Comparative Performance of Breast Cancer Human Epidermal Growth Factor Receptor 2 Fluorescence In Situ Hybridization and Brightfield In Situ Hybridization on College of American Pathologists Proficiency Tests

Katherine B. Geiersbach, MD; Julia A. Bridge, MD; Michelle Dolan, MD; Lawrence J. Jennings, MD, PhD; Diane L. Persons, MD; Rhona J. Souers, MS; Karen D. Tsuchiya, MD; Patricia H. Vasalos, BS; Joel T. Moncur, MD, PhD

• ***Context.*—Fluorescence in situ hybridization (FISH) and brightfield in situ hybridization (ISH) are 2 clinically approved laboratory methods for detecting ERBB2 (HER2) amplification in breast cancer.**

***Objective.*—To compare the performance of FISH and brightfield ISH on proficiency testing administered by the College of American Pathologists Laboratory Accreditation Program.**

***Design.*—Retrospective review was performed on 70 tissue core samples in 7 separate proficiency testing surveys conducted between 2009 and 2013.**

***Results.*—The samples included 13 consensus-amplified tissue cores, 53 consensus-nonamplified cores, and 4 cores that did not reach consensus for FISH and/or brightfield ISH. There were 2552 individual responses for FISH and 1871 individual responses for brightfield ISH. Consensus response rates were comparable for FISH (2474 of 2524; 98.0%) and brightfield ISH (2135 of 2189; 97.5%). The FISH analysis yielded an average HER2 copy number per cell that was significantly higher (by 2.86; $P = .02$) compared with brightfield ISH for amplified cores. For nonamplified cores, FISH yielded slightly, but not significantly, higher (by 0.17; $P = .10$) HER2 copy numbers per cell. There was no significant difference in the average HER2 to control ratio for either consensus-amplified or consensus-nonamplified cores. Participants reported "unable to analyze" more frequently for brightfield ISH (244 of 2453; 9.9%) than they did for FISH (160 of 2684; 6.0%).**

***Conclusions.*—Our study indicates a high concordance rate in proficiency testing surveys, with some significant differences noted in the technical performance of these assays. In borderline cases, updated American Society of Clinical Oncology/College of American Pathologists cutoff thresholds that place greater emphasis on HER2 copy number per cell could accentuate those differences between FISH and brightfield ISH.**

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From the Department of Laboratory Medicine and Pathology, Mayo Clinic, Rochester, Minnesota (Dr Geiersbach); the Department of Pathology and Microbiology, University of Nebraska Medical Center, Omaha (Dr Bridge); the Department of Laboratory Medicine and Pathology, University of Minnesota Medical School, Minneapolis (Dr Dolan); the Department of Pathology, Ann & Robert H. Lurie Children's Hospital of Chicago, Illinois (Dr Jennings); the Department of Pathology and Laboratory Medicine, University of Kansas Medical Center, Kansas City (Dr Persons); the Departments of Biostatistics (Ms Souers) and Proficiency Testing (Ms Vasalos), College of American Pathologists, Northfield, Illinois; the Department of Laboratories, Seattle Children's Hospital, Seattle, Washington (Dr Tsuchiya); and the Department of Pathology, Walter Reed National Military Medical Center, Bethesda, Maryland (Dr Moncur).

The authors are either College of American Pathologists (CAP) employees or are members/past members of the CAP Cytogenetics Resource Committee or the CAP Molecular Oncology Committee. The authors have no relevant financial interest in the products or companies described in this article.

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Corresponding author: Joel T. Moncur, MD, PhD, Department of Pathology, Walter Reed National Military Medical Center, 8901 Wisconsin Ave, Bldg 9, Basement, Room 0849, Bethesda, MD 20889 (email: joel.t.moncur.mil@mail.mil).

Amplification or overexpression of the human epidermal growth factor receptor 2 (*ERBB2*, *HER2*) gene is an important oncogenic driver in up to 20% to 25% of breast cancers. Breast cancers with *HER2* amplification or HER2 overexpression tend to have a higher histologic grade, and before the use of HER2-targeted therapy, HER2$^+$ cancers had a poor prognosis.[1] The first HER2-targeted therapy, trastuzumab (Genentech, South San Francisco, California), was introduced in 1998, and early clinical trials demonstrated the efficacy of trastuzumab for treating HER2$^+$ breast cancers.[2–9] Most HER2$^+$ breast cancers demonstrate overexpression of the HER2 tyrosine kinase receptor protein on the cell surface; that overexpression is driven at the DNA level by amplification of the *HER2* gene.[10] HER2 overexpression by immunohistochemistry was found to be closely correlated with a high copy number (amplification, usually >10 copies of *HER2* per cell) in several fluorescence in situ

hybridization (FISH) studies in the 1990s.[11,12] Early clinical trials from the late 1990s, which led to the US Food and Drug Administration (FDA) approval of trastuzumab in 2006, used immunohistochemistry and FISH testing to determine eligibility for targeted therapy.

