Assessment of HER2 amplification status in breast cancer using a new automated HER2 IQFISH pharmDx™ (Dako Omnis) assay

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**1. Introduction**

Within the last 10–15 years assessment of human epidermal growth factor receptor 2 (HER2) status has become increasingly important for treatment of breast cancer. Determination of HER2 status serves as a critical predictive test for HER2 targeted therapy. The monoclonal antibody trastuzumab (Herceptin\(^\text{®}\), Roche/Genentech), together with the IHC assay HercepTest™ (Dako Denmark A/S), was the first drug-diagnostic combination to obtain United States Food and Drug Administration (FDA) approval in 1998 [1]. Since then several other drugs for treatment of HER2-positive breast cancer have been introduced and implemented in the clinic such as the tyrosine kinase inhibitor lapatinib (Tykerb\(^\text{®}/\text{Tyverb}\(^\text{®}\), Novartis), the HER dimerization inhibitor pertuzumab (Perjera\(^\text{®}\), Roche/Genentech), and recently the antibody-drug conjugate ado-trastuzumab emtansine (Kadcyla\(^\text{®}\), Roche/Genentech) [2–5].

A positive HER2 status is found in approximately 20% of all women with breast cancer, and assessment of the HER2 status is currently one of the most frequently performed companion diagnostics (CDx) tests in the pathology laboratory [6]. Different slide-based assays are available for assessment of HER2 status in patients with breast cancer. Overexpression of the HER2 receptor is assessed by immunohistochemistry (IHC) whereas amplification of the HER2 gene is assessed by fluorescence in situ hybridization (FISH) or other in situ hybridization (ISH) methods. For determination of the HER2 status in breast cancer the IHC and ISH assays are considered equally valuable methods [7].

In many laboratories FISH testing is regarded as time-consuming and technically challenging with an increased risk of human processing errors, which calls for automation of the staining procedure [8]. The HER2 IQFISH pharmDx™ (Dako Omnis) (Dako Denmark A/S) is a newly developed assay for the automated staining platform Dako Omnis (Dako Denmark A/S). This assay uses a non-toxic buffer that significantly reduces the hybridization time, which results in

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2. Materials and methods

The performance of the HER2 IQFISH pharmDx (Dako Omnis) assay using the Dako Omnis staining platform was investigated in a number of different studies.

2.1. Tissue specimens

The breast cancer specimens used in the different studies were residual formalin-fixed, paraffin-embedded (FFPE) blocks originating from individual patients. Furthermore, these specimens needed to fulfill the pre-analytical requirements as specified in the package insert for the HER2 IQFISH pharmDx (Dako Omnis) assay, which means fixation for 18–24 h using neutral buffered formalin [12]. The specimens were obtained from commercial providers or local hospitals, and the identity of the patients was not traceable. For each of the described studies, tumor specimens were derived from at least two different hospitals to address the variability in tissue procurement and processing. The specimens were cut in serial sections of 5 μm and mounted on glass slides. For each specimen a pathologist identified the tumor area, which was indicated directly on a hematoxylin and eosin stained slide. The tumor specimens represented a wide range of HER2/CEN–17 ratios representing both amplified and non-amplified cases. The studies were conducted in accordance with the current version of the World Medical Association Declaration of Helsinki. In the United States the inter-laboratory reproducibility protocol was reviewed and approved by an Institutional Review Board (Western International Review Board, Puyallup, WA). In Europe the study protocols were not submitted to Ethics Committees (EC) as this type of analytical study is exempt from EC approval.

2.2. Dako Omnis

The Dako Omnis instrument is a fully automated staining platform, which manages the slide staining processes of FFPE specimen sections for both IHC and ISH independently. The staining process requires no manual user interaction from loading the FFPE slides to unloading the stained slides, minimizing human error. The instrument supports continuous loading and unloading of slide racks and can run different staining protocols for individual slides at the same time, including simultaneous staining of IHC and ISH slides. The turn–around-time for IHC slides is approximately 2.5 h, and for ISH it is 3½ to 4 h. The instrument uses Dako Omnis ready-to-use (RTU) antibodies and Dako Omnis RTU reagents for the automated staining processes; however, it can also run customized staining protocols if necessary. The instrument is operated by the user from a touch screen interface [13].

