Production of polyacrylamide gradient gels for the electrophoretic resolution of lipoproteins

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Summary We describe a protocol to cast nondenaturing polyacrylamide gradient gels (SFBR3/31) for the size resolution of lipoproteins. The protocol yields gels with minimal lot-to-lot variation in length and electrophoretic properties. Absorbance profiles of cholesterol-stained lipoproteins in baboon sera were used to estimate the relative amounts of stain in four lipoprotein size classes (VLDL+LDL, HDL_1, HDL_2, and HDL_3). When compared with gels from a commercial source, the SFBR3/31 gels gave very similar results in terms of precision (coefficients of variation) and of estimated amounts of lipoproteins in the four size classes. In other studies, we estimated peak diameters of protein-stained human lipoproteins after calibrating the gels with size standards. Peak diameters estimated using SFBR3/31 gels were highly correlated (r^2 = 0.99, n = 33) with those estimated using gels from a commercial source. We conclude that the protocol reliably produces gradient gels that are suitable for the analysis of lipoprotein phenotypes.—**Rainwater, D. L., D. W. Andres, A. L. Ford, W. F. Lowe, P. J. Blanche, and R. M. Krauss.** Production of polyacrylamide gradient gels for the electrophoretic resolution of lipoproteins. *J. Lipid Res.* 1992. **33:** 1876–1881.

Supplementary key words gradient gel electrophoresis • baboon • VLDL • LDL • HDL

For many years, nondenaturing 4–30% polyacrylamide gradient gels have been used extensively for the study of size-resolved lipoproteins. Recently, we became interested in casting such gels for long term studies of lipoprotein phenotypes. To accomplish this, it was necessary that lipoprotein phenotypes be repeatable across gels and that the gradient be precisely controlled between gel lots cast on different occasions. In this report, we describe a protocol that will produce such gels.

MATERIALS AND METHODS

Gel casting (SFBR3/31 gels)

The stock solutions were: *1)* 292.95 g/l acrylamide (Bio-Rad Laboratories, Richmond, CA) and 17.05 g/l bisacrylamide (Bio-Rad) (31.0% total, 5.5% cross-linker), and *2)* 32 g/l acrylamide (3.2% total, 0% cross-linker). The solutions were vacuum-filtered and stored at room temperature for less than 1 month. The high limit solution was made immediately prior to casting the gradient, and contained one volume of solution *1,* 0.0015 volumes of freshly prepared ammonium persulfate (100 g/l; Sigma

Abbreviations: VLDL+LDL, very low density plus low density lipoproteins; HDL, high density lipoproteins; SFBR3/31, laboratory-produced gradient gels; SD, standard deviation; CV, coefficient of variation; SE, standard error.

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Chemical Company, St. Louis, MO), and 0.00025 volumes of 3-dimethylaminopropionitrile (Sigma). The low limit solution was also made immediately prior to casting the gradient, and contained one volume of solution *2*, 0.0046 volumes of ammonium persulfate, and 0.0006 volumes of 3-dimethylaminopropionitrile.

A Wiz dual-pump gradient controller (ISCO, Lincoln, NE) was used to cast the gradients. In this system, two pumps are separately controlled by an Apple IIe computer (Apple Computer, Inc., Cupertino, CA) via a ChemInterface unit and software (ISCO) to define the characteristics of any gradient that can be described adequately in less than 20 linear segments. This system was selected because the solutions are retained in disposable plastic tubing, the pumps are easily calibrated for each occasion using the software, and the gradients are reproducibly generated by a computer. **Table 1** provides the characteristics of the gradient that were used by the ChemInterface gradient controller software.

The solutions from the two pumps were mixed in an external mixing chamber (**Fig. 1**) before entering the casting stand. The mixing chamber was constructed by layering three sheets of plastic that were sealed with acetone. The mixing volume of 0.76 ml was made by boring a 12.7-mm diameter hole in the central 6-mm-thick piece prior to sealing. A 2.8 × 10 mm stir bar was placed inside the chamber and inlet/outlet ports (1/8" ID/10-32 barbed fitting, Ark-Plas, Flippin, AR) were tapped into the central piece of plastic on opposite sides of the chamber (Fig. 1).

