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# Technical Advance

## Analysis of *HER2* Gene Amplification Using an Automated Fluorescence *in Situ* Hybridization Signal Enumeration System

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**The *HER2* gene, amplified in 10 to 35% of invasive human breast carcinomas, has prognostic and therapeutic implications. Fluorescent *in situ* hybridization is one method currently used for assessing *HER2* status, but fluorescent *in situ* hybridization involves the time-consuming step of manual signal enumeration. To address this issue, Vysis has developed an automated signal enumeration system, Vysis AutoVysion. A multicenter, blinded study was conducted on 39 formalin-fixed, paraffin-embedded invasive breast carcinoma specimens, including 20 *HER2* nonamplified and 19 *HER2* amplified (weakly to highly amplified), provided in duplicate to each study site for analysis. Calculation of the *HER2*/CEP17 ratio and the hands-on time of both manual and automated enumeration approaches were compared. Overall agreement of *HER2* classification results (positive and negative) was 92.5% (196 of 212). The Vysis AutoVysion System requires manual enumeration for cases with scanner results within the ratio range of 1.5 to 3.0. When the data in this range are excluded, the agreement between manual and scanner results is 98.8% (169 of 171). The average Vysis AutoVysion System hands-on time per slide was 4.59 versus 7.47 minutes for manual signal enumeration (savings of 2.88 minutes/slide). These data suggest that the Vysis AutoVysion System can correctly classify specimens and may increase the overall efficiency of *HER2* testing.** *(J Mol Diagn 2007, 9:144–150; DOI: 10.2353/jmoldx.2007.060102)*

The *HER2* gene (*ERBB2*) is located on chromosome 17 (q11.2-q12) and encodes a 185-kd transmembrane glycoprotein with intracellular tyrosine kinase activity, which is closely related to the epidermal growth factor receptor.[1–6] Overexpression of the *HER2* oncogene seems to stimulate growth and cellular motility and has been implicated in several malignancies.[4,6,7]

Approximately 10 to 35% of invasive human breast carcinomas are associated with HER2 protein overexpression.[2,6,8] HER2 overexpression is considered an unfavorable prognostic factor and is associated with better response rates to trastuzumab (Herceptin) therapy and anthracycline-based chemotherapy regimens.[9] Most breast carcinomas that overexpress HER2 are invasive ductal adenocarcinomas (95.5%) and are high-grade tumors [histopathological grades 2 (28%) or 3 (69%)]. Overexpression of HER2 is rare in invasive lobular carcinoma (0.8%) and other specialized types of breast carcinomas.[8,10]

Studies have shown that the most common mechanism (90 to 96%) of HER2 overexpression is gene amplification.[2,9,11,12] Widely used approaches to assess *HER2* status include immunohistochemistry (IHC) to determine protein expression and fluorescent *in situ* hybridization (FISH) to determine *HER2* gene copy number.[4,11,13–15] Reportedly, FISH has shown greater interlaboratory concordance and has been found to be a better predictor of response to trastuzumab (Herceptin) therapy, as compared with IHC.[1,11,14,16–21] One drawback to the FISH assay is the time-consuming process of manual signal enumeration. In an effort to determine whether the efficiency of FISH *HER2* gene copy num-

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ber analysis could be improved, Vysis (Downers Grove, IL) developed an automated signal enumeration system (Vysis AutoVysion System), which uses a scanner, a computer with scanning and analysis software, and an automated fluorescence microscope with a motorized stage. The goal of this study was to validate the use of an automated signal enumeration system in the *HER2* Vysis PathVysion FISH assay and to assess its utility in the laboratory setting.

