One-Day FISH Approach for the High-Speed Determination of HER2 Gene Copy Status in Breast Carcinoma

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Abstract: Fluorescence in situ hybridization (FISH) is a commonly used method to detect chromosomal aberrations, for example, to assess human epidermal growth factor receptor 2 (HER2) gene status in breast carcinoma. The classical FISH approach requires overnight incubation for proper hybridization result. Tissue morphologic features are varying because of aggressive pretreatment and application of high temperatures. To eliminate some of the methodological problems, a new 1-day FISH method was recently introduced. The aim of our study was to evaluate the utility of the Instant Quality FISH with the conventional FISH kit from the same provider (Dako pharmDx) for determination of HER2 status.

We performed in situ hybridization on the same 40 invasive breast carcinoma samples with both probe kits, and HER2/CEN17 and chromosome 17/cell nucleus ratios were calculated. FISH signal stability was also tested by the reassessment of the slides after 2 months storage. The accordance regarding HER2 gene amplification status between the 2 FISH kits tested was 100%. There was an excellent correlation between HER2/CEN17 ratios with a concordance correlation coefficient of 0.958 and correlation coefficient (R) of 0.959. The 1-day *HER2* Instant Quality FISH diagnostic kit points with fast and stable reaction showing the same result in the diagnostic practice when compared with the conventional overnight FISH method.

Key Words: HER2, IQFISH, FISH, breast cancer, high-speed FISH

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The human epidermal growth factor receptor 2 (HER2, c-erbB-2) gene localizes at 17q21 and encodes a transmembrane tyrosine kinase receptor protein that is involved in the process of cell growth.[1]

Amplification of HER2 gene occurs in 15% to 25% of invasive breast cancers.[2] The gene amplification is associated with overexpression of the HER2 protein, a feature with strong prognostic impact for increased risk of recurrence and short survival.[3] As the HER2 status predicts therapeutic response to cyclosporamide, methotrexate fluorouracil,[4] and trastuzumab[5,6] as well, the accurate determination of the gene status has great clinical significance.

In situ hybridization is a powerful molecular genetic technique allowing the assessment of gene copy numbers by microscopic visualization in interphase nuclei in formaldehyde-fixed and paraffin-embedded sections and tissue microarray samples.[7] Fluorescence in situ hybridization (FISH) is a commonly used method for determining HER2 gene amplification status in breast cancer.[8] Unfortunately, the conventional FISH method performed on histologic specimens is rather time consuming as an overnight incubation is required for proper probe annealing and target DNA hybridization.[9] Moreover, tissue integrity is sometimes influenced by aggressive pretreatment and hybridization reagents. To overcome these problems, a modified FISH method was recently introduced, which requires only 1.5 to 2 hours of hybridization time; thanks to the adjustment of the hybridization buffer allowing also for the reduction of the denaturation temperature. The aim of our study was to evaluate the performance of the new 1-day FISH approach (*HER2* IQFISH pharmDx, Glostrup, Dako) and compare the data with those obtained by the conventional overnight HER2 FISH setting supplied by the same manufacturer.

MATERIALS AND METHODS

Patients and Samples

A total of 40 cases diagnosed with invasive breast carcinoma at the Department of Pathology, University of Debrecen, were prospectively selected. Routine formaldehyde-fixed and paraffin-embedded specimens consisted of surgically resected carcinomas and tumor biopsies. The sample set contained 38 invasive ductal and 2 invasive lobular carcinomas (Table 1). All evaluated carcinomas showed scores of 2 or 3 HER2 positivity by immunohistochemistry.

FISH Procedure

In situ hybridization was performed on 5 μm thick sections with both *HER2* FISH pharmDx (Dako) and *HER2* IQFISH pharmDx (Dako) probe kits on serial

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TABLE 1. Histopathologic Characteristics of the Breast Carcinoma Samples Evaluated for Conventional HER2 and 1-Day IQFISH (n = 40)

Variables	No. Cases
Histotype	
Ductal	38
Lobular	2
ER status	
Negative	4
Positive	35
Unknown	1
PR status	
Negative	8
Positive	32
c-erb-B2 score	
−	0
+	0
++	25
+++	15
HER2 amplification	
Negative	20
Positive	20

ER indicates estrogen receptor; HER2, human epidermal growth factor receptor 2; IQFISH, Instant Quality fluorescence in situ hybridization; PR, progesterone receptor.

sections according to the manufacturer's protocol. The main differences in the 2 protocols are shown in Figure 1.