Gel cassettes were assembled as suggested by Pharmacia (Pharmacia technical bulletin, Polyacrylamide Gel Electrophoresis, revised edition, Uppsala, Sweden) with modifications to stretch the waterproof tape and make the seal tighter. For the first seal, the two plates were placed on the flattened tape, each about 0.5 mm from the spacer. Drawing the two plates to a parallel position caused the tape to stretch. For the second seal, the spacer was placed in the middle of the plate and parallel to the first spacer, the plates were slightly compressed, and the edges were taped. Then the spacer was forced to the newly taped edge to cause the tape to stretch. Tight seals on the cassettes at this stage virtually eliminated the formation of bubbles between the plates and the gel during electrophoresis.

TABLE 1. Characteristics of the gradient segments used in the production of SFBR3/31 gradient gels

Segment Number	Flow rate	% High[a] Start	% High[a] End	Duration
	ml/min			*min*
1	7.0	0.0	5.0	1.7
2	7.0	5.0	10.8	1.7
3	7.0	10.8	16.6	2.5
4	7.0	16.6	22.6	2.5
5	7.0	22.6	29.0	2.4
6	7.0	29.0	36.9	2.4
7	7.0	36.9	45.7	2.4
8	7.0	45.7	56.7	2.4
9	7.0	56.7	71.9	1.0
10	7.0	71.9	100.0	1.0

[a] Percent of total flow that is from the high limit solution at the start and the end of each gradient segment.

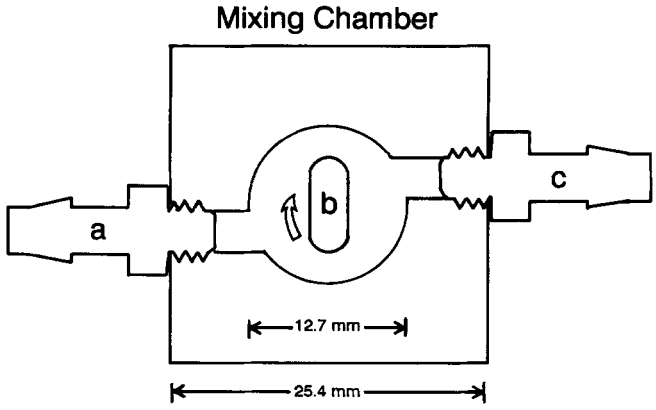

Fig. 1. Scaled drawing of the mixing chamber. The code for parts is: a) inlet port, b) stir bar, and c) outlet port.

Eight gels were cast at a time using the GSC-8 Gel Slab Casting Apparatus (Pharmacia, Piscataway, NJ). For the gradient described in Table 1, we routinely prepared 105 ml of the low limit solution and 48 ml of the high limit solution. The gradient was pumped at 7.0 ml/min from the bottom under a 10 ml layer of ethanol–water 1:4 (v/v). Total time for the gradient was 20 min, at the end of which the computer specified a pause and then 18 ml of sucrose solution (600 g/l) was pumped into the chamber which displaced the gradient upward to the bottom of the glass plates. After polymerization (approximately 2 h at room temperature), the cassettes were separated. The polymerized gel never extended more than 1 mm below the glass plates. Gels were stored in plastic bags with electrophoresis buffer for up to 2 weeks with no apparent alterations in electrophoretic properties.

Electrophoresis and densitometry

For analyses of protein-stained human lipoproteins, a $d \leq 1.20$ g/ml fraction was isolated by ultracentrifugation of plasma. Samples (each derived from 10 μl plasma) were electrophoresed in a Pharmacia GE-4 Gel Electrophoresis Apparatus as described previously (1). Gels were fixed in 10% sulfosalicylic acid for 1 h, stained with Coomassie Blue G-250 (0.4 g/l in 3.5% perchloric acid), and destained in 5% acetic acid. Lipoprotein profiles were analyzed by computer-automated densitometry using a model RFT densitometer (Transidyne Corporation, Ann Arbor, MI) at 600 nm. Scan data were processed on a VAX station 3200 (DEC, Maynard, MA). Peak particle

sizes were calculated from migration distances, expressed as R_f (migration relative to bovine serum albumin), obtained from the standard protein mixture (HMW Calibration Kit, Pharmacia) (1).

