HER2 IQFISH pharmDx™
Improved Quality and Efficiency

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Improved Quality and Efficiency in Manual. Preparation and Analysis of Formalin-Fixed, Paraffin-Embedded Fluorescence In Situ Hybridization (FISH) Specimens: A Comparison of Two FDA Approved HER2 Kits

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Introduction
Fluorescence in situ hybridization (FISH) with a DNA probe targeting HER2 (epidermal growth factor receptor 2, ERBB2) is used to detect amplification of the HER2 gene in breast and gastric cancers. HER2 amplification in patients with breast or gastric cancer has been correlated to poor outcomes and a more aggressive disease (1, 2). However, HER2 positive cancers typically respond to monoclonal antibody therapies specific for human HER2 proteins (e.g. trastuzumab [Herceptin®]) (3). Numerous manufacturers offer FISH DNA probe sets composed of a probe targeting HER2 and a second probe targeting the centromere of chromosome 17, which serves as a control for assessing the copy number status of HER2 in interphase nuclei. Only two of these FISH probe sets are approved by the U. S. Food and Drug Administration (FDA); PathVysion HER-2 DNA Probe Kit (Abbott Molecular) and HER2 IQFISH pharmDx™ (Dako, An Agilent Technologies Company). Here we describe the experience of our clinical lab in testing more than one thousand patient samples over a period of 8 months with each of the FDA-approved HER2 FISH testing platforms.

We compare several aspects of our tests including
- Concordance
- Preparation and Processing Steps
- Interpretation and Analysis Time
- Cost and Time Savings
  - Re-run frequency
  - Turnaround time
  - Hands-on time
- Staining Quality

Concordance
Correlation Study
We first performed a head-to-head comparison study of the two FDA-approved probe sets to assess the concordance between the two assays. Slides were prepared from 21 clinical breast tumor specimens using consecutive sections of tissue. Each assay was performed per the manufacturer’s instructions. There was 100% concordance of the HER2 assessment result from both assays, with six HER2-amplified specimens, and 15 HER2-non-amplified specimens with both assays. The HER2/CEN-17 ratios demonstrated a good correlation (slope 1.1, intercept 0.05, R² value 0.94, p<0.0001) (Figure 1). We next compared some of the practical considerations and the workflow of the two assays.

Figure 1. Correlation studies of HER2/CEN-17 centromere ratios using HER2 IQFISH pharmdX and PathVysion showed a high correlation between the two probes sets.
Preparation Steps

Solution Preparation
Both FDA-approved HER2 FISH assays required preparation of solutions that were used for pre-treatment and post-hybridization processing steps. The PathVysion assay required preparation of a stock solution of 20X SSC, adjusted to a pH of 5.3 and then filtered. This solution was used to make the pre-treatment and post-hybridization solutions, both of which require the pH to be checked and adjusted. The protease solution was prepared fresh by weighing the enzyme powder and adding it to a measured amount of protease buffer solution prior to each run. In contrast, the Pre-Treatment Solution, Wash Buffer, and Stringent Wash Buffer for HER2 IQFISH pharmDx are prepared by simply diluting the concentrated stock solution in an appropriate volume of deionized water. Similarly, the pepsin solution was made fresh prior to each run by preparing a dilution of the stock solution. Thus, solution preparation for the HER2 IQFISH pharmDx assay did not require the use of a balance or pH meter and required less time compared to the PathVysion assay. Because the solutions used in the HER2 IQFISH pharmDx assay did not require weighing, or assessing and adjusting pH, there was a reduced risk for errors to occur during solution preparation.

Processing
The overall processing steps are similar for both FDA-approved HER2 test kits, with a few key differences (Figure 2). The PathVysion procedure started with incubation in hydrochloric acid solution, followed by pre-treatment and protease incubations with washing between each step. The time required for each of these steps varied depending on

Figure 2. Comparison of timing and processing steps of HER2 IQFISH pharmDx and PathVysion. The workflow was based on information in the product insert for each FDA-approved assay (4, 5). Overall assay time is ~3½ hours and ~20 hours, respectively.
the tissue source (15 additional minutes each for gastric versus breast) and tissue processing conditions (3 to 5 additional minutes each depending on the processing conditions used). The pre-hybridization processing procedure for the HER2 IQFISH pharmDx assay required fewer steps (six steps) compared to the PathVysion assay (eight steps), and less time to perform (65 minutes compared to 92 minutes). Perhaps more importantly, the pre-hybridization processing steps for the HER2 IQFISH pharmDx assay was standardized regardless of the tissue type or processing conditions.

Hybridization

The hybridization time required for the two HER2 FISH kits is significantly different. The PathVysion assay required 17 hour hybridization, while the HER2 IQFISH required 1.5 hour hybridization. Thus, the HER2 IQFISH pharmDx allowed for a faster turnaround time, and made same day results for HER2 FISH feasible.

