Liquid-Based Fluorescence In Situ Hybridization Assay for Detection of ERBB2 Gene Amplification in Patients with Breast Cancer†

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BACKGROUND: Current reference methods for evaluating gene amplification and expression of ERBB2 (also known as HEP-2)—cell-based fluorescence in situ hybridization (FISH) and immunohistochemistry (IHC)—are subjective and influenced by methods of tissue preparation and fixation. We developed and evaluated a novel, quantitative liquid-based FISH (L-FISH) assay that uses flow cytometry to detect ERBB2 gene amplification in breast cancer patients.

METHODS: DNA was extracted from serum or tissue, biotinylated, hybridized to differentially labeled probes for ERBB2 and a chromosome 17–specific single-copy sequence (17-SSC), and immobilized to streptavidin-coated microspheres. The ERBB2/17-SSC signal ratio measured by flow cytometry was used to evaluate ERBB2 amplification. We used L-FISH to test 122 stored formalin-fixed, paraffin-embedded (FFPE) tissue samples and 22 serum samples from randomly selected breast cancer patients; results were compared with those obtained with conventional FISH and IHC.

RESULTS: The inter- and intraassay imprecisions were 3.7%–18.9% for FFPE tissue and 2.8%–6.3% for serum. Overall, L-FISH analyses of FFPE tissues demonstrated 84.4% concordance with results obtained with conventional FISH (P < 0.001) and 78.8% concordance with IHC results (P < 0.001). L-FISH analyses of serum samples showed 91% concordance with tissue-based IHC/FISH results (P = 0.038).

CONCLUSIONS: Our data indicate that this PCR-free L-FISH method can be used to evaluate ERBB2 amplification in both cell-containing (paraffin-embedded tissue) and cell-free (serum) samples. This approach provides more objective results and is amenable to automation and quantitative measurement.

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Human epidermal growth factor receptor 2 [(ERBB2, v-erb-b2 erythroblastic leukemia viral oncogene homolog 2, neuro/glioblastoma derived oncogene homolog (avian); also known as HER-2], an orphan receptor tyrosine kinase, is involved in the regulation of a wide spectrum of cell activities, including cell proliferation, differentiation, adhesion, and migration (1, 2). ERBB2 overproduction occurs in 20%–30% of patients with invasive breast cancer (3) and is considered an adverse prognostic factor for clinical outcome (4, 5). Amplification of the ERBB21 gene, located on chromosome 17q21, is the major underlying mechanism and accounts for approximately 97% of protein overproduction (6, 7). Deregulated ERBB2 expression has also been shown to have potential prognostic implications in patients with ovarian, gastric, lung, colon, pancreatic, and prostate cancers (6, 8–12).

The approval of immunotherapy based on trastuzumab (Herceptin), a humanized anti-ERBB2 monoclonal antibody, has created an increasing clinical demand for a reliable laboratory assay to accurately measure ERBB2 status. An evaluation of ERBB2 status not only is relevant in monitoring trastuzumab therapy but also appears to be a predictive indicator of clinical responsiveness to a variety of treatment regimens. For example, ERBB2-overproducing breast cancers are often resistant to endocrine therapy (13, 14); ERBB2 amplification is correlated with resistance to radiation therapy (15) and may be associated with a clinical benefit from cyclophosphamide, methotrexate, and fluorouracil chemotherapy (16); and ERBB2 positivity

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1 Human genes: ERBB2, v-erb-b2 erythroblastic leukemia viral oncogene homolog 2, neuro/glioblastoma derived oncogene homolog (avian).
may also be predictive of an enhanced response to doxorubicin and paclitaxel treatment modalities (17, 18). Given the prognostic and therapeutic implications, ERBB2 testing is an indispensable prerequisite for good management of breast cancer patients.

