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Comparing the Sensitivity of HER2 Epitope Detection of HercepTest mAb pharmDx (Dako Omnis, GE001) and Ventana PATHWAY Anti-HER-2/neu (4B5) Using IHC Calibrators

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Abstract: Accurate assessment of HER2 expression levels is paramount for determining eligibility for targeted therapies. HER2 immunohistochemistry provides a semiquantitative measurement of HER2 protein overexpression. Historically, little focus has been on the lower end of the HER2 expression range. The advent of novel therapeutic molecules that require fewer membrane epitopes to be effective has prompted a reevaluation of the current immunohistochemistry testing protocols, with special emphasis on the detection limit. Here, we have used Boston Cell Standards technology to determine the sensitivity of 2 commercially available HER2 immunohistochemistry assays, including a lower limit of detection.

Key Words: HER2, immunohistochemistry, sensitivity, lower limit of detection, pharmDx

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The assessment and therapeutic significance of human epidermal growth factor receptor 2 (HER2) in breast cancer have historically been based on a standard binary system, that classifies patients according to their immunohistochemistry (IHC) and in situ hybridization (ISH) results: HER2-negative (IHC0, IHC1+, and IHC2+/ISH-); and HER2-positive (IHC2+/ISH+ and IHC3+).[1] The recent approval of Trastuzumab-Deruxtecan, a third-generation antibody-drug conjugate (ADC) for HER2-low (IHC1+ and IHC2+/ISH-) breast cancer patients has highlighted the need to adapt current HER2 assessment, to integrate the clinical needs of this new category of patients.[2,3] Updated guidelines for HER2-low assessment and reporting have now been issued by both the American Society of Clinical Oncology—College of American Pathologists (ASCO-CAP), and the European Society for Medical Oncology—Expert Consensus. These new guidelines affirm the previous ASCO-CAP 2018 HER2 scoring algorithm and maintain consistency in the nomenclature used to report HER2, according to the results of approved IHC and ISH assays.[2,3] The proper treatment course which derives from the interpretation of the HER2 IHC scoring result into HER2 over expressed, HER2 low, and HER2 not over expressed, is determined by the oncologist.[1,4] Current HER2 IHC testing is based on semiquantitative assays, that were developed to detect HER2 overexpression (IHC2+/ISH+, IHC3+). The interest in the lower end of the HER2 expression spectrum considering new ADC approvals raises questions on the capability of these assays to effectively classify patients across the range of HER2 expression, and more importantly to discriminate between HER2-negative (IHC0), HER2-low (IHC1+, IHC2+/ISH-), and HER2-positive (IHC2+/ISH+ and IHC3+/ISH+).[5,6] These questions are clinically relevant. Some patients classified in the IHC0 category following the ASCO/CAP guidelines for HER2 scoring, show a faint to weak incomplete membrane staining in $<10\%$ of tumor cells, which is equivalent to a score of $<1+$ and could fit in the new HER2-ultralow category. Recent data suggests that these patients could equally benefit from T-DXd therapy.[6] Although the potential mechanism of action of these new ADCs in HER2 IHC0 is not yet well understood, some studies demonstrate a quantitative relationship between the uptake amount of the cytotoxic payload found in ADCs and the level of expression of the targeted antigen.[7,8] In vitro studies have demonstrated that threshold densities for efficient binding and internalization of HER2-targeted ADCs are between 50,000 to 200,000 HER2 receptor molecules on the cell membrane.[7,9] However, given multiple reports of a potentially beneficial response from T-DXd in some patients with IHC scores between 0 and 1+

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(0 < IHC < 1+), it is likely that the number of HER2 receptors necessary for therapeutic efficacy is lower.[6,10] From the perspective of a pathologist routinely performing HER2 scoring on breast cancer specimens, the fundamental question is how to accurately identify and differentiate "true" HER2-negative (IHC0) cases from "borderline" (0 < IHC < 1+) ultralow HER2-expressor patients, that have sufficient levels of HER2 protein necessary to achieve therapeutic efficacy.[10] The effectiveness of the HER2 assays available in the market to detect HER2 expression in the lower range is not the same, with multiple reports suggesting a higher sensitivity of some over others.[11,12] This variability arises from disparities in preanalytical procedures, antibody selection, staining conditions, and interpretation criteria, among other factors.

