Rapid Research Communication

Comparison of Dako fluorescence in situ hybridization assays (FISH and IQFISH) in the assessment of HER2 amplification in breast cancer

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Determining human epidermal growth factor receptor 2 (HER2) status in breast cancer patients can be attained by fluorescent in situ hybridization (FISH) analysis, which prompts targeted treatment options. This study was performed to compare the staining results of Dako’s manual overnight HER2 FISH pharmDx™ assay with the manual (4 hours) HER2 IQFISH pharmDx™ assay and the Dako Omnis automated (4 hours) HER2 IQFISH assay, for assessment of HER2 amplification. Thirty-four formalin-fixed paraffin-embedded (FFPE) tissue blocks from patients diagnosed with invasive breast cancer were used to compare three FISH assays in three experiments. All assays included Texas Red-labeled HER2 and fluorescein-labeled centromere 17 (CEN-17) probes. First, 20 blocks with known HER2 immunohistochemistry (IHC) and FISH results from a reference laboratory were used in the validation of HER2 FISH pharmDx™ assay. Secondly, 19 of the same blocks were used in a comparison of HER2 FISH pharmDx™ assay and HER2 Instant Quality FISH pharmDx (IQFISH pharmDx™) assays. The third experiment, which included 23 blocks, compared the HER2 FISH pharmDx™ assay with the Dako Omnis IQFISH assay (stained on a Beta version of Dako’s new Omnis platform). Stained slides were counted following American Society of Clinical Oncology/College of American Pathologists (ASCO/CAP) guidelines (2007 version), with three categories of labeling: <1.8 (negative), 1.8–2.2 (equivocal), and >2.2 (positive) calculated HER2/CEN-17 ratio. The results showed high agreement between all assays employed. Initial validation of HER2 FISH pharmDx™ assay to the results provided by a reference laboratory had a concordance of 95%. The concordance between HER2 FISH pharmDx™ assay and HER2 IQFISH pharmDx™ assay was 100%. There was also 100% concordance between manual and Omnis IQFISH staining. A high level of concordance was obtained in all three experiments, indicating that manual and automated IQFISH are comparable to HER2 FISH pharmDx™. IQFISH has a faster turn-around-time (TAT) and showed comparable results in quality and quantitation.

Keywords: FISH validation, HER2 amplification, Fluorescence in situ hybridization, HER2 FISH pharmDx™ assay, HER2 IQFISH pharmDx™ assay

Introduction

The human epidermal growth factor receptor 2 (HER2/neu) gene is found in normal cells located on chromosome 17 (17q12). The HER2 gene encodes the HER2 protein, a transmembrane receptor that helps regulate the division, growth and migration of cells. An oncogenic activation of the HER2 receptor can occur, resulting in an overexpression of the HER2 gene in breast cancer cells. Amplification of the HER2 gene has been recognized in 18–20% of breast cancers1 and influences prognosis and treatment. Research and clinical trials have shown that drug therapies such as trastuzumab (Herceptin®), which target the HER2 protein, improve the outcome patients with HER2-amplified invasive breast tumors.2

Determining HER2 status in breast cancer patients can be attained by fluorescent in situ hybridization (FISH) analysis, which prompts targeted treatment options. There are numerous assays available to
determine HER2 status through FISH analysis, and validating an assay for accuracy and reproducibility is imperative. This study was performed to validate and compare the staining results of three FISH assays for assessment of HER2 amplification: Dako’s manual overnight HER2 FISH pharmDx™ assay, manual (4 hours) HER2 IQFISH pharmDx™ assay, and the Dako Omnis automated (4 hours) HER2 IQFISH assay.

**Materials and Methods**

Formalin-fixed paraffin-embedded (FFPE) tissue blocks from patients diagnosed with invasive breast cancer were used to compare three FISH assays in three experiments. Tissue sections were cut at 4 μm and picked up from a water bath on Silanized Dako slides. Slides were dried overnight and baked for 30 minutes at 60°C. First, 20 blocks with known HER2 IHC and FISH results from a reference laboratory were used in the validation of HER2 FISH pharmDx™ assay. The HER2 FISH pharmDx™ kit reagents were used following the recommended manufacturer overnight protocol. On day 1, pre-treatment at 95–99°C for 10 minutes and enzymatic digestion with pepsin at 37°C for 3 minutes were followed by application of Dako HER2/CEN-17 probe mix consisting of Texas Red-labeled deoxyribonucleic acid (DNA) probes covering a 218 kb region for HER2 gene and fluorescein-labeled peptide nucleic acid (PNA) probes targeting the centromeric region of chromosome 17 (CEN-17). Sealed slides were then put on a Dako hybridizer programed for 5 minutes denaturation at 82°C and an overnight (20 hours) hybridization at 45°C. Day 2 consisted of stringent washes for 10 minutes at 65°C and the application of a fluorescence mounting medium containing 4,6-diamidino-2-phenylindole (DAPI). Slides were evaluated and enumerated using a Leica DMRE microscope with DAPI filter cube and Texas Red/fluorescein isothiocyanate duo filter cube. Enumeration was completed by two medical laboratory technicians (MLTs) and slides were reviewed by a pathologist. Secondly, 19 of the same blocks were used in a comparative of HER2 FISH pharmDx™ assay and HER2 IQFISH pharmDx™ assay. The HER2 IQFISH pharmDx™ kit reagents were used following the recommended manufacturer protocol. The probe provided in the IQFISH kit is HER2/CEN-17 IQISH Probe Mix, consisting of Texas Red-labeled HER2 DNA probes and fluorescein-labeled CEN-17 PNA probes supplied in IQISH hybridization buffer. The HER2/CEN-17 IQISH Probe Mix is stored at −18°C and was thawed.