For analyses of cholesterol-stained baboon lipoproteins, fresh serum was prepared from baboons bled for the purposes of other studies. Serum samples (8.3 μl) were prestained for 6 h with Sudan black B (final concentration was 2.8 g/l) and subjected to electrophoresis (2750 V • h) as described previously (2). Individual lanes were analyzed by densitometry at 610 nm (Cliniscan, Helena Instruments, Beaumont, TX). A baseline (lowest absorbance value) was subtracted from each lipoprotein absorbance profile. Using a serum standard placed in one lane of each gel, absorbance profiles were cut, using a dropline approach, into four lipoprotein size classes: VLDL+LDL, HDL_1, HDL_2, and HDL_3. The diameters of particles at the cutpoints were estimated to be 20 nm and larger for VLDL+LDL, 14 to 20 nm for HDL_1, 11 to 14 nm for HDL_2, and 8 to 11 nm for HDL_3 using the standard protein mixture. These cutpoint diameters corresponded with those previously described for baboon HDL size classes (3, 4). Absorbance in each lipoprotein size class was expressed as a fraction of total absorbance.

For comparisons with gels commonly used in other studies, we also analyzed the samples using PAA4/30 gels (Pharmacia). These gels were not commercially available at the time of this study which was done with gels purchased previously. Samples and electrophoresis conditions were identical to those described above.

Data analysis

Coefficients of variation were calculated as the square root of the pooled variance divided by the mean; pooled variance was calculated as the sum of the squared differences between paired samples divided by 2n (n = number of different samples). Linear regression analyses and paired t tests were done with a commercial statistics package.

RESULTS AND DISCUSSION

Protocol development

For many years, nondenaturing gradient gels, based on the method of Margolis and Kenrick (5), have been used for the study of lipoprotein phenotypes. While some investigators have developed protocols to cast gradient gels for specialized tasks such as immunoblotting (6) and resolving larger lipoproteins (7), most have relied on commercial sources for gradient gels. Because of the uncertain supply of gels from commercial sources, we developed a method to produce nondenaturing gradient gels for the resolution of lipoproteins. As long-term studies often exceed the shelf life of gradient gels from a single lot, the principal requirement that we imposed on the method was that it be highly reproducible. In addition, the gels had to provide lipoprotein resolutions comparable to those previously obtained. To assess this we ran samples on SFBR3/31 and PAA4/30 gels. **Fig. 2** shows a comparison of cholesterol-stained baboon lipoproteins run on the two gel types. Many distinctive features of lipoprotein patterns in the samples were observed in both gel types.

The solutions, gradients, and procedures that we developed for casting gels were based on information supplied by Pharmacia (Pharmacia technical bulletin, Polyacrylamide Gel Electrophoresis, revised edition, Uppsala, Sweden). One major difference between the SFBR3/31

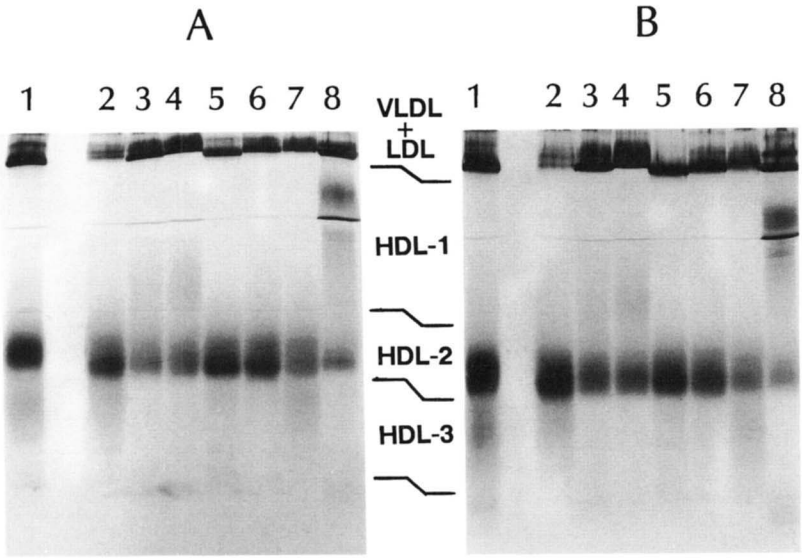

Fig. 2. Comparison of cholesterol-stained lipoproteins in baboon serum run on PAA4/30 (panel A) and SFBR3/31 (panel B) gradient gels. Gels are oriented with the largest pore sizes at the top. Numbers indicate sample pairs from different baboons.