Addressing the need to establish a consensus-driven protocol that spans preanalytical procedures and analytical interpretation, Boston Cell Standards, a leading authority in cell-based assay standardization has developed an FDA approved,[13,14] harmonized HER2 IHC controls panel. These HER2 IHC reference standards not only integrate meticulous technical guidelines but also encompass a rigorously characterized set of reference materials. We conducted this study to clarify the effectiveness of 2 commercially available HER2 IHC assays to detect various levels of HER2 protein using standardized reference microbeads.

MATERIALS and METHODS

HER2 IHC Reference Calibrators

HER2 IHCalibrators slides were purchased from Boston Cell Standards (Boston, MA). The principle of IHCalibrators has been extensively described in previous publications.[13,15–17] These HER2 calibrators are provided as a microscope slide with 10 dots. Each dot comprises 2 different microbeads: analyte-coated glass test microbeads (7 to 8 μm in diameter) and color standard microbeads (4.5 μm in diameter). The different bead types are shown in Figure 1.

The HER2 analyte-coated microbeads are constituted of HER2 peptides that are 18 to 30 amino acids long, comprising the regions targeted by different anti-HER2 antibodies (GE001/CB11/4B5 and 1E3SP3 antibodies). Each peptide incorporates end-capped amino acids, that is, N-terminal acetylation and C-terminal amidation. The analyte-coated microbeads are present in 10 different coupling levels, each of which is placed on the IHCalibrators slide in a pellet of around 5000 analyte-coated microbeads, together with around 1500 color control beads. The coupling range was from $\sim 4\times10^4$ to $\sim 2\times10^6$ HER2 peptide epitopes/microbead on average (Table 1).[18] A single FITC molecule is present on each HER2 peptide and used to quantify the number of coupled peptides per microbead. The concentrations are traceable to a NIST SRM 1934 standard used to assign fluorescence intensity values to calibration microparticles.[15,19,20] Both types of microbeads are suspended in a proprietary clear liquid that hardens after application to the glass microscope slide, thereby retaining the microbeads on the glass slide during baking, deparaffinization, antigen retrieval, and staining. The manufacturer provides instructions on how to interpret IHC calibrators (Fig. 2).[18]

Immunohistochemistry

Two triplicate glass slides with IHC reference calibrators (from level 1 to level 10) were stained using 2