In an effort to provide guideline recommendations for when and how to test for overexpression and/or amplification of *HER2*, a joint committee of the College of American Pathologists (CAP) and the American Society of Clinical Oncology (ASCO) convened and published a guideline for HER2 testing in breast cancer in 2007.[13] New test methods have emerged since the 2007 guideline was released. One of those new methods is brightfield in situ hybridization (ISH), and several FDA-approved brightfield ISH assays are now available. In the 2013 update to the original ASCO/CAP guideline, the joint committee approved brightfield ISH for evaluating HER2 status in breast.[14] Moreover, FISH and brightfield ISH are interpreted using the same criteria, and several studies have indicated similar performance with these methods.[15–29] However, some studies suggest that brightfield ISH might be less sensitive for detecting low-level amplification and might have a lower concordance than FISH in challenging samples.[15,19,30,31]

Since 2007, the CAP Laboratory Accreditation Program has required that laboratories performing HER2 testing on breast cancer participate in external proficiency testing, according to ASCO/CAP guidelines. The Cytogenetics Resource Committee, comprising members of CAP and the American College of Medical Genetics and Genomics, has administered proficiency testing surveys for *HER2* FISH on formalin-fixed, paraffin-embedded breast cancer tissue since 2000.[32] The CAP Molecular Oncology Committee has offered *HER2* proficiency testing for brightfield ISH since 2009. We compared the performance of FISH and brightfield ISH on samples that were shared between these proficiency testing surveys. The samples used for proficiency testing during the study period were selected to represent routine cases that had unambiguously negative or positive results for *HER2* amplification.

MATERIALS AND METHODS

This study includes data from CAP *HER2* proficiency testing surveys from 2009 to 2013 for FISH (survey designation, CYH) and brightfield ISH (survey designation, ISH2). The CAP offered the CYH and ISH2 surveys biannually in the form of 2 tissue-microarray slides; each slide had 5 cores of formalin-fixed, paraffin-embedded breast cancer tissue and 1 negative control (nonneoplastic liver tissue). The CAP randomly assigned tissues from 1 of 4 material groups (A–D) to CYH survey participants, and the CAP assigned tissue from material group D to all ISH2 survey participants. Of 9 CYH and 9 ISH2 surveys from 2009 to 2013 conducted before the release of the 2013 updated ASCO/CAP guideline, 7 (78%) shared material between CYH (Material Group D) and ISH2 and were compared. Survey participants provided the mean number of *HER2* signals per nucleus, the ratio of *HER2* to the chromosome 17 control (centromere) signals (if applicable), and an interpretation of *not amplified*, *equivocal*, or *amplified* according to the 2007 ASCO/CAP guideline. Consensus for each tissue core required a minimum 80% participant agreement on the interpretation of nonamplified, equivocal, or amplified. This study focused on survey performance for FDA-approved probes, excluding results from 2 participating laboratories that used laboratory-developed probe sets.

Stepwise logistic-regression models were used to analyze survey performance according to the consensus interpretation. The initial model included a length-of-participation factor, but because that factor was not significant, it was excluded from the final stepwise logistic-regression model. The model was fit with 4 factors: (1) method (FISH or brightfield ISH), (2) annual test volume (5 levels), (3) amplification status (amplified or not amplified), and (4) probe (single or dual color). The model also included a strata specification to compare the specimen/core results between CYH and ISH2. In addition, the CYH and ISH2 average *HER2* copies/cell and the average *HER2* to control ratios were tested for systematic positive or negative differences with the sign test. A significance level of $\alpha = 0.05$ was used for those analyses. Statistical analyses were performed using SAS 9.3 software (SAS Institute, Cary, North Carolina).

RESULTS

The analysis included a total of 70 breast cancer tissue samples from 14 surveys (CYH [material group D] and ISH2 09B, 10B, 11A, 11B, 12A, 12B, 13A). There were 13 consensus-amplified tissue samples, 53 consensus-nonamplified tissue samples, and 4 tissue samples that did not reach consensus for CYH and/or ISH2 surveys. One tissue sample did not reach consensus for CYH, 2 did not reach consensus for ISH2, and 1 did not reach consensus for either survey. The cumulative results of the study period included 2552 individual responses to FISH (CYH) challenges and 1871 individual responses to brightfield ISH (ISH2) challenges. The difference in the number of responses for FISH versus brightfield ISH was primarily due to differences in participant enrollment. During the 5-year period, 245 to 300 laboratories participated in the CYH surveys, 32 to 56 of which received material group D in any given survey. Participation in the ISH2 survey grew from 20 to 63 laboratories during that 5-year period (Figure 1).

The overall rate of consensus interpretations was comparable for FISH and brightfield ISH (Table 1). Likewise, there was no significant difference in consensus rates based on dual-color versus single-color probe strategy. The duration of participation in CAP surveys was included in the logistic-regression models, and there were no statistically significant performance differences based on that factor.

