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From Cell Biology to Tissue Engineering

Analysis of HER2 status in gastroesophageal tumor specimens using a new automated *HER2* IQFISH pharmDx™ (Dako Omnis) assay

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Summary. The human epidermal growth factor receptor 2 (HER2) is an important target for treatment of gastroesophageal cancer. Different slide-based assays are available for assessment of HER2 status. Overexpression of the HER2 protein 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. Here we report a summary of the validation data on *HER2* IQFISH pharmDx™ (Dako Omnis), a newly developed assay for the automated staining platform Dako Omnis. This assay uses a non-toxic buffer that significantly reduces the hybridization time, which results in a total turnaround time of less than 4 hours from deparaffinization to counting of the gene and centromere signals. The data reported in the current summary cover method comparison, assessment of staining quality, observer-to-observer reproducibility as well as reproducibility within and between laboratories. Based on data from the different studies it was concluded that *HER2* IQFISH pharmDx (Dako Omnis) is a reliable and robust assay, with high precision and at least comparable to the manual *HER2* IQFISH pharmDx™ assay. The *HER2* IQFISH pharmDx (Dako Omnis) assay is currently not commercially available outside the European Union.

Key words: *HER2,* IQFISH, Gastric cancer, Automation, Companion diagnostic

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Introduction

The human epidermal growth factor receptor 2 (HER2) is an important target for treatment of both gastroesophageal and breast cancer. For gastroesophageal cancer the clinical utility of HER2 targeted therapy was demonstrated in the ToGA trial, in which HER2-positive patients with advanced disease were randomized to receive 5-FU/capecitabine and cisplatin, either alone or in combination with trastuzumab (Herceptin®, Roche/Genentech). A statistically significant gain in overall survival was observed in patients who received the combined treatment of trastuzumab plus chemotherapy (Bang et al., 2010). Based on the positive results from the ToGA trial HER2 testing is now routinely performed in most pathology laboratories. In general, a positive HER2 status is found in 15% to 20% of patients with gastroesophageal cancer (Jørgensen and Hersom, 2012).

Different slide-based assays are available for assessment of HER2 status in patients with gastroesophageal cancer. Overexpression of HER2 protein 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. *HER2* IQFISH pharmDx™ (Dako Omnis) (Dako Denmark A/S) is a newly developed assay for the automated staining platform Dako Omnis. This assay uses a non-toxic buffer that significantly reduces the hybridization time, which results in a total turnaround time of less than 4 hours from deparaffinization to counting the gene and centromere signals (Matthiesen and Hansen, 2012; Franchet et al., 2014). The *HER2* IQFISH pharmDx

(Dako Omnis) assay is currently not commercially available outside the European Union.

Due to the differences in tumor biology, HER2 testing in gastroesophageal cancer differs from breast cancer. Gastric cancer tissue more frequently shows HER2 heterogeneity and incomplete membrane staining, and as a consequence of this a specific gastric cancer scoring system has been developed (Hofmann et al., 2008; Rüschoff et al 2012). In the ToGA trial, HER2 positivity was defined as being either IHC3+ or FISH+ and, based on the results from this study, a specific testing algorithm was developed. Here IHC was regarded as the primary test, with FISH as a reflex test in borderline cases of IHC2+ (Hofmann et al., 2008). However, as almost all patients in the ToGA trial had a FISH and IHC test performed, the United States FDA recommends that both IHC2+ and IHC3+ cases are reflex tested with FISH (Jørgensen, 2014).

For therapies such as trastuzumab and a number of other HER2-targeted drugs under development for gastroesophageal cancer, the HER2 companion diagnostic (CDx) assays have an important role to inform the treatment choice (Jørgensen, 2014). This position requires that the CDx assays have been clinically and analytically validated and have demonstrated both accuracy and precision. The current article presents a summary of the analytical validation studies that have been conducted for the new *HER2* IQFISH pharmDx (Dako Omnis) assay in order to fulfill the requirements for CE marking of In Vitro Diagnostic Medical Devices in the European Union per Directive 98/79/EC.