Deparaffinized sections (NeoClear, Merck, Darmstadt) were pretreated with MES [2-(N-morpholino) ethanesulfonic acid, provided by the 2 kits] buffer followed by proteolytic digestion using pepsin (provided by the kits). Ready-to-use pepsin solution was dropped on slides for performing conventional FISH, and diluted pepsin solution was applied in cuvette for Instant Quality FISH (IQFISH). After washing in ×1 wash buffer (provided by the kits), slides were dehydrated through a graded series of ethanol and the ready-to-use probe mix was applied to the sections, coverslipped, and sealed.

Denaturation and hybridization were performed in a hybridization chamber (StatSpin ThermoBrite, Abbott Molecular). Slides were denatured at 82°C for 5 minutes for conventional HER2 pharmDx, and at 66°C for 10 minutes for the IQFISH probe. Hybridization was performed overnight for the conventional HER2 FISH and 90 minutes for HER2 IQFISH protocol at 45°C in both options. Stringent wash was performed at 65°C for conventional FISH and at 63°C for IQFISH for 10 minutes for both kits. Slides were dehydrated and covered with DAPI containing antifade solution and coverglass. Storage was at 4°C in the dark until evaluation.

Evaluation of FISH Results

Signals were counted using Zeiss Axio Imager Z2 fluorescence microscope equipped with DAPI, FITC, and Texas Red filters in a blind fashion. Fluorescence images were archived with the Isis imaging system (MetaSystems, Altlussheim, Germany).

Microscopic evaluation was carried out on the basis of the current guidelines,[10] including the overview of the whole hybridization area to determine relevant tumor-containing areas and to exclude heterogeneity. After the general assessment of the HER2 status, chromosome 17 (green) and HER2 (red) FISH signals were counted in 20 nuclei in representative areas using a ×63 immersion oil objective for all samples and HER2/CEN17 ratios were calculated. Samples with ratio above 2.2 were considered HER2 amplified, ratios between 1.8 and 2.2 were considered as borderline, whereas ratios below 1.8 as nonamplified. If the HER2/CEN17 ratio was in the borderline range, additional 20 nuclei were counted and ratios were recalculated. Chromosome 17 copy numbers were considered as normal between 1.5 and 2.3 CEN17 signals per nucleus, partial monosomy was stated below 1.5, and polysomy was stated above 2.3 signals per nucleus.

Reassessment of the same slides after 2 months storage at 4°C was performed to obtain information on FISH signal stability. The reassessment was carried out according to the same technique as described for the first evaluation.

Statistical Analysis

Comparative study of Dako HER2 FISH and IQFISH pharmDx was performed determining the concordance correlation coefficient and confidence interval. MedCalc and SPSS statistical softwares were used for analysis.

RESULTS

All 40 breast carcinoma samples gave appropriate results for both the conventional HER2 FISH and for the new HER2 IQFISH. The accordance between the results by the 2 kits regarding HER2 gene amplification status was 100%. Seventeen of the 40 cases were stated as HER2 nonamplified and showed chromosome 17 disomy, 3/40 were stated as HER2 nonamplified with chromosome 17 polysomy, 16/40 cases were HER2 amplified with chromosome 17 disomy, and 4/40 cases proved to be HER2 amplified with chromosome 17 polysomy. The correlation between HER2/CEN17 ratios were found to be high, with a concordance correlation coefficient of 0.958 and a correlation coefficient (R) of 0.959 (Fig. 2).

In 3 cases the result of in situ hybridization using the conventional HER2 FISH kit was difficult to interpret because of poor cellular morphology and/or background fluorescence. The extended evaluation of additional areas was necessary and the signal ratios had to be recalculated to determine amplification status. In all these 3 cases, a clear tissue architecture and fluorescence signals and less autofluorescence was seen using HER2 IQFISH kit. Accordingly, unambiguous FISH result was obtained after the standard evaluation of the 20 tumor cell nuclei (Fig. 3).

