

Delineation of *HER2* Gene Status in Breast Carcinoma by Silver *in Situ* Hybridization is Reproducible among Laboratories and Pathologists

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An automated enzyme metallographic silver *in situ* hybridization method (SISH) has been reported to successfully determine human epidermal growth factor receptor 2 (*HER2*) gene amplification. We evaluated the staining and interpretative reproducibility of the *HER2* SISH assay at five laboratories and compared SISH results with other *in situ* hybridization (ISH) methods. The *HER2* gene status of 89 breast carcinomas was analyzed in parallel using manual dual-color fluorescence ISH, manual chromogenic ISH, and bright-field automated SISH. A total of 1098 SISH-stained slides were evaluated. For comparison, all specimens were stained by 4B5 immunohistochemistry for *HER2* protein expression. Interpretation was performed by pathologists at five different laboratories using the algorithms provided by the manufacturers and the guidelines of American Society of Clinical Oncology/College of American Pathologists. Staining and interpretative reproducibility were measured through the computation of weighted kappa statistics. Following the optimization of SISH staining, 1077/1098 (98%) of slides were evaluable. Excellent reproducibility and efficacy of *HER2* SISH staining, and interobserver interpretation ($K_w = 0.91$), were observed among five sites. For the 89 invasive breast cancer cases, the overall rate of concordance between consensus 4B5 and consensus SISH, fluorescence ISH, and chromogenic ISH was 96.6% (86/89), 97.8% (87/89), and 96.6% (86/89), respectively. Overall concordance between positive and

negative SISH and fluorescence ISH results, as well as between individual and consensus positive and negative SISH results, was excellent ($P < 0.001$). (J Mol Diagn 2008, 10:527-536; DOI: 10.2353/jmoldx.2008.080052)

The identification of breast carcinomas that are *HER2* (*ERBB2*) amplified and potentially responsive to targeted therapies relies on accurate and reproducible clinical laboratory assessment.¹⁻⁵ Currently, *HER2* status is determined by detection of the encoded *HER2* protein by immunohistochemistry (IHC) and/or by detection of the presence or absence of *HER2* gene amplification assessed by *in situ* hybridization (ISH).⁶⁻¹² At present, fluorescence *in situ* hybridization (FISH) is the reference standard technique for the assessment of the amplification state of the *HER2* gene.

Despite attempts to improve the status of *HER2* testing in routine clinical practice, testing inaccuracy remains a major issue with both IHC and FISH.¹³⁻¹⁵ Importantly, the use of laboratory assays as the sole determinant for therapy eligibility represents a significant challenge to pathologists performing and interpreting the results. The American Society of Clinical Oncology (ASCO) and the College of American Pathologists (CAP) therefore published important guidelines regarding *HER2* testing in breast cancer.¹⁶

Recently, new bright field methods of ISH (chromogenic ISH, or CISH) have been developed.¹⁷⁻¹⁹ Preliminary studies have also illustrated a promising high-sensitivity ISH technique based on enzymatic metallography and metallic silver deposition (SISH), which has been

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used for genotyping *HER2* in breast cancer.^{20,21} In the present study, the *HER2* gene amplification status of a series of breast cancer cases was examined in parallel by using FISH, CISH, and SISH. Pathologists at five Italian National Cancer Institutes reviewed and scored the slides following the guidelines of ASCO/CAP¹⁶ to compare the genetic assays. Specific aims of the study were: 1) to demonstrate feasibility of the *HER2* SISH assay using multiple breast carcinoma samples from five sites, assessing staining efficacy, portability, and robustness; 2) to evaluate interlaboratory interpretative reproducibility of the *HER2* SISH assay at five laboratories; 3) to compare SISH results with other ISH methods; and 4) to compare gene amplification status with protein *HER2* expression.

Materials and Methods

Study Design

The first phase of the study was based on the selection of infiltrating breast carcinomas and assessment of the state of amplification of the *HER2* gene by *HER2* FISH and *HER2* CISH, followed by IHC analysis of *HER2* protein expression using the 4B5 (Ventana) rabbit monoclonal antibody. In the second phase, the same cases were tested by using *HER2* SISH. Comparison between the *HER2* bright field ISH (CISH and SISH) and with *HER2* FISH on the same cases, was performed to validate the SISH scoring systems. This phase of the study also included a test of interlaboratory interpretative reproducibility. The tests were performed by pathologists and experts (A.C., Milan; G.B., Naples; A.G., Aviano; G.S., Bari; M.T., Genoa) participating in the *HER2* evaluation study for the Oncotipo Mammario ER2 Overesprimente (OMERO) project of the Alliance Against Cancer, the Association of the Italian National Cancer Institutes. OMERO is a multicenter, retrospective study of the biology and prognosis of 1647 patients with primary breast carcinomas diagnosed in Italy in 2000/2001 and scored as *HER2*-positive by IHC. A control for each case was identified as the first consecutive patient with *HER2*-negative breast cancer. A website dedicated to the OMERO study is available at <http://www.progettomero.org> (in Italian), where newsletters compiled during the project are also available.

