

Bimodal Population or Pathologist Artifact?

TO THE EDITOR: In a recent Comments and Controversies article Dr Schnitt¹ makes a series of observations regarding the variability and lack of reproducibility in the common methods for assessment of estrogen receptor (ER) expression in the current clinical practice in breast cancer. Although he compares the value of immunohistochemistry favorably to radio ligand binding assays (LBA), he notes that the continuity of expression seen in the LBA has generally not been reproduced in the largest population-based studies.^{2,3} Schnitt then asks whether this is a biologic phenomenon that should alert us to be more attentive in our selection of endocrine therapy, or whether the fault is in the IHC assay. He goes on to cite a number of articles that illustrate substantial nonreproducibility in IHC ER assays with particular emphasis on work with variable sensitivity. In particular, he cites the work of Umemura et al⁴ showing good correlation between a low sensitivity IHC assay with the biochemical assays, but the correlation was diminished with a highly sensitive IHC test.

We share Schnitt's concern regarding the IHC test and have recently reported the potential for artifacts when using assays with dynamic range that is insufficient for the protein being measured.⁵ However, techniques are available that provide accurate and reproducible measurement of protein expression. AQUA (version 1.2; HistoRx, New Haven, CT) is a fluorescence-based method for quantitative assessment of in situ protein concentration with accuracy comparable to an enzyme-linked immunosorbent assay, without loss of spatial information.⁶ AQUA, combined with the use of standard curves produced from well-characterized cell lines prepared as tissue microarrays demonstrates both the reproducibility and dynamic range of ER expression (Fig 1). Then once the range is appreciated, large cohorts can be tested to address the bimodality question. In our cohort of 650 patients, scored by the Hscore method (intensity X area),⁷ we observed a bimodal pattern similar to the works cited by Schnitt (Fig 2). On the contrary, AQUA-based quantitative analysis of the same cohort (calculated as the average of five unique histospot AQUA scores) produces a continuous pattern similar to that seen in the biochemical assays (Fig 3).

Thus, we believe the noncontinuity or bimodality of ER expression may be an artifact of the subjective scoring process. The preanalytic factors or antibody-related issues raised in the Schnitt article as alternative explanations for the variability are also present in our cohort, but these problems do not appear to alter data reproducibility (average *r* value between redundancies, *r* = 0.822). Furthermore, our previous studies have shown that the human eye is worst in the low intensity range, where scores show the greatest variability with respect to quantitative assessment.⁸ Therefore, we suggest that a quantitative approach to analysis, with reproducible standard curve controls incorporated in each test, could provide the advantages of the LBA where

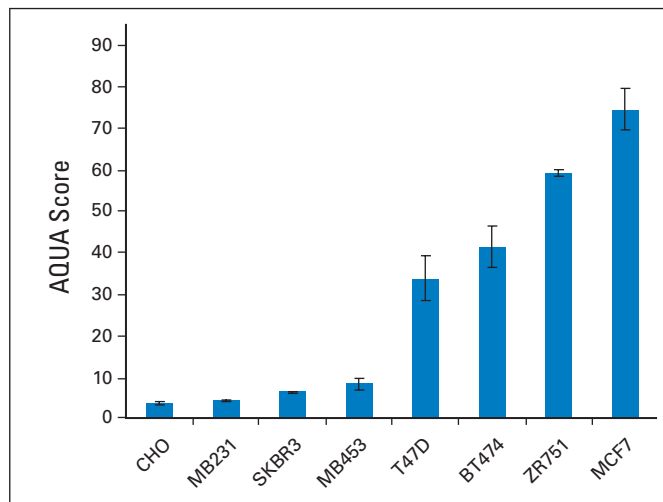


Fig 1. Cell lines that are grown, then formalin fixed and paraffin embedded into tissue microarrays are used as standard controls. Each read is a four-fold redundancy the average score is shown with error bars showing the SE of the mean. The choice of cell lines was suggested by work by DeFazio et al.⁹ Although the exact concentration for each line has not yet been determined, AQUA (version 1.2; HistoRx, New Haven, CT) scores have been shown to be linear with concentration in previous works.^{5,10}

the magnitude of benefit was proportional to the quantity of ER in the tumor, but also the advantages of IHC, where the test can be done on a very small amount of tissue.