gels and the PAA4/30 gels was at the gel top. Serum VLDL+LDL particles migrated farther into the SFBR3/31 gels, and resolution of these lipoproteins was improved (Fig. 2B). These differences were probably due to the use of the 3.2% low limit solution which specified larger pore sizes than in PAA4/30 gels. We retained the larger pore sizes at the gel top in order to better visualize the heterogeneity of apolipoprotein B-containing lipoproteins.

Repeatability of gel gradient

We obtained a high degree of gradient repeatability by using the computer-controlled dual-pump system. With this system, gradients were easily programmed, and flow rates were reliably calibrated. To assess lot-to-lot reproducibility of gel volume, we measured the length of polymerized gel in one cassette from thirteen lots of gels cast over a 1-month period. The average gel length was 74.5 mm (SD = 0.5 mm, n = 13). Similarly, we measured the mean migration distances from the gel top for three consistently identifiable bands in the standard serum lane on one gel from each of five lots: LDL was 6.6 mm, SD 1.1 mm; an unidentified band was 15.8 mm, SD 1.3 mm; albumin was 66.4 mm, SD 2.6 mm. Inspection of various serum proteins across lanes indicated that pore sizes were very similar horizontally (Fig. 2B). Also, we compared lipoprotein particle diameters (see below) that were estimated from gels cast in separate lots to determine whether there were lot-to-lot differences in calibration. Lipoproteins from three human plasma samples were applied in replicate to two SFBR3/31 gels cast on different days. Particle diameters were estimated for a total of nine distinct peaks in these samples. There were no significant differences (using a paired t test) in estimated particle sizes among gel lots. These observations indicated that gradient shape and volume were reproducible among gel casting lots.

Precision

Precision was estimated using the absorbance profiles taken from cholesterol-stained baboon lipoproteins that were cut into four size class intervals. We loaded aliquots of the same sample onto each of two PAA4/30 gels and two SFBR3/31 gels; we loaded samples from 42 different baboons in this manner. After electrophoresis, sample lanes were densitometrically scanned to determine the proportional distribution of stain among the four major lipoprotein size classes. **Table 2** shows the means and coefficients of variation for replicated measurements of each lipoprotein size class for the two types of gels. The coefficients of variation for each lipoprotein class were very similar for the two gel types. These data suggested that the SFBR3/31 gels were similar in precision to the PAA4/30 gels.

TABLE 2. Mean proportion of absorbance and the coefficients of variation (CV) for each baboon lipoprotein size class estimated for the two gel types

Lipoprotein Size Class	PAA4/30		SFBR3/31	
	Mean	CV	Mean	CV
VLDL + LDL	0.1994	0.1219	0.2304	0.0996
HDL_1	0.2260	0.1023	0.1959	0.0900
HDL_2	0.3849	0.0559	0.4182	0.0547
HDL_3	0.1898	0.0595	0.1557	0.0694

To calculate coefficients of variation, serum aliquots from 42 different baboons were run on two gels of each type. For this experiment only, the SFBR3/31 gels were cast using a slightly different gradient in which each gradient segment in Table 1 was run for 2 min.

TABLE 3. Results of regression analyses comparing proportion of total absorbance in each lipoprotein size class estimated in two gel types

Lipoprotein Size Class	Slope	SE	Intercept	SE	r^2
VLDL + LDL	1.056	0.070	0.011	0.018	0.858
HDL_1	1.206	0.057	−0.075	0.014	0.922
HDL_2	1.149	0.056	−0.030	0.021	0.906
HDL_3	1.389	0.082	−0.089	0.016	0.883

Serum samples from 40 different baboons were run. The value from the PAA4/30 gel was set as the independent variable and the value from the SFBR3/31 gel was the dependent variable.