Materials and methods

In a number of different studies we investigated the performance of the *HER2* IQFISH pharmDx (Dako Omnis) assay applied to nearly 800 formalin-fixed, paraffin-embedded (FFPE) gastroesophageal tumor specimens sections using the new Dako Omnis staining platform.

Tissue specimens

The gastroesophageal specimens used in the different studies were residual, de-identified FFPE blocks originating from individual patients. The specimens were cut in serial sections of 4 µm and mounted on glass slides. 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. For each specimen a pathologist identified the tumor area which was indicated directly on a hematoxylin and eosin stained slide. The tumor specimens represented both resections and biopsies with a homogeneous or heterogeneous signal distribution. The studies were conducted in accordance with the current version of the World Medical Association Declaration of Helsinki. In the United States the reproducibility protocol was reviewed and approved by an Institutional Review Board (Western International Review Board). In Europe the study protocols were not submitted to Ethics Committees (EC) as this type of analytical study is exempt from EC approval.

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, which also include simultaneously staining of IHC and ISH slides. The turn-around-time for IHC slides is approximately 2.5 hours and for ISH less than 4 hours. The instrument uses Dako Omnis ready-to-use (RTU) antibodies for automated IHC staining processes; however it can also run customized staining protocols as needed. The instrument is operated by the user from a simple touch screen interface (Dako Denmark, 2014a).

HER2 IQFISH pharmDx assay

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 minutes at 66°C followed by hybridization at 45°C for 90 minutes 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 minutes 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 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 40 additional nuclei and the

ratio was then recalculated for these 40 nuclei to determine if amplification was present or not. Normal cells within the specimen taken from the tumor site served as an internal control for the staining (Matthiesen and Hansen, 2012; Dako Denmark, 2013).

HER2 IQFISH pharmDx (Dako Omnis) assay

HER2 IQFISH pharmDx (Dako Omnis) is an assay similar to the manual *HER2* IQFISH pharmDx assay with the same DNA (*HER2*) and PNA (CEN-17) probes; however, it was developed specifically to run on the automated Dako Omnis staining platform. After staining onboard the Dako Omnis platform, the specimen sections were mounted with Fluorescence Mounting Medium containing DAPI and cover slipped. The stained slides were evaluated as described above for *HER2* IQFISH pharmDx assay (Dako Denmark, 2014b).

Method comparison

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 gastroesophageal cancer specimens were included in the study, which covered the full range of non-amplified, 'extended borderline' (ratios 1.5 to 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 IHC2+ category. The slides were stained according to the working procedures for the two assays, as described above (Dako Denmark, 2013, 2014b). 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 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.

Staining quality

The slides in the method comparison study stained with either the manual *HER2* IQFISH pharmDx assay or the *HER2* IQFISH pharmDx (Dako Omnis) assay 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) and the centromere signal (fluorescein) were scored using a 0 to 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 to 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 structure 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.

Reproducibility – Inter-Observer

The observer-to-observer reproducibility for *HER2* IQFISH pharmDx (Dako Omnis) was also investigated as part of the method comparison study. The same 140 gastroesophageal 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.

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 gastroesophageal specimens with different levels of *HER2* gene amplification were included in the study. The specimens selected for 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. The stained sections were evaluated by one certified observer. The overall agreement with the median was calculated as well as the total CV's for the lot-to-lot and the day-to-day reproducibility. The calculation of the different CV's was based on Box-Cox transformed data.

Reproducibility - Inter-Laboratory

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 USA). The study was

designed as a stratified and blinded study on consecutive specimen sections from 12 different FFPE gastroesophageal 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 laboratory performed seven automated staining runs in a minimum of seven non-consecutive days over a period of at least 28 days. The stained sections were evaluated by one blinded trained observer at each of the three laboratories. 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.