Interpretation and Analysis Time

Interpretation and scoring

The most important factor to consider when comparing FISH assays is the quality of final product (stained slide). Viewing through a 100X objective the PathVysion HER2 probe kit had an orange/red haze over the entire preparation and the orange signal for HER2 was often not much brighter than the background (Figure 3). The control probe at the centromere of chromosome 17 was diffuse and sometimes difficult to enumerate. In comparison, HER2 IQFISH pharmDx had very little background and the probe signals were bright and punctate for both HER2 and the control probe at the centromere of chromosome 17 (Figure 4). These findings are consistent with other studies comparing signal intensity for FISH assays (6-8).

Analysis Time

Our lab uses the CytoVision FISH Imaging System to capture, clean and save a minimum of 3 images using a 100X objective and one DAPI image using a 10X objective per case. The cameras are very sensitive and detect even the smallest signals when capturing the field using the individual filters for orange (or red), green and DAPI. Because of the high background observed using PathVysion (Figure 3), the technologists had to go back and forth from the microscope to the image in order to clean up the image by removing the background signals. This process added anywhere from 10 to 40 minutes to the analysis of the case. Capturing images using the HER2 IQFISH pharmDx assay rarely required the captured image to be cleaned up (Figure 4), and when it was required, it generally took less than a minute to complete (Table 1).

Figure 3. PathVysion HER-2 DNA Probe Kit staining of HER2 non-amplified tissue. A representative image captured with threshold turned off for all filters (unaltered image).

Figure 4. Dako HER2 IQFISH pharmDx staining of HER2 non-amplified tissue. A representative image captured with threshold turned off for all filters (unaltered image).
ASCO Guidelines for Interpretation

The 2013 ASCO Guidelines Data Supplement 8: ISH Interpretation Criteria states that “counting can be done by a trained technologist, but pathologist must confirm that result (count) is correct and that invasive tumor was counted. Pathologist must survey entire tumor before counting to define whether more than one population of cells is present and the percentage of the tumor that this population represents. This survey can also be done using IHC protein expression to select the area for ISH counting” (9).

Many of the specimens that are submitted to our laboratory for HER2 FISH testing do not include an IHC stained slide. Therefore, the entire tumor must be scanned to fulfill this criterion. Scanning at a 400X magnification (using a 40X objective) is preferred for speed and efficiency. In order to quickly scan the tumor at this magnification, the signals must be bright and the cells must have very low background. The HER2 IQFISH pharmDx met these two requirements. Scanning the entire tumor area with a 40X objective took approximately 10 to 20 minutes depending on the tumor size. In contrast, the high background and comparatively dull signals generated using the PathVysion made it difficult to discern signal from background using a 40X objective. In order to meet the ASCO Guideline requirements using PathVysion, the tumor had to be scanned using a 100X objective. This took two to three times longer (20 to 60 minutes) to accomplish, depending on the tumor size.

Cost and Time Savings

Repeat Rate

The repeat rate using PathVysion during the 8 month evaluation period was 2.69% (37 of 1375 specimens). The repeat rate using HER2 IQFISH pharmDx was 0.4% (6 of 1491 specimens). We estimate that the markedly reduced repeat rate using HER2 IQFISH pharmDx resulted in a cost savings of approximately $6,500 over an 8 month period or approximately $4.7 per slide (Table 1). The low re-run frequency of HER2 IQFISH pharmDx agrees with findings from other research groups even when using different fixation methods (6, 7).

Turnaround Time

The HER2 IQFISH pharmDx can be completed in less than half of a day; however several factors outside of the process affected the turnaround time of the test. These included the number of tests being processed, number of runs per day, other testing in the lab (e.g., chromosome analysis, hematologic FISH, etc.) and weekends. The availability of pathologists to review and sign-out the report also affected the time to report. However, given that the HER2 IQFISH pharmDx required less time for the pre-hybridization processing steps, hybridization, and scoring, it is not surprising that our lab achieved a major reduction in time to report using the HER2 IQFISH pharmDx process compared to PathVysion (Figure 5). For IQFISH, 37% of all samples were reported in one day or less, while only 13% of samples were reported in the same time frame when using PathVysion HER-2 DNA Probe Kit (Table 2). Thus the amount of cases reported within a day was almost three-fold higher using IQFISH.

Table 1. Comparison of hands on manual processing times for HER2 IQFISH pharmDx versus PathVysion HER-2 DNA probe kit. Time reduction was calculated to 27-58 min per slide (best/worst case scenario). With an annual throughput of 2000 FISH slides this accounts to ~900-2000 hours of manual labor. The lower repeat rate of IQFISH adds a saving of $4.7 pr slide. Calculated on 5-slide batch runs.