Although the overall performance of FISH and brightfield ISH was similar, FISH tended to yield higher average *HER2* copies per cell for consensus-amplified cores; on average, the *HER2* copy number per cell was higher for FISH by 2.86 ($P = .02$). For nonamplified cores, the average *HER2* copy number was slightly, but not significantly, greater for FISH than brightfield ISH; on average, the *HER2* copy number per cell was higher for FISH by 0.17 ($P = .10$) (Figure 2; Table 2). For the average *HER2* to control ratios, there was no significant difference between FISH and brightfield ISH for either amplified or nonamplified cores (Figure 3; Table 2). Only 4 cores failed to reach consensus; in those cases, FISH assays showed a higher average number of *HER2* signals per cell and 3 of 4 (75%) showed a higher average *HER2* to control ratio than did brightfield ISH on the same sample.

Participant responses of "unable to analyze" were more frequent for brightfield ISH (244 of 2453; 9.9%) than they were for FISH (160 of 2684; 6.0%). Data obtained from participant results indicated that an "absent or weak probe signal" was the most common reason specimens examined by either method could not be analyzed. The percentage of unsatisfactory samples affected by that issue was greater for brightfield ISH (124 of 239; 51.9%) versus FISH (62 of 159; 39.0%) for those participants who provided a reason why a sample could not be analyzed.

The proficiency testing summarized here was performed from 2009 to 2013; during which time, the 2007 ASCO/CAP guideline was in effect. To compare the performance of

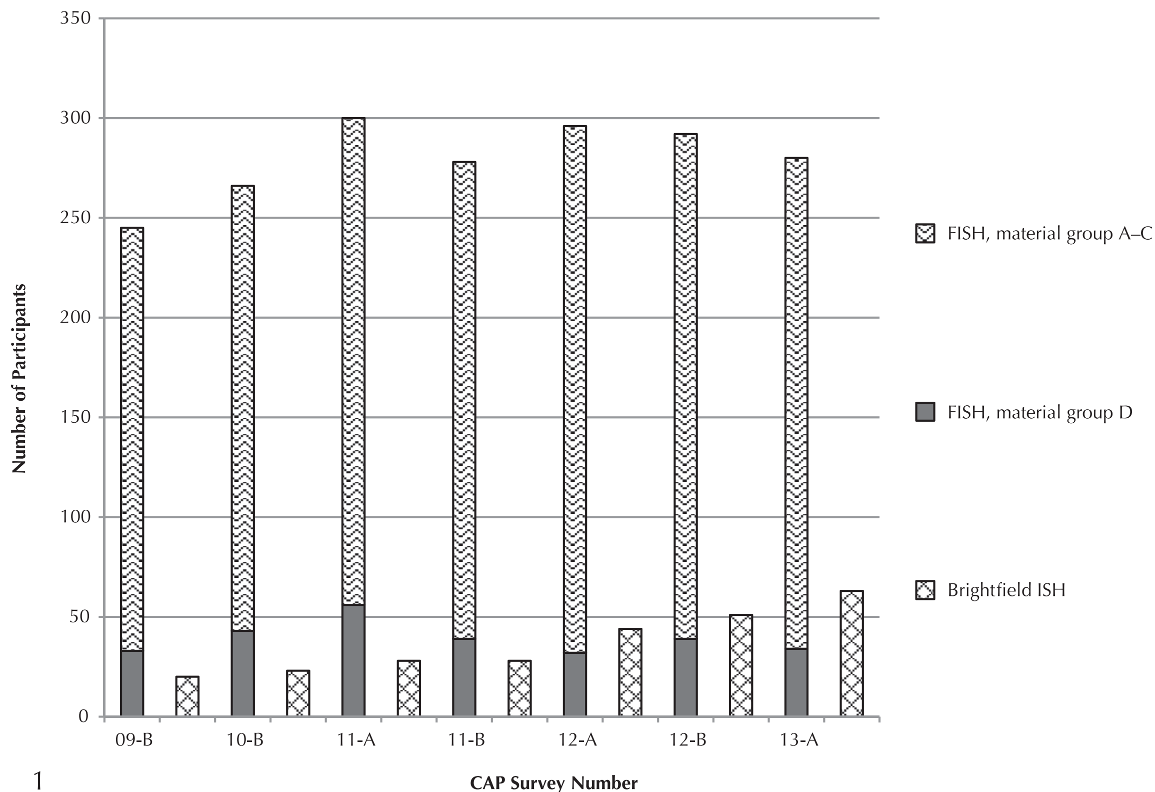

Figure 1. *Number of laboratories participating in College of American Pathologists (CAP) surveys for HER2 fluorescence in situ hybridization (FISH) and brightfield in situ hybridization (ISH) for surveys included in the study. The tissue cores included in the study were from material group D and were shared between FISH and brightfield ISH surveys.*

FISH and brightfield ISH according to the 2013 ASCO/CAP guideline, we performed a scoring simulation based on the participant data for the *HER2* to control ratio and on average *HER2* copies per cell for all tissue samples, including both consensus and nonconsensus samples (Table 3). In cases in which the classification changed, most changes were from *not amplified* to either *equivocal* or *amplified*, or from *equivocal* to *amplified*, reflecting the changes in the cutoff values instituted in the 2013 ASCO/CAP guideline update.

DISCUSSION

This study evaluated CAP proficiency testing data generated by more than 300 accredited laboratories participating in FISH and brightfield ISH surveys for *HER2* in breast cancer. The breast cancer tissue samples used for proficiency testing were selected to represent routine, nonamplified or amplified cases typically encountered in clinical laboratory practice.