## Materials and Methods

### Study Design

A blinded study was conducted at three separate sites, including the University of Nebraska Medical Center, Omaha, NE; Advocate Lutheran General Hospital, Park Ridge, IL; and the Mayo Clinic, Rochester, MN. Duplicate study slide sets of 39 breast cancer specimens and corresponding control slides were sent to the different sites for 10 scanning days. For standardization, Vysis performed the slide preparation including the hybridization of fluorescent probes before distribution to the different study sites. FISH assays were performed according to the Vysis PathVysion *HER2* DNA probe package insert and the VP2000/HYBrite procedure as specified by the manufacturer's instructions (PathVysion *HER2* DNA Probe Kit package insert. Vysis, 1998). Each set of slides was analyzed at the different study sites within 24 hours of receipt. Automated enumeration was performed first with the scanner (Vysis AutoVysion System) followed by manual enumeration, all within a single 8-hour time period. The *HER2* and CEP17 signal counts, *HER2*/CEP17 ratios, the time to produce a result, and the average hands-on time were calculated and recorded for each method.

### Specimen Selection

This study used formalin-fixed, paraffin-embedded human breast tissue specimens from 40 different invasive breast carcinomas (ductal and lobular) with varying degrees of *HER2* gene amplification as determined by Vysis (Abbott Laboratories Inc., Des Plaines, IL) using FISH. The specimens included 20 *HER2* nonamplified breast carcinomas and 20 *HER2* amplified breast carcinoma specimens including normal, weakly amplified (ratios, 2.0 to 3.0), moderately amplified (ratios, 3.0 to 6.0) and highly amplified (ratios, $>$6.0) specimens. One specimen within the group of amplified specimens consistently failed the Vysis PathVysion assay and was subsequently excluded from the study. Each slide was labeled with a one-way identifier. Each set of slides included an average of eight specimen slides (each with a corresponding hematoxylin and eosin slide) and two control slides (Vysis ProbeChek). The control slides consisted of 4- to 6-$\mu$m sections of pelleted paraffin-embedded cell lines exhibiting *HER2* to CEP17 ratios of $\sim$1.8 (cutoff control) and 1.0 (normal control). An independent pathologist identified and marked the area of invasive tumor on each slide using a diamond tip stylus. Each study site received randomly selected sets of 10 slides on separate study days. The 78 specimen slides analyzed at each site represented duplicate slides of each of the 39 different breast carcinoma specimens.

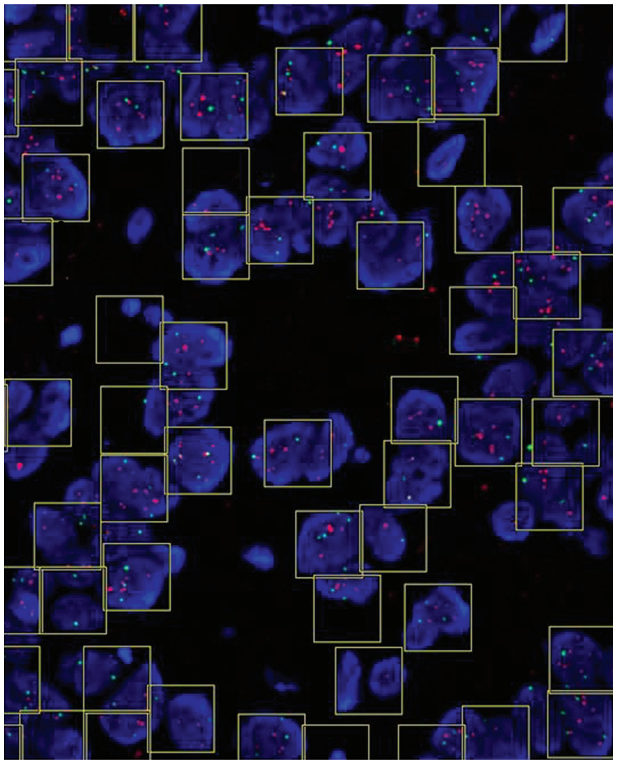

**Figure 1.** Software placement of nonoverlapping tiles (**yellow open squares**) in areas with DAPI fluorescence captures the majority of nuclei in each field of view.