The reevaluation of the same FISH slides after 2 months storage at 4°C following the original study was carried out to check fluorescence stability and reproducibility of the reaction obtained by the new FISH kit. In this blinded analysis, we could not find significant

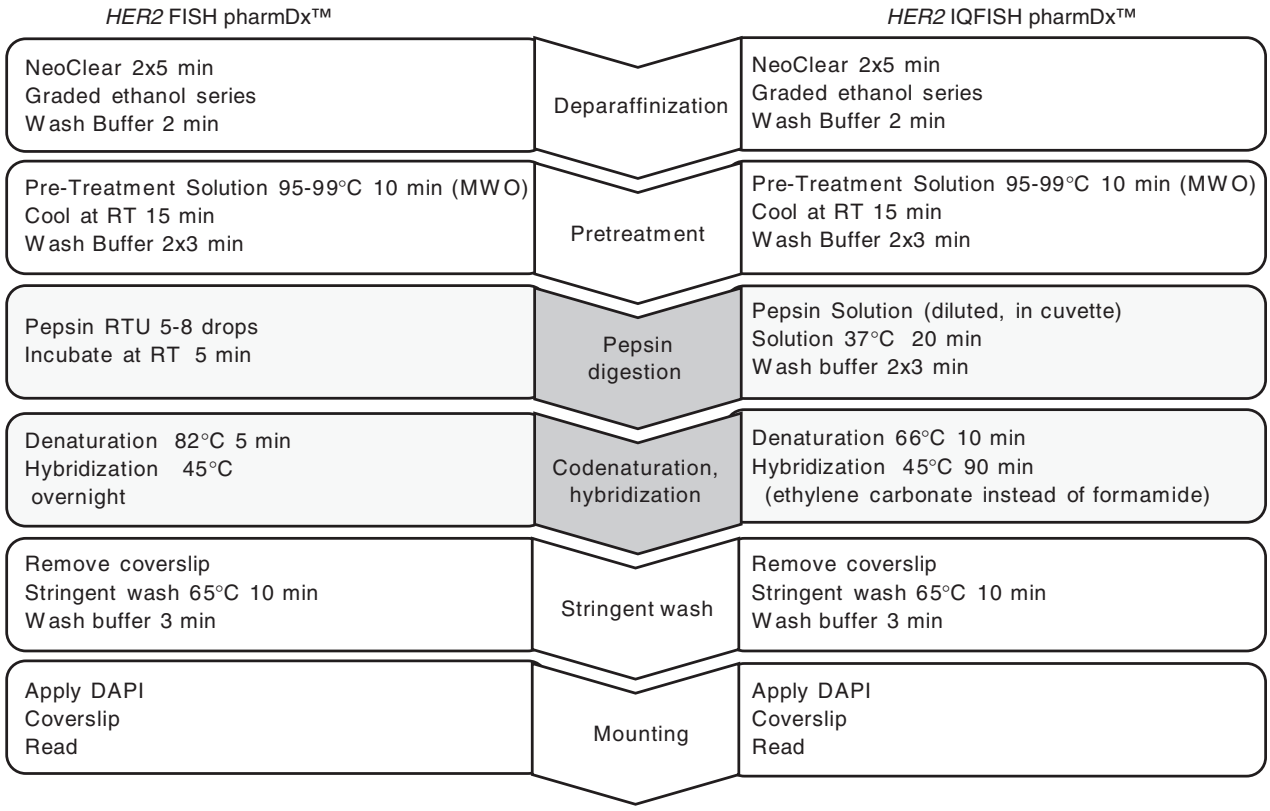

FIGURE 1. Main differences in the reaction protocols between the conventional HER2 FISH and the new 1-day IQFISH compared in this study. FISH indicates fluorescence in situ hybridization; HER2, human epidermal growth factor receptor 2; IQFISH, Instant Quality FISH.

differences in the final interpretation (Table 2). Tissue morphology was well preserved after 2 months storage; however, 2 slides (2/40, 5%) from the conventional FISH series were no longer appropriate for microscopic evaluation because of almost complete disappearance of the signals. In general, the fading of the HER2 and the centromere 17 signals could be seen in both FISH approaches during reevaluation. Interestingly, mean HER2 signal copy/nucleus dropped only by 0.95 copy/nucleus when IQFISH was used in contrast with 1.82 copy/nucleus difference following the conventional FISH method (Fig. 4). Even more fading could be stated for the chromosome 17 signals that was dominant in the slides approached by the conventional FISH method (0.01 copy/nucleus loss for IQFISH when compared with 0.17 copy/nucleus loss for the conventional assay) (Fig. 4). After reassessment, a different HER2 gene amplification status was stated in 5 cases for both kits; all 5 cases originally stated as HER2 low-grade amplified shifted over to the no amplification group after reassessment. In 1 additional case, only low-grade amplification was observed after reevaluation in an originally highly amplified sample after the use of the conventional HER2 FISH kit.