Table 1. Fluorescence In Situ Hybridization (FISH) and Brightfield In Situ Hybridization (ISH) Consensus Rates by Method, *HER2* Status, Probe Type, and Laboratory Testing Volume

Factor	FISH		Brightfield ISH		Wald χ^2 *P* Value
	Responses, No.	Consensus No. (%)	Responses, No.	Consensus No. (%)	
Method	2524	2474 (98.0)	2189	2135 (97.5)	.15
Consensus *HER2* interpretation					.99
Amplified	532	519 (97.6)	460	452 (98.3)	
Not amplified	1992	1955 (98.1)	1729	1683 (97.3)	
Equivocal	0	—	0	—	
Probe type					.99
Dual	2297	2249 (97.9)	1777	1740 (97.9)	
Single	37	37 (100.0)	351	341 (97.2)	
Volume					.14
<100	595	583 (98.0)	617	593 (96.1)	
100–199	509	500 (98.2)	369	366 (99.2)	
200–299	313	303 (96.8)	262	259 (98.9)	
300–500	370	360 (97.3)	250	240 (96.0)	
>500	663	654 (98.6)	578	566 (97.9)	

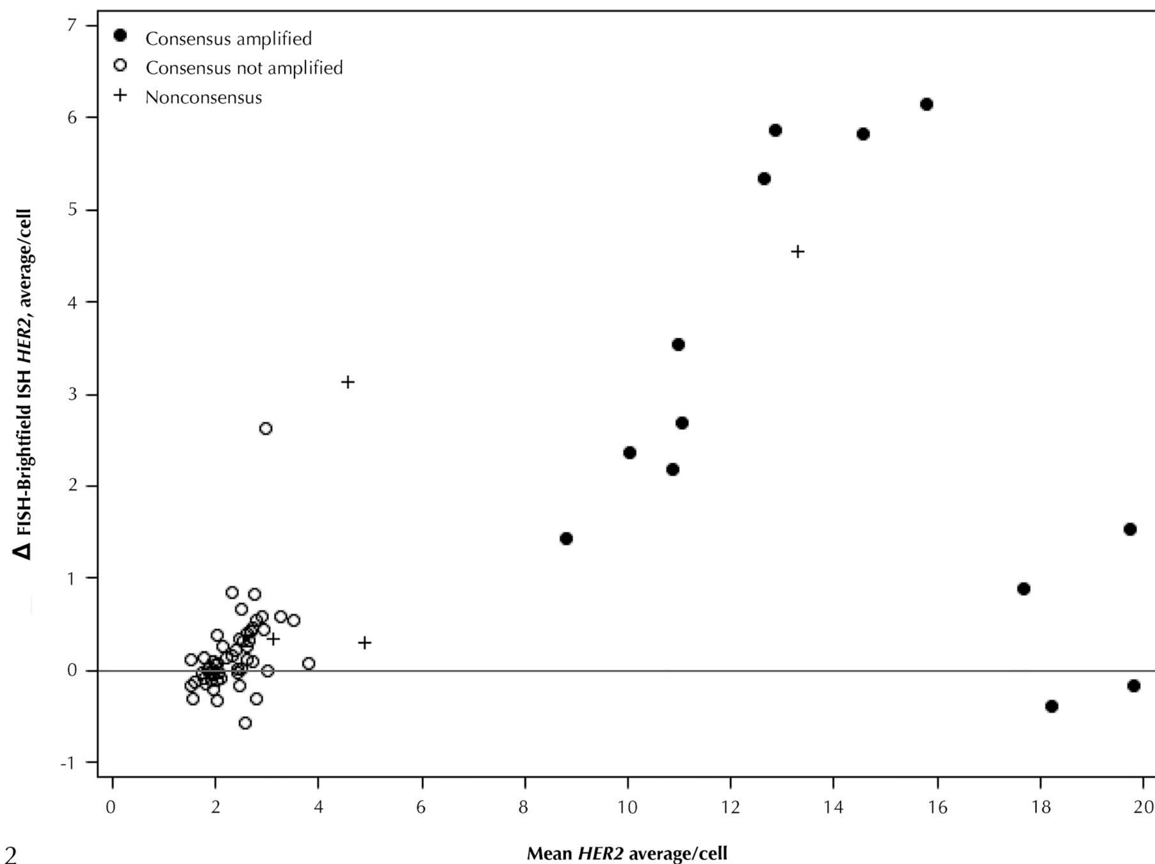

Figure 2. *Bias chart demonstrating the difference between the mean participant value for HER2 copies per cell for fluorescence in situ hybridization (FISH) and the mean participant value for HER2 copies per cell for brightfield in situ hybridization (ISH) on 70 tissue cores as a function of the overall consensus number of HER2 copies per cell.*