### Scanner Software

The Vysis AutoVysion System utilizes software that addresses three potential concerns when enumerating FISH signals via an automated system. First, it is difficult for automated systems to reliably separate overlapping nuclei, which is commonly seen in tissue sections. Second, it is difficult for automated systems to consistently differentiate normal cell nuclei from tumor cell nuclei, especially when cell nuclei overlap. Third, tissue sections, although thin, are three-dimensional and often require focusing up and down through the tissue to determine accurate signal counts.

To overcome these issues, the prototypic Vysis AutoVysion System software uses a targeted tiles sampling method (Figure 1). In this method, each field of view in the area selected for analysis is sampled by placing a set of nonoverlapping square patches or tiles of equal size (approximately the size of a large tumor cell nucleus) on the image. The distribution of the tiles maximizes the 4′,6-diamidino-2-phenylindole (DAPI) fluorescence contained within each tile. This results in a set of tiles that cover the majority of the nuclear material in each field of view, while leaving the background areas unsampled. Each tile may include a single tumor nucleus, a single normal nucleus, or portions of one or more nuclei of either type. The system scans in three

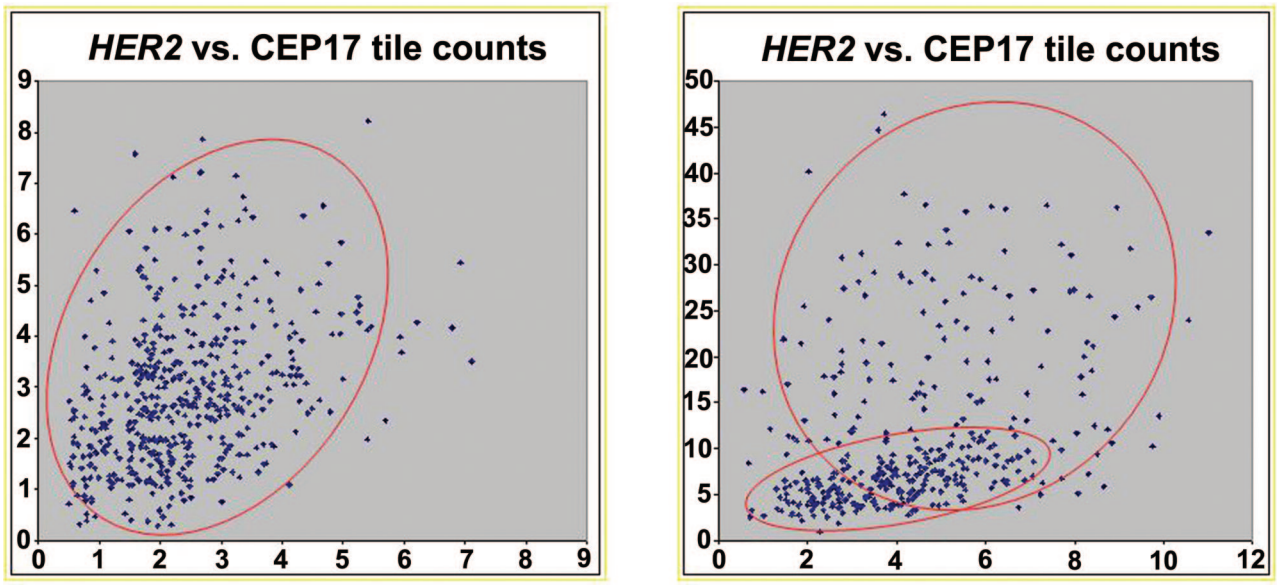


**Figure 2.** Two-dimensional per tile *HER2*/CEP17 spot count distributions. The *HER2*/CEP17 spot count distribution typical of a homogenous set of cells (all nonamplified or all amplified cells; left) contrasts with that of a mixed population of cells (nonamplified and amplified cells; right).

dimensions, including nine separate planes of focus (*Z* plane) through the thickness of the tissue, accounting for signals in multiple planes of sections. The software then determines the spot counts in each tile irrespective of whether the tile contains a whole nucleus, part of a nucleus, or multiple nuclei. Image files can be stored and reviewed at a later date if needed.

