the two competitive assays were quite significantly correlated ($r = 0.630$, $P = 0.0029$). Our studies with affinity chromatography-purified anti-ox-LDL antibodies have shown significant cross-reactivity with MDA-LDL, and the two assays apparently detect overlapping populations of antibodies to modified LDL.

In conclusion, the main problem with using differential assays to compare reactivities with MDA-LDL or with ox-LDL with reactivities with native LDL is their lack of specificity. The results reflect a combination of specific antibody binding and nonspecific interactions, in proportions that appear to vary from sample to sample. The competitive assays are likely to be biased in favor of antibodies with a relatively higher affinity, and as such may underestimate the real antibody concentration, but they have the significant advantage of measuring a better defined analyte.

This research was supported in part by the Research Service of the Ralph H. Johnson Department of Veteran Affairs Medical Center, by the State of South Carolina Appropriation for Biomedical Research, and by Grant HL46815 from the National Institutes of Health.

References

Gabriel Virella
Marina Mironova
Dept. of Microbiol. and Immunol.
Med. University of South Carolina
Charleston, SC

Maria F. Lopes-Virella$^{1,2}$

Ralph H. Johnson VA Med. Center
109 Bee St.
Charleston, SC 29403
and $^1$ Dept. of Med.
Div. of Endocrinol.
Med. University of South Carolina
Charleston, SC

$^2$ Address correspondence to this author at the Ralph H. Johnson VA Med. Center.

Modification of NCCLS EP10 to Include Interference Screening

To the Editor:

The National Committee for Clinical Laboratory Standards (NCCLS) protocol EP10 efficiently estimates five parameters each time it is run (1). We wished to increase the number of parameters estimated with EP10 to include detection of interferences. A preliminary account of this work appeared as an abstract (2); our approach is an outgrowth of previous work (3).

EP10 includes assay of three replicates of three concentration values. The nine-sample sequence is: high, low, mid, low, low, high, high, mid. (For this paper, we exclude primers from any discussion relating to sample numbering.) In addition to the intercept, a series of independent variables are constructed: concentration, carryover, drift, and nonlinearity. Multiple regression is used, with the assay response data serving as the dependent variable. The statistical significance of each parameter estimate is determined. The rationale for the sample ordering and the properties of the design are explained by Daniel (4).

To explain how the modification of EP10 (EP10$_3$) works, we first consider the effect of introducing an interfering substance in the EP10 design. In that design, the mid level is repeated at sample positions 3, 4, and 9. Assume that an interfering substance is added to the fourth sample, producing a deviation of the fourth value from its expected value. In the standard EP10 analysis, this will produce a "large" residual for sample 4. This large residual is a signal that the EP10 model cannot account for this interference—which is reasonable because the terms in EP10 do not include anything to estimate interference for the mid level at sample number 4. A standard way to handle this problem is to construct a dummy variable for the fourth sample (5). The merit of this approach is that, if the candidate interfering substance does interfere, it can be estimated directly in the regression.

In our method, to test for interferences, we add interfering substances A–C to samples 4, 5, and 8. Users can decide how best to add interfering substances. For example, one could use the same concentration of the same interfering substance at positions 4, 5, and 8, or different concentrations corresponding to different interfering substances, or other combinations. As in EP10, we recommend multiple runs (at least five), since only very large effects will be detected as significant in one run.

To estimate parameters, we need to add appropriate columns to the design matrix. This is done by creating dummy variables for interferences A, B, and C. To create a dummy variable D4 for interference A, set D4 equal to 1 for sample 4, and 0 otherwise. This is repeated for the other two interfering substances, where interference B is at sample 5 and interference C at sample 8. These columns are added to the usual columns required by EP10, although we use actual reference values for concentration and carryover, and concentration squared for nonlinearity rather than coded values.

To obtain estimates, we perform the following regression: $y = \beta_0 + \beta_1 \text{slope} + \beta_2 \text{nonlin} + \beta_3 \text{carry} + \beta_4 \text{drift} + \beta_5 \text{D5} + \beta_6 \text{D6}$. Percent carryover is expressed as $(\beta_3/\beta_2) \times 100$ (4).