We noted comparable performance for both methods in the interpretation of those nonamplified and amplified specimens that reached consensus. However, some differences between methods were apparent. The FISH analyses showed a statistically significant greater number of *HER2* copy numbers per cell in consensus-amplified cores. For consensus-nonamplified cases, there was a trend toward greater *HER2* copy numbers per cell for FISH, which did not reach statistical significance. For the few samples that failed to reach 80% consensus, the mean *HER2* copy number per cell and *HER2* to control ratio by FISH trended higher compared with brightfield ISH. There was no significant difference in the average *HER2* to control ratios for consensus responses for either amplified or nonamplified cores. Similar findings have been demonstrated previously in a cohort of challenging cases.[30]

Table 2. Aggregate Data for All Consensus Tissue Core Samples According to *HER2* Status

Factor	Cores, No.	Δ FISH-Brightfield ISH[a] (95% CI)	Sign Test *P* value
Average *HER2*/cell			
Amplified	13	2.86 (1.48–4.25)	.02
Not amplified	53	0.17 (0.05–0.30)	.10
Average *HER2* to control ratio			
Amplified	13	0.31 (−0.43–1.06)	.99
Not amplified	53	−0.02 (−0.06–0.03)	.09

Abbreviations: FISH, fluorescence in situ hybridization; ISH, in situ hybridization.

[a] Δ FISH-Brightfield ISH refers to the difference between the average value reported for FISH and the average value reported for brightfield ISH.

When we analyzed the participant data according to the updated guideline, the appropriate interpretation changed from *nonamplified* to *equivocal HER2* status in 4.3% (100 of 2327) of the FISH cases and from *nonamplified* to *equivocal* status in 1.2% (25 of 2126) of the brightfield ISH cases, reflecting the tendency of FISH to generate higher average *HER2* copy numbers per cell, which sometimes fell in the equivocal range (4.0–5.9 average *HER2* copies per cell). *HER2* copy number has become a more important factor following the release of the 2013 updated ASCO/CAP guidelines for *HER2* testing.[14] In the original 2007 guideline, an *equivocal* result for ISH was defined as a *HER2* to chromosome 17 control ratio of 1.8 to 2.2 for dual-color probe assays or an average *HER2* copy number per cell of 4 to 6 for single-color probe assays. In the updated 2013 guideline, an *equivocal* result was defined as a *HER2* copy number of 4.0 or greater and less than 6.0 signals/cell and, for dual-color probes, a *HER2* to CEP17 ratio of less than 2.0. Additionally, cases with 6.0 or more average *HER2* copies per cell were reported as positive for *HER2* amplification regardless of the *HER2* to CEP17 ratio. These changes caused a shift toward more *equivocal* and *positive* results in HER2 testing since 2013,[33–38] particularly with the use of alternative probes for reflex testing on equivocal cases.[39,40]

