Silver-enhanced radial immunodiffusion assay of plasma apolipoproteins

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Summary Silver-staining of immunoprecipitates extends the sensitivity of the radial immunodiffusion assay by tenfold. This modification permits the quantification of apolipoproteins A-I, A-II, C, and E at levels of 0.2–1.0 mg/dl in plasma samples at a sensitivity threshold of 10 ng. The silver-enhanced radial immunodiffusion method is readily adapted from the standard method, simple and inexpensive to perform, and does not require costly instrumentation. These advantages make the modified RID assay an attractive alternative to other forms of immunoassay.—**Ishida, B. Y., and B. Paigen. Silver-enhanced radial immunodiffusion assay of plasma apolipoproteins.** *J. Lipid Res.* 1992. **33:** 1073–1078.

Supplementary key words apoA-I • apoA-II • apoC • apoE • plasma apolipoproteins • radial immunodiffusion assay • immunoassay • silver staining

Immunoassays provide for sensitive quantitative measurement of a variety of biological compounds. Enzyme-linked immunosorbent assay (ELISA) (1, 2), radioimmunoassay (RIA) (3), nephelometry (4), electroimmunoassay (EIA) (5–7), and radial immunodiffusion (RID) (8–11) are often used. ELISA, RIA, nephelometry, and electroimmunoassay usually require expensive and sophisticated instrumentation such as microplate readers, gamma counters, nephelometers, and electrical power supplies.

In comparison, the RID method is performed using materials commonly available in most laboratories. On the other hand, the standard RID technique consumes large quantities of antibodies, which can make the method impractical and costly to perform. However, the sensitivity of detection in the RID technique is dependent upon the ability to detect an immunoprecipitate formed at the antigen/antibody equivalence point (9). The opaque immunoprecipitant rings are generally visible at antigen assay levels of 0.1–0.5 μg. The detection sensitivity of immunoprecipitates can be increased two to fivefold with protein stains such as Coomassie R-250. We routinely use this stain since the accurate localization of the ring boundary is critical in determining the immunoprecipitate ring size (diameter).

Among apolipoproteins in mouse plasma, apoC is one of the lowest in concentration. During nutritional manipulations such as the feeding of a high fat and high cholesterol diet, apoC is further reduced in concentration (12). Hence the SERID method was developed, in part, to detect changes in apoC levels. Previously, we have assayed apolipoproteins from ultracentrifugally purified lipoproteins using Coomassie R-250 densitometry of sodium dodecyl sulfate polyacrylamide gels (SDS-PAGE) (12). Since SDS-PAGE is a chemical assay, it forgoes the need of specific antisera to each apolipoprotein. In situations where antisera is unavailable, species-specific (13, 14), or difficult to produce, SDS-PAGE is a useful alternative. Accordingly, this method has been used to measure apolipoprotein A-I in experimental animals such as the inbred and transgenic mouse (12, 15), and the baboon, dog, gerbil, goat, and rat (16). In many studies little or no attempt is made to correct for differences in chromogenicity when proteins are stained with dyes such as Coomassie blue. In our own study (12) we have found that the chromogenicity among mouse apolipoproteins can differ by fivefold, which necessitated the use of homologous protein calibrators. When using SDS-PAGE to measure apoC, the plasma concentration was estimated as 8 ± 6 mg/d (mean ± SE); however, the poor sensitivity of the technique and the low content of plasma apoC contributed to the large standard error. Our current attempts to measure apoC by the standard RID methodology were similarly hampered by inadequate sensitivity.

As protein detection in agarose and polyacrylamide gels is enhanced 10- to 100-fold by silver-staining (17–20), we reasoned that its use in the RID techniques may lead to similar increased sensitivities. In this report, we describe a silver-enhanced RID (SERID) method that results in a 10-fold increased sensitivity over the standard method in sensitivity, and allows immunoquantitation at nanogram protein levels.

MATERIALS AND METHODS

All chemicals and reagents used were of the highest analytical grade obtainable. Gels were formulated to contain 0.8% agarose (w/v) (DNA grade, Bio-Rad, Richmond, CA), 3% polyethylene glycol (w/v) (8000 MW, Polysciences, Warrington, PA), in RID buffer (0.02 M Tris (pH 7.4), 1 mM EDTA, and 0.05% sodium azide). Antisera in 10 ml molten agarose solution (50°C) was cast as an open gel on a 7.5 × 7.5 cm chemically activated polyester film (GELBOND™, FMC, Rockland, ME) adhered by surface tension (distilled water) to a glass plate of similar dimensions. Each gel required 20–50 μl antisera in the SERID assay or 10-fold more in the standard assay.