We illustrate the method with an example involving simulated cholesterol data. The assay was given the following values: concentrations = 2.59, 6.47, and 10.34 mmol/L; imprecision = 0.05 mmol/L; linear drift = $-0.0078$ mmol/L per sample; nonlinearity and sample carryover effects = 0; intercept = 0; slope = 1.00; and three interfering substances (A–C) with effects of $A = 0.13$ mmol/L, $B = 0.13$ mmol/L, and $C = 0$ mmol/L. The data were simulated by using SAS with deterministic effects added first,
followed by imprecision from a normal distribution.

The EP10 design recommends five runs. Table 1 shows the mean of 10 sets of five runs, as well as the overall average of the same 50 runs. To assess statistical significance for multiple runs, we used the parameter estimates for data as a t-test. For example, for interference A, a set of five runs produced five estimates of the interference due to A. These estimates were then averaged and a two-sided t-test was performed with the hypothesis that the effect of interference A = 0. Similar tests were performed for all parameters, except slope, where the hypothesis is slope = 1.

In Table 1 the parameter estimates are the same; however, the significance and the 95% confidence intervals are not. For five runs, interferences A and B are borderline significant, and drift (−0.07 mmol/L across nine samples) is not significant. This analysis shows that for the cholesterol case chosen, multiple runs are required to reliably assess performance. For this case, or any other assay, one could estimate the number of runs required on the basis of the within-run standard deviation and on the desired detection magnitude of each parameter (6).

The protocol described here is intended as a screening design, which is appropriate when one can assume a sparsity of effects (7). In one run, very large effects for up to eight different parameters will be detected. If no effects are statistically significant, however, one has not proved that effects do not exist. This key point is often not understood. In fact, this mistake can be found in EP10, where nonlinearity was concluded not to be an effect for the blood urea nitrogen (BUN) data used as an example. This misinterpretation happened because in each of five runs nonlinearity was not statistically significant. By the procedure recommended here—taking the five nonlinearity estimates, and treating them as data for a t-test—the result is t = −8.3 with P < 0.0001; i.e., nonlinearity has been detected. A simple plot of the EP10 data would confirm the nonlinearity. Thus, we recommend analyzing multiple runs for multifactor protocols by simply performing the t-test as described.

The addition of three extra terms to the model has added imprecision to the list of original parameters estimated in EP10. The magnitude of the increase in imprecision can be assessed by calculating the ratio of the variance for the terms common to both EP10 and EP10a, where the variance of an estimate equals the variance of the observations times the corresponding diagonal element of the [X'X]⁻¹, where X is the design matrix. This computation resulted in the following variance inflations: intercept 1.4, slope 1.7, nonlinearity 1.7, carryover 1.3, and drift 1.3.

Of the 27 possible combinations of three locations to add interferences (where each concentration level is added only once), the choice of samples 4, 5, and 8 inflated the variance the least. Given that samples 4, 5, and 8 will be altered, one must decide which interference to use to add to which sample. For example, interferences A, B, and C can be combined six ways with positions 4, 5, and 8 (one is 4A, 5B, 8C; a second is 4B, 5A, 8C; etc.) Each combination is identical with respect to the variance of the parameter estimates.

In a previous paper (3) we recommended a different calculation procedure involving the residuals of dummy variables. We now recommend using dummy variables as described here as a much simpler calculation procedure, with only one regression and no need to center design variables. No additional calculations are needed to transform the slope and intercept to their expected values of one and zero.

We have written Windows-based software that performs all calculations (supplied on request to address below or by E-mail to krouwer@cibadiag.com).

References

Jan S. Krouwer1
Katherine L. Monti
Ciba Corning Diagnostics Corp.
63 North St.
Medfield, MA 02052

1 Author for correspondence.

Circadian Rhythms of Lipoproteins

To the Editor:

The report by Rivera-Coll et al. (1) presents interesting aspects about the chronobiology of lipoproteins. Given the establishment by several