2.3. HER2 IQFISH pharmDx

The HER2 IQFISH pharmDx™ assay for manual staining contains all key reagents required to complete a FISH procedure for sectioned FFPE specimens. Briefly, the specimen sections were exposed to heat pre-treatment using a microwave oven and pepsin digestion at 37 °C to prepare the tissue for probe hybridization. Denaturation was performed for 10 min at 66 °C followed by hybridization at 45 °C for 90 min using a Hybridizer (Dako Denmark A/S). The hybridization was performed using the RTU FISH Probe Mix based on a combination of a Texas Red-labeled DNA probe (HER2) and a fluorescein-labeled PNA probe (CEN-17). The specimen sections were subjected to stringent wash at 63 °C for 10 min before dehydration and drying. The dried slides were subsequently mounted using Fluorescence Mounting Medium containing 4',6-diamidino-2-phenylindole (DAPI) and cover slipped. The HER2 IQFISH pharmDx stained slides were evaluated and enumerated using a fluorescence microscope with 20 ×, 40 × and 100 × objectives and equipped with appropriate fluorescence filters for detection of the DNA and PNA probe signals. The HER2/CEN-17 ratio was calculated based on the enumeration of 20 nuclei from the invasive tumor area. Based on the ratio, the specimens were categorized into amplified (HER2/CEN-17 ≥ 2.0) or non-amplified (HER2/CEN-17 < 2.0) categories. Specimens with a ratio between 1.8 and 2.2 (borderline cases) were subjected to enumeration of 20 additional nuclei, and the ratio was then recalculated for the 40 nuclei to determine if amplification was present or not. Normal cells within the specimens served as an internal control for the staining [14]. The interpretation of the assay results was performed in accordance with the US FDA approved package inserts for HER2 FISH Tests [14,15].

2.4. HER2 IQFISH pharmDx (Dako Omnis)

The HER2 IQFISH pharmDx (Dako Omnis) assay is an assay similar to the manual HER2 IQFISH pharmDx assay with the same DNA (HER2) and PNA (CEN-17) probes; however, it is developed specifically to run on the automated Dako Omnis staining platform. After staining on the Dako Omnis platform, the specimen sections were mounted with Fluorescence Mounting Medium containing DAPI and coverslipped. The stained slides were evaluated as described above for the manual HER2 IQFISH pharmDx assay [12].

2.5. Method Comparison – HER2 IQFISH pharmDx versus HER2 IQFISH pharmDx (Dako Omnis)

In order to compare the manual HER2 IQFISH pharmDx assay with the HER2 IQFISH pharmDx (Dako Omnis) assay for automated staining, a method comparison study was performed. In this study the concordance between the two assays with regards to the HER2 gene status (amplified/non-amplified) as well as HER2/CEN-17 ratio was investigated. The study was designed as a blinded head-to-head comparison between the manual and the automated assay. A total of 140 FFPE breast cancer specimens were included in the study, which covered the full range of non-amplified, ‘extended borderline’ (ratios between 1.5 and 2.5) and amplified cases. Furthermore, the IHC categories of the included specimens were known, and in the study protocol it was pre-specified that at least 30% should represent the IHC 2+ category. The slides were stained according to the working procedures for the two assays, as described above [12,14]. The evaluation and enumeration of the manual and automated stained slides were performed by one blinded certified observer. Based on the HER2 gene status obtained for the two assays, the overall percent agreement (OPA), positive percent agreement (PPA), and negative percent agreement (NPA) were calculated. Furthermore, the means and the 95% confidence interval
intervals (CI) were calculated for the HER2/CEN-17 ratios for the two assays. A weighted linear regression plot of the HER2/CEN-17 ratios for the two assays was made, and the squared correlation coefficient ($R^2$) was calculated. The 95% CI for the mean difference between the two assays at cut-off ($HER2$/CEN-17 = 2) was also calculated.