Statistical analyses

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

Results

Method comparison

The method comparison study included 140 representative gastroesophageal specimens. The characteristics of the specimens with respect to type, signal distribution, and anatomic location are reported in Table 1. The study specimens represented all the IHC scoring categories (0; 1+; 2+; 3+) as well as the *HER2*/CEN-17 'extended borderline' cases (ratios 1.5 to 2.5). A relatively large proportion of the specimens were either IHC2+ (32%) and/or *HER2*/CEN-17 ratio 'extended borderline' cases (21%). Table 2 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 cross tabulation of the *HER2* amplification status obtained by the two assays is shown in Table 3. This shows a high concordance between the two assays and disagreement was only observed for one specimen resulting in an OPA of 99.3%. The PPA and NPA were 98.4% and 100%, respectively. The mean *HER2*/CEN-17 ratios for the two assays for all 140 specimens were close to identical. For the manual *HER2* IQFISH pharmDx assay the mean and 95% CI were 4.80 [3.77; 5.83] and for the *HER2* IQFISH pharmDx (Dako Omnis) assay the corresponding figures were 4.79 [3.77; 5.81]. 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 [0.007; 0.022], which means that a difference higher than 2% is not expected.

Staining quality

Each of 280 specimen sections in the method

Table 1. The characteristics and distribution of the gastroesophageal specimens included in the method comparison study.

Gastric Cancer Type	Specimens (N)
Stomach	113
Gastroesophageal junction	27
Total	140
Signal Distribution	
Homogeneous	58
Heterogeneous - focal	61
Heterogeneous - mosaic	21
Total	140
Type of specimens	
Resections	134
Biopsies	6
Total	140

Table 2. 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.

HER2 IHC staining score	0	1+	2+	3+	Total
N	39	14	45	42	140
HER2 FISH status					
Amplified	4	1	19	40	64
Non-amplified	35	13	26	2	76
Total FISH-tested samples	39	14	45	42	140

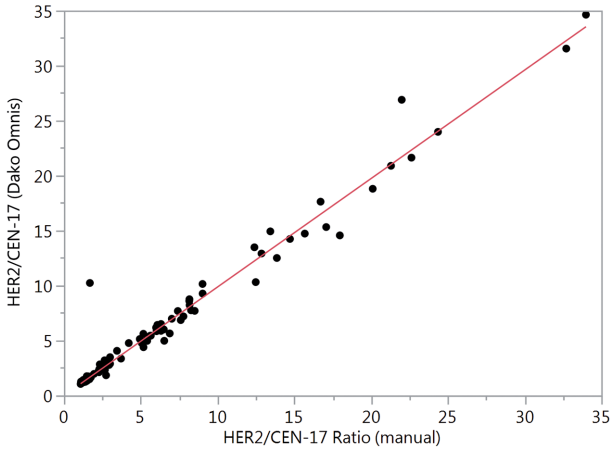

Fig. 1. Correlation between HER2/CEN-17 ratios for the HER2 FISH pharmDx (Dako Omnis) and the manual HER2 IQFISH pharmDx assay on 140 gastroesophageal cancer specimens (R2=0.97).

