Letter to the Editor

Correspondence re: C. Chen, K. E. Malone, J. Prunty, and J. R. Daling, Measurement of Urinary Estrogen Metabolites Using a Monoclonal Enzyme-linked Immunoassay Kit: Assay Performance and Feasibility for Epidemiological Studies

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Before dealing with specific issues raised by the results reported in this paper (1), it is important to note that the authors' view that the new assay kit is only a variant of the kit they studied is totally incorrect. As part of improving sensitivity and ease of manufacture, a different methodology is employed in the new kits. In the kit type they used, the wells in the ELISA plates are coated with antibodies specific for the 2 or for the 16 antibody, which is then dried and shipped as such. After being activated, the hydrolyzed unknowns to be analyzed and the conjugated ligands are sequentially added to the wells. This is a time-variant system that requires a rigid protocol and experience to achieve satisfactory results. Because aging of the specific antibodies attached to the plates is possible, we have never used these kits for more than 2 weeks, nor was any time guarantee ever offered by the manufacturer. This kit model is no longer available.

The new kit uses a modified methodology, in which the wells are initially coated with a proprietary reagent to which the specific antibodies will bind. The hydrolyzed unknowns, a specific conjugated ligand, and the corresponding specific antibody are then added to these wells, resulting in a time-independent system. Specific assays are carried out for each of the metabolites. During the incubation period, the specific antibodies bind to the plates, and the unknowns and the conjugated ligands compete in binding to the specific antibodies. This results is tighter triplicate values and better inter- and intra-assay CV[2] values (Tables 1 and 2). In addition, because the sensitivity has been extended to 0.625 ng/ml, the assay of postmenopausal urines with a satisfactory degree of accuracy is now possible at a level that was not possible with the old kit, which could not measure samples below 2 ng/ml satisfactorily. Although the upper end of the range is now only 20 ng/ml, premenopausal urines can be measured readily at an appropriate dilution. It should be noted that concentrated samples need to be diluted until the values are in the linear part of the sigmoid curve.

Although the results with the new and the old kits are comparable in premenopausal samples, the results on postmenopausal samples are clearly superior with the new kit.[3] Similar findings have also been observed by Dr. Giske Ursin at the University of Southern California.[4]

Table 1 Within- and between-assay CV with the old and new assays

Within-assay variation (old assay)	7.8% (n = 72)
Within-assay variation (new assay)	7.6% (n = 72)
Between-assay variation (old assay)	8.1% (n = 35)
Between-assay variation (new assay; low control)	11.8% (n = 15)
Between-assay variation (new assay; medium control)	13.0% (n = 123)

Table 2 Stability of measurements over time

Correlation	Number	Mean[2]	Intraclass correlation
Duplicate urine samples within individuals collected at baseline			
Measured at baseline	24	0.43	0.95
Measured 6 months later	23	0.145	0.81
Duplicate urine samples within individuals collected at baseline and 6 months later			
Measured at baseline and 6 months later	20	0.27	0.93
Comparison of baseline and 6-month samples	171	0.39	0.67

Although we cannot comment on the aging of kits, because we made it a policy to use kits for no more than 2 weeks, we have not found a secular change or other changes in assay values with different lots of the older assay kits. Our Levy-Jennings plots (Figs. 1 and 2) show the stability of our own standards over multiple kit lots to be satisfactory and that the variance was much lower than the values reported by these authors. In the analysis of samples from more than 2000 women, primarily carried out with samples supplied by other investigators who routinely included 10% of blind duplicates, we obtained good replication.

Serial repeats of the same set of National Cancer Institute-supplied samples in different random orders at monthly intervals using different kit lots showed good agreement in premenopausal samples, which also correlated well with the gas chromatography-mass spectroscopy measurements of Dr. Adlercreutz.[5] Postmenopausal samples in this set showed greater variance with the old kit model, which was one of the reasons for turning to the new kit design. The new kit also

Received 6/13/96; revised 10/4/96; accepted 10/13/96.

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[2] The abbreviation used is: CV, coefficient of variation.

[3] R. Zeigler, manuscript in preparation.

[4] G. Ursin, personal communication.

[5] H. Adlercreutz, personal communication.