ERBB2 expression and/or amplification can be detected by immunologic methods such as Western blotting, immunohistochemistry (IHC), and ELISA, or by molecular approaches such as Southern blotting, fluorescence in situ hybridization (FISH), chromogenic or bright-field in situ hybridization, and real-time quantitative PCR (19, 20). Most of these assays are cell based and require tissue-sample preparation, whereas ELISA and real-time quantitative PCR have the potential to be carried out on cell-free samples. IHC and FISH are the 2 most commonly used testing approaches in the clinical setting. FISH is more sensitive, specific, and accurate, but IHC is less labor-intensive, quicker, and more cost-effective and therefore has been adopted as the first-line method for routine and archival analysis of tissue samples (20–22). The prognostic evaluation of ERBB2 status with these tissue-based methods has been hampered by the limited availability of samples for testing. Once tumor tissues have been surgically removed, tissue-based methods are no longer applicable for detecting early recurrence, testing for metastasis, or monitoring therapeutic outcome. This fact narrows the ERBB2-testing window to the time of diagnosis. A test with the potential to accurately evaluate ERBB2 status from blood samples will remedy this situation.

We describe a novel molecular diagnostic assay, liquid-based FISH (L-FISH), that can be used to quantitatively measure ERBB2 amplification in both tissue and blood samples. This approach involves solution hybridization of sample DNA to labeled probes, DNA capture on streptavidin-coated microspheres, and fluorescence quantification via flow cytometry. In this study, we compared L-FISH results with those obtained with 2 US Food and Drug Administration (FDA)-approved methods—conventional FISH and IHC—for tissue sections and serum samples from breast cancer patients. Tissue- and serum-based L-FISH results showed high concordance with both conventional FISH and IHC results.

Materials and Methods

COLLECTION OF PATIENT SAMPLES
The study materials consisted of stored tissues from 144 randomly selected patients with breast cancer: 22 stored serum samples and 122 formalin-fixed, paraffin-embedded (FFPE) samples of primary tumor tissues. We cut 6 sections (4–5 μm) for IHC and FISH and sliced 3 additional 10-μm sections from the same block to extract DNA for L-FISH. All tissue samples contained invasive carcinoma. There was no evidence of tumor cell dissemination into the blood for any of the blood samples used for L-FISH. Tissue samples were fixed in neutral buffered formalin for 6–48 h; all testing was performed in a high-throughput testing laboratory.

DNA EXTRACTION AND NICK TRANSLATION
Genomic DNA samples from human breast cancer cell lines BT-474, SK-BR-3, and MCF-7 and from chronic myelogenous leukemia cell line K562 were purchased from the ATCC. To isolate breast tissue DNA, we used the QiAamp MinElute Virus Spin Kit (Qiagen) with some modifications. We deparaffinized FFPE tissue sections with xylene/ethanol, treated the sections with proteinase K, inactivated the proteinase by heating at 96 °C for 10 min, and then purified the DNA by centrifugation through the kit’s silica membrane–containing spin columns.

Cell-free circulating DNA from serum (200 μL) was purified with the same Qiagen kit according to the manufacturer’s protocol. All DNA samples were eluted in PCR-grade water (Ambion/Applied Biosystems). The quality and concentrations of all isolated DNA samples were estimated on a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies) from the ultraviolet absorbance spectra between 220 and 320 nm. Samples were analyzed further only when the DNA was of high purity (i.e., an A260/A280 ratio >1.6).

We used Biotin-Nick Translation Mix (Roche Applied Science) to biotinylate purified DNA with biotin-16-dUTP. To obtain the highest sensitivity for detection, we adjusted the molar ratio of biotin-16-dUTP to dTTP in the mix to ensure that every 20th to 25th nucleotide in the newly synthesized DNA was labeled with biotin. The labeled fragments obtained in a typical nick-translation reaction showed a size distribution of 200–500 bp.

L-FISH ASSAY
Fluorescein isothiocyanate (FITC)-labeled ERBB2 probes were generated with the Universal Linkage System (ULS™) (Kreatech Diagnostics). This ULS system allows efficient, nonenzymatic, and direct labeling of fluorochromes to the DNA molecule. For an internal reference, we had Sigma–Aldrich custom-synthesize a chromosome 17–specific single-copy oligonucleotide (17-SSC) end-labeled with Cy5 at the 5’ end. The coordinates of this sequence are Chr17: 43964239–43964288. We chose this sequence because of its single-copy sequence composition, GC content (40%–55%), lack of potential secondary structure, and length (23).