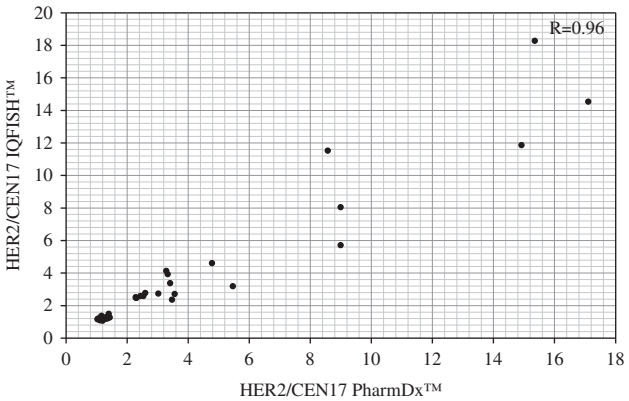

FIGURE 2. Correlation between the HER2/CEN17 relative copy number ratios determined by the 2 FISH kits. Strong correlation was found with concordance correlation coefficient 0.958 and correlation coefficient (*R*) 0.959. FISH indicates fluorescence in situ hybridization; HER2, human epidermal growth factor receptor 2.

DISCUSSION

In situ hybridization is the gold standard used in the everyday practice for the determination of the HER2 status in breast carcinoma.[11] The conventional FISH technique on histology samples requires 2 days because of overnight hybridization of the probes. The new IQFISH method was developed to shorten the procedure and allow a 1-day application requiring only 1.5 to 2 hours

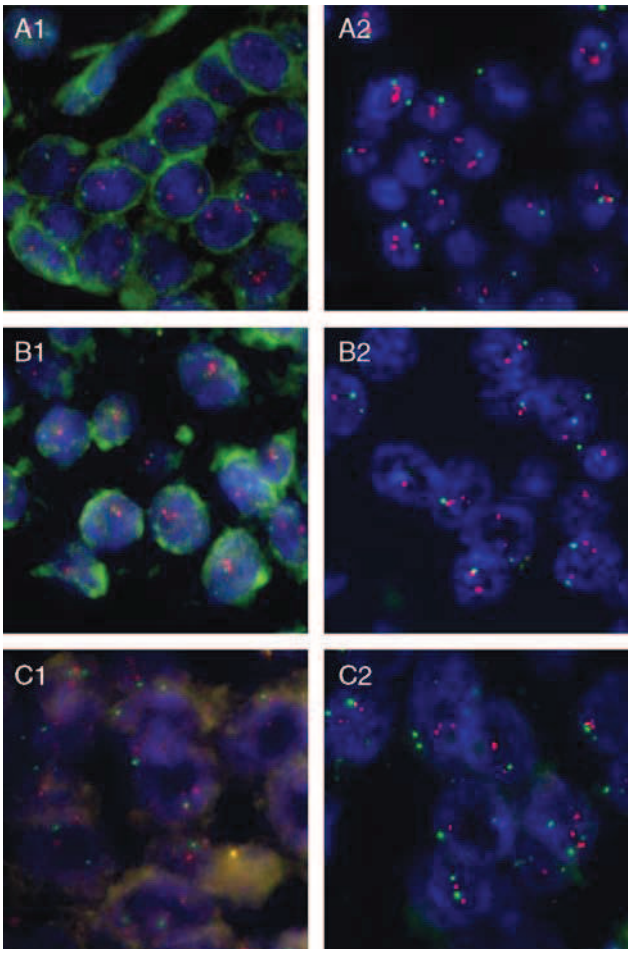

FIGURE 3. FISH images illustrating differences between conventional HER2 FISH (A1, B1, C1) and IQFISH (A2, B2, C2) of the same breast carcinoma tissue material. High fluorescence background was detected in these samples by the conventional method (left column), whereas clear signals and good morphology was obtained after IQFISH (right column). FISH indicates fluorescence in situ hybridization; HER2, human epidermal growth factor receptor 2; IQFISH, Instant Quality FISH.

for proper probe binding. The modification in the hybridization chemistry resulted in 2 major qualitative changes: both (1) reduced temperature (only 66°C) heat denaturation, and (2) short hybridization time (only 1.5 h) significantly improve sample handling and tissue morphology.