Cohort Selection

For the study, each of the five participating cancer institutes provided breast carcinoma cases, consecutively identified from the series of the OMERO project of Alliance Against Cancer. Cases were diagnosed and surgically treated during the years 2000 and 2001 at the five Italian national Cancer Institutes in Milan (pilot site), Aviano, Bari, Genoa and Naples (hereafter randomly referred to as sites A, B, C, D, and E).

At each site, a consecutive case selection was made to develop a cohort for which 25% cases over-expressed *HER2* (IHC score 3+ in the OMERO study) and a bal-

anced distribution of IHC score 0, 1+ and 2+ for the remaining cases. A total cohort of 94 cases was identified: 20 from site A, 19 from site B, 20 from site C, 20 from site D, and 15 from site E; 24 cases of the cases were IHC score 3+.

All cases were fixed in 4% neutral buffered formalin for 6 to 48 hours at room temperature, followed by processing and paraffin-embedding according to the consolidated standard routine practice adopted by the laboratory of the institute that provided each case.

Tissue Sectioning and Slide Preparation

At four of the five sites participating in the study, 5 μ m tissue sections were cut from paraffin blocks of the respective cases, according to the recommendations of the INFORM *HER2* SISH assay manufacturer (Ventana). As two objectives of the study were evaluation of the *HER2* SISH assay robustness and its reliability and portability in a multicentric environment at laboratories with different routine practices, tissue sections of cases from site E were cut at 3 to 4- μ m thickness.

Similarly, the sections from three of the five sites were mounted on SuperFrost ++ slides, according to Ventana's indications, but the sections of cases from sites B and E were mounted on different types of slides (ProbeOn+ and SuperFrost Polylysine respectively).

A set of 41 sequentially numbered slide samples was prepared for each of the 94 cases, and allocated for the different staining procedures: five slides (one for each site) for H&E staining, three slides for FISH (performed at Milan), three slides for CISH (performed at Aviano), five slides (one for each site) for Pathway *HER2* (clone 4B5) IHC staining, and 25 slides (five for each site) for SISH *HER2* DNA and Chr 17 protocols setup and staining.

At each site, the slides were processed in parallel for H&E staining, IHC (Pathway anti-*HER2*/neu (4B5) Rabbit Monoclonal Antibody, Ventana), and SISH (INFORM *HER2* DNA and Chromosome 17 probes and ultraVIEW SISH Detection Kit, Ventana).

The FISH (PathVysion *HER2* DNA Probe Kit, Abbott/Vysis, Downers Grove, IL) and CISH (Zymed Spot-Light *HER2* CISH Kit, Zymed Laboratories Inc., South San Francisco, CA) assays were performed and evaluated at Milan and Aviano respectively.

Staining Procedures

The FISH procedures for *HER2* were performed according to the recommendations of the probe manufacturer (Abbott/Vysis PathVysion, Downers Grove, IL), with some modifications.

Staining Procedures: FISH

Tissue sections were deparaffinized in xylene, rehydrated with graded ethanol, air dried, and heated in 5 mmol/L Tris and 1 mmol/L EDTA, pH 7.0, at 96°C for 15 minutes. Samples were pepsin digested (4% pepsin in 0.01N HCl) for 6 to 10 minutes at 37°C, with monitoring of

the progression of the enzymatic digestion using a phase contrast microscope. The slides were then washed (twice for 3 minutes each) in distilled water, dehydrated with graded ethanols and air dried. After application of the hybridization mixes (PathVysion HER2 DNA Probe Kit), the specimens were codenatured at 85°C for 1 minute, and then hybridized at 37°C overnight using Vysis Hybrite equipment.

After hybridization, the slides were washed in 2× standard saline citrate/0.3% NP-40 at 72°C for 2 minutes, air dried, and counterstained with 4,6-diamidino-2-phenylindole. A minimum of 60 nuclei of invasive tumor cells were scored using Olympus epifluorescence microscope equipped with an ×100 oil immersion objective and 4,6-diamidino-2-phenylindole/Spectrum Green/Orange single and triple bandpass filters.

The CISH procedures for HER2 were performed according to the recommendations of the probe manufacturer (Zymed Laboratories Inc., South San Francisco, CA), with some modifications.

Staining Procedures: CISH

Sections were deparaffinized and heated in a 1M NaCNS solution 95°C for 10 minutes. After two 5-minute washes at 4°C in distilled water, the slides were incubated with 3 to 10 µg/ml proteinase K (EC 3.4.21.64) (Sigma, St. Louis, MO) for 10 to 15 minutes at 37°C. The slides were then washed (twice at 5 minutes each, at 4°C) in distilled water, dehydrated with graded ethanols, and air dried. The digoxigenin-labeled HER2 probe (double-stranded) (Zymed Laboratories Inc.) was applied to the slides, covered with coverslips, and denatured at 96°C for 6 minutes on a heat block. Hybridization was performed overnight at 37°C in a humid chamber. The slides were then washed for 5 minutes with 0.5× standard saline citrate at 75°C, followed by a brief rinsing in phosphate buffered saline/0.25% Tween20. Immunodetection was performed according to the manufacturer's instructions. Finally, sections were lightly counterstained with hematoxylin.