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2 Nonstandard abbreviations: IHC, immunohistochemistry; FISH, fluorescence in situ hybridization; L-FISH, liquid-based FISH; FDA, Food and Drug Administration; FFPE, formalin-fixed, paraffin-embedded, FITC, fluorescein isothiocyanate; 17-SSC, chromosome 17–specific single-copy sequence.
Labeled probes were hybridized to the appropriate amount of biotinylated DNA in the presence of Cot-1 and salmon sperm DNA (300 ng each) in Hybrisol VII hybridization solution (MP Biomedicals), denatured for 15 min at 87 °C, and reannealed at 45 °C for 2 h. To reduce nonspecific binding, we sequentially treated streptavidin-coated microspheres (Bangs Laboratories) with BlockAid (Invitrogen) and sheared salmon sperm DNA (100 μg/mL) before conjugation.

The annealed DNA mixture was then coupled to the streptavidin-coated microspheres in conjugation buffer (100 mmol/L Tris-HCl, pH 8.0, 1 g/L Tween 20, and 1 mol/L LiCl) for 1 h at room temperature. The coupled microspheres were washed once with 100 mL/L formamide/0.2× standard saline citrate (0.03 mol/L NaCl and 0.003 mol/L sodium citrate) and twice with 0.2× standard saline citrate. The amount of bead-bound DNA was estimated with the aid of SYBR Green (Invitrogen). We used the Alexa Fluor 488 Signal Amplification Kit (Invitrogen) to enhance FITC fluorescence. This kit uses 2 antibodies tagged with Alexa Fluor 488—a rabbit anti-FITC antibody and a goat anti-rabbit IgG antibody. Microspheres were washed 3 times with 1× PBS containing 10 g/L BSA and analyzed on a FACS Canto flow cytometer (BD Biosciences). We measured the fluorescence emissions of FITC and allophycocyanin (which has the same spectrum as Cy5) and computed the mean fluorescence intensity of at least 5,000 events for each sample.

**FISH ANALYSIS**

For FISH analysis of ERBB2 amplification, we used the PathVysion HER-2 DNA Probe Kit (Vysis/Abbott Molecular) according to manufacturer’s instructions. In brief, we prepared several paraffin sections (4–5 μm thick) from each breast tissue sample and subjected them to deparaffinization, rehydration, and protease digestion (37 °C, 1–2 h) before we hybridized the sections overnight with fluorescently labeled probes for the ERBB2 gene and α-satellite DNA for chromosome 17 (CEP17). After appropriate posthybridization washing steps, we visualized dual-color fluorescence signals by fluorescence microscopy. We counted and scored the ERBB2 (orange) and CEP17 (green) signals in each of 30 nuclei. We interpreted samples with ERBB2/CEP17 signal ratios >2.2 as positive for amplification and signal ratios <1.8 as negative for amplification. Ratios between 1.8 and 2.2 were considered borderline results, and such samples were excluded from concordance studies. We also excluded cases with aneuploidy. Aneuploidy was defined as a mean chromosome 17 signal outside the range of 1.76–2.25/cell.

**IHC ASSAY**

ERBB2 protein overexpression was measured with an FDA-approved HercepTest (Dako Corp.) and scored as recommended by experienced pathologists. Samples subjected to ERBB2 testing were fixed in 10% neutral buffered formalin for at least 6 h and not longer than 48 h. Careful attention was paid to the recommended exclusion criteria for performing and interpreting ERBB2 IHC assays (e.g., artifactual distortion of tissues, apoptotic or necrotic areas). IHC-stained slides were interpreted and scored on a scale of 0 to 3+ in accordance with the FDA-approved guidelines. IHC immuno- staining of 3+ was considered to indicate ERBB2 overproduction, and 0/1+ immunostaining was considered negative for ERBB2 amplification. Samples with scores of 2+ were interpreted as inconclusive (equivocal) and were excluded from concordance studies.