We found a 100% accordance regarding HER2 amplification and chromosome 17 copy number between the new 1-day FISH and the conventional FISH tests indicating the absence of bias between the 2 methods. The correlation between the HER2/CEN17 ratios was very good. Reaction interpretation problems were virtually absent in case of the 1-day approach in the evaluated series of cases but a high fluorescence background interfered with the FISH signal in 3 samples resulting in ambiguous values by the conventional HER2 pharmDX kit. Such technical issues are repeatedly observed in routine FISH diagnostics and are usually associated with suboptimal fixation and tissue handling. These cases could be cleared after exhaustive counting of additional 20 tumor cell nuclei and recalculation of the ratios as described. Two of these cases were found to be HER2 nonamplified with chromosome 17 disomy and 1 case was found to be HER2 amplified with chromosome 17 disomy. In contrast, the use of the IQFISH approach eliminated most of the autofluorescence and tissue damage was also much less obvious in any of the slides. These observations led us to conclude that the modified hybridization conditions favor tissue morphology and avoid the generation of autofluorescence when IQFISH was used.

Reassessment of the slides provided us with further data regarding the stability of the FISH signals during long-term storage. IQFISH performed clearly better in the signal intensity and reproducibility after 2 months storage. Although this feature seems to have relatively little value in the current setting, stability of fluorescence preparations, for example, for quality assurance purposes, is a recurrent problem and is a major argument for chromogenic FISH applications. A long-term effect of storage on IQFISH signals was not tested in the frame of our study but an improved preservation of FISH signals over a 2-month period was well stated. As an interesting observation in our setting, both the red (HER2) and the green (centromere 17) signal intensities were significantly reduced when conventional FISH approach was used. This resulted in different interpretation of the relative HER2 copy number in selected cases primarily having an impact on quality assurance approaches in the future. In this context, it is of special interest that FISH signal reading proved to be much easier after reevaluation because of enhanced fluorescence stability when IQFISH was used.

In summary, the 1-day HER2 IQFISH approach is a potent method for the determination of HER2 status

TABLE 2. Relative Copy Numbers per Cell Nucleus of HER2 and Chromosome 17 Signals Evaluated Within 24 Hours After the FISH Reaction (First Assessment) and After 2 Months Storage of the FISH Preparations at 4°C (Second Assessment)

	HER2/Nucleus (Mean ± SD, n = 40)			Chromosome 17/Nucleus (Mean ± SD, n = 40)		
	First Assessment	Second Assessment	*P*	First Assessment	Second Assessment	*P*
Conventional HER2 FISH	7.14 ± 8.04	5.32 ± 6.11	0.27	1.97 ± 0.40	1.8 ± 0.64	0.18
IQFISH	6.85 ± 7.64	5.9 ± 6.64	0.56	1.99 ± 0.44	1.98 ± 0.51	0.92

FISH indicates fluorescence in situ hybridization; HER2, human epidermal growth factor receptor 2; IQFISH, Instant Quality FISH.