A CISH procedure for Chr 17 was performed on parallel sections using a digoxigenin-labeled probe (ZytoDot CEN 17 probes; ZytoVision GmbH, Bremerhaven, Germany) under conditions similar to those adopted for HER2.

The SISH assay (INFORM HER2 DNA and Chr 17 probes and ultraVIEW SISH Detection Kit, Ventana) was performed on Ventana's Benchmark XT (sites A, B, C, and D) and Benchmark IHC/ISH (site E) automated platforms. Before proceeding to run the SISH assays, all of the instruments were decontaminated, calibrated, and verified by qualified personnel.

As the study was multicentric, at each site the instrument operating protocols were appropriately optimized and calibrated, following Ventana's indications, to compensate and minimize the potential heterogeneity of results, which could be generated by slight differences in the hardware performances and/or the preanalytical procedures at the five sites. Fixation conditions among the cases, aging of the case blocks, thickness of tissue sec-

tioning (site E), and tissue adhesion on different types of slides (sites B and E) were particularly considered.

Automated SISH of consecutive slides from the same paraffin blocks as for H&E were stained for the INFORM HER2 DNA and Chr 17 probes.

Staining Procedures: SISH

For HER2 and Chr 17 SISH assay processing, Ventana's Benchmark series of automated slide stainers were used. Automated SISH of consecutive slides from the same paraffin blocks as for H&E were stained for the INFORM HER2 DNA and Chr 17 probes. Both probes were labeled with dinitrophenol and optimally formulated for use with the ultraVIEW SISH Detection Kit and accessory reagents on Ventana's Benchmark series of automated slide stainers. The HER2 DNA probe was denatured at 95°C for 12 minutes and hybridization was performed at 52°C for 2 hours. After hybridization, appropriate stringency washes (three times at 72°C) were performed. The Chr 17 probe was denatured at 95°C for 12 minutes and hybridization was performed at 44°C for 2 hours. After hybridization, appropriate stringency washes (three times at 59°C) were performed. The HER2 and Chr 17 dinitrophenol-labeled probes were visualized using the rabbit anti-dinitrophenol primary antibody and the ultraVIEW SISH Detection Kit. The detection kit contains a goat anti-rabbit antibody conjugated to horseradish peroxidase used as the chromogenic enzyme. The chemistry of the SISH reaction, briefly described, is driven by the sequential addition of silver A (silver acetate), silver B (hydroquinone), and silver C (H₂O₂). Here, the silver ions (Ag⁺) are reduced by hydroquinone to metallic silver atoms (Ag). This reaction is fueled by the substrate for horseradish peroxidase, hydrogen peroxide (silver C). The silver precipitation is deposited in the nuclei and a single copy of the HER2 gene is visualized as a black dot. The specimen is then counterstained with Ventana Hematoxylin II for interpretation by light microscopy.

Staining Procedures: IHC

For automated IHC staining with Pathway anti-HER2/neu (4B5) rabbit monoclonal antibody and ultraVIEW universal diaminobenzidine detection kit (Ventana), Ventana's Benchmark series of automated slide stainers were used.

Group Training

In a 5-hour specific training session pathologists were shown the variety of staining patterns that may be observed in breast biopsies when stained with INFORM HER2 DNA and Chr 17 Probes. A set of photomicrographs allowed the readers to become familiar with the spectrum of staining patterns, including single copy staining of HER2 and Chr 17, amplified gene copies and clusters of HER2 staining, as well as staining artifacts that might be encountered.

Additionally, the images allowed the five readers to become familiar with the determination of slide adequacy, enumeration methods, and troubleshooting of the assay.

Finally, the scoring methods proposed by Ventana for the HER2 SISH assay evaluation were explained.

Determining HER2 Gene Status with SISH

To provide a scoring algorithm that fits within the workflow of a pathology laboratory and generates results that are reproducible among readers, Ventana has developed a two-part approach. Method 1 allows the reader to rapidly and semiquantitatively determine HER2 gene status for approximately 85% of all cases, which are either clearly negative or positive for HER2 gene amplification (ie, cases with an HER2/Chr 17 ratio less than 1.4 or greater than 4.0). This is obtained by analyzing the overall staining patterns of HER2 and Chr 17 within a target area. Alternatively, a reader may choose to skip Method 1 and proceed directly to Method 2, the quantitative method, for enumeration of the HER2/Chr 17 ratio.

For the minority of cases in which the HER2/Chr 17 ratio falls within the range of 1.4 to 4.0, the reader must proceed to Method 2, a quantitative method. This is recommended to ensure accuracy in determining HER2 gene status. As cited above, approximately 15% of breast carcinoma cases fall within the range of 1.4 and 4.0. Using Method 2 the reader records the quantitative enumeration of HER2 and Chr 17 signals in 20 nuclei within a target area, and calculates the HER2/Chr 17 ratio. Cases with an HER2/Chr 17 ratio lower than 1.8 are negative for HER2 gene amplification whereas cases with an HER2/Chr 17 ratio higher than 2.2 are positive for HER2 gene amplification and can be reported as such. Cases with an HER2/Chr 17 ratio that is either equal to or falls between 1.8 and 2.2 must be enumerated using Method 2A to ensure accuracy.