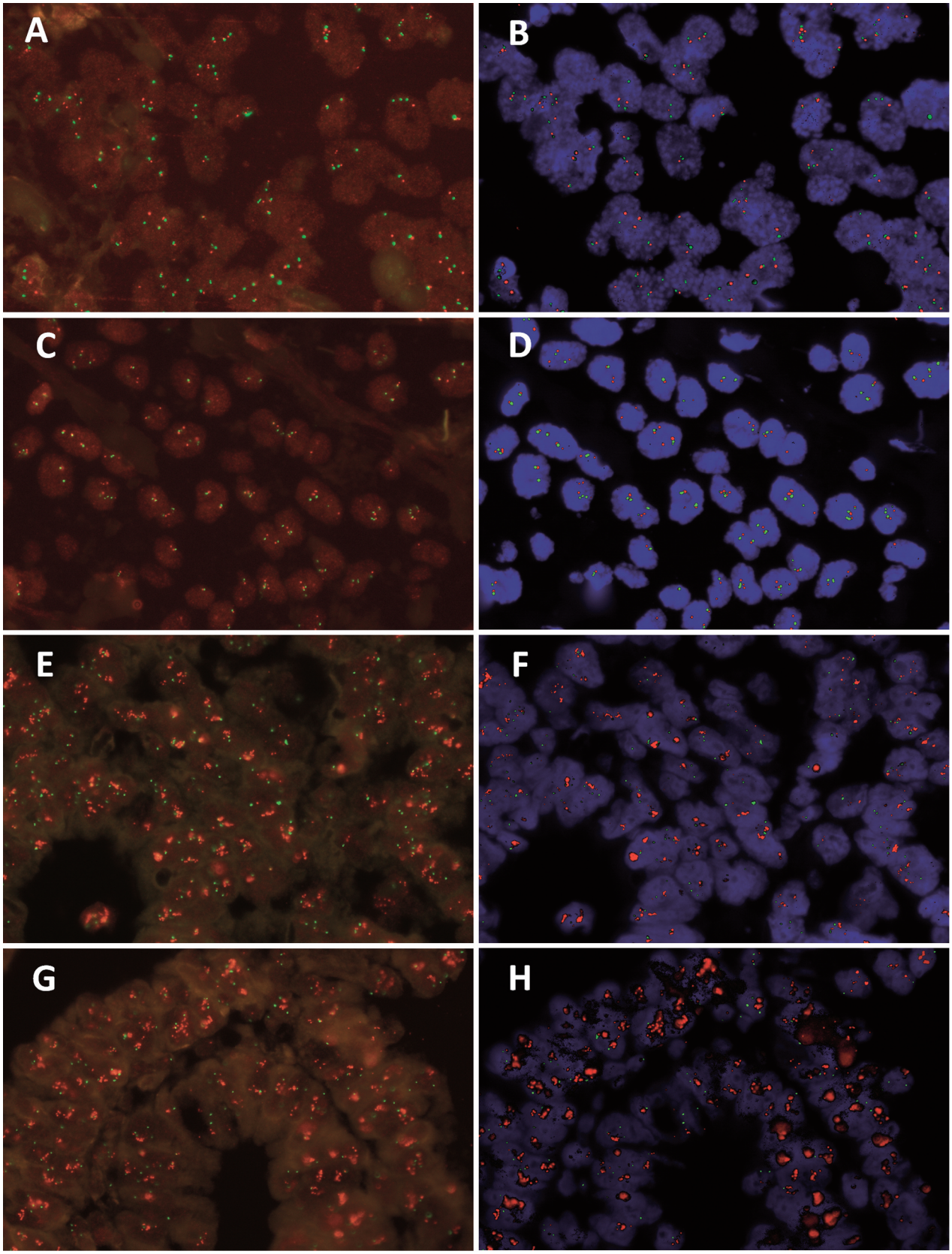

Fig. 2. Examples of gastric adenocarcinoma specimen sections stained with the manual HER2 IQFISH pharmDx assay **(A, B, E, F)** or the HER2 IQFISH pharmDx (Dako Omnis) assay **(C, D, G, H)**. For the images in the left column **(A, C, E, G)** a double filter was used and for the images in the right column **(B, D, F, H)** a triple filter was used. Both HER2 non-amplified cases **(A, B, C, D)** as well as amplified cases **(E, F, G, H)** are shown.

comparison study, 140 sections stained with the manual *HER2* IQFISH pharmDx assay and 140 stained with the *HER2* IQFISH pharmDx (Dako Omnis) assay, were 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 IQFISH 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. Table 4 provides the mean staining quality scores and 95% CI for both the manual *HER2* IQFISH pharmDx assay and the *HER2* IQFISH pharmDx (Dako Omnis) assay. Examples of stainings with the manual *HER2* IQFISH pharmDx assay and the *HER2* IQFISH pharmDx (Dako Omnis) are shown in Fig. 2.