**IMPRECISION STUDY**

We used 15 tissue and 15 serum control samples (5 samples each of low, medium, and high ERBB2 expression) to evaluate the imprecision of the L-FISH assay. We assessed intraassay (within-run) variation by analyzing 5 control samples on the same day and evaluating interassay (between-run) variation by measuring ERBB2/internal control ratios over a 5-day period. We then calculated within- and between-run CVs. CVs of <20% were considered to indicate acceptable performance for both within-run imprecision and between-run imprecision.

**STATISTICAL ANALYSIS**

Data were compared with the Fisher exact and non-parametric tests as appropriate. Tests included Spearman correlation, Wilcoxon matched pairs, Kruskal–Wallis, and χ² tests. All tests were 2-tailed, and P values <0.05 were considered statistically significant. To assess the relationship between L-FISH ERBB2/17-SSC ratios and results for conventional FISH and IHC analyses, we also calculated 95% confidence intervals for the means in some studies. We used SigmaPlot (Systat Software) and Statistica (StatSoft) software for all statistical analyses.

**Results**

**DETECTION OF ERBB2 AMPLIFICATION**

To determine whether we could detect an ERBB2 signal by flow cytometry, we first tested DNA extracted from breast cancer cell lines BT-474 and MCF-7. ERBB2 signals were easily detected in BT-474 samples, as shown by the dramatic increase and upward shift of the FITC mean fluorescence intensity and the percent positivity of the microspheres (Fig. 1A). The L-FISH assay showed a strong linear relationship between the FITC index and target DNA concentrations across 3 orders of magnitude (up to 100 ng DNA, Fig. 1B); the signal reached a plateau as the amount of input DNA in-
Fig. 1. The L-FISH assay.
(A) Flow cytometry analysis of the L-FISH ERBB2 signal in breast cancer cell line DNA. The panels show representative scatter plots of the entire microsphere population for the blank control (no DNA, left panels), cell line BT-474 (middle panels), and cell line MCF-7 (right panels). A marked increase in the FITC-positive population (upward shift from the Q3 to the Q1 quadrant) was noted in BT-474 samples, compared with MCF-7 samples and the control (84.2% vs 6.1% and 0.6%, respectively). FSC, forward scatter; SSC, side scatter. (B), Linearity of the curve of input-DNA amount vs output fluorescence signal in L-FISH assays. Increasing amounts of DNA from 2 breast cancer lines, BT474 (high ERBB2 copy number) and MCF-7 (low ERBB2 copy number), analyzed by L-FISH. The amount of biotinylated target DNA varied from 0.1–100 ng. Sample DNA was hybridized to FITC-labeled ERBB2 probes and conjugated to streptavidin-coated microspheres. The fluorescence signal was expressed as an FITC index [mean fluorescence intensity (MFI) times the percent positive population].
increased above 100 ng (data not shown). With SYBR Green staining, we consistently estimated the maximum capacity of the microspheres at approximately 1 pg DNA/microsphere (data not shown). The curve of breast cancer cell line BT-474 also had a steeper slope than that of MCF-7, indicating a higher degree of ERBB2 gene amplification in BT-474, an observation in accord with previous reports (24, 25).

To expand on our initial findings, we used L-FISH to quantitate ERBB2 gene status in 3 well-established tumor cell lines with different ERBB2 copy numbers per nucleus: BT-474 (high amplification, approximately 50–60 ERBB2 copies/nucleus), SK-BR-3 (moderate amplification, approximately 10–20 ERBB2 copies/nucleus), and MCF-7 (little or no amplification, approximately 2–3 ERBB2 copies/nucleus) (24, 25). Leukemia line K562 (2 ERBB2 copies) was used as a control. The ERBB2/17-SSC ratios obtained corresponded with the ERBB2 copy number for each cell line and did not change appreciably with increased DNA concentration (Table 1), providing proof-of-concept evidence that L-FISH could be a valid, sensitive assay for assessing ERBB2 status.