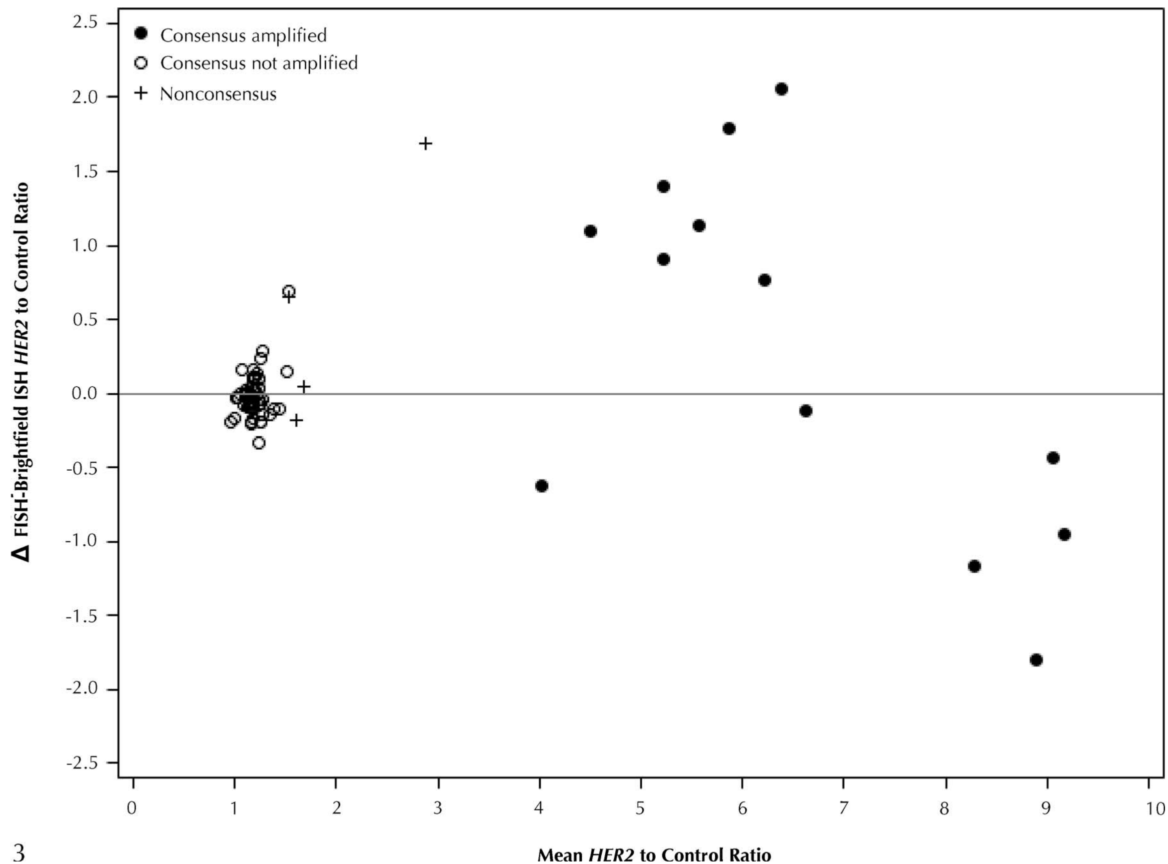

Figure 3. *Bias chart demonstrating the difference between the mean participant HER2 to control ratio value for fluorescence in situ hybridization (FISH) and the mean participant HER2 to control ratio value for brightfield in situ hybridization (ISH) on 70 tissue cores as a function of the overall consensus HER2 to control ratio value.*

Several technical differences between FISH and brightfield ISH might contribute to the differences in the average *HER2* to control ratio and *HER2* copy number per cell. At the level of the cell nucleus, *HER2* amplification frequently occurs intrachromosomally in homogenously staining regions of 1 or more chromosomes, typically generating 1 or more clusters of signals.[41,42] Although FISH generates discrete fluorescent *HER2* signals for each locus, brightfield ISH can generate aggregates of silver particles that coalesce in a manner that makes enumeration more difficult.[43] Moreover, FISH is usually scored at a higher magnification (×60–×100) than brightfield ISH (×40–×60), and the use of a single-pass filter to enumerate HER2 and control signals individually in dual-color probe assays is possible for FISH but not for brightfield ISH. These factors can generate differences in the estimated *HER2* copy number, as was shown in a recent study of a cohort enriched for equivocal cases.[31]

Table 3. Scoring Simulation[a] Using Submitted Data for the *HER2* to Control Ratio and the Average *HER2* Copies Per Cell to Generate Assigned Interpretation Under 2007 or 2013 Guideline

2007 Versus 2013 Guideline	FISH, n = 2327, No. (%)	Brightfield ISH, n = 2126, No. (%)
No change	2169 (93.2)	2011 (94.6)
Not amplified to equivocal	100 (4.3)	25 (1.2)
Not amplified to amplified	11 (0.5)	10 (0.5)
Equivocal to amplified	31 (1.3)	39 (1.8)
Equivocal to not amplified	16 (0.7)	41 (1.9)
Amplified to equivocal	0	0
Amplified to not amplified	0	0

Abbreviations: FISH, fluorescence in situ hybridization; ISH, in situ hybridization.
[a] χ^2; $P < .001$.

Another difference we observed between the 2 methods was the rate of assay failure; laboratories using brightfield ISH responded "unable to analyze" at a greater rate (9.6%) than did laboratories using FISH (6.0%). That might be related to technical challenges that are unique to brightfield ISH, such as the presence of black precipitate ("black silver dust") that can obscure *HER2* signals and large chromosome 17 centromere (control) signals that can mask smaller *HER2* signals.[43]

There are some limitations to this study. First, the study was not enriched for breast cancers considered problematic or challenging, such as samples that are close to the *equivocal* or *amplified* thresholds. Second, the samples used for proficiency testing were small tissue cores and would not be reflective of larger tissue samples that are also frequently encountered in clinical practice. Third, result entry for proficiency testing differs from clinical test reporting and can result in clerical errors.

In conclusion, we found no significant differences in the overall assignment of *HER2* status for FISH and brightfield ISH in routine breast cancer tissue core samples used for proficiency testing. However, FISH yielded a significantly higher average *HER2* copy number per cell compared with

brightfield ISH for amplified tissue cores, and FISH had a somewhat lower rate of technical failures. These differences could affect the evaluation of cases with values near the thresholds for *HER2 equivocal* or *amplified* status and, thus, possibly affect the choice of therapy.