2.6. Method Comparison – HER2 IQFISH pharmDx (Dako Omnis) versus PathVysion HER-2 DNA Probe Kit

A method comparison study was likewise performed with PathVysion® HER-2 DNA Probe Kit (Abbott Molecular Inc.) [15], similar to the one described above for the two Dako assays. The evaluation and enumeration of the stained slides were performed by the same certified observer who performed the scoring of the slides in the study that compared the manual HER2 IQFISH pharmDx assay with the HER2 IQFISH pharmDx (Dako Omnis) assay for automated staining. This study was conducted in order to compare the HER2 IQFISH pharmDx (Dako Omnis) assay with the PathVysion HER-2 DNA Probe Kit. The staining of the breast cancer sections with the PathVysion HER-2 DNA Probe Kit was performed using a validated procedure previously used in another method comparison study [16]. The statistical evaluation of the data from this study was similar to the one performed for the comparison of the manual and the automated HER2 IQFISH pharmDx assays.

2.7. Staining quality

The slides stained in the method comparison study with either the manual HER2 IQFISH pharmDx assay, the HER2 IQFISH pharmDx (Dako Omnis) assay or the PathVysion HER-2 DNA Probe Kit were evaluated with respect to staining quality based on an assessment of the signal intensity grade and morphology. For the signal intensity grade, the gene signal (Texas Red/Spectrum Orange) and the centromere signal (fluorescein/Spectrum Green) were scored using a 0–3 categorical scale with increments of 0.5. The extremes on the scale were “signals cannot be distinguished” (score 0) and “signals are clear and distinguishable” (score 3). Similarly, the morphology was scored using a 0–3 categorical scale with increments of 0.5. The extremes on this scale were “tissue structures and nuclear boundaries are completely destroyed” (score 0) and “tissue structures and nuclear boundaries are preserved and can be clearly seen” (score 3). Descriptive statistics such as mean and 95% CI were calculated for the different staining quality scores.

2.8. Reproducibility – inter-observer

The inter-observer reproducibility for HER2 IQFISH pharmDx (Dako Omnis) was investigated as part of the method comparison study. The same 140 breast cancer specimens, stained with HER2 IQFISH pharmDx (Dako Omnis), were evaluated by three blinded certified observers independently of each other. The overall agreement with the median was calculated based on the HER2 gene status for the three observers. Furthermore, the total coefficient of variation (CV) was calculated based on the Box-Cox transformed HER2/CEN-17 ratios. Box-Cox data transformation was performed in order to obtain data variance homogeneity.

2.9. Reproducibility – intra-laboratory

The day-to-day and lot-to-lot reproducibility of the HER2 IQFISH pharmDx (Dako Omnis) assay were investigated in a blinded randomized intra-laboratory study. Eight FFPE breast cancer specimens with different levels of HER2 gene amplification were included in the study. The specimen selection criteria for inclusion in the study were based on either predetermined HER2 IHC scores using the HercepTest assay (Dako Denmark A/S), or the HER2/CEN-17 ratio using the manual HER2 IQFISH pharmDx assay. Each specimen was stained with three different lots of the HER2 IQFISH pharmDx (Dako Omnis) assay on five non-consecutive days. A total of 240 specimen sections were processed for the entire study. Each specimen was stained with the HER2 IQFISH pharmDx (Dako Omnis) assay 30 times (5 days × 3 lots × 2 duplicates). The stained sections were evaluated by one blinded certified observer. The overall agreement with the median was calculated as well as the total CVs for the lot-to-lot and the day-to-day reproducibility. The calculation of the different CVs was based on Box-Cox transformed data.

2.10. Reproducibility – inter-laboratories

The inter-laboratory and day-to-day reproducibility of the HER2 IQFISH pharmDx (Dako Omnis) assay were investigated in a three-site multicentre study (two laboratories in Europe and one in the USA). The study was designed as a stratified and blinded study on consecutive specimen sections from 11 different FFPE breast cancer specimens. The specimens in the study were selected based on their predetermined HER2 IHC score using the HercepTest assay, or the HER2/CEN-17 ratio using the manual HER2 IQFISH pharmDx assay. Each specimen was stained a minimum of five times on five non-consecutive days and scored by one blinded certified observer at each of the three laboratories. A total of 192 sections were stained and included in the statistical analysis. Based on the HER2/CEN-17 ratios obtained at the three laboratories, the total CV was calculated based on the Box-Cox transformed data and reported with 95% CI. Furthermore, the overall agreement with the median was calculated.