All tiles are analyzed and included in the ratio analysis unless they are determined to be of poor quality (not based on ratio calculation). A minimum of 34 high-quality tiles is needed to produce a valid scanner result (not determined from this study). Depending on the degree of heterogeneity (amplified versus nonamplified) of the cells in the scanned areas, different two-dimensional per tile *HER2*/CEP17 spot count distributions will be obtained (Figure 2). When the tiles sample a homogeneous set of cells, the spot count distributions will be unimodal. In contrast, if the tiles sample a mixed set of cells containing both amplified and nonamplified cells, a bimodal distribution, or at least a significantly skewed shape, will be seen. In the latter case, the spot counts of the amplified cells can be extracted using an expectation maximization algorithm producing a *HER2*/CEP17 ratio estimation that is not falsely lowered by the nonamplified cells. Expectation maximization algorithm, a well-known iterative procedure for solving distribution mixture problems, is used to fit a mixture of two distributions to the observed two-dimensional spot count distribution. It is able to exploit the constraint that one of the fitted distributions (representing the nonamplified component) must have an overall *HER2*/CEP17 ratio of 1. The goodness of fit of the two-distribution model can be compared with the goodness of fit of a single distribution, to ensure that truly homogeneous samples are not erroneously fitted by two separate distributions. The final *HER2*/CEP17 ratio is then obtained directly from the parameters of the fitted distribution(s). In cases in which homogeneous staining regions (cytogenetic phenomenon representing gene amplification) are encountered and individual signals cannot be counted, the software shifts to a different spot-counting algorithm, which measures the signal area (instead of individual spot counts). The measured signal areas are then converted to spot count numbers based on a predetermined conversion factor.

## *Signal Enumeration*

Automated signal enumeration was achieved using the Vysis AutoVysion System, which combines a scanner, an automated fluorescence microscope, a motorized scanning stage holding up to eight slides, a large-format charge-coupled device camera, and a computer with scanning and analysis software. The fluorescence microscope contains a mercury arc lamp for epi-illumination; three single-pass filter sets for DAPI, Spectrum Green, and Spectrum Orange and a triple-pass filter set for DAPI/Spectrum Green/Spectrum Orange mounted in a motorized filter turret; ×10 and ×40 objectives in a motorized objective turret; a ×10 eyepiece; and camera port. Individual slides or up to eight slides can be batched and analyzed in a given run. According to separate internal studies conducted solely by Vysis (data not derived from the current study), in serial analysis the system is ready for the next eight slides in ~85 minutes, and in batched analysis the system is ready for the next eight slides in ~35 minutes.

For the study, slides were loaded onto the motorized stage and scanned by the technologist in the previously marked tumor area. Ten distinct (nonoverlapping) fields of view with acceptable hybridization quality were selected by the technologist for automatic slide coordinate recording. The fields of view for all eight slides were selected before the system scanned any of the slides, only requiring technician input at the start of a run. These

preselected fields of view were subsequently analyzed by the system independent of the technologist. First, two control slides were used at the beginning of a run to confirm the system was producing results within the expected limits. If the system determined the *HER2*/CEP17 ratio was between 1.5 to 3.0, it notified the technician that the results were equivocal. In addition, if the system detected a large number of poor quality tiles, the entire sample was rejected.

After automated enumeration, each slide was manually scored within the same previously marked tumor area by two technologists, according to the manufacturer's instructions. These technologists were blinded to the automated system results. For each slide, the *HER2* and CEP17 signal counts were recorded for at least 20 nuclei and the average *HER2*/CEP17 ratio was calculated. Each technologist evaluated 20 additional nuclei if the *HER2*/CEP17 ratio was in the 1.8 to 2.2 range. The 1.8 to 2.2 range is the specified borderline range for reflex counting of additional nuclei by the PathVysion *HER2* DNA probe kit package insert when performing manual signal enumeration. The time to obtain a result for both the manual and automated methods was monitored with stopwatches with an accuracy of better than ±6 seconds.