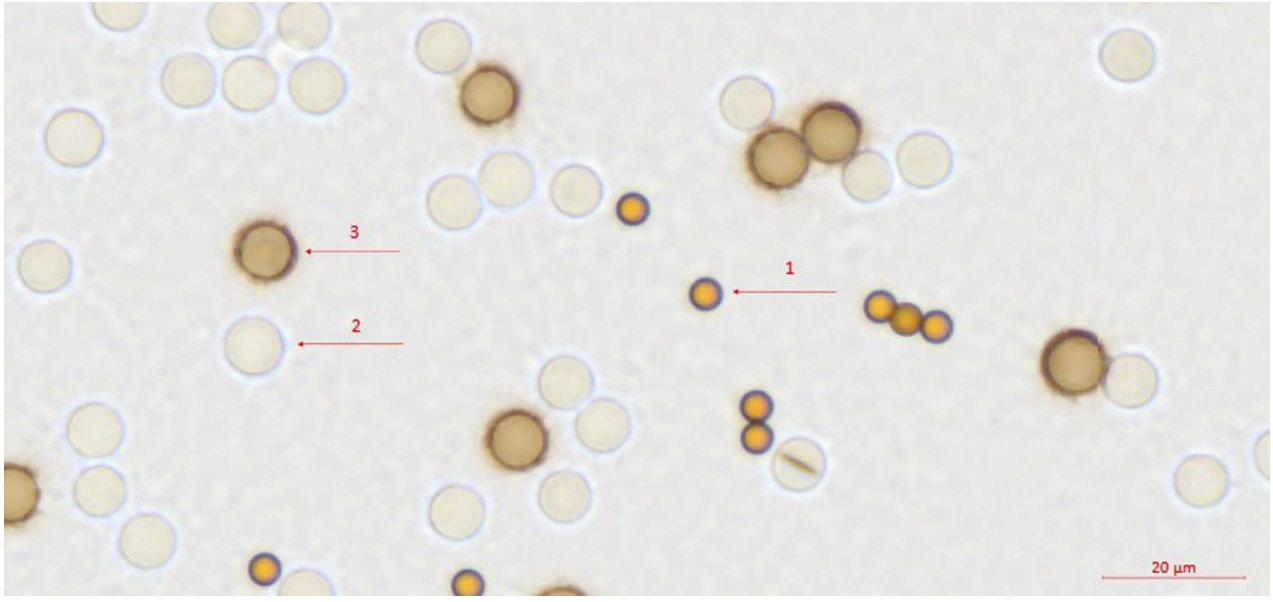

FIGURE 1. High-resolution image of HER2 IHCalibrators beads stained with HercepTest mAb PharmDx. The arrows depict color (1) standard color calibrator microbeads with a diameter of ~4.5 μm, (2) glass test microbeads negative for HER2, and (3) glass test microbeads positive for HER2. Both glass test microbeads have a diameter of ~7 to 8μm.

TABLE 1. IHCalibrators Product Concentrations (Molecules/microbead) as Reported in the Certificate Analysis of Each Lot

Pellet number	Average HER2 epitopes/microbead
1	39,279
2	65,486
3	110,912
4	208,754
5	379,976
6	631,568
7	1,096,314
8	1,396,827
9	1,711,317
10	1,749,755

commercially available HER2 assays. Triplicates were evaluated to account for relevant variation within each testing system. The first assay used was the HercepTest mAb pharmDx (Agilent Technologies Inc., Santa Clara, CA).[21] Staining was performed by Agilent R&D laboratories (Glostrup, Denmark) on the Dako Omnis platform (Agilent Technologies Inc.), using 3 separate stainer modules. The second assay was the PATHWAY anti-HER2/NEU, clone 4B5 (Ventana Medical Systems; Roche Diagnostics, Tucson, AZ).[22] Staining was performed by NordiQC (Aalborg, Denmark) on the Ventana BenchMark ULTRA (Ventana Medical Systems; Roche Diagnostics), using 3 different staining chambers. IHC staining using either assay was performed using a validated protocol as described by the manufacturer.[21,22] Since the IHCalibrators beads did not require hematoxylin as a counterstain, no counterstain was included in either of the protocols. Slides were not baked before staining.

Scanning and Digital Analysis

All IHCalibrators slides were scanned in 20X/0.8NA brightfield using a Zeiss Axioscan Z1 (Carl Zeiss, Oberkochen, Germany). A semantic segmentation network was trained on the Zeiss APEER platform,[23] to segment pixels into 3 classes: epitope-coated microbeads, control beads, and background by providing annotated examples of each class. The network was imported into Zeiss Zen 3.4 software (Carl Zeiss) and a custom analysis pipeline was set up, the objective of which was first to segment out IHCalibrators beads and control beads, followed by measurement of the greyscale intensity of both classes of beads. The training network is available from the authors upon request. The stain intensity was calculated as a ratio of the intensity of the epitope-coupled microbeads to the intensity of the color control beads as previously described.[15] The algorithm measures the image intensity of the test microbeads relative to an internal color intensity standard bead. Because of its smaller size, the color standard bead is easily distinguished from a test bead. Consequently, IHC reference calibrator stain intensity is expressed as a ratio. A score of 1.0 means that the test microbeads, stained for HER2 are equally intense in color as the color standard microbeads. Thus, the value of ≥ 1 is more intense than the color control beads.

Generation of Dose-Response Curves

Analysis for the results and graphs were performed and generated using SAS JMP 17.0 (SAS Institute Inc., Cary, NC). The dose-response curve of microbead intensity ratios against the log (10) HER2 concentration was fitted using a 3-parameter logistic model with the formula:

$$\text{Intensity ratio} = \frac{c}{1 + e^{-a(\log 10(\text{average HER2 per bead}) - b)}} \quad (1)$$

where c is the asymptote, a is the growth rate and b is the inflection point.