**Table 1. Quantitation of ERBB2 signal by a chromosome 17–specific single-copy sequence (17-SSC).**

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<tr>
<th>ERBB2&lt;sup&gt;a&lt;/sup&gt;</th>
<th>17-SSC&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Ratio</th>
<th>ERBB2 copies/nucleus&lt;sup&gt;d&lt;/sup&gt;</th>
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<tr>
<td>25 ng DNA</td>
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<tr>
<td>BT-474</td>
<td>14 876</td>
<td>413</td>
<td>36.02</td>
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<tr>
<td>SK-BR-3</td>
<td>9775</td>
<td>407</td>
<td>24.02</td>
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<tr>
<td>MCF-7</td>
<td>5636</td>
<td>398</td>
<td>14.16</td>
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<tr>
<td>K562</td>
<td>3268</td>
<td>387</td>
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<td>50 ng DNA</td>
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<td>BT-474</td>
<td>15 952</td>
<td>422</td>
<td>37.80</td>
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<td>SK-BR-3</td>
<td>9923</td>
<td>411</td>
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<td>MCF-7</td>
<td>7313</td>
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<td>K562</td>
<td>3651</td>
<td>395</td>
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<sup>a</sup> Shown are representative results of 3 experiments.

<sup>b</sup> Values are expressed as the mean fluorescence intensity of Alexa Fluor 488/FITC.

<sup>c</sup> Values are expressed as the mean fluorescence intensity of Cy5.

<sup>d</sup> Data are from published studies that used Southern blotting, quantitative PCR, or FISH [Kallioiniemi et al. (24), Grushko et al. (25)].

**Fig. 2. Cutoff values for ERBB2 gene amplification from tissue and serum samples.**

Quantification of ERBB2/17-SSC ratios by L-FISH from 26 nonpathologic tissue and 52 nonpathologic serum samples. The data distributions for 10 IHC 3+ FFPE cases and 7 ERBB2 ELISA–positive cases are included for comparison. The dashed lines indicate the optimal cutoff (mean + 3 SDs) of ERBB2/17-SSC ratios for tissue (2.34) and serum (2.13).

**DETERMINATION OF CUTOFF VALUES FOR CLINICAL SAMPLES**

To establish cutoff values for clinical samples, we used L-FISH to test 26 nonneoplastic FFPE tissues (11 breast, 5 lung, 5 lymph node, and 5 tonsil samples) and 52 nonneoplastic serum samples. The control groups were from individuals with nonneoplastic diseases, and their ages were similar to those of the patients with breast cancer. Tissue samples were carefully selected to exclude areas of necrosis or infection. The mean (SD) ERBB2/17-SSC ratios obtained with L-FISH were 1.38 (0.32) for nonneoplastic tissue samples and 1.35 (0.26) for serum samples from individuals without cancer. The optimal cutoff ratio for ERBB2 gene amplification was 2.13 for serum and 2.34 for tissue (3 SDs above the mean for nonneoplastic samples; Fig. 2). Notably, ERBB2/17-SSC ratios derived from cell lines were much higher than those from FFPE tissues or sera, probably because of the poorer quality and lower quantity of tumor DNA available from clinical samples, particularly when tumors are small and contaminated with nonneoplastic or apoptotic/necrotic elements (26).

**ANALYTICAL ASSESSMENT OF IMPRECISION**

The imprecision results obtained for the ERBB2 L-FISH assay with 3 levels of controls (low, medium, and high) from tissue and serum samples are presented in Table 2. The interassay and intraassay reproducibilities were very high for all 30 data points (CVs of <20%, Table 2). Interassay CVs for tissue samples were 3.7%–13%, and serum samples exhibited higher interassay CVs (12%–19%). Intraassay CVs were relatively low for both sample types (2.8%–6.3%).