### *Statistical Analysis*

The *HER2* and CEP17 signal counts, *HER2*/CEP17 ratios, the time to obtain a result and the average hands-on time were calculated and recorded for both the automated and manual approaches. The time to obtain a result and the average hands-on time for the manual enumeration method were both defined as the average elapsed time between the time analysis began and when a result was created. The time to obtain a result for the scanner enumeration method was defined as the average elapsed time between the time field of view selection began and the time a result was created. The average hands-on time for the scanner enumeration method was defined as the average time required for fields of view selection.

Only cases with informative results for both the manual and automated methods were included in the data analyses. Bias calculations were restricted to cases with duplicate informative results. The optimum number of fields of view to be analyzed for each sample was 10. One sample had poor cellularity, and only eight fields of view were obtained. This case still provided informative data, however, which were included in the results. *HER2* status assessment was conducted with respect to percentage of cases correctly classified (ie, positive or negative for *HER2* gene amplification as determined by manual signal enumeration).

All statistical analyses were performed by Vysis, Abbott Laboratories (Des Plaines, IL) as required by the Food and Drug Administration for 510K submission by Vysis. For quantitative analysis, the manufacturer's design specifications used the Clinical and Laboratory Standards Institute (CLSI) guideline EP9-A2 Section 6.2, "Method Comparison and Bias Estimation Using Patient Samples, Approved Guideline" as a guide for data analysis. Identification of within method outliers and average bias throughout a specified range were calculated in accordance with the CLSI document. The point value ±15% was considered acceptable error for evaluating bias.[22] In addition, a poolability analysis using Fischer's exact test was conducted on the manual enumeration results to determine whether the data collected across the three study sites could be pooled. An analysis of variance was performed on results from the automated enumeration method to determine whether there were significant differences among the means from the three sites and from day to day using Levene's test for homogeneity of variances.

## *Results*

A total of six slides (two slides from each case for each of the three study sites) from each of the initial 40 cases were prepared (total 240 slides). Of the initial slides, 20 slides, including all six slides from one case, failed to hybridize or produced no results. Of the 220 slides with results, bias analysis identified four within-method outliers (eight data points) and were removed from the data set (per CLSI guideline EP9-A2), for a total of 212 slides with informative results. Among specimens with informative results for both methods, classification of results (ie, positive or negative for *HER2* gene amplification) were concordant in 92.5% (196 of 212) of slides tested. Scanner and manual results were considered positive if the ratio was >2.0 and negative if <2.0. However, the manufacturer stipulates that manual rescoring be performed for scanner results in the 1.5 to 3.0 *HER2*/CEP17 ratio range. When the scanner data in this range and corresponding manual data for each slide are excluded (41 data points), the concordance rate between the automated and manual classification increases to 98.8% (169 of 171). The distribution of ratio results for each enumeration method within specified *HER2*/CEP17 ratio ranges is illustrated in Table 1. Positive agreement (slides with both scanner and manual results >2.0), negative agreement (slides with both scanner and manual results <2.0), and discordant results (slides with one result >2.0 and one <2.0) as well as the totals for data sets with scanner results in the 1.5 to 3.0 *HER2*/CEP17 ratio range are summarized in Table 2. Table 3 displays the positive agreement, negative agreement, and discordant results after slides with scanner results in the 1.5 to 3.0 range are removed. (Note: The corresponding manual results of slides with scanner results in the 1.5 to 3.0 range are removed, and therefore the data in Tables 1 and 3 are not directly interchangeable.)