Using Method 2A the reader selects a second target area for each of HER2 and Chr 17 and counts the number of signals in 20 additional nuclei. The HER2/Chr 17 ratio using counts from both target areas is then calculated. A total of 40 nuclei will have been read between both target areas. The HER2/Chr 17 ratio is then calculated by dividing the total number of HER2 signals in both target areas (40 cells) by the total number of Chr 17 signals in both target areas (40 cells). Results are reported as: negative for HER2 gene amplification, defined as an HER2/Chr 17 ratio lower than 1.8; equivocal for HER2 gene amplification, defined as an HER2/Chr 17 ratio equal to or between 1.8 and 2.2; positive for HER2 gene amplification, defined as an HER2/Chr 17 ratio higher than 2.2.

Scoring

All cases were scored independently by readers as follows: HER2 FISH slides were reviewed by the reader at the FISH reference site only (Milan); HER2 CISH slides were reviewed by the reader at the CISH reference site only (Aviano); H&E, HER2 SISH, chromosome 17 (Chr 17)

SISH, and CONFIRM HER2 4B5 slides were reviewed by the readers at the sites where the slides were stained. H&E-stained slides were available to each scoring pathologist to evaluate the histological adequacy of tissue sections, and for identification of tumor areas for SISH and CISH scoring.

Evaluation was performed by pathologists at the five different laboratories according to the algorithms provided by the manufacturers and the guidelines of ASCO/CAP.

Scoring Criteria and Algorithms: FISH

According to ASCO/CAP guidelines,¹⁶ HER2 gene amplification status was classified applying the following criteria: negative for HER2 gene amplification was defined as an HER2/Chr 17 ratio lower than 1.8; equivocal for HER2 gene amplification was defined as an HER2/Chr 17 ratio between 1.8 and 2.2; positive for HER2 gene amplification was defined as an HER2/Chr 17 ratio higher than 2.2 on an average of 60 cells. The detailed evaluation was performed following the instructions provided by the manufacturer (ABBOTT/Vysis PathVysion CE Package Insert Revision I (2002)).

Scoring Criteria and Algorithms: CISH

Interpretation of CISH results was based on the interpretation guide provided by the HER2 probe manufacturer (Zymed Laboratories Inc.). Briefly, tumors were classified depending on the number of gene copies in the nuclei as (a) nonamplified, those with 1 to 5 brown intranuclear dots of HER2 gene present per nucleus in >50% of tumor cells in the chosen area for enumeration; and (b) amplified those with >6 brown intranuclear dots or when small or large clusters were seen in at least 50% of nuclei. Though Chr 17 is not part of the aforementioned interpretation guide, on equivocal cases with results at or near the cutoff point between amplification and nonamplification (ie, between 4.8 and 5.5 dots) a CISH for Chr 17 was performed to confirm/exclude polysomy, as recently recommended during an Italian Consensus Workshop on the different methods for the study of HER2 gene status (Consensus Workshop on the Use of the Different Methods for Determination of the Status of HER2. Palermo, 26–27 May 2006; manuscript in preparation).

Scoring Criteria and Algorithms: SISH

According to ASCO/CAP guidelines,¹⁶ HER2 gene amplification status was classified applying the following criteria: negative for HER2 gene amplification was defined as an HER2/Chr 17 ratio lower than 1.8; equivocal for HER2 gene amplification was defined as an HER2/Chr 17 ratio between 1.8 and 2.2; positive for HER2 gene amplification was defined as a HER2/Chr 17 ratio higher than 2.2.

A detailed evaluation was performed following the instructions provided by the manufacturer (Ventana

INFORM HER2 DNA Probe CE Package Insert 16034EN Revision 1 [2007]).

The SISH slides were read without knowledge of the HER2 results obtained by IHC and FISH. FISH results were not disclosed to the readers before a consensus diagnosis was established.

Furthermore, to better explore the relationship between SISH consensus and FISH, the pertinent quantitative results were classified in three different categories defined as follows: a category with HER2/Chr17 ratio <1.5 , representing cases negative for HER2 gene amplification; a category with HER2/Chr17 ratio >3.0 representing cases positive for HER2 gene amplification; a wide category with HER2/Chr17 ratio between 1.5 and 3.0, including also the equivocal cases.

Scoring Criteria and Algorithms: IHC

According to ASCO/CAP guidelines,¹⁶ HER2 protein expression status was classified applying the following criteria: negative for HER2 protein was an IHC staining of 0 or 1+ with no staining or weak, incomplete membrane staining in any proportion of tumor cells; equivocal for HER2 protein was an IHC staining of 2+ with complete membrane staining either non-uniform or weak in intensity but with obvious circumferential distribution in at least 10% of cells; positive for HER2 protein was an IHC staining of 3+ with uniform intense membrane staining of $>30\%$ of invasive tumor cells.