RESULTS

In Figure 3, representative images of stained beads at various HER2 levels are shown. There is a clear increase in staining intensity as the average number of

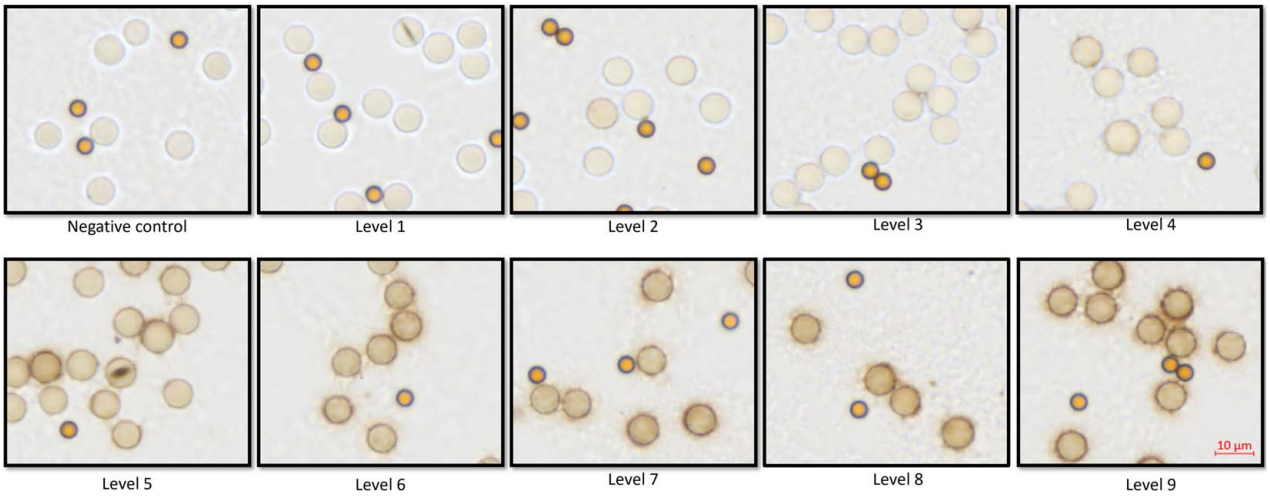

FIGURE 2. Highly magnified images of IHCalibrators levels 1 to 10 stained with HercepTest mAb PharmDx.