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We tested 122 FFPE tissue samples from breast cancer patients with L-FISH, IHC, and conventional FISH methods. According to recent guidelines (22), only samples with either IHC results of 3+/H11001 or ratios ≥2.2 in conventional FISH analysis can be considered positive for ERBB2 amplification. Samples with IHC 0/1+ staining or ratios of <1.8 in conventional FISH are interpreted as negative for ERBB2 amplification. For the concordance study, we excluded samples with equivocal IHC results (i.e., 2+) or borderline results in conventional FISH analysis (i.e., ratios of 1.8–2.2). These borderline cases are important; however, we excluded them because clinical follow-up data on these cases are not available. Therefore, we used a clean set of results to compare the methods. Overall, the L-FISH results demonstrated 84.4% concordance with results obtained with conventional FISH (Table 3, upper panel). Four cases showed ERBB2 amplification by conventional FISH but not by L-FISH, and 15 cases identified as unamplified by conventional FISH showed ERBB2 gene amplification by L-FISH.

The L-FISH and IHC results were concordant in 78.8% of the paired samples (Table 3, top). Of the 17 discordant paired samples, 8 scored as 3+ by IHC were negative for amplification by L-FISH, and 9 samples scored as 0/1+ by IHC were positive by L-FISH.

We also used L-FISH to test sera from 22 unselected breast cancer patients for ERBB2 gene amplification and compared the results with those obtained with conventional FISH and IHC (IHC 3+ samples reflexed to FISH) done on FFPE tissues from the same patients. Serum L-FISH results exhibited 91% concordance with tissue-based IHC/FISH results (Table 3, bottom). L-FISH showed ERBB2 gene amplification in 3 cases, 2 of which were considered positive by IHC and conventional FISH. Only one of the 19 cases considered negative by L-FISH was scored positive by IHC and conventional FISH.

We drew ROC curves to further confirm the concordance between L-FISH and conventional FISH or

<table>
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<th>Table 2. Imprecision of the ERBB2 L-FISH assay. a</th>
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<tr>
<td>Mean ERBB2/17-SSC ratio</td>
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<td>SD</td>
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<td>Interassay CV, %</td>
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<td>Intraassay CV, %</td>
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* Results were collected and analyzed from 2 sources (tissue and serum samples) each day over a 5-day period.
DISTRIBUTION OF L-FISH ERBB2 RATIOS ACCORDING TO AMPLIFICATION STATUS AS ASSESSED BY IHC AND CONVENTIONAL FISH

We segregated the results of L-FISH ERBB2 analyses by IHC scores and conventional FISH results. L-FISH mean ratios were significantly higher in both IHC-positive samples (mean, 2.28; range, 1.02–7.24) and conventional FISH-positive samples (mean, 2.36; range, 1.27–2.93) than in the corresponding IHC-negative samples (mean, 1.48; range, 0.95–4.34) and FISH-negative samples (mean, 1.58; range, 0.99–4.34). Together, these results indicate a close association between L-FISH and the 2 conventional methods.

Discussion

We have described a novel approach that can evaluate ERBB2 amplification in both cell-free and tissue/cell extracts. This approach uses probes in a fashion similar to that of conventional FISH, but hybridization occurs in solution (liquid format) rather than on a slide (solid support).

Multiple methods are currently used for determining ERBB2-amplification status. Tissue-based testing, including conventional FISH and IHC, has the advantage of assessing ERBB2 amplification in intact cells; however, these methods can be subjective, laborious, and time-consuming if large numbers of clinical samples are to be tested. Neither method is applicable to cell-free samples. L-FISH, on the other hand, has the advantages of automation and a high-throughput capacity and allows levels of ERBB2 gene amplification to be measured in either tissue or cell-free samples. The sensitivity and accuracy of L-FISH could be further enhanced by macro- or microdissection of tumor tissues to eliminate dilution of the apparent ERBB2 copy number by nontumor cells in the samples.

We first evaluated the L-FISH assay by assessing 4 cell lines: BT-474, SK-BR-3, MCF-7, and K562. The ERBB2/17-SSC ratios obtained for all 4 cell lines were consistent with the ERBB2 gene dosages reported for other methods (24, 25) and had a broader dynamic range, indicating that L-FISH has the potential to distinguish between low and nonpathologic ERBB2 copy numbers. These results demonstrate the degree of correctness that can be achieved with this assay. Interestingly, the optimal cutoff ratios for L-FISH with FFPE and serum samples were 2.34 and 2.13, respectively, similar to the arbitrary cutoff value of 2.2 for conventional FISH.