References

1. Slamon DJ, Clark GM, Wong SG, Levin WJ, Ullrich A, McGuire WL. Human breast cancer: correlation of relapse and survival with amplification of the HER-2/neu oncogene. *Science*. 1987;235(4785):177–182.
2. Cobleigh MA, Vogel CL, Tripathy D, et al. Multinational study of the efficacy and safety of humanized anti-HER2 monoclonal antibody in women who have HER2-overexpressing metastatic breast cancer that has progressed after chemotherapy for metastatic disease. *J Clin Oncol*. 1999;17(9):2639–2648.
3. Slamon DJ, Leyland-Jones B, Shak S, et al. Use of chemotherapy plus a monoclonal antibody against HER2 for metastatic breast cancer that overexpresses HER2. *N Engl J Med*. 2001;344(11):783–792.
4. 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*. 2002;20(3):719–726.
5. Konecny GE, Thomssen C, Luck HJ, et al. *Her-2/neu* gene amplification and response to paclitaxel in patients with metastatic breast cancer. *J Natl Cancer Inst*. 2004;96(15):1141–1151.
6. Romond EH, Perez EA, Bryant J, et al. Trastuzumab plus adjuvant chemotherapy for operable HER2-positive breast cancer. *N Engl J Med*. 2005; 353(16):1673–1684.
7. Piccart-Gebhart MJ, Procter M, Leyland-Jones B, et al; Herceptin Adjuvant (HERA) Trial Study Team. Trastuzumab after adjuvant chemotherapy in HER2-positive breast cancer. *N Engl J Med*. 2005;353(16):1659–1672.
8. Pritchard KI, Shepherd LE, O'Malley FP, et al; National Cancer Institute of Canada Clinical Trials Group. HER2 and responsiveness of breast cancer to adjuvant chemotherapy. *N Engl J Med*. 2006;354(2):2103–2111.
9. Joensuu H, Kellokumpu-Lehtinen PL, Bono P, et al; FinHer Study Investigators. Adjuvant docetaxel or vinorelbine with or without trastuzumab for breast cancer. *N Engl J Med*. 2006;354(8):809–820.
10. Slamon DJ, Godolphin W, Jones LA, et al. Studies of the *HER-2/neu* proto-oncogene in human breast and ovarian cancer. *Science*. 1989;244(4905):707–712.
11. Pauletti G, Godolphin W, Press MF, Slamon DJ. Detection and quantitation of *HER-2/neu* gene amplification in human breast cancer archival material using fluorescence in situ hybridization. *Oncogene*. 1996;13(1):63–72.
12. Press MF, Bernstein L, Thomas PA, et al. *HER-2/neu* gene amplification characterized by fluorescence in situ hybridization: poor prognosis in node-negative breast carcinomas. *J Clin Oncol*. 1997;15(8):2894–2904.
13. Wolff AC, Hammond ME, Schwartz JN, et al. American Society of Clinical Oncology/College of American Pathologists guideline recommendations for human epidermal growth factor receptor 2 testing in breast cancer. *Arch Pathol Lab Med*. 2007;131(1):18–43.
14. Wolff AC, Hammond ME, Hicks DG, et al; American Society of Clinical Oncology; College of American Pathologists. Recommendations for human epidermal growth factor receptor 2 testing in breast cancer: American Society of Clinical Oncology/College of American Pathologists clinical practice guideline update. *Arch Pathol Lab Med*. 2014;138(2):241–256.
15. Hanna WM, Kwok K. Chromogenic in-situ hybridization: a viable alternative to fluorescence in-situ hybridization in the HER2 testing algorithm. *Mod Pathol*. 2006;19(4):481–487.
16. Sáez 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(4):519–527.
17. Vocaturo A, Novelli F, Benevolo M, et al. Chromogenic in situ hybridization to detect *HER-2/neu* gene amplification in histological and ThinPrep-processed breast cancer fine-needle aspirates: a sensitive and practical method in the trastuzumab era. *Oncologist*. 2006;11(8):878–886.
18. Sínczak-Kuta A, Tomaszewska R, Rudnicka-Sosin L, Okoń K, Stachura J. Evaluation of *HER2/neu* gene amplification in patients with invasive breast carcinoma: comparison of in situ hybridization methods. *Pol J Pathol*. 2007;58(1): 41–50.
19. van de Vijver M, Bilous M, Hanna W, et al. Chromogenic in situ hybridisation for the assessment of HER2 status in breast cancer: an international validation ring study. *Breast Cancer Res*. 2007;9(5):R68.
20. Di Palma S, Collins N, Faulkes C, et al. Chromogenic in situ hybridisation (CISH) should be an accepted method in the routine diagnostic evaluation of HER2 status in breast cancer. *J Clin Pathol*. 2007;60(9):1067–1068.
21. Carbone A, Botti G, Gloghini A, et al. Delineation of *HER2* gene status in breast carcinoma by silver in situ hybridization is reproducible among laboratories and pathologists. *J Mol Diagn*. 2008;10(6):527–536.
22. Capizzi E, Gruppioni E, Grigioni AD, et al. Real time RT-PCR approach for the evaluation of ERBB2 overexpression in breast cancer archival samples: a comparative study with FISH, SISH, and immunohistochemistry. *Diagn Mol Pathol*. 2008;17(4):220–226.
23. Mayr D, Heim S, Weyrauch K, et al. Chromogenic in situ hybridization for *Her-2/neu*-oncogene in breast cancer: comparison of a new dual-colour chromogenic in situ hybridization with immunohistochemistry and fluorescence in situ hybridization. *Histopathology*. 2009;55(6):716–723.
24. Pedersen M, Rasmussen BB. The correlation between dual-color chromogenic in situ hybridization and fluorescence in situ hybridization in assessing *HER2* gene amplification in breast cancer. *Diagn Mol Pathol*. 2009; 18(2):96–102.