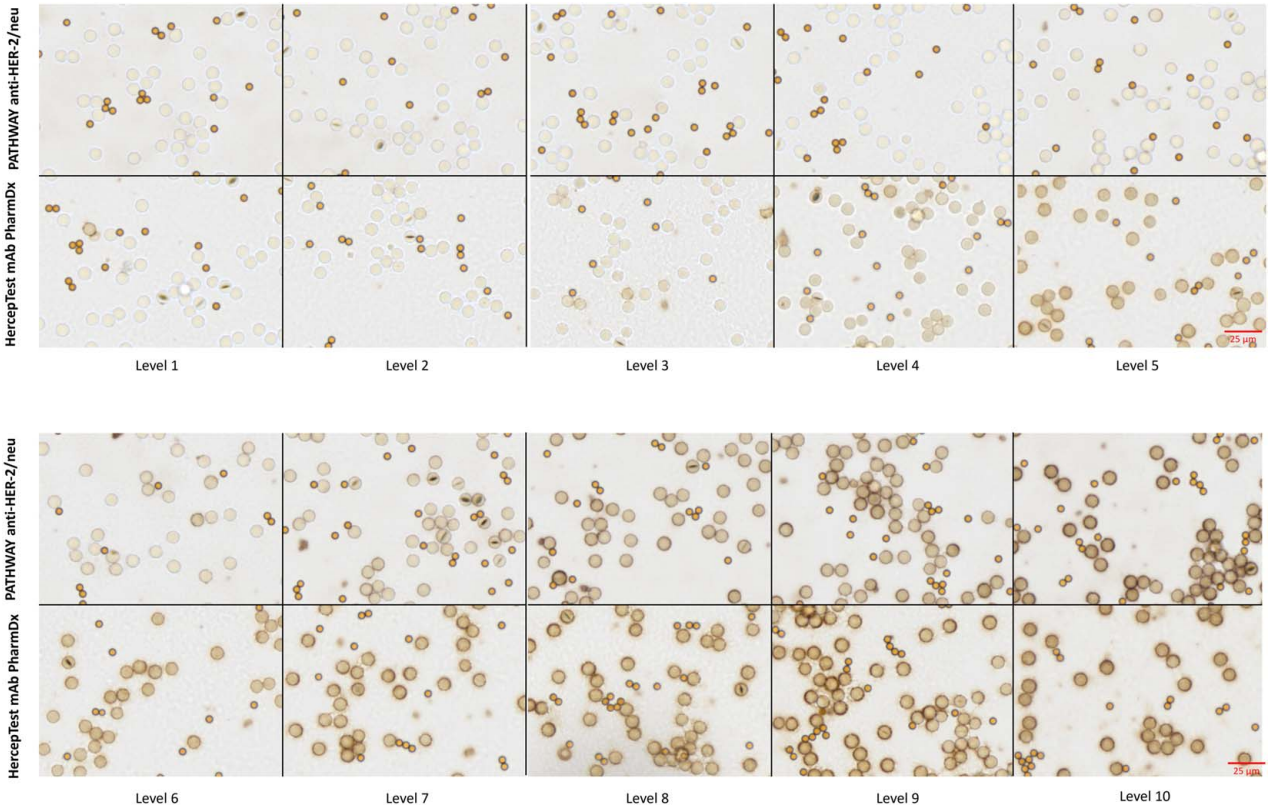

FIGURE 3. Images of each glass bead pellet from concentration level 1 to 10 stained with PATHWAY anti-HER2/neu and HercepTest mAb PharmDx, respectively.

HER2 epitopes per microbead increases. In Figure 4, the triplicate measurements of the intensity ratio for each assay are fitted against the IHC calibrator level.

Figure 5 shows the dose-response curve of log10 (average HER2/bead) versus the intensity ratio generated using a 3-parameter sigmoid model described in Table 2.

The detection limits of the 2 assays show noticeable differences. The response curve of PATHWAY anti-HER2/NEU shifts to the right concerning the response curve of HercepTest mAb PharmDx. While the limit of detection for

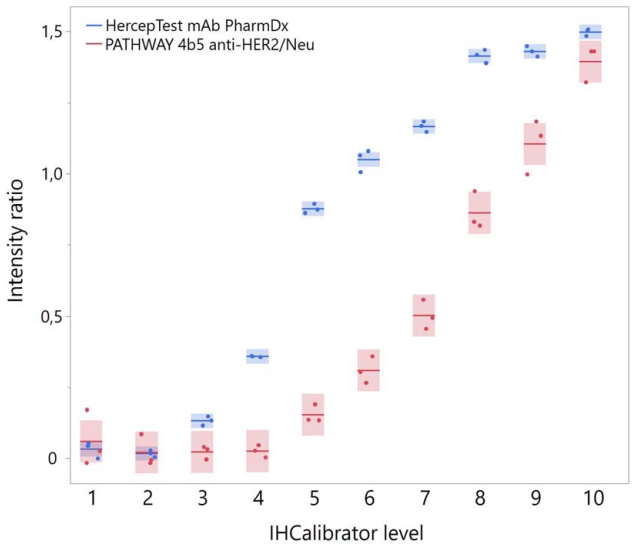

FIGURE 4. The intensity ratio is distributed in discrete IHCalibrators levels. The horizontal lines show the mean of triplicate measurement, and the shaded area depicts the 95% CI of the mean.