Abbreviations: apo, apolipoprotein; CV, coefficient of variation; SERID, silver-enhanced radial immunodiffusion.

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The solidified gel was further cured on ice for 10 min. Sample wells of 5 μl capacity were cut 1 cm apart with a 4-mm diameter punch (Bio-Rad). Agarose plugs were removed by gentle aspiration with a Pasteur pipet connected by tubing to a vacuum flask. Staggered rows of 7 and 6 wells were alternately cut to provide a total of 46 wells per plate. This allowed for 5 purified apoprotein calibrator wells, 3 control plasma wells, and 38 sample wells.

Calibrator apolipoproteins were purified to homogeneity by column chromatography from ultracentrifugally isolated mouse plasma lipoproteins as previously described (21). Apolipoproteins were quantitated for total protein as described by Peterson (22). Antisera were prepared in New Zealand White rabbits immunized with purified mouse apolipoproteins A-I, A-II, C, and E. The latter three apolipoproteins were coupled to keyhole limpet hemocyanin prior to immunization. Each apolipoprotein (50 μg) was emulsified in complete Freund's adjuvant and immunized intradermally at 2- to 4-week intervals. The antisera employed in this study were obtained 4 months after the initial immunization. The antibody titers varied among the antisera requiring the following final dilutions (standard, SERID): anti-A-I (1:40, 1:500), anti-A-II (1:20, 1:250), anti-C (1:18, 1:200), anti-E (1:40, 1:360).

Apolipoprotein calibrators and plasma samples were diluted 2- to 150-fold in RID buffer containing deionized 10 M urea (RG501-X8, Bio-Rad) except for apolipoprotein E samples which were diluted in RID buffer containing a final concentration of 1% Triton X-100 (v/v) (Sigma, St. Louis, MO). A control reference plasma consisting of pooled fasting plasma from chow-fed C57BL/6 mice was aliquoted and stored at −70°C. Freshly thawed aliquots were previously assayed by the standard RID method and subsequently included in each assay of uncharacterized plasma samples. Blood in this study was obtained by cardiac puncture and mixed with a cocktail (100 ×) containing 200 mM EDTA (pH 7.4), 5 mg/ml gentamicin sulfate, and 5% sodium azide were added to the blood prior to plasma separation by centrifugation. The plasmas were aliquoted and stored at −70°C for assay at weekly intervals. Samples (5 μl) were pipetted in duplicate or triplicate into the wells and allowed to diffuse for 24 h at 37°C in a 10 × 10 cm polystyrene culture dish (American Scientific Products, McGraw Hill Park, IL). The culture dish was humidified with a distilled water-saturated filter paper (#05080, Schleicher and Schuell, Keene, NH) placed on the bottom. Following incubation, nonspecific proteins were removed by overlaying the RID gel with a 1-cm stack of filter paper and a glass plate, then pressed with a 1-kg weight for 1 h. The gel (attached to GelBond) was washed overnight at room temperature in 0.15 M NaCl, 1 mM EDTA (pH 7.4), 0.05% NaN_3, 0.1% Triton X-100 with gentle agitation. Gels were pressed as before, washed for 30 min in 5% aqueous glycerol, pressed for 30 min, washed in distilled water for 15 min, and dried under a forced stream of warm air. Gels were stained by *a)* the standard method with 0.2% Coomassie R-250 (w/v) (Sigma Chemical Co.) dissolved in 45% methanol, 10% glacial acetic acid (v/v), followed by destaining for 1 min in 95% ethanol; or *b)* the SERID method using commercial silver-staining reagent kit (GS-25, Isolab, Akron, OH). While the kit's reagent formulation is not fully disclosed, the manufacturer does cite the silver-staining method published by Willoughby and Lambert (17) whose study reported the silver-staining of proteins in agarose gels with a solution consisting of sodium carbonate, silver nitrate, ammonium nitrate, tungstosilicic acid, and formaldehyde. The manufacturer's kit lists sodium carbonate as an ingredient of its Solution I and the latter four chemicals in its preparation of Solution II. The kit's staining process is also procedurally similar to the published method. With these similarities, we presume that the described SERID method can be duplicated with a chemically defined system, but for consistency and convenience the commercial kit was used. With the kit, we extended the capacity of stainable gels per kit by proportionally reducing the quantity of each reagent. This action did not affect the staining characteristics. In this method, the gel is subjected to two sequential staining cycles in Solution III prepared immediately before use by slowly adding 5.0 ml Solution II to 5.0 ml Solution I in a disposable polystyrene weighing dish. Upon combining the latter reagents, the color of Solution III rapidly progresses from opaque white to a dark grey over 5 min at room temperature. At minutes 2 through 4 of this period, the gel is floated face-down on the surface of Solution III when the immunoprecipitates become faintly visible. The gel is removed and rinsed for 30 sec under a gentle stream of distilled water. A second staining cycle is immediately initiated to enhance the staining intensity. Staining is allowed to continue until the immunoprecipitant rings are well defined, or until a light grey background staining is apparent. The reaction is stopped by transferring the gel to a solution of 1% glacial acetic acid (v/v) for 1 min. Occasionally, an extremely high background can result due to prolonged contact with Solution III, or due to the use of an outdated reagent set. This background can be reduced by agitating the stained gel in a 20% sodium thiosulfate solution for 30 min. Destaining should be frequently monitored as total destaining can result. If this occurs, however, the gel can be restained as before. Upon completion of staining, the gel is rinsed in distilled water for 5 min and totally dried under a stream of warm air. Dried gels can be conveniently attached by transparent tape to laboratory worksheets for measurement of the immunoprecipitant rings and for permanent storage.