Reproducibility Analyses

Reproducibility between each laboratory and the consensus (see below) as well as the interlaboratory reproducibility related to the clinical interpretation of both HER2 SISH and IHC assay were evaluated by the computation of the weighted kappa statistic (K_w).²² This approach makes it possible to adjust the observed agreement for chance by making allowance for the relative seriousness of disagreement (ie, the distance between the categories). K_w values lie between zero (absence of agreement) and 1 (absolute agreement). Observed values of K_w were considered satisfactory if equal to or greater than 0.80. Only samples that were fully assessed by all of the five sites were included in the reproducibility analyses.

Consensus, Resolution of Discrepant Cases and Comparison between Methods

To compare SISH with the other ISH methods and gene amplification status with protein HER2 expression, a consensus value (consensus) was defined for both SISH and IHC methods as the agreement of three or more members representing either a simple or greater majority. Resolution of discrepancies and consensus were discussed during a common microscopy session, which included slide reviews of the discrepant samples stained at the five sites. Finally FISH results were compared with

CISH results. Comparisons between methods results were performed by the Fisher exact test.

Results

Case Evaluation

Of the 94 cases entering the study, four cases were not assessable with any of the three different ISH techniques (FISH, CISH, and SISH), and therefore were excluded. Another case was not assessable due to absence of adequate SISH signals and was excluded from the study.

FISH and SISH results were scored according to ASCO/CAP guidelines,¹⁶ interpretation of CISH results was based on the interpretation guide provided by the HER2 probe manufacturer (see also Materials and Methods).

Interlaboratory SISH Staining

Assessment of Efficacy

Analysis of the interlaboratory assessment of staining efficacy performed on 89 cases at 5 sites with different routine practices ranged from 93.3% to 98.9%, and revealed that 429 SISH stained cases of 445 (96.4%) were assessable with HER2 SISH. Every case with an inadequate HER2 DNA or CHR 17 slide sample was considered not assessable.

Assessment of Portability

The SISH assay interlaboratory portability, expressed as the number of cases successfully processed and assessed through five, four, three, or two sites, demonstrated that 76 of 89 cases (85.4%) were fully assessed by all of the five sites, independently of their routine practices; 11 cases (12.4%) were assessable in four of the five sites; 1 case (1.1%) was assessable in three sites; and one case (1.1%) was assessable in two sites.

Assessment of Robustness

Our data demonstrated that within a total set of 1098 slides processed with the HER2 SISH assay (HER2 DNA and CHR 17 probes) the most recurring issue during protocol optimization was excessive background (101 samples, corresponding to 9.2%), followed by poor morphology (68 samples, corresponding to 6.2%). Tissue loss from slides and absence of signal occurred less frequently (each point on 25 samples, each corresponding to 2.3%). After protocol optimization, the samples inadequate due to excessive background decreased significantly to one slide (0.1%); the morphological inadequacy was still present on three slides (0.3%). The slide inadequacy after protocol optimization due to tissue fall was reduced to three slides (0.3%), and the lack of signal decreased to 21 slides (1.3%) (see Table 1). Thus, after

Table 1. Inadequacy Issues Found during SISH Automated Protocol Optimization

Problem occurred during protocol optimization	No. of slides repeated during protocol optimization		No. of inadequate slides after protocol optimization	
		%		%
Excessive background	101	9.2%	1	0.1%
Morphology issues	68	6.2%	3	0.3%
Tissue fall	25	2.3%	3	0.3%
Inadequate SISH signal	25	2.3%	14	1.3%
Total # of inadequate slides	219	20.0%	21	2.0%

In total, 1098 slides were processed for HER2 SISH assay, including both HER2 DNA and Chromosome 17 SISH probes.

staining optimization, 1077 of 1098 SISH stained slides (98%) were evaluable.

SISH Reproducibility Analyses

Assessment of interobserver reproducibility was determined by performing all possible pairwise comparisons. Our results showed that in general the level of agreement was excellent (Supplemental Table 1 at <http://jmd.amjpathol.org/>), with a median K_w value of 0.91 (range, 0.75 to 1.00). For each laboratory a median K_w value greater than 0.80 was observed (Supplemental Table 2 at <http://jmd.amjpathol.org/>).

Table 2 details results of SISH *HER2* gene status obtained from 89 invasive breast cancer cases and the corresponding 429 SISH assays assessable at the five laboratories. The agreement between SISH consensus and individual site SISH assessment ranged from 97.6% to 100% corresponding to K_w values ranging between 0.86 and 1.00.

Among the 429 SISH assessable assays, there were discrepant results relating to two cases. Case 1 was equivocal by SISH (see Figure 1). It was equivocal also for the other ISH methods (FISH and CISH) (Table 3). Case 2, which showed high polysomy, revealed the following discrepancies: no *HER2* gene amplification for the readers at sites B and D, *HER2* gene amplification for the readers at sites C and E, and equivocal for the reader at site A.

Comparison of SISH Results with Other ISH Methods

By using the Fisher exact test we found a statistically significant association ($P < 0.001$) between individual

site SISH assessment and each of the other considered ISH methods (FISH and CISH) (Table 3).

By comparing in the 89 cases SISH consensus with FISH and CISH, 88 (98.9%) and 87 (97.8%) cases were classified in the same categories, respectively (Figure 2 and Supplemental Figure 1 at <http://jmd.amjpathol.org/>).