2.11. Statistical analysis

The statistical analysis described for the individual studies was conducted using the JMP® software from SAS and/or Excel from Microsoft.

3. Results

3.1. Method comparison – HER2 IQFISH pharmDx versus HER2 IQFISH pharmDx (Dako Omnis)

The method comparison study between the manual and automated HER2 IQFISH pharmDx assays included 140 representative breast cancer specimens. Table 1 shows the distribution of the specimens based on the IHC scoring categories and corresponding HER2 gene status obtained with the manual HER2 IQFISH pharmDx assay. The study specimens represented all the IHC scoring categories (0; 1+; 2+; 3+) as well as HER2/CEN-17 ‘extended borderline’ cases. A relatively large proportion of the specimens were either IHC 2+ (38%) and/or HER2/CEN-17 ratio ‘extended borderline’ cases (14%). The cross tabulation of the HER2 amplification status obtained by the two assays is shown in Table 2. A perfect concordance between the two assays was observed with an OPA of 100%. Both the PPA and NPA were likewise 100%. The mean HER2/CEN-17 ratios for the two assays for all 140 specimens were close to each other. For the manual HER2 IQFISH pharmDx assay the mean and 95% CI were 3.75 [3.12; 4.39] and for the HER2 IQFISH pharmDx (Dako Omnis) assay the corresponding figures were 3.67 [3.07; 4.26]. A high degree of correlation, with regards to the HER2/CEN-17 ratios, was shown between the HER2 IQFISH pharmDx (Dako Omnis) assay and the manual HER2 IQFISH pharmDx assay ($R^2 = 0.97; p < 0.0001$). The result of the weighted linear regression plot is shown in Fig. 1. The 95% CI for the mean difference between the two assays at the
cut-off (HER2/CEN-17 = 2) was found to be [0.002; 0.031], which indicates that a difference higher than 3% is not expected.

3.2. Method comparison – HER2 IQFISH pharmDx (Dako Omnis) versus PathVysion HER-2 DNA probe kit

The method comparison study between the HER2 IQFISH pharmDx (Dako Omnis) assay and the PathVysion HER-2 DNA Probe Kit included likewise 140 representative breast cancer specimens. Table 3 shows the distribution of the specimens based on the IHC scoring categories and corresponding HER2 gene status obtained with the manual HER2 IQFISH pharmDx assay. The study specimens represented all the IHC scoring categories (0; 1+; 2+; 3+) as well as HER2/CEN-17 ‘extended borderline’ cases. A relatively large proportion of the specimens were either IHC 2+ (39%) and/or HER2/CEN-17 ratio ‘extended borderline’ cases (14%). The cross tabulation of the HER2 amplification status obtained by the two assays is shown in Table 4. Also for this comparison a perfect concordance between the two assays was observed for all specimens resulting in an OPA of 100%. Both the PPA and NPA were likewise 100%. The mean HER2/CEN-17 ratios for the two assays for all 140 specimens were very close to each other. For the HER2 IQFISH pharmDx (Dako Omnis) assay the mean and 95% CI were 3.63 [3.02; 4.23] and for the PathVysion HER-2 DNA Probe Kit the corresponding figures were 3.65 [3.05; 4.26]. A high degree of correlation, with regards to the HER2/CEN-17 ratios, was shown between the HER2 IQFISH pharmDx (Dako Omnis) assay and the PathVysion HER-2 DNA Probe Kit (R² = 0.96; p < 0.0001). The result of the weighted linear regression plot is shown in Fig. 2. The 95% CI for the mean difference between the two assays at the cut-off (HER2/CEN-17 = 2) was found to be [0.001; 0.031], which indicates that a difference higher than 3% is not expected.