Although conventional FISH is generally considered the comparison method for detecting ERBB2 gene amplification, our L-FISH assay could provide an attractive alternative to FISH, especially in laboratories that are familiar with flow cytometry. In our study, we first compared L-FISH with conventional FISH and IHC methods with a retrospective series of FFPE breast cancer samples. L-FISH showed good concordance with both conventional FISH and IHC methods (84.4% and 78.8%, respectively). Our data are consistent with the majority of correlation results reported for several validated ERBB2 assays of FFPE tissues. For example, the FISH and IHC methods have a reported concordance of 85%–95% (27, 28), chromogenic in situ hybridization has shown a 83–100% association with FISH (29, 30), and quantitative PCR and FISH/IHC methods exhibit a concordance of 79%–100% (31–33). A recent large study designed to address the reproducibility of FISH and IHC also revealed a certain degree of discordance between central and local laboratories, with concordance rates of 77.5% for IHC to IHC, 92% for FISH to IHC, and 79% for FISH to FISH (34). The discrepancies between L-FISH and FISH/IHC results (false-negative cases) could mainly be ascribed to the dilution of cells carrying amplified genes by nontumor cells (35, 36). Thus, use of a laser-assisted microdissection step should enhance the reliability of L-FISH detection of ERBB2 gene amplification. Several FFPE cases classified as positive by L-FISH but negative by FISH/IHC could have resulted from difficulties in detecting and counting the signals on histologic sections because of the effects of the fixative, tissue permeability of antibody or probe, and antigen- or DNA-accessibility problems (21, 22, 27). The isolation of sample DNA in

![Fig. 3. ROC plot.](image)
L-FISH should allow a more accurate and robust signal readout than a fluorescent or chromogenic signal on histologic slides in FISH/IHC, where overlapping cell layers and tissue debris are often prevalent. The application of a dual-color L-FISH technique, with both a probe for the ERBB2 gene and a probe for a single-copy sequence on chromosome 17, has the advantage of providing an accurate estimate of gene amplification. Nevertheless, a second reference sequence could be added to our assay to address aneuploidy (37) and to make L-FISH analysis more consistent with the conventional FISH methodology.

Accumulating evidence has demonstrated the presence of substantially higher amounts of free circulating DNA in the plasma or serum of cancer patients than in healthy individuals (38, 39). A noninvasive assay for tumor-related genetic aberrations that uses serum/plasma DNA is thus desirable for assessing prognosis and therapy stratification. Our investigation of the use of L-FISH with a single-copy reference gene has demonstrated that the ERBB2/17-SSC ratio in serum is a valuable indicator of ERBB2 gene amplification and that serum L-FISH assay results are strongly concordant with tissue IHC/FISH data (91%).

In summary, the L-FISH assay is fast, objective, cost-effective, and easily automated; it therefore can offer a high degree of standardization and reproducibility. Furthermore, it is easily applicable to clinical samples from breast cancer patients and produces results that have good concordance with IHC results for corresponding histologic sections or with data obtained by conventional FISH. This noninvasive assay can be used when primary tumor samples are unavailable and eliminates the need for biopsy. L-FISH could also be an attractive alternative method for determining ERBB2 status when tissue results are discordant; however, flow cytometry instrumentation and expertise must be available to perform this type of testing. In addition, morphologic evaluation of the FISH findings is not possible when L-FISH is used. Although there is a need to prospectively collect clinical data from a large set of patients (especially those with equivocal results for IHC and conventional FISH) to provide clinical verification of the accuracy of L-FISH, our results are promising and warrant pursuing such study.

Author Contributions: All authors confirmed they have contributed to the intellectual content of this paper and have met the following 3 requirements: (a) significant contributions to the conception and design, acquisition of data, or analysis and interpretation of data; (b) drafting or revising the article for intellectual content; and (c) final approval of the published article.

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