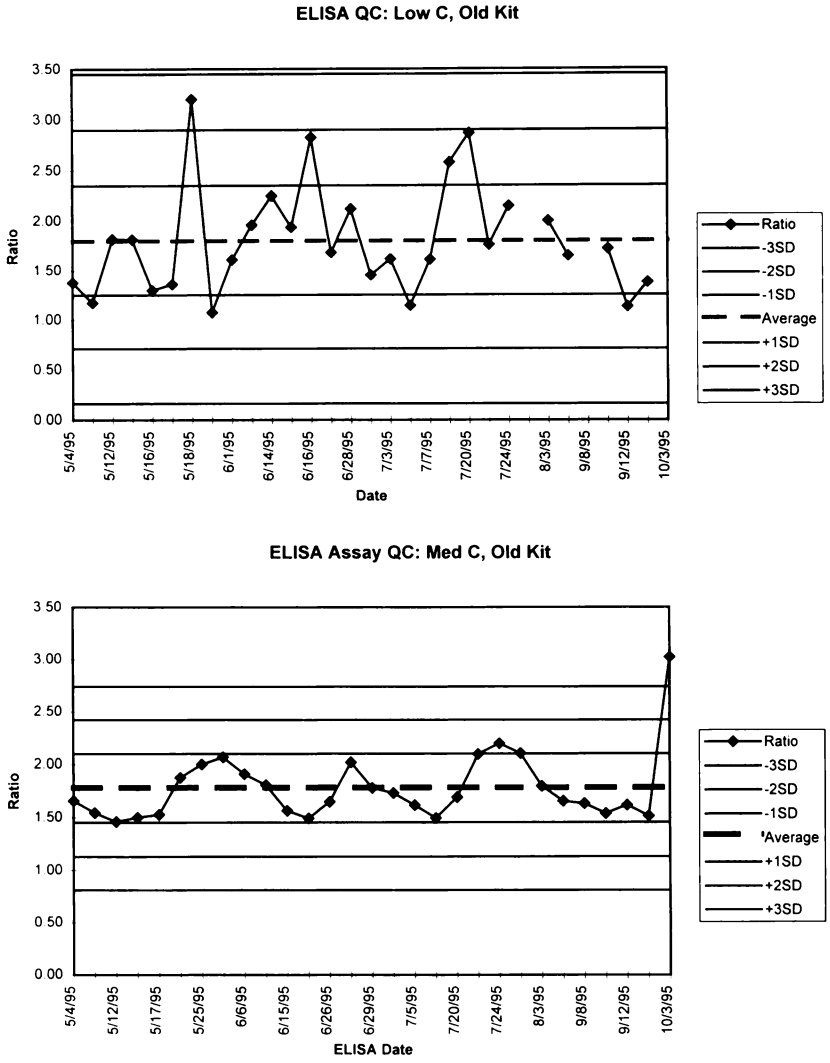

Fig. 1. ELISA assay quality control Levy-Jennings charts. Control sample values plotted against time for low- and medium-level samples. All of the sample values are the ratio of 2OHE1:16αOHE1.

minimized the number of samples that could not be assayed because their levels were below the assay limits.

The only limitation that we have found with this assay is that boric acid, which is occasionally used as a urine preservative at higher concentrations, tends to lower both values (2). We have also found that increasing sample size beyond 20 μl does not result in a linear increase in the values.

When the postmenopausal samples were analyzed with the new kit, very satisfactory results were obtained that correlated well with the gas chromatography-mass spectroscopy values obtained by Dr. Adlercreutz.[5]

Overall, the results that we have obtained bear no resemblance to the results Chen *et al.* (1) have reported in their paper, even though we are both using plates from the same manufacturing lots. Specifically, in the time frame in which they reported highly irregular values, we obtained satisfactory results based on reproducibility and our Levy-Jennings plots. We also had good reproducibility from kit to kit, as illustrated by our inter-assay CV values. Contrary to their claims, the assay is adaptable to epidemiological studies, which we are currently carrying out.

In one study using sample supplied by Pasagian-Macauley *et al.*, close agreement was observed in samples measured 6 months later (3).

It should also be noted that, using the same kits, Dr. Kim Westerlind at the AMC Cancer Center at Denver also found no diurnal variation in the ratio measurements using morning spot urine *versus* spot samples at intervals during the day *versus* 24-hr collections.[6] In her patients, she also found no monthly variation in the ratio values.

Finally, in two case control studies in which 10–15% of the samples had blind duplicates, close agreement was found in independent measurements of the two samples. In both studies, a decrease in 2-hydroxylation in cases was observed.[7,8]

For anyone interested in exploring this method, we would be happy to carry out cross-matching of samples and

[6] K. Westerlind, personal communication.

[7] M. M. Crane, D. W. Sepkovic, N. K. Robertson, A. Lopez, D. W. Blackhurst, R. P. Sticca, A. L. Coker, and H. L. Bradlow. Association of estrogen urinary metabolites and breast cancer: pilot study, submitted for publication.

[8] G. C. Kabot, C. J. Chang, J. A. Sparano, D. W. Sepkovic, X. P. Hu, A. Khalil, R. Rosenblatt, and H. L. Bradlow. Urinary estrogen metabolites and breast cancer: a case-control study, submitted for publication.