3.3. Staining quality

In the method comparison studies 131 specimen sections were stained with all three assays; the manual HER2 IQFISH pharmDx assay, the HER2 IQFISH pharmDx (Dako Omnis) assay, and the PathVysion HER-2 DNA Probe Kit. Each of these 393 specimen sections was evaluated with respect to staining quality. Both the signal intensity grade for the gene and the centromere as well as the morphology were evaluated by one blinded certified observer. The two HER2 IQFISH pharmDx assays showed very little background staining, and both the gene and the centromere signals were found to be bright and punctuate or as clusters in the nuclei of the tumor cells. Somewhat more background staining was observed with the PathVysion HER-2 DNA Probe Kit; however, this background staining did not significantly impact the ability to score the individual slides. Table 5 provides the mean staining quality scores and 95% CI for all three HER2 assays. Examples of staining with the manual...
Table 3
Distribution of the specimens based on the HER2 IHC scoring categories and the corresponding HER2 gene status obtained with the manual HER2 IQFISH pharmDX assay. A total of 140 breast cancer specimens were included in the method comparison study between the HER2 IQFISH pharmDX (Dako Omnis) assay and the PathVysion HER-2 DNA Probe Kit.

| HER2 IHC staining score | N  | 0 | 1+ | 2+ | 3+ | Total |
|-------------------------|----|---|----|----|----|------|
| HER2 FISH status        |    |   |    |    |    |      |
| Amplified               |    |   |    |    |    |      |
| Non-amplified           |    |   |    |    |    |      |
| Total FISH-tested samples | 28 | 10| 54 | 48 |140 |

Table 4
Cross tabulation of the HER2 gene status obtained by the HER2 IQFISH pharmDX (Dako Omnis) assay and the PathVysion HER-2 DNA Probe Kit (N = 140).

| HER2 gene status (PathVysion) | Non-amplified | Amplified | Total |
|-------------------------------|---------------|-----------|-------|
| HER2 gene status (Dako Omnis) |               |           |       |
| Non-amplified                | 80            | 0         | 80    |
| Amplified                    | 0             | 60        | 60    |
| Total                         | 80            | 60        | 140   |

Table 5
Staining quality scores for the manual HER2 IQFISH pharmDX assay, the HER2 IQFISH pharmDX (Dako Omnis) assay, and the PathVysion HER-2 DNA Probe Kit (N = 131).

| Mean [95% CI] | HER2 IQFISH pharmDX | HER2 IQFISH pharmDX (Dako Omnis) | PathVysion HER-2 DNA Probe Kit |
|--------------|---------------------|---------------------------------|--------------------------------|
| HER2 Signal  | 2.61 [2.56–2.66]    | 2.56 [2.51–2.61]                | 2.46 [2.39–2.52]               |
| CEN-17 Signal| 2.63 [2.59–2.68]    | 2.55 [2.51–2.59]                | 2.31 [2.25–2.37]               |
| Morphology   | 2.36 [2.32–2.40]    | 2.39 [2.35–2.42]                | 2.24 [2.20–2.28]               |

HER2 IQFISH pharmDX assay and the HER2 IQFISH pharmDX (Dako Omnis) assay are shown in Fig. 3.

3.4. Reproducibility – inter-observer

The 140 breast cancer specimen sections stained with the HER2 IQFISH pharmDX (Dako Omnis) assay in the method comparison study were also used for an inter-observer reproducibility study. All slides were evaluated by three blinded independent certified observers. Based on the HER2 gene status, the agreement between the three observers was calculated. For 4 out of 140 specimens, disagreements were observed with regard to the HER2 gene status, which resulted in an overall agreement with the median of 98.8%. The total CV and 95% CI for the inter-observer reproducibility was found to be 8.4% [7.5; 9.3].

3.5. Reproducibility – intra-laboratory

Based on 240 stained specimen sections, the lot-to-lot and day-to-day reproducibility were evaluated. Fig. 4 shows the variation in the HER2/CEN-17 ratios obtained in the study for the three different lots of the HER2 FISH pharmDX (Dako Omnis) assay used for testing on the five non-consecutive days. The overall agreement in the study was calculated to be 97.1%. The total CV and 95% CI in the study was 4.3% [3.7; 4.9]. Furthermore, a variance component analysis showed that the day-to-day and lot-to-lot variations accounted for 1.5% and 0.0%, respectively.