The areas of immunoprecipitates were measured as a function of diameter (9) under 2× binocular magnification with a digital electronic caliper micrometer (Cole-

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Parmer, Chicago, IL) capable of resolving 0.01 mm; however, a resolution of 0.1 mm is adequate. The squared diameter values of the calibrator immunoprecipitates were linearly dependent on the concentration. This function was used to calculate the control and sample apoprotein concentrations. Interassay variation was corrected by normalizing the sample values to previously determined values of the control reference plasma. Control plasma was assayed in triplicate with unknown samples and their apoprotein values were obtained from the apoprotein calibration curve. Each unknown sample value was multiplied by the reference value to current value ratio of the control plasma to obtain normalization.

RESULTS AND DISCUSSION

Porro et al. (18) described the use of silver-staining to extend the detection sensitivity of "rocket" immunoprecipitates. We reasoned that silver-staining would similarly extend the sensitivity of the RID technique. In agreement with their results, we found that the staining of RID gels with silver substantially improved the detection sensitivity by at least tenfold. As illustrated in **Fig. 1**, purified mouse apoC calibrators and control reference plasma were assayed at two concentration ranges, and stained with Coomassie blue or with silver. One RID gel contained apoprotein calibrators ranging from 2 to 10 mg/dl (0.1 to 0.5 μg per well), and replicates of a control plasma sample diluted twofold. The second gel contained a tenfold lower calibration range of 0.2 to 1.0 mg/dl (0.01 to 0.05 μg per well), and replicates of a control plasma diluted fivefold. The first gel was stained with Coomassie blue R-250 (standard method) and the second with silver (SERID method). Even when the calibrators were assayed at a tenfold lower concentration, the silver-stained immunoprecipitates were comparable in size to the Coomassie-stained series. This comparison of the two methods demonstrates the enhanced sensitivity imparted by silver-staining.