Reproducibility – Inter-Observer

The 140 gastroesophageal 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; however, during the course of the study, one observer could not identify 20 nuclei for counting in one of the specimen sections, and consequently this specimen had to be excluded. Hence, 139 specimens were available for the analysis in the inter-observer reproducibility study. Based on the *HER2* gene status, the agreement between the three observers was calculated. For 6 out of 139 specimens, disagreements were observed with regards to the *HER2* gene status which resulted in an overall agreement with the median of 98.6%. The total CV and 95% CI for inter-observer reproducibility was found to be 16.5% [15.0; 18.0].

Reproducibility - Intra-Laboratory

Based on the 240 stained specimen sections, the lot-to-lot and day-to-day reproducibilities were evaluated. Fig. 3 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 99.2%. The total CV and 95% CI in the study was 6.5% [5.9; 7.1]. Furthermore, a variance component analysis showed that the lot-to-lot and day-to-day variations accounted for 0.8% and 0.7%, respectively.

Reproducibility - Inter-laboratory

In the inter-laboratory and day-to-day reproducibility study, the three participating laboratories

Table 3. Cross tabulation of the *HER2* gene status obtained by the manual *HER2* IQFISH pharmDx and the *HER2* IQFISH pharmDx (Dako Omnis) assays.

	HER2 gene status (manual)	
	Non-amplified	Amplified
HER2 gene status (Dako Omnis)		
Non-amplified	76	1
Amplified	0	63
Total	76	64

Table 4. Staining quality scores for the manual *HER2* IQFISH pharmDx assay (N=140) and the *HER2* IQFISH pharmDx (Dako Omnis) assay (N=140).

Mean [95% CI]	Manual *HER2* IQFISH pharmDx assay	*HER2* IQFISH pharmDx (Dako Omnis) assay
HER2 Signal	2.69 [2.64; 2.75]	2.70 [2.65; 2.76]
CEN-17 Signal	2.82 [2.77; 2.87]	2.75 [2.70; 2.81]
Morphology	2.35 [2.31; 2.40]	2.50 [2.47; 2.54]

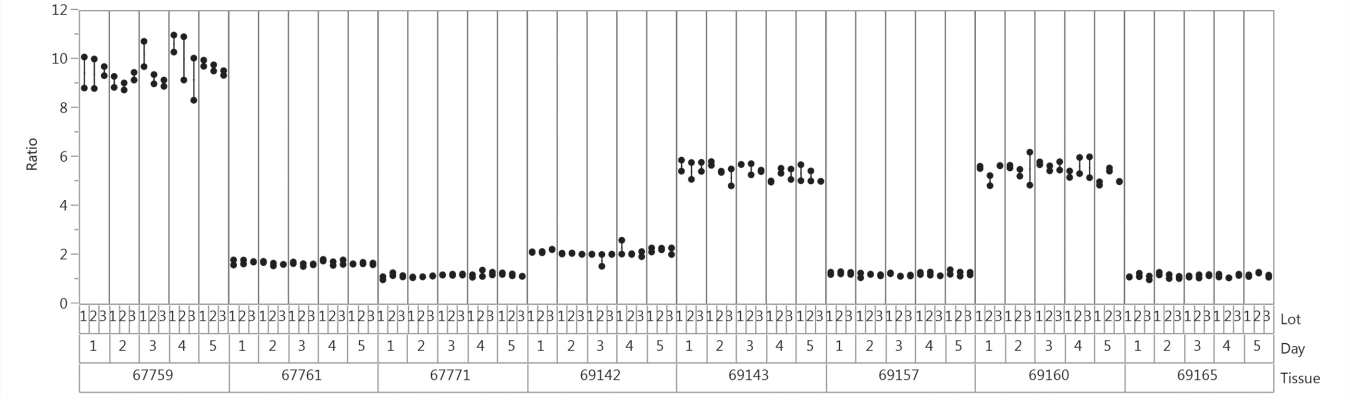

Fig. 3. The HER2/CEN-17 ratios obtained in the intra-laboratory reproducibility study with 3 different lots of the HER2 FISH pharmDx (Dako Omnis) assay tested on 5 non-consecutive days. The horizontal lines indicate the mean ratio for the 8 gastroesophageal specimens tested.

stained and evaluated 12 different FFPE gastroesophageal specimens, which represented all of the IHC scoring categories (0; 1+; 2+; 3+) as well as the *HER2*/CEN-17 'extended borderline' cases (ratios 1.5 to 2.5). Each specimen was stained and evaluated a minimum of seven times at each laboratory. A total of 314 stained sections were 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. Figure 4 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 15.2% [6.1; 24.3]. A variance component analysis showed that 38% of the total variance comes from the laboratory, and the remaining 62% is residual variation which corresponds to biological variation between the specimen sections and repeatability factors. The overall agreement in this study was 86.3%.