<table>
<thead>
<tr>
<th></th>
<th>IQFISH (N = 1491)</th>
<th>PathVysion (N = 1375)</th>
<th>Saving per slide (5-slide batch runs)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reagent Prepar.</td>
<td>15 min</td>
<td>82 min</td>
<td>13 min</td>
</tr>
<tr>
<td>Pre-Treatment</td>
<td>65 min</td>
<td>92 min</td>
<td>5 min</td>
</tr>
<tr>
<td>Digital Imaging</td>
<td>0-1 min</td>
<td>10-40 min</td>
<td>9-40 min</td>
</tr>
<tr>
<td>Post Processing</td>
<td>0.4%</td>
<td>2.7%</td>
<td>$4.7</td>
</tr>
<tr>
<td>Total Saving</td>
<td></td>
<td></td>
<td>27-58 min* + $4.7 (re-runs)</td>
</tr>
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*Overall financial saving not calculated and depends on hourly tech/pathologist rates.
Table 2. Comparison of time to result for IQFISH and PathVysion. The reduced hybridization time of HER2 IQFISH pharmDx results in a marked reduction in assay turnaround time leading to almost three times more patient cases to be reported within a day.

<table>
<thead>
<tr>
<th></th>
<th>IQFISH</th>
<th>PathVysion</th>
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<tbody>
<tr>
<td>Hybridization Time</td>
<td>1.5 hours</td>
<td>17 hours</td>
</tr>
<tr>
<td>Assay Turnaround Time*</td>
<td>~3.5 hours</td>
<td>~20 hours</td>
</tr>
<tr>
<td>Time to Report ≤ 1 day</td>
<td>37% of cases</td>
<td>13% of cases</td>
</tr>
</tbody>
</table>

*Includes pre- and post-processing steps.

Staining Quality

In our opinion, the improved signal to background ratio generated by the HER2 IQFISH pharmDx assay allowed for a more accurate determination of copy numbers for both HER2 and the control probe. The HER2 IQFISH pharmDx assay also required considerably less time to score.
FISH signals must be bright and the cells must have very low background for quick scanning of the tumor at 40X magnification. These two requirements are met by the HER2 IQFISH pharmDx assay. It was more difficult to discern signal from background using a 40X objective for signal generated with the PathVysion assay due to the high background and comparatively dull signals.

Summary
Overall, the performance of the HER2 IQFISH pharmDx was superior to PathVysion. The time required for pre-hybridization processing, hybridization, and analysis was shorter using the HER2 IQFISH pharmDx, allowing for a considerable reduction in the turnaround time for clinical test results. The slides generated with the HER2 IQFISH pharmDx assay demonstrated bright signals with minimal background (Figure 4), which decreased technologist’s frustration and facilitated a more accurate analysis. The lack of background enabled the technologists to scan the entire tumor area using a 40X objective to detect signal patterns, as per the new ASCO Guidelines, saving a notable amount of technologist time. The reduction in the number of repeat tests, and the increased efficiency in processing resulted in pronounced savings in our lab. Our laboratory now uses the HER2 IQFISH pharmDx assay for all HER2 FISH testing.

Table 3. Summary of results from testing more than one thousand patient samples over a period of 8 months with each of the FDA-approved HER2 FISH testing platforms.

<table>
<thead>
<tr>
<th>Data Summary</th>
<th>Dako HER2 IQFISH pharmDx</th>
<th>Abbott PathVysion</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reagent Preparation Time*</td>
<td>15 min</td>
<td>82 min</td>
</tr>
<tr>
<td>Pre-Hybridization Processing Time</td>
<td>65 min</td>
<td>92 min</td>
</tr>
<tr>
<td>Hybridization Time</td>
<td>1.5 h</td>
<td>17 h</td>
</tr>
<tr>
<td>Post-Hybridization Processing Time</td>
<td>32 min</td>
<td>13 min</td>
</tr>
<tr>
<td>Total Processing Time</td>
<td>3 hours, 22 minutes</td>
<td>20 hours, 7 minutes</td>
</tr>
<tr>
<td>Digital Imaging, Post Processing</td>
<td>0-1 min</td>
<td>10-40 min</td>
</tr>
<tr>
<td>Tumor Scan Time</td>
<td>10-20 min</td>
<td>20-60 min</td>
</tr>
<tr>
<td>Repeat Rate</td>
<td>0.4%</td>
<td>2.7%</td>
</tr>
<tr>
<td>Average Time to Report</td>
<td>2.6 days</td>
<td>3.3 days</td>
</tr>
<tr>
<td>Time to Report ≤ 1 Day</td>
<td>37% of cases</td>
<td>13% of cases</td>
</tr>
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*Reagent preparation time includes filtering of the PathVysion solutions.

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Disclosure/Conflict of interest
The authors declare that they have no conflict of interest. Support for third-party graphical design and printing assistance for this manuscript was provided by Dako Denmark A/S.
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