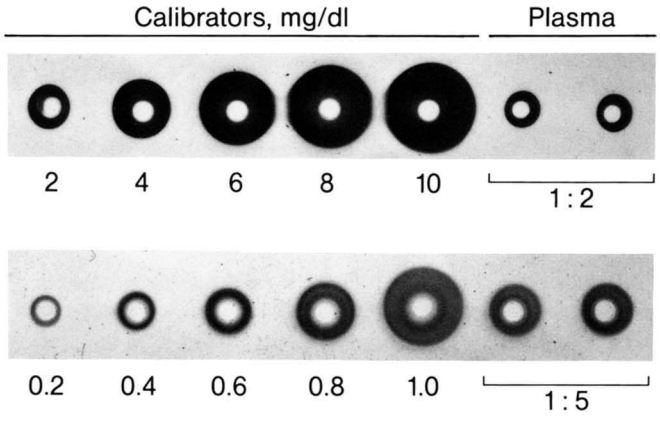

Fig. 1. Comparison of radial immunodiffusion by the standard and the SERID methods. In the standard method, purified mouse apolipoprotein C calibrators were diluted in a buffer containing 10 M urea, 0.02 M Tris (pH 7.4), 0.05% NaN_3 at protein concentrations ranging from 2 to 10 mg/dl, and mouse plasma samples were diluted twofold in triplicate (only two wells are shown) in the same buffer (top row). For the SERID method, calibrator concentrations ranged from 0.20 to 1.0 mg/dl, and mouse plasma samples were diluted fivefold (bottom row). The immunoprecipitates were visualized with Coomassie R-250 (standard method) or by silver-staining (SERID method) following the removal of unreacted plasma and antisera components. Immunoprecipitate diameters (mean ± SE, n = 4) for the lowest and highest calibrators were 4.41 ± 0.08 and 9.83 ± 0.12 mm (standard method), and 3.23 ± 0.04 and 7.97 ± 0.14 mm (SERID method), respectively.

For the control plasma sample stained with Coomassie (Fig. 1, top row), a twofold dilution resulted in a small immunoprecipitate that fell slightly out of the calibration range. However, an extrapolated value (mean ± SE) of 2.8 ± 0.2 mg/dl was calculated. This value for apoC agrees closely with the value, 8 ± 6 mg/dl, previously determined in similar mouse plasma by SDS-PAGE/densitometry (12). In the latter study, the precision of the technique was poor. In the standard RID assay, a larger immunoprecipitate would have resulted if the plasma sample was not diluted, but we experimentally found it necessary to dilute the plasma at least twofold in the 10 M urea buffer (an effective concentration of 4 M) to expose all immunoreactive apolipoprotein sites. Cryptic apolipoprotein epitopes that may result from secondary protein structure and protein-lipid interaction have been suggested in studies by others (23–25). Alternatively, an attempt was made to avoid extrapolation by the lowering the calibration range in the standard assay to 0.75–1.00 mg/dl. In this situation, however, the inability to accurately measure very small ring diameters (<3 mm) was not a viable solution. These measurement difficulties were not a problem in the SERID assay. ApoC immunoprecipitates were easily measured even when the identical plasma sample was diluted fivefold in the 10 M urea buffer (Fig. 1, bottom row). This plasma dilution placed the ring diameter at the midrange of the 0.20–1.00 mg/dl calibration curve.

Although apolipoproteins A-I, A-II, and E are present at adequate plasma concentrations to use the Coomassie-staining method, apoprotein measurement at a lower level would allow the quantity of antisera to be correspondingly reduced by tenfold, thus conserving valuable antisera. A comparison of the standard and the SERID assays of apolipoproteins A-I, A-II, C, and E displays the feasibility and the increased sensitivity achieved by silver-staining (**Table 1**). For this study a single plasma pool from five C57BL/6 mice and a concentration series of purified apolipoprotein calibrators were aliquoted, quickly frozen in liquid nitrogen, and stored at −70°C for later assay at three to four weekly intervals. Calibration curves for all measured apolipoproteins displayed an excellent linear dependence of immunoprecipitant areas (diameters) as a

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TABLE 1. Radial immunodiffusion assay of plasma apolipoproteins by the standard and SERID methods

	Standard					SERID				
			CV[d]					CV		
Apoprotein[a]	Equation[b]	r[c]	Intra[e]	Inter[f]	Plasma[g]	Equation	r	Intra	Inter	Plasma
A-I	y = 113.37 × + 4.77	0.999	3.8	10.2	112.0 ± 5.7	y = 754.41 × + 0.71	0.989	7.7	4.6	170.0 ± 4.5
A-II	y = 145.59 × + 3.13	0.999	5.0	5.1	33.8 ± 0.9	y = 1664.89 × − 7.52	0.994	3.3	5.6	70.4 ± 2.3
C	y = 196.74 × − 0.79	0.999	4.8	11.6	2.8 ± 0.2[h]	y = 1355.51 × − 7.50	0.986	1.9	11.8	5.6 ± 0.3
E	y = 126.39 × + 9.65	0.998	4.2	12.6	3.9 ± 1.8	y = 1218.89 × + 13.61	0.998	10.4	4.7	5.1 ± 0.1