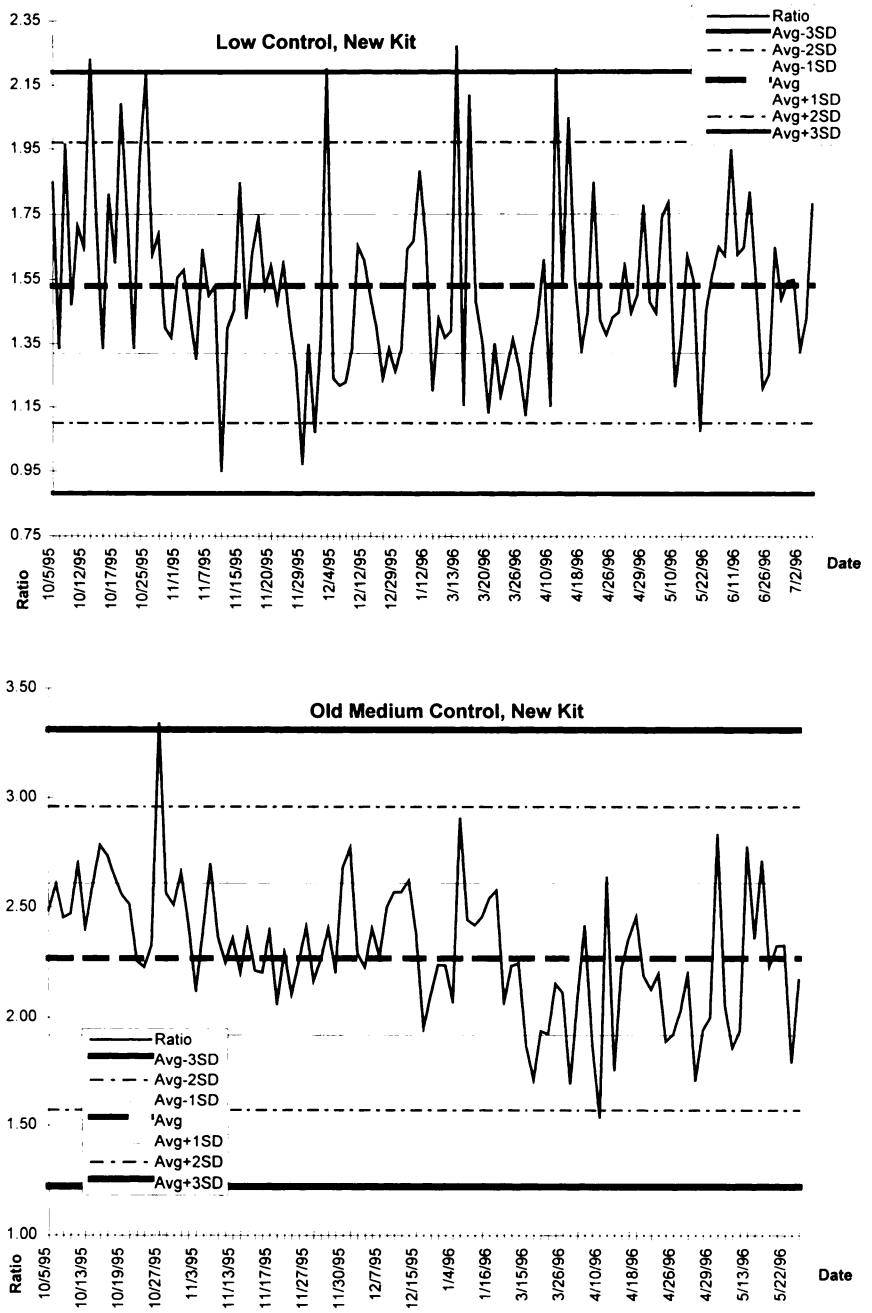

Fig. 2. ELISA assay quality control Levy-Jennings charts. Control sample values plotted against time for low- and medium-level samples. All of the sample values are the ratio of 2OHE1:16αOHE1.

results, which is the final test of any assay. Attempts to carry out such cross-matching with this group were not successful. We have successfully trained new college graduates to carry out the assay in a 2-week period and to obtain satisfactory results.

References

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3. Pasagian-Macauley, A., Meilahn, E. N., Bradlow, H. L., Sepkovic, D. W., Buhari, A. M., Simlin-Silverman, L., Wing, R. R., and Kuller, L. H. Urinary markers of estrogen metabolism 2- and 16α-hydroxylation in premenopausal women. Steroids, *61:* 461–467, 1996.