Table 3 (without scanner results in the 1.5 to 3.0 *HER2*/CEP17 ratio range) shows two false-positive scanner results. In the study, each tumor was enumerated in a blinded manner six times with the scanner and six times manually (two times by each method at each of the three different sites). For each of the two tumors categorized as false-positive scanner results, five of the six manual and five of the six scanner results were concordantly classified as amplified (only one of six manual results was classified as nonamplified). These findings suggest that the single nonamplified manual result for each of these two specimens represent false negatives, possibly sec-

**Table 1.** Distribution of Scanner and Manual *HER2*/CEP17 Ratios

| Scanner r*HER2*/ CEP17 ratio | Manual *HER2*/CEP17 ratio | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | <1.5 | 1.5 to <2.0 | 2.0 to <2.5 | 2.5 to <3.0 | 3.0 to <5.0 | 5.0 to <10 | ≥10.0 | Total |
| <1.5 | 77 | 2 | 0 | 0 | 0 | 0 | 0 | 79 |
| 1.5 to <2.0 | 17 | 3 | 3 | 1 | 0 | 0 | 0 | 24 |
| 2.0 to <2.5 | 7 | 0 | 2 | 1 | 0 | 1 | 0 | 11 |
| 2.5 to <3.0 | 3 | 0 | 0 | 1 | 0 | 2 | 0 | 6 |
| 3.0 to <5.0 | 1 | 0 | 3 | 4 | 16 | 14 | 4 | 42 |
| 5.0 to <10.0 | 0 | 1 | 0 | 1 | 5 | 19 | 20 | 46 |
| =10.0 | 0 | 0 | 0 | 0 | 0 | 1 | 3 | 4 |
| Total | 105 | 6 | 8 | 8 | 21 | 37 | 27 | 212 |

ondary to poor hybridization or scoring of nonamplified tumor cells, which can falsely lower the overall *HER2*/CEP17 ratio used for HER-2 status determination in manual scoring. In contrast, the software used by the system can identify a population of amplified cells within a background of nonamplified cells, and therefore is, in theory, less susceptible to false-negative results attributable to background nonamplified cells.

The average time to obtain a result for the automated enumeration method (Vysis AutoVysion System) was 8.28 minutes. The average time to obtain a result for the manual enumeration method was 7.47 minutes. The average hands-on time of the automated system was 4.59 minutes, in contrast to 7.47 minutes for the manual method, a time-saving difference of 2.88 minutes.

Average bias for the manual enumeration *HER2*/CEP17 ratio range of 1.18 to 4.39 was 0.472 with a SD of 1.24. The average bias is 11.7%, which is within the manufacturer's design specifications of ±15% point value criteria for acceptable error. Statistical analysis for poolability resulted in a *P* value of 0.862, indicating the results from the three sites could be pooled. The scanner analysis of variance produced a *P* value for the F-test of 0.8650, which indicates that there were not significant differences among the means at the different study sites.

Reproducibility was evaluated using four formalin-fixed, paraffin-embedded breast tumor specimens sent to the three study sites for 3 scanning days. Each study site received four slides each day (three slides total for each specimen) for 3 days (12 total slides reviewed at each site). Analysis of the day-to-day reproducibility did not detect a statistically significant difference (all *P* values >0.05) in variations of the mean observed *HER2*/CEP17 ratios throughout 3 study days (Table 4). Analysis of the site-to-site reproducibility did not detect a statistically significant difference (all *P* values >0.05) in variations of the mean observed *HER2*/CEP17 ratios between the three study sites (Table 5).

## Discussion

The purpose of this study was to evaluate the use of an automated signal enumeration system (Vysis AutoVysion System) with the Vysis PathVysion *HER2* FISH assay. Three separate study sites compared automated and manual signal enumeration approaches by examining slides from 39 invasive breast cancer specimens. The results showed a high concordance rate of correct *HER2* status classification. Among all tissue specimens with informative results for both methods, overall agreement of *HER2* classification results (positive and negative) was 98.8% when the scanner data collected in the 1.5 to 3.0 ratio range were excluded. The average bias was within the manufacturer's design specifications point value criteria based on CLSI guideline EP9-A2 for acceptable error. The average hands-on time per slide was 7.47 minutes for the manual method versus 4.59 minutes with the automated method, a time savings of 2.88 minutes per slide.