Discussion

In many laboratories FISH testing is regarded as time-consuming and technically challenging with an increased risk of human processing errors (van der Logt et al., 2015). This calls for an automation of the staining procedure, which in fact also has been discussed in the 2007 guideline for HER2 testing in breast cancer by the American Society of Clinical Oncology (ASCO)/College of American Pathologists (CAP) (Wolff et al., 2007). Recently, Dako has developed a fully automated staining platform, Dako Omnis, for both ISH and IHC (McClanaghan et al., 2014). In a number of different analytical validation studies, we have evaluated the performance of the new *HER2* IQFISH pharmDx (Dako Omnis) assay developed for the Dako Omnis platform, on FFPE specimens from patients with gastroesophageal adenocarcinoma.

The aim of one of the validation studies was to compare the manual *HER2* IQFISH pharmDx assay with the automated *HER2* IQFISH pharmDx (Dako Omnis) assay. This study showed a high concordance between the two assays with regards to the *HER2* gene status. Disagreement was only observed for one of the 140 specimens resulting in an OPA of 99.3%. When the mean *HER2*/CEN-17 ratios for the two assays were compared, they proved to be remarkably close to one another. That the two assays gave similar results at the ratio level was further confirmed by the high correlation between the ratios for the two assays, with a close to perfect correlation coefficient of 0.99. Based on the results from the method comparison study, it can be concluded that both with respect to the *HER2* gene status and the *HER2*/CEN-17 ratios the two assays give close to identical results.

The 280 specimen sections in the method comparison study were also evaluated with regard to staining quality. The two assays showed a quite similar performance with very little background staining and bright and distinguishable signals for both gene and centromere. Concerning the morphology, the two assays also gave similar results with a slight trend towards a higher quality score for the *HER2* IQFISH pharmDx (Dako Omnis) assay. The findings with regard to the staining quality are consistent with another study that has evaluated the *HER2* IQFISH pharmDx (Dako Omnis) assay (McClanaghan et al., 2014).

In order to evaluate the inter-observer reproducibility the 140 specimen sections stained with the *HER2* IQFISH pharmDx (Dako Omnis) assay in the