Even if we could count every molecule accurately every time, does it matter? Schnitt suggests that it does and that the LBA data and limited data with IHC suggest we should attempt to do so. But given the limitations of both assays, can that be done? We would argue that the data herein, along with reproducible standard curves using cell

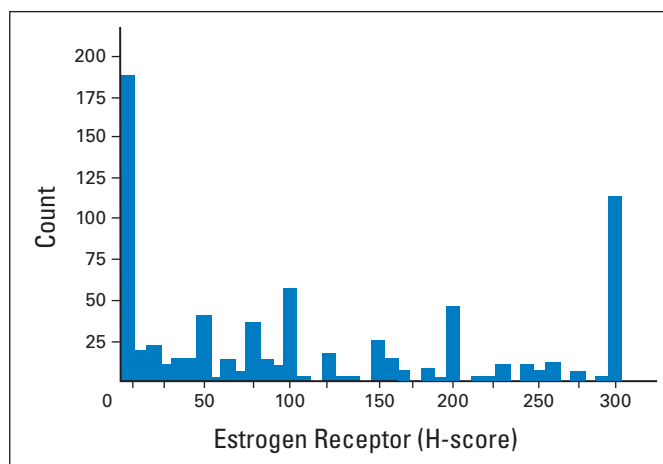


Fig 2. The frequency distribution of H-scores from a cohort of 651 patients which were used for the construction of the tissue microarrays for the quantitative analysis in Figure 3. More than one half of the patients fall into the category of 0 or 300. The distribution is clearly bimodal.

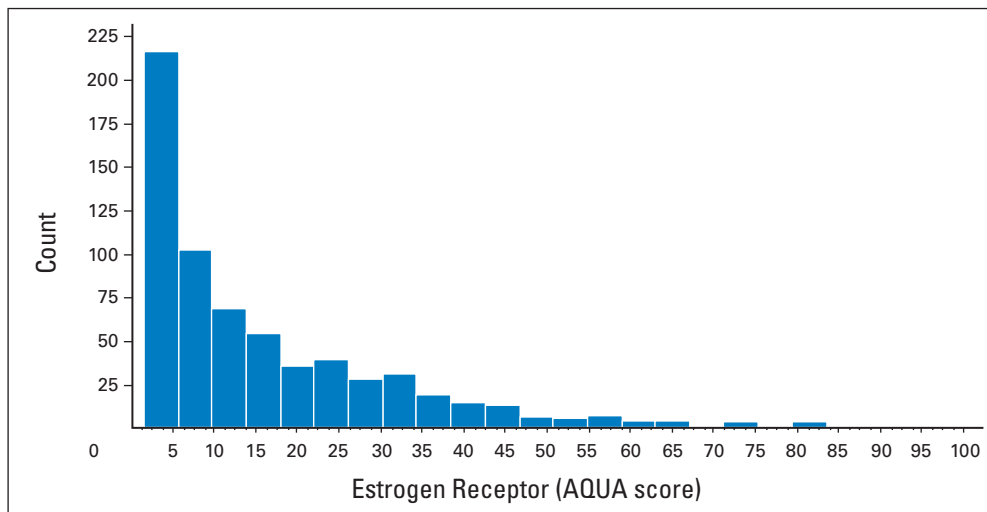


Fig 3. The frequency distribution by average AQUA (version 1.2; HistoRx, New Haven, CT) scores of estrogen receptor in five histospots from a cohort of 651 patients shows no evidence of bimodality.

lines should allow the best of both worlds—accurate quantification and analysis of tiny tissue specimens. However, our data are currently limited to disease-specific survival outcomes, which may not be predictive of response to therapy. We are in the process of looking at a series of ongoing prospective trials, including a Tamoxifen trial collected by Richard Love, a Tamoxifen trial conducted by the Southern and Southeastern Swedish Cancer Groups, in collaboration with Lisa Rydén, and National Surgical Adjuvant Breast and Bowel Project B-14, in collaboration with Soon Paik. When these studies are complete we may be able to provide a more definitive answer to Dr Schnitt.

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