25. Gong Y, Sweet W, Duh YJ, et al. Performance of chromogenic in situ hybridization on testing HER2 Status in breast carcinomas with chromosome 17 polysomy and equivocal (2+) HercepTest results: a study of two institutions using the conventional and new ASCO/CAP scoring criteria. *Am J Clin Pathol*. 2009; 132(2):228–236.
26. Shousha S, Peston D, Amo-Takyi B, Morgan M, Jasani B. Evaluation of automated silver-enhanced in situ hybridization (SISH) for detection of *HER2* gene amplification in breast carcinoma excision and core biopsy specimens. *Histopathology*. 2009;54(2):248–253.
27. Garcia-Caballero T, Grabau D, Green AR, et al. Determination of HER2 amplification in primary breast cancer using dual-colour chromogenic in situ hybridization is comparable to fluorescence in situ hybridization: a European multicentre study involving 168 specimens. *Histopathology*. 2010;56(4):472–480.
28. Papouchado BG, Myles J, Lloyd RV, et al. Silver in situ hybridization (SISH) for determination of HER2 gene status in breast carcinoma: comparison with FISH and assessment of interobserver reproducibility. *Am J Surg Pathol*. 2010; 34(6):767–776.
29. Bartlett JM, Campbell FM, Ibrahim M, et al. A UK NEQAS ISH multicenter ring study using the Ventana HER2 dual-color ISH assay. *Am J Clin Pathol*. 2011; 135(1):157–162.
30. Mansfield AS, Sukov WR, Eckel-Passow JE, et al. Comparison of fluorescence in situ hybridization (FISH) and dual-ISH (DISH) in the determination of HER2 status in breast cancer. *Am J Clin Pathol*. 2013;139(2):144–150.
31. Shao T, Wood M, Wing A, et al. Comparison of HER2 dual-color and fluorescence in situ hybridization in breast cancer: a cohort study emphasizing equivocal cases. *Am J Clin Pathol*. 2016;146(3):339–345.
32. Persons DL, Tubbs RR, Cooley LD, et al. HER-2 fluorescence in situ hybridization: results from the survey program of the College of American Pathologists. *Arch Pathol Lab Med*. 2006;130(3):325–331.
33. Bethune GC, Veldhuijzen van Zanten D, MacIntosh RF, et al. Impact of the 2013 American Society of Clinical Oncology/College of American Pathologists guideline recommendations for human epidermal growth factor receptor 2 (HER2) testing of invasive breast carcinoma: a focus on tumours assessed as 'equivocal' for HER2 gene amplification by fluorescence in situ hybridization. *Histopathology*. 2015;67(6):880–887.
34. Long TH, Lawce H, Durum C, et al. The new equivocal: changes to HER2 FISH results when applying the 2013 ASCO/CAP guidelines. *Am J Clin Pathol*. 2015;144(2):253–262.
35. Muller KE, Marotti JD, Memoli VA, Wells WA, Tafe LJ. Impact of the 2013 ASCO/CAP HER2 guideline updates at an academic medical center that performs primary HER2 FISH testing: increase in equivocal results and utility of reflex immunohistochemistry. *Am J Clin Pathol*. 2015;144(2):247–252.
36. Tchrakian N, Flanagan L, Harford J, Gannon JM, Quinn CM. New ASCO/CAP guideline recommendations for HER2 testing increase the proportion of reflex in situ hybridization tests and of HER2 positive breast cancers. *Virchows Arch*. 2016;468(2):207–211.
37. Fan YS, Casas CE, Peng J, et al. HER2 FISH classification of equivocal HER2 IHC breast cancers with use of the 2013 ASCO/CAP practice guideline. *Breast Cancer Res Treat*. 2016;155(3):457–462.
38. Lim TH, Lim AS, Thike AA, Tien SL, Tan PH. Implications of the updated 2013 American Society of Clinical Oncology/College of American Pathologists guideline recommendations on human epidermal growth factor receptor 2 gene testing using immunohistochemistry and fluorescence in situ hybridization for breast cancer. *Arch Pathol Lab Med*. 2016;140(2):140–147.
39. Shah MV, Wiktor AE, Meyer RG, et al. Change in pattern of HER2 fluorescent in situ hybridization (FISH) results in breast cancers submitted for fish testing: experience of a reference laboratory using US Food and Drug Administration criteria and American Society of Clinical Oncology and College of American Pathologists guidelines. *J Clin Oncol*. 2016; 34(29):3502–3510.
40. Hui L, Geiersbach KB, Downs-Kelly E, Gulbahce HE. RAI1 alternate probe identifies additional breast cancer cases as amplified following equivocal HER2 fluorescence in situ hybridization testing: experience from a national reference laboratory. *Arch Pathol Lab Med*. 2017;141(2):274–278.
41. Kallioniemi OP, Kallioniemi A, Kurisu W, et al. ERBB2 amplification in breast cancer analyzed by fluorescence in situ hybridization. *Proc Natl Acad Sci USA*. 1992;89(12):5321–5325.
42. Muleris M, Almeida A, Gerbault-Seureau M, Malfoy B, Dutrillaux B. Identification of amplified DNA sequences in breast cancer and their organization within homogeneously staining regions. *Genes Chromosomes Cancer*. 1995;14(3):155–163.
43. Gao FF, Dabbs DJ, Cooper KL, Bhargava R. Bright-field HER2 dual in situ hybridization (DISH) assay vs fluorescence in situ hybridization (FISH): focused study of immunohistochemical 2+ cases. *Am J Clin Pathol*. 2014;141(1):102–110.