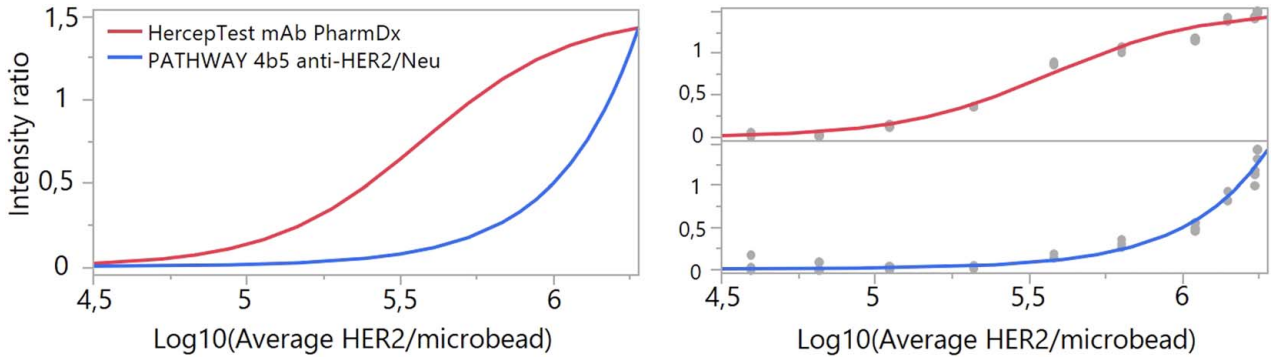

FIGURE 5. The 3-parameter logistic fit of intensity ratio against the log10 (average HER2/microbead) for both assay types (HercepTest mAb PharmDx in red, PATHWAY anti-HER2/NEU in blue).

PATHWAY anti-HER2/NEU was at level 5 (~380.000 HER2 epitopes per bead), it was at level 3 for HercepTest mAb PharmDx (~110.000 HER2 epitopes per bead). The dynamic range of HercepTest mAb PharmDx is broader than that of PATHWAY anti-HER2/NEU at the low range of the IHCalibrators levels, whereas PATHWAY anti-HER2/NEU has a broader dynamic range at the high end of IHCalibrators levels (from level 8 to 10, 1.3×10^6 to 1.7×10^6 HER2 epitopes/microbead). Tumor cells expressing above 1×10^6 HER2 epitopes per cell typically stain 3+,[24] indicating that the end of the dynamic range of HercepTest mAb PharmDx is set around the 3+ level.

While HercepTest mAb PharmDx flattens out at the higher end of the dose-response curve, the PATHWAY anti-HER2/NEU dose-response curve has not reached a point of saturation. This is seen graphically in Figure 5, but it is also clear from the CIs of the asymptote and inflection point parameters that it is not possible to accurately estimate these because of the lack of saturation. This has been observed previously with PATHWAY anti-HER2/NEU.[15,18]

DISCUSSION

In this study, we have compared the sensitivity of 2 widely used HER2 assays to assess their dynamic range of detection, using standard reference commercially available microbeads coated with various levels of HER2 proteins. Given the emergence of new ADC therapies targeting patients with HER2-low expression levels, it is imperative to explore the quantitative relationship between the level of HER2 target expression and assay results, to enhance accuracy in the classification of patients and their access to the most beneficial therapy. IHC interpretation of HER2 testing is based on 3 fundamental elements: the intensity of staining (absence, faint/barely perceptible, weak to moderate, intense); the appearance of the tumor cell membrane (absence, incomplete, complete); and the percentage of cells showing staining (0%, $\leq$10%, $>$10%).[1,4] The number of HER2 molecules/cell corresponding to each IHC grade has been estimated at fewer than 20,000 HER2 molecules/cell for IHC 0, ~100,000 HER2 molecules/cell for IHC1+; ~500,000 HER2 molecules/cell for IHC2+, and ~2,300,000 HER2 molecules/cell for IHC3+ (Table 3).[22]