The *HER2* FISH assay has been reported to be a better predictor of response to trastuzumab (Herceptin) therapy as compared with IHC.[1,11,14,17–21] A drawback of the FISH test is the time required for signal enumeration. Many steps in FISH processing have been automated and include deparaffinization, pretreatment, routine slide washing steps, and the denaturing (heating) and annealing (cooling) aspects of the hybridization process.

Narath and colleagues[23] examined the utility of an automated signal enumeration system, which used a Metafer4 scanner, in FISH assays for *MYCN* and *HER2* by

**Table 2.** Automated Versus Manual Classification of *HER2* Gene Amplification Including Automated Results in the 1.5 to 3.0 *HER2*/CEP17 Ratio Range

| | | Manual | | |
|---|---|---|---|---|
| | | + | − | Total |
| Scanner | + | 97 | 12 | 109 |
| | − | 4 | 99 | 103 |
| | Total | 101 | 111 | 212 |

Positive, scanner or manual ratio results >2.0; negative, scanner or manual ratio results <2.0.

**Table 3.** Automated Versus Manual Classification of *HER2* Gene Amplification Excluding Slides with Automated Results in the 1.5 to 3.0 *HER2*/CEP17 Ratio Range

| | | Manual | | |
|---|---|---|---|---|
| | | + | − | Total |
| Scanner | + | 90 | 2 | 92 |
| | − | 0 | 79 | 79 |
| | Total | 90 | 81 | 171 |

Positive,= scanner or manual ratio results >2.0; negative, scanner or manual ratio results <2.0.

**Table 4.** Mean *HER2*/CEP17 Ratio Results by Study Day

| Level of amplification | Day 1 (mean/SD/CV/*n*) | Day 2 (mean/SD/CV/*n*) | Day 3 (mean/SD/CV/*n*) | *P* value* |
|---|---|---|---|---|
| Normal | 1.17/0.33/28.04/3 | 1.11/0.14/12.39/3 | 1.14/0.06/5.12/3 | 0.1152 |
| Low/borderline | 2.08/0.61/29.34/3 | 2.38/0.64/26.82/3 | 2.45/NR/NR/2 | 0.9049 |
| Moderate | 5.70/0.86/15.01/3 | 5.55/0.33/5.87/3 | 5.47/0.66/12.09/3 | 0.2788 |
| High | 6.42/0.81/12.55/3 | 7.42/0.17/2.24/3 | 8.01/0.35/4.38/3 | 0.1205 |

*The *P* value from Levene's test for homogeneity of variances.[31]
NR, no result.

analyzing signal intensities. They concluded that identification and quantification of gene amplification consisting of either double minutes or homogenous staining regions could be reliably performed using the automated system. One disadvantage was the need for well-separated nuclei, which limited the system's use in tissue sections.[23] The software included with the Vysis AutoVysion System incorporates a targeted tiles sampling method, which corrects for overlapping nuclei and allows its use in tissue sections. Two studies by Ellis and colleagues[24] and Iourov and colleagues[25] found that computer-assisted signal enumeration of FISH signals was both reproducible and accurate. In a recent article discussing FISH assessment of *HER2*, Hicks and Tubbs[26] postulate that automated FISH scoring will enhance reproducibility of the FISH analysis and facilitate incorporation into pathology laboratories.

Automated methods have also been applied to IHC assays for HER2 status. Some studies have found significant interobserver variability between pathologists in grading the intensity of HER2 staining by IHC.[3,10,16,19,27] High concordance of results between FISH and IHC with strong (3+) staining and negative (0 to 1+) staining are generally seen. However, a marked discordance in the classification of results is seen between the two methods in cases with weak (2+) HER2 staining by IHC.[11,14,16,28] Approximately 23 to 26% of cases with weak (2+) HER2 staining by IHC show gene amplification by FISH testing.[1,5,10,29,30] Studies by Bloom and Harrington[3] and Lehr and colleagues[7] found that computer-assisted image analysis significantly increased the correlation of IHC and FISH results.