By comparing in the 89 cases FISH with CISH assay, 88 (98.9%) cases were classified in the same categories.

Challenging Cases

Table 4 summarizes aberrant ISH patterns. Fourteen cases (15.7%) showed aberrant ISH patterns. These included samples with a heterogeneous tumor population and with Chr 17 aneusomy (monosomy or polysomy). One case showed a very small clone of invasive cancer cells (<5%) with high amplification levels (>21 copies) of *HER2*, whereas the remaining tumor cells had two normal centromere 17 signals each, and only one copy of the *HER2* gene. When the signals from all of the nuclei of this sample were averaged, the ratio was lower than 2.0. Also, nine cases were observed with incidence of extra copies of *HER2* in approximately 50% of the nuclei of the cell population, associated with low or high polysomy of Chr 17. Three cases showed monosomy of Chr 17: in each cell only one signal for *HER2*, and only one signal of centromere 17 were seen. Finally, one case showed in each cell a cluster of innumerable *HER2* signals and, at the same time, clusters of centromere 17 signals. The formal *HER2*/CEP17 ratio score was 1, even though the number of *HER2* signals was consistent with amplification but the *HER2*/CHR17 ratio did not reflect the real *HER2* status. A normal pattern of endogenous *HER2* and CHR17 signals was present.

Table 2. Concordance of Individual Site SISH Assessment Versus Consensus SISH

Individual site SISH assessment	Site A		Site B		Site C		Site D		Site E		Total	
	Assays	%	Assays	%	Assays	%	Assays	%	Assays	%	Assays	%
Concordance versus consensus SISH												
Concordant cases	86	97.7%	85	100.0%	84	98.8%	87	98.9%	81	97.6%	423	98.6%
Discordant cases	2	2.3%	0	0.0%	1	1.2%	1	1.1%	2	2.4%	6	1.4%
Cases not assessable	1	1.1%	4	4.5%	4	4.5%	1	1.1%	6	6.7%	16	3.6%
K_w	1.00		1.00		1.00		0.86		1.00		1.00	

The discordant cases include both discrepant and equivocal assays. According to ASCO/CAP guidelines, equivocal cases could not be unequivocally assessed as amplified or nonamplified at one or more sites. Discordant cases showed discordant assessments at the sites. The assays not assessable by SISH were 16 in total (3.6%).

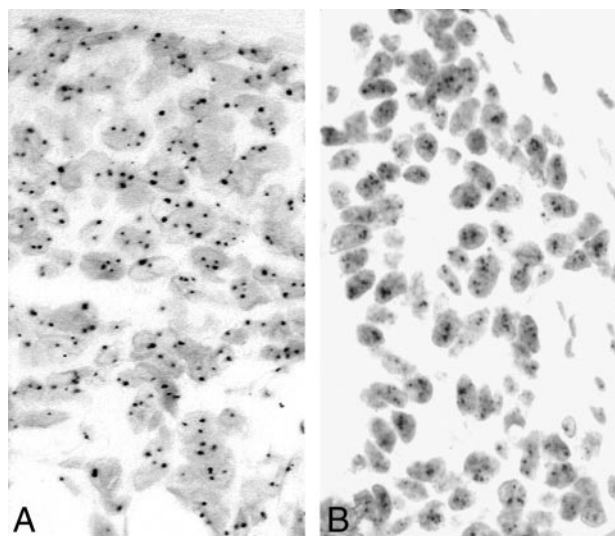


Figure 1. A and B: This case is equivocal for SISH *HER2* gene amplification. Note that chromosome 17 is polysomic. **A:** In the nuclei there are two or more copies of chromosome 17. The corresponding FISH and CISH results were equivocal (not shown).

Table 5 reports SISH consensus results and FISH *HER2* results obtained by considering three different categories defined by a *HER2*/Chr17 ratio <1.5, between 1.5 and 3.0 and >3.0. In this case the overall concordance rate was 91.0%. The highest concordance rate was observed for the categories with ratio <1.5 and >3.0 (95.0% and 90.9% respectively) whereas for the category with ratio ranging from 1.5 and 3.0 was observed the lowest concordance rate (57.1%).

IHC Assay Reproducibility Analyses

The results of 4B5 antibody immunostaining obtained from the examined 89 invasive breast cancer cases and the corresponding 439 immunostaining assays assessable at the five laboratories showed that the interobserver interpretative reproducibility was excellent ($K_w = 1.00$) for all of the considered pairwise comparisons. As regards the comparison between the 4B5 IHC consensus value and individual site 4B5 assessment, our results showed that the level of excellent was satisfactory for all sites ($K_w = 1.00$). Among the 439 assessable IHC assays, there was only one equivocal case (in terms of