3.6. Reproducibility – inter-laboratory

In the inter-laboratory and day-to-day reproducibility study the three participating laboratories stained and evaluated 11 different FFPE breast cancer specimens, which represented all the IHC scoring categories (0; 1+; 2+; 3+) as well as HER2/CEN-17 ‘extended borderline’ cases. Each specimen was stained and evaluated a minimum of five times at each laboratory. A total of 192 sections were stained and included in the statistical analysis. In order to illustrate the inter-laboratory and day-to-day variability, the individual HER2/CEN-17 ratios were plotted in a variability gauge chart. Fig. 5 shows that the variance for the individual specimens increased with increasing HER2/CEN-17 ratios, which illustrates the need for data transformation to obtain variance homogeneity before calculation of the CV. The total CV and 95% CI based on the Box-Cox transformed data was 11.6% [7.9; 15.3]. A variance component analysis showed that 23% of the total variance comes from the laboratories, and the remaining 77% is residual variation which corresponds to biological variation between the specimen sections and repeatability factors. The overall agreement in this study was 96.4%.

4. Discussion

The HER2 IQFISH pharmDX assay for manual staining has been available for clinical use for several years and has shown to be a reliable method for assessment of the HER2 gene status [9,10,17,18]. In a number of different validation studies we have evaluated HER2 IQFISH pharmDX (Dako Omnis), a newly developed assay for the automated staining platform Dako Omnis, in order to secure reliable and robust performance. One aspect of this validation was to compare the new assay with one or more already validated and regulatory approved FISH assays. For the HER2 IQFISH pharmDX (Dako Omnis) assay, method comparison studies were performed with both the manual HER2 IQFISH pharmDX assay and the PathVysion HER-2 DNA Probe Kit. For both of these assays a perfect concordance with the HER2 IQFISH pharmDX (Dako Omnis) assay was demonstrated. For the HER2 gene status the OPA, PPA and NPA was 100%. When a weighted linear regression analysis of the HER2/CEN-17 ratios between the HER2 IQFISH pharmDX (Dako Omnis) assay and the PathVysion HER-2 DNA Probe Kit was performed, the correlation was close to perfect (R = 0.98). The situation was similar for the two Dako assays; here also a close to perfect correlation was found (R = 0.99). Based on the results from these two studies it was concluded that the performance of the HER2 IQFISH pharmDX (Dako Omnis) assay, with regard to the HER2 gene status, was very close to being identical to the manual HER2 IQFISH pharmDX assay and the PathVysion HER-2 DNA Probe Kit. The results obtained here with regards to method comparison are similar to what have been
Fig. 3. Examples of the same breast cancer specimen sections stained with the manual HER2 IQFISH pharmDx assay (A, B, E, F) or the HER2 IQFISH pharmDx (Dako Omnis) assay (C, D, G, H). For the images in the left column (A, C, E, G) a double filter was used, and for the images in the right column (B, D, F, H) a triple filter was used. Both HER2 non-amplified cases (A–D) as well as amplified cases (E–H) are shown.

Fig. 4. Variability gauge charts with the HER2/CEN-17 ratios obtained in the intra-laboratory reproducibility study with 3 different lots of the HER2 FISH pharmDx (Dako Omnis) assay tested on 5 non-consecutive days using 8 different breast cancer specimens.
shown in other studies that have compared automated \textit{HER2 FISH} to manual \textit{HER2 FISH} [19,20].

An important factor to consider when comparing FISH assays is the quality of the stained slides. The 131 specimen sections that were stained with all three assays in the method comparison studies were evaluated with regard to staining quality. The two Dako assays showed similar performance with very little background staining and bright and distinguishable signals for both gene and centromere. Also, the evaluation of the morphology resulted in similar quality scores for these two assays. For the PathVysion \textit{HER-2} DNA Probe Kit the quality score for the gene signal was similar to the Dako assays. However, with regard to the centromere signal and the morphology, a trend towards lower quality scores was observed for the PathVysion \textit{HER-2} DNA Probe Kit compared with the Dako assays, which might be due to the slightly higher background staining. Overall, it could be concluded that the staining quality of the slides stained with the \textit{HER2 IQFISH pharmDx} (Dako Omnis) assay is similar to the manual Dako assay and at least comparable to the PathVysion \textit{HER-2} DNA Probe Kit. The findings with regard to the quality of the slides stained with the \textit{HER2 IQFISH pharmDx} (Dako Omnis) are in agreement with another study that evaluated the staining quality of the assay [17].