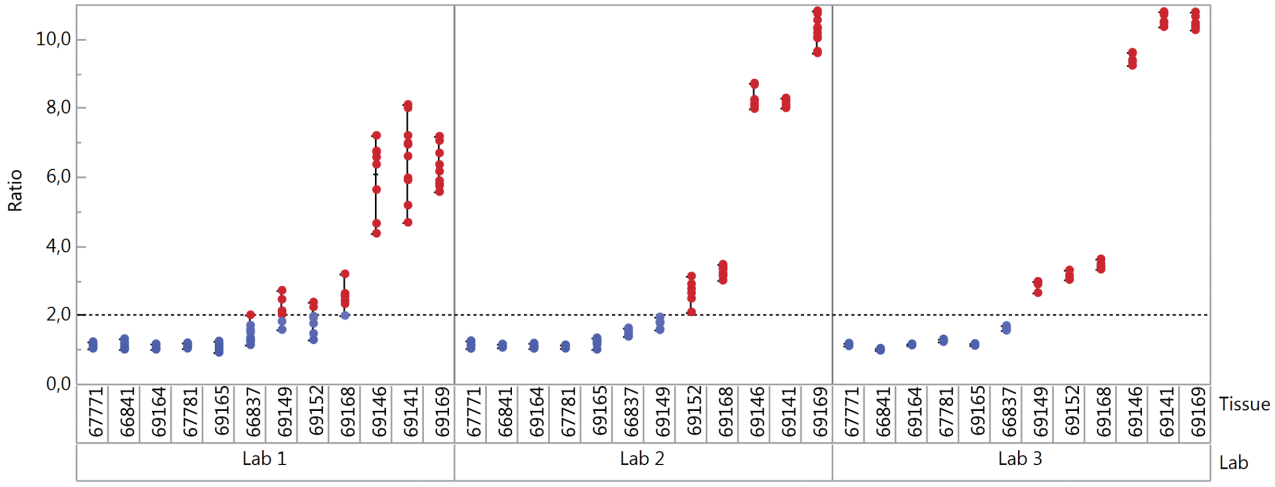

Fig. 4. Variability gauge charts with the HER2/CEN-17 ratios from each of the 12 tissues for the three laboratories in the study. The horizontal dotted line indicates the clinical decision point.

method comparison study were evaluated by three blinded independent observers. The overall agreement for the three observers based on the 420 readings was 98.6%, which is regarded as excellent. The total CV was found to be 16.5% with an upper CI limit of 18.0%, which was well below the study acceptance criteria of 25.0%. Likewise, a separate study on the day-to-day and the lot-to-lot reproducibility showed a very high overall agreement of 99.2% and a total CV of 6.5%. 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 0.7% and 0.8% of the total variance, respectively. The remaining variance was largely related to the repeatability. Based on this study, it can be concluded the *HER2* IQFISH pharmDx (Dako Omnis) assay has a high reproducibility with very low day-to-day and lot-to-lot variation.

The 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 for this study was 86.3%, which is lower than for the other reproducibility studies. The total CV for the study was 15.2% with an upper CI limit of 24.3%. The broader 95% CI also indicates a higher variation in this reproducibility study compared to the other studies described in this summary. However, a somewhat higher variability would normally be expected for a multi-site study. Furthermore, a partial explanation for the higher variability can be found by looking at Figure 4. As it appears from the figure it is mainly Laboratory 1 that contributed to this increased variability. Here the *HER2*/CEN-17 ratios varied somewhat between the days, especially for three of twelve specimens. Another contributing factor was the increased intra-observer variation seen with increased *HER2*/CEN-17 ratios, which to some extent were also observed for the two other laboratories. The increased intra-observer variation seen with increased *HER2*/CEN-17 ratios was also observed in the intra-laboratory reproducibility study and has been previously described for *HER2* FISH testing in breast cancer (Umemura et al., 2008). Finally, intratumoral heterogeneity of these tissues is also a likely contributing factor. Despite the increased variability the study met the pre-specified acceptance criteria of an upper CI limit <25% for total CV.

As a number of new HER2-targeted compounds for treatment of gastroesophageal cancer are currently under development the demands for HER2 testing will likely increase in the future (Jørgensen, 2014). As a reliable and robust assay with a short turn-around time, the *HER2* IQFISH pharmDx (Dako Omnis) assay combined with the automated Dako Omnis staining platform will be a valuable tool to cope with the increasing testing demands. In conclusion, the different validation studies summarized in this article have shown that the performance of the automated *HER2* IQFISH pharmDx (Dako Omnis) assay was at least comparable to the manual *HER2* IQFISH pharmDx assay. Furthermore, the automated assay has demonstrated excellent precision which will contribute to a more consistent and standardized staining of FFPE gastroesophageal specimen sections.

Conflict of Interest Statement. Giuseppe Viale has a consultant agreement with Dako, and received honoraria for participation in Advisory Boards from Roche, Astra Zeneca, Merck Sharp & Dohme, and Dompe'. Jennifer Paterson, Miriam Bloch, George Csathy, David Allen, Patrizia Del'Orto have no conflicts to declare.
Gitte Kjærsgaard 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 lectures at meetings sponsored by AstraZeneca, Merck Sharp & Dohme, and Roche.

