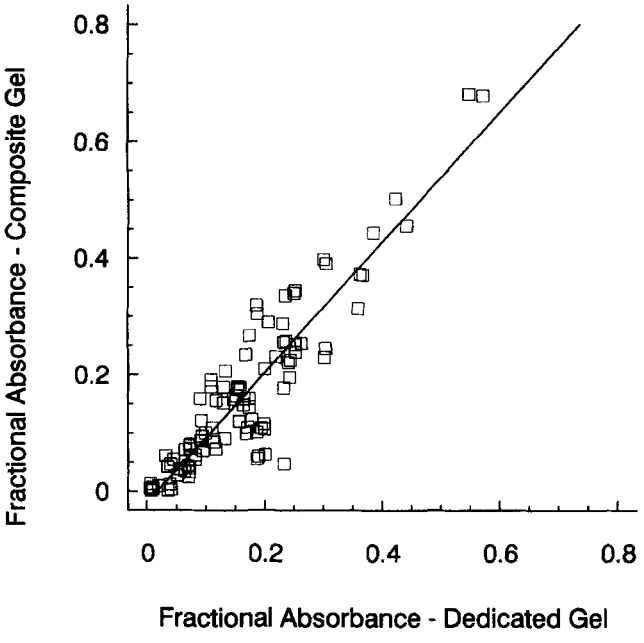

Fig. 4. Comparison of fractional absorbances for six lipoprotein size intervals estimated using the Composite and the two dedicated gels. Lipoprotein absorbance profiles were cut into the following fractions: VLDL, IDL and Lp[a], 29–38 nm; large LDLs, 25.5–29 nm; small LDLs, 21–25.5 nm for human samples and 24.3–25.5 nm for baboon samples; HDL_1, 12.9–21 nm for human samples and 12.9–24.3 for baboon samples; HDL_2, 8.8–12.9 nm; HDL_3, 7.2–8.8 nm. To calculate fractional absorbance for the six fractions from the dedicated gels, absorbance area of the first three fractions was obtained from runs on LDL gels and of the last three fractions, from runs on HDL gels. Eight samples were run twice in each gel format, leading to 96 fractions in the plot (slope was 1.11 ± 0.05 and Y-intercept was −0.02 ± 0.01; $r^2 = 0.828$).

The slope was 1.11 ± 0. 05, close to the expectation of unity, and the Y-intercept was not significantly different from zero. In Fig. 4 we have included data from all fractions, although the nature of fractional data dictates that only five of the six fractions contain unique information (i.e., one minus the sum of five fractions exactly predicts the value of the sixth). Accordingly, we also compared raw absorbance in the six lipoprotein size intervals run on Composite or dedicated gels. Neither the slope (0.92 ± 0.05) nor the Y-intercept (−1987 ± 2073) were significantly different from expectations of one and zero (data not shown). However, at 0.86 (r^2 = 0.742), the correlation coefficient for the comparison of raw absorbance was lower than that for the fractional absorbance. This degradation of correlation was due to the inclusion of variation from additional sources, particularly individual gel differences in staining and destaining characteristics.

In this report we have described the characteristics of a new gradient gel format that we designed for evaluations of both LDL and HDL phenotypes. We used the same gel casting protocol that was previously shown to produce gels with highly repeatable properties. Measurements, derived from the new gel format, of peak and median diameters and of fractional absorbance for both LDLs and HDLs were very similar to the same measures made from specialized gels dedicated to the separate analyses of LDLs and HDLs. This new gel format should help increase analytical efficiency and improve comparability of measures made for the various species of lipoproteins found within a sample.

The authors are grateful for the advice of Drs. C. M. Kammerer and L. D. Atwood in developing the computer program for analyzing the gradient gel profiles. This work was supported in part by grants from the National Institutes of Health (HL-28972, HL-45522, and RR-09919).

Manuscript received 16 October 1996 and in revised form 12 February 1997.

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