Correlation of lipoprotein profiles

To compare lipoprotein patterns in the two gel types, we tested for correlation of estimated amounts in lipoprotein classes using cholesterol-stained lipoprotein profiles from 40 different baboons. We performed regression analysis of estimates for each lipoprotein size class. **Table 3** presents the regression values for the lipoprotein classes. Slopes were greater than one for each lipoprotein class comparison; this may have resulted from a slightly nonlinear background frequently observed for PAA4/30 gels. Although significantly different ($P < 0.05$) in some cases, the slopes and intercepts approached the expected values of one and zero, respectively. Absorbance values from the two gels were highly correlated, with the correlations explaining between 86 and 92% of the variation. Thus, the lipoprotein profiles in PAA4/30 gels predicted very well the distributional patterns of the major lipoprotein subclasses in SFBR3/31 gels.

Calibration using size standards

Pharmacia high molecular weight standard proteins were used to calibrate gels after electrophoresis and staining for protein. **Fig. 3** shows densitometric scans of the standard protein mixture subjected to electrophoresis on PAA4/30 (A) and SFBR3/31 (B) gels. The two absorbance profiles in Fig. 3 were plotted on the same scale to illustrate the similarities in peak heights and areas. Peak shape and location (R_f) were very similar in the two types of gel.

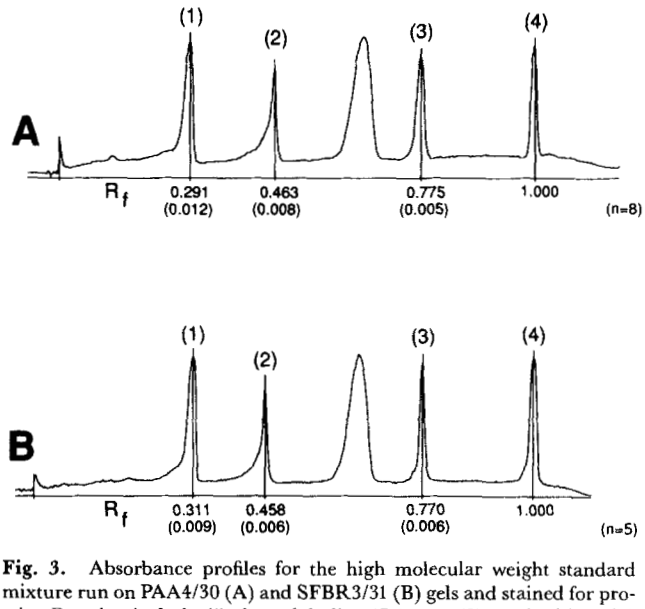

Fig. 3. Absorbance profiles for the high molecular weight standard mixture run on PAA4/30 (A) and SFBR3/31 (B) gels and stained for protein. Proteins include (1) thyroglobulin, 17.0 nm, (2) apoferritin, 12.2 nm, (3) lactate dehydrogenase, 8.16 nm, and (4) bovine serum albumin, 7.1 nm; the unmarked peak is catalase which is not routinely used in gel calibration. R_f migration units and standard deviations are shown below each scan. Absorbance profiles are plotted on the same scale in the two panels.