References

Bang Y.J., Van Cutsem E., Feyereislova A., Chung H.C., Shen L., Sawaki A., Lordick F., Ohtsu A., Omuro Y., Satoh T., Aprile G., Kulikov E., Hill J., Lehle M., Rüschoff J., Kang Y.K. and ToGA Trial Investigators (2010). Trastuzumab in combination with chemotherapy versus chemotherapy alone for treatment of HER2-positive advanced gastric or gastro-oesophageal junction cancer (ToGA): a phase 3, open label, randomised controlled trial. Lancet 376, 687-697.

Dako Denmark AS (2013). HER2 IQFISH pharmDx™, Code K5731, Working Procedure 9th edition. (http://www.dako.com/us/ar39/p235733/prod_products.htm). Accessed April 8, 2015.

Dako Denmark AS (2014a). Dako Omnis Data Sheet. (http://www.dako.com/dk/29067_dako-omnis-data-sheet.pdf). Accessed April 8, 2015.

Dako Denmark AS (2014b). HER2 IQFISH pharmDx™ (Dako Omnis), Code GM333, Working Procedure 1st edition.

Franchet C., Filleron T., Cayre A., Mounié E., Penault-Llorca F., Jacquemier J., Macgrogan G., Arnould L. and Lacroix-Triki M. (2014). Instant-quality fluorescence in-situ hybridization as a new tool for HER2 testing in breast cancer: a comparative study. Histopathology 64, 274-283

Hofmann M., Stoss O., Shi D., Büttner R., van de Vijver M., Kim W., Ochiai A., Rüschoff J. and Henkel T. (2008). Assessment of a HER2 Scoring system for gastric cancer: results from a validation study. Histopathology 52, 797-805.

Jørgensen J.T. and Hersom M. (2012). HER2 as a prognostic marker in gastric cancer - A systematic analysis of data from the literature. J. Cancer 3,137-144.

Jørgensen J.T. (2014). Role of human epidermal growth factor receptor 2 in gastric cancer: biological and pharmacological aspects. World J. Gastroenterol. 20, 4526-4535.

Matthiesen S.H. and Hansen C.M. (2012). Fast and non-toxic in situ hybridization without blocking of repetitive sequences. PLoS One 7, e40675

McClanaghan M., Vandenberg J., Colly E. and Stead R. (2014). Comparison of Dako fluorescence in situ hybridization assay (FISH and IQFISH) in the assessment of HER2 amplification in breast cancer. J. Histotechnol. 37, 90-94.

Rüschoff J., Hanna W., Bilous M., Hofmann M., Osamura R.Y., Penault-Llorca F., van de Vijver M. and Viale G. (2012). HER2 testing in gastric cancer: a practical approach. Mod. Pathol. 25, 637-650.

Umemura S., Osamura R.Y., Akiyama F., Honma K., Kurosumi M., Sasano H., Toyoshima S., Tsuda H., Rüschoff J. and Sakamoto G. (2008). What causes discrepancies in HER2 testing for breast cancer? A Japanese ring study in conjunction with the global standard. Am. J. Clin. Pathol. 130, 883-891.

van der Logt E.M., Kuperus D.A., van Setten J.W., van den Heuvel M.C., Boers J.E., Schuuring E. and Kibbelaar R.E. (2015). Fully automated fluorescent in situ hybridization (FISH) staining and digital analysis of HER2 in breast cancer: A validation study. PLoS One 10, e0123201.

Wolff A.C., Hammond M.E., Schwartz J.N., Hagerty K.L., Allred D.C., Cote R.J., Dowsett M., Fitzgibbons P.L., Hanna W.M., Langer A., McShane L.M., Paik S., Pegram M.D., Perez E.A., Press M.F., Rhodes A., Sturgeon C., Taube S.E., Tubbs R., Vance G.H., van de Vijver M., Wheeler T.M., and Hayes D.F. (2007). American Society of Clinical Oncology/College of American Pathologists guideline recommendations for human epidermal growth factor receptor 2 testing in breast cancer. J. Clin. Oncol. 2007, 25118-25145.

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