Although the overall time to obtain a result was longer with the automated signal enumeration system (Vysis AutoVysion System), the actual time a technologist spends with a slide (hands-on time) showed an average time savings of 2.88 minutes per slide. In high-volume FISH laboratories, this may increase the overall efficiency of HER2 testing. The system software also has a report-generating feature that may further contribute to overall time saved in processing a specimen; however, this attribute was not assessed in the current study. One important caveat to consider is that the manufacturer specifies that specimens exhibiting scanner results in the 1.5 to 3.0 *HER2*/CEP17 ratio range be rescored manually. With this in mind, it is possible that an increase in the overall hands-on time per slide could occur depending on the number of cases requiring rescoring.

Several large studies have demonstrated that 14.3 to 34% of all breast carcinomas display gene amplification by FISH.[19,28–30] Of these tumors, a substantial number showed borderline to low-level amplification. Specifically, in three studies, 48 to 51% of cases exhibited borderline to low-level amplification (*HER2*/CEP17 ratios defined as 2.0 to 5.0)[19,28,30] and in another study, 47% demonstrated borderline to low-level amplification as defined by *HER2*/CEP17 ratios of 2.0 to 3.9).[29] A common algorithm for determining *HER2* status uses initial evaluation with IHC, followed by confirmatory testing by FISH of 2+ positive cases.[8,14,15,27,28] The incidence of positive gene amplification by FISH in cases with 2+ staining by IHC ranges from 17 to 42%.[19,28] Of these FISH-positive/IHC 2+ cases, 63 to 74% showed borderline to low-level amplification (*HER2*/CEP17 ratios 2.0 to 5.0).[19,28,30] It is important to note that the 2.0 to 5.0 *HER2*/CEP17 ratio range for borderline to low-level amplification found in the literature is different from the range specified by the manufacturer for manual re-enumeration (1.5 to 3.0 *HER2*/CEP17 ratio range). The manufacturer estimates that only 7% of cases would require manual rescoring (cases falling within 1.5 to 3.0 *HER2*/CEP17 ratio; PathVysion Her-2 DNA Probe Kit package insert. Vysis, 1998). The actual number of cases requiring manual rescoring would probably vary from one laboratory to the next, depending on how cases are selected for *HER2* gene FISH analysis (for example, if selection is based on 2+ IHC findings the number of cases requiring manual rescoring may be higher). Because this study was sponsored by Vysis, independent studies are needed to exclude any potential bias. Moreover, although the current study focused on *HER2* FISH testing, the system could theoretically be applied to FISH testing for other genes

**Table 5.** Mean Observed *HER2*/CEP17 Ratios for the Three Study Sites

| Level of amplification | Site 1 (mean/SD/CV/*n*) | Site 2 (mean/SD/CV/*n*) | Site 3 (mean/SD/CV/*n*) | *P* value* |
|---|---|---|---|---|
| Normal | 1.24/0.26/21.09/3 | 1.05/0.05/4.52/3 | 1.14/0.18/15.97/3 | 0.1833 |
| Low/borderline | 2.09/0.58/27.84/3 | 2.42/NR/NR/2 | 2.39/0.75/31.23/3 | 0.5436 |
| Moderate | 5.41/0.58/10.70/3 | 5.88/0.06/1.02/3 | 5.44/0.88/16.12/3 | 0.1612 |
| High | 6.95/1.32/19.03/3 | 7.51/0.81/10.72/3 | 7.39/0.30/4.04/3 | 0.1665 |

*The *P* value from Levene's test for homogeneity of variances.[31]
NR, no result.

such as *EGFR* amplification in lung cancer, among others.

In conclusion, these data suggest that the Vysis AutoVysion System reliably and correctly classifies *HER2* gene amplification status in FISH assays when used according to the manufacturer's instructions. A decrease in the average hands-on time of 2.88 minutes per slide may translate into significant time savings in high-volume laboratories. The prototypical software used by the Vysis AutoVysion System has report-generating capabilities and allows its application to tissue sections by overcoming the problems of HSR interpretation, overlapping nuclei, and distinction of amplified and nonamplified cells.

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