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**Figure 1** Validation of HER2 FISH pharmDx™ assay. There was 95% concordance of the data from both laboratories. One equivocal case in the reference laboratory (2.0 ratio) was just positive in the Gamma Dynacare Medical Laboratory (GDML) data (2.3 ratio).
and mixed just prior to use and then returned to −18°C freezer immediately following use. The IQFISH assay was completed manually and a Dako hybridizer was used for denaturation at 66°C for 10 minutes and hybridization at 45°C for 120 minutes. Slides were evaluated and enumerated using a Leica DMRE microscope with DAPI filter cube and Texas Red/fluorescein isothiocyanate duo filter cube. Slides from the two assays were enumerated separately and then compared back to back to evaluate differences in the staining between kits. Enumeration was completed by two MLTs and slides were reviewed by a pathologist. The third experiment, which included 23 blocks, compared the HER2 FISH pharmDx™ assay with the Dako Omnis IQFISH assay (stained on a Beta version of Dako’s new automated Omnis platform).
Due to limited tissue availability, nine of these blocks were part of the original validation series for the HER2 FISH pharmDx™ kit, and 14 were breast tumor blocks with known HER2 IHC values. The HER2 FISH was completed using the same protocol as in the initial validation; the slides for the Dako Omnis IQFISH assay had the same tissue preparation and pre-handling as the slides treated with the HER2 FISH pharmDx™ kit. The Dako Omnis IQFISH assay was completely automated on the Omnis platform (Beta version); hands-on time was limited to pre-handling of slides, cover-slipping, and slide review. Slides from the two assays were enumerated separately and then compared back to back to evaluate differences in the staining between kits. Enumeration was completed by two MLTs and slides were reviewed by a pathologist.

Stained slides were evaluated using a Leica DMRE fluorescent microscope with DAPI and Texas Red/FITC dual filter cube, and signals were counted following American Society of Clinical Oncology/College of American Pathologists (ASCO/CAP) guidelines5 (2007 version) with three categories of labeling: <1.8 (negative), 1.8–2.2 (equivocal), and >2.2 (positive) calculated HER2/CEN-17 ratio. Images were captured using a Leica DC300F camera.

Results
Data from the three experiments was charted and graphed to determine concordance.

The validation of HER2 FISH pharmDx™ assay using 20 FFPE invasive breast cancer tissue with known HER2 results, provided by a reference laboratory, had a concordance of 95% (Fig. 1). One equivocal case in the reference laboratory data (2.0 ratio) was just positive in the GDML data (2.3 ratio). Some of the highly amplified samples had dense clustering of the HER2 signals, so the number of signals per cell had to be estimated; these cases all remained highly amplified but in the graph (Fig. 1), those cases are visible as they fall further away from trend line.

The comparative validation between HER2 FISH pharmDx™ assay and HER2 IQFISH pharmDx™ manual assay used 19 FFPE invasive breast cancer blocks and had a concordance of 100% (Fig. 2). There were slight differences in the staining appearance between assays which included slightly higher red nuclear staining in HER2 IQFISH pharmDx™ assay. The signal strength of probes in both assays was bright and distinguishable.

The comparative validation between HER2 FISH pharmDx™ assay and Dako Omnis IQFISH automated assay included 23 FFPE invasive breast cancer blocks had a 100% concordance (Fig. 3). There were slight differences in the staining appearance between assays, which included higher red nuclear staining in the Dako Omnis IQFISH automated assay. The signal strength of probes in both assays was bright and distinguishable.

Images were captured during microscopic review of all three assays, allowing comparison of staining variations by multiple viewers (Fig. 4). The validation of HER2 FISH pharmDx™ assay entailed 62 individual tests run and evaluated throughout this study. This evaluation showed concordance with results from a reference laboratory and also reproducibility of the assay. After completion of this study, supplemental validation of the HER2 FISH pharmDx™ assay was run with additional tissue.

This study was completed following the 2007 version of the ASCO/CAP guidelines, which were updated in 2013. All future HER2 testing completed in the investigators laboratory will follow the 2013 version of the ASCO/CAP recommendations and guidelines.

Discussion
There are numerous assays available to perform HER2 analysis. There are many different factors that can influence the choice of validating a specific assay that include; but are not limited to: cost, availability, turn-around-time (TAT), workflow, and automated instrumentation. It is important to also factor in the assay performance.

This study has shown that the assays tested are all candidates, with appropriate validation protocols, for HER2 analysis.

This study has enabled our laboratory to assess these assays, which will allow us to confidently choose the assay that best suits our current testing needs, and has provided options for change in the future.

Conclusion
In this validation study, statistical analysis confirms that the HER2 IQFISH pharmDx™ manual and automated assays provide an alternative assay to HER2 FISH pharmDx™ assay in determining the HER2/CEN-17 ratio of HER2 gene amplification in invasive breast carcinoma samples. The manual HER2 IQFISH pharmDx™ assay has a faster TAT with a 1-day protocol that takes under 4 hours, as compared to a 2-day protocol that takes 22 hours, and showed a comparable result in quality and quantitation.

The automated Dako Omnis IQFISH assay (at the time of Beta trial) offered a high TAT with an almost completely automated procedure. This assay also showed comparable results in quality and quantification.
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References