[a]Apolipoprotein.
[b]Linear regression equation, x = μg, y = mm^2.
[c]Correlation coefficient.
[d]Coefficient of variation.
[e]Intra-assay, n = 3 replicates.
[f]Interassay, n = 4 determinations (except for apolipoproteins A-I and A-II SERID method, n = 3).
[g]Plasma was obtained from pooled fasting blood of five female C57BL/6 mice fed a normal chow diet (Purina) and stored at −70°C. Concentration, mg/dl ± SE.
[h]Value extrapolated from standard curve (assay value uncorrected for dilution = 1.4 mg/dl; calibrators range: 2.0–10.0 mg/dl).

function of the apolipoprotein concentration. The calibrator linearity as measured by the least square regression coefficient, *r*, was overall slightly better in the standard RID method. Nevertheless, the regression coefficient obtained using the SERID method was highly acceptable ranging from 0.986 (apoC) to 0.998 (apoE). In both assay methods, the intra-assay (CV = 2–10%, n = 3) and the interassay variations (CV = 5–13%, n = 3–4) were acceptable and similar to variances reported for other apolipoprotein immunoassays (23).

Despite excellent precision, the SERID method produced discrepantly higher plasma values for apolipoproteins A-I, A-II, C, and E (Table 1). The plasma apoprotein values obtained by the standard RID method were considered accurate as they have been previously verified by an independent method (12). Except for apoC whose plasma value measured by the standard RID assay was slightly extrapolated, we cannot explain the observed differences in the apoA-I, A-II, and E values as their measurements by the standard and the SERID methods were within the respective calibration ranges, and both the intra- and interassay variations were the lowest among the apolipoproteins assayed. That similar plasma A-I/A-II mass ratios were calculated for each RID method suggested a systematic positive bias in the SERID determination for these apolipoproteins. We speculate that an error of this type could result from an increased epitope exposure in the SERID method, perhaps caused by a higher urea concentration (due to a larger sample dilution) that effectively promotes total protein denaturation and reduces intermolecular interactions. Similar effects have been observed in the EIA technique where samples treated with urea can result in an overestimation of apolipoprotein A-I (Ishida, B. Y., unpublished results, and reference 6). The use of other denaturants, such as nonionic detergents, has been explored for the assay of mouse apolipoproteins (data not shown). In the case of apoA-I, A-II, and C, Triton X-100 at concentrations up to 1% (v/v) was inferior to urea, producing indistinct immunoprecipitant boundaries. Conversely, urea treatment was found to prevent the formation of anti-apoE/apoE immunoprecipitates. This led us to use 1% Triton X-100 in the assay of apoE which yielded sharp precipitant rings. Incomplete delipidation can also affect epitope exposure. However, the apoprotein standards applied to the RID gels were similarly affected, and yet were extensively delipidated by ethanol–diethyl ether 3:1 (v/v). Thus, the discrepancy is probably not explained by an interaction with lipids. We have also noted that apoB-48 and apoB-100 can be measured by the standard RID method when plasma and lipoprotein samples are allowed to diffuse freely without prior treatment with urea or Triton X-100. These observations make it clear that conditions that optimize epitope exposure and stability vary for the apolipoprotein classes.

If a systematic bias is responsible, then corrective measures can be incorporated into the assay. Normalization has been achieved in studies by others through the use of a plasma reference calibrator (6). Applied here, this approach was used to correct values from another plasma sample that gave positively biased SERID values for apoA-I (+75%), apoA-II (+78%), apoC (+53%), and apoE (+4%). These values were multiplied by the ratio of values ($Reference_{Standard}/Reference_{SERID}$) obtained by each method for a control reference plasma. As shown in **Fig. 2**, an excellent correlation was achieved by this correction for apolipoproteins that differ by two orders of magnitude in plasma concentration. Overall, the SERID method resulted in −11% average bias after the normalization process, which is not unusual even among identical immunoassay methods. For example, Albers and Adolphson in evaluating the accuracy of commercial and re-