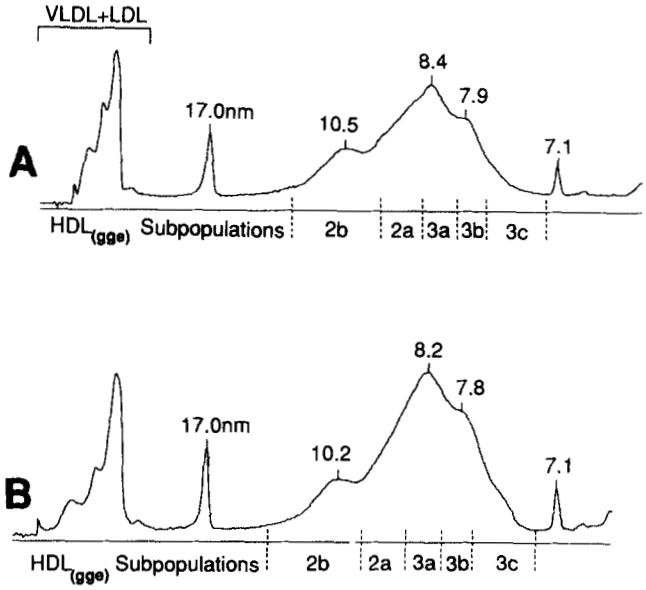

Fig. 4. Particle size distribution in a representative human plasma lipoprotein sample electrophoresed in PAA4/30 (A) and SFBR3/31 (B) gels. HDL subpopulation intervals are shown below each scan profile and peak particle diameters are given in nm. Migration region of VLDL+LDL and peak diameters of thyroglobulin (17.0 nm) internal standard and human serum albumin (7.1 nm) are also indicated. Absorbance profiles are plotted on the same scale in the two panels.

Fig. 4 shows densitometric scans of a protein-stained human lipoprotein sample run on PAA4/30 (A) and SFBR3/31 (B) gels. Particles in the VLDL+LDL region had greater mobilities and were better resolved in the SFBR3/31 gel when compared with the PAA4/30 gel. However, distributions (i.e., peak shapes and relative heights) of stained proteins among the HDL size classes were similar for the two gel types, and estimated peak diameters were also very similar. After calibration, we analyzed 15 protein-stained human lipoprotein samples that were electrophoresed on the two gel types. Thirty three discrete peaks within the HDL particle size range (1) were identified in these samples, and their molecular diameters were measured. **Fig. 5** shows a plot of the calculated peak diameters measured on the two gel types. While the SFBR3/31 gels consistently gave lower estimates of peak diameters, the estimates were highly correlated (r^2 = 0.991), and the slope (0.96 ± 0.02) and the intercept (0.10 ± 0.15) were close to the expected values of one and zero, respectively. These data suggest that particle sizes may be estimated using the SFBR3/31 gels, and that the values are highly correlated with those derived using PAA4/30 gels.

The present protocol was designed to produce gradient gels that would substitute for those that have been supplied commercially. In this respect the protocol differs from other protocols (6, 7) that were designed to overcome certain limitations. For example, Lefevre, Goudey-Lefevre, and Roheim (6) documented the failure of PAA4/30 gels to permit adequate transfer of lipoproteins to nitrocellulose paper for immunoblotting procedures, while their own gels permitted nearly complete transfer. An advantage of the present gels is that they not only reproduce the resolutions obtained with PAA4/30 gels, but they also permit nearly complete transfer of lipoproteins (D. L. Rainwater, unpublished observations).

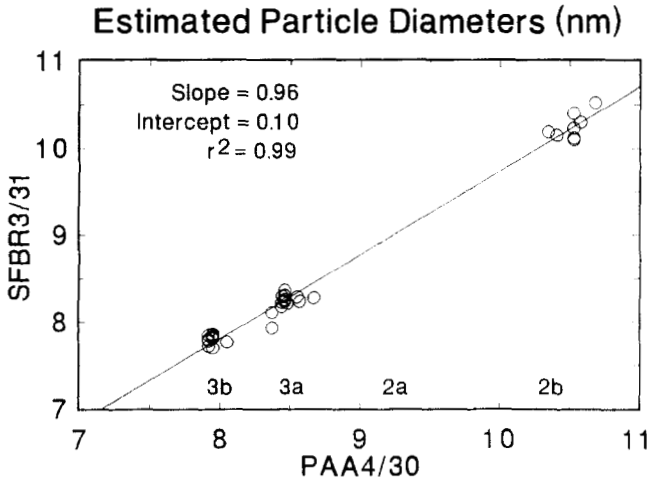

Fig. 5. Comparison of HDL peak particle diameters (n = 33) from human plasma lipoproteins run on PAA4/30 and SFBR3/31 gradient gels and stained for protein. HDL subclass designations and their size intervals are taken from reference 1.

In summary, we have described a protocol that will reproduce the gradient gels that have been supplied by Pharmacia. The protocol was demonstrated to produce gels with similar properties from casting to casting. The gels yielded lipoprotein profiles and estimated peak diameters that were very similar to those obtained using PAA4/30 gels, and the two gel types showed equal precision.

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