In order to evaluate the inter-observer reproducibility, the 140 specimen sections stained with the \textit{HER2 IQFISH pharmDx} (Dako Omnis) assay in the method comparison study were evaluated by three blinded independent observers. The overall agreement for the three observers based on the 420 readings was 98.8%, which is regarded as excellent. The total CV was found to be 8.4% with an upper CI limit of 9.3%, which is substantially below the study acceptance criteria of 25.0%. Likewise, the separate study on the day-to-day and the lot-to-lot reproducibility showed an overall agreement of 97.1% and a total CV as low as 4.3%. When the total CV was broken down in day-to-day and lot-to-lot reproducibility, it was shown that these two variables only accounted for 1.5% and 0.0% of the total variance, respectively. The remaining variance was largely related to repeatability and biological variation. It was quite remarkable that the lot-to-lot variance in this study did not contribute to the total CV, which indicates a very low and negligible variation between the individually produced lots. Based on this study, it can be concluded that the \textit{HER2 IQFISH pharmDx} (Dako Omnis) assay has a high reproducibility with very low day-to-day and lot-to-lot variation.

Inter-laboratory and day-to-day reproducibility were evaluated in a multi-site study using one laboratory in the United States and two in Europe. The overall agreement in this study was estimated to be 96.4%, which was at the same level as seen in the other reproducibility studies reported here for the \textit{HER2 IQFISH pharmDx} (Dako Omnis) assay. The total CV for the study was 11.6% with an upper CI limit of 15.3%, which again was well below the study acceptance criteria of 25.0%. Only one disagreement with regards to the \textit{HER2} gene status was observed between the 3 study sites. This specimen (ID 68122) was a ‘true borderline’ case (HER2/CEN-17 ratio 1.8–2.2), which was evaluated as amplified by two of the study sites and non-amplified by the third. The increased intra-observer variation seen with increased \textit{HER2/CEN-17} ratios was also observed in the intra-laboratory reproducibility study and has previously been described for \textit{HER2 FISH} testing in breast cancer [21].

It is important that assay procedures are standardized and one way to achieve this is through automation. In the 2007 ASCO/CAP guideline for \textit{HER2} testing in breast cancer it was stated that an optimal assay performance was more easily obtained using automated staining platforms rather than manual methods [22]. Beside advantages in relation to standardization and assay performance it has also been proposed that automation could result in a more rapid processing of samples, fewer laboratory errors as well as decrease personnel and operating costs [20].

In conclusion, the different validation studies summarized in this article have shown that the performance of the automated \textit{HER2 IQFISH pharmDx} (Dako Omnis) assay is at least comparable to the manual \textit{HER2 IQFISH pharmDx} assay and the PathVysion \textit{HER-2} DNA Probe Kit. Furthermore, the assay has demonstrated excellent precision which will contribute to more consistent and standardized \textit{HER2 FISH} staining of FFPE breast cancer specimens.

\textbf{Conflict of interest}

Giuseppe Viale has a consultant agreement with Dako, and received honoraria for participation in Advisory Boards from Roche, Astra Zeneca, Merck Sharp & Dohme, and Dompé’. Jennifer Pater-son, Miriam Bloch, George Csathy, David Allen, Patrizia Del’Orto have no conflicts to declare. Gitte Kjaersgaard and Yaron Y Levy are employees of Dako/Agilent. Jan Trøst Jørgensen is working as a consultant for Dako/Agilent and Euro Diagnostica, and has given

Fig. 5. Variability gauge charts for the \textit{HER2}/CEN-17 ratios from each of the 11 breast cancer specimens for the three laboratories in the inter-laboratory reproducibility study with the \textit{HER2 FISH pharmDx} (Dako Omnis) assay.
lectures at meetings sponsored by AstraZeneca, Merck Sharp & Dohme, and Roche.

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