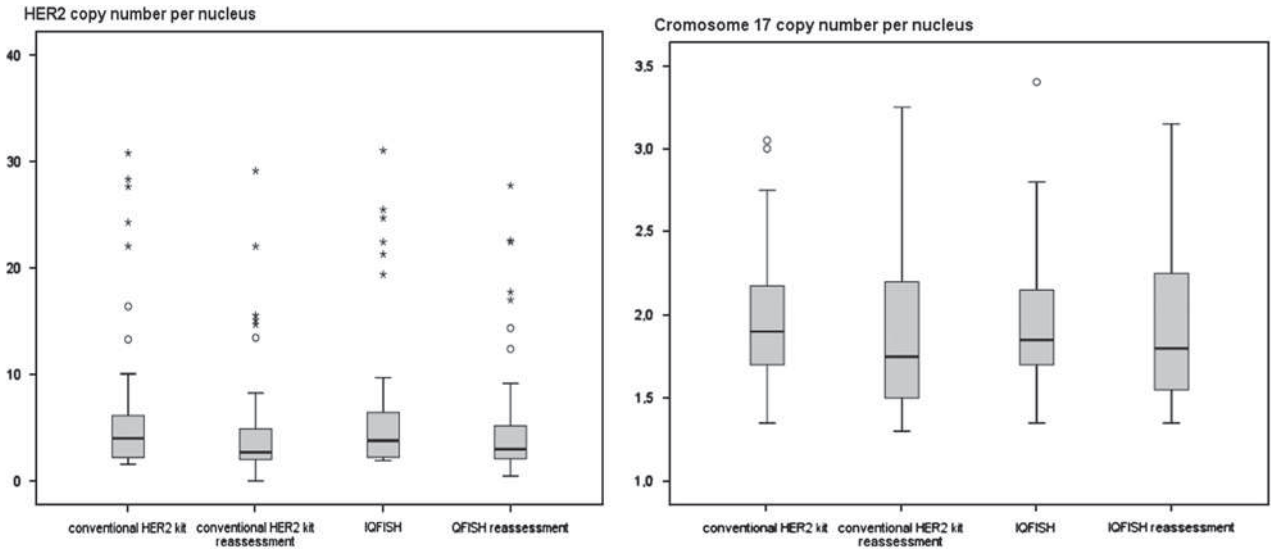

FIGURE 4. Relative mean copy numbers of HER2 and chromosome 17 signals per cell nucleus in 40 breast carcinoma samples calculated after the initial evaluation and a reassessment after 2 months storage (4°C) of the slides. At the first assessment, the mean HER2/nucleus ratio was found to be 7.14 ± 8.04 by conventional HER2 kit and 6.85 ± 7.64 by IQFISH; these values were observed as 5.32 ± 6.11and 5.9 ± 6.64 after reevaluation. Relative chromosome 17 mean copy number were 1.97 ± 0.40 using conventional kit and 1.99 ± 0.44 using IQFISH kit; these values were found as 1.8 ± 0.64 and 1.98 ± 0.51 after 2 months storage. HER2 indicates human epidermal growth factor receptor 2; IQFISH, Instant Quality fluorescence in situ hybridization.

offering some relevant advances further to the short incubation time, including enhanced tissue integrity and preserved fluorescent signal intensity when compared with the conventional HER2 FISH kit. All these features make fluorescence detection more competitive in the area of light microscopy-based in situ hybridization approaches.

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REFERENCES

1. Ross JS, Fletcher JA, Bloom KJ, et al. Targeted therapy in breast cancer: the HER-2/neu gene and protein. *Mol Cell Proteomics*. 2004;4:379–398.
2. Yang YL, Fan Y, Lang RG, et al. Genetic heterogeneity of HER2 in breast cancer: impact on HER2 testing and its clinicopathologic significance. *Breast Cancer Res Treat*. 2012;134:1095–1102.
3. Ross JS, Fletcher JA. HER-2/neu (c-erb-B2) gene and protein in breast cancer. *Am J Clin Pathol*. 1999;112(suppl 1):S53–S67.
4. Menard S, Valagussa P, Pilotti S, et al. Response to cyclophosphamide, methotrexate, and fluorouracil in lymph node-positive breast cancer according to HER2 overexpression and other tumor biologic variables. *J Clin Oncol*. 2001;19:329–335.
5. Vogel CL, Cobleigh MA, Tripathy D, et al. Efficacy and safety of trastuzumab as a single agent in first-line treatment of HER2-overexpressing metastatic breast cancer. *J Clin Oncol*. 2001;20:719–726.
6. Joensuu H, Kellokumpu-Lehtinen PL, Bono P, et al. Adjuvant docetaxel or vinorelbine with or without trastuzumab for breast cancer. *N Engl J Med*. 2006;354:809–820.
7. Lambros MB, Natrajan R, Reis-Filho JS. Chromogenic and fluorescent in situ hybridization in breast cancer. *Hum Pathol*. 2007;38:1105–1122.
8. Penault-Llorca F, Bilous M, Dowsett M, et al. Emerging technologies for assessing HER2 amplification. *Am J Clin Pathol*. 2009;132:539–548.
9. Walker RA, Bartlett JM, Dowsett M, et al. HER2 testing in the UK: further update to recommendations. *J Clin Pathol*. 2008;61:818–824.
10. Bartlett JM, Starczynski J, Atkey N, et al. HER2 testing in the UK: recommendations for breast and gastric in-situ hybridisation methods. *J Clin Pathol*. 2011;64:649–653.
11. Saez A, Andreu FJ, Segui MA, et al. HER-2 gene amplification by chromogenic in situ hybridisation (CISH) compared with fluorescence in situ hybridisation (FISH) in breast cancer—a study of two hundred cases. *Breast*. 2006;15:519–527.