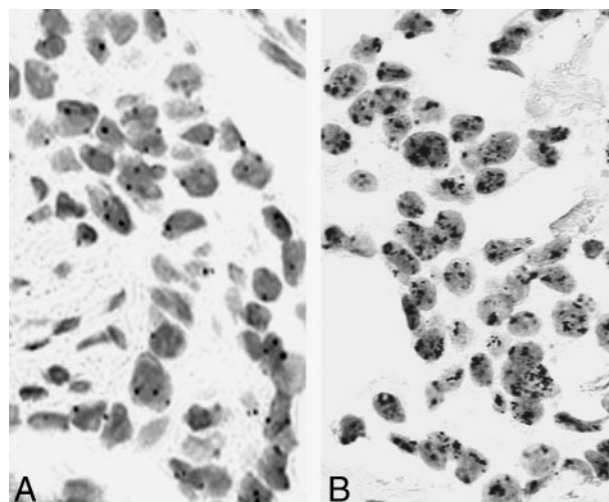


Figure 2. The figure shows a typical example of an amplified case as demonstrated by SISH. **A:** Less than 3 chromosome 17 signals in tumors cells can be seen. **B:** same case, *HER2*. Clusters of *HER2* signals in tumor cells are recognizable.

HER2 protein expression), with discrepant results. The case analysis revealed the following scoring disagreement: no *HER2* protein overexpression for the readers at sites A and D, *HER2* protein overexpression for the readers from sites C and E, and sample not assessable for the reader at site B.

Comparison between Gene Amplification Status and *HER2* Protein Expression

By using the Fisher exact test we found a statistically significant association ($P < 0.001$) between *HER2* protein expression detected by IHC with 4B5 antibody and gene amplification status obtained by each of the ISH methods (Supplemental Figure 2 at <http://jmd.amjpathol.org/>).

By considering the 89 invasive breast cancer cases, the overall rate of concordance between consensus 4B5 and consensus SISH, FISH, and CISH was 96.6% (86/89), 97.8% (87/89), and 96.6% (86/89), respectively (Supplemental Table 3 at <http://jmd.amjpathol.org/>).

When consensus 4B5 was compared to FISH, two cases were discrepant: the afore mentioned case (discrepant 4B5, FISH-amplified), and case 1, previously described, which resulted equivocal for all three molec-

Table 3. Individual Site SISH Results Versus FISH and CISH Results

Individual site SISH assessment	Site A			Site B			Site C			Site D			Site E		
	P	EQ	N	P	EQ	N	P	EQ	N	P	EQ	N	P	EQ	N
FISH															
P	22	0	0	20	0	0	22	0	0	22	0	0	19	0	0
EQ	1	0	0	0	0	1	0	0	1	0	0	0	1	0	0
N	0	1	64	0	0	64	1	0	61	0	1	65	1	0	62
P	22	0	0	20	0	0	22	0	0	22	0	0	19	0	0
CISH															
EQ	1	0	1	0	0	1	0	0	1	0	0	1	1	0	1
N	0	1	63	0	0	64	1	0	61	0	1	64	1	0	61
Cases not assessable		1			4			4			1			6	

EQ, equivocal for *HER2* gene amplification; N, negative for *HER2* gene amplification; P, positive for *HER2* gene amplification.

Table 4. Challenging Cases with Aberrant Genetic Patterns

Case origin	Aberrant ISH patterns
Site A	High polysomy 17
Site A	High polysomy 17; heterogeneous; small number of cells with amplification
Site A	Monosomy
Site B (*)	Low polysomy 17; heterogeneous; low amplification
Site B	High polysomy 17
Site B	High level HER2 amplification in a very small area of invasive tumor
Site B	High level HER2 amplification with centromeric sequences included
Site B	Monosomy
Site B	Monosomy
Site C	High polysomy 17
Site C (*)	High polysomy 17
Site D (*)	High polysomy 17; heterogeneous; low amplification?
Site D	Polysomy 17; about 50% of cells with low amplification
Site E	High polysomy 17; small number of cells with amplification

Three cases (*) were also equivocal.

ular methods (FISH, CISH, and SISH), and showed a consensus on non-overexpression of *HER2* protein detected by IHC with 4B5 antibody.

Discussion

In the present study, the *HER2* gene amplification status of a series of breast cancer cases, which were diagnosed at five separate laboratories, was examined in parallel by using FISH, CISH, and SISH. Results of the study demonstrated interlaboratory staining efficacy of the *HER2* SISH assay on samples from five sites. Overall, the results of the study indicated good efficacy and portability, and excellent robustness of the *HER2* SISH assay in the interlaboratory environment.

From a performance standpoint, the overall assessment of efficacy, evaluated on a heterogeneous set of cases surgically treated in 2000 and 2001 at different cancer institutes and processed at five different sites, was excellent (96.4%). The strict application of the most recent ASCO/CAP¹⁶ guidelines to more standardized preanalytical routine practices should further increase the efficacy rate for the assay.