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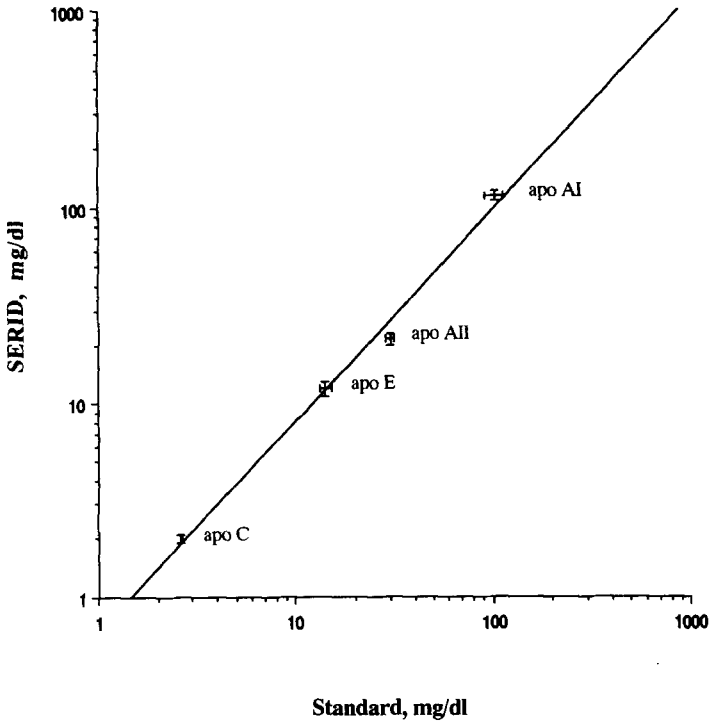

Fig. 2. Correlation of apolipoprotein measurements between the standard and SERID assay methods. A pooled mouse plasma sample was measured for apoA-I, apoA-II, apoC, and apoE content by the standard and the SERID methods. The SERID values were normalized by multiplication with the appropriate $Reference_{Standard}/Reference_{SERID}$ ratio where $Reference_{Standard}$ and $References_{SERID}$ are the control reference plasma values obtained by the standard and SERID assay methods, respectively. The averaged paired apoprotein values (n = 3) for the two methods are plotted against each other and the standard error for each technique is indicated by bars (where absent the error was too small to indicate). Least square linear regression analysis was used to calculate the line of best fit and resulted in a correlation coefficient of 0.996.

search apoA-I RID assays found both positively and negatively biased values (3). They concluded that discordant values can result from compositional differences in the RID gel and sample diluent, and in the formulation of the reference calibrator.

The SERID assay was further tested for its sensitivity to determine diet-induced plasma changes in apoC levels. Our previous study suggested that an atherogenic diet high in fat and cholesterol composition causes a reduction in lipoprotein apoC content (12). In that study the apoprotein levels were measured by gel densitometry, which required very precise sample manipulation and a chromogenicity correction for each Coomassie blue-stained apoprotein. The low level and the poor sensitivity of Commassie blue-staining contributed to imprecision in the measurement of apoC. These factors did not allow us to show statistically significant apoC reductions by the diet. In the current study, the SERID method was used to compare apoprotein levels in fasting plasmas of mice fed chow or the atherogenic diet for 4 weeks. SERID measurements readily detected plasma level changes for apoA-II ($P \leq 0.005$) and apoE ($P \leq 0.005$) (**Table 2**). These results corroborated our previous findings which showed that the atherogenic diet consumption caused a reduction in apoA-II and an elevation in apoE. In addition, plasma apoC levels in the female C57BL/6 mice were significantly reduced ($P \leq 0.005$). Thus, due to the increased precision and sensitivity of the SERID assay, we were able to discern changes of 1.6 mg/dl (5.6 ± 0.3 to 4.0 ± 0.1 mg/dl) in plasma apoC levels.

TABLE 2. Plasma apolipoprotein levels in mice fed chow[a] and atherogenic[b] diets

Apolipoprotein	Chow[c]	Atherogenic[d]	Significance[e]
		mg/dl ± SE	
A-II	70.4 ± 2.3	53.4 ± 1.8	$P \leq 0.005$
C	5.6 ± 0.3	4.0 ± 0.1	$P \leq 0.005$
E	5.1 ± 0.1	14.7 ± 1.0	$P \leq 0.005$

[a]Five female C57BL/6 mice were fed normal chow (Purina) containing 4% fat for 4 weeks.
[b]Fasted plasma from five C57BL/6 mice fed an atherogenic diet (ref. 26) containing 0.5% cholic acid, 1.25% cholesterol, and 15% fat for 4 weeks.
[c]n = 3-4 (data from Table 1).
[d]n = 4.
[e]Student's *t*-test.

In conclusion, this study describes the general use of silver-staining in the development of a sensitive, nonradiometric immunoassay. Like the standard method, the SERID method retains the operational simplicity, uses readily available reagents, does not require sophisticated or costly instrumentation, and avoids the use of highly toxic or radiochemicals. The SERID method, which provides nanogram sensitivity and lowered antibody consumption, should promote a renewed interest for the development of radial immunodiffusion assays.

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