Multiple authors have reported a direct correlation between the number of HER2 proteins/molecules and the quantity of antibodies binding those targets. Using FACS analysis, DeFazio-Eli et al[24] demonstrated no to minimal signal intensity in cells expressing ~1000 HER2 molecules, intermediate signal intensity in cells expressing ~50,000 HER2, and highest signal intensity in cells expressing $>$500,000 HER2. Using a similar approach, Osum et al[26] not only confirmed this relationship but demonstrated the correlation between the mean HER2 receptor numbers per cell and the HER2 scoring categories as defined by IHC (using HercepTest) and FISH. Our results demonstrated that HercepTest produces a distinct and demonstrable IHC staining of HER2 protein on the microbead at a lower concentration than PATHWAY (~380,000 HER2/cell vs. 110,000 HER2/cell). One could argue that our results because obtained in a well-controlled laboratory environment, cannot be representative of real-world laboratory experience and the impact of preanalytical variables when assessing the sensitivity of various assays in

TABLE 2. Description of the Estimate, Standard Error on the Estimates, and the Upper and Lower 95% Confidence Limits of the 3-parameter Sigmoid Model Used to Generate Dose-response Versus Intensity Ratio Curves

Parameter	Group	Estimate	SE	Lower 95%	Upper 95%
Growth rate	HercepTest mAb PharmDx	4.10	0.43	3.25	4.94
Inflection point	HercepTest mAb PharmDx	5.57	0.03	5.51	5.64
Asymptote	HercepTest mAb PharmDx	1.51	0.06	1.40	1.62
Growth rate	Pathway anti-HER2/NEU	3.81	0.93	1.98	5.63
Inflection point	Pathway anti-HER2/NEU	8.93	2955.97	−5784.67	5802.52
Asymptote	Pathway anti-HER2/NEU	34,894.55	3.93E+08	-7.70E+08	7.70E+08

TABLE 3. Correlation Between IHC Staining Intensity and Estimated Number of HER2 Molecules/Cell[25]

Intensity of staining	Appearance of the tumor cell membrane	% stained cells	IHC grade	Number of HER2 molecules/cell
Absence	Absence	0	0	< 20,000
Faint/barely perceptible	Incomplete	≤ 10		
		> 10	1+	~100,000
Weak to moderate	Complete	> 10	2+	~500,000
Intense			3+	~2,300,000

the lower ranges of HER2 expression. However, in a recent study to evaluate reproducibility across 42 laboratories using the same HER2 calibrator microbeads, HercepTest anti-HER2 monoclonal antibody showed a lower interlaboratory variation of the limit of detection as compared with PATHWAY (coefficient of variation of 5% vs. 55%, respectively). This was observed despite the need to consider the disparities in the number of laboratories utilizing each antibody.[27] While we cannot assume that the assay sensitivity level observed using microbeads artificially coated with HER epitopes will be similar if using actual patient specimens,[13] we can; however, speculate that the difference in sensitivity in the lower end of the HER2 expression spectrum can explain some of the discordances reported previously. Rüschoff et al[11] found complete concordance in only 69.7% of the cases (83/119) when comparing the performance of HercepTest and PATHWAY in preselected breast cancer samples covering the entire range of HER2 IHC expression scores.[11] For all discordant cases, a higher score was obtained by HercepTest as compared with PATHWAY. This difference was more noticeable in the lower end of HER2 expression, with HercepTest identifying a significantly larger proportion of HER2-low specimens (HER2 score 2+ or 1+ /not amplified) as compared with PATHWAY (35% vs. 19%, $P < 0.01$).

The phase 2 DAISY trial, to better understand the performance of T-DXd across the entire spectrum of HER2 expression levels, evaluated the efficacy of T-DXd in patients with HER2 overexpressing, HER2 low, and HER2 nonexpressing metastatic breast cancer using PATHWAY. In the final population analysis, an objective response rate (ORR) was achieved in 11/37 (29.7%) patients classified as HER2-negative (IHC0), as compared with 27/72 (37.5%) for HER2-low and 48/68 (70.6%) for HER2-positive.[10] The question of whether these patients identified as HER2-negative (IHC0), should have a different IHC rate by using other antibodies considering these results, remains unknown and needs further investigation. Correlation between the lower limit of detection of current assays and clinical outcomes of responder patients previously classified as HER2-negative will potentially provide more rationale for the decision to provide appropriate therapy for this group of patients in the future. Such a trial could objectively highlight the minimum cutoff required for maximal therapeutic benefits, as the HER2 expression threshold that predicts HER2-targeted ADC therapy efficacy is still underdetermined.

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