Table 5. SISH Consensus Versus FISH for Three Different Categories Defined by a *HER2*/Chr17 Ratio <1.5, between 1.5 and 3.0, and >3.0, Respectively

	Consensus SISH			Total
	Ratio <1.5	Ratio 1.5–3.0	Ratio >3.0	
FISH				
Ratio <1.5	57	3	0	60
Ratio 1.5–3.0	3	4	0	7
Ratio >3.0	1	1	20	22
Total	61	8	20	89

The full interlaboratory portability of the assay was 85.4%, expressed as the percentage of cases that were successfully processed and scored in all of the five sites involved in the study; the assay portability through four sites out of five was 12.4%. In total, high portability features were found on 97.8% of the cases, whereas poor portability occurrences accounted for 2.2%. These figures are compatible with a definition of the *HER2* SISH assay as portable and reproducible in a multicentric context involving laboratories with different routine practices.

As regards assay robustness, when applying the standard initial operating protocol (protease III for 8 minutes) evaluated on the complete set of 1098 slides processed revealed an initial 80% staining success rate. The major sources of inadequate staining were excessive background and the morphological alterations of the tissue sections (15.4% total inadequacy). Inadequacies due to tissue loss and absence of assessable SISH signals had a minor impact on the study (4.6% total inadequacy). For slides failing initial staining, the manufacturer's recommendation for staining a second slide using protease II for four minutes, which improved the successful staining rate to 98%. This improvement was particularly effective for resolving excessive background and morphological alterations (rate after protocol optimization 0.4%). These two issues proved to be inversely correlated with the strength of the enzymatic digestion step, and indicated that when the protocol optimization is carefully matched to fixation and processing conditions, the percentage of slide staining failures is very low.

Inadequate SISH staining for the 1.6% of samples inadequate after protocol optimization was attributable to tissue loss and absence of endogenous *HER2* and/or CHR17 signal; both depend on laboratory preanalytical variables, and do not reflect the robust performance of the SISH assay.

Results of our study showed high SISH interobserver reproducibility at the five laboratories. In fact, the K_w values related to interobserver reproducibility between the consensus SISH and individual site SISH results ranged from 0.86 to 1.00. A similar high level of agreement was found in the two previous studies that examined interobserver interpretation of SISH *HER2* status.^{20,23} These observations, together with the present high level of reproducibility, validate the reliability of the SISH method for assessing *HER2* status. Only two cases proved discrepant among the pathologists at the five laboratories. Discrepancy in observers' SISH scoring among these cases was attributable to CHR17 polysomy. One of these cases was equivocal for *HER2* gene amplification by SISH, FISH, and CISH.

Notably, there was excellent agreement among SISH, CISH, and FISH with regard to *HER2* status assessment (Fischer exact test, $P < 0.001$). Discrepancy in results between SISH and FISH was encountered in two cases with polysomy of CHR 17, which resulted in SISH scores of low-level amplification/equivocal, whereas the corresponding FISH results showed no amplification. The rate of concordance between the CISH and FISH results was excellent (88/89, 98.9%), as also shown by previous studies.^{24–29} Also the rate of concordance between SISH

consensus and CISH results was excellent (87/89, 97.8%). However, the rate of concordance between SISH and FISH was lower for an intermediate group of cases with *HER2*/Chr ratio between 1.5 and 3.0 (see Table 5). While SISH may provide what is needed for clinical decisions about Herceptin therapy, SISH would not be able to provide very much information about the actual degree of amplification as is provided by FISH. Some papers suggest that relatively low levels of amplification, as low as 1.5, (that may not be easily observed by SISH or not be reproducibly quantitated by SISH) may predict response to Herceptin.³⁰

Challenging cases with monosomic and polysomic CHR 17, *HER2* monoallelic deletion, and genotypic heterogeneity comprised 15.7% of the evaluated cases. These aberrant ISH patterns were encountered in all of the cases with a major scoring discrepancy and in three equivocal cases (Table 4). Most cases with aberrant ISH patterns (9/14) showed extra copies of *HER2* associated with polysomy of CHR17. Some investigators have interpreted this level of *HER2* copy number elevation as a low level amplification^{31–33} or as a duplication of *HER2*.³⁴ Nuclei with extra copies of *HER2* frequently contained from 2 to 9 *HER2* signals and from 1 to 4 CHR17 signals. These cells were uniformly distributed within the tumor population. We assume that these tumor cells are in the S phase of the cell cycle, and that duplication at the 17q *HER2* sequence has already taken place, whereas the relative DNA sequences of centromere 17 will duplicate later, or this pattern may represent real tumor heterogeneity.

Using FISH as the reference standard, it has recently been shown that the anti-*HER2* 4B5 rabbit monoclonal antibody provides high sensitivity, specificity, and interlaboratory reproducibility for the detection of *HER2* status in breast cancer.³⁵ In our study, 4B5 demonstrated excellent interlaboratory reproducibility for the detection of *HER2* status for each performed comparison ($K_w = 1.00$). The level of agreement between the consensus 4B5 and individual site assessment was satisfactory for all sites ($K_w = 1.00$). Moreover, there was statistically significant association of FISH, CISH, and SISH results with *HER2* protein expression. These conclusions must also be tempered by the IHC preselection inclusion criteria for the samples used in this study.

Based on the results of this study, methods of bright field ISH—SISH and CISH—may be implemented in many routine pathology laboratories. Due to the stable metallic silver signal as a permanently archived slide, a cell-by-cell discussion to achieve consensus using a multithread microscope